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Leptin enhances porcine preimplantation embryo development in vitro

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Abstract

Recent studies have suggested that leptin plays an important role in reproduction. Ob-R is expressed in the murine embryo, and is suggested to play a role in embryo development, although contradictory results have been reported. In the present study, Ob-R expression was observed both at the mRNA and protein levels in porcine early embryos. We have also demonstrated that leptin is produced in the porcine oviduct, making it spatially available to interact with its receptor during preimplantation development. When included at 10 ng/ml in embryo culture medium, leptin significantly increased the proportion of cleaved embryos ($P < 0.01$). At day 7 of in vitro culture, leptin at 10 and 100 ng/ml increased the proportion of embryos reaching the blastocyst stage ($P < 0.01$). We have previously observed that leptin increases oocyte maturation in vitro, and here we report that inclusion of leptin in both IVM and embryo culture medium further increased blastocyst development ($P < 0.05$), compared to when leptin was included in the embryo culture alone, suggesting leptin has a synergistic role on both oocyte maturation and preimplantation embryo development.

Keywords: Leptin; Leptin receptor; Preimplantation embryo; Embryo development

1. Introduction

The leptin hormone, encoded by the *obese* (*ob*) gene is produced primarily in adipose tissue (Zhang et al., 1994), and is acknowledged to play an important role in nutritional control (reviewed in Houseknecht et al., 1998). Recently, the importance of leptin in reproductive function has also become evident (Holness et al., 1999; Gonzalez et al., 2000). Obese mice exhibiting a congenital leptin deficiency (*ob*/*ob*) are sterile (Ingalls et al., 1950; Jones and Harrison, 1957). However, this sterility can be reversed by exogenous leptin administration, but not by the restriction of food intake (Barash et al., 1996), indicating that infertility is directly related to the deficiency of this hormone. In addition, return to reproductive competence of *ob*/*ob* mice is associated with increased ovarian and uterine weight, increased serum lutenizing hormone (LH) concentrations and an increased number of primary and Graafian follicles (Barash et al., 1996). In women, serum leptin concentrations are correlated with menstrual cycle and ovulation; leptin levels are lowest in the early follicular phase and are elevated during luteal phase (Hardie et al., 1997; Lukaszuk et al., 1998), suggesting leptin may play a role during early embryo development.

The timely requirement of leptin for conception and early pregnancy has recently been demonstrated (Malik et al., 2001). When leptin-null mice were given the minimum daily doses of leptin required to maintained normal body weight and sexual maturation until at least 6.5 days post coitum (dpc), pregnancies were retained and carried to term. However, pregnancy was disrupted if exogenous leptin administration was stopped at 0.5 or 3.5 dpc (Malik et al., 2001), indicating that leptin is essential for either early embryo development or implantation. In wild-type mice, leptin is expressed in both the oviduct and uterus during early pregnancy, and is thus temporally and spatially available for early embryo development (Kawamura et al., 2002). Moreover, the human endometrium secrets leptin and secretion is influenced by the

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blastocyst (Gonzalez et al., 2000), suggesting that the actions of leptin may be important during the preimplantation and implantation time window.

Recent reports of leptin's influence on early embryo development in mice have been controversial (Kawamura et al., 2002; Fedorcsak and Storeng, 2003). At concentrations ranging from 10 to 1000 ng/ml, leptin significantly promoted the development of murine 56 h expanded blastocysts and 72 h hatched blastocysts (Kawamura et al., 2002). In contrast, it has been reported that at 16 ng/ml, leptin decreased the proportion of embryos reaching the hatched blastocyst stage at day 5 of in vitro culture, and increased the rate of DNA fragmentation (Fedorcsak and Storeng, 2003). In light of these contradictory results, we thought it important to perform additional studies to understand the role of leptin during embryo preimplantation development in vitro. Here we report that the leptin receptor is expressed in the porcine embryo at different stages of early development. Embryo development was stimulated when cultured in the presence of leptin, and development was further enhanced when leptin was present during both oocyte maturation and embryo development.

2. Materials and methods

2.1. Oocyte collection and in vitro maturation (IVM)

Porcine ovaries were collected from 100 to 150 kg gilts at a local abattoir and transported to the laboratory in 1× PBS at 35 °C. Cumulus–oocyte complexes (COC) were isolated from antral follicles between 3 and 6 mm diameter using a 21-gauge needle. COCs were washed three times with maturation medium [Tissue Culture Medium-199; TCM199 (Gibco) supplemented with 5 IU/ml follicle stimulating hormone (FSH; Sioux Biochemicals), 5 IU/ml LH (Sioux Biochemicals), 0.1 mg/ml cysteine (Sigma), 10 ng/ml epidermal growth factor (EGF; Sigma)] and incubated at 38.5 °C for 42–44 h.

