Temporal regulation of *BMP2, BMP6, BMP15, GDF9, BMPR1A, BMPR1B, BMPR2* and *TGFBR1* mRNA expression in the oocyte, granulosa and theca cells of developing preovulatory follicles in the pig

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Abstract

This study aimed to describe the abundance and localization of *BMP2, BMP6, BMP15, GDF9, BMPR1A, BMPR1B, BMPR2* and *TGFBR1* mRNA during pig preovulatory follicular development and to evaluate their implication in improving follicular maturity in the preovulatory period preceding the second versus first post-weaning oestrus. Oocytes, granulosa (GC) and theca cells (TC) were recovered from antral follicles of primiparous sows at day 1, 2 and 4 after weaning and at day 14, 16 and 20 of their subsequent oestrous cycle. Real-time PCR analysis revealed that with the exception of *BMP6* mRNA, which was absent in GC, all genes were expressed in every cell type. Although *BMP6, BMP15* and *GDF9* mRNA were most abundant in the oocyte, their expression remained relatively constant during follicular development. By contrast, receptor *BMPR1B* and *TGFBR1* expressions in the GC and TC were temporally regulated. *BMPR1B* mRNA abundance was positively correlated with plasma oestradiol (E_2) suggesting that its regulation by oestrogen may be implicated in normal folliculogenesis. Interestingly, the increase in *BMPR1B* mRNA and protein abundance during the periovulatory period in GC and TC suggests a role for bone morphogenetic protein (BMP) 15 in the ovulatory process. Finally, expression of these ligands and receptors was not associated with potential differences in follicle maturity observed during the second versus first post-weaning preovulatory follicular wave. In conclusion, our results clearly demonstrate the presence of a complex signalling system within the pig follicle involving the transforming growth factor-β superfamily and their receptors, and provide evidence to support a role for BMP15 and BMPR1B during ovulation.

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Introduction

Ovarian follicular development in large domestic mammals is a long and intricate process that ultimately results in the ovulation of a subset of one or more oocytes potentially competent to support fertilization and embryonic development. Since ovulation rate and oocyte quality can be important determinants of reproductive efficiency, it is essential to understand the regulation of follicular growth leading to ovulation. It is now widely accepted that during the later phases of follicular growth, the processes of recruitment and selection establish a preovulatory follicle population characteristic of each species (Hunter *et al.* 2004). At the onset of the follicular phase in the pig, a pool of ∼50 follicles are recruited to actively grow, from which the preovulatory population of 12–20 follicles will be selected for ovulation (Foxcroft & Hunter 1985, Hunter *et al.* 2004). These processes are closely regulated by endocrine and paracrine factors, including the gonadotrophins, metabolic factors and several local growth factors.

Nutritional manipulations modify sow fertility and increased lactational catabolism in primiparous sows has detrimental consequences for embryonic survival by day 30 of gestation in the subsequent litter (Foxcroft 1997). Nutritional restriction during the last week of lactation was also shown to exert detrimental effects on follicle and oocyte quality (Zak *et al.* 1997). Using *in vitro* techniques, these authors demonstrated that 1) the oocytes recovered from the presumptive preovulatory follicles of feed-restricted sows were less capable of

undergoing nuclear maturation and 2) the follicular fluid obtained from the same follicles was less able to support nuclear maturation of pools of oocytes obtained from prepubertal gilts. Later studies established a relationship between the extent of protein catabolism during lactation and follicle quality in the sow (Yang *et al.* 2000*a*, Clowes *et al.* 2003*a*, 2003*b*). Interestingly, delaying breeding of primiparous sows until the second oestrus post-weaning results in an increase in litter size compared with animals bred at their first oestrus (Clowes *et al.* 1994). This difference in litter size likely results from the increased embryo survival in the sows bred at their second post-weaning oestrus and potentially originates from differences in follicle maturity (size) during the periovulatory period (Foxcroft *et al.* 2007). The mechanisms controlling such differences in follicular maturity are, therefore, of considerable practical significance.

Traditionally, studies investigating the control of follicular development have focused primarily on endocrine regulation by LH and FSH, and on local regulation involving insulin-like growth factor-I and activin/inhibin/follistatin systems. However, the recent findings that the bone morphogenetic proteins (BMP) and growth and differentiation factor 9 (GDF9) are key regulators of follicular development and might have a determinant role in establishing the ovulation quota has raised an entirely new set of questions regarding the control of follicle/oocyte maturation (Shimasaki *et al.* 2004, Juengel & McNatty 2005, Gilchrist *et al.* 2008). This is especially true in the pig where there is a paucity of information available. Therefore, the objectives of this study were: 1) to confirm the presence and determine the localization of the ligands *BMP2, BMP6, BMP15, GDF9* and their receptors *BMPR1A, BMPR1B, BMPR2* and *TGFBR1* mRNA in developing preovulatory follicles in the pig, 2) to establish the temporal changes in mRNA abundance during the preovulatory wave of follicular development and 3) to determine whether these ligands and receptors could play a role in creating the differences in follicle characteristics observed between the first and second post-weaning preovulatory wave of follicular development in primiparous sows. To our knowledge, this is the first experiment to report the spatial and temporal changes in mRNA expression for these members of the transforming growth factor-β (TGF-β) superfamily and their associated receptors in pig preovulatory follicles.

Results

Sow and follicle characteristics

The reproductive characteristics of the sows euthanized during the first and second post-weaning preovulatory wave of follicular development were very similar. The average number of follicles observed per ovary, the range of follicle size observed and the concentration of follicular fluid oestradiol (E_2) were not different between cycles (Table 1). As expected, the sows killed during the second post-weaning preovulatory wave of follicular development were heavier ($P \leq 0.01$). Although, overall, the average diameter of the three largest follicles was 0.5 mm larger ($P \leq 0.05$) in the second preovulatory wave of follicle development, these sows had lower overall concentration of plasma E_2 ($P \leq 0.05$). There were no interactions between cycle and stage of follicular development for any of the parameters studied; therefore, the data from both cycles were grouped to analyse the effect of stage of follicular development (Table 2). The number of follicles per ovary was lower during the final selection (FS) phase pre- and post-LH surge compared with the recruitment (R) and mid-selection (MS) phase ($P \leq 0.001$). The average size of the three largest follicles showed a concomitant increase between the R and MS phases ($P \leq 0.05$) and was higher during the FS phase pre- and post-LH surge when compared with the previous two phases ($P \leq 0.001$). Plasma E_2 concentrations were low during the R and MS phases, increased during the FS phase pre-LH surge and then declined in the post-LH surge period ($P \leq 0.001$). Finally, follicular fluid E_2 concentrations were maximal in the pre-LH surge period and the subsequent decline in follicular fluid E_2 ($P \leq 0.001$) was used to identify sows exposed to the preovulatory LH surge.

Table 1 Overall characteristics of the sows and follicles for each cycle irrespective of the phase of follicular development.

						Follicular fluid E_2 concentration (ng/ml)*	
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Cycle	Sow weight (kg)*,[†]	No. of follicles per ovary*,[†]	Range of follicle size (mm)[†]	Average size of the three largest follicles (mm)*,[†]	Plasma E_2 concentration (pg/ml)*,[†]	FS[‡]	FS post-LH[‡]
First	174 ± 5^a	26 ± 4	1–10	6.9 ± 0.3^a	13.0 ± 3.1^b	265 ± 57	15 ± 8
Second	189 ± 4^b	29 ± 5	1–10	7.4 ± 0.3^b	10.6 ± 4.3^a	466 ± 99	10 ± 2

*Data are expressed as lsmean±s.e.m. Different letters within columns indicate significant differences ($P \leq 0.05$).
[†]Represent the means for each cycle across all phases of follicular development (first cycle $n=14$; second cycle $n=14$). [‡]Follicular fluid concentration measured during the final selection phase (FS; first cycle $n=3$; second cycle $n=5$) and the final selection phase post-LH surge (FS post-LH; first cycle $n=5$; second cycle $n=3$).

Table 2 Characteristics of the sows and follicles from each stage of follicle development, irrespective of the cycle.

Stage of follicle development	No. of follicle per ovary[*][†]	Range of follicle size (mm)[*][‡]	Average size of the three largest follicles (mm)[*]	Plasma E_2 concentration (pg/ml)[*]	Follicular fluid E_2 concentration (ng/ml)[*]
Recruitment ($n=6$)	43 ± 4^b	1–6	5.0 ± 0.2^a	5.2 ± 1.1^b	NA
Mid-selection ($n=6$)	40 ± 2^b	1–7	5.9 ± 0.2^a	5.9 ± 1.6^b	NA
Final selection ($n=8$)	15 ± 1^a	3–10	8.8 ± 0.3^b	33.1 ± 2.4^c	365.5 ± 72.0^b
Final selection+LH ($n=8$)	11 ± 1^a	2–10	8.9 ± 0.2^b	3.2 ± 0.9^a	12.2 ± 4.8^a

[*]Data are expressed as lsmean±S.E.M. Different letters within columns indicate significant differences ($P\leq0.05$). NA, not applicable.
[†]The number of follicles reported for the recruitment and mid-selection phase represents the total number of visible follicles, while the numbers reported for the final selection pre- and post-LH represent the number of healthy preovulatory follicles. [‡]The range in follicle size reported represents the range of all visible follicles.

