Granulosa cells of the cumulus oophorus are different from mural granulosa cells in their response to gonadotrophins and IGF-I

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Abstract

There are two types of granulosa cells: those which surround the oocyte are cumulus cells (CC) and those which surround the antrum are mural granulosa cells (MGC). These cells are under the influence of several hormones and growth factors, the most important of which are gonadotrophins and IGF-I. In this article, we report novel observations on the differences between these two types of granulosa cells and their interaction with gonadotrophins and IGF-I. We were able to conduct physiological studies on the role of IGF-I by using an analogue of IGF-I which does not bind to IGF-I-binding proteins (LR³-IGF-I). Immature rats received saline, equine chorionic gonadotrophin (eCG), LR³-IGF-I or eCG plus LR³-IGF-I by infusion using a pump from 24–29 days of age. The rats were killed and the ovaries removed. Surface follicles were punctured and MGC and oocyte cumulus complexes were removed. These were cultured in saline (control) and in three different doses of FSH. Cell repli-

cation was assessed by ³H-thymidine incorporation and differentiation was evaluated by the measurement of progesterone secretion. It was noted that CC replicated ten times more than MGC. Similarly, progesterone secretion by CC was six times more than by MGC. *In vivo* exposure to gonadotrophins (eCG) positively influenced *in vitro* treatment with FSH in both cell types. This phenomenon was observed in both cell replication and progesterone secretion. The IGF-I analogue had a positive effect on cell replication of MGC but a negative effect on the cell replication of CC. With respect to progesterone secretion, the IGF-I analogue had a negative effect on CC but a positive effect on MGC. In conclusion, CC behaved differently from MGC in response to gonadotrophins and the IGF-I analogue. IGF-I and FSH acted additively, synergistically or antagonistically in different circumstances.

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Introduction

As the Graafian follicle matures, there are several concentric layers of granulosa cells surrounding the oocyte. When the antrum develops and enlarges, the granulosa cells divide into two functional groups: the cells in immediate contact with the oocyte which are called the cumulus cells (cumulus oophorus, CC) and the mural granulosa cells (MGC) which line the follicular wall around the antrum (Greenwald & Terranova 1988). There has been evidence showing that CC provide nutrition for the oocyte and influence oocyte development in a paracrine fashion (Brower & Schultz 1982, Moor 1983). In turn, the oocyte produces materials that influence CC function (Vanderhyden *et al.* 1990, 1992, Einspanier *et al.* 1997, Eppig *et al.* 1997). Until recently, all the investigations reported were with MGC but CC play an important role in oocyte maturation and deserve similar meticulous studies. We have therefore conducted a number of

studies using bovine, porcine, murine and human models (Xia *et al.* 1994, Armstrong *et al.* 1996, Khamsi & Armstrong 1997, Khamsi *et al.* 1999, 2001, Khamsi & Roberge 2000, 2001) which investigated the physiology and pathology of CC.

Initially, gonadotrophins and insulin-like growth factor-I (IGF-I) were investigated as major factors in follicular development. Although this area has been extensively reviewed (Adashi *et al.* 1985) we wished to use physiological circumstances in our current murine model to compare CC and MGC and their interactions with gonadotrophins and IGF-I. A problem encountered with parenteral administration of IGF-I is that it becomes bound to its circulating binding proteins and is essentially neutralized. We therefore used an analogue of IGF-I, LR³-IGF-I, which binds very little to IGF-I-binding proteins and therefore is free to simulate the role of IGF-I in a physiological fashion (Francis *et al.* 1992, Ballard *et al.* 1993).

Figure 1 Diagram of the experimental design of this study. Rats (24 days of age) were treated *in vivo* with eCG, LR³-IGF-I, a combination of eCG and LR³-IGF-I or saline only. CC and MGC were cultured with different concentrations of FSH or saline before being challenged with ³H-thymidine for measurement of replication. This experiment was repeated twice.

