Leptin has concentration and stage-dependent effects on embryonic development *in vitro*

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Abstract

There is accumulating evidence that leptin may be directly involved in pre-implantation embryonic development, however, it is unclear whether there is a concentration and stage-dependent regulatory pattern. In this study, the addition of 10 ng/ml human recombinant leptin to the culture medium significantly increased the percentage of two-cell mouse embryos that developed into blastocysts and hatched blastocysts, whereas in the presence of 100 ng/ml leptin, the development rate was significantly inhibited. The total cell numbers in the hatched blastocysts were significantly higher in the presence of 10 ng/ml leptin compared with controls and higher concentrations. The differential sensitivity to leptin was found to vary among embryos at different stages of development. Supplementation of leptin (10 ng/ml) to culture medium at two- to eight-cell stages resulted in a consistent stimulatory effect on embryo development. Most interestingly, the inhibitory effect of high leptin concentration (100 ng/ml) on embryo development was diminished when it was added to the culture medium at the eight-cell stage of development. The concentration-dependent regulation pattern was confirmed using sheep embryos, under similar conditions although sheep embryos appeared to be more sensitive in responding to leptin. Having established the effect of exogenous leptin on embryo development, the expression pattern of leptin and its receptors were also investigated. Leptin mRNA was not detected in mouse two-, four-, eight-cell and blastocyst stage embryos, whereas three isoforms of leptin receptor (Ob-Ra, Ob-Rb and Ob-Re) were identified in these cells, indicating that leptin is likely to modulate embryo development via a paracrine signalling system.

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Introduction

There is increasing evidence that, in addition to its action on food intake and energy expenditure, leptin plays an important role in reproduction and development (Cunningham *et al.* 1999, Holness *et al.* 1999, Cervero *et al.* 2005). The *ob/ob* mouse is deficient in leptin and is obese and infertile. Fertility can be restored in both female and male *ob/ob* mice by the exogenous provision of leptin, which is characterized by an increase in basal luteinizing hormone (LH) and follicle-stimulating hormone, (FSH) (Chehab *et al.* 1996, Mounzih *et al.* 1997). The onset of puberty in humans and other animals is often associated with an increase in fat and consequent increase in circulating leptin (Arslanian *et al.* 1998, Ahmed *et al.* 2001, Urbanski 2001). Leptin treatment of normal pre-pubertal female mice accelerates the onset of puberty (Ahima *et al.* 1997, Chehab *et al.* 1997). During the menstrual cycle, there are variations in serum leptin levels, with higher concentration reported in the pre-ovulatory and mid-luteal phases and lower in

the early follicular phase (Hardie *et al.* 1997, Lukaszuk *et al.* 1998, Henson & Castracane 2005).

Leptin (Bennett *et al.* 1996) and leptin receptors (Chen *et al.* 2000) have been identified in a number of foetal tissues at different stages of development, indicating that leptin may be important in foetal growth, a role which is distinct from its activity as a satiety factor. A leptin surge has been detected in mice during the early post-natal period, which is independent of fat mass and not acutely regulated by food restriction (Devaskar *et al.* 1997). In addition, leptin is ineffective in regulating food intake and energy expenditure in the neonatal mouse (Mistry *et al.* 1999). Taken together, these studies have provided evidence that leptin has a developmental role. Indeed, the *ob/ob* mouse exhibits numerous reproductive abnormalities including unusually small ovarian weight in females and a small amount of interstitial tissue in males (Jones & Ainsworth-Harrison 1957). Treatment of *ob/ob* mice with leptin results in the normalization of testicular and ovarian weight and function (Chehab *et al.* 1996, Mounzih *et al.* 1997), thus suggesting that

leptin may be required for normal growth and development of reproductive organs.

The restoration of fertility in the female *ob/ob* mouse by the exogenous administration of leptin has demonstrated that leptin is essential for normal pre-implantation and/or implantation processes (Malik *et al.* 2001). As recently reviewed by Cervero *et al.* (2005), leptin has been detected by immunofluorescence in meiotically mature mouse, human, pig and bovine oocytes, and early cleavage embryos (Antczak & Vanblerkom 1997, Cioffi *et al.* 1997, Madeja *et al.* 2005, Kim *et al.* 2005). Moreover, leptin mRNA and protein have been identified in human and mouse blastocysts and hatched blastocysts (Gonzalez *et al.* 2000, Kawamura *et al.* 2002, Kawamura *et al.* 2003), and leptin appears to be regulated in a paracrine manner when blastocysts are co-cultured with human endometrial cells (Gonzalez *et al.* 2000).

Supplementation of culture medium with leptin significantly enhanced the formation of blastocyst and hatched blastocysts in mice and pigs (Kawamura *et al.* 2002, Kawamura *et al.* 2003, Craig *et al.* 2005). Conversely, Swain *et al.* (2004) reported that increasing concentrations of murine leptin had no effect on one-cell embryo development, while the study by Fedorcsak & Storeng (2003) showed that even low concentrations of leptin inhibited the proportion of embryos reaching the hatched blastocyst stage as the result of induced DNA fragmentation. These conflicting results may have reflected the differences in the strain or species of experimental animal, the culture conditions of the embryos at different developmental stages or the type of exogenous leptin used.

