Dynamic changes in leptin distribution in the progression from ovum to blastocyst of the pre-implantation mouse embryo

Laura C. Schulz\textsuperscript{1} and R. Michael Roberts\textsuperscript{2,3}

\textsuperscript{1}Department of Obstetrics, Gynecology and Women’s Health, N625A Health Sciences Center, 1 Hospital Dr. Columbia, MO 65212.

\textsuperscript{2}Division of Animal Sciences and \textsuperscript{3}Division of Biochemistry, Bond Life Sciences Center, 1201 Rollins St, University of Missouri, Columbia, MO 65211, U.S.A.

**Short title:** Leptin in the pre-implantation mouse embryo

**Correspondence and reprint requests**

Laura C. Schulz, N625A Health Sciences Center, 1 Hospital Dr. Columbia, MO 65212

schulzL@missouri.edu
Abstract

The hormone leptin, which is primarily produced by adipose tissue, is a critical permissive factor for multiple reproductive events in the mouse, including implantation. In the CD1 strain, maternally-derived leptin from the oocyte becomes differentially distributed among blastomeres of pre-implantation embryos to create a polarized pattern, a feature consistent with a model of development in which blastomeres are biased towards a particular fate as early as the 2-cell stage. Here, we have confirmed that embryonic leptin is of maternal origin and re-examined leptin distribution in two distinct strains in which embryos were derived after either normal ovulation or superovulation. A polarized pattern of leptin distribution was found in the majority of both CD1 and CF1 embryos (79.1 % and 76.9 %, respectively) collected following superovulation, but was reduced, particularly in CF1 embryos (29.8 %; p < 0.0001), after natural ovulation. The difference in leptin asymmetries in the CF1 strain arose between ovulation and the first cleavage division, and was not affected by removal of the zona pellucida. Presence or absence of leptin polarization was not linked to differences in ability of embryos to develop normally to blastocyst. In the early blastocyst, leptin was confined subcortically to trophectoderm but upon blastocoel expansion it was lost from cells. Throughout development leptin co-localized with LRP2, a multi-ligand transport protein, and its patterning resembled that noted for the maternal-effect proteins OOEP, NLRP5, and PADI6, suggesting that it is a component of the subcortical maternal complex with as yet unknown significance in pre-implantation development.
Introduction

There is considerable evidence that leptin is required for one or more of the developmental processes that allow a conceptus to develop and implant successfully. For example, the obese Lep\textsuperscript{ob/ob} mouse, which produces only a truncated leptin protein, is infertile, yet fertility may be restored by injecting leptin into Lep\textsuperscript{ob/ob} dams to initiate their reproductive cycles. Moreover, pregnancies can be maintained in such mice by continuing leptin injections through day 6.5 post-coitus (Malik et al. 2001), a result consistent with the prevention of implantation by intrauterine injection of a leptin antagonist in wild-type mice (Ramos et al. 2005). Leptin has beneficial \textit{in vitro} effects on blastocyst formation rates and cell number and ability to form outgrowths, particularly with respect to trophectoderm, in several species, including cattle (Boelhauve et al. 2005), pig (Kim et al. 2006) and mouse (Kawamura et al. 2003, Herrid et al. 2006, Yang et al. 2006). In one case, human leptin was found to inhibit mouse embryo development, but the cross-species comparison complicates interpretation (Fedoresak & Storeng 2003). Treatment with an antibody to leptin receptor (LEPR) neutralized the beneficial effect of leptin on blastocyst development in the mouse, thereby implicating signal transduction through LEPR in mediating leptin action (Kawamura et al. 2002). By contrast, however, Lepr\textsuperscript{db} mouse conceptuses, which lacked the long form of the receptor, and Lepr\textsuperscript{3J} mouse conceptuses, null for all LEPR isoforms, were born at expected ratios to heterozygote mothers (Hummel et al. 1966, Leiter et al. 1980), indicating that there may be no absolute requirement for leptin interaction with its cognate receptors in the embryo for a pregnancy to succeed. This paradox remains unresolved, but might imply that an alternative receptor to LEPR is involved in whatever beneficial effects leptin has on the embryonic component of implantation.

