Ontogeny of the expression of leptin and its receptor in the murine fetus and placenta

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Leptin is a 167-amino acid protein that is secreted from adipose cells and expressed in placental tissues. It is important nutritionally in the regulation of energy balance, but also has other functions such as a role in reproduction. To investigate the function of the leptin system in fetal development we examined, primarily by in-situ hybridization and immunohistochemistry, the expression (both mRNA and protein) of leptin and its receptor (including the signalling splice variant) in tissues from 11.5, 13.5, 16.5 and 18.5 d postcoitus murine fetuses and associated placentas. We detected leptin mRNA (at low levels) and protein predominantly in the cytotrophoblasts of the labyrinth part of the placenta, an area of nutrient exchange between the developing fetus and the placenta, and in the trophoblast giant cells situated in the junctional zone at the maternal interface. In addition, leptin was strongly expressed in the fetal cartilage–bone and at a lower level in the hair follicles, heart, and liver of the murine fetus at differing stages of development. The leptin receptor, including the signalling splice variant, was also identified in specific fetal tissues. The physiological importance of expression of both leptin and the leptin receptor (OB-R and OB-Rb) in the placenta remains to be determined. In addition, the high levels of expression of leptin and its receptor in discrete areas of the murine fetus suggest that leptin has a critical role in fetal development.

Leptin: Placenta: Fetal tissues

The Obese (ob) gene product, leptin, is a 167-amino acid protein, and until recently adipose tissue was recognized as the major, if not exclusive, site of ob gene expression and leptin secretion (Campfield et al. 1996). The leptin receptor gene has been shown to have at least six splice variants, ob-R(a–e) (Chen et al. 1996; Lee et al. 1996) and muB219 (Cioffi et al. 1996). The ob-Rb variant encodes a receptor with a long intracellular domain that is thought to be essential for intracellular signal transduction (Tartaglia et al. 1995). Leptin is deficient in obese (ob/ob) mice, in which daily administration of recombinant leptin causes reductions in body weight, body fat and food intake, as well as an increase in physical activity (Campfield et al. 1995; Halaas et al. 1995; Pelleymonter et al. 1995). Such observations demonstrate that leptin is nutritionally important in the regulation of body weight and fat deposition. This does not, however, appear to be the only function of this hormone. Leptin treatment has also been shown to restore fertility to ob/ob mice (Barash et al. 1996; Chehab et al. 1996) and to accelerate the onset of puberty in normal female rodents (Ahima et al. 1997; Chehab et al. 1997).

In keeping with a role for leptin in reproduction, the hormone rises in the maternal circulation towards the end of pregnancy in human subjects (Hardie et al. 1997) and rodents (Chien et al. 1997; Kawai et al. 1997), with a full in titre to below pre-pregnancy levels at about the time of birth. This effect is particularly pronounced in the mouse which has been reported to show a 25-fold rise (Tomimatsu et al. 1997) in maternal leptin. The source of this peak in leptin in late pregnancy remains to be established, particularly as there is no positive correlation between leptin concentration and BMI in pregnant women.

One possible explanation for the increase in leptin production towards the end of pregnancy is synthesis by the placenta. Recently, both the human (Green et al. 1995; Masuzaki et al. 1997) and murine placentas (Hoggard et al. 1997a) have been shown to express leptin, albeit at different levels. Expression by the human placenta occurs at a level detectable by Northern blotting while expression in the murine placenta is detectable by in-situ hybridization and reverse transcriptase-polymerase chain reaction (RT-PCR). It is interesting to note that the human leptin gene has a placental specific upstream enhancer (Bi et al. 1997). This implies that placental leptin is differentially regulated from leptin of adipose origin.

We recently identified a number of discrete sites of leptin

Abbreviations: bp, base pair; IgG, immunoglobulin G; RT-PCR, reverse transcriptase-polymerase chain reaction.

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expression in the 14.5-d-old murine fetus, including the fetal cartilage–bone tissues and hair follicles (Hoggard et al. 1997a). Together with the placental expression of leptin, this raised important questions regarding the function of leptin in these tissues during fetal development. To increase our understanding of the role of leptin in fetal development we examined the expression of leptin and its receptor in the murine fetus and placenta at 11.5, 13.5, 16.5 and 18.5 d postcoitus.