2.2. Parthenogenetic embryo activation and culture

After 42–44 h maturation, cumulus cells were removed from COCs by gentle vortexing in 0.1% hyaluronidase (Fisher) in TCM199. Metaphase II (MII) status was confirmed by the presence of the first polar body, and was consistently reached by 75–80% of oocytes. Denuded oocytes (DO) were washed three times in TCM199 containing 10% FBS (fetal bovine serum), placed between two platinum electrodes (0.5 mm apart) and overlaid with 50 μl of fusion medium consisting 297 mM mannitol (CalB), 0.001 mM $CaCl_2$ (Sigma), 0.05 mM $MgCl_2$ (Sigma), 0.1% BSA (Fraction V, Sigma). Two electrical pulses were applied to the oocytes at 2.6 kV/cm, generated by a BTX ElectroCell 2001 manipulator to activate the MII oocytes as parthenogenetic embryos. Following activation, embryos were cultured in NCSU-23 (North Carolina State University medium-23; supplemented with 4 mg/ml BSA and overlayed with light mineral oil for 7 days in the presence (10, 100 ng/ml) or absence of leptin to evaluate preimplantation embryo development (day 2 cleavage rate, day 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. Over 150 embryos were analyzed per treatment group over six independent experiments.

2.3. In vitro fertilization and embryo culture

Denuded MII oocytes were washed three times in PBS containing 10% FBS, and once in fertilization medium containing 60 μM glucose, 34 μM sodium citrate, 12.4 μM EDTA, 17 μM citric acid, 54 μM trizma base and 1 mM caffeine-sodium benzoate (Sigma). The semen was collected from a boar, washed twice in PBS containing 10% FBS, and resuspended in fertilization medium. Approximately 50 oocytes were incubated in NCSU-23 (as described by Lee et al., 2003) with sperm at 38.5 °C for 10 min. Oocytes and attached sperm were then transferred to NCSU-23 supplemented with 4 mg/ml BSA and covered with light mineral oil. Embryos were cultured for 7 days in vitro as described above. Over 150 embryos were analyzed per treatment group over six independent experiments.

2.4. Superovulation and in vivo produced embryo collection

Selected Yorkshire gilts between 70 and 80 kg were superovulated by intramuscular injection of 10 mg PGF2α, followed 48 h later by 2000 IU of pregnant mare's gonadotropin (PMSG, Ayerst Veterinary Laboratories). An amount of 1000 IU human chorionic gonadotropin (hCG, Ayerst Veterinary Laboratories) was administered 72 h later to induce ovulation. Gilts were artificially inseminated twice on the day following onset of estrous using semen from a Yorkshire boar. Gilts were slaughtered the next morning, reproductive tracts recovered, and one cell embryos flushed from the oviduct. Embryos were washed three times in TCM199 plus 10% FBS, once in NCSU-23 and then cultured in NCSU-23 supplemented with 4 mg/ml BSA. Embryos were collected at the 2-, 4-, 8-cell, morula and blastocyst stages for real time RT-PCR in lysis buffer as described below.

2.5. RNA isolation and RT-PCR

Single embryos were lysed in 10 μl lysis buffer containing 2 U/μl porcine RNase Inhibitor (Amersham) and 5 mM dithiothreitol (DTT; Invitrogen) by boiling followed by vortexing for 1 min each. Cell lysates were DNase I treated according to manufacturer's directions (Invitrogen). RT-PCR was performed in a total volume of 25 μl containing 10 μl of DNase-treated cell lysate, 1× RT-PCR buffer, 2 U/μl MMLV-RT, (Moloney Murine Leukemia Virus Reverse Transcriptase; Invitrogen), 2.5 μM random hexamer primers (Applied

Table 1
Primers used for PCR and nested PCR for detection of leptin, Ob-R, HPRT and H2A

Transcript	PCR round		Primer sequence (5′–3′)	Product size (bp)	Primer location	Gene Bank 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	ctc ttg cct gct gga atc tc	250	Exon 4	AF092422
		Antisense	ttc cag ttt gca cct gtt tg		Exon 5	
HPRT	1	Sense	cca gtc aac ggg cga tat aa	130	Exon 4	U69731
		Antisense	ctt gac caa gga aag caa gg		Exon 6	
H2A	1	Sense	gtg gca aac aag gag gaa ag	226		BP459633
		Antisense	atg cgg gtc ttc ttg ttg tc			

Biosystems), and 0.2 mM dNTP. The RT reaction was carried out at 25 °C for 10 min, and 37 °C degrees for 50 min, followed by 15 min at 75 °C to inactivate MMLV.