Therefore, in the present study, we used mice and sheep embryos in an *in vitro* culture system to examine the effects of leptin on pre-implantation embryonic development and to determine if there were any difference in sensitivities to leptin among embryos at different stages of development or embryos from different breeds of animal. To eliminate environmental effects on an embryo's gene expression, mouse embryos that were fertilized and developed *in vivo* were collected at different developmental stages. Leptin and its receptor expression in pre-implantation embryos were then investigated to provide molecular evidence for the leptin effect on embryo development.

Materials and Methods

Animals

Female mice were housed individually in an air-conditioned room under a 12 h light:12 h darkness cycle (0700–1900) and fed a commercial diet and given water *ad libitum*. Random-bred Swiss albino mice, 6–8-week-old, were superovulated by i.p. injection of 5 IU pregnant mares serum gonadotrophin (PMSG; Pregnenol, Bioniche Animal Health, Armidale,

NSW, Australia) and followed 48 h later by i.p. injection of 5 IU human chorionic gonadotrophin (hCG) (Sigma). They were paired overnight with males of proven fertility.

Sheep embryos were collected from fine wool Merino ewes (UNE rural properties, NSW, Australia). The oestrous cycle of ewes was synchronized by the insertion of controlled internal drug release pessaries (CIDR, containing 0.3 g progesterone; Horizon Pty Ltd, Vic., Australia) for a 13-day period with a renewal on the sixth day of treatment. To induce superovulation, a combination of both PMSG and ovine FSH (in-house preparation) was administered intramuscularly during the last 3 days before the removal of CIDR. Two rams of proven fertility harnessed with marking crayons were placed with a group of 5 ewes 1 day after CIDR removal. Embryos were surgically collected 48 h after the ewes had been marked. Ewes were deprived of food and water 12 h prior to the surgery. On the day of surgery, animals were injected with 0.2 ml Rompun (Bayer, Australia Ltd., Botany, NSW, Australia) and 0.1 ml Ketamine (Parke-Davis, Caringbah, NSW, Australia) 5 min prior to surgery. Gas anaesthesia was induced using oxygen and 4% halothane to the animal. When the ewe had reached a satisfactory depth of anaesthesia and had stabilized, this state was maintained by reducing the halothane to 2.5% gas mixture. The uterine horns and ovaries were then exteriorized and an 18-gauge needle was inserted in the uterus at the utero-tubule junction and approximately 10 ml of Dulbecco's phosphate buffered solution (Horizon Pty Ltd, Australia) containing 3% BSA (v/v) was flushed through the oviduct into a tube (1.5 mm in diameter optical density, OD), which had been introduced via the fimbriae. Flushing fluids were collected in 5 cm Petri dishes and embryos were recovered. The flushing medium and dishes were pre-warmed in an incubator at 37 °C.

All the animal experimentation in this study was approved by the University of New England Animal Ethics Committee and in accordance with the National Health and Medical Research Council of Australia guidelines for animal experimentation.

Embryo culture *in vitro*

Human tubal fluid (HTF) medium (Quinn *et al.* 1985) was prepared in advance using chemicals from Sigma. Mouse embryos at the two-cell stage were collected by flushing the oviducts of mated animals 42 h after hCG injection. Embryos were collected and then washed in three changes of M2 (Sigma) media. The embryos were randomly divided into groups of 18–25 and transferred into a 25 µl drop of HTF medium. Drops of culture medium were placed in a culture dish (Nunc, Roskilde, Denmark) and covered with mineral oil (Sigma) and pre-incubated in a humidified atmosphere of 5% CO₂ at 37 °C for 30 min. In each experiment, 45–60 embryos were employed and the experiment was

repeated five times. Media and leptin were replaced every 24 h by removing 20 µl of media and replacing it with 20 µl media supplemented with fresh leptin.

Sheep embryos at the two-cell stage were collected surgically and washed in three changes of synthetic oviduct fluid (SOF) medium supplemented with 8 mg/ml BSA (Steeves & Gardner 1999). Embryos were cultured in 25 µl droplets of culture medium in a 24-well plate covered with mineral oil in groups of 3–5. All media were equilibrated in the culture dishes in a humidified atmosphere containing 5% CO₂ in air at 39 °C for 30 min. For the first 48 h of culture, SOF+1 mM Glu+1% non-essential amino acids was used, and for the last 72 h of culture, SOF+1 mM Glu+1% non-essential amino acids+1% Eagle's minimum essential medium essential amino acids without Glu was used.

