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Leptin Enhances Oocyte Nuclear and Cytoplasmic Maturation via the Mitogen-Activated Protein Kinase Pathway

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Recent studies have suggested that leptin has a central role in female reproduction, including ovarian function. The leptin receptor (Ob-R) has six isoforms and can signal through either the MAPK or the Janus-activated kinase/signal transducer and activator of transcription signal-transduction pathway, depending on the isoform. Expression of Ob-R has been reported in human and mouse oocytes; however, the physiological role of leptin during follicular development and oocyte maturation is largely unknown. In the current study, expression of Ob-R during oocyte growth and maturation was investigated in porcine oocytes from small, medium, and large follicles and in oocytes in the germinal vesicle (GV), GV breakdown, and metaphase II (MII) stages at both the mRNA and protein levels. The proportion of oocytes expressing Ob-R was maximal in oocytes from medium follicles and at the GV breakdown stage ($P < 0.05$), whereas the proportion of oocytes expressing the long isoform, Ob-Rb, was found to be consistently low throughout growth and maturation. When included in oocyte maturation medium, leptin significantly increased the proportion of oocytes reaching MII ($P < 0.01$), elevated cyclin B1 protein content in MII-stage oocytes ($P < 0.05$), and enhanced embryo developmental potential ($P < 0.05$), suggesting that leptin plays a role in both nuclear and cytoplasmic maturation. During oocyte maturation, leptin increased phosphorylated MAPK content by 2.8-fold ($P < 0.05$), and leptin-stimulated oocyte maturation was blocked when leptin-induced MAPK phosphorylation was suppressed by a specific MAPK activation inhibitor, U0126 ($P < 0.01$), demonstrating that leptin enhances nuclear maturation via activation of the MAPK pathway. (*Endocrinology* 145: 5355–5363, 2004)

LEPTIN IS A 16-kDa peptide hormone that is secreted mainly from adipose tissue and plays an integral role in the regulation of body weight and energy expenditure (1–3). Recent evidence suggests that leptin is also an important signal in female reproduction, including control of ovarian function (reviewed in Ref. 4). Mice lacking either leptin (ob/ob) or the leptin receptor (db/db) are both obese and infertile (5, 6). Treatment of ob/ob mice with exogenous leptin restores their fertility; however, diet restriction alone has no effect (6, 7), suggesting that the influence of leptin on reproduction is a direct action.

The leptin receptor (Ob-R), product of the diabetes (db) gene, is a member of the class I cytokine receptor superfamily, with six known isoforms (8). Ob-R expression has been observed in many tissues, including the hypothalamus and peripheral tissues including the ovary (reviewed in Ref. 4). Of the known isoforms, only the full-length form (Ob-Rb) contains the intracellular domains necessary to mediate signal transduction through both the signal transducer and activator of transcription 3 (STAT3) pathway (9, 10) and the MAPK pathway (10). Lacking the binding site for STAT3 because of truncated intracellular domains, the short isoforms (Ob-Rs) can signal only through the MAPK pathway (10). Recently, it has been suggested that leptin signaling through the STAT3 pathway may be less important in reproduction because mice with disrupted Ob-Rb STAT3 signaling are obese yet fertile (11).

A significant body of work has shown that Ob-R is expressed in the ovary. Leptin receptor transcripts are present in both human and rat granulosa and theca cells (12–15), as well as in porcine granulosa and theca cells (16). Ob-Ra and Ob-Rb levels in the ovary vary during the estrous cycle (17), and it has recently been demonstrated that expression of Ob-Ra and Ob-Rb in the rat ovary changes in response to human chorionic gonadotropin administration and peaks at ovulation, suggesting a possible role of leptin in ovarian function at this point (15).

The presence of Ob-R mRNA (18, 19) and protein (15, 18, 20, 21) has been detected in mouse, rat, and human oocytes, suggesting that the oocyte may be capable of responding to leptin. Leptin protein has been detected in the oocyte by immunofluorescence (20, 21); however, others have been unable to detect its mRNA transcript in the oocyte by nested RT-PCR (18, 19, 21), suggesting it may be produced elsewhere and transported into the oocyte. In the human ovary, leptin is produced by ovarian somatic cells (14, 22) and is present in follicular fluid at concentrations similar to serum (12–14), making leptin spatially available for the oocyte. Although leptin has been shown to increase the rate of meiotic resumption in preovulatory follicle-enclosed oocytes, presumably via indirect actions on the theca (21), its role in

Abbreviations: COC, Cumulus-oocyte complex; GV, germinal vesicle; GVBD, GV breakdown; HPRT, hypoxanthine phosphoribosyltransferase; IVM, *in vitro* maturation; MEK, MAPK kinase; MII, metaphase II; MPF, maturation promoting factor; Ob, leptin; Ob-R, leptin receptor; STAT, signal transducer and activator of transcription; TCM, tissue culture medium.

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oocyte maturation and the signal-transduction pathways involved are still unclear.

Materials and Methods

Oocyte collection and in vitro maturation (IVM)

Porcine ovaries were collected from gilts weighing between 100 and 150 kg at a local slaughterhouse and transported to the laboratory in 1× PBS (37 C). Cumulus-oocyte complexes (COCs) were aspirated from small (1–2 mm in diameter), medium (2–3 mm), and large (>3 mm) follicles, and washed three times in PBS + 1× antibiotics (penicillin G sodium, streptomycin sulfate, and amphotericin B; Invitrogen, Burlington, Ontario, Canada). Oocytes from large follicles were used for subsequent investigation of oocyte nuclear and cytoplasmic maturation. COCs were removed from the follicular fluid, and granulosa cells were collected by centrifuging the aspirated follicular fluid. Cell pellets were stored at −80 C until RNA isolation. Immature oocytes in the germinal vesicle (GV) stage (intact nucleus) were denuded and collected immediately after aspiration, whereas oocytes in the germinal vesicle breakdown (GVBD) and metaphase II (MII) stages were collected after 24 and 48 h IVM, respectively. Oocytes were matured in tissue culture medium (TCM) 199 supplemented with 5 IU/ml FSH (Sioux Biochemicals, Sioux City, IA), 5 IU/ml LH (Sioux Biochemicals), 0.1 mg/ml cysteine (Sigma Chemical Co., St. Louis, MO), and 10 ng/ml epidermal growth factor (Sigma) at 38.5 C with 5% CO_2 and 95% air. After maturation, cumulus cells were removed from the oocytes with 0.1% hyaluronidase and washed three times. Cumulus cells were collected by centrifuging and stored at −80 C until RNA isolation.