Materials and Methods

Animals and in vivo treatments

Immature Sprague–Dawley rats were purchased at 20 days of age (Charles River, St Constant, Canada) and were housed in an animal care facility, maintained on a 14 h light:10 h darkness cycle, and provided with food and water available *ad libitum* in accordance with guidelines on the handling of laboratory animals. A diagram of the experimental design is presented in Fig. 1.

At 24 days of age, rats were stratified by body weight into four groups with equal mean body weights. They were then randomized to receive continuous s.c. infusion of an IGF-I analogue, long Arg³-IGF-I (LR³-IGF-I; Gro-Pep Pty Ltd, Adelaide, Australia) at the rate of 1 mg/kg per day with Alzet mini-osmotic pumps (Alza Corp., Vacaville, CA, USA) or saline. Installation of the pump consisted of one ‘jab’ of a very fine needle into the subcutaneous tissue. The infusion rate was selected on the basis of previous dose–response studies in rats employing stimulation of growth and anabolic responses as end points of biological efficacy (Francis *et al.* 1992, Ballard *et al.* 1993). Infusion of LR³-IGF-I was continued until the rats were killed at 29 days of age.

At 27 days of age, rats were randomized to receive either an s.c. injection of 15 IU equine chorionic

gonadotrophin (eCG; Ayerst, Montreal, Canada) or saline only. The injection was given at 0900 h and the rats were killed (by decapitation) 48 h later which is the equivalent of pro-oestrus for eCG-treated rats.

Rats were weighed immediately before they were killed and their reproductive tracts removed under aseptic conditions. Ovaries of untreated or eCG-treated rats were dissected from the oviducts and adherent adipose and connective tissues, weighed and placed in HEPES-buffered tissue culture medium 199 (HTCM-199; Sigma, St Louis, MO, USA) for recovery of CC and MGC.

Table 1 Effects of different doses of FSH on ³H-thymidine incorporation on cultured CC and MGC of immature rats. The rats were treated *in vivo* with saline before being killed. Values are means ± S.E.M. (*n*=six per group)

FSH concentration (ng/ml)	³ H-Thymidine incorporation (dpm)	
	CC (dpm/CC)	MGC (dpm/10 ³ MGC)
0	603 ± 368	43 ± 4
2	1548 ± 326*	50 ± 5
10	1855 ± 341*	72 ± 6*
50	1053 ± 341	77 ± 4*

*Values are significantly (*P*<0.05) different from control (no FSH).

Figure 2 Thymidine incorporation of (a) OCC and (b) MGC from rats treated *in vivo* with eCG ($\blacktriangle$), LR³-IGF-I ($\blacksquare$), both ($\bullet$) or saline ($\times$). Cultured OCC were treated *in vitro* with different concentrations of FSH. Thymidine incorporation mean values are reported (a) per each OCC and (b) for 10³ MGC. Data are mean values $\pm$ S.E.M. for pooled data from two experiments, with six *in vitro* replicates per FSH concentration. Broken lines represent groups that received LR³-IGF-I (either LR³-IGF-I or eCG plus LR³-IGF-I). Asterisks indicate values significantly different from control (no IGF-I), within the same *in vivo* treatment ($P < 0.05$).

Cell culture and *in vitro* treatments

Surface follicles (approximately 30 follicles per rat) on the ovaries were punctured with a hypodermic needle and subsequently the ovaries were gently compressed with forceps and agitated in the culture medium to release oocyte cumulus complexes (OCC) and MGC. CC were the cells surrounding the oocyte which were adhered together and to the oocyte. MGC were the loose cells in the follicular fluid. CC had a 'shiny' appearance which was distinct from MGC. Pooled OCC from several rats in each *in vivo* treatment were rinsed through two successive washes in HTC-M-199 and a final rinse in bicarbonate-buffered medium (BT-CM-199). Ten to fifteen OCC were then transferred at random to each well of a 96-well tissue culture plate (Nalge Nunc International,

Table 2 Effects of different doses of FSH on ³H-thymidine incorporation on cultured CC and MGC of immature rats. The rats were treated *in vivo* with eCG before being killed. Values are means $\pm$ S.E.M. ($n =$ six per group)

FSH concentration (ng/ml)	³ H-Thymidine incorporation (dpm)	
	CC (dpm/CC)	MGC (dpm/10 ³ MGC)
0	797 $\pm$ 301	86 $\pm$ 4
2	1448 $\pm$ 48*	82 $\pm$ 2
10	2447 $\pm$ 399*	131 $\pm$ 9*
50	2315 $\pm$ 628*	126 $\pm$ 4*

*Values are significantly ($P < 0.05$) different from control (no FSH).