\textit{In vivo}, the embryo is exposed to leptin in both the oviduct and uterus (Kawamura et al. 2002). In addition to their exposure to exogenous leptin, pre-implantation embryos contain leptin, originating from maternal deposition into the oocyte (Cioffi et al. 1997), although there have been conflicting reports as to whether or not the blastocyst itself expresses Lep mRNA (Kawamura et al. 2003, Herrid et al. 2006) a question that we address again in this paper. Controversially, leptin has been observed to be localized
asymmetrically in oocytes and zygotes (Antczak & Van Blerkom 1997) of the CD-1 mouse strain. In 2-cell embryos, it was confined to the cortical cytoplasm just below the plasma membrane and absent from the zone of cell-to-cell contact between the two blastomeres. In addition, in some embryos, fluorescence intensity was greater in one blastomere of the 2-cell stage embryo than the other. The asymmetry in distribution persisted as blastomeres continued to cleave. At the 4-cell stage, there was one blastomere with high leptin content, one with low, and two of intermediate status. From the 8-cell stage onwards, leptin was predominantly localized beneath the outer surface plasma membrane of cells on the exterior and absent from inner cells, so that by the blastocyst stage it was confined to the trophectoderm, with most intense localization to mural trophectoderm. The same authors (Antczak & Van Blerkom 1999) reported that STAT3, VEGF, BCL-X, BAX, TGFβ2, VEGF, C-KIT, and EGF-R, were also subcortically localized and distributed asymmetrically among early blastomeres.

These data, showing the early embryo to be polarized in terms of protein composition, have been held as evidence that the cytoplasm of the mouse oocyte and zygote is pre-patterned and that the 2-cell stage embryo itself is biochemically asymmetrical as a result of differential inheritance of zygotic cytoplasm (Edwards & Beard 1999). On the other hand, the findings have been controversial (Johnson & McConnell 2004a, Johnson 2009) and, if correct, tend to support the hypothesis that, in mammals, as in invertebrates and amphibians, cell lineage determination in the early embryo is influenced by asymmetries in informational macromolecules, a theory that implies that the fate of blastomeres from the 2-cell stage onwards is somewhat predictable. Others hold a contrary view, namely that all blastomeres are essentially equivalent through the 8-cell stage, at which time location (outer versus inner) and polarization of outside cells becomes the determinant of differentiation fate. However, both theories recognize that there is flexibility in mammalian embryo development, as separating, moving, or damaging individual blastomeres does not necessarily prevent formation of a viable blastocyst.
Recent evidence suggests embryos derived via superovulation show a far greater tendency to
demonstrate asymmetries in the allocation of cells to trophoderm and inner cell mass than embryos derived
by natural ovulation and that there are also differences among mouse strains with regard to these properties
(Katayama & Roberts 2010). It is also known that the properties of the zona pellucida, especially its shape,
might restrict the ability of embryo to rotate as it advanced towards the blastocyst stage, and that this feature
might cause apparent asymmetries without evoking potential fate differences among early blastomeres
(Kurotaki et al. 2007a, Honda et al. 2008, Katayama & Roberts 2010). Conceivably such variables could have
contributed to the leptin immunolocalization patterns observed by Antczak and Van Blerkom (Antczak & Van
Blerkom 1997). There have been four main goals of the present study. The first was to examine whether the
polarized pattern of leptin distribution in conceptuses could be confirmed and, if it exists, whether or not it is
dependent on strain and on the ovulation method utilized to generate the zygotes, or on the zona pellucida.
The second was to settle the controversy over whether or not Lep transcripts can be detected in mouse
conceptuses. The, third was to find whether evidence existed for a second mode of leptin mobilization by the
conceptus that did not involve the classical cognate receptor LEPR. The final goal was to determine whether
there was any link between leptin partitioning into blastomeres and their subsequent developmental fate.

Materials and Methods

Mouse strains:

CD-1 mice are an outbred Swiss-derived, strain, also known as ICR, and were derived by Charles
River Laboratories in 1959 from mice donated by the Institute for Cancer Research (ICR) in Philadelphia,
which, in turn, had imported the mice from the Rockefeller Institute in 1926. The CF-1 strain was produced
by Carworth Farms in the UK from mice obtained via a Missouri laboratory and thought to have been derived
from wild albino mice (Charles River, www.criver.com). Accordingly, CD1 and CF1 mice are believed to be
genetically distinct. Harlan Swiss Webster mice (ND4) originated from the same Rockefeller Swiss strain
used to derive CD-1, and were later re-derived at the University of Notre Dame (Chia et al. 2005). They are,
therefore, likely to be more related to the CD1 strain than to the CF1 strain. All animal experiments
conformed to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the
University of Missouri-Columbia Animal Care and Use Committee.

