Leptin and reproduction

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Fertility in mammals requires adequate nutrition and a certain reserve of metabolic fuel (Van Der Spuy, 1985). Individuals experiencing wasting diseases (e.g. insulin-dependent diabetes), severe dietary restriction (e.g. anorexics), or who are high-performance athletes (e.g. marathon runners or ballet dancers) may have severely impaired reproductive systems (De Souza & Metzger, 1991; Stewart, 1992; Griffen *et al.* 1994). The effect of nutritional status on reproduction is postulated to reflect the action of unknown metabolic signal(s) that are recognized by the brain and serve as indices of metabolic state (Kennedy & Mitra, 1963). Leptin has been postulated to be either the, or one such, signal. Thus, if metabolic reserves are low or the system is stressed, reproduction will be inhibited (Barash *et al.* 1996; Chehab *et al.* 1996).

Restoration of fertility in *ob/ob* mice

The sterility of male and female homozygous *ob/ob* mice is a recognized feature of the *ob* mutation (Ingalis *et al.* 1950). The *ob/ob* males can occasionally reproduce if maintained on a restricted diet, but *ob/ob* females are always sterile (Lane & Dickie, 1954). Reproductive hormones are reduced in *ob/ob* females, suggesting a functional defect in the hypothalamic–pituitary axis (Batt, 1972; Batt *et al.* 1982).

The repeated administration of recombinant human leptin into female *ob/ob* mice corrects their sterility; thus ovulation, pregnancy, parturition and lactation are each restored (Chehab *et al.* 1996). In initial studies, it was determined that leptin withdrawal at day 14 of pregnancy did not prevent continued gestation and parturition. However, subsequent generations of the female *ob/ob* pups were unable to reproduce following leptin withdrawal, demonstrating that correction of their sterility requires continuous leptin treatment (Chehab *et al.* 1996).

The administration of leptin for 14 d to female *ob/ob* mice results in increased ovarian weight compared with pair-fed female *ob/ob* controls. The histology of the leptin-treated ovary showed greater amounts of follicular development consistent with activation of ovarian function. The trophic action of leptin on gonadal function apparently led to an increase in sex steroids, as shown by increased uterine weights. This appeared to be due to the proliferative growth of the uterine glands, epithelium and endometrium, which is a typical response to stimulation by oestrogen. In addition to the responses in females, male *ob/ob* mice treated with leptin showed an elevation in testicular weight compared with pair-fed male *ob/ob* controls. Testicular histology suggested that leptin stimulated cellular activity in the seminiferous tubules. The increased weight of the seminal vesicles was accompanied by an increase in epithelial height, indicating increased circulating levels of testosterone (Barash *et al.* 1996).

A signal triggering the onset of puberty?

The ability of leptin to restore fertility to mice that are genetically deficient in leptin suggests that this hormone may be a signal triggering the onset of reproductive function. Indeed, leptin has been shown to accelerate the onset of puberty in normal female mice (Ahima *et al.* 1997; Chehab *et al.* 1997). Normal pre-pubertal mice injected with leptin reproduce earlier, showing earlier maturation of the reproductive tract as determined by: (1) timing of vaginal opening, (2) progress toward the first oestrus cycle and (3) the weights of uteri, ovaries and oviducts. In mice treated with leptin there was a concomitant decrease in luteinizing hormone and 17β-oestradiol compared with the saline-treated mice. Leptin treatment did not appear to interfere with successful ovulation, pregnancy or delivery of pups (Ahima *et al.* 1997; Chehab *et al.* 1997). Importantly, the effect of leptin on puberty can occur in the absence of any effect on body weight (Ahima *et al.* 1997). This suggests that the actions of leptin to regulate neuroendocrine and reproductive function in normal mice are not secondary to effects on energy balance.