Napierville, IL, USA) containing BT-CM-199. OCC consisted of one oocyte and approximately 30 granulosa cells.

Pooled MGC from all rats of the same *in vivo* treatment were suspended and washed twice in HTC-M-199 and resuspended in BT-CM-199 after the second wash, with centrifugation at 400 *g* for 10 min after each wash. The number of MGC was counted with a haemocytometer, and their viability was assessed by trypan blue dye exclusion (Sigma). The final MGC suspension was delivered into wells of a 96-well tissue culture plate and the volume was adjusted to 0.25 ml to give final MGC concentrations of 4×10^5 viable cells/ml. Each culture well received ovine follicle-stimulating hormone (FSH; NIADDK-oFSH-17) at one of the following concentrations: 2, 10, 50 ng/ml or no hormone (control). After the addition of the *in vitro* treatments, CC and MGC were incubated for 20 h in a humidified atmosphere of 5% CO₂ in air at 37.5 °C in a water-jacketed tissue culture incubator (Forma Scientific, Marietta, OH, USA). At the end of the incubation period, 1 μ Ci ³H-thymidine (ICN Biochemicals, Irvine, CA, USA; 20 Ci/mmol) was added to each well and cultures were continued for a further 8 h. Cells were then harvested with a Skatron cell harvester (Fisher Scientific Ltd, Nepean, ON, Canada). Radioactivity not incorporated into DNA was washed off the OCC and MGC by two rinses with BT-CM-199. The filter disks containing the cells were air-dried, and incorporated radioactivity was counted with a liquid scintillation spectrometer for the measurement of ³H-thymidine incorporation. This was interpreted as an index of DNA synthesis.

In vitro dose-response with FSH was performed using six rats per *in vivo* treatment, and three *in vitro* replicates per FSH concentration. Progesterone secretion was utilized as an index of steroidogenic differentiation. Progesterone was measured by a specific radioimmunoassay of unextracted aliquots of culture medium removed immediately before the cells were harvested (Leung & Armstrong 1979).

Table 3 Effects of different doses of FSH on ^3H -thymidine incorporation on cultured CC and MGC of immature rats. The rats were treated *in vivo* with LR ^{3} -IGF-I before being killed. Values are means $\pm$ S.E.M. (n =six per group)

FSH concentration (ng/ml)	^3H -Thymidine incorporation (dpm)	
	CC (dpm/CC)	MGC (dpm/10 ^{3} MGC)
0	931 $\pm$ 175	39 $\pm$ 1
2	1031 $\pm$ 210	46 $\pm$ 6
10	1184 $\pm$ 56	80 $\pm$ 7*
50	891 $\pm$ 370	110 $\pm$ 2*

*Values are significantly ($P<0.05$) different from control (no FSH).

Table 4 Effects of different doses of FSH on ^3H -thymidine incorporation on cultured CC and MGC of immature rats. The rats were treated *in vivo* with eCG+LR ^{3} -IGF-I before being killed. Values are means $\pm$ S.E.M. (n =six per group)

FSH concentration (ng/ml)	^3H -Thymidine incorporation (dpm)	
	CC (dpm/CC)	MGC (dpm/10 ^{3} MGC)
0	408 $\pm$ 150	54 $\pm$ 5
2	1467 $\pm$ 194*	58 $\pm$ 6
10	1973 $\pm$ 602*	96 $\pm$ 2*
50	1358 $\pm$ 362*	83 $\pm$ 2*

*Values are significantly ($P<0.05$) different from control (no FSH).