Embryo culture

Embryos were collected from CD1 (Harlan), CF1 (Harlan and Charles River) and Swiss Webster
(Harlan) mice. For superovulation, females were injected intraperitoneally with 5 IU PMSG (Calbiochem,
San Diego, CA), followed 46-47 h later by 5 IU hCG (Sigma, St. Louis, MO), at which time they were
introduced to male mice of the same strain. Naturally ovulated and superovulated mice were sacrificed for
embryo collection at the 2 cell stage, approximately 24 h after detection of a copulatory plug, i.e. ~36 h post-
coitus. Oviducts were dissected in CZB-Hepes medium on a warming stage, then embryos were transferred
to drops of KSOM medium with amino acids (KSOM-aa) (Millipore, Billerica, MA) overlain with mineral
oil and cultured at 37°C under 5% CO₂ and atmospheric oxygen. Ova were collected from the oviducts of
CF1 mice following superovulation on the morning after hCG injection, and, following natural ovulation, on
the morning after successful mating with a vasectomized male. Cumulus cells were removed with
hyaluronidase (Sigma) (Katayama & Roberts 2010).

For comparison of leptin gradients in embryos with and without a zona pellucida, CF1 mice were
superovulated as described above, and zygotes were collected from the oviduct. Cumulus cells were removed
with hyaluronidase, and then zygotes washed in CZB-Hepes medium. Zona pellucidae were removed by
exposure to acid Tyrode’s solution (Sigma), followed by pipetting in CZB-Hepes solution (Katayama &
Roberts 2010). One zona-intact embryo and two to four embryos with zona removed were cultured in 5 µl
drops of KSOM-aa medium. All embryos were fixed at day 3.5 post coitus. All those at the morula or
blastocyst stage at the time of fixation were immunostained for leptin.

Immunohistochemistry
Embryos and ova were fixed in 4 % paraformaldehyde for 15 min immediately after collection (ova and 2 cell) or after culture to the 4 cell (~57h), 8 cell (~64 h), morula (~84 h), early (~89 h) and expanded (~108 h) blastocyst stages, and then washed and stored in PBS/0.1 % BSA (w/v) at 4° C. After fixation, zona pellucidae were removed with acid Tyrode’s solution (Sigma, St Louis, MO). The embryos or ova were then incubated for 30-60 minutes at room temperature in PBS containing 5 % (w/v) IgG-free BSA , and either 5% (v/v) normal goat serum or 5 % normal donkey serum (v/v) to reduce non-specific binding of antibodies in subsequent treatments, and 1 % (v/v) Triton-X-100 to permeabilize cell membranes. Embryos were incubated with antibodies to leptin (catalog # 15-288-20031F chicken anti-leptin, Genway Biotech, San Diego, CA; now available from Sigma, catalog # GW20031F), CDX2 (mouse-anti-CDX2, Biogenex, San Ramon, CA) and megalin (LRP2) (goat anti-megalin, Santa Cruz Biotechnology, Santa Cruz, CA, and rabbit anti-megalin, gift of Joachim Herz) at dilutions of 1:700, 1:100 and 1:100, respectively, overnight at 4°C in PBS containing 1% serum, 1% BSA and 0.1% Triton-X-100. Embryos were washed three times in PBS, 0.1% BSA. Secondary antibodies (goat anti-chicken Alexa Fluor 488 A11039, goat anti-mouse Alexa Fluor 568 A11031, goat anti-rabbit Alexa Fluor 568 A11036, donkey anti-goat Alexa Fluor 568 A11057, Invitrogen, Carlsbad, CA; donkey anti-chicken -FITC, Chemicon, Temecula, CA) were used at a 1:500 dilution for 1-2h at room temperature. Nuclei were stained with DAPI (Invitrogen, Carlsbad, CA). Embryos were washed three times in PBS, 0.1% BSA, then mounted in a drop of this solution sealed between two coverslips separated by a rubber gasket (catalog #C18156, Invitrogen).

Embryos were examined either on a Zeiss LSM 510 two photon confocal system or a Zeiss LIVE confocal microscope. Embryos exposed only to secondary antibody were used to determine microscope settings so that non-specific signals were subtracted during image acquisitions. Similarly, settings were adjusted so that no part of the image was saturated in order to maximize detection of differences in staining intensity. For each confocal microscopy session, settings were maintained for all embryos imaged on a particular microscope. For the analysis of leptin staining gradients, 3D projections of stained embryos were pseudocolored with LSM Image Examiner software (Zeiss) to envisage differences in staining intensity. Two
independent observers, one blinded to strain and ovulation method, scored embryos as having “polarized” or “non-polarized” leptin expression. In the rare cases of disagreement, results were averaged. The proportion of embryos showing polarization was compared between natural ovulation and superovulation by using a Fisher exact test (GraphPad Prism software).