Gene localization and expression

With the exception of *BMP6*, which could not be detected in the granulosa cells (GC) with the current primer-probe set, the mRNA for the ligands *BMP2*, *BMP6*, *BMP15* and *GDF9* was present in every cell type throughout the different stages of follicular development (Table 3, Fig. 1). Similarly, the transcripts for the receptors *BMPR1A*, *BMPR1B*, *BMPR2* and *TGFBR1* were also detected in all three cell types and in every follicle class (Table 3, Fig. 1). The oocyte was found to exhibit the highest transcript abundance for all ligands and the relative mRNA abundance for *BMP15* and *GDF9* respectively, were 50- and 9-fold higher than *BMP6*, the next most abundant gene observed in the oocyte, and at least 110- and 18-fold higher than any other gene

across all cell types (Fig. 1). Although a statistical analysis across cell type was not possible because of the difference in endogenous control mRNA abundance, some of the differences observed in mRNA abundance between cell type (per example BMP15 and GDF9) were so large that they could not be solely explained by the differences in cyclophilin mRNA abundance. In addition, the relative mRNA abundance of the various receptors also appears higher in the oocyte than in the GC and theca cells (TC; Fig. 1). Interestingly, the mRNA abundance for the receptors in GC and TC was, in most cases, higher than the ligands (Fig. 1). Finally, no differences in mRNA localization or abundance were observed in the oocyte, GC or TC studied during the first versus second preovulatory wave of follicle growth after weaning (Fig. 2).

Table 3 Summary of the statistical analysis of oocyte, granulosa cell and theca cell mRNA abundance (ΔC_t) at specific stages of follicular development.

Gene	Cell type	Recruitment	Mid-selection	Final selection	Final selection +LH	*P* value
BMP2	Oocyte	5.18 ± 0.13	5.31 ± 0.13	5.14 ± 0.11	5.12 ± 0.12	NS
	Granulosa cell	11.63 ± 0.42^a	9.89 ± 0.42^b	9.14 ± 0.59^b	10.71 ± 0.59^{ab}	<0.05
	Theca cell	9.25 ± 0.18^a	7.89 ± 0.18^b	7.65 ± 0.25^b	8.69 ± 0.25^a	<0.001
BMP6	Oocyte	2.42 ± 0.17	2.31 ± 0.17	2.14 ± 0.15	1.94 ± 0.19	NS
	Granulosa cell	ND	ND	ND	ND	ND
	Theca cell	9.82 ± 0.22^a	8.42 ± 0.22^b	8.88 ± 0.31^b	8.85 ± 0.31^b	<0.01
BMP15	Oocyte	-3.85 ± 0.16^a	-3.66 ± 0.16^a	-3.45 ± 0.14^{ab}	-3.19 ± 0.14^b	<0.05
	Granulosa cell	13.66 ± 0.69	11.37 ± 0.69	13.38 ± 0.97	14.17 ± 0.97	NS
	Theca cell	11.19 ± 0.47	9.59 ± 0.47	9.77 ± 0.67	9.73 ± 0.67	NS
GDF9	Oocyte	-1.15 ± 0.12	-0.97 ± 0.12	-0.98 ± 0.10	-0.74 ± 0.11	NS
	Granulosa cell	8.28 ± 0.18^a	7.18 ± 0.18^b	7.62 ± 0.25^b	8.39 ± 0.25^a	<0.01
	Theca cell	8.47 ± 0.25^a	7.33 ± 0.25^b	7.67 ± 0.36^{ab}	7.62 ± 0.36^{ab}	<0.05
BMPR1A	Oocyte	4.67 ± 0.12	4.64 ± 0.12	4.59 ± 0.11	4.49 ± 0.14	NS
	Granulosa cell	6.78 ± 0.15^a	6.00 ± 0.15^b	6.22 ± 0.22^{ab}	6.30 ± 0.22^{ab}	<0.05
	Theca cell	6.37 ± 0.35	5.49 ± 0.35	5.82 ± 0.50	6.16 ± 0.50	NS
BMPR1B	Oocyte	3.41 ± 0.20	3.11 ± 0.20	3.17 ± 0.17	3.33 ± 0.22	NS
	Granulosa cell	5.17 ± 0.49^a	4.39 ± 0.49^b	2.36 ± 0.70^c	3.27 ± 0.70^{bc}	<0.001
	Theca cell	9.75 ± 0.42^a	8.75 ± 0.42^a	6.90 ± 0.59^b	8.30 ± 0.59^{ab}	<0.05
BMPR2	Oocyte	4.96 ± 0.11^a	4.56 ± 0.11^b	4.62 ± 0.11^b	4.38 ± 0.13^b	<0.05
	Granulosa cell	6.34 ± 0.25	5.39 ± 0.25	5.51 ± 0.35	6.10 ± 0.35	NS
	Theca cell	7.58 ± 0.28^a	6.37 ± 0.28^b	6.56 ± 0.40^{ab}	6.56 ± 0.40^{ab}	<0.05
TGFBR1	Oocyte	5.39 ± 0.18^{bc}	5.24 ± 0.18^c	5.91 ± 0.17^a	5.88 ± 0.20^{ab}	<0.05
	Granulosa cell	6.09 ± 0.19^a	5.20 ± 0.19^b	5.39 ± 0.27^b	4.74 ± 0.27^b	<0.01
	Theca cell	7.21 ± 0.14^a	6.41 ± 0.14^b	6.41 ± 0.19^b	6.61 ± 0.19^b	<0.01

NS, non-significant; ND, not detected. ΔC_t are expressed as lsmean±S.E.M. Different letters within rows indicate significant differences ($P\leq0.05$).
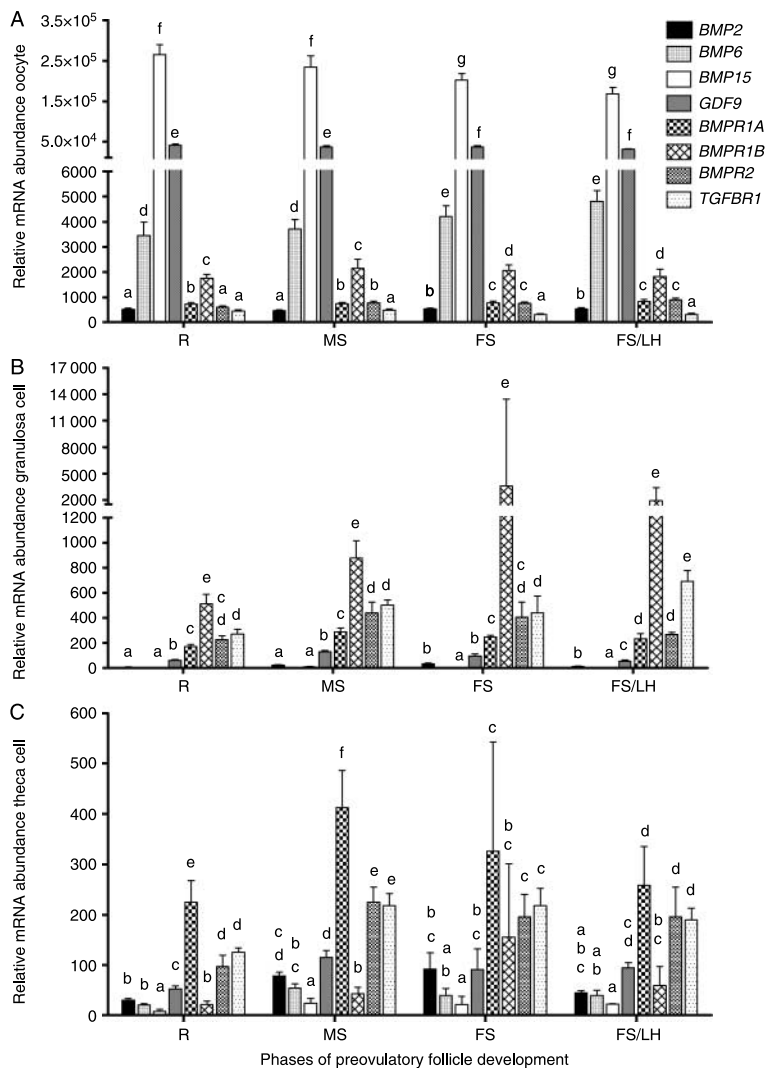
Figure 1 Comparison of *BMP2*, *BMP6*, *BMP15*, *GDF9*, *BMPR1A*, *BMPR1B*, *BMPR2* and *TGFBR1* mRNA abundance in pig oocytes (A), granulosa cells (B) and theca cells (C) for each phase of preovulatory follicle development (recruitment (R), mid-selection (MS), final selection (FS) and final selection post-LH surge (FS/LH)), irrespective of cycle. Relative mRNA abundance is expressed as lsmean±s.e.m. Different letters between genes within phases represent significant differences ($P \leq 0.05$).