Statistical analysis

Six rats were utilized for each *in vivo* treatment. The OCC were pooled from the six rats and divided into four groups for each of the four *in vitro* treatments (saline and three different doses of FSH). Each of the aliquots containing a specific dose of FSH was divided into three equal parts and the reading of ^3H -thymidine incorporation was obtained in three different samples. The whole experiment was repeated once more which yielded six readings for each dose group. The data were subjected to analysis of variance (ANOVA) which produced F statistics to compare variation attributed to specific treatment, e.g. LR ^{3} -IGF-I, eCG etc. with the calculation of random error. We show comparisons in the figures and tables.

Results

Cell replication

Dose-response curve to FSH comparing MGC with CC Table 1 and Fig. 2 show that the baseline ^3H -thymidine incorporation was more than ten times higher in CC than in MGC per cell. Both cell types

showed a response to FSH but the dose-response curves were different. With CC, it peaked at 10 ng/ml FSH whereas with MGC it continued to rise up to the highest dose of FSH tested (50 ng/ml).

Dose-response curve to FSH with prior treatment with eCG Table 2 and Fig. 2 demonstrate that prior exposure to eCG increased the rate of ^3H -thymidine incorporation at all dose levels of FSH in MGC and most dose levels of FSH in CC. The response curve was different between CC and MGC. With both cell types the dose-response curves flattened after the dose of 10 ng/ml FSH.

Dose-response curve to FSH with prior treatment with LR ^{3} -IGF-I Table 3 and Fig. 2 demonstrate that prior exposure to IGF-I analogue resulted in quite different responses to FSH *in vitro* of CC compared with MGC. There was an apparent increase in the MGC, whereas in the CC there was a reduction in ^3H -thymidine incorporation. In the MGC there was a stimulatory effect of the IGF-I analogue which appeared to act synergistically with *in vitro* FSH. In the CC there was a reduction in cell replication at all dose levels of FSH, i.e. IGF-I analogue *in vivo* appeared to act antagonistically with *in vitro* FSH exposure.

Dose-response curve to FSH with prior exposure to LR ^{3} -IGF-I and eCG Table 4 and Fig. 2, when compared with Table 1, show a more modest effect of combined LR ^{3} -IGF-I and eCG compared with eCG alone on MGC replication. This combination also appeared to have a more modest effect than eCG on CC. Looking at the curves, LR ^{3} -IGF-I plus eCG appeared to reduce the stimulating effect of eCG on CC or MGC proliferation. *In vivo* exposure to the IGF-I analogue appeared to act antagonistically with respect to gonadotrophin (eCG) also administered *in vivo*. The curves of eCG alone or eCG plus IGF-I analogue are fairly similar but it is of note that after a dose of 10 ng/ml FSH the response declined. It appears that higher doses of FSH *in vitro* have a further antagonistic interaction with IGF-I analogue given *in vivo*.

Progesterone secretion

Progesterone secretion by CC The results are shown in Fig. 3b. The raw means for CC, displayed for all treatment groups and dosages of FSH, indicated some strong patterns of difference among the three factors (Table 5). ANOVA (Table 6) indicated significant differences among many of the contrasts tested. In particular, the interactions between the dose of FSH compared with eCG or IGF-I were significant at the $P<0.001$ level. In the presence of an interaction between all three factors, eCG, LR ^{3} -IGF-I and dose of FSH, one cannot make

Figure 3 Progesterone secretion of (a) MGC and (b) OCC from rats treated *in vivo* with eCG (■), LR³-IGF-I (◆), both (▲) or saline (●). Cultured CC were treated *in vitro* with different concentrations of FSH. Progesterone secretion mean values are reported (a) for 10³ MGC or (b) per each OCC. Data are mean values $\pm$ S.E.M. for pooled data from two experiments, with six *in vitro* replicates per FSH concentration.

simple statements about the main effect of any factor. However, the following deductions can be made.