**Materials and methods**

**Animals**

Pregnant lean MF-1 mice were obtained, following timed mating, from Schaw’s Farm, Blackthorn, Oxon., UK. The mice, maintained on a Harlan Teklad diet (Harlan; Blackthorn, Oxon., UK), were killed by cervical dislocation. The fetuses and placentas were removed at 11.5, 13.5, 16.5 and 18.5 d postcoitus and frozen immediately on solid CO₂, three for each time point.

**Reverse transcriptase-polymerase chain reaction**

Total RNA was extracted from tissues using RNAzol (GibcoBRL, Paisley, UK) and cDNA were generated by reverse transcription using the Superscript Pre-amplification system (GibcoBRL) as previously described (Hoggard et al. 1997b). The cDNA primers to the leptin gene were 5’-TGAGTTTGTCCAAGATGGACC-3’ and 5’-GCCATCCAGGCTCTCCG-3’ (Genbank U18812); a 191 base pair (bp) fragment was generated. The cDNA primers to the leptin receptor sequence, which is common to all the known splice variants (473 bp product), and ob-Rb to the short form common to all the known splice variants (237 bp product) and to the long form of the leptin receptor sequence, which is corresponding to amino acids 32–51 mapping at the amino terminus of the human OB-R (Santa Cruz Biotechnology, CA), were previously described (Hoggard et al. 1997b). The quality of each cDNA and mock cDNA was determined by the relative level of amplification of the mouse β-actin gene (542 bp product) (Hoggard et al. 1997b). The polymerase chain reaction was performed on a Hybaid Touchdown thermal cycler (Hybaid, Ashford, Middlesex, UK) using the same conditions as previously described (Hoggard et al. 1997b). The leptin cDNA used the same amplification conditions as ob-R (Hoggard et al. 1997b). Agarose gel electrophoresis (20 g/l) in the presence of ethidium bromide confirmed the presence of a band of the expected size for each of the polymerase chain reaction primer pairs.

**In-situ hybridization**

Using the same specific primers used for RT-PCR, with the exception of leptin for which the primers are described elsewhere (Hoggard et al. 1997a), cDNA were cloned for the generation of in-situ hybridization probes. Probes to leptin, the common leptin receptor sequence ob-R, and the long form of the leptin receptor ob-Rb, were produced as previously described (Mercer et al. 1996b). Polymerase chain reaction products were purified using Wizard PCR preparations (Promega, Southampton, Hants., UK) and cloned directly into pGEM-T (Promega). The sequence and orientation of the inserts were confirmed by automated sequencing. Plasmids were linearized with Sac I or Apa I for transcription with T7 or SP6 RNA polymerase to generate antisense and sense riboprobes. In-situ hybridization techniques and sample preparation have been described in detail elsewhere (Mercer et al. 1996a,b). For comparison across the different stages of fetal development for each individual probe, all fetuses and placentas were hybridized with the same labelled probe at the same time and exposed to Hyperfilm (Amersham, Bucks., UK) in the same cassette.

**Placental content of murine immunoreactive leptin protein**

Placental wet weight was noted before homogenization. Tissues were homogenized in five volumes (w/v) of extraction buffer (100 mM-NH₄HCO₃, 10 mM-EDTA, 10 mM-EGTA, pH 9.3) and centrifuged at 15 000 g for 15 min. The supernatant fraction was stored at −70 °C until assay. The leptin concentration of the samples was determined in a solid-phase sandwich ELISA as previously described (Hardie et al. 1996). Briefly, high-affinity microtitre plates were coated with 1 μg/ml rabbit anti-leptin immunoglobulin G (IgG), washed and blocked as previously described (Hardie et al. 1996). Following incubation with 100 μl/well recombinant murine leptin standard (25–0.05 ng/ml) or test sample the plates were incubated with 100 μl/well biotinylated rabbit anti-leptin IgG (50 ng/ml) and detected as previously described (Hardie et al. 1996). Leptin levels were expressed as recombinant leptin equivalents (detection limit 100 pg/ml) by comparison with murine recombinant leptin standards.