Determination of cell numbers in embryo

Embryo cell numbers were determined by the air-drying method. Briefly, embryos were put into a hypotonic solution 1 and 0.5% sodium citrate for 20 and 10 min respectively. They were then transferred to a methanol:acetic acid (3:1, v:v) solution for a further 5–10 min. Embryos were transferred to a microscope slide in a minimal amount of mixture of methanol and acetic acid. Before drying of fixative was complete, 1–2 drops 60% acetic acid was added to facilitate embryo spreading. After staining with 5% Giemsa (Curr's R66 Improved; BDH Chemical, Poole, Dorset, UK) solution for 5 min, the total cell numbers were counted under a microscope.

RNA extraction and RT-PCR

Mice embryos at the two-, four- and eight-cell stages were collected by flushing the oviducts of mated animals at 42, 52 and 66 h after hCG injection with M2 medium. The blastocysts were collected from the uterus at 90 h of hCG injection. Fifty to 100 embryos were washed in

three changes of the medium and then transferred into 0.8 ml of TRIzol reagent containing 50 µg of carrier RNA (yeast tRNA; Sigma). Samples were then homogenized by vigorous shaking by hand (Stojanov & O'Neill 1999). The detection of mRNA for leptin and leptin receptor in embryos was subjected to 45 cycles of PCR condition. The primers for leptin, Ob-Ra and Ob-Rb, were made based on published sequences as shown in Table 1 and all PCR conditions were listed in Table 2. Experiments were repeated three times on three separate pools of embryos at each stage and the tissues from three different animals. All PCR products were sequenced to confirm their identity and negative controls were run with water substituted for cDNA and PCR performed prior to RT (data not shown).

Experiment 1: effects of increasing concentrations of leptin

Two-cell stage mouse embryos were randomly placed in microdrops of HTF containing recombinant human leptin (R&D Systems, Minneapolis, MN, USA) at concentrations of 0, 0.1, 1, 10 and 100 ng/ml. The numbers of two-cell embryos that developed to four-cell embryos, eight-cell embryos, blastocysts and hatched blastocysts were counted at 70, 96, 120, and 144 h post-hCG respectively and the percentages of two-cell embryos that developed to the different embryonic stages calculated.

Experiment 2: determination of the sensitivity of mouse embryos to leptin

The aim of this experiment was to determine the stage of embryo development, which was affected by leptin. Embryos at the two-cell stage were cultured in HTF medium *in vitro* for 0, 24, or 48 h to obtain two-, four-, or eight-cell embryos respectively. Embryos at each developmental stage were then cultured in HTF-containing leptin at concentrations of 0, 10 or

Table 1 Sequence of primers for mouse leptin, leptin receptors and GAPDH.

Target gene (GeneBank accession no.)	Sequence	PCR product size (bp)
Leptin (U18812) (Hoggard <i>et al.</i> 2000)		
Sense	TGAGTTTGTCCAAGATGGACC (208–229)	
Antisense	GCCATCCAGGCTCTCTGG (381–399)	191
Ob-Ra (U49106) (Hoggard <i>et al.</i> 1997)		
Sense	ACACTGTTAATTCACACCAGAG (2568–2591)	
Antisense	AGTCATTCAAACCATAGTTTAGG (2781–2805)	237
Ob-Rb (U49107) (Hoggard <i>et al.</i> 1997)		
Sense	GTGTGAGCATCTCTCCTGGAG (2829–2849)	
Antisense	ACCACACCAGACCCTGAAAG (3362–3343)	533
Ob-Re (AF0078195)		
Sense	GCAGAATCAGCACACACTGTT (422–462)	
Antisense	GTAAAAGCACAGTACACATACC (721–742)	297
GAPDH (XO2231)		
Sense	CGTCTTCACCAACATGGAAGA (365–385)	
Antisense	CGGCCATCACGCCACAGTTT (645–664)	300

Table 2 PCR conditions for leptin, leptin receptors and GAPDH.

Leptin and Ob-Rb	94 °C (4 min), 1-cycle; 94 °C (40 s), 55 °C (40 s), 72 °C (40 s), 35-cycles; 72 °C (10 min) -1 cycle
Ob-Ra	94 °C (4 min), 1-cycle; 94 °C (40 s), 51 °C (40 s), 72 °C (40 s), 35-cycles; 72 °C (10 min) -1 cycle
Ob-Re	94 °C (4 min), 1-cycle; 94 °C (40 s), 56 °C (40 s), 72 °C (40 s), 35-cycles; 72 °C (10 min) -1 cycle
GAPDH	94 °C (4 min), 1-cycle; 94 °C (40 s), 60 °C (40 s), 72 °C (40 s), 35-cycles; 72 °C (10 min) -1 cycle

100 ng/ml. The numbers of embryos that developed to four-cell stage embryos, eight-cell stage embryos, blastocysts and hatched blastocysts were counted at 72, 96, 120, and 144 h post-hCG respectively. A preliminary experiment showed that leptin treatment at the eight-cell stage tended to accelerate blastocyst formation, so the percentages of eight-cell stage embryos that developed to blastocysts were assessed at 116 and 120 h post-hCG and hatched blastocysts were counted at 138 and 144 h post-hCG respectively. A sub-sample of the blastocysts ($n=5$) that developed in each treatment was collected at 120 h post-hCG and their total number of cells counted, as previously described.