1. Levels of progesterone increased with dose of FSH; the rate of increase was linear with log dose of FSH.
2. Rates of increase were consistently higher when eCG was present than when it was absent (Table 7).
3. The effect of LR³-IGF-I on the slope when eCG was absent was negative; the slope was lower when LR³-IGF-I

was present than when it was absent. The effect of LR³-IGF-I on the slope when eCG was present was positive; the slope was higher when LR³-IGF-I was present than when it was absent.

Progesterone secretion by MGC The results are shown in Fig. 3a. The table of means for MGC cells also indicated strong patterns for eCG, LR³-IGF-I and dose

Table 5 Table of means for CC

Group	Dose of FSH (ng/ml)				Mean square error	Total error
	0	2	10	50		
eCG	137·00	226·00	430·83	536·33	332·54	S ² =408·1
IGF-I	123·83	133·17	177·17	316·67	187·71	S=20·2
Combination	147·17	278·00	476·50	596·33	374·50	
Control	69·17	138·83	201·17	336·00	186·29	
Total	119·29	194·00	321·42	446·33	270·26	

S, square root of mean; S², mean square of the error.

of FSH (Table 8). In this instance, however, ANOVA (Table 9) indicated no statistically significant interactions except with dose of FSH. The term dose, eCG and IGF-I were not significant, indicating that changes in progesterone secretion for different levels of dose were less complex for MGC than for CC. Also the term eCG or IGF-I were not significant ($P=0\cdot057$), suggesting that these factors do not modify each other's effects. The following general statements can therefore be made.

1. Increasing doses of FSH increased the level of progesterone secretion in each of the four combinations of treatments (Table 9). There was a strong linear component to this change. There was also evidence of significant departure from linearity for all but the eCG group.

2. The presence of eCG was associated with a consistently higher slope.

3. The presence of LR³-IGF-I was also associated with a consistently higher slope. The effect of LR³-IGF-I was, however, less dramatic than the effect of eCG. The magnitude of these effects could be estimated from the table of means since no interactions have been demonstrated (Table 10).

Discussion

Innate replication and progesterone secretion of OCC compared with MGC

The first obvious finding was that cell replication, as judged by ³H-thymidine incorporation, was tenfold higher in CC compared with MGC (comparing Fig. 2*a* with *b*). This phenomenon was in agreement with results of radioautographic studies in rats, in which ³H-thymidine

Table 6 ANOVA table for CC. Number of observations=96; R-squared=0·9900; root MSE=20·2023; adjusted R-squared=0·9842

Source	Partial SS	df	MS	F	P
Main					
ID	18 669·9271	5	3733·98542	2·86	0·0524
eCG	665 500·51	1	665 500·51	1630·59	<0·001
IGF	11 288·3438	1	11 288·3438	27·66	<0·001
eCG*IGF	9861·76042	1	9861·76042	24·16	<0·001
Error (A)	19 602·1979	15	1306·81319		
Subplots					
Dose	1 493 420·36	3	497 806·788	1219·71	<0·001
Dose*eCG	193 064·031	3	64 354·6771	157·68	<0·001
Dose*IGF	1421·69792	3	473·899306	1·16	0·3321
Dose*IGF*eCG	14 817·6146	3	4939·20486	12·10	<0·001
Error (B)	24 488·0417	60	408·134028		
Total	2 452 134·49	95	25 811·942		

MSE, mean square of error; ID, control; eCG, equine chorionic gonadotrophin; SS, sum of squares; MS, mean sum of squares. The errors represent the SS for the error in each of the ANOVAs.

Table 7 Table of slopes for CC. Rate of change per fivefold increase in dose of FSH

	eCG	
	Absent	Present
LR³-IGF-I		
Absent	98.6	155.2
Present	91.8	159.2

incorporation in antral follicles was much higher in CC than in MGC (Takaoka *et al.* 1985, Hirshfield 1986).