**Antisera**

The murine leptin antiserum was a rabbit anti-leptin antiserum that was raised at the Rowett Research Institute against recombinant murine leptin (Pepro Tech EC, London, UK). This antiserum was used for immunohistochemistry and ELISA. The human leptin receptor antiserum was a goat anti-leptin receptor antiserum that binds to the epitope corresponding to amino acids 32–51 mapping at the amino terminus of the human OB-R (Santa Cruz Biotechnology, Heidelberg, Germany). The murine OB-Rb rabbit receptor antiserum was a rabbit anti-leptin receptor antiserum that binds to the epitope corresponding to amino acids 996–1009 mapping at the carboxy terminus of the murine OB-Rb, raised at the Rowett Research Institute. These antisera were used for immunohistochemistry.

**Immunohistochemistry**

Sections were prepared as described for in-situ hybridization. Non-specific binding was blocked by incubating in normal serum for 20 min. Tissue sections were incubated overnight at 4 °C with primary antiserum either directed against murine leptin, the human leptin receptor (OB-R) or the signalling form of the leptin receptor (OB-Rb). The leptin antiserum was diluted 1 : 2000. OB-R antiserum was diluted 1 : 300 and OB-Rb was diluted 1 : 200. Sections were washed for 15 min in PBS buffer and then incubated for 30 min with biotinylated secondary antibody (either anti-rabbit or anti-goat conjugated to biotin) according to the manufacturer’s
Leptin expression in murine tissues

Results

Leptin mRNA was detected in the murine placenta at days 11-5, 13-5, 16-5 and 18-5 postcoitus as determined by RT-PCR (Fig. 1). The leptin receptor (ob-R) and specific splice variants ob-Ra and ob-Rb were also expressed in the placenta from day 11-5 onwards (Fig. 1).

RT-PCR is a highly sensitive technique for the detection of mRNA. In order to determine whether the encoded protein was also present, placental leptin content was assessed by ELISA. The levels of immunoreactive leptin detected in the placenta were relatively high (between 23 and 31 ng murine leptin equivalents/g wet weight tissue) and showed a small but statistically significant decrease from day 11-5 to day 18-5 (Fig. 2). However, overall, taking into account the increase in the size of the placentas with development, there was no significant change in the total leptin content of the placentas (results not shown).

Leptin mRNA was localized by in-situ hybridization to the placenta and specific sites in the mouse fetus at different stages of development (Figs. 3 and 4(a, b)). Within the 11-5 and 13-5-d-old placenta, expression was in the junctional zone, mainly associated with the trophoblast giant cells (Fig. 4(a)), with some low expression in the cytotrophoblasts (Fig. 4(b)) of the labyrinthine part of the placenta. The trophoblast giant cells and the cytotrophoblasts are terminally differentiated cells with both endocrine and invasive functions in close proximity to the maternal and fetal sides of the placenta respectively. Leptin mRNA expression within the placenta in the latter stages of gestation was below the limits of detection using this technique (Fig. 3(a)). Leptin mRNA expression was also detected in 11-5-d-old maternal myometrium and decidua (results not shown). In the fetus, high levels of leptin gene expression were observed in numerous cartilage–bone structures as shown for the vertebrae (Fig. 3(a)). Hybridization of the leptin probe was also observed over the fetal heart, liver, and hair follicles including the vibrissae (Fig. 3(a, b)). Hybridization to the hair follicles was clearly demonstrated by photomicroscopy (Fig. 3(b)). Hybridization with the sense probe to leptin resulted in a weak, general or undetectable signal, although there was some association, albeit weak, with the cartilage–bone structures late on in gestation (Figs. 3 and 4(d, e)). Expression of leptin mRNA in the cartilage–bone, liver and hair follicles was observed from day 13-5 and throughout fetal development (Fig. 3(a, c)). The liver was not present in the whole fetal section chosen from day 18-5 (Fig. 3(a)), but was readily observed in other sections (see photomicrograph Fig. 3(c)). Leptin gene expression was detected in the heart in day 16-5 and 18-5 fetuses (Fig. 3).