Oocyte mRNA expression profiles

Despite their abundance, little or no changes were observed in the mRNA abundance of the four ligands throughout the follicular phase (Fig. 3A). *BMP15* was the only ligand whose transcript abundance was affected by the stage of follicular development ($P \leq 0.05$), with a 1.6-fold decrease in abundance between the R phase and the FS phase post-LH surge (Fig. 3A). Interestingly, both *BMP15* and *GDF9* mRNA abundance were negatively correlated with the average size of the largest follicles ($r = -0.49$, $P \leq 0.01$ and $r = -0.38$, $P \leq 0.05$ respectively).

The expression of mRNA for the receptors *BMPR1A* and *BMPR1B* in the oocyte also remained constant across the different phases of follicle development (Fig. 3B). By contrast, mRNA abundance for *BMPR2* and *TGFBR1* was affected by the stage of follicular development ($P \leq 0.05$; Fig. 3B). *BMPR2* mRNA increased by ∼1.3-fold between the R and MS phases and remained high thereafter. Oocyte-derived *TGFBR1*

showed a temporal mRNA expression profile dissimilar to that observed for any other gene, with relatively constant mRNA abundance during the R and MS phases, followed by a 1.6-fold fall in expression during both the FS phase pre- and post-LH surge (Fig. 3B). *TGFBR1* mRNA abundance was negatively correlated with the average size of the largest follicles ($r = -0.48$, $P \leq 0.05$).

Granulosa cell mRNA expression profiles

In comparison with the oocyte, *BMP2*, *BMP15* and *GDF9* transcripts appeared to be much less abundant in the GC. Among the ligands, GDF9 was the most abundant transcript in the GC with an average relative mRNA abundance 30-fold higher than *BMP15*, which had the lowest abundance in this cell type (Fig. 4A). Moreover, *BMP2* and *GDF9* mRNA abundance was affected by the stage of follicular development ($P \leq 0.05$ and $P \leq 0.01$ respectively) and both genes showed a similar temporal mRNA expression profile (Fig. 4A).
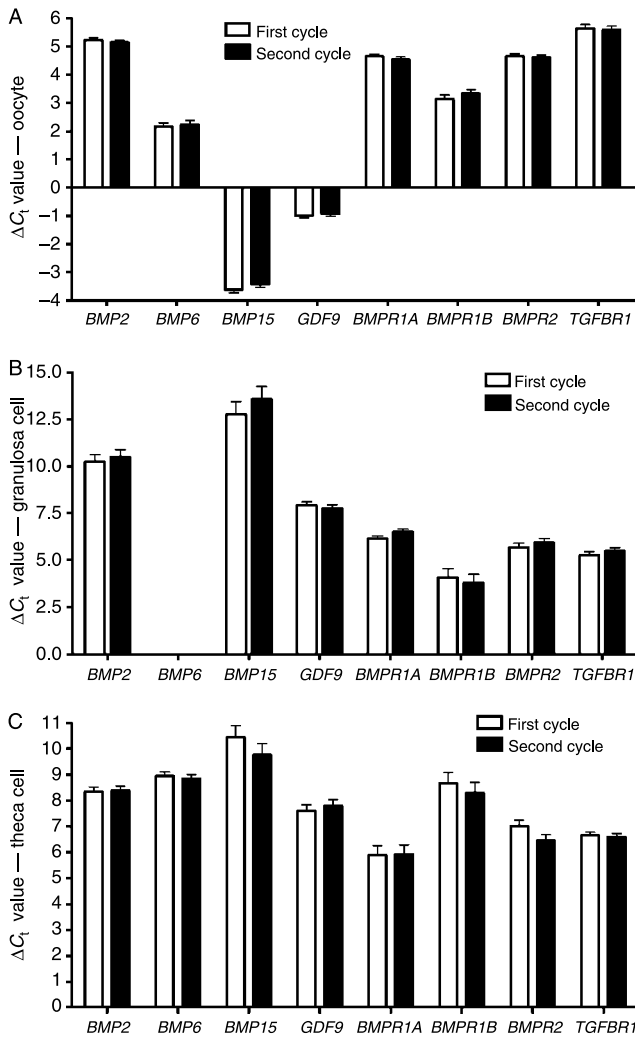
Figure 2 Quantification via real-time RT-PCR of *BMP2, BMP6, BMP15, GDF9, BMPR1A, BMPR1B, BMPR2* and *TGFBR1* mRNA abundance in pig oocytes (A), granulosa cells (B) and theca cells (C) between first and second cycle, irrespective of the phase of the cycle. The ΔC_t values are expressed as lsmean$\pm$s.e.m.

In the case of *BMP2*, a 3.3-fold increase in mRNA abundance was observed between the R and MS phases; mRNA abundance remained unchanged during the FS phase but showed a tendency to decrease following the LH surge ($P=0.08$; Fig. 4A). Interestingly, *BMP2* mRNA abundance also tended to be positively correlated with plasma E_2 ($r=0.42$, $P=0.08$). *GDF9* mRNA also increased 2.1-fold during the MS phase, remained constant during the FS phase and decreased 1.7-fold following exposure to the LH surge (Fig. 4A). The abundance of *GDF9* mRNA in the GC obtained from large preovulatory follicles was strongly and positively correlated with follicular fluid E_2 concentration ($r=0.87$, $P\leq0.05$).

The relative mRNA abundance for all receptors was found to be higher than those of the ligands. *BMPR2*

mRNA remained relatively constant in each follicle category; however, *BMPR1A, BMPR1B* and *TGFBR1* mRNA were temporally regulated during follicular development ($P\leq0.05$, $P\leq0.01$ and $P\leq0.01$ respectively; Fig. 4B). *BMPR1A* mRNA abundance increased by 1.7-fold during the MS phase, decreased to an intermediate level in the preovulatory follicle population and was not affected by exposure to the LH surge (Fig. 4B). *TGFBR1* expression showed a twofold increase during the MS phase and remained high in the preovulatory follicles, even after exposure to the LH surge (Fig. 4B). *TGFBR1* mRNA abundance was positively correlated with follicle size ($r=0.54$, $P\leq0.05$) and in the GC of the preovulatory population was strongly and negatively correlated with follicular fluid E_2 ($r=-0.84$, $P\leq0.05$). Finally, *BMPR1B* expression increased gradually over the first three phases of follicular development to peak during the FS phase prior to the LH surge (Fig. 4B). At that point, *BMPR1B* mRNA abundance was sevenfold higher than that during the R phase and fourfold higher than during the MS phase. Following exposure to the LH surge, *BMPR1B*
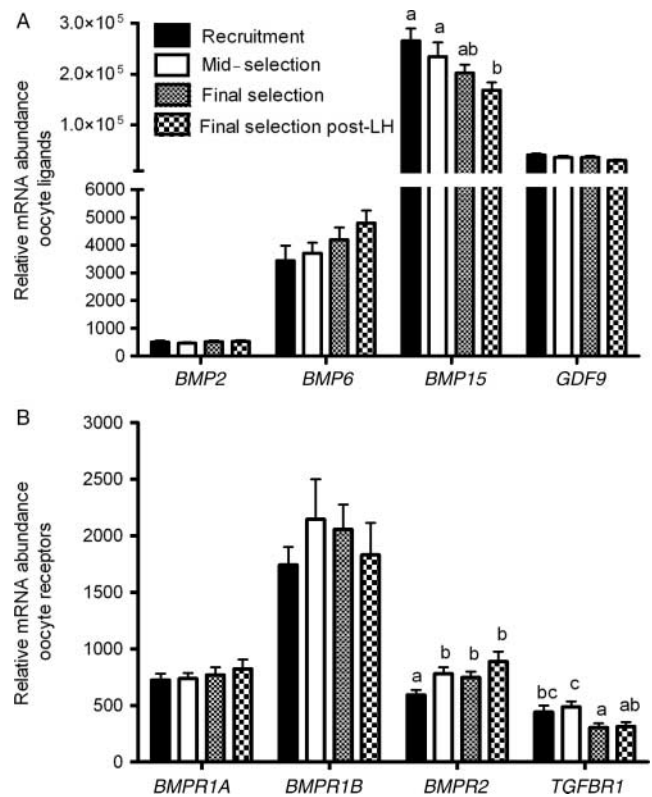