Hypothalamic–pituitary–gonadal axis

The presence of functionally-active leptin receptors in the hypothalamus (Mercer *et al.* 1996*b*; Schwartz *et al.* 1996) and the fact that leptin increases luteinizing hormone in female *ob/ob* mice and fasted normal mice suggested that leptin acts at a central level primarily through the hypothalamic– pituitary–gonadal axis (Chehab *et al.* 1996, 1997; Ahima *et al.* 1997). There is strong evidence both from functional studies and from co-localization of the leptin receptor with preproneuropeptide Y gene expression that hypothalamic neuropeptide Y is an important neuroendocrine target for leptin (Stephens *et al.* 1995; Mercer *et al.* 1996*a*; Schwartz *et al.* 1996). Interestingly, neuropeptide Y has been widely implicated in the regulation of gonadotropin-releasing hormone secretion (Kalra, 1993). It has also been

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https://doi.org/10.1079/PNS19980061 Published online by Cambridge University Press

demonstrated that rats treated intracerebroventricularly with leptin antiserum showed a marked impairment in luteinizing hormone pulsatility, compatible with leptin regulating the reproductive axis by influencing gonadotropin-releasing hormone pulsatility (Carro *et al.* 1997).

Leptin at very low concentrations has been shown to stimulate luteinizing hormone-releasing hormone release from hypothalamic explants and follicle-stimulating hormone and luteinizing hormone release from anterior pituitaries of adult male rats. This may indicate that leptin plays an important role in controlling gonadotropin secretion by stimulating both hypothalamic and/or pituitary actions (Yu *et al.* 1997).

In addition, leptin receptors have been reported in the ovary and testis, so leptin may act as a metabolic signal to the reproductive system via a direct action on the gonads (Cioffi *et al.* 1996). Indeed, leptin has been shown to inhibit insulin-induced progesterone and oestradiol production by granulosa cells (Spicer & Francisco, 1997; Zachow & Magoffin, 1997). Overall, it seems likely that the action of leptin on the reproductive system arises from a combined influence on the brain and the gonads.

Leptin and its role in pregnancy

The role of leptin in the restoration of fertility in *ob/ob* mice and also in the onset of puberty in lean female mice, coupled with its effects on appetite and thermogenesis, suggested that the hormone may play an important role during pregnancy. Indeed, circulating leptin levels appear to rise towards the end of pregnancy in human subjects and in rodents, with a fall in titre to below pre-pregnancy levels at around birth. This effect is particularly pronounced in the mouse, which has been reported to show a 25-fold rise (Tomimatsu *et al.* 1997) in leptin on day 17 in the maternal circulation compared with a 2·7-fold and 1·8-fold maximum rise in human subjects (Hardie *et al.* 1997) and rats (Chien *et al.* 1997) respectively, relative to the pre-pregnancy levels.

There appear at present to be three possible explanations for the increase in leptin levels in pregnancy: (1) increased production by maternal fat, (2) expression by the placenta and (3) increased levels of binding protein(s) in the maternal circulation.

Increased production by maternal fat

In rodents, leptin mRNA levels have been shown to increase during pregnancy in the parametrial adipose tissue, as determined by Northern blotting, compared with the non-pregnant rodent (Tomimatsu *et al.* 1997). Another report, however, suggests there is no change in expression from this fat depot (Gavrilova *et al.* 1997). This contradiction remains to be resolved and does not rule out differential expression by other fat depots in the pregnant rodent. During pregnancy a number of hormones increase in concentration, such as cortisol, insulin and oestradiol, and it is interesting to note that these have been shown to stimulate leptin expression in adipocytes from *in vivo* studies on laboratory animals and/or from cell-culture studies (Shimizu *et al.* 1997; Trayhurn *et al.* 1998).

Expression by the placenta

A recent finding shows that leptin, which was previously thought to be exclusively produced by adipocytes, is also produced at a significant level in the placenta (Green *et al.* 1995; Hoggard *et al.* 1997*a*; Masuzaki *et al.* 1997). Expression of the leptin gene in the human placenta can be detected by Northern blotting, but this technique is not sensitive enough to detect leptin mRNA in the rodent placenta. However, we have shown leptin gene expression can be detected by reverse transcription–polymerase chain reaction and by *in situ* hybridization in the murine placenta (Figs. 1 and 2; Hoggard *et al.* 1997*a*). Interestingly, high levels of immunoreactive leptin protein can be detected in the murine placenta at levels comparable with those in the human placenta (Fig. 3; Hoggard *et al.* 1997*a*; Tomimatsu *et al.* 1997). Leptin appears to be expressed by the placental trophoblasts in both the rodent (Fig. 4) and human placenta (Masuzaki *et al.* 1997).