RNA was isolated from the oviducts of day 3–4 pregnant sows using Trizol reagent according to manufacturer's directions (Invitrogen). Up to 1 μg RNA was incubated in a final volume of 10 μl containing DNase I (1 U) and DNase buffer (1×), and DNase treatment and RT-PCR were performed as described above.

Real time RT-PCR was performed using SYBR Green (Qiagen) and the Smart Cycler thermocycler (Cepheid). Briefly, 12.5 μl SYBR Green 2× mix (contains HotStar *Taq* DNA polymerase, QuantiTect SYBR Green PCR buffer, dNTP mix, SYBR Green 1, ROX and 5 mM $MgCl_2$), 0.3 μM each of forward and reverse primer (Table 1) and 2.5 μl cDNA was included in a 25 μl PCR reaction. To detect the presence of leptin expression in oviduct tissue, nested PCR was performed. Reverse transcription and PCR was performed as described above, followed a second round of PCR using nested primers (Table 1). Melt curve analysis was preformed to confirm the specificity of the products amplified. All PCR products were sequenced to confirm their identity. Negative controls were performed in which water was substituted for cDNA, or reverse transcription was not performed prior to PCR.

2.6. Immunocytochemistry

Embryos were fixed in 4% paraformaldehyde in PBS for 30 min, followed by permeabilization in 0.1% Tween 20 for 10 min, and 0.1% TritonX-100 for 20 min. Embryos were then blocked in PBS supplemented with 1% skim milk and 5% goat serum. Primary antibody (1:100; rabbit anti-Ob-R; Santa Cruz Biotechnology) was incubated with the blocked oocytes overnight at 4 °C, followed by goat anti-rabbit FITC (fluorescein isothiocyanate) secondary antibody incubation (1:500; Sigma) for 1 h at room temperature. Embryos were counterstained with DAPI (4′,6-diamidino-2-phenylindole), and mounted on glass slides with fluorescent mounting medium (DakoCytomation). Fluorescence was examined using an Olympus BX-UCB microscope and MetaMorph image analysis software (Universal Imaging Corporation). Over 30 embryos at each of the 2-, 4-, 8-cell, morula and blastocyst stages were analyzed for expression of Ob-R.

2.7. Immunohistochemistry

Oviduct tissue was obtained from day 3 to 4 pregnant sows and fixed with 10% formalin for 20–24 h. Paraffin-embedded tissue was sectioned at 5 μM and sections were adhered to glass slides. Tissue sections were defaraffinized by passage through three consecutive Xylene (Fisher) washes and were rehydrated in decreasing concentration of ethanol. Tissue sections were treated with sodium borohydrate (0.2%; Sigma) for 20 min, followed by blocking for 1 h in 5% FBS. Samples were incubated with primary antibody (1:100 rabbit anti-leptin, Santa Cruz Biotechnology) overnight at 4 °C. After two washes with 1× PBS, secondary antibody (1:500; goat anti-rabbit IgG conjugated FITC; Sigma) was added to the samples for 1 h at room temperature in the dark. Following two washes in 1× PBS, cell nuclei were stained with DAPI and coverslips were mounted using fluorescent mounting medium (DakoCytomation). For negative controls, control slides were subjected to the same method, except for the primary antibodies were omitted. Fluorescence was examined under UV light using an Olympus BX-UCB microscope and MetaMorph Image Analysis software (Universal Imaging Corporation).

2.8. Statistical analysis

Cleavage and blastocyst rates, as well as total cell number per embryo, were analyzed by ANOVA. Significant results 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 represents the average of all repeats.

3. Results

3.1. Ob-R is expressed in the porcine preimplantation embryo

We first studied whether Ob-R is expressed in the porcine preimplantation embryo, using RT-PCR. To ensure amplification of all isoforms of Ob-R, primers were designed between exons 4 and 5, which are common to all isoforms of