Figure 3 Quantification via real-time RT-PCR of (A) *BMP2, BMP6, BMP15* and *GDF9* and (B) *BMPR1A, BMPR1B, BMPR2* and *TGFBR1* mRNA abundance in pig oocytes during follicle recruitment ($n=6$), mid-selection ($n=6$), final selection ($n=8$) and final selection post-LH surge ($n=8$). Data are expressed as a relative mRNA abundance $\pm$s.e.m. for each phase, irrespective of cycle. Different letters within gene represent significant differences among phases ($P\leq0.05^{a,b,c}$; $P\leq0.01^{x,y,z}$).
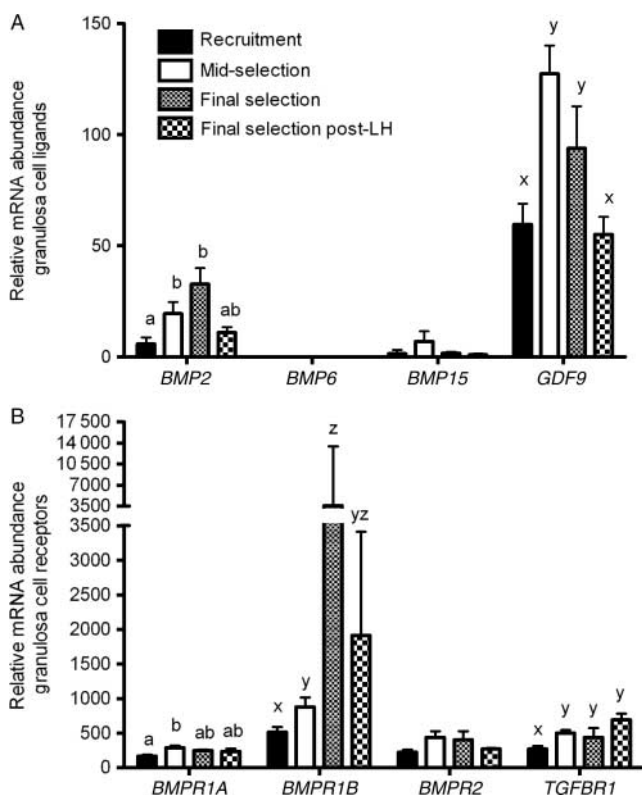
Figure 4 Quantification via real-time RT-PCR of (A) *BMP2*, *BMP6*, *BMP15* and *GDF9* and (B) *BMPR1A*, *BMPR1B*, *BMPR2* and *TGFBR1* mRNA abundance in pig granulosa cells during follicle recruitment ($n=6$), mid-selection ($n=6$), final selection ($n=3$) and final selection post-LH surge ($n=3$). Data are expressed as a relative mRNA abundance $\pm$ s.e.m. for each phase, irrespective of cycle. Different letters within gene represent significant differences among phases ($P \leq 0.05^{a,b,c}$; $P \leq 0.01^{x,y,z}$).

Theca cell mRNA expression profiles

The mRNA for all four ligands was expressed in the TC and mRNA abundance was comparable with that found in the GC. Moreover, as with the GC, *GDF9* was found to be the most abundant ligand mRNA in the TC, while *BMP15* had the lowest expression (Fig. 5A). Moreover, *BMP2*, *BMP6* and *GDF9* mRNA abundance in the TC were affected by the stage of follicular development ($P \leq 0.01$, $P \leq 0.01$ and $P \leq 0.05$ respectively). *BMP6* mRNA abundance increased 2.7-fold between the R and MS phases, and remained high thereafter (Fig. 5A). *BMP2* mRNA was also upregulated 2-fold during the MS phase and then downregulated 2.1-fold post-LH surge (Fig. 5A). *BMP2* mRNA abundance was positively

correlated with plasma E_2 concentration ($r=0.56$, $P \leq 0.05$), and in the TC from the large preovulatory follicles, a strong positive correlation was also observed with follicular fluid E_2 concentration ($r=0.94$, $P \leq 0.01$). Finally, *GDF9* mRNA abundance increased 2.3-fold from the R phase to the MS phase and returned to an intermediate level in the TC of the preovulatory follicle population (Fig. 5A).

TC receptor *BMPR1B*, *BMPR2* and *TGFBR1* mRNA expression was also affected by the stage of follicular development ($P \leq 0.05$, $P \leq 0.05$ and $P \leq 0.01$ respectively; Fig. 5B). The temporal patterns of *BMPR1B* and *TGFBR1* expression paralleled those observed in the GC. *BMPR1B* mRNA abundance increased 3.6-fold during the FS phase prior to the LH surge relative to the mRNA abundance observed during the MS phase (Fig. 5B). *BMPR1B* mRNA in the TC was also positively correlated with plasma E_2 concentration ($r=0.51$, $P \leq 0.05$) and with follicle size ($r=0.71$, $P \leq 0.001$). *TGFBR1* mRNA expression showed a 1.7-fold increase in abundance during the MS phase (Fig. 5B) and also tended to be
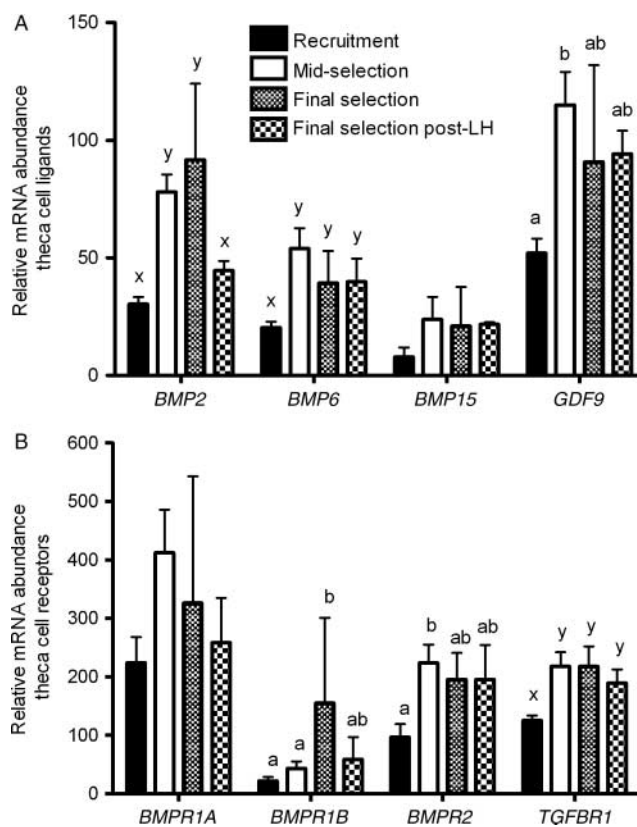

Figure 5 Quantification via real-time RT-PCR of (A) *BMP2*, *BMP6*, *BMP15* and *GDF9* and (B) *BMPR1A*, *BMPR1B*, *BMPR2* and *TGFBR1* mRNA abundance in pig theca cells during follicle recruitment ($n=6$), mid-selection ($n=6$), final selection ($n=3$) and final selection post-LH surge ($n=3$). Data are expressed as a relative mRNA abundance $\pm$ s.e.m. for each phase, irrespective of cycle. Different letters within gene represent significant differences among phases ($P \leq 0.05^{a,b,c}$; $P \leq 0.01^{x,y,z}$).
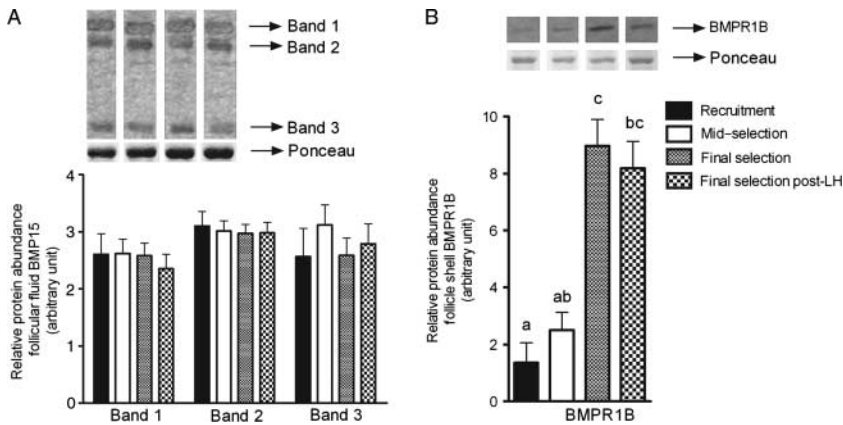
expression fell to an intermediate level. *BMPR1B* mRNA abundance tended to be positively correlated with plasma E_2 concentration ($r=0.44$, $P=0.07$) and was strongly and positively correlated with follicle size ($r=0.73$, $P \leq 0.0001$).

Figure 6 Western blot analysis for the presence of (A) BMP15 in follicular fluid and (B) BMPR1B in follicle hemisections during follicle recruitment (BMP15 $n=3$, BMPR1B $n=6$), mid-selection (BMP15 $n=6$, BMPR1B $n=6$), final selection (BMP15 $n=8$, BMPR1B $n=3$) and final selection post-LH surge (BMP15 $n=6$, BMPR1B $n=3$). Relative protein abundance is expressed as lsmean ± s.e.m. Different letters represent significant differences among phases ($P \leq 0.05$).

positively correlated with follicle size ($r=0.45$, $P=0.063$). Finally, *BMPR2* mRNA expression increased 2.3-fold between the R phase and the MS phase (Fig. 5B).