The leptin-like immunoreactivity detected in the human placenta is considered to be identical to human leptin of adipose origin on the basis of size, charge and

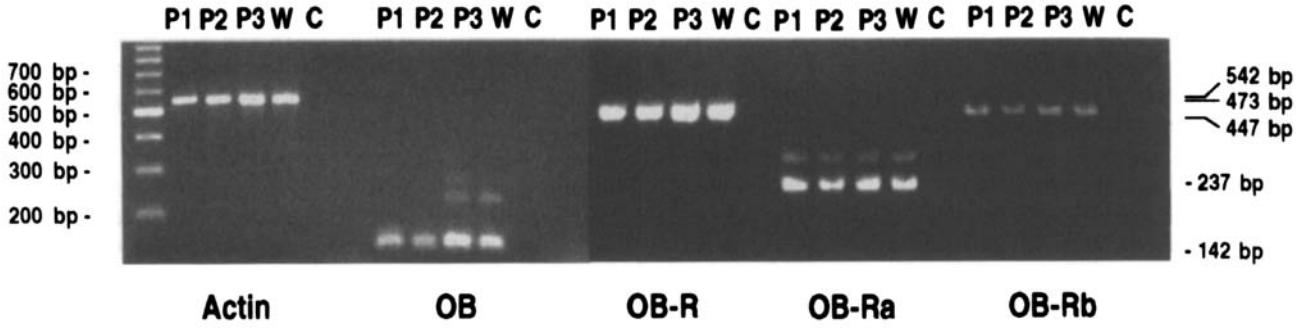

Fig. 1. Reverse transcription–polymerase chain reaction expression analysis of leptin and alternatively-spliced leptin receptors in mouse placentas (P1-P3) and mouse adipose tissue (W). The expression of leptin (OB) was compared with the common extracellular domain of the leptin receptor (OB-R), the short splice variant (OB-Ra) and the signalling form of the leptin receptors (OB-Rb). β-Actin amplification was used as a control. The tissues were all extracted and reverse transcribed at the same time. No bands were observed with the mock complementary DNA (data not shown) or in the absence of complimentary DNA (C). Molecular markers (100 base pair (bp) ladder) and size of reverse transcription–polymerase chain reaction products are shown.

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immunoreactivity (Senaris *et al.* 1997). The same promoter is used for both human adipose and placental transcription of the leptin gene. However, an upstream enhancer has been identified which functions in the JEG-3 and JAR choriocarcinoma cell lines but not in adipocytes or HeLa cells. Binding analyses demonstrated three protein-binding sites in this enhancer region, designated placental leptin-enhancer elements 1–3. The protein binding to the placental leptin-enhancer element 3 motif appears to be a novel placenta-specific transcription factor (Bi *et al.* 1997). This implies that human placental leptin is differentially regulated from leptin of adipose origin.

Increased levels of binding protein(s) in the maternal circulation

In addition to leptin expression in the placenta, we have also shown that the murine placenta expresses high levels of leptin receptor mRNA (Figs. 1 and 2) and protein (Fig. 3). We have identified a number of different receptor splice variants in the murine placenta (Fig. 1). These include OB-Rb, the signalling form of the leptin receptor, and OB-Ra, the proposed transport form of the receptor (Hoggard *et al.* 1997*a*). This suggests that the placenta may be a target organ for leptin action. In addition, OB-Re, the soluble form of the