Experiment 3: effect of leptin on sheep embryo

Experiment 3 was conducted to determine the effect of leptin on early sheep embryonic development. Two-cell stage sheep embryos were cultured in SOF medium containing leptin at concentrations of 0, 1, 10 and 100 ng/ml. The percentage of two-cell stage embryos that developed to four-, eight-cell embryo, morula, blastocysts and hatched blastocyst was assessed at 24, 48, 96, 120 and 144 h of culture respectively.

Statistical analysis

Overall effects of treatments were evaluated by the General Linear Model procedure and followed by Student–Newman–Keuls test for multiple comparisons using the SAS statistical software (SAS Institute, Inc.,

Cary, NC, USA). Differences of $P<0.05$ were considered significant.

Results

Effect of leptin on mouse embryo development

To determine the effects of leptin on the *in vitro* development of mouse pre-implantation embryos, two-cell stage embryos were cultured in the presence of 0.1, 1, 10 and 100 ng/ml human recombinant leptin and the percentage of two-cell embryos that developed to the different embryonic stages are shown Fig. 1. The lowest concentrations of leptin (0.1 and 1 ng/ml) had no effect on the number of two-cell embryos that developed into four-, eight-cell stage embryos and blastocyst or hatched blastocyst ($P>0.05$) compared with the control. At a concentration of 10 ng/ml, leptin had no effect on the four- and eight-cell embryo formation when compared with the control, whereas this concentration significantly increased the proportion of embryos developing to blastocysts from 60 to 72% and to hatched blastocysts from 39 to 51%, compared with the control ($P<0.05$). The rates of development into blastocysts and hatched blastocysts in the 10 ng/ml treatment were significantly higher than that for embryos in 0.1 or 1 ng/ml treatment.

Increasing the concentration of leptin to 100 ng/ml resulted in an inhibitory effect on all stages of embryo development, with the proportion of four-, eight-cell, blastocyst and hatched blastocyst stage embryos decreasing significantly ($P<0.05$), when compared with the control and the other treatment groups.

Figure 1 The effects of increasing concentrations of leptin on the development of mouse two-cell embryo to the four-cell, eight-cell, blastocyst, and hatched blastocyst stages. Data are presented as means $\pm$ S.E.M. of 5 replicates. Embryos were observed at specific time points, and the proportion of embryos at each stage was recorded: four-cell at 70 h post-hCG; eight-cell at 96 h post-hCG; blastocyst at 120 h post-hCG; hatched blastocyst at 144 h post-hCG. Different letters above the bars within the one developmental stage indicate significant differences ($P<0.05$). Hatched B: hatched blastocyst.

Sensitivity of embryos to leptin

To determine whether the sensitivity to leptin varied at different stages of development, embryos at two-, four- and eight-cell stages were treated with leptin at concentrations of 10 and 100 ng/ml. When embryos were exposed to leptin treatment at the two-cell stage, results similar to those observed in Experiment 1 were obtained (data not shown). As shown in Fig. 2A, the addition of 10 ng/ml leptin at the four-cell stage had no effect on the eight-cell stage embryo formation compared with the control. However, leptin stimulated the development rate of the blastocyst and hatched blastocyst and the corresponding rates were 85 and 69% respectively, which differed significantly ($P < 0.05$) from the control. A leptin concentration of 100 ng/ml inhibited the formation of eight-cell embryos although this was not significant. The development into the

Figure 2 Effects of 10 ng/ml and 100 ng/ml leptin on the development rate of mouse embryos, added at the four-cell stage (panel A) and 8-cell stage (panel B). Data are presented as means $\pm$ S.E.M. in 5 replicates. Embryos were observed at specific time points, and the proportion of embryos at each stage was recorded: 8 cell at 96 h post-hCG; blastocyst at 120 h post-hCG, hatched blastocyst at 144 h post-hCG. Different letters above the bars within the one developmental stage indicate significant differences ($P < 0.05$).

blastocyst as well as blastocyst hatching was significantly impaired compared with the control.

The addition of 10 ng/ml leptin at the eight-cell stage significantly increased ($P < 0.05$) the proportion of blastocysts and hatched blastocysts when compared with the control (Fig. 2B). This result was similar to that obtained from the two- and four-cell stage embryos. However, most interestingly, the treatment of 100 ng/ml leptin had no effect on the development rates when the embryos were added at the eight-cell stage, with the blastocysts and hatched blastocysts formation being similar to that of the control.