Western blot analysis

Western blot analysis of follicular fluid BMP15 revealed three immunoreactive bands located at ∼65, 55 and 25 kDa (Fig. 6A). The three bands appeared to be specific, as the secondary antibody alone and the preimmune rabbit IgG did not produce any cross-reactivity (data not shown). Furthermore, the same bands were also detected using a different specific antibody directed against BMP15 (data not shown). These bands likely correspond to the uncleaved promature protein (∼65 kDa), the cleaved proregion (∼55 kDa) and the mature protein (∼25 kDa), which correspond to the different forms of BMP15 recently reported in the mouse (McIntosh *et al.* 2008). Densitometric analysis of each immunoreactive band showed that BMP15 protein abundance did not change between the different phases of follicular development studied (Fig. 6A). In addition, western blot analysis for BMPR1B in follicle hemisections revealed one immunoreactive band located at ∼45 kDa (Fig. 6B). This band also appeared to be specific for BMPR1B, as the secondary antibody alone and the preimmune rabbit IgG did not produce any cross-reactivity (data not shown). Densitometric analysis of BMPR1B confirmed that protein abundance peaked during the periovulatory period prior to the LH surge confirming the mRNA expression profile observed in both GC and TC (Fig. 6B).

Discussion

As members of the TGF-β superfamily, BMP and GDF9, and their receptors, have been shown to play important roles during mammalian folliculogenesis (Shimasaki *et al.* 2004, Juengel & McNatty 2005, Gilchrist *et al.* 2008). However, little information is available on the role of these genes during the

preovulatory wave of follicular development. In order to better understand the physiological role of these genes in the developing porcine ovarian follicle and to assess their role in determining the differences in follicle quality observed between first and second post-weaning preovulatory wave of follicle development, the expression pattern of four relevant ligands with their known receptors was determined by real-time PCR. In the light of the real-time PCR results, the protein abundance for BMP15 in follicular fluid and its receptor, BMPR1B, in follicle hemisections were evaluated to confirm their potential role during the periovulatory period.

Few differences were observed between the first and second post-weaning preovulatory wave of follicle development. However, consistent with the results of a previous experiment, follicle size was bigger during the second wave of follicular development (Foxcroft *et al.* 2007). Interestingly, no cycle by stages interactions were observed, suggesting that the members of the TGF-β superfamily investigated in the present study are unlikely to be involved in the differences in follicle maturity leading to the differences in embryo survival and litter size observed in this and/or in earlier studies in the weaned sows (Clowes *et al.* 1994, Foxcroft *et al.* 2007).

Notwithstanding this lack of differences between follicular 'cycles', our results showed that, with one exception, *BMP2*, *BMP6*, *BMP15* and *GDF9* mRNA were expressed in all three cell types throughout the follicular phase. The exception was *BMP6* mRNA expression, which could not be detected in the GC using the current primer probe set. As anticipated, the oocyte was found to be the main site of transcription for all four ligands, with *BMP15* and *GDF9* being the most abundantly expressed genes in the oocyte. These results are for the most part consistent with previous studies done in the pig. First, *GDF9* mRNA and protein were shown to be highly expressed in the oocyte of 3–5 mm follicles from immature gilts, but were also detected at lower abundance in the cumulus and mural granulosa cells (CGC and MGC; Prochazka *et al.* 2004, Lee *et al.*

2008, Zhu *et al.* 2008). To our knowledge, no other studies in the pig have reported *GDF9* mRNA expression in the TC. However, in goat ovaries, the GDF9 protein was occasionally detected in the TC of follicles larger than 3 mm (Silva *et al.* 2005). This contrast with the cow where *GDF9* mRNA could not be detected in the TC from small or large follicles (Spicer *et al.* 2008). Brankin *et al.* (2005) reported a strong signal for BMP2 and BMP6 proteins in the oocyte of healthy 2–6 mm follicles from mature gilts, and BMP2 was also detected in the GC and TC from the same follicles. Interestingly, these authors also reported BMP6 expression in GC but not in TC. This discrepancy could be explained in part by the maturity of the animals used (gilts versus sows in our study) and/or the sensitivity and specificity of the detection technique (western blot versus real-time PCR in the present study). It is also probable that the differences in isolation techniques between the two studies might yield different TC populations (theca interna vs theca interna and externa in our experiment). Finally, Zhu *et al.* (2008) also detected *BMP6* mRNA in oocytes and cumulus cells from prepubertal gilts, whereas *BMP15* mRNA could be detected only in the oocyte and *BMP2* failed to produce a PCR amplicon in both the oocyte and its surrounding cumulus cells. Interestingly, despite the widespread interest in BMP15 in other species, no other studies have investigated the localization of *BMP15* mRNA or the BMP15 protein in porcine follicles. Our results are consistent with the observations made in goat follicles in which *BMP15* mRNA and protein were found to be expressed in oocytes and GC of all antral follicles while *BMP15* mRNA was also detected in TC (Silva *et al.* 2005). This is in contrast to mouse, rat, human and sheep follicles in which *BMP15* mRNA and/or BMP15 protein were localized exclusively to the oocyte (Dube *et al.* 1998, Laitinen *et al.* 1998, Aaltonen *et al.* 1999, Galloway *et al.* 2000, Juengel *et al.* 2002, Erickson & Shimasaki 2003). Generally, our results confirm observations made in mouse, rat, human, sheep and goat follicles (Dube *et al.* 1998, Laitinen *et al.* 1998, Aaltonen *et al.* 1999, Galloway *et al.* 2000, Juengel *et al.* 2002, 2006, Erickson & Shimasaki 2003, Glister *et al.* 2004, Fatehi *et al.* 2005, Silva *et al.* 2005) and support the general conclusion that the oocyte is the primary source of BMP6, BMP15 and GDF9.

Interestingly, little changes in ligand mRNA abundance were observed in the oocyte throughout the last week of follicular development. By contrast, although ligand expression in GC and TC was much lower than in the oocyte, in many cases their mRNA abundance was affected by the phase of follicular development. This is particularly intriguing, because BMP6, BMP15 and GDF9 are generally considered to be oocyte-derived ligands and BMP15 and GDF9 are exclusively expressed in the oocyte of most species (Juengel & McNatty 2005, Gilchrist *et al.* 2008). One could suggest that the expression of these ligands in GC in the present experiment is due to a cross-contamination from oocytes. Although, it is not possible to firmly rule out a potential cross-contamination, the abundance of *BMP15* and *GDF9* mRNA observed in the oocyte and GC suggests otherwise. In the oocyte, *BMP15* mRNA was found to be approximately six times more abundant than *GDF9* mRNA, while the converse was true for expression in the GC. The same argument against a cross-contamination of the TC by the GC is supported by the relative abundance of *BMPR1A* and *BMPR1B* mRNA. On the other hand, the relatively low abundance and the changing expression of these ligands in the GC and TC could reflect phase-specific interactions between the GC and/or the TC populations necessary to regulate their response to the follicle microenvironment. This has been suggested in rat in which the GC-derived *BMP6* mRNA is lost during the selection of the dominant follicle (Erickson & Shimasaki 2003). Knowing that BMP6 can prevent FSH action (Otsuka *et al.* 2001), the authors suggested that this loss of *BMP6* mRNA in GC may be necessary for FSH to affect the development of dominant follicles. Similar mechanisms have been shown for other members of the TGF-β superfamily such a BMP4 and BMP7, which can potently suppress androgen production by bovine TC (Glister *et al.* 2005). The major difference in this case is that BMP4 and BMP7 are TC-derived ligands. Although this hypothesis is attractive, the potential roles for the GC and TC-derived BMP2, BMP6, BMP15 and GDF9 have yet to be confirmed in any species, including the pig.

Our results also showed that the mRNA for the receptors of the TGF-β superfamily were detected in all three cell types and at every time point studied. Our findings confirmed observations made in the pig, sheep, goat and cow in which *BMPR1A*, *BMPR1B* and *BMPR2* mRNA and/or protein were expressed in the oocyte and in both GC and TC of antral follicles (Wilson *et al.* 2001, Souza *et al.* 2002, Glister *et al.* 2004, Quinn *et al.* 2004, Fatehi *et al.* 2005, Silva *et al.* 2005, Feary *et al.* 2007). The only exception appears to be the rat follicle in which *BMPR2* mRNA could not be detected in the TC (Erickson & Shimasaki 2003). The expression of *TGFBR1* mRNA has only been studied in antral follicles of mouse, sheep and cow ovaries and, consistent with our findings, was expressed in the oocyte, GC and TC (Juneja *et al.* 1996, Juengel *et al.* 2004, Jayawardana *et al.* 2006, Feary *et al.* 2007). Similar to the observations made for the ligands in our study, the oocyte was found to abundantly express *BMPR1A*, *BMPR1B*, *BMPR2* and *TGFBR1* mRNA but little variation in mRNA abundance was observed between the different time points. On the other hand, the GC and TC were found to express the mRNA for each receptor at slightly lower levels than the oocyte. However, in both the GC and TC, three out of the four receptors showed at least a 1.7-fold change in mRNA abundance between time points.