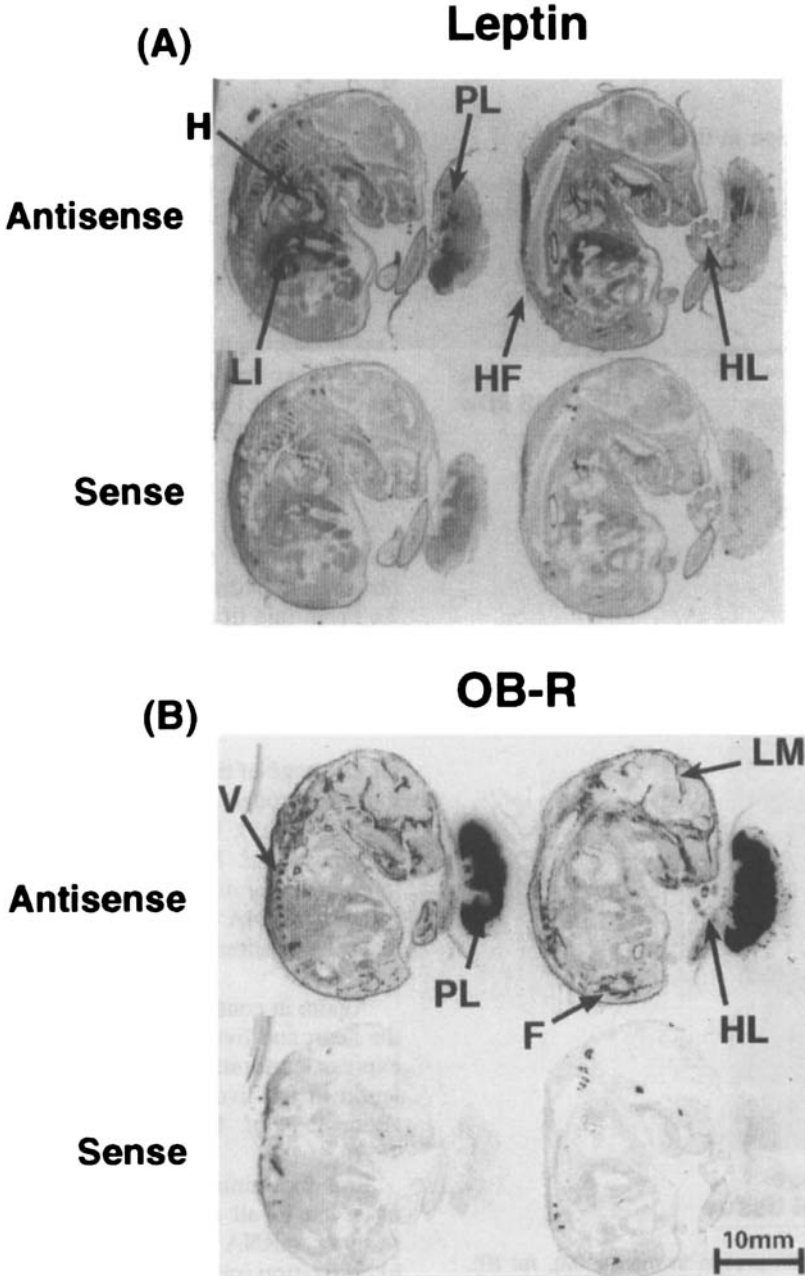

Fig. 2. *In situ* hybridization to sections of murine fetus and placenta hybridized with a ^{35}S-labelled antisense (upper panel) and sense (lower panel) riboprobes to (A) leptin and (B) the common extracellular domain of the leptin receptor (OB-R) mRNA. PL, placenta; LM, leptomeninges; V, vertebrae; F, femur; HL, hindlimb; LI, liver; H, heart; HF, hair follicle.

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leptin receptor has been identified (Gavrilova *et al.* 1997). The soluble form appears to be secreted into the maternal serum where it is suggested that it binds circulating leptin. This may protect the leptin from degradation and/or excretion while preventing it from binding to the signalling form of the leptin receptor, thereby giving rise to leptin resistance (Gavrilova *et al.* 1997). This proposal has the advantage of explaining the contradiction of a peak in leptin, an appetite suppressant, at a time of increased nutritional requirement. However, the increase in bound circulating leptin during late pregnancy, like the large increase in murine maternal leptin, appears to be restricted to mice and does not occur in human subjects or rats (Gavrilova *et al.* 1997).

The role of placental leptin and its receptor clearly requires further investigation. The leptin system may play a role in the control of pregnancy, parturition, or in the development of the fetus.

Leptin expression in the fetus

We have also identified leptin gene expression and mature leptin protein in a number of tissues in the 16·5-d-old murine fetus, by *in situ* hybridization (Fig. 2) and immunohistochemistry (Fig. 4). The major areas of leptin receptor expression are discussed elsewhere in this symposium.