**Real-time RT-PCR**

For RNA isolation, CF1 embryos were collected at the 2-cell stage and cultured to the morula or blastocyst stage. They were then placed in “RNA Later” (Ambion, Austin, TX) at 4°C for up to 72 h. Trireagent (Sigma, St. Louis, MO) was added, and, after phase separation, the aqueous layer was applied to an RNEasy Mini column (Qiagen, Valencia, CA), and RNA purified according to manufacturer’s instructions. The RNA was DNase treated and reverse transcribed by using the Quantiscript Kit (Qiagen, Valencia, CA). To provide a standard, leptin transcripts were PCR-amplified from mouse adipose tissue, the band of amplified DNA excised and purified, and DNA concentration measured on a nanodrop spectrophotometer. The PCR product was then serially diluted to create a standard curve. Standard and sample Lep cDNA were amplified by using gene specific primers (F-CAGCCTGCCTTCCCAAAT and R-ATGGAGGAGGTCTCGGAGAT and a FAM-labeled probe (TGCTGCAGATAGCCAATGACCTGG) on an ABI 7500 real-time thermal cycler.

**Results**

*Immunostaining Controls*

Multiple controls were used to ensure the specificity of immunofluorescent staining patterns. When primary antibody was omitted, faint background staining was observed. The background fluorescence from these controls was used to establish image exposure times for the samples co-processed with the controls, such that non-specific background was reduced or eliminated (Suppl. Fig. 1a,b). Embryos incubated with primary antibody in the presence of 50-fold (w/w) excess leptin showed only faint background or non-detectable staining (Suppl. Fig. 1c). Controls in which detergent was eliminated showed punctate staining on
the exterior of the embryo, very different from the diffuse peripheral cytoplasmic staining observed when samples had been permeabilized with detergent (Suppl. Fig. 1e). Finally, if the zona pellucida were not removed, antibody adhered to it non-specifically, and there was a general failure to stain the blastomeres enclosed within it (Suppl. Fig. 1f).

**Polarization of leptin expression**

Leptin staining was most intense near the periphery of the blastomeres but was absent at cell-cell junctions in embryos examined from the two-cell stage up to the expanded blastocyst stage (Fig. 1). In addition to this subcortical staining pattern found in all embryos, leptin expression was classified as polarized when it displayed a similar pattern to that described earlier by Antczak and Van Blerkom (Antczak & Van Blerkom 1997) (Fig. 1 III-IV). Specifically, in 2-cell embryos, leptin staining was considered polarized when it was more intense at one pole of the embryo in both of the blastomeres or more rarely, more intense in one blastomere relative to the other (Fig. 1b, III-IV). In 4-cell embryos, polarized expression was defined as differences in leptin intensity among the 4 cells, generally with one cell being particularly strongly or weakly stained (Fig. 1c, III-IV). At the 8-cell through early blastocyst stages, staining was considered polarized if there was a gradient of intensity across the embryo, with a strongly stained region associated with one pole (Fig. 1d-f, III-IV). Conversely, staining was considered not polarized if it were of approximately equal intensity at the periphery of all outer blastomeres in the embryo, with no single pole exhibiting markedly increased expression compared to the rest of the circumference (Fig. 1 I-II).

Consistent with the findings of Antczak and Van Blerkom (Antczak & Van Blerkom 1997), we found that in both CF1 and CD1 mouse strains almost 80 % of embryos derived from superovulation showed polarized leptin expression (Table 1). On the other hand, these values were reduced when the embryos were obtained after natural ovulation. In the case of the CD1 strain, there was a tendency for fewer embryos from natural ovulation (61.3 % versus 80 %; P = 0.1) to show a polarized leptin distribution, while in the CF1 strain the value dropped from 79.1 % following superovulation to 29.8 % following natural ovulation, a
change that was highly significant ($P < 0.0001$). It should be noted that we observed a slightly lower prevalence of polarization in CD1 embryos than Antczak and Van Blerkom (Antczak & Van Blerkom 1997) who reported values >90%. This discrepancy may be due to minor technical differences. For example, we were unable to detect a gradient when it happened to be oriented precisely in the z-axis of the confocal microscope image, because there is a slight loss of signal intensity with increasing depth of field.

Shortly after cavitation, leptin immunofluorescence became limited to the trophectoderm and again was most intense immediately beneath the exterior plasma membrane (Fig. 1f). As at earlier stages, nearly all superovulated (81.8 %) and the majority of the naturally derived CD1 early blastocysts (60.0 %) showed polarized leptin expression, whereas in the CF1 strain polarization was observed in a majority of early blastocysts from superovulated (60%) but not in those from naturally ovulated dams, where the value was 37.5 % (Table 1). Among those early blastocysts in which polarization was observed, intense leptin expression was concentrated in the abembryonic pole in 7 of 9 superovulation-derived CD1 blastocysts, 1 of 3 naturally derived CD1 blastocysts, 2 of 3 naturally-derived CF1 blastocysts, and 3 of 6 superovulation-derived CF1 blastocysts.