Assessment of oocyte nuclear maturation

Oocytes were collected after 24 and 48 h IVM in the presence (10, 100, 1000 ng/ml) or absence of human recombinant leptin (Sigma), and nuclear maturation status was determined. At 24 h, cumulus cells were removed by 0.1% hyaluronidase and washed in 1× PBS plus 0.1% polyvinyl alcohol. Oocytes were incubated with 1 μg/ml Hoechst 33342 for 1 h at 38.5 C, mounted on glass slides with fluorescent mounting medium (DakoCytomation, Carpinteria, CA), and examined under UV light for intact nucleus (GV) or GVBD. MII-arrested oocytes were identified after 48 h IVM based on the presence of the first polar body under a phase-contrast microscope.

Single-cell RT-PCR and nested PCR

Single oocytes were lysed in 10 μl lysis buffer containing 2 U/μl porcine RNase Inhibitor (Amersham, Piscataway, NJ) and 5 mM dithiothreitol (Invitrogen) by boiling followed by vortexing for 1 min each. One-step RT-PCR was performed in a total volume of 25 μl containing 10 μl of cell lysate, 1× RT-PCR buffer, 200 U Moloney murine leukemia virus reverse transcriptase; Invitrogen), 2.5 μM random hexamer primers (Applied Biosystems, Foster City, CA), 0.2 mM deoxynucleotide triphosphate (Invitrogen), and 0.05 U/μl *Taq* polymerase (Invitrogen). The RT reaction was carried out at 25 C for 10 min and 37 C for 50 min, followed by 15 min at 75 C to inactivate the Moloney murine leukemia virus.

After RT, the resulting cDNA was split into two aliquots, and 0.3 μM of the respective primers for Ob-R, Ob-Rb, and hypoxanthine phosphoribosyl-transferase (HPRT) were added (Table 1). All other components necessary for PCR were added during reverse transcription. Thermocycling parameters for Ob-R and Ob-Rb were as follows: 95 C for 2 min and then 35 cycles consisting of 94 C for 20 sec, 55 C for 30 sec, and 72 C for 40 sec, followed by an extension of 72 C for 10 min. Conditions for HPRT amplification were as follows: 95 C for 2 min and then 30 cycles consisting of 94 C for 30 sec, 52 C for 30 sec, and 72 C for 30 sec, followed by an extension of 10 min at 72 C.

Nested PCR was performed in a mix containing 1× PCR buffer; 1.5 mM $MgCl_2$; 0.1 μM deoxynucleotide triphosphate; 0.2 μM each of the nested primers for Ob-R, Ob-Rb, and HPRT; and 0.05 U/μl *Taq* polymerase. For Ob-R, 2.5 μl of a 1:500 dilution of primary PCR product was added to a total volume of 25 μl PCR mix. For Ob-Rb and HPRT, 2.5 μl of primary product was added. Thermocycling conditions were the same as for the primary reactions.

Total RNA was isolated from oocytes and from granulosa and cumulus cells using the RNAeasy Mini kit (QIAGEN, Valencia, CA), whereas RNA was isolated from ovarian tissue using the Trizol reagent (Invitrogen). Two-step RT-PCR was performed as above with the modification that *Taq* polymerase was not included in the reverse transcription reaction. Nested PCR was performed as described above. The expression of HPRT was used to confirm the presence of RNA in each oocyte sample.

Parthenogenetic embryo activation and culture

After 44–48 h IVM of oocytes from antral follicles more than 3 mm in diameter, cumulus cells were removed from COCs by gentle pipetting in TCM199 supplemented with 0.1% hyaluronidase. Maturation of oocytes to MII was confirmed visually by the presence of the first polar body. MII oocytes were washed three times in TCM199 containing 10% FBS, placed between two platinum electrodes (1 mm apart), and overlaid with 1 ml of fusion medium consisting of 297 mM mannitol (Calbiochem, Mississauga, Ontario, Canada), 0.001 mM $CaCl_2$ (Sigma), 0.5 mM $MgCl_2$ (Sigma), and 0.1% BSA (fraction V, Sigma). They were then subjected to two pulses at 2.6 kV, generated by a BTX ElectroCell 2001 manipulator (BTX Instrument Division, Holliston, MA). After activation, embryos were washed twice with TCM199-HEPES medium and cultured in North Carolina State University 23 medium, supplemented with 4 mg/ml BSA, and overlaid with light mineral oil. Embryos were cultured for 7 d to evaluate preimplantation embryo development (d 2 cleavage rate, d 7 blastocyst rate). Day 7 blastocysts were stained with 1 μg/ml Hoechst 33342 to visualize cell nuclei. The total cell number of each blastocyst was counted under UV light.

TABLE 1. Primers used for PCR and nested PCR for detection of leptin, Ob-R, Ob-Rb, hypoxanthine phosphoribosyltransferase (HPRT), and histone H2A

Transcript	PCR round		Primer sequence (5′–3′)	Product size (bp)	Primer location	GenBank accession no.
Leptin	1	Sense	ttg gcc cta tct gtc cta cg	277	Exon 1	AF102856
		Antisense	gag gtt ctc cag gtc att cg		Exon 3	
	2	Sense	caa gac gat tgt cac cag ga	184	Exon 1	
		Antisense	ttg gat cac att tct gga agg		Exon 2	
Ob-R	1	Sense	tgt gat aac tgc att tga ctt gg	356	Exon 4	AF092422
		Antisense	tcc tct ttc atc cag cac tgt		Exon 5	
	2	Sense	ctc ttg cct gct gga atc tc	250	Exon 4	
		Antisense	ttc cag ttt gca cct gtt tg		Exon 5	
Ob-Rb	1	Sense	aag atg ttc caa acc cca ag	312	Exon 19	
		Antisense	cct cac gag tta tct cca tgc		Exon 20	
	2	Sense	gaa ctg ttc ctg ggc aca ag	215	Exon 19	
		Antisense	ttt caa ggt ccg gag ttg tc		Exon 20	
HPRT	1	Sense	cca gtc aac ggg cga tat aa	130	Exon 4	U69731
		Antisense	ctt gac caa gga aag caa gg		Exon 6	

Leptin treatment

For detection of phosphorylated MAPK, groups of 50 denuded GV, GVBD, and MII oocytes, as well as GV oocytes from small, medium, and large follicles, were incubated in the presence (10 ng/ml) or absence of recombinant human leptin (Sigma) diluted in TCM199 at 38.5 C for 30 min. Oocytes were collected and frozen at −80 C until use.