Using the ob-R probe to the extracellular domain of the leptin receptor sequence (which is common to all the known splice variants) for in-situ hybridization, high levels of gene expression were observed in the placenta. Within the placenta, ob-R mRNA was localized predominantly to the junctional zone at the maternal interface mainly associated with the trophoblast giant cells and over the cytrophoblast

![Fig. 1. Reverse transcriptase-polymerase chain reaction (RT-PCR) expression analysis of leptin and alternatively spliced leptin receptors in day 11-5 (lanes 2–3), 13-5 (lanes 4–5), 16-5 (lanes 6–7) and 18-5 (lanes 8–9) placentas and mouse adipose tissue (AT; lanes 10–11) from two individual animals (one per lane). 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 long form of the leptin receptor (ob-Rb). β-Actin (β-ACT) 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 cDNA (results not shown) or in the absence of cDNA (lane 1). Molecular markers (M; 100 base pair (bp) ladder) and sizes of RT-PCR products are shown.](https://www.cambridge.org/core/core/image/10.1017/S0007114500000398)

![Fig. 2. Tissue content of leptin from 11-5, 13-5, 16-5 and 18-5-d-old murine placentas (ng leptin equivalents/g wet weight placenta). Placentas (n 3) were weighed, homogenized in extraction buffer and leptin protein concentration determined by a specific ELISA. Mean values for days 11-5 and 18-5 of pregnancy were significantly different: *P < 0.05 (ANOVA).](https://www.cambridge.org/core/core/image/10.1017/S0007114500000398)
cells of the labyrinthine part of the placenta (Figs. 5(a) and 4(c)). The expression of \( \text{ob-R} \) mRNA in these areas was much more intense than that of leptin mRNA. In addition, compared with leptin mRNA expression, \( \text{ob-R} \) gene expression was much more widespread over the labyrinthine part of the placenta (Figs. 3(a), 4(a–f) and 5(a)). Within the fetus, \( \text{ob-R} \) expression was detected in the same cartilage–bone structures that expressed leptin mRNA, as well as at a lower intensity in the lung, medulla of kidney, testis and hair follicles at different stages of development (Fig. 5). 

\( \text{Ob-R} \) gene expression was also observed in the leptomeninges and choroid plexus in the fetal brain, though we could not detect expression in the hypothalamus (Fig. 5(a)). \text{In-situ} \) hybridization with the probe for the long splice variant, \( \text{ob-Rb} \), revealed a similar hybridization pattern to \( \text{ob-R} \), although the autoradiographic signal was less intense (results not shown). Autoradiographic signals generated by the sense probes to \( \text{ob-R} \) or \( \text{ob-Rb} \) were similar to that described for the sense probe to leptin (Figs. 4(f) and 5).

Expression of the leptin receptor mRNA (both \( \text{ob-R} \) and \( \text{ob-Rb} \)) was observed in day 11.5 placenta and throughout placental development (Fig. 5(a)). A similar pattern of leptin receptor mRNA expression during development was observed in the fetus for the cartilage–bone structures (Fig. 5). The leptin receptor mRNA (both \( \text{ob-R} \) and \( \text{ob-Rb} \)) was detected in the leptomeninges, choroid plexus and hair follicles in 13.5-d-old fetuses and throughout fetal development (Fig. 5(a, d)). Expression in the lung was also detected in 13.5-d-old fetuses and throughout fetal development, albeit at low levels in the day 13.5 and 16.5 fetuses compared with day 18.5 (Fig. 5(a, b, c)). The lung was not present in the whole fetal sections chosen from day 13.5 and day 16.5 (Fig. 5(a)), but was readily observed in other sections (see photomicrograph Fig. 5(b, c)). In the kidney, the leptin receptor mRNA was detected only at days 16.5 and 18.5 (Fig. 5(a, e)), while gene expression in the testes was detected only in the day 18.5 male fetus (Fig. 5(a)).

In contrast to the low levels of leptin mRNA detected by
in-situ hybridization, marked immunostaining for leptin and the leptin receptor was observed, mainly in the trophoblast giant cells (Fig. 4(g–i)) and the labyrinthine cytotrophoblasts (Fig. 4(j–o)) within the placenta at all gestational time points (Figs. 3(a) and 4; e.g. day 13-5). In the fetus, immunostaining for leptin (OB), the leptin receptor (OB-R) and the signalling form of the leptin receptor (OB-Rb) was observed in all tissues shown to be positive for the appropriate mRNA by in-situ hybridization. A selection of the immunostaining for leptin and the leptin receptor in different tissues at different gestational time points is given in Fig. 6 (OB-Rb results not shown). The specificity of the immunoreaction was confirmed by the absence of primary antibody (Fig. 4(m)), incubation with human IgG (Figs. 4(n, o) and 6(b, d, f, j, l, n)) or preabsorption with respective protein (results not shown).