Preliminary experimental results showed that leptin treatment at the eight-cell stage tended to accelerate blastocyst formation, the percentage of eight-cell stage embryos that developed to blastocysts were assessed at 116 and 120 h post-hCG and hatched blastocysts were counted at 138 and 144 h post-hCG respectively. As shown in Fig. 3, 10 ng/ml leptin treatment significantly accelerated the formation of blastocysts ($P < 0.05$), with 35% of total blastocyst formed by 116 h post-hCG, while only 10% developed to blastocyst in the control group. Treatment with 100 ng/ml leptin tended to promote cell proliferation and advance blastocyst formation, with 16% of blastocyst formed by this time, which was not significantly ($P > 0.05$) different from the control.

Similar to the effects on blastocyst development, the 10 ng/ml leptin treatment significantly advanced the formation of hatched blastocyst by 138 h post-hCG ($P < 0.05$), 66% of total hatched blastocyst developed at this time point, while the corresponding percentage for the control was 46%. However, there was no significant difference ($P < 0.05$) between the control group and the 100 ng/ml treatment group.

The total numbers of cells in blastocysts obtained by cultures containing 10 or 100 ng/ml leptin, when supplemented at the two-, four- and eight-cell stages are shown in Table 3. The supplementation of 10 ng/ml leptin at all three stages significantly increased ($P < 0.05$) the total cell number of blastocysts in the treatment group when compared with the control. In contrast, the supplementation of 100 ng/ml leptin at the two- and four-cell stages significantly reduced ($P < 0.05$) the total cell number of blastocysts when compared with the control; however, this concentration had no effect on the total cell number of blastocysts when added at the eight-cell stage.

Effect of leptin on sheep embryo development

To determine the effects of leptin on the *in vitro* development of the sheep embryo, two-cell stage embryos were cultured in the presence of 1, 10 and 100 ng/ml human recombinant leptin; results are shown in Fig. 4. In each experiment, 5–6 embryos were tested in each group and the experiment was repeated five times. The percentage of embryos that developed into the four-

Figure 3 Rate of blastocyst and hatched blastocyst formation at 116 h and 138 h post-hCG in exposure of 10 ng/ml and 100 ng/ml leptin at the eight-cell stage. Data are presented as means $\pm$ S.E.M. in 6 replicates. Embryos were observed at specific time points, and the proportion of embryos at each stage was recorded: blastocyst at 116 h post-hCG, hatched blastocyst at 138 h post-hCG. Different letters above the bars within the one time point indicate significant differences ($P < 0.05$).

and eight-cell stages was not affected by leptin at any of the treatment levels tested ($P > 0.05$) except that the 100 ng/ml treatment significantly lowered the eight-cell embryo formation when compared with the control, 1 and 10 ng/ml groups ($P < 0.05$). The percentage of embryos that developed into morula, blastocysts and hatched blastocysts when treated with 10 ng/ml leptin was significantly higher ($P < 0.05$) than the control. No effect on embryo development from the four-cell stage to the morula was found in the 1 ng/ml treatment, however, it significantly increased ($P < 0.05$) the blastocyst and hatched blastocyst rates of development when compared with the control. The lowest morula, blastocyst and hatched rates were obtained in the 100 ng/ml treatment group, which was significantly lower ($P < 0.05$) than the control.

Expression of leptin and leptin receptor in pre-implantation mouse embryo

In the preliminary experiments using adipose tissue cDNA, the RT-PCR assays were optimised by testing the different annealing temperatures for leptin and leptin receptor mRNA expression (data not shown) and optimal

conditions were used for the following experiments. The mRNA pattern of expression for leptin and the three isoforms of leptin receptor (Ob-Ra, Ob-Rb and Ob-Re) were studied in mouse pre-implantation embryos at different stages (two-, four-, eight-cell and blastocyst) using RT-PCR. All embryos at each developmental stage as well as the adipose tissue expressed GAPDH mRNA (Fig. 5, GAPDH, lane 2, 3, 4, 5 and 6), confirming the integrity of the RNA and the RT-PCR. Leptin mRNA was not detected in embryos at the two-, four-, eight-cell and blastocyst stage embryos with leptin-specific primers (Fig. 5, Leptin, lane 3, 4, 5 and 6). RT-PCR analysis with leptin primers and mRNA isolated from adipose tissue gave rise to the expected fragments (191 bp) (Fig. 5, Leptin, lane 2). No band was detected in the negative controls (Fig. 5, Leptin, lane 1).

Electrophoresis of RT-PCR products for Ob-Ra, Ob-Rb and Ob-Re were identified in two-, four-, eight-cell and blastocyst stage embryos (Fig. 5, Ob-Ra and Ob-Rb), with a weaker intensity for Ob-Re when compared with both Ob-Ra and Ob-Rb for all embryo stages tested (Fig. 5, Ob-Re). The mRNA for mouse GAPDH was detected in embryos at all developmental stages tested (Fig. 5, GAPDH). No bands were detected in the negative controls (Fig. 5, Ob-Ra, Ob-Rb and Ob-Re, lane 1).