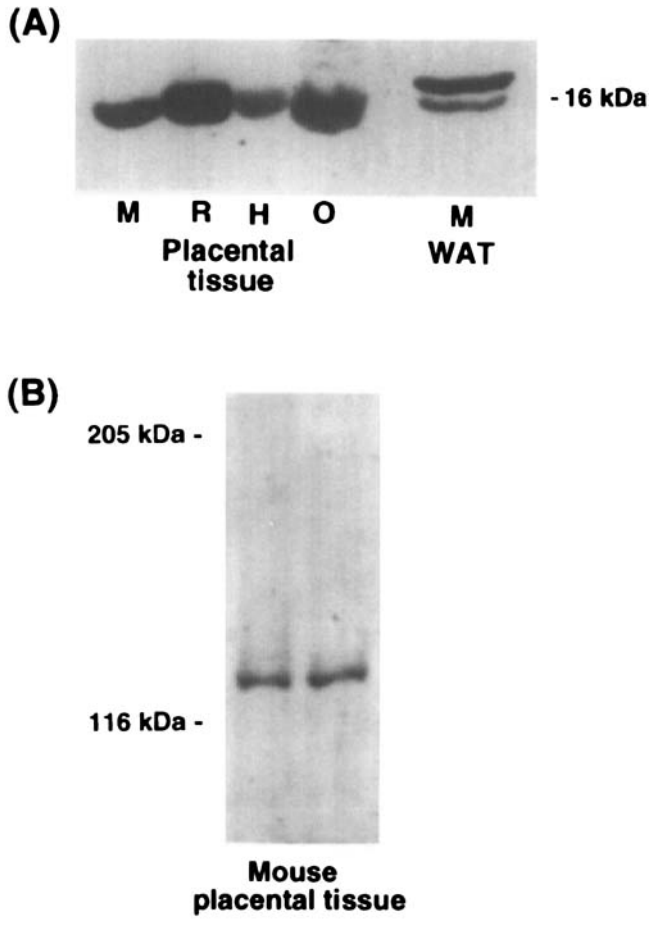

Fig. 3. (A) Western blot of leptin protein in mouse (M), rat (R), human (H) and ovine (O) placentae compared with mouse white adipose tissue (WAT). A major band was observed at 16 kDa. (B) Western blot of leptin receptor protein in two mouse placentas. Markers are as shown. 10 μg of protein was loaded onto each lane.

Fetal cartilage and bone

High levels of leptin gene expression were observed in the fetal cartilage and bone; in particular the vertebrae, ribs and digits of the hindlimb (Fig. 2). Similarly, using a probe to the leptin receptor for *in situ* hybridization, high levels of expression were observed in the same cartilage and bone structures that expressed leptin mRNA (Fig. 2), but in a different cell population (data not shown). This suggests that leptin may function in an autocrine or paracrine manner in the fetus (Fig. 2).

The expression of high levels of leptin and its receptor (both mRNA and protein) in the fetal bone and cartilage implies a role for leptin in fetal bone and/or cartilage development, which may be linked to its influence on haematopoiesis in the adult (Bennett *et al.* 1996; Cioffi *et al.* 1996; Gainsford *et al.* 1996) and/or represent a new function for leptin in bone development.

Brain

Leptin receptor mRNA and leptin receptor protein were identified in the leptomeninges and choroid plexus of the fetal brain (Fig. 2), confirming earlier studies in the adult rodent (Mercer *et al.* 1996*b*; Schwartz *et al.* 1996).

Hair follicles

Hybridization of the leptin probe and the receptor probe was observed over the hair follicles, including the verbrisae (Fig. 2). From this finding it is tempting to speculate that leptin plays a role in thermoregulation (Trayhurn *et al.* 1998).

Other fetal tissues

Of the range of tissues that express leptin receptor mRNA in the adult rodent, with the exception of the brain tissues (Tartaglia *et al.* 1995; Ghilardi *et al.* 1996; Lee *et al.* 1996; Hoggard *et al.* 1997*b*), only the lung and kidney express leptin receptor mRNA in the murine fetus (Fig. 2). No leptin receptor mRNA or protein expression was identified in the heart, liver, adrenal, or pancreatic primordium of the murine fetus.

Again, in contrast to previous published data in the adult, the heart and liver of the 16·5-d-old murine fetus appear to express leptin mRNA and protein (Fig. 2). The presence of leptin in the liver may relate to its role in haematopoesis (Bennett *et al.* 1996; Cioffi *et al.* 1996; Gainsford *et al.* 1996).

Immunostaining for leptin and the leptin receptor was observed in all tissues examined where leptin and leptin receptor mRNA had previously been detected by *in situ* hybridization (placenta only shown; Fig. 4).