It was possible to count the number of ICM and trophectoderm cells in confocal images of 30 of 35 of these early blastocysts. The relative number of ICM and trophectoderm cells was no different in blastocysts with or without polarized leptin expression, with the ICM comprising 26.9% of all cells in blastocysts with polarized leptin expression ($n=19$) and 27.1% of cells in blastocysts with a non-polarized leptin pattern ($n=11$). There was no indication of any effect of strain or ovulation method on this percentage.

**Leptin expression in expanded blastocysts**

There was a sudden transition in the pattern of leptin immunofluorescence as the blastocyst expanded. In particular, leptin expression was largely lost from the majority of trophectoderm cells. Instead, a region of intense staining, approximating the area of 1-2 cells was observed in >90% of all expanded blastocysts.
examined (n = 22) (Fig. 2e) regardless of strain or ovulation method. This “spot” was often located close to the junction between trophectoderm, which stained for CDX2, and the unstained ICM. It was not possible to determine with any certainty whether the spot was inside a cell or located at the periphery of the blastocoel cavity, but it in most instances it appeared to be juxtaposed to 1-2 trophectoderm nuclei (Fig. 2e).

Although it was not possible to follow leptin immunoreactivity in one blastocyst over time, because immunostaining was performed after fixation, some insight into the timing of the phenomenon was gained by fixing groups of CF1 embryos every few hours from the afternoon of d 3.5 until the afternoon of d 4.5. The diffuse cortical leptin staining present in trophectoderm of all early blastocysts began to fade during the early expansion of the blastocoel cavity, and bright punctate staining became evident (Fig. 2, a-d). Multiple larger spots were observed in some embryos on the morning of d 4.5 (Fig. 2b & d), whereas a single very large and intensely labeled spot was observed in most fully expanded blastocysts fixed from cultures on the morning or afternoon of day 4.5 (Fig. 2e & f). This intense spot was mostly, but not entirely, eliminated by incubation with excess leptin (Suppl. Fig. 1d), suggesting that it contained either concentrated leptin or leptin remnants. The spot did not appear either when primary antibody was omitted or when other primary antibodies, such as ones against CDX2, (Fig. 2e) were used. The exception to this rule was anti-LRP2 (Fig. 2f, discussed below).

Leptin localization in Swiss Webster embryos in vivo during early pregnancy:

To determine whether the peripheral localization of leptin observed during in vitro embryo culture was an experimental artifact, embryos obtained from natural ovulation were fixed and stained immediately after flushing at the 4-cell, 8-cell, and morula stages from Swiss Webster dams (Fig. 3). The leptin staining patterns were similar to those observed in cultured embryos, with leptin predominately located at the periphery of blastomeres, and absent from sites of cell-cell contact, and was asymmetrically distributed in some cases.

Origin and maintenance of leptin polarization
Leptin immunostaining was performed in ova collected from superovulated or naturally ovulated CF1 mice. As in embryos, leptin was localized predominately to the subcortical region of the ova (Fig. 1a, top images). A gradient of leptin expression, with intense staining at one pole and diminished staining intensity at the opposite pole was also observed in some but not all ova. In contrast to embryos, there was no difference in the prevalence of polarization between naturally and superovulated ova at 70.0 % and 66.7 % respectively (Table 1). Thus, polarized leptin expression is apparently lost in naturally ovulated but not superovulated ova between the time between when the eggs are ovulated and the first cell division.

Role of the zona pellucida

To determine whether the zona pellucida (ZP) is involved in maintenance of polarized leptin expression, and thus, whether differences in the ZP could account for staining differences between embryos of naturally ovulated and superovulated dams, the ZP was removed from fertilized, superovulated CF1 ova, which were then cultured with ZP-intact controls until day 3.5 when they were at the blastocyst stage. At the 4-cell stage, 10 of 21 embryos from which the zona had been removed were in either the more common tetrahedral or less common planar configuration, while the other 11 had unusual conformations, with all four blastomeres either in a line, or in a Y- or L-shape as observed previously (Katayama & Roberts 2010). At the morula or early blastocyst stage, the prevalence of polarization in zona intact controls was similar to that observed previously, 73.3% (n=15), and was only slightly lower, 63.3% (n=15), in embryos from which the zona had been removed (Fig. 4). These differences were not significant. In other words, the asymmetries observed did not appear to be due to whether or not a ZP was present.