Discussion

Mouse embryos

The results of this study confirm that leptin improves early embryonic development at physiological concentrations *in vitro* (Tomimatsu *et al.* 1997, Kawamura *et al.* 2002). In contrast, the presence of high leptin concentrations (100 ng/ml) exerts an inhibitory effect on the two- and four-cell stage embryos developing into advanced stages when cultured *in vitro*. However, the inhibitory impact of high leptin concentration on the embryo development was diminished by the eight-cell stage. Collectively, these data indicate that leptin has a concentration and developmental stage-dependent effect on early mouse embryo development.

Contradictory findings have been reported with respect to leptin's effects on embryo development in the *in vitro* culture. In agreement with our observations, it has been reported that the 10 ng/ml leptin significantly increased the development rate of two-cell stage

Table 3 Effects of addition of 10 and 100 ng/ml leptin at the mouse 2-, 4-, and 8-cell stages on the total cell number of blastocyst. Blastocysts were collected and stained at 120 h post-hCG.

Treatment	Control	10 ng/ml Leptin	100 ng/ml Leptin
Two-cell stage	65.9 $\pm$ 2.6 ^a (n=30)	76.0 $\pm$ 3.4 ^b (n=28)	54.0 $\pm$ 2.7 ^c (n=30)
Four-cell stage	64.1 $\pm$ 2.8 ^a (n=28)	75.4 $\pm$ 3.1 ^b (n=28)	54.3 $\pm$ 3.5 ^c (n=27)
Eight-cell stage	64.8 $\pm$ 2.9 ^a (n=29)	78.0 $\pm$ 3.5 ^b (n=25)	71.0 $\pm$ 3.5 ^{a,b} (n=25)

Significant difference was compared within rows. Data was analyzed by ANOVA and SHK multiple comparison test. Different letters indicate significant difference between values within rows ($P < 0.05$).

Figure 4 Effects of various concentrations of leptin on the development of sheep two-cell embryo to the 4-cell, 8-cell, morula, blastocyst and hatched stages. Data are presented as means $\pm$ S.E.M. in 5 replicates. Embryos were observed at specific time points, and the proportion of embryos at each stage was recorded, with 4-cell, 8-cell embryo, morula, blastocysts and hatched blastocyst assessed at 24 h, 48 h, 96 h, 120 h and 144 h of culture, respectively. Different letters above the bars within the one developmental stage indicate significant differences ($P < 0.05$).

embryos into blastocysts and hatched blastocysts (Kawamura *et al.* 2002, 2003). In the present study, the 100 ng/ml leptin treatment exhibited an inhibitory effect on two-cell embryos, while Kawamura and co-workers (2002, 2003) found that leptin, at concentrations of 100 and 1000 ng/ml, produced a further stimulatory effect on embryo development. One possible explanation for the difference is that the Kawamura *et al.* (2002, 2003) studies used the IVCS strain of mouse, in contrast to the Swiss random-bred strain used in our study. Serum

leptin concentrations are reported to differ between various strains of rats, with the variation up to two times, suggesting that different genetic background can affect the circulating leptin levels (Landt *et al.* 1998). If this was the case in the mouse, it is possible that there are different thresholds existing in tissues and cells with respect to leptin sensitivity in various strains of mice. Another possible explanation is that the different types of leptin used in the two experiments, since exogenous recombinant human leptin has a half-life period of 1 h in the circulation of mice (Chehab *et al.* 1997), whereas injected mouse recombinant leptin in fasted mice had a half-life period of 3 h (Ahima *et al.* 1996). Interestingly, in complete contrast to the findings of Kawamura *et al.* (2002, 2003) and present study, two recent studies showed that leptin treatment neither influences embryo development or degeneration (Swain *et al.* 2004), nor inhibited the proportion of embryos reaching the hatched blastocyst stage through increased apoptosis (Fedorcsak & Storeng 2003). Therefore, the discrepancies of results in those studies could not be simply interpreted by the above explanations as these utilized mouse embryos and mouse recombinant leptin (Fedorcsak & Storeng 2003, Swain *et al.* 2004). Particularly puzzling are the results by Fedorcsak & Storeng (2003), who showed that leptin concentrations of 1.6 to 160 ng/ml exhibited an inhibitory effect on embryo development, which cover the range of physiological levels of leptin in mice. These are in contrast with the concept that there is a timely requirement of leptin for a successful pregnancy in mice (Malik *et al.* 2001).