For detection of cyclin B1, COCs were matured in the presence (100 ng/ml) and absence of leptin for 48 h. Surrounding cumulus cells were stripped from the oocytes with 0.1% hyaluronidase. The oocytes were washed three times, collected, and stored at −80 C until Western blotting.

U0126 treatment

Oocytes were cultured as COCs for 24 h to GVBD in maturation medium described above. Cumulus cells were then removed from the oocytes, which were washed three times in 1× PBS to remove any contaminating cells. Groups of 50 denuded oocytes were incubated in the presence of leptin (10 ng/ml) and varying concentrations of U0126 (0, 1, 2.5, 5, and 10 μM) diluted in TCM199 at 39 C for 30 min. U0126 is a specific inhibitor of MAPK kinase (MEK) 1/2 activation and activity, which prevents MAPK activation. After challenge with leptin and U0126, oocytes were collected and frozen at −80 C until use for Western blotting.

For investigation of blockage of leptin-induced increases in phosphorylated MAPK protein, denuded oocytes were cultured in the presence of leptin (10 ng/ml) and U0126 (1, 2.5, 5, and 10 μM) for 48 h, and the proportion of oocytes reaching MII was determined on the basis of the presence of the first polar body under a phase-contrast microscope.

Western blotting

Protein was isolated from oocytes (50 for MAPK, 200 for cyclin B1) in a lysis buffer [50 mM Tris (pH 7.5); 150 mM NaCl; 1 mM EDTA; 0.5% Nonidet P-40; 1 mM sodium orthovanadate; 1 mM NaF; 0.75 mM phenylmethylsulfonylfluoride; 15% glycerol; and 10 μg/ml each of aprotinin, pepstatin, and leupeptin] as described (18). Samples were boiled for 3 min and centrifuged for 3 min at 13,000 rpm. The supernatant containing the soluble protein was subjected to 10% SDS-PAGE. Proteins were transferred to polyvinylidine difluoride membranes that were blocked overnight in 5% skim milk at 4 C before antibody detection. Membranes were incubated with primary antibodies (rabbit antiphosphorylated MAPK, Cell Signaling Technology, Beverly, MA; mouse anti-cyclin B1, PharMingen, San Diego, CA) diluted in 1× PBS, 0.1% Tween 20, and 5% skim milk powder for 1 h at room temperature, followed by incubation with secondary antibody (for MAPK, antirabbit IgG horseradish peroxidase; for cyclin B1, antimouse IgG horseradish peroxidase; Cell Signaling Technology) for 1 h at room temperature. Proteins were detected by the enhanced chemiluminescence Advance Western Blotting Detection Kit (Amersham). For subsequent protein detection, primary and secondary antibodies were stripped from the membrane in a stripping buffer containing 62.5 mM Tris-HCl (pH 6.7), 2% SDS, and 100 mM β-mercaptoethanol at 50 C for 30 min. Membrane blocking and antibody incubation was repeated as above with rabbit anti-MAPK (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) for normalization of protein loading. Densitomitry of band intensities was performed using GeneTools (Syngene, Frederick, MD).

Immunocytochemistry

Oocytes were fixed in 4% paraformaldehyde in PBS for 30 min, followed by permiabilization in 0.1% Tween 20 for 10 min and 0.1% Triton X-100 for 20 min. Oocytes were then blocked in PBS supplemented with 1% skim milk and 5% goat serum. Primary antibody (1:100; rabbit anti-Ob-R and rabbit antileptin, Santa Cruz Biotechnology) was incubated with the blocked oocytes overnight at 4 C, followed by goat antirabbit fluorescein isothiocyanate secondary antibody incubation (1:500; Sigma) for 1 h at room temperature. Oocytes were counterstained with 4′,6-diamidino-2-phenylindole and mounted on glass slides with fluorescent mounting medium (DakoCytomation). Fluorescence was examined using an Olympus (Melville, NY) BX-UCB microscope and MetaMorph image-analysis software (Universal Imaging Corp., Downingtown, PA). Using integrated metamorphometric analysis (MetaMorph), the average area of the oocyte that stained positive was measured for each oocyte and was compared between oocytes from different-sized follicles and at different maturation stages.

Leptin RIA

For RIA, follicular fluid was collected during aspiration of ovarian follicles. Fluid was pooled from multiple ovaries and centrifuged to remove contaminating cells and debris. The RIA was performed by Linco Diagnostics (St. Charles, MO), using their multispecies leptin RIA kit. The presence of leptin was detected by using radiolabeled human leptin as a tracer and an antihuman leptin antibody. The specificity of this antibody for porcine leptin is 67%.

Statistical analysis

Differences in the number of oocytes expressing Ob-R or Ob-Rb were detected using Fisher's exact test with a Bonferroni correction. Western blot data were analyzed by ANOVA (MAPK) or *t* test (cyclin B). The number of oocytes reaching MII and the cleavage, blastocyst, and total cell numbers of blastocysts were analyzed by ANOVA. Significant results from ANOVA were further analyzed by Tukey test. Results were considered significant at $P < 0.05$ for all tests. Each experiment was repeated at least three times, and data represent the average of all repeats.

Results

Oocyte leptin receptor expression is dependent on follicular development and maturation

Alternate splicing at the 3′ end of the mRNA is responsible for producing the different isoforms of the leptin receptor. Each differentially spliced mRNA has a unique 3′-terminal coding sequence, whereas the common extracellular region for all membrane-bound subtypes is the product of exons 1–15 (23). To study the expression of Ob-R in single porcine oocytes at varying stages of follicular development and oocyte maturation via RT-PCR and nested PCR, primers were designed on the basis of exons 4 and 5 of the receptors (Table 1). Ob-R mRNA was detectable in oocytes during follicular development and oocyte maturation (Fig. 1). During follicular development, there was no difference in the percentage of oocytes expressing Ob-R in small or large follicles (11.5 and 7.7%, respectively), whereas the proportion of oocytes expressing Ob-R was significantly increased in oocytes from medium follicles (38.5%, $P < 0.05$). During IVM of oocytes from large follicles, Ob-R was expressed in a significantly higher percentage of GVBD-stage oocytes (45%, $P < 0.05$) than in GV- or MII-stage oocytes (7.7 and 11.8%, respectively; Fig. 1B). To study whether the leptin receptor protein is present in porcine oocytes, immunocytochemistry was performed (Fig. 2A). Leptin receptor was present in the oocyte, irrespective of follicle size and oocyte maturation stage. Similar to the trends observed at the mRNA level, Ob-R protein was suggested to be increased in oocytes from medium follicles and at GVBD, as shown by the average area stained immunopositive in each oocyte (Fig. 2, C and D).