The same difference between CC and MGC was seen in progesterone secretion when CC (per cell) secreted about sixfold higher levels of progesterone than MGC. Were these differences exclusively related to the different cells or were the CC influenced by the oocyte? We are

Table 8 Table of means for MGC

Group	Dose of FSH (ng/ml)				Mean square error	Total error
	0	2	10	50		
eCG	31.17	34.50	82.50	126.00	68.54	S ² =56.30
IGF-I	20.67	28.33	48.33	92.83	47.54	S=7.50
Combination	35.83	48.00	97.50	166.17	86.88	
Control	13.38	20.50	34.83	71.50	35.17	
Total	25.38	32.83	65.79	114.12	59.53	

Table 9 ANOVA table for MGC. Number of observations=96; R-squared=0.9805; root MSE=7.50375; R-squared=0.9692

Source	Partial SS	df	MS	F	P
Main					
ID	792.59375	5	158.51875	1.44	0.2668
eCG	31 719.0104	1	31 719.0104	563.33	0.001
IGF	5658.01042	1	5658.01042	100.49	0.001
eCG*IGF	213.010417	1	213.010417	3.78	0.0565
Error (A)	1651.53125	15	110.102083		
Subplots					
Dose	117 578.365	3	39 192.7882	696.06	0.001
Dose*eCG	10 142.6146	3	3380.87153	60.04	0.001
Dose*IGF	2114.78125	3	704.927083	12.52	0.001
Dose*IGF*eCG	377.614583	3	125.871528	2.24	0.0933
Error (B)	3378.375	60	56.30625		
Total	173 625.906	95	1827.64112		

For definitions of terms see Table 6.

Table 10 Table of slopes for MGC. Rate of change per fivefold increase in dose of FSH

	eCG	
	Absent	Present
LR³-IGF-I		
Absent	25.5	45.8
Present	32.3	59.1

undertaking further studies removing the oocytes from OCC to investigate the possibilities.

The difference between OCC compared with MGC in their response to in vivo LR³-IGF-I treatment

There was a difference as judged by ³H-thymidine incorporation or progesterone secretion. LR³-IGF-I given *in vivo* increased ³H-thymidine incorporation and progesterone secretion in MGC whereas it had a reverse effect

with OCC. One possible explanation may be that LR³-IGF-I loosens the connection of CC from the oocyte and therefore results in this reverse effect.

Shape of the response curves

It can be seen that CC and MGC had different response curves to FSH. For example, the effect of FSH peaked at the 10 ng/ml dose for CC whereas it continued to climb up to the 50 ng/ml dose for MGC, as judged by ³H-thymidine incorporation.

Interaction of LR³-IGF-I and eCG

Progesterone secretion data showed that these two factors had an additive effect in MGC compared with a synergistic effect in CC. When the ³H-thymidine incorporation data were reviewed (Fig. 2) it could be seen that for both CC and MGC, LR³-IGF-I had a negative effect on eCG stimulation. What can be the explanation for the difference in cell replication compared with progesterone secretion (an indicator of maturation) in the interaction of LR³-IGF-I and eCG? It is conceivable that IGF-I encourages cell maturation in eCG-treated animals and that it therefore causes reduced cell replication but increased progesterone secretion.

In conclusion, a rather complex picture of the relationship between gonadotrophins (eCG) and IGF-I (LR³-IGF-I) is emerging. These two chemicals may act synergistically (progesterone secretion by CC), additively (progesterone secretion by MGC) or antagonistically (cell replication of CC and MGC).