The first general observation emerging from the current study is that pig oocytes produce high but relatively constant amounts of the TGF-β superfamily ligand mRNA during the follicular phase, while mRNA expression for the receptors was temporally regulated in the surrounding somatic cells. Based on the current concept that the oocyte regulates its own microenvironment by secreting soluble factors, it is tempting to hypothesize that the regulation of the type I and II receptor mRNA expression in the GC and TC plays a stage-dependent role in the interaction between the oocyte and its surrounding cells (Gilchrist *et al*. 2008). Consistent with our hypothesis, several other studies have shown that the receptors associated with the TGF-β superfamily are hormonally regulated. In bovine GC, oestrogen used alone and in combination with FSH has been shown to upregulate the expression of the activin receptor type I (*ACVR1*), activin receptor type IIA (*ACVR2A*), *TGFBR1* (*ALK5*) and *BMPR2* mRNA (Jayawardana *et al*. 2006, Shimizu *et al*. 2006). Furthermore, FSH alone was shown to downregulate *TGFBR1* and *BMPR2* mRNA (Jayawardana *et al*. 2006) and in a study using human granulosa-like tumour cell line (KGN) FSH upregulated *BMPR1A*, *BMPR1B*, *BMPR2* and *ACVR2A* (Miyoshi *et al*. 2006). These results provide a basis for the temporal regulation of the receptor mRNA in porcine GC and TC and would explain the observed correlation between plasma E_2 and *BMPR1B* mRNA abundance.

Finally, each ligand of the TGF-β superfamily is known to signal through specific receptor complexes composed of a type I and type II serine–threonine kinase receptors. In the context of our study, BMPR2 has been identified as one of the potential type II receptors involved in BMP2, BMP6, BMP15 and GDF9 signalling (Shimasaki *et al*. 2004, Juengel & McNatty 2005). In addition, BMPR1A has been identified as a potential type I receptor for BMP2 and BMP6, BMPR1B has been found to be involved in BMP6 and BMP15 signalling and TGFBR1 was identified as the receptor for GDF9 (Shimasaki *et al*. 2004, Juengel & McNatty 2005). Given this information, the temporal changes in expression of each receptor in the ovarian follicle could be indicative of a functional role for its ligand(s) at a precise stage of follicular development.

In the context of recent literature, results from the present study on the expression pattern of *TGFBR1* mRNA in the GC and TC are consistent with a role for GDF9 in follicle selection. Spicer *et al*. (2008) reported that bovine TC isolated from small follicles were more responsive to GDF9 than their counterparts isolated from larger follicles, and that GDF9 stimulated proliferation, while inhibiting progesterone and androstenedione production, by the TC of small antral follicles. Similarly, building on the observations of Vitt *et al*. (2000) in the rat suggesting that GDF9 controls proliferation of GC, Shimizu *et al*. (2008) reported that intra-ovarian injection of GDF9 promoted the development of medium-sized antral follicles. Collectively, these observations in other species suggest that GDF9 modulates aspects of GC and TC function important for follicle selection.

Finally, particularly in the context of the pig follicular development, the most interesting findings among our data are the pattern of *BMPR1B* mRNA and protein expression in GC and TC, along with that of *BMP15* mRNA in the oocyte and its protein in the follicular fluid. First, in the oocyte, *BMP15* mRNA marginally decreased during the periovulatory period, but no change in BMP15 protein abundance was observed in the follicular fluid. However, the upregulation of *BMPR1B* mRNA in the GC and TC during the same period, accompanied by a similar increase in BMPR1B protein in follicle hemisections, suggests that the oocyte-derived BMP15 may play a key role in the periovulatory period in the pig follicle. This is in accordance with the phenotype observed in *Bmp15* null mice where no apparent defects in follicular development were observed; but ovulation rate was lower and the ovulated oocytes showed reduced developmental potential (Yan *et al*. 2001, Su *et al*. 2004). Interestingly, the *Bmpr1b* null mice exhibited defects in cumulus expansion which, in turn, prevented *in vivo* fertilization (Yi *et al*. 2001). Moreover, recent results obtained in two different mouse studies showed that BMP15 is likely involved in cumulus expansion (Gueripel *et al*. 2006, Yoshino *et al*. 2006). Although the precise timing was different, both studies showed that the mature form of BMP15 protein appeared in the periovulatory period following gonadotrophin stimulation. This is in contrast to our study, in which levels of the mature form of BMP15 remained constant between the different phases of follicular development. However, as the current preovulatory follicle population was exposed to both LH and FSH, we hypothesized that the receptor rather than the ligand is subjected to regulation in the pig. The correlation observed between plasma E_2 and *BMPR1B* mRNA abundance is another good indicator that this signalling pathway might be under the influence of FSH and/or LH.

In conclusion, the findings of the present study clearly demonstrate the presence of a complex signalling system within the porcine follicle involving members of the TGF-β superfamily and their associated receptors. To our knowledge, this is the first study to investigate the spatial and temporal regulation of those ligands and receptors during the final week of follicular development preceding ovulation in the pig. Our results clearly showed that what are generally considered to be oocyte-derived ligands are also expressed in the GC and TC of pig follicles, potentially reflecting species-specific somatic cell interactions. Finally, although additional studies will be required to further assess the exact role of these genes in the porcine follicle, this study provides strong evidence to support a role for BMP15 during ovulation.

Materials and Methods

Chemicals and media

Unless otherwise stated, all chemicals were obtained from Sigma–Aldrich. The media used for washing the cumulus–oocyte complexes (COC) and during follicle dissection was modified Tyrode lactate (TL)-HEPES medium supplemented with 0.1% (w/v) polyvinyl alcohol (PVA; Funahashi *et al*. 1997), 50 U/ml penicillin and 50 μg/ml streptomycin (Invitrogen, #15070-063). The PBS (pH 7.4) was composed of 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.47 mM KH_2PO_4.

Animals

This experiment was conducted in accordance with the guidelines of the Canadian Council on Animal Care and with the approval of the University of Alberta, Faculty Animal Policy and Welfare Committee (Protocol #2005-40B). A total of 28 primiparous F1 sows (Large White×Landrace, Hypor, Regina, SK, Canada) were used for follicle dissection and oocyte aspiration and an additional nine sows were required for follicular fluid collection. Within 48 h after farrowing, litter size was standardized to between 9 and 11 piglets through cross-fostering and routine piglet processing procedures (tail-docking, teeth clipping, ear notching and iron injection) were performed. During lactation, sows were offered fresh feed three times daily and permitted access to fresh water *ad libitum*. Sows were offered 3.5 kg feed on the day of farrowing, and during the remainder of lactation, the amount of feed offered was increased by 0.5 kg daily until the sows daily consumption was exceeded by 0.5 kg. Sows were weaned 20.8 ± 3.2 (mean$\pm$s.d.) days after farrowing and were randomly allocated to treatment at this time. After weaning, sows were moved to a common weaned-sow room, housed in individual sow stalls and were fed to appetite twice daily until the day of killing. From the day after weaning, sows were actively heat-checked using fenceline boar contact twice daily (at 0800 and 1400 h). Onset of standing heat was determined as the midpoint between the time of first detection of standing oestrus and the last time that oestrus was detected. Similarly, the end of standing heat was calculated as the midpoint between the last time that signs of oestrus were detected and the first time after standing heat that no signs of oestrus were detected. Sows were weighed on days 1, 6 and 13 of lactation, at weaning and at the time of killing.

Sows used for follicle dissection and oocyte aspiration were killed on day 1 (D1; $n=3$), 2 (D2; $n=3$) or 4 (D4; $n=8$) after weaning, or day 14 (D14; $n=3$), 16 (D16; $n=3$) or 20 (D20; $n=8$) after the first post-weaning oestrus. The additional nine sows used for follicular fluid collection were killed on D2 ($n=2$), D14 ($n=3$) and D16 ($n=4$) respectively. These time frames correspond to the first and second post-weaning preovulatory wave of follicular development and D1/D14 corresponds to the period of R of the growing cohort of follicles, D2/D16 corresponds to the MS phase at which time follicles either keep growing or, if not selected, start to undergo atresia and D4/D20 corresponds to the FS phase where the preovulatory follicle population has been established (Grant

et al. 1989, Hunter & Wiesak 1990). The preovulatory follicle population from the FS phase was further divided between the follicles in pre-LH surge (FS) or post-LH surge (FS/LH) stages based on follicular fluid E_2 concentrations (see procedure below). The sows used in the experiments were killed on-site in a purpose-built necropsy facility according to the Swine Research and Technology Centre standard operating procedures. Prior to killing, a single blood sample was collected into a 10 ml heparinized Vacutainer (Becton Dickinson, Franklin Lakes, NJ, USA) by jugular puncture, centrifuged at 1700 *g* for 15 min at room temperature and the plasma was then stored at −20 °C until assayed for plasma E_2 concentration.