Embryo leptin production

To quantify Lep mRNA at the morula and blastocyst stages, real-time PCR was used to construct a standard curve in which 257 - 2,570,000 copies of Lep cDNA could be measured over a linear scale (Suppl. Fig. 2). The data indicate that Lep mRNA, if present at all, was below the limit of detection in small pools of embryos. Five to eight morulae or blastocysts were pooled in lysis buffer, and total RNA extracted. Lep
mRNA was not detected in any of these pools by real-time RT-PCR (Table 2). Transcripts for a control gene 
(β-actin/Actb) were easily assayed in these embryo RNA pools. Moreover, an adipose tissue sample 
containing a comparable concentration of Actb mRNA showed Lep expression near the upper end of the 
standard curve (Table 2, Suppl. Fig. 2).

Colocalization with LDL receptor related protein (LRP2)

The staining pattern for leptin, with predominant localization to the outer surface of outer 
blastomeres, resembles the pattern reported for LRP2, a protein also known by the names megalin and gp330 
(Gueth-Hallonet et al. 1994, Assemat et al. 2005). When CF1 mouse embryos were co-stained for LRP2 and 
leptin to determine whether the two proteins were co-localized (Fig. 5), both were found in the outer 
subcortical regions. Areas that that stained most intensely for leptin were also those that stained most 
intensely for LRP2, confirming the likely association of the two antigens within the same region of the 
peripheral cytoplasm. LRP2 was detectable at all embryonic stages, including the zygote, suggesting that, like 
leptin, its presence there was due to maternal import. At the expanded blastocyst stage, LRP2 staining, 
unlike that of leptin, was predominately localized near the outer surface of trophectoderm, as it had been at 
earlier stages, but it was also associated with the “spot” of intense leptin staining (Fig. 2f).

Discussion

The experiments reported here largely confirm the leptin distribution data of Antczak & Van Blerkom 
(Antczak & Van Blerkom 1997) on CD1 mouse pre-implantation embryos. In our confirmatory experiments, 
we noted two distinct aspects of leptin localization. First, from the 2-cell stage onwards leptin was localized 
to the cytoplasm just beneath the plasma membrane but absent from regions of cell to cell contact. By the 
morula stage leptin was concentrated in outer and absent from inner cells, and by the early blastocyst stage it 
was confined to trophectoderm. This pattern was independent of strain and ovulation method. Second, like 
Antczak & Van Blerkom (Antczak & Van Blerkom 1997), we noted that the majority of CD1 embryos from 
the 2-cell stage onwards demonstrated asymmetries in leptin distribution. It was equally clear however, that
the asymmetries were influenced according to how ovulation was controlled and also by the strain of mouse employed. Embryos from naturally ovulated eggs, particularly of the CF1 strain, frequently showed a fairly uniform distribution of leptin among outer blastomeres, even though the protein had originally been asymmetrically distributed in the ovum (Table 1). This observation, which is novel, has important implications for the developmental significance of leptin asymmetries and adds to the growing body of evidence of significant differences between embryos derived by natural and superovulation.

Before exploring the distribution of leptin, it was important for us to establish that the leptin observed in our study was of maternal and not embryonic origin. Unlike another report, which used a nested PCR approach (Kawamura et al. 2003), our experiments suggest that the Lep gene is not transcribed from the embryonic genome through the blastocyst stage of development, in agreement with results from conventional PCR used by Herrid et al (2006). Real-time PCR, which allowed detection of as few as 250 copies of Lep mRNA, provided no signal above background from pools of morula or blastocyst cDNA. As there was a minimum of 150 cells (5 embryos x 32 cells) contributing to each blastocyst pool, there were less than 2 copies of Lep mRNA per cell. Separate microarray studies performed on 2-cell stage blastomeres also indicate that transcripts were below the limits of detection (R.M. Roberts, M. Katayama, unpublished data). Thus, while we cannot resolve the question of whether there is any embryonic Lep mRNA in mouse embryos, we conclude that the transcript levels present cannot account for the amount of leptin protein observed, thereby supporting the previous contention that all of the leptin present in mouse embryos from the zygote stage to blastocyst is of maternal origin (Antczak & Van Blerkom 1997, Cioffi et al. 1997).