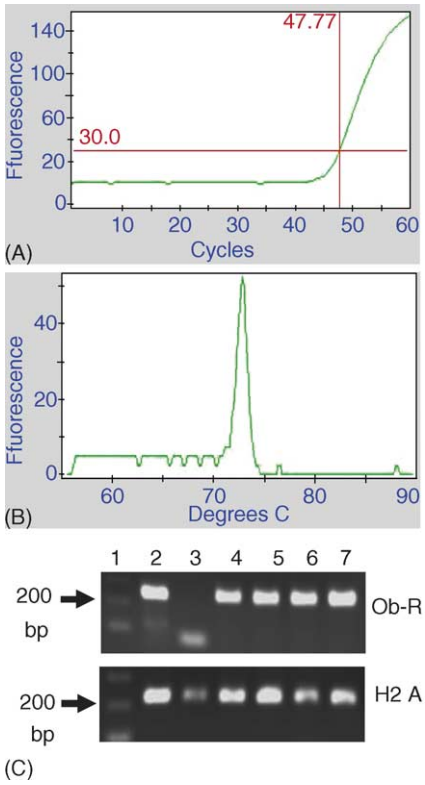

Fig. 1. The leptin receptor is expressed in the porcine preimplantation embryo. Total RNA was isolated from in vivo produced porcine embryos, reverse transcribed and 10% of cDNA used as template in real time PCR. (A) Representative real time PCR amplification, (B) melt curve analysis showing specificity of the product amplified, (C) agarose gel image to confirm the size of amplified products (Ob-R: 250 bp, H2A: 226 bp), lane 1: 100 bp marker, lane 2: lung (positive control), lane 3: 2-cell embryo, lane 4: 4-cell embryo, lane 5: 8-cell embryo, lane 6: morula stage embryo, lane 7: blastocyst stage embryo. H2A was amplified from each sample to confirm presence of RNA in the reaction.

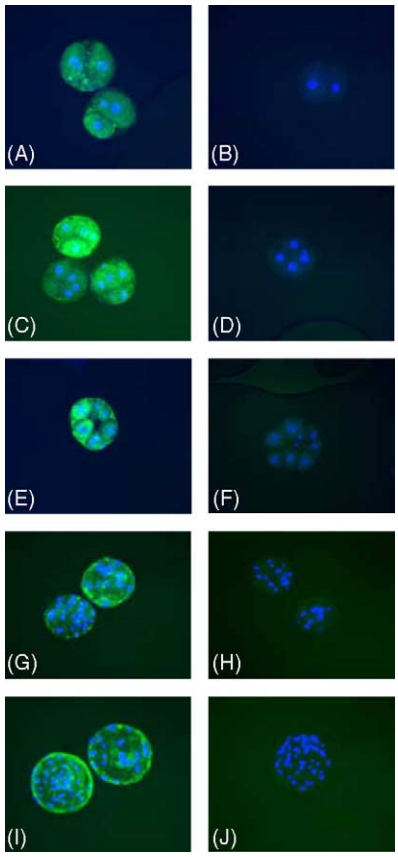

Fig. 2. Representative image of detection of leptin receptor protein in porcine embryos during preimplantation development. Individual embryos were fixed in 4% paraformaldehyde, and immunocytochemistry was performed. Ob-R was detected using an anti-Ob-R antibody, and anti-IgG conjugated FITC. (A, C, E, G, I) 2, 4, 8, morula and blastocyst stage embryos, (B, D, F, H, J) negative control embryos where primary antibody was omitted. Fluorescence images were obtained using an Olympus BX-UCB microscope and MetaMorph software (Universal Imaging Corporation).

the receptor (Chua et al., 1997). As shown in Fig. 1, Ob-R mRNA was detected in 4-, 8-cell, morula and blastocyst stage embryos, but was not detectable at the 2-cell stage. Histone 2A (H2A) was amplified to confirm the presence of RNA in each sample. The expression of Ob-R at the protein level was also studied. As shown in Fig. 2, Ob-R protein was detected in embryos from the 2-cell stage through to the blastocyst stage.

3.2. Leptin is expressed in the porcine oviduct

To exert a physiological influence on the preimplantation embryo, leptin must be spatially available in the oviduct. To investigate whether leptin protein is indeed present in the oviduct, immunohistochemistry was performed using oviduct tissue from day 3 to 4 pregnant pigs. As shown in Fig. 3D, the epithelium of the mucosal folds was strongly stained, as well the muscularis layer also showed staining. No staining was detected in negative control slides that were not exposed to primary antibody (Fig. 3E). To further investigate whether leptin is expressed in the oviduct or if the leptin protein had migrated to the oviduct from another source, real time RT-PCR was performed. Leptin mRNA was detected in RNA isolated from oviducts of day 3–4 pregnant sows, but not in oviducts from non-pregnant sows (Fig. 3C).