Tissue collection

Within 20 min after killing, the ovaries were moved to an adjacent laboratory suite in 50 ml Falcon tube containing 0.9% (w/v) warm saline when the ovaries were intended for oocyte aspiration and follicular fluid collection, or in 0.9% (w/v) ice-cold saline when the ovaries were intended for follicle dissection. The ovaries from the 28 sows intended for follicle dissection and/or oocyte aspiration were allocated as followed: for each phase of follicular development (R, MS and FS), three sows from each cycle group (first or second cycle) were killed. One ovary from each sow was chosen for follicle dissection and the other ovary was used for oocyte aspiration. In order to account for the low number of follicles present in the ovary of D4 and D20 animals and the risk of ovulation occurring, an additional five sows were allocated to each of these days. Both ovaries from these animals were used for oocyte aspiration. Before processing the ovaries, the number and size of all visible follicles were recorded and the follicular fluid from the largest follicle(s) (one to three pooled follicles depending on the volume recovered) of D4 and D20 animals was collected using an 18 gauge needle attached to a 1 ml disposable syringe for assay of E_2 concentration. An aliquot of follicular fluid from the D4 and D20 sows as well as the follicular fluid collected from the additional nine sows was used for western blot analysis. The follicular fluid was centrifuged for 5 min at 13 000 *g* to remove any cellular debris, diluted 11-fold in Medium 199 (M199) and stored at −20 °C for either assaying E_2 concentrations or for western blot analysis.

Follicle dissection

A total of 18 ovaries, each originating from a different animal and representing each phase of follicular development, were sliced in half longitudinally and washed twice in ice-cold PVA-TL-HEPES to remove blood contamination. The half ovaries were then placed into Petri dishes containing ice-cold PVA-TL-HEPES and follicles representative of the population present on the ovary were dissected free of stromal tissue under a dissecting microscope using fine scissors and forceps. Intact dissected follicles were then placed into RNAlater (Ambion, Austin, TX, USA) to preserve RNA integrity during the remaining procedure. Depending on the size of the follicles, between five and ten follicles were dissected within a

1-h period. The intact follicles were then placed back into ice-cold PVA-TL-HEPES and cut into half using a scalpel blade. One half of each follicle (hemisection) from an individual sow containing GC and TC was transferred to a 1.5 ml microcentrifuge tube in ice-cold media and centrifuged for 5 min at 200 *g* at room temperature. The hemisections were washed twice with 1 ml ice-cold PBS followed by centrifugation at 200 *g* for 5 min. Following the last wash, the supernatant was removed and the cells were snap frozen in liquid nitrogen. The hemisections were stored at −80 °C until protein extraction. The MGC were then gently scraped from the inner wall of the remaining hemisection using a fine glass loop and the oocytes were removed to ensure a pure MGC population. The MGC were transferred to a 1.5 ml microcentrifuge tube in ice-cold media and centrifuged for 5 min at 200 *g* at room temperature. The MGC pellets were washed twice with 1 ml ice-cold PBS followed by centrifugation at 200 *g* for 5 min. Following the last wash, the supernatant was removed and the cells were snap frozen in liquid nitrogen. The remaining follicle shells contained mainly TC and were vigorously agitated by repeated pipetting to ensure that all MGC were removed. The TC were then transferred to a 1.5 ml microcentrifuge tube in ice-cold media and centrifuged for 5 min at 200 *g* at room temperature. The TC pellets were washed twice with 1 ml ice-cold PBS followed by centrifugation at 200 *g* for 5 min. Following the last wash, the supernatant was removed and the cells were snap frozen in liquid nitrogen. The MGC and TC were finally stored at −80 °C until RNA extraction.

Oocyte aspiration

COC were collected from the remaining ovaries by aspiration using an 18 gauge needle attached to a 5 ml disposable syringe and the COC from each sow were processed as a group. The COC were transferred to a petri dish containing 15 ml warm PVA-TL-HEPES. The recovered COC were washed three times in warm PVA-TL-HEPES to remove any cellular debris. The oocytes were then denuded by vortexing at low speed for 5 min in 150 μl PBS in a 1.5 ml microcentrifuge tube. The denuded oocytes were observed under a dissecting microscope to ensure that they were free of cumulus cells and then washed twice in PBS before transfer to a fresh 1.5 ml microcentrifuge tube in a minimum volume of PBS and snap freezing on dry ice. The remaining CGC were transferred to a 1.5 ml tube and centrifuged for 5 min at 200 *g* in a table top microcentrifuge. The pellets were washed twice with PBS followed by centrifugation at 200 *g* for 5 min. Following the last wash, the supernatant was discarded and the cells were snap frozen on dry ice. The oocytes and the CGC were finally stored at −80 °C until RNA extraction.

RIA

Plasma E₂

E₂ concentrations were determined in all plasma samples in triplicate in a single RIA using the method of Yang *et al.* (2000*b*). Extraction efficiency was $63 \pm 4\%$ and estimated

potencies were not corrected for recovery. Assay sensitivity, defined as 90% of total binding, was 0.35 pg/ml. The intra-assay coefficient of variation (CV) was 9%.

Follicular fluid E₂

Follicle fluid (FF) E₂ concentrations were quantified in a single RIA using a double antibody kit (Diagnostic Products Corporation # KE2D1, Diagnostic Products Corporation, Los Angeles, CA, USA) without extraction, with the following modifications to the manufacturer's protocol. In-house E₂ reference standards (E₂, Sigma # E8875, Sigma–Aldrich) in PBS gel (0.1% (w/v) gelatin, Sigma #G2500, Sigma–Aldrich) buffer (PBS), containing 2.77 mM monobasic phosphate (Fisher # S369-500, Fisher Scientific, Nepean, ON, Canada), 7.22 mM dibasic phosphate (Fisher # 374B-500), 15 mM sodium azide (Fisher # S369-500) and 139 mM sodium chloride (BDH #ACS 783, BDH Inc., Toronto, Canada; pH 7.0) were used, rather than the standards provided by the kit. Standards ranged from 0.1 to 50 pg per tube. The primary antiserum was reconstituted as directed by the manufacturer, but then further diluted threefold with distilled and deionized water to improve assay sensitivity. Centrifugation time was increased to 30 min to improve pelleting. The volume of sample taken to assay was 0.1 ml + 0.1 ml PBS gel buffer. Serial dilutions with PBS gel buffer of a control FF pool diluted 11-fold in M199 showed parallelism to the standard curve. Samples were assayed in triplicate in a single assay at two dilutions: FF diluted 11-fold in M199 and FF further diluted 200-fold in PBS gel buffer for a final 2200-fold dilution. The intra-assay CV for the single assay run was 10.2%. Sensitivity estimated at 95% of total binding was 0.127 pg/tube, equivalent to 2.79 ng/ml for 11-fold diluted samples. The recovery of a known amount of E₂ when added to a sample of known potency was $94.5 \pm 2.7\%$.

RNA isolation and real-time RT-PCR

Total RNA was extracted from the pooled CGC, MGC and TC of individual animals using TRIzol reagent (Invitrogen) following the manufacturer's instructions, with the following modification. The cells were thawed on ice directly in TRIzol and homogenized with a Polytron. The homogenized samples were then incubated for 10 min at room temperature before further processing. The homogenized CGC and MGC originating from the same animals were then pooled together prior to the extraction and will be further referred to as GC. The GC and TC total RNA was precipitated with 1/10 volume of 5 M ammonium acetate, 1 volume of isopropanol and linear acrylamide (Ambion) was also added to the RNA as a carrier at a final concentration of 10 μg/ml. The total RNA was resuspended in nuclease free H₂O (Ambion) and was DNase treated using DNA-free (Ambion) following the manufacturer's instructions. The samples were quantified using the spectrophotometer ND-1000 (NanoDrop, Wilmington, DE, USA) and RNA integrity was evaluated on a 1% (w/v) denaturing agarose gel. All samples were stored at −80 °C until cDNA synthesis. Oocyte total RNA was extracted from pools of between 15 (for D4 and D20 animal) and 150 (for D1 and D14 animals) oocytes recovered from individual sows

Table 4 Details of primers and probes used for real-time PCR.