Maternally supplied leptin must presumably be accumulated through some kind of receptor-mediated uptake into the oocyte and possibly the early embryo (Cioffi et al. 1997). Such transport seems unlikely to depend upon the high affinity, low capacity cognate receptor, LEPR. Additionally embryos with mutated Lepr genes develop normally (Hummel et al. 1966, Leiter et al. 1980). Conceivably, LRP2, a multi-ligand endocytic receptor associated with clathrin-coated pits and essential for uptake of a wide range of circulating
proteins by absorptive epithelia (Fisher & Howie 2006) and for leptin transport at the blood-brain barrier and
in the kidney (Hama et al. 2004, Dietrich et al. 2008), is the responsible transporter in the oocyte and embryo
rather than LEPR, which mediates leptin hormonal effects. Consistent with this hypothesis, the most intense
regions of leptin staining coincided with equivalently intense regions of LRP2 staining, at least up to the
blastocyst stage. In contrast to previous studies (Gueth-Hallonet et al. 1994, Assemat et al. 2005), in which
LRP2 was first detected at the 8-cell and 16-cell stages, respectively, we observed its presence at all
embryonic stages, although LRP2 staining intensity did increase as development progressed.

Some LRP2 even co-localized with leptin to the apparently extracellular “spots” observed in
expanded blastocysts, suggesting that these structures could represent clumps of protein formed after
vesicular transport out of trophectoderm and into the blastocoel cavity. Though this is the first report of leptin
being distributed in this manner, similarly located “spots” have been observed for the protein OOEP (Herr et
al. 2008). Moreover, the blastocoel is known to contain other proteins that have been selectively released into
the blastocoel cavity (Dardik & Schultz 1991). It is well established that the endocytic and exocytotic systems
of the embryo become progressively more active and polarized as development proceeds, and are, of course,
essential during blastocyst expansion (Fleming et al. 1986, Pemble & Kaye 1986). It seems likely that
exocytosis of proteins into the blastocoel cavity accompanies bulk transport of fluids, but we can only
speculate on why there might be a need to exclude all existing maternal leptin from the trophectoderm at this
time. Conceivably, it is important for the embryo to rid itself of once essential proteins to undergo yet another
transition, in this case one associated with hatching and implantation.

The subcortical localization pattern we report for leptin has also been observed for LRP 2(Gueth-
Hallonet et al. 1994, Assemat et al. 2005), NLRP5 (previously known as MATER) ((Tong et al. 2000, Ohsugi
et al. 2008), OOEP (also called FLOPED and MOEP19) (Pierre et al. 2007, Herr et al. 2008, Li et al. 2008,
Tashiro et al. 2010), PADI6 (Yurttas et al. 2008) and UCHL1 (Sekiguchi et al. 2006). That this pattern is
observed for multiple proteins, and is independent of strain and ovulation method suggests functional
significance of leptin in pre-implantation embryo development. Some have dubbed this suite of proteins the “subcortical maternal complex” (SCMC), and others have suggested that it is associated with “cytoplasmic lattices”, filamentous structures unique to the oocyte and early embryo (Capco & McGaughey 1986, Wright et al. 2003, Li et al. 2008, Kim et al. 2010, Tashiro et al. 2010). *Ooep*, *Uchl1*, *Nlrp5*, and *Padi6*, in particular, are maternal-effect genes; that is, they are not transcribed by the embryo itself, but embryos from null mothers do not develop beyond the 2- to 4- cell stage (Yurttas et al. 2008, Li et al. 2010). Unfortunately, it is not possible to test whether leptin is a maternal-effect gene in the same way, as Lep<sup>ob/ob</sup> dams are anovulatory unless supplied with ectopic leptin.

The reports of asymmetric localization of LEP, STAT3, and a few other proteins within the oocyte, the 4- and 8-cell stage embryo, the morula, and the blastocyst in the CD1 mouse (Antczak & Van Blerkom 1997, Antczak & Van Blerkom 1999), have been received with skepticism (Johnson & McConnell 2004b). However, the fact that the pattern for leptin is reproducible in our hands suggests that the phenomenon deserves some further explanation in terms of more recent models for cell fate decisions in the mouse embryo, such as those described by Zernicka-Goetz and colleagues (Piotrowska-Nitsche & Zernicka-Goetz 2005, Zernicka-Goetz 2006). This model depicts three cell allocation patterns (Fig. 6). The leptin localization pattern observed in the majority of CD1 embryos, whether obtained by superovulation or by natural ovulation, is consistent with the first pattern of cleavage, i.e. a meridional cleavage occurring first and an equatorial cleavage second (Path 1, illustrated in Fig. 6). The leptin localization patterns observed in CF1 embryos derived from superovulation is consistent with the second cleavage pattern described by Zernicka-Goetz and colleagues (Piotrowska-Nitsche et al. 2005, Piotrowska-Nitsche & Zernicka-Goetz 2005). It predicts that when the first division at the 2 cell stage is equatorial and the second is meridional, the leading blastomere will be equally likely to contribute to the abembryonic or embryonic pole, which is similar to the patterns we observed in CF1 embryos derived by superovulation where the leptin rich domain in the blastocyst was equally likely to be located at either pole. This observation is also consistent with previous dye labeling experiments with CF1 embryos derived by superovulation. In these studies, when the progeny of
a single blastomere tagged with dye at the 2-cell stage was segregated predominantly to one blastocyst pole, it contributed to the embryonic pole 42% of the time and to the abembryonic pole 58% of the time (Katayama & Roberts 2010).