We next investigated Ob-Rb expression in the oocyte, and whether the proportion of oocytes expressing Ob-Rb was also dependent on follicular development and oocyte maturation, using primers specific to exons 19 and 20 of the receptor. These exons are only present in the b isoform (23). During follicular development, Ob-Rb transcripts were not detected in oocytes from small or medium follicles (Fig. 1A).

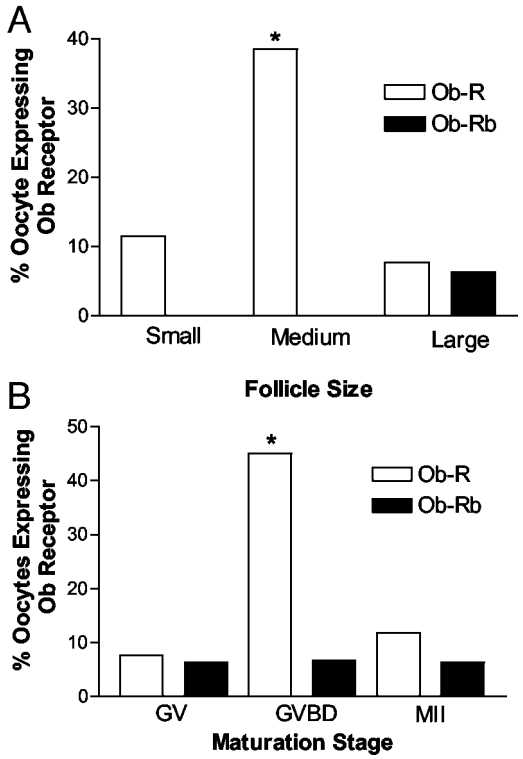

FIG. 1. Expression of Ob-R and Ob-Rb in individual oocytes during follicular development (A) and oocyte maturation (B). Total RNA was isolated from individual denuded oocytes, and nested RT-PCR was performed to detect total Ob-R (*open bars*) and the b isoform (Ob-Rb; *solid bars*). A total of 15–26 oocytes from each group was examined individually. *, $P < 0.05$.

Expression was low in oocytes from large follicles (6.3%; Fig. 1A) and did not change significantly during oocyte maturation: 6.3% at GV, 6.7% at GVBD, and 6.3% at MII (Fig. 1B).

Leptin is expressed in the porcine oocyte and is present in follicular fluid

To study whether leptin is spatially available to influence oocyte function, expression of leptin in the porcine ovary was investigated. Using total RNA extracted from porcine ovaries, nested RT-PCR revealed the presence of leptin mRNA transcripts. Further study demonstrated that leptin mRNA was also detectable in the oocyte via nested RT-PCR at all stages of follicular development and oocyte maturation investigated (Fig. 3), although leptin mRNA was not detectable from cumulus or granulosa cells (data not shown). In addition to being expressed in the oocyte at the transcriptional level, leptin protein was also detected in the oocyte (Fig. 3B) and in follicular fluid. Follicular fluid from small, medium, and large follicles was tested for the presence of leptin using a RIA. Leptin was detected in follicular fluid pooled from each size of follicle, as follows: small follicles, 1.21 ± 0.28 ng/ml; medium follicles, 1.24 ± 0.06 ng/ml; and large follicles, 1.13 ± 0.24 ng/ml. Given that the human leptin antibody used in the RIA is 67% specific for porcine leptin and will recognize approximately 67% of leptin in the sample, the level shown here could be a slight underestimation of the actual leptin level in porcine follicular fluid.

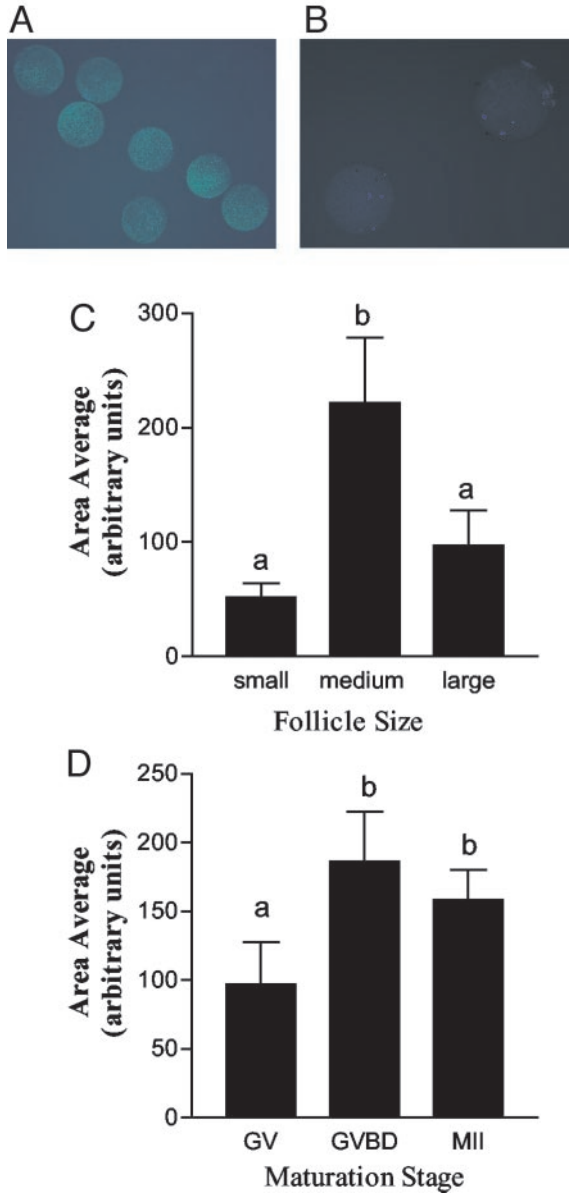

FIG. 2. Detection of Ob-R protein in individual denuded oocytes via immunocytochemistry. Images were obtained using an Olympus BX-UCB microscope, and the average area of immunofluorescence was determined using MetaMorph 5.0 (Universal Imaging Corp.). A, Representative Ob-R detection in individual oocytes. B, Immunocytochemistry-negative control in which antibody against Ob-R was omitted from the assay. C and D, Relative Ob-R protein in oocytes from small, medium, and large follicles (C) and during oocyte maturation (D). Values are mean ± SEM of observations of more than 30 oocytes per group. *a* and *b* indicate significant differences ($P < 0.05$).