3.3. Leptin promotes porcine preimplantation development

The influence of leptin on preimplantation in vitro embryo development was also studied. Following in vitro fertilization, embryos were cultured in NCSU-23 supplemented with leptin (10, 100 ng/ml) for 7 days to evaluate preimplantation development. Cleavage of embryos was significantly increased in the presence of 10 ng/ml leptin (85%; Fig. 4A; $P < 0.01$) compared to embryos cultured without leptin (70.7%). Interestingly, a higher concentration of leptin (100 ng/ml) did not increase cleavage rates (71.9%). Blastocyst development was also influenced by leptin. Following culture for 7 days in the presence or absence of leptin, starting at 10 ng/ml lep-

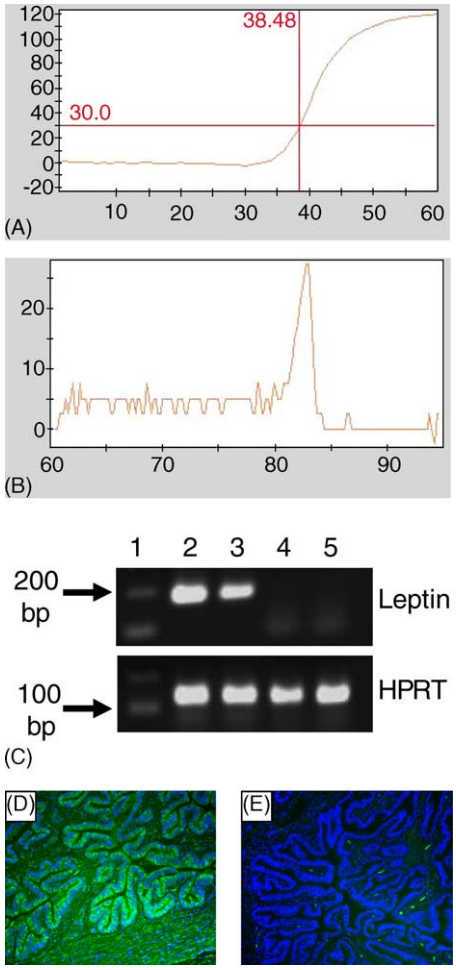

Fig. 3. Expression of leptin in the porcine oviduct. RNA was isolated from sow oviducts, reverse transcribed and 10% of the cDNA was used as a template for nested PCR. (A) Representative real time amplification curve, (B) melt curve showing specificity of produce, (C) agarose gel image confirm the size of amplified products (leptin: 184 bp, HPRT: 130 bp). Lane 1: 100 bp marker, lane 2: fat (positive control), lane 3: day 3–4 pregnant pig, lane 4: non-pregnant pig, lane 5: liver (negative control). (D) Immunofluoresence staining of leptin in the oviduct of a day 3–4 pregnant pig. Paraffin embedded tissue sections were deparaffinized, blocked, and incubated overnight with anti-leptin antibody, followed by anti-rabbit IgG conjugated to FITC. Images were obtained with an Olympus BX-UCB microscope and MetaMorph Software (Universal Imaging Corporation), (E) negative control in which primary antibody was omitted.

tin, significantly more embryos reached the blastocyst stage (Fig. 4B; $P < 0.01$).

3.4. Synergistic influence of leptin on oocyte maturation and embryo development

We have previously reported that leptin enhanced oocyte cytoplasm and nuclear maturation. To investigate whether there is a synergistic influence of leptin on oocyte maturation and embryo development, we investigated preimplantation development of embryos derived from oocytes matured in the presence or absence of leptin, and cultured in the presence of leptin during preimplantation development. As shown in

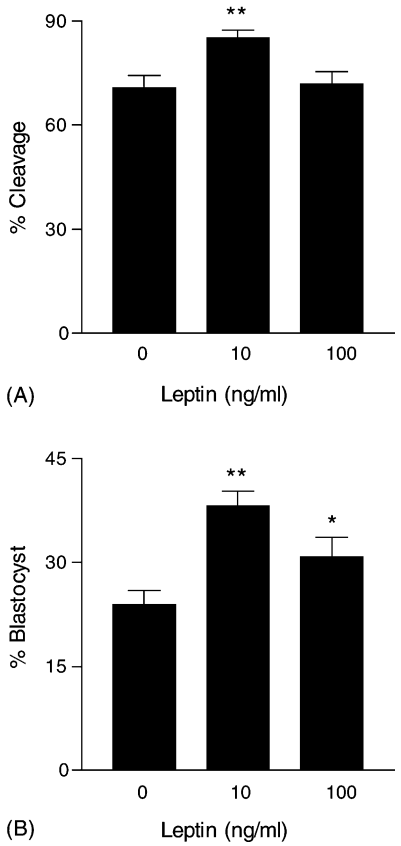

Fig. 4. Preimplantation development of IVF embryos cultured in the presence or absence of leptin. Cumulus–oocyte complexes isolated from large antral follicles (>3 mm) were in vitro matured for 48 h, fertilized and cultured for 7 days, in the presence (10, 100 ng/ml) or absence of leptin. Cleavage rate (A) was evaluated at day 2 of culture, blastocyst rate (B) was determined at day 7 of culture. Data is the mean ± S.E.M. of six independent experiments and over 150 embryos were analyzed per treatment. $^{*}P < 0.05$, $^{**}P < 0.01$.