The high levels of expression of both leptin and leptin receptors in the fetus suggest that this cytokine plays a key role in fetal development. One possible role is that of a fetal

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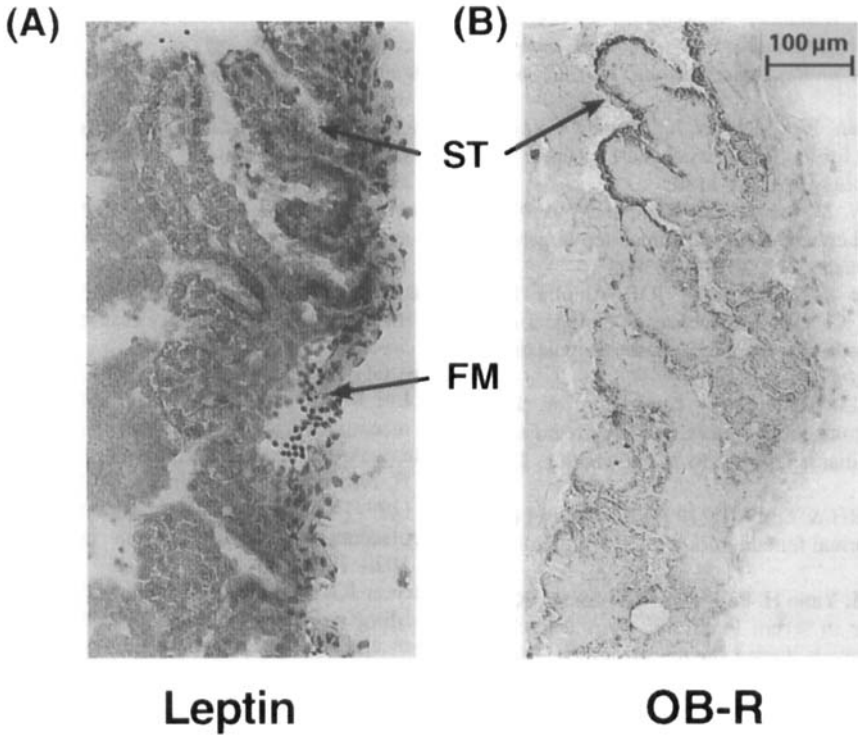

Fig. 4. Murine tissue sections of placenta were incubated with (A) leptin antiserum and (B) leptin receptor (OB-R) antiserum. The immunoreaction is visualized by 3,3′-diaminobenzidine, positive cells giving a brown colour at the site of reaction. The specificity of the immunoreaction of leptin and OB-R was confirmed by omission of the primary antibody (not shown). In case of leptin specificity, this was further confirmed by preabsorption of the leptin antiserum with synthetic leptin (not shown). Sections were counter-stained with toluidine blue. ST, syncytial trophoblast; FM, fetal mesenchyme.

growth factor, or as a signal to the fetus of maternal energy status. Alternatively, fetal leptin could provide a signal to the mother of fetal growth and development. However, it should be noted that leptin-deficient *ob/ob* murine fetuses appear to develop normally. In addition, two very obese children with a frameshift mutation in the leptin gene (who essentially display the same phenotype as *ob/ob* mice) also appear to be developing normally (in respects other than the obesity) and were born with normal birth weights. They do, however, have abnormalities of growth in the long bones of their legs and this may relate to the leptin expression detected in the fetal bone and cartilage (Montague *et al.* 1997). In summary, leptin expression in the fetus appears to be multifunctional, with both paracrine and endocrine effects.

Conclusion

Leptin has an extensive role to play in reproduction. It has been shown to restore the fertility of infertile leptin-deficient *ob/ob* mice, and it is also involved in the onset of puberty in normal mice. Recently, attention has focused on the increase in circulating maternal leptin towards the end of pregnancy and the placental expression of leptin. We have shown that although leptin gene expression is not detectable by Northern blot analysis in the murine placenta, it is still expressed at levels high enough to be detected by *in situ* hybridization. The question still remains as to the function of placental leptin, particularly when leptin in the human placenta appears to be differentially regulated. Is it secreted to the maternal or fetal circulation? Is it involved in maternal appetite regulation? Leptin and its receptor are also present in the murine fetus, where leptin appears to be multifunctional, with both paracrine and endocrine effects which require further investigation.

Acknowledgements

This work is supported by the Scottish Office Agriculture, Environment and Fisheries Department. We are grateful to Mr R. A. Duthie for graphics expertise.

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