Influence of leptin on oocyte nuclear maturation

To determine whether leptin plays a role in oocyte maturation *in vitro*, COCs were cultured in maturation medium with the addition of leptin at 0, 10, 100, and 1000 ng/ml. The nuclear maturation status of oocytes was assessed at 24 and 48 h. After 24 h maturation, there is an increasing trend in the proportion of oocytes reaching GVBD in the presence of leptin (64 *vs.* 54%; 100 *vs.* 0 ng/ml); however, this difference was not statistically significant (data not shown). However,

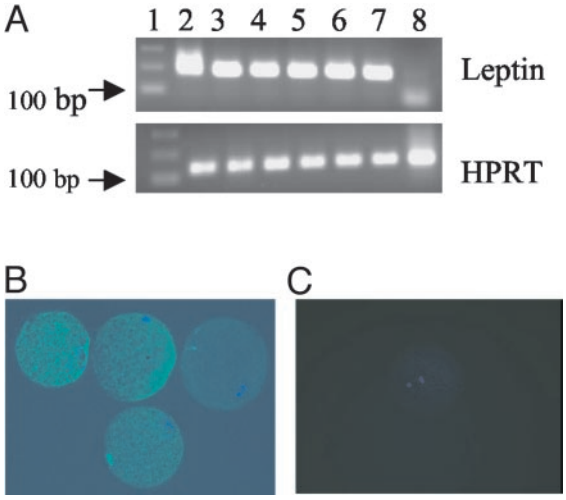

FIG. 3. Leptin is expressed in the porcine oocyte during follicular development and oocyte maturation. RNA was isolated from 100 oocytes at each stage of follicle growth and oocyte maturation and reverse transcribed, and 10% of the cDNA was used as a template for nested PCR. A, Agarose gel picture showing amplified product for leptin and HPRT. Lane 1, 100-bp marker; lane 2, fat (positive control); lane 3, oocytes from small follicle; lane 4, oocytes from medium follicle; lane 5, oocytes from large follicle (GV); lane 6, GVBD-stage oocytes; lane 7, MII-stage oocytes; lane 8, liver (negative control). HPRT housekeeping gene expression indicates the presence of RNA in each sample. Expression of leptin protein via immunocytochemistry is shown in panel B, whereas panel C, as negative control, lacks primary antibody.

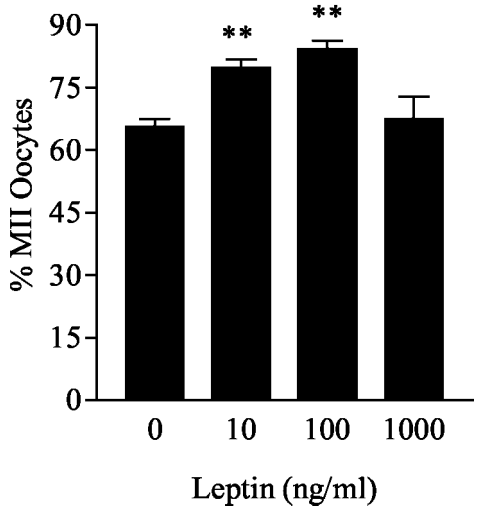

FIG. 4. The influence of leptin on the proportion of oocytes in COCs from large antral follicles reaching MII after 48 h IVM cultured in the presence or absence of leptin. Data shown are percentage of oocytes reached MII stage. Values are mean ± SEM of four independent experiments. **, $P < 0.01$, compared with the 0-ng/ml leptin group.

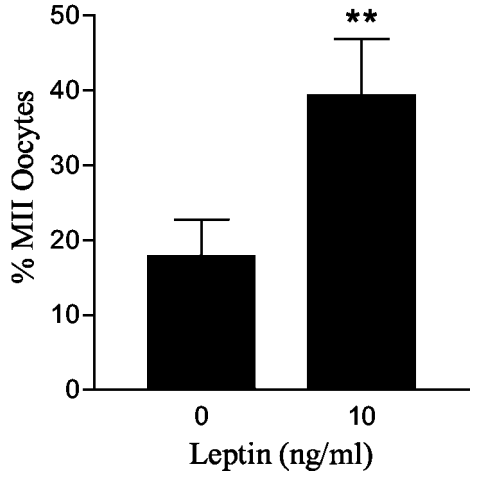

FIG. 5. Direct influence of leptin on oocyte nuclear maturation. Denuded oocytes isolated from large antral follicles were cultured in the presence or absence of leptin for 48 h. The percentage of oocytes at MII stage was evaluated under a phase-contrast microscope. Values are mean ± SEM of six independent experiments. **, $P < 0.01$.

at 48 h of maturation, the proportion of oocytes reaching MII in the presence of leptin was elevated. The percentage of oocytes reaching MII, indicated by extrusion of the first polar body, was significantly increased in the presence of 10 and 100 ng/ml leptin (80.1 and 85.0%, respectively, *vs.* 65.9% control; Fig. 4, $P < 0.01$), whereas a higher concentration of leptin (1000 ng/ml) failed to stimulate oocyte maturation.

Because the leptin receptor is present in both cumulus cells (our unpublished data) and the oocyte, it was unknown from our COC study whether the effect of leptin on meiotic progression is a direct action on the oocyte or whether leptin exerts an indirect influence on the oocyte by influencing cumulus cells. To clarify this, cumulus cells were removed from the COCs and denuded oocytes were cultured in maturation medium for 48 h before nuclear status assessment. In the presence of leptin (10 ng/ml), 39% of denuded oocytes progressed to MII, which is a significantly higher percentage than among those matured without leptin (18%; Fig. 5), suggesting that leptin enhances oocyte nuclear maturation, at least partly via its direct action on the oocyte.