Fig. 5, consistent with what we found previously, presence of leptin during oocyte maturation alone enhanced both cleavage and blastocyst formation (20 and 23.2% increase over control without leptin, $P < 0.05$; Fig. 5). The stimulation of blastocyst formation was further enhanced if leptin was included in both IVM and embryo culture (EC) medium (40.5% increase over control, $P < 0.05$ compared leptin in IVM group alone), although no further improvement on cleavage was observed. This data suggests a synergistic influence of leptin on IVM and EC. In addition, the total cell number per blastocyst was increased in the presence of leptin (31%) compared to control embryos not exposed to leptin during either IVM or EC (31% versus 23% control; $P < 0.01$), however there was no difference between IVM and EC treated groups (30.7, 31.2%, respectively).

4. Discussion

The present study has demonstrated that (1) the leptin receptor is expressed in the porcine preimplantation embryo,

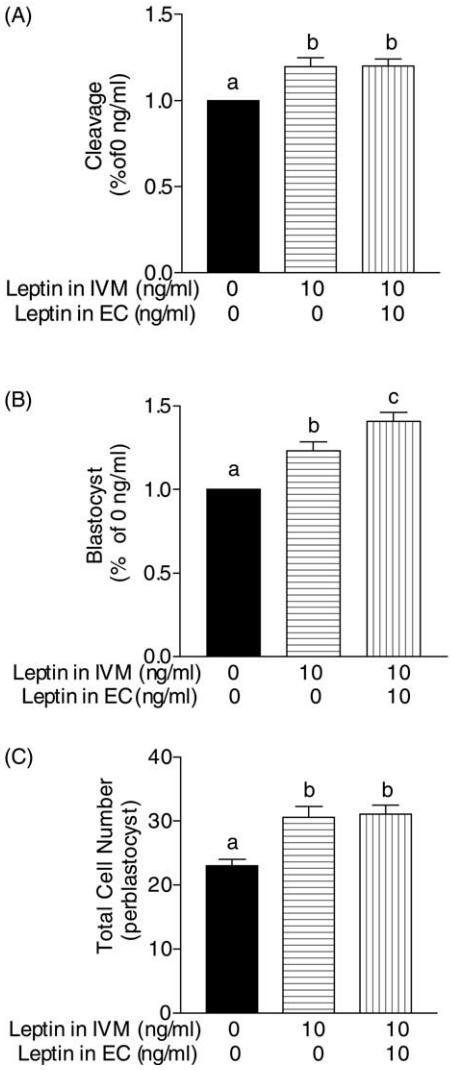

Fig. 5. Synergistic influence of leptin on oocyte maturation and embryo development when present during both IVM and EC. Oocytes were cultured in the presence (10 ng/ml) or absence of leptin and were electro-activated. Embryos were cultured in the presence (10 ng/ml) or absence of leptin and cleavage was evaluated at day 2 (A), while blastocyst rate (B) and total cell number per blastocyst (C) were evaluated at day 7. Solid bar: control, horizontal lined bar: leptin in IVM, vertical lined bar: leptin in both IVM and EC. Data is the mean ± S.E.M. of six independent experiments, with over 150 embryos analyzed per treatment. Different letters indicate significant differences.

(2) leptin is spatially available to the embryo within the oviduct, and (3) leptin has a positive influence on preimplantation embryo development in vitro.

Ob-R mRNA was detected in the porcine embryo from the 4-cell stage through to the blastocyst stage. Previously, we have shown expression of Ob-R in the porcine oocyte (Craig et al., in press). The inability to detect Ob-R mRNA transcripts in the 2-cell stage embryo may suggest that maternal transcripts are being degraded at this stage. In the pig, genome activation occurs at the 4-cell stage (Jarrell et al., 1991), suggesting that Ob-R transcripts observed from the 4-cell stage onwards are embryonic in origin. In a previous study in mice, Ob-R expression was detected at the 2-cell, morula and blastocyst stage embryo (Kawamura et al., 2002). Genome activation occurs at the 2-cell stage in the mouse, suggesting Ob-R expression from the embryonic genome is not activated until the morula stage of development. The earlier expression of Ob-R from the embryonic genome in the pig may reflect the earlier involvement of leptin during preimplantation development in this species. In the present study, Ob-R protein was detectable in embryos from the 2-cell stage onwards. The presence of Ob-R protein at the 2-cell stage is likely to be derived from maternal transcripts.