Gene	Accession number	Primer	Sequence 5′→3′	Product size (bp)	Annealing/extension temperature (°C)
Cyclophilin	AY266299	Forward	AATGCTGGCCCCAACACA	56	60
		Reverse	TCAGTCTTGGCAGTGCAAATG		
		Probe	ACGGTTCCCAGTTTT		
BMP2	AY669080	Forward	CCCCACGGAGGAGTTTATCA	59	60
		Reverse	CCTGTGTCTGTTCCCGAAAGA		
		Probe	CTCAGCAGAACTTC		
BMP6	EU693015	Forward	CCAAACTTTTCTTATCAGCATTTATCA	75	62
		Reverse	TCCAACAGAAACAGGTCAGAGTCT		
		Probe	CTTACAGCAGCGTCAACA		
BMP15	NM001005155	Forward	TTCCCAGAGGCCTGGAAGA	58	60
		Reverse	GCCTTCCGCAAAAGAAGAGA		
		Probe	TTTATGGCAAGAGACCC		
GDF9	AY649763	Forward	ATGTGACGGCCATCCTTCAG	63	60
		Reverse	CGATGGACATGTGAATCTCTCT		
		Probe	CCCTAGTGGTCTCCAAC		
BMPR1A	EU693016	Forward	GTGGATCTGGACTACCCTTGTTG	65	60
		Reverse	TTGCCGAACCATCTGTATCTGT		
		Probe	TTCAGCGAACTATTGCCA		
BMPR1B	AY065994	Forward	TGGTTCCGAGAGACAGAAATATATCA	68	60
		Reverse	GCAATGAAGCCCAAAATGTTTT		
		Probe	ACAGTGTTGATGAGGCAT		
BMPR2	EU693017	Forward	GGGTCGGGTGAAAAGATCAA	60	60
		Reverse	GCGCCACCGCTTAAGAGA		
		Probe	AAACGTGTGAAAACTCCCTA		
TGFBR1	DQ519378	Forward	GTCTGCATCTCACTCATGTTGATG	67	60
		Reverse	GCACTCGATGGTGAATGACTGT		
		Probe	TCTATATCTGCCATAACCG		

using the PicoPure RNA isolation kit (Arcturus, Mountain View, CA, USA) following the manufacturer's instructions. All samples were DNase treated as suggested in the protocol and the RNA was eluted in 30 μl. Oocyte total RNA was stored at −80 °C until use. Due to the large difference in the number of oocytes recovered between animals, the efficiency of RNA extraction and cDNA synthesis was tested and was found to be consistent when ≥15 oocytes were used (data not shown).

Oocyte, GC and TC total RNA was reverse transcribed with Superscript III RT (Invitrogen) according to the manufacturer's instructions, using a combination of 5 μM oligo dT and 5 ng/μl random hexamer. RNaseOUT (Invitrogen) was also added to the reaction at a concentration of 2 U/μl. cDNA synthesis was performed using 2 μg GC and TC total RNA and with 10 μl of the oocyte total RNA. After RT, GC and TC cDNA were diluted to 20 ng/μl and the oocyte cDNA to an equivalent of 0.25 oocytes/2 μl with nuclease-free H_2O (Ambion).

Real-time PCR was performed in duplicate using 20 ng GC or TC cDNA, or the cDNA equivalent to 0.25 oocytes, in 96-well fast plates using the Taqman Fast Universal PCR Master Mix and the ABI 7900HT thermocycler (Applied Biosystems, Foster City, CA, USA). The primers and Taqman-MGB probes (Table 4) were designed using the Primer Express software v3.0 (Applied Biosystems) using species-specific sequences found on GenBank. The amplification efficiency for each gene was determined using serial dilution of ovarian cDNA and was found to be ≥90% for all genes (data not shown). Moreover, the amplification efficiency slopes for all nine genes were found to be identical. As reported by Bettegowda *et al.* (2006), cyclophilin was used as the endogenous control to correct for RNA extraction and RT efficiency within cell type.

Cyclophilin transcript abundance was found to be stable within each cell type throughout the different stages of follicle development, confirming its usefulness as a good endogenous control. However, cyclophilin abundance differed between the cell types studied and limited our ability to statistically compare the abundance of each gene of interest across cell types.

Western blot analysis

BMP15

Aliquots of follicular fluid (200 μl) diluted 11-fold in M199 were precipitated using 3 volumes of ice-cold acetone. The protein pellets were resuspended in 150 μl cell extraction buffer (Invitrogen) supplemented with 1 mM phenylmethylsulphonyl fluoride (PMSF; Sigma–Aldrich) and 5% (v/v) of protease inhibitor cocktail (Sigma–Aldrich) as recommended by the manufacturer. Protein concentration was determined by bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) according to the manufacturer's instructions and 7.5 μg follicular fluid protein from each sample was resolved onto 12% SDS-PAGE gels. A control sample was also loaded on each gel to correct for interblot variability. The proteins were transferred at 4 °C overnight at 150 mA onto nitrocellulose membranes. The membranes were stained with Ponceau S to evaluate total protein loading and the image was acquired with an ImageScanner (GE Healthcare, Piscataway, NJ, USA). The membranes were blocked at room temperature for 1 h in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBS-T) and 5% (w/v) non-fat dry milk. The membranes were then incubated for 1 h with the primary anti-BMP15 antibody

(sc-28911, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) using a 1:400 dilution in the blocking solution. Following the primary antibody, the membranes were washed three times for 5 min with the blocking solution and hybridized with the secondary antibody (HRP-conjugated donkey anti-rabbit, cat # 711-035-152, Jackson ImmunoResearch Laboratory, West Grove, PA, USA) diluted 1:10 000 in the blocking solution. The membrane was finally washed once for 15 min, followed by three 5-min washes in TBS-T.

BMPR1B

Hemisections from each animal were ground in liquid nitrogen and the proteins extracted on ice for 2 h in cell extraction buffer supplemented with 1 mM PMSF (Sigma–Aldrich) and 5% (v/v) of protease inhibitor cocktail (Sigma–Aldrich). The samples were then centrifuged at 18 000 ***g*** for 10 min at 4 °C to remove any insoluble material and protein concentration was determined by BCA assay (Pierce) according to the manufacturer's instructions. Immunoblotting was performed exactly as described for BMP15 with the following modifications. A total of 10 μg protein from each sample was resolved onto 12% SDS-PAGE gels. The blocking solution used was TBS-T supplemented with 3% (w/v) non-fat dry milk and 5% (v/v) donkey serum. The primary anti-BMPR1B antibody (sc-25455, Santa Cruz Biotechnology, Inc.) was diluted 1:200 in the blocking solution and the secondary antibody (HRP-conjugated donkey anti-rabbit, cat # 711-035-152, Jackson ImmunoResearch Laboratory) was diluted 1:20 000 in the blocking solution.

To ensure antibody specificity, preimmune rabbit IgG (Jackson ImmunoResearch, cat# 011-000-003) at a concentration identical to each primary antibody and the secondary antibody alone were tested on follicular fluid and hemisection protein samples. Immunoreactive proteins were detected with ECL plus (GE Healthcare) and the images were acquired on a Typhoon Trio (GE Healthcare). Finally, the abundance of each immunoreactive protein, as well as a predetermined band stained with Ponceau S, was determined by densitometric analysis using the ImageQuant software (GE Healthcare).

Statistical analysis

The sow's reproductive data including weight, number of follicles per ovary, average size of the three largest follicles, plasma E_2 and follicular fluid E_2 concentrations were analysed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC, USA). With the exception of the weight data that were normally distributed, all reproductive data were RANK transformed and the analysis was performed on the transformed data. The model for the experiment included phases of follicular development and cycle (first and second preovulatory wave after weaning) as the independent variables and sow as the random variable. Differences between means were analysed using a least significant difference (LSD) test at a 95% confidence level.

Real-time PCR data for the genes of interest (GOI) were normalized against their respective means for cyclophilin using the ΔC_t method ($\Delta C_t = C_{t\,GOI} - C_{t\,cyclophilin}$). The cycle threshold ($C_t$) is defined as the PCR cycle where the fluorescence reaches a determined threshold. Consequently, the C_t and corrected C_t (ΔC_t) value are inversely related to the copy number of the targeted gene initially present in the sample. For the analysis of mRNA abundance between each GOI within day and within cell type, the ΔC_t values of the oocyte and GC were RANK transformed and the analysis was performed on the transformed data. The ΔC_t for each GOI were analysed using the MIXED procedure of SAS and the model for the experiment included GOI as the independent variables. Differences between means were analysed using a LSD test at a 95% confidence level. For the analysis of the temporal changes in mRNA abundance, the ΔC_t values for all GOI were normally distributed except for BMPR1B in the GC, which was RANK transformed and the analysis was performed on the transformed data. The individual ΔC_t for each GOI were analysed using the MIXED procedure of SAS. The model for the experiment included phases and cycle as the independent variables, and sow as the random variable. Since neither an effect of cycle nor an interaction between phase and cycle was found, the data were further analysed irrespective of cycle. Differences between means were analysed using a LSD test at a 95% confidence level. For ease of interpretation of the expression profiles, the data were converted using the formula $2^{-(\Delta C_t - \Delta C_{t\,calibrator})}$ and are expressed as relative mRNA abundance±S.E.M. The ΔC_t value obtained for BMP15 in the GC during the FS phase post-LH surge was used as the calibrator value, thus maintaining the relative mRNA abundance for the GOI within and, to a limited extend, between cell type. Finally, correlations analyses were performed across all sows to determine relationships between the expression of each GOI and follicular fluid and plasma E_2 concentration and follicle size.

Western blot data were corrected for loading differences using the abundance of a predetermined band from the total protein stain and then corrected for inter-blot variation using the abundance of each specific immunoreactive band from the loading control. The corrected data were analysed using the MIXED procedure of SAS (SAS Institute Inc). The data for BMP15 band 2 were normally distributed; however, the data for BMP15 bands 1 and 3 were log transformed and the data for BMPR1B were RANK transformed and analyses were then performed using the transformed data. The model for BMP15 included only phase, while the model for BMPR1B included phase, cycle and their interaction, as independent variables. Differences between means were analysed using a LSD test at a 95% confidence level. Data are expressed as relative protein abundance±S.E.M.

Declaration of interest

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