Leptin influences oocyte cytoplasmic maturation and embryonic developmental competency

To study the influence of leptin on cytoplasmic maturation, cyclin B1 protein levels were used as a marker as described (24). COCs were cultured in maturation medium in the presence (100 ng/ml) or absence of leptin for 48 h, and protein was extracted from the oocytes for Western blot analysis to determine the level of cyclin B1 protein expression. As shown in Fig. 6, the level of the 62-kDa cyclin B1 protein was increased by 1.9-fold in oocytes matured in the presence of leptin as compared with the control group matured without leptin ($P < 0.05$).

The quality of the oocyte is a key factor in determining the development of embryos to the blastocyst stage (25). To compare the quality of oocytes matured in the presence (10 and 100 ng/ml) or absence of leptin, the preimplantation development of parthenogenetic embryos was evaluated. COCs were matured in the presence or absence of leptin for 46 h and denuded before activation via electrical pulse. The resulting parthenogenetic embryos were cultured *in vitro* for 7 d to evaluate preimplantation development. Cleavage rates were assessed 24 h after activation, and on d 7 the percentage of parthenogenetic embryos that developed to the blastocyst stage and the total cell number were evaluated. As shown in

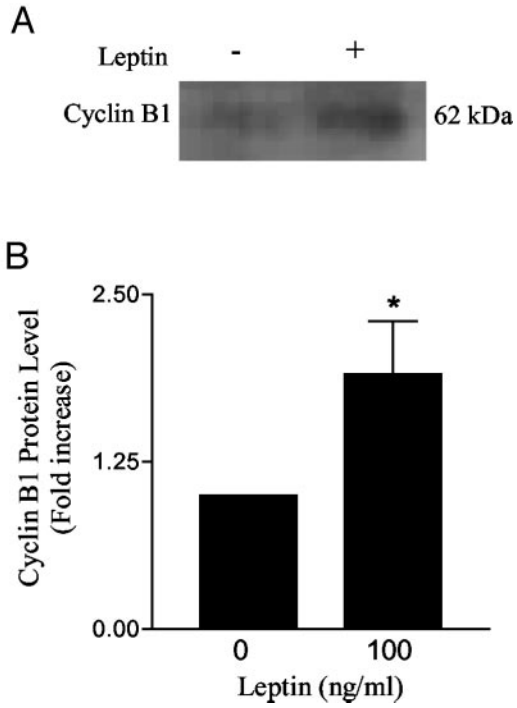

FIG. 6. Cyclin B1 protein content is increased in porcine oocytes matured in the presence of leptin. GV oocytes from large follicles (200 oocytes/group) were cultured as COCs in the presence or absence of leptin for 48 h, proteins were extracted, and Western blot analysis was performed with an anti-cyclin B1 antibody. A, Representative immunoblot. B, Densitometry analysis of cyclin B1 levels was performed using Image Analysis System (Syngene). Data are expressed as fold of control (0 ng/ml of leptin) and are mean ± SEM of six independent experiments. *, $P < 0.05$.

Fig. 7, the percentage of parthenogenetic embryos reaching the two-cell stage (cleavage) was increased at 10 ng/ml leptin, whereas development to the blastocyst stage and the cell number per blastocyst were significantly higher when oocytes were matured in the presence of 10 or 100 ng/ml leptin. Thus, oocytes matured in the presence of leptin show higher developmental competency to develop to the later stages of preimplantation development and are of better quality (higher cell number).

Leptin influences oocyte maturation via the MAPK pathway

Because the short form of the leptin receptor appears to be the dominant form expressed in the oocyte (Fig. 1), and these isoforms are capable of signaling through the MAPK pathway in other cell systems (26–29), we investigated whether leptin can activate the MAPK signal-transduction pathway during oocyte maturation. Denuded oocytes from different stages of maturation were incubated in the presence (10 ng/ml) or absence of leptin for 30 min. Lysates from GV-, GVBD-, or MII-stage oocytes were immunoblotted with antiphosphorylated MAPK antibody and subsequently reprobed with anti-MAPK for normalization of densitometry results. MAPK appears as a doublet at 44 and 42 kDa (Fig. 8A), representing ERK1 and ERK2. Basal phosphorylated MAPK was not detectable in oocytes from the GV stage, and it increased during oocyte maturation from GVBD to MII

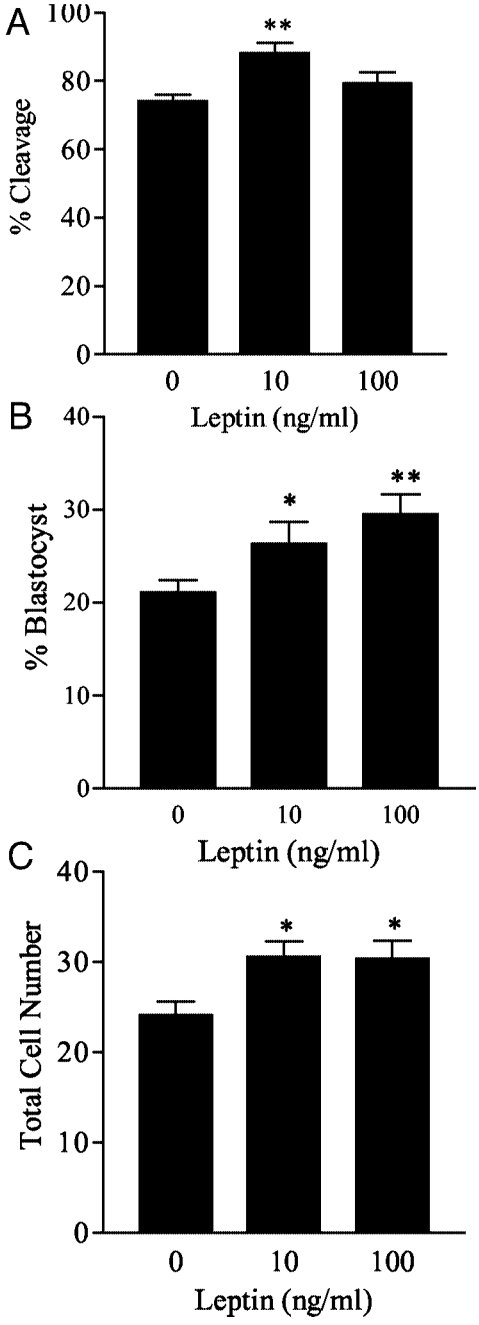

FIG. 7. Preimplantation development of parthenogenetic embryos derived from oocytes matured in the presence or absence of leptin *in vitro*. COCs from large follicles were *in vitro* matured in the presence or absence or leptin for 48 h, and parthenogenetic embryos were generated via electroactivation and cultured for 7 d. Cleavage rate (A) was evaluated at d 2 of culture, and blastocyst rate (B) and cell number per blastocyst (C) were determined at d 7 of culture. Data are mean ± SEM of six independent experiments. *, $P < 0.05$, **, $P < 0.01$.