Discussion

We have demonstrated that leptin mRNA expression and mature leptin protein are co-expressed in a number of tissues during the development of the murine fetus and placenta. The major areas of expression of leptin and its receptor, and the pattern of expression that occurs during fetal development are discussed here.

Placenta

We were unable to detect leptin mRNA in the murine placenta by Northern blotting in agreement with other studies (Kawai et al. 1997; Tomimatsu et al. 1997). However, we could detect leptin expression in murine placenta by RT-PCR from as early as day 11-5, the earliest gestational time point examined, and throughout fetal gestation (Fig. 1), as well as by in-situ hybridization in the placenta at days 11-5 and 13-5 (Fig. 3).

In contrast to the relatively low levels of leptin gene expression in the placenta, high levels of leptin protein were detected by ELISA (Fig. 2). This apparent discrepancy between the leptin mRNA and protein levels has been observed elsewhere in a study which reported a leptin concentration of 46 ng/g wet tissue, but no leptin mRNA expression, as determined by Northern blotting, in day 17 placenta (Tomimatsu et al. 1997). The reason for the discrepancy in the apparent mRNA:protein ratios remains to be determined, but may reflect to some extent leptin binding to its receptor.

The main sites of both leptin and, at a higher concentration, leptin receptor (OB-R and OB-Rb; Figs. 3(a), 4 and 5(a)) expression were the trophoblast giant cells situated at the maternal interface and the cytotrophoblasts of the labyrinthine part of the placenta in close proximity to the developing fetus, suggesting a role for leptin in both fetal and maternal physiology. However, one should note that the expression of leptin in the placenta is low and it remains to be determined if it is physiologically significant. Furthermore, placental leptin may also have an autocrine role in the placenta given the high levels of leptin receptor. In addition there may be a contribution from the maternal and/or fetal circulation to leptin binding to the placenta, particularly given the apparent discrepancy between placental leptin mRNA and protein levels. Earlier in gestation (day 11-5), leptin immunostaining in maternal tissues (myometrium, metrial gland, decidua and luminal epithelium) may indicate a role of leptin in the process of implantation and/or early placentation.

Another leptin receptor splice variant ob-Re, the so-called soluble form of the leptin receptor, has been identified in the murine placenta (Gavrilova et al. 1997). The soluble form appears to be secreted into the maternal serum where it is suggested that it binds circulating leptin. Although we did not look specifically for expression of ob-Re in the placenta, these results are consistent with the high levels of leptin receptor (ob-R) expression in the placenta (Fig. 5(a)).

Fetal cartilage–bone and liver

High levels of leptin and its receptor gene expression and protein were observed in the fetal cartilage–bone, e.g. vertebrae (Figs. 3(a), 5(a) and 6(a, i)). These observations are in agreement with our previous report (Hoggard et al. 1997a). Later on in development, expression in the bone may be linked to leptin’s influence on haematopoiesis (Bennett et al. 1996; Cioffi et al. 1996; Gainsford et al. 1996). Given the likely role of this hormone in haematopoiesis it is perhaps not surprising to find expression of leptin in the murine liver from day 13-5 (Figs. 3(a, c) and 6(h)), since from day 12 through to day 16 this tissue is the almost exclusive site of haematopoietic activity (Rugh, 1994). However, we could not detect leptin expression in the fetal spleen, another site of haematopoiesis.

Hair follicles

Expression of leptin and its receptor (ob-R and ob-Rb), both mRNA and protein, was observed over hair follicles including the vibrissae (Figs. 3(a, b); 5(a, d) and 6(c, p)). Expression occurred from day 13-5 and continued throughout gestation, the former being the earliest point at which the skin can be differentiated from aggregations of cells which are the forerunners of the hair follicles.

Heart

In contrast to previous published data in the adult mouse (Zhang et al. 1994), the heart of the 16-5 and 18-5-d-old murine fetus appears to express leptin mRNA and protein (Figs. 3 and 6(e, g)). At this stage of fetal development the heart is completely formed. Although leptin has not been detected in the adult rodent heart it should be noted that leptin mRNA was detected at low levels in the human heart by Northern blotting (Green et al. 1995). However, no leptin receptor mRNA or protein expression was detected in the heart during fetal development. The significance of this remains to be determined.