To study the spatial availability of leptin for the developing embryo, the presence of leptin in the oviduct was investigated via immunohistochemistry. Strong staining of leptin was detected in the luminal epithelium of the oviduct of pigs 3–4 days pregnant, a time which corresponds to the presence of preimplantation embryos in the oviduct, thus indicating the spatial availability of leptin during early embryo development. To determine whether leptin protein is indeed produced in the oviduct, or transported from elsewhere, RT-PCR was performed. As shown in Fig. 3, while leptin mRNA transcripts were not detectable in the oviducts of non-pregnant pigs, leptin transcripts were detected in oviduct RNA isolated from pigs 3 to 4 days pregnant. The up-regulation of Ob-R expression in pregnant pigs compared to non-pregnant pigs suggests that leptin expression is regulated in stage specific manner. The up-regulation of leptin expression in the oviduct during early pregnancy was also reported in mice (Kawamura et al., 2002). Interestingly, leptin expression has been observed to be up-regulated in cultured endometrial endothelial cells by the presence of blastocyst stage embryos (Gonzalez et al., 2000).

To further study whether leptin plays a role in preimplantation development, in vitro produced embryos were cultured in the presence or absence of leptin. Enhanced preimplantation development was observed in the presence of leptin starting at 10 ng/ml, as indicated by increased cleavage and blastocyst rates, as well as increased total cell numbers of day 7 blastocysts. Thus, our findings lend further support to the report of a beneficial role of leptin in murine preimplantation development (Kawamura et al., 2002). Our study also provides in vitro evidence to support the finding that leptin is important for the early implantation time window (Malik et al., 2001). We have previously shown that leptin has an influence on cytoplasmic maturation of the oocyte, which enhances implantation embryo development (Craig et al., in press). Leptin is present in porcine follicular fluid and thus, oocytes are exposed to leptin in the follicle during maturation (Craig et al., in press), and embryos are exposed to leptin during preimplantation development in the oviduct in vivo (our current finding and Kawamura et al., 2002). We therefore hypothesized that the inclusion of leptin in both oocyte in vitro maturation medium and embryo culture medium would further enhance preimplantation embryo development. Con-

sistent with our previous observations, inclusion of leptin in oocyte in vitro maturation medium enhanced preimplantation development (Craig et al., in press). Interestingly, preimplantation development was further enhanced by the inclusion of leptin in the embryo culture medium in addition to IVM medium, suggesting a synergistic effect of leptin on these two processes.

Leptin is known to be capable of activating both the JAK/STAT and MAPK signal transduction pathways in many cell types (Hegyi et al., 2004). In the oocyte, leptin enhances the maturation of oocyte via the MAPK pathway (Craig et al., in press). It is possible that leptin may also activate the MAPK pathway in the preimplantation embryo. Although the role of MAPK in the oocyte has been extensively studied, comparatively little is known about the role of MAPK in the early embryo. Activation of this pathway in the embryo may contribute to enhance preimplantation development via increased cell proliferation, as MAPK signal transduction is a key regulator of cell proliferation (reviewed in Roux and Blenis, 2004). Indeed, we observed that exposure of either oocytes or embryos to leptin increased the total cell number of day 7 blastocysts, suggesting enhanced proliferation.

The JAK/STAT pathway has also been suggested to be activated by leptin in the murine embryo (Fedorcsak and Storeng, 2003). STAT3 has previously been shown to be an essential pathway in embryonic development, as STAT3 null mice die before day 7.5 of gestation (Takeda et al., 1997). STAT3 signaling is activated by a wide range of cytokines and growth factors (Raz et al., 1994) and may contribute to embryo development via activation of genes resulting in increased proliferation or protection against apoptosis.

Our findings in the present study suggest that leptin plays a positive role in the development of porcine preimplantation embryos. In addition to adding to knowledge on regulation of early embryo development, these results may also have clinical applications. In the pig, the current success rate of IVF and nuclear transfer techniques remains low. The finding that the inclusion of leptin in IVM and embryo culture mediums enhances early development suggests that leptin may aid in the optimization of these techniques, possibly leading to improved success rates.