(Fig. 8A). In the presence of leptin, the level of phosphorylated MAPK significantly increased in GVBD-stage oocytes compared with unstimulated oocytes from the same maturation stage (Fig. 8; 2.8-fold over the respective control, $P < 0.05$). Phosphorylated MAPK was not detectable in GV oocytes from small or medium follicles (data not shown).

If the increase of phosphorylated MAPK protein content

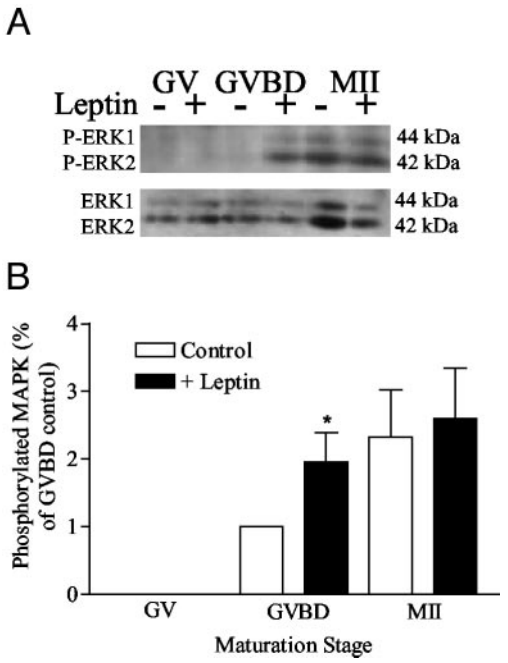

FIG. 8. Influence of leptin on phosphorylated MAPK (ERK1/ERK2) protein content during porcine oocyte maturation. Oocytes from large antral follicles were matured to GV, GVBD, or MII as COCs; were challenged with leptin (50 ng/ml) or without leptin for 30 min after removal of cumulus cells; and were subjected to immunoblotting. A, Representative immunoblot with anti-MAPK and antiphosphorylated MAPK antibodies. B, Densitometry analysis of phosphorylated MAPK content was performed using Image Analysis System (Syngene) and normalized to total MAPK protein levels. Data are expressed as percentage of control (0 ng/ml leptin) from GVBD-stage group and are mean ± SEM of three independent experiments. *, $P < 0.05$ compared with control of the respective stage.

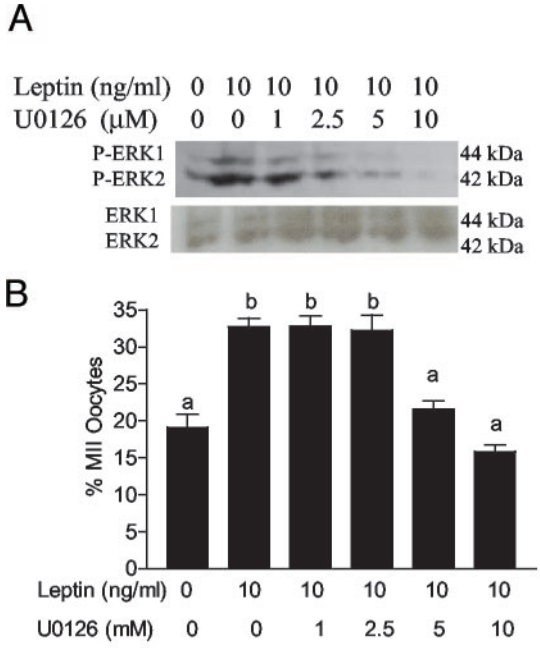

FIG. 9. Influence of leptin on oocyte nuclear maturation is mediated through the MAPK pathway. The leptin-induced increase of phosphorylated MAPK content is blocked by the MEK inhibitor U0126. Denuded oocytes (50 oocytes/group) from large antral follicles at GVBD were challenged with indicated concentrations of leptin and U0126 for 30 min and were subjected to immunoblotting. A, Representative immunoblot with anti-MAPK and antiphosphorylated MAPK antibody. B, Denuded oocytes from large follicles were cultured in the presence of indicated concentrations of leptin and the MEK inhibitor U0126 for 48 h, and the nuclear maturation status was evaluated. Data are mean ± SEM of three independent experiments. *a* and *b* indicate significant differences ($P < 0.01$).

and increased MII rates are causally related, inhibition of increased phosphorylated MAPK content in the presence of leptin should block the stimulatory effect of leptin on oocyte maturation. To test this hypothesis, U0126, the inhibitor of MAPK activation, was added to the IVM medium along with 10 ng/ml leptin. As shown in Fig. 9A, U0126 at 5 and 10 μM blocked the increase in phosphorylated MAPK protein content induced by leptin treatment of denuded oocytes. At these concentrations of U0126, the proportion of denuded oocytes reaching MII in the presence of leptin was reversed to a proportion similar to that of the control group without leptin (Fig. 9B), indicating that leptin enhances oocyte maturation via MAPK pathway.

Discussion

In the present study, we have demonstrated that 1) the leptin receptor is expressed in the porcine oocyte, and its expression is dependent on the stage of follicular development and oocyte maturation; 2) leptin is expressed by the porcine oocyte and facilitates oocyte maturation via stimulation of the oocyte to progress to MII and up-regulation of cyclin B1 protein levels, as well as enhancing the developmental competency of parthenogenetic embryos; and 3) the action of leptin on oocyte nuclear maturation is mediated via the MAPK pathway.