This study demonstrated that, while leptin may have a regulatory role in pre-implantation embryo development, it also modulates cell proliferation in mouse embryos. Blastocysts cultured with 10 ng/ml of leptin at two-, four-, and eight-cell stages had a significantly higher total cell number as compared with blastocysts cultured in the control group. Furthermore, we also observed that the 10 ng/ml leptin treatment advanced the formation of blastocysts when leptin was added at the eight-cell stage. This may be due to the effect of leptin in promoting cell proliferation throughout the early development of mouse embryos. These results are consistent with the observations in this study that leptin influences the development rates, suggesting that leptin regulates embryo development via cell proliferation. It has also been reported that leptin increased the total cell number of blastocysts, especially the trophectoderm cells, which are necessary for implantation and form the placenta and extrayembryonic membranes (Kawamura *et al.* 2002, Craig *et al.* 2005). Collectively, these results provide the evidence for the mechanism by which leptin regulates implantation.

The developmental period during which the addition of leptin to culture medium affected mouse embryo development *in vitro* in the present study (prior to the

Figure 5 RT-PCR detection of leptin, Ob-Ra, Ob-Rb and Ob-Re mRNAs from the different stages of mouse embryo. The mouse adipose tissue was used as positive control. The quality of each cDNA was determined by the PCR products of amplification of the mouse GAPDH primers. The tissues were all extracted and reverse transcribed at the same time in each experiment. No bands were observed in the absence of cDNA (negative control). MM: Molecular marker; lane 1: negative control; lane 2: adipose tissue (positive control); lane 3: 2-cell embryos; lane 4: 4-cell embryos; lane 5: 8-cell embryos; lane 6: blastocysts.

blastocyst) seems to coincide with the period during which mouse embryos are present in the oviduct *in vivo*. Previous studies (Gonzalez *et al.* 2000, Kawamura *et al.* 2002) have shown that leptin is produced by oviduct epithelial cells in mice, so the addition of leptin to embryo culture *in vitro* may reproduce the environment of the cleavage-stage embryos in the oviduct *in vivo*. The requirement of leptin for embryo development changes during the gestational period, with lower levels being beneficial in the early embryogenesis and higher levels in the later stages. This concentration and stage-specific effect of leptin may explain, at least in part, why women with a low leptin body mass index ratio had significantly more superior quality embryos on day 3 post-retrieval (Brannan *et al.* 2001), while the patients with higher leptin concentrations at 12 days after embryo transfer had a higher potential for pregnancy (Unkila-Kallio *et al.* 2001). This may also explain in part why the pre-implantation uterine environment is normally hostile to pre-morula-stage embryos and blastocysts fail to survive in the non-receptive uterine environment (Paria *et al.* 1993).

Sheep embryos

The time of embryonic genome activation differs among rodents and ruminant embryos and it is believed that this is associated with the *in vitro* culture-induced developmental block (Thompson 2000). In addition, leptin and the leptin receptor system in rodent pregnancy differ significantly from that in both human, non-human primates (Henson & Castracane 2005) and ruminants (Chilliard *et al.* 2005). Therefore, we examined the leptin effect on sheep embryo development *in vitro* and obtained similar results as that seen in the mouse embryo culture experiment. The leptin treatment (10 ng/ml) was found to be more effective in stimulating the two-cell stage sheep embryos developing into morula, blastocyst and hatched blastocyst. The treatment of 1 ng/ml leptin also significantly increased the percentage of blastocyst and hatched blastocyst formation in sheep embryo culture, which was not observed in mouse embryo, suggesting that there may be a different sensitivity to leptin existing among embryos from different species.

Considerable information is available concerning the effect of nutrition on ovulation and embryo development in the sheep. Increased dietary intake for a relatively short time prior to mating increased the ovulation rate and oocyte quality, and consequently produced good quality embryos in sheep. However, high nutrition resulted in a negative effect on oocyte quality and resulted in decreased pregnancy rates (Boland & O'Callaghan 1999, 1999). *in vivo* leptin levels are influenced by energy balance with an elevation in plasma leptin following an increase in the plane of nutrition of ewes (Blache *et al.* 2000b). The nutritional switch-over study in sheep also revealed that maternal

leptin levels increased 65% within 48 h after the dietary switch, and is independent of the changes in adipose tissue mass (Thomas *et al.* 2001). These data suggest that leptin may be an important factor in regulating early pregnancy and the requirement of this hormone appears to be within a limited range, with too much or too little being detrimental to oocyte quality and embryo development. Similar to mouse embryo, our results of leptin effects on sheep embryo development provide evidence for this concept by showing that physiological levels of leptin promote early embryo development, whereas high concentrations of leptin inhibit this process.

Leptin and leptin receptors in the embryo

In the present study, leptin mRNA was not detected in the two-, four-, eight-cell stages and blastocyst stage mouse embryos by RT-PCR. Using *in situ* hybridisation and immunohistochemistry, a study on the ontogeny of leptin expression in murine foetus has shown that leptin mRNA and protein are expressed in bone, liver and hair follicles as early as day 13.5 post-coitus, indicating that leptin expression in the foetus starts from mid-gestation (Hoggard *et al.* 1997). However, leptin mRNA expression was detected in the mouse blastocyst and hatched blastocyst stage embryos (Kawamura *et al.* 2002).