Other fetal tissues

Of the range of tissues that express leptin receptor mRNA in the adult rodent (Ghilardi et al. 1996; Lee et al. 1996; Hoggard et al. 1997b), only the brain (from day 13-5; Figs. 5(a) and
Fig. 4. (a–f) In-situ hybridization to sections of 13.5-d-old murine placenta hybridized with 35S-labelled antisense (AS) and sense (S) riboprobes to leptin receptor (ob-R) and leptin mRNA (ob-R and ob-Rb). Riboprobes to leptin receptor mRNA are shown hybridized to the junctional zone (c, f) from day 13.5 incubated with antisense to leptin, leptin receptor (OB-R) and the signalling form of the leptin receptor (OB-Rb). The immunoreaction was visualized by diaminobutyric acid, positive cells giving a brown colour at the site of reaction. Sections were counterstained with toluidine blue.

Fig. 5. (a) In-situ hybridization to sections of 11.5-, 13.5-, 16.5- and 18.5-d-old murine fetus and placenta hybridized with 35S-labelled antisense (AS) and sense (S) riboprobes to leptin receptor (ob-R) mRNA. Photomicrographs of the lung from day 13.5 (b) and day 16.5 (c) fetus, as well as the hair follicle (d) and kidney (e) from day 16.5 are also shown. PL, placenta; LU, lung; LM, leptomeninges; V, vertebrae; HL, hindlimb; HF, hair follicle; L, liver; K, kidney; T, testes; R, rib.

6(o)), lung (from day 13.5: Fig. 5(a, b, c)) and medulla of the kidney (from day 16.5: Fig. 5(a, e)) express leptin receptor mRNA (ob-R and ob-Rb) and protein in the murine fetus (Figs. 5 and 6(k, m)). No leptin receptor mRNA or protein expression was identified in the liver, adrenal gland, or pancreas of the murine fetus (Fig. 5(a)). In the adult mouse, leptin has been shown to be actively secreted or degraded by the kidney (Cumin et al. 1997) and also to increase renal Na and water excretion (Jackson & Li, 1997). In the 18.5-d-old male fetus, leptin receptor (ob-R) expression was observed in the testes (Fig. 5(a)) which is consistent with leptin receptor expression in the stratified epithelium of spermatic...
Fig. 6. Murine fetal tissue sections from different stages of gestation incubated with antiserum to leptin (a, c, e, g, h), leptin receptor (i, k, m, o, p) or, as a control, human immunoglobulin G (IgG) (b, d, f, j, l, n). Immunoreactive leptin (brown colour) is observed over the rib (a, day 16.5) compared with the human IgG control (b), vibrissae (c, day 13.5; d, control), heart (e, day 18.5 and g, day 18.5; f, control) and liver (h, day 18.5). Immunoreactive leptin receptor (OB-R) is observed over the rib (i, day 13.5; j, control), lung (k, day 18.5; l, control), kidney (m, day 16.5; n, control), brain (o, day 18.5) and vibrissae (p, day 18.5). For details of procedures, see legend to Fig. 4.
cells and in the Leydig cells of the adult mouse testes (Hoggard et al. 1997b).

**Conclusion**

Leptin is important nutritionally in the regulation of energy balance, but also has other functions such as a role in reproduction. To investigate the leptin system in fetal development we examined the expression of leptin and its receptor during development in murine fetuses and associated placentas. We indicate that placental leptin may play a role in both fetal and maternal physiology during pregnancy although it remains to be determined if the low levels of expression are physiologically significant. We also demonstrate that the placenta is an important target organ for leptin. Early speculations as to the function of leptin in the placenta suggest that it may be a signal of fetal growth and/or a monitor of fetal–maternal energy status. However, the demonstration that leptin is an angiogenic factor may also indicate a role for leptin in the microvascularization of the placenta (Sierra-Honigmann et al. 1998). In addition, leptin and its receptor were identified in a number of fetal tissues at different stages of development. This is critical in our understanding of the role leptin plays during development of the fetus. Future studies need to determine the function of leptin in these specific tissues during development and whether leptin has a role in these tissues in the neonatal animal.

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