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References

Barash, I.A., Cheung, C.C., Weigle, D.S., Ren, H., Kabigting, E.B., Kuijper, J.L., Clifton, D.K., Steiner, R.A., 1996. Leptin is a metabolic signal to the reproductive system. Endocrinology 137, 3144–3147.

Chua Jr., S.C., Koutras, I.K., Han, L., Liu, S.M., Kay, J., Young, S.J., Chung, W.K., Leibel, R.L., 1997. Fine structure of the murine leptin receptor gene: splice site suppression is required to form two alternatively spliced transcripts. Genomics 45, 264–270.

Craig, J.A., Zhu, H., Dyce, P.W., Petrik, J., Li, J. Leptin enhances oocyte nuclear and cytoplasmic maturation via the mitogen-activated protein kinase pathway. Endocrinology 145, in press.

Fedorcsak, P., Storeng, R., 2003. Effects of leptin and leukemia inhibitory factor on preimplantation development and STAT3 signaling of mouse embryos in vitro. Biol. Reprod.

Gonzalez, R.R., Caballero-Campo, P., Jasper, M., Mercader, A., Devoto, L., Pellicer, A., Simon, C., 2000. Leptin and leptin receptor are expressed in the human endometrium and endometrial leptin secretion is regulated by the human blastocyst. J. Clin. Endocrinol. Metab. 85, 4883–4888.

Hardie, L., Trayhurn, P., Abramovich, D., Fowler, P., 1997. Circulating leptin in women: a longitudinal study in the menstrual cycle and during pregnancy. Clin. Endocrinol. (Oxf.) 47, 101–106.

Hegyi, K., Fulop, K., Kovacs, K., Toth, S., Falus, A., 2004. Leptin-induced signal transduction pathways. Cell Biol. Int. 28, 159–169.

Holness, M.J., Munns, M.J., Sugden, M.C., 1999. Current concepts concerning the role of leptin in reproductive function. Mol. Cell. Endocrinol. 157, 11–20.

Houseknecht, K.L., Baile, C.A., Matteri, R.L., Spurlock, M.E., 1998. The biology of leptin: a review. J. Anim. Sci. 76, 1405–1420.

Ingalls, A.M., Dickie, M.M., Snell, G.D., 1950. Obese, a new mutation in the house mouse. J. Hered. 41, 317–318.

Jarrell, V.L., Day, B.N., Prather, R.S., 1991. The transition from maternal to zygotic control of development occurs during the 4-cell stage in the domestic pig *Sus scrofa*: quantitative and qualitative aspects of protein synthesis. Biol. Reprod. 44, 62–68.

Jones, N., Harrison, G.A., 1957. Genetically determined obesity and sterility in the mouse. Proc. Soc. Study Fertil. 9, 51–64.

Kawamura, K., Sato, N., Fukuda, J., Kodama, H., Kumagai, J., Tanikawa, H., Nakamura, A., Tanaka, T., 2002. Leptin promotes the development of mouse preimplantation embryos in vitro. Endocrinology 143, 1922–1931.

Lee, J.W., Wu, S.C., Tian, X.C., Barber, M., Hoagland, T., Riesen, J., Lee, K.H., Tu, C.F., Cheng, W.T., Yang, X., 2003. Production of cloned pigs by whole-cell intracytoplasmic microinjection. Biol. Reprod. 69, 995–1001.

Lukaszuk, K., Liss, J., Kusiak, E., Wojcikowski, C., 1998. Serum leptin concentration increase during luteal phase in healthy premenopausal women. Horm. Metab. Res. 30, 172–173.

Malik, N.M., Carter, N.D., Murray, J.F., Scaramuzzi, R.J., Wilson, C.A., Stock, M.J., 2001. Leptin requirement for conception, implantation, and gestation in the mouse. Endocrinology 142, 5198–5202.

Raz, R., Durbin, J.E., Levy, D.E., 1994. Acute phase response factor and additional members of the interferon-stimulated gene factor 3 family integrate diverse signals from cytokines, interferons, and growth factors. J. Biol. Chem. 269, 24391–24395.

Roux, P.P., Blenis, J., 2004. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiol. Mol. Biol. Rev. 68, 320–344.

Takeda, K., Noguchi, K., Shi, W., Tanaka, T., Matsumoto, M., Yoshida, N., Kishimoto, T., Akira, S., 1997. Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. Proc. Natl. Acad. Sci. U.S.A. 94, 3801–3804.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., Friedman, J.M., 1994. Positional cloning of the mouse obese gene and its human homologue. Nature 372, 425–432.