We have detected both Ob-Ra and Ob-Rb mRNA expressions in the two-cell to blastocyst stage embryos. Similarly, mRNA for Ob-Ra and Ob-Rb was reported in mouse oocytes, one-, two-cell, morula, blastocyst and hatched blastocyst stages but not four- and eight-cell stage embryos (Kawamura *et al.* 2002, 2003). However, we were able to detect the expression of mRNAs for Ob-Ra and Ob-Rb in the four- and eight-cell stage mouse embryos in the present study. Several lines of evidence from mouse and cattle data indicate that gene expression patterns in the embryo are affected by *in vitro* culture conditions, especially by the presence or absence of serum in the culture medium (Niemann & Wrenzycki 2000). We employed embryos developed *in vivo*, whilst these authors collected *in vivo* two-cell stage embryos and cultured them to four- and eight-cell stages *in vitro*, from which embryo mRNA was extracted. Therefore, it is likely that the environment which the embryos find themselves in, may modulate leptin receptor expression and have contributed to the differing results where there was approximately a 30% blastocyst formation rate in their study (Kawamura *et al.* 2002), compared with 65% in our experiment. This is supported in part by the observation that improved media such as KSOM yield higher blastocyst formation rates (Erbach *et al.* 1994).

The secreted form of the leptin receptor, Ob-Re, circulates in plasma and is capable of binding to leptin (Gavrilova *et al.* 1997). Leptin binds to Ob-Re with a high affinity thereby delaying the clearance of leptin

from circulation and increasing the half-life period of leptin. Consequently, hyperleptinemia is generally observed in late pregnancy in the mouse (Gavrilova *et al.* 1997). To our knowledge, the results of this study show for the first time that mRNA for the leptin-soluble form receptor (Ob-Re) is present in the two-, four-, eight- and blastocyst stage mouse embryos. Considering the presence of leptin protein in both mouse and human oocytes and embryos, which is suggested to be critical for the establishment of inner cell mass and trophoblast development, in conjunction with the absent expression of leptin mRNA in embryos during this period (Antczak & Vanblerkom 1997, present study), the availability and transportation of leptin seems to be important for embryo development before the blastocyst stage. Therefore, our results present the possibility that leptin modulates embryo development via a paracrine signalling system, with the assistance of Ob-Re to delay the clearance of leptin, or indeed increase the local concentration of leptin in these cells.

Signal transducers and activator of transcription (STAT) proteins, known to be involved in transcriptional regulation, are implicated in the mechanism of leptin signal transduction (Antczak & Vanblerkom 1997). Leptin may influence tissue- and cell-specific gene expression within the reproductive systems through the JAK/STAT pathway (Baumann *et al.* 1996, Bendinelli *et al.* 2000). Leptin and STAT-3 are differentially distributed within the cells of pre-implantation stage embryos (Antczak & Vanblerkom 1997, Fedorcsak & Storeng 2003), hence leptin may regulate embryo development by influencing the activity of STAT-3 to control DNA-directed mRNA synthesis or DNA fragmentation.

Since the mRNA expression for Ob-Ra, Ob-Rb and Ob-Re in two-, four-, eight-cell and blastocyst stage embryos was observed consistently, we could not provide a satisfactory explanation, in the context of leptin and leptin receptor interaction, for the mechanism by which leptin affects embryo development in a concentration and stage-dependent manner. Unfortunately, we were unable to measure quantitative change in the mRNA expression for these different isoforms. It is important to acknowledge, however, that the changes in the number of mRNA copies for these different isoforms of leptin receptor in embryo during pre-implantation developmental stages, may contribute to the concentration and stage-dependent leptin effect on the embryos growth. However, a similar stimulatory and inhibitory response to leptin was observed by Yu *et al.* (1997) in a study on the release of gonadotrophins from pituitary cultures. Finally, Wang *et al.* (2004) and Hamatani *et al.* (2004) using microarray analysis of RNA amplified from one-cell mouse embryos through to blastocysts have demonstrated the expression of leptin and its receptor in these samples. However, confirmation of the expression of leptin and its receptor by real-time PCR or *in situ* hybridisation assays were not reported in these studies.

Conclusion

In summary, it is evident that leptin has the ability to regulate the development of the pre-implantation embryo *in vitro*. In the mouse, supplementation of leptin, at physiological concentrations (10 ng/ml), to culture medium exhibited a beneficial effect on the embryo development at two-, four- and eight-cell stages. However, the high concentration of leptin (100 ng/ml) in culture medium showed an inhibitory effect on the embryo growth at two- and four-cell stages, but had no effect at eight-cell stage. Therefore, the influence of leptins on embryo development appears to be dependent on both concentration and the developmental stage of the embryo. The concentration-dependent regulation pattern of leptin on the embryo development was also confirmed by the sheep embryo experiment. By comparing the development rates of embryo at various leptin concentrations in mice and sheep, there seems to be species differences existing in embryos with regard to the sensitivity to leptin.

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