Although it has been shown previously that the leptin receptor is expressed in human, mouse, and rat oocytes (14, 18–20), whether its expression is stage dependent and whether leptin plays a role in oocyte maturation are largely unknown. The present study demonstrated that Ob-R is expressed in oocytes from all stages of follicular development and oocyte maturation, with the highest level of expression occurring in oocytes from medium follicles and at GVBD, indicating that its expression is dependent on follicular stage and oocyte maturation. Interestingly, the expression of Ob-Rb, the only known isoform capable of activating the Janus-activated kinase/STAT3 signal-transduction pathway, was very low and was not significantly different at any stage of oocyte maturation. This suggests that the increase in Ob-R expression in oocytes from medium follicles and GVBD was due to increased expression of the short isoforms, which are capable of MAPK signal transduction (10). Leptin has previously been shown to activate the MAPK pathway in several cell types, including mouse embryonic cells (26), pancreatic B cells (27), human blood mononuclear cells (28), rat aortic smooth muscle cells (29), and placental cells (30). In mouse embryonic cells, leptin increased both phosphorylated MAPK content and its substrate ELK-1, leading to increased cell proliferation, suggesting that leptin may act as a mitogen (26), whereas in placental cells, leptin increased transcription of c-*fos* and proliferation through MAPK, independent of JAK/STAT signal transduction (30). In the present study, we

have also confirmed the spatial availability of leptin to the developing oocyte by its expression in the ovary and oocyte and its presence in follicular fluid.

The MAPK pathway is activated and is believed to play an important role during meiotic maturation of vertebrate oocytes (reviewed in Ref. 31). In porcine oocytes, MAPK is activated at GVBD and retains a high level of activity until the oocyte reaches MII (32). Oocyte maturation involves the activation of various signal-transduction pathways that converge to activate maturation-promoting factor (MPF), which is composed of cyclin B and Cdc2 kinase (33). Microinjection of antisense c-*mos* RNA, a kinase upstream of MAPK, suppressed MPF activity in porcine oocytes, suggesting the role of MAPK in promoting MPF activity (34). In the current study, consistent with the highest level of Ob-R expression at GVBD, an increase in phosphorylated MAPK was observed at this stage when the oocytes were challenged with leptin. These data provide the first evidence that leptin can activate the MAPK pathway in the oocyte. Furthermore, suppression of leptin-induced phosphorylated MAPK protein content by the MAPK activation inhibitor, U0126, blocked the simulating effect of leptin on oocyte nuclear maturation. This indicates that the influence of leptin on oocyte maturation occurs via the MAPK pathway.

Although the precise physiological role of MAPK in oocyte maturation remains to be fully established, it is known that MAPK phosphorylates cyclin B1 in *Xenopus* oocytes (35). When the activity of c-*mos*, the upstream kinase of MAPK, was inhibited, MPF activity was also suppressed, and *Xenopus* oocytes were unable to reach meiosis II (36). In addition, MAPK plays a role in the regulation of cyclin B expression, given that microinjection of antisense c-*mos* RNA into mouse oocyte results in suppression of cyclin B translation (37). Interestingly, leptin also increased cyclin B1 protein levels in MII oocyte in the current study (Fig. 7).

Both MAPK and MPF are involved in the control of oocyte maturation via suppression of DNA replication, the segregation of meiotic chromosomes, and the prevention of parthenogenetic activation (reviewed in Ref. 38). The amount of cyclin B is the principal factor of MPF activity (39). In the pig, cyclin B protein accumulates during interphase, peaks during M-phase, and is subsequently destroyed, which results in the inactivation of MPF and exit of the cells from metaphase (40). The importance of cyclin B levels in oocyte maturation was also shown in a recent study in which the inhibition of porcine oocyte maturation by dexamethasone was found to be associated with decreased cyclin B but not p34cdc2 levels (41). It was also reported that microinjection of cyclin B1 antisense RNA into porcine oocytes prevented arrest at the second meiotic metaphase, whereas antisense cyclin B2 RNA microinjection had almost no effect on oocyte maturation (42). Thus, of the two subforms of cyclin B, cyclin B1 is the principal molecule involved in regulation of mammalian oocyte maturation, whereas the role of cyclin B2 is only accessory in this process. MAPK phosphorylation and cyclin B1 levels have been suggested to be important markers for evaluating cytoplasmic maturation (24). The fact that leptin increased the level of phosphorylated MAPK and cyclin B1 protein expression in our current study also suggests that leptin influences oocyte cytoplasmic maturation through this pathway.

Embryonic developmental competency (cleavage and blastocyst rate) has been used as an indicator of oocyte quality (reviewed in Ref. 25), and the number of cells per blastocyst is positively correlated to the quality of the blastocyst-stage embryos (43). The fact that oocytes matured in the presence of leptin showed enhanced developmental competency and higher blastocyst cell numbers after activation further confirms the stimulating effect of leptin on oocyte maturation.

Nuclear maturation is marked by progression through three distinct phases of the meiotic cell cycle; GV, GVBD, and MII. In the current study, when oocytes were cultured as COCs, there seemed to be an increase in the number of oocytes reaching GVBD when leptin was included in the maturation medium; however, this increase was not significant (data not shown). Starting at 10 ng/ml, leptin significantly increased the proportion of oocytes that reached MII after 48 h IVM. Although this concentration of human recombinant leptin used appears to be higher than the leptin concentration in porcine follicular fluid (1.2 ng/ml; current study), it should still be considered as a concentration close to the physiological level, considering that heterospecies ligand was used in the study.

In mice, preovulatory follicles cultured in the presence of leptin promoted GVBD, and it was suggested that leptin may have induced GVBD via its action on the theca cells rather than via direct action on the oocyte or cumulus cells (21). To further clarify whether the enhancing effect of leptin on oocyte maturation is a direct action on the oocyte or an indirect effect, we investigated nuclear maturation in denuded oocytes cultured in the presence or absence of leptin. A significantly higher proportion of denuded oocytes reached MII in the presence of leptin. To our knowledge, this finding provides the first indication that leptin facilitates cell cycle progression to MII via direct action on oocyte.

The developmental competency of the oocyte is acquired during the follicular growth period (24, 44, 45), and follicular development is dependent on the action of hormones, growth factors, and cytokines. The physiological relevance of higher leptin receptor expression in oocytes from medium follicles is still unclear. Because the b isoform of the receptor and phosphorylated MAPK are not detectable in oocytes from medium-sized follicles, it is unlikely that the STAT3 and MAPK pathways are involved in the function of leptin in this group of oocytes. It has been suggested that leptin may be transported to the oocyte via endocytosis after production elsewhere (14, 20, 21), although the physiological function of this influx is yet unknown. The leptin receptor (both long and short isoforms) is known to mediate transport of leptin across cellular membranes *in vitro* (46, 47). It is possible that the high expression of the short form of the leptin receptor in oocytes from medium follicles may play a role in the influx of leptin into the oocyte at this stage.

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