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References

- Adashi EY, Resnick CE, D'Ercole AJ, Svoboda ME & Van Wyk JJ 1985 Insulin-like growth factors as intraovarian regulators of granulosa cell growth and function. *Endocrine Reviews* **6** 400–420.
- Armstrong DT, Xia P, de Gannes G, Tekpetey FR & Khamsi F 1996 Differential effects of insulin-like growth factor-I and follicle-stimulating hormone on proliferation and differentiation of bovine cumulus cells and granulosa cells. *Biology of Reproduction* **54** 331–338.
- Ballard FJ, Francis GL, Walton PE, Knowles SE, Owens PC, Read LC & Tomas FM 1993 Modification of animal growth with growth hormone and insulin-like growth factors. *Australian Journal of Agricultural Sciences* **44** 567–577.
- Brower PT & Schultz RM 1982 Intercellular communication between granulosa cells and mouse oocytes: existence and possible role during oocyte growth. *Developmental Biology* **90** 144–153.
- Einspanier R, Lauer B, Gabler C, Kamhuber M & Schams D 1997 Egg–cumulus–oviduct interactions and fertilization. *Advances in Experimental Medicine and Biology* **44** 279–289.
- Eppig JJ, Chesnel F, Hirao Y, O'Brein MJ, Pendola FL, Watanabe S & Wigglesworth K 1997 Oocyte control of granulosa cell development: how and why. *Human Reproduction* **12** 127–132.
- Francis GL, Ross M, Ballard FJ, Milner SJ, Senn C, McNeil KM, Wallace JC, King R & Wells JRE 1992 Novel recombinant fusion protein analogues of insulin-like growth factor (IGF)-I indicate the relative importance of IGF-binding protein and receptor binding for enhanced biological potency. *Journal of Molecular Endocrinology* **8** 213–223.
- Greenwald GS & Terranova PF 1988 Follicular selection and its control. In *The Physiology of Reproduction*, vol 1, pp 387–445. Eds E Knobil & J Neill. New York: Raven Press.
- Hirshfield AN 1986 Patterns of ³H thymidine incorporation differ in immature rats and mature cycling rats. *Biology of Reproduction* **34** 229–235.
- Khamsi F & Armstrong DT 1997 Interactions between follicle-stimulating hormone and growth factors in regulation of deoxyribonucleic acid synthesis in bovine granulosa cells. *Biology of Reproduction* **57** 684–688.
- Khamsi F & Roberge S 2000 Novel studies on influence of gonadotrophins and insulin-like growth factor-I on growth of cumulus oophorus in the rat. *Endocrine* **12** 41–45.
- Khamsi F & Roberge S 2001 Differential effects of insulin-like growth factor-I (IGF-I) and gonadotrophins on the proliferative activity of two subgroups of granulosa cells: cumulus oophorus and mural granulosa cells. *Fertility and Sterility* **75** 997–1002.
- Khamsi F, Roberge S, Lacanna I C, Wong J & Yavas Y 1999 Effects of granulosa cells, cumulus cells, and oocyte density on *in vitro* fertilization in women. *Endocrine* **10** 161–166.
- Khamsi F, Roberge S & Wong J 2001 Novel demonstration of a physiological/pharmacological role of insulin like growth factor-1 (IGF1) in ovulation in rats and action on cumulus oophorus. *Endocrine* **14** 175–180.
- Leung PCK & Armstrong DT 1979 Estrogen treatment of immature rats inhibits ovarian androgen production *in vitro*. *Endocrinology* **104** 1411–1417.
- Moor RM 1983 Contact, signalling and cooperation between follicle cells and dictyate oocytes in mammals. In *Current Problems in Germ Cell Differentiation*, pp 307–324. Eds A McLaren & CC Wylie. Cambridge: University Press.
- Takaoka H, Satoh H, Makinoda S, Moriya S & Ichinoe K 1985 Granulosa cells growth factor in oocyte and its transport system. *Acta Obstetrica et Gynaecologica Japonica* **37** 92–98.
- Vanderhyden BC, Caron PJ, Buccione R & Eppig JJ 1990 Developmental pattern of the secretion of cumulus-expansion enabling factor by mouse oocytes and the role of oocytes in promoting granulosa cell differentiation. *Developmental Biology* **140** 307–317.

- Vanderhyden BC, Telfer EE & Eppig JJ 1992 Mouse oocytes promote proliferation of granulosa cells from preantral and antral follicles *in vitro*. *Biology of Reproduction* **46** 1196–1204.
- Xia P, Tekpetey FR & Armstrong DT 1994 Effect of IGF-I on pig oocyte maturation, fertilization rate and early embryonic development *in vitro*, and on granulosa and cumulus cell

biosynthetic activity. *Molecular Reproduction and Development* **38** 373–379.

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