However, the model seems less useful in explaining the leptin distribution in CF1 blastocysts obtained by natural ovulation. In both the dye labeling experiments performed earlier (Katayama & Roberts 2010) and the leptin staining experiments described here, ~70% of blastocysts from natural ovulation showed a randomized pattern of the marker, with no obvious polarization to one or the other pole. The model of Zernicka-Goetz and colleagues (Piotrowska-Nitsche et al. 2005, Piotrowska-Nitsche & Zernicka-Goetz 2005, Zernicka-Goetz 2006) predicts that this mixed patterning would occur mainly in embryos that showed a planar configuration at the 4-cell stage and generally not in ones that had been tetrahedral (Fig. 6, Path 3). However, the majority of 4-cell stage, zona-enclosed, CF1 embryos derived from natural ovulation retain a tetrahedral configuration (Katayama & Roberts 2010).

One possible explanation for the different leptin patterns in embryos from natural and superovulation is that the ZP of superovulated ova places more restrictions on planes of cell division and embryo movement within its confines than the ZP of naturally ovulated eggs (Katayama & Roberts 2010) and is responsible for the predominantly polarized localization of progeny derived from blastomeres tagged with markers at the 2-cell stage, which intermix after the 8-cell stage in the absence of the ZP (Motosugi et al. 2005, Kurotaki et al. 2007b, Honda et al. 2008). If the properties of the ZP underpinned differences in leptin distribution, removal of the ZP from CF1 zygotes derived by superovulation would result in a loss of leptin polarization. However, this outcome was not observed. One possibility is that leptin patterning is actively maintained and is not solely dependent on the sequence of cleavage divisions. Certainly the localization of other cortically concentrated proteins such as OOEP and LRP2 are actively regulated, as only in this way could be excluded from sites of cell-cell contact (Gueth-Hallonet et al. 1994, Herr et al. 2008, Li et al. 2008). Moreover, the
maintenance of leptin polarization in zona-free blastocysts derived from 4-cell stage embryos that exhibited
the aberrant open chain conformations would also appear to require active relocalization of leptin.

In conclusion, we suggest that the distinctive leptin patterning seen in the CD1 and CF1 mouse strains
is due primarily to three factors: first the asymmetric loading of leptin into the oocyte, second the sequence
and orientation of cleavage divisions in the embryo, and third, active localization of leptin by as yet
unidentified binding partners. Superovulation confers a more restrictive pattern of lineage allocation than
natural ovulation, particularly in the CF1 strain, but this predictability confers no apparent developmental
advantage in terms of increased cell numbers to either ICM or TE (Katayama & Roberts 2010). Accordingly,
there is no evidence to suggest that an asymmetric distribution of leptin (or absence of asymmetry) is linked
to abnormal embryonic development. Nevertheless, as maternal leptin is a necessity for normal pregnancy, its
essentiality may be linked to its characteristic subcortical location within outwardly facing blastomeres,
possibly as a component of the SCMC. Its sudden loss just prior to the time of implantation may reflect the
necessary destruction of the complex and its components at that time.

**Declaration of interest**

The authors have no conflict of interest to declare that could be perceived as prejudicing the impartiality of
the research reported.

**Funding**

This work was supported by NIH grants K99 HD055231 (to L.C.S.) and R01 HD21896 (to R.M.R.)

**Acknowledgements**

The authors wish to thank Mika Katayama for technical assistance and helpful scientific discussion. Jessica
Schlitt, Amber Wiggins and Lisa Mao assisted with analysis of leptin polarity and cell counts.
References

Antczak M & Van Blerkom J 1997 Oocyte influences on early development: the regulatory proteins leptin and STAT3 are polarized in mouse and human oocytes and differentially distributed within the cells of the preimplantation stage embryo. *Mol Hum Reprod* 3 1067-1086.


Motosugi N, Bauer T, Polanski Z, Solter D & Hiiragi T 2005 Polarity of the mouse embryo is established at blastocyst and is not prepatterned. *Genes Dev* 19 1081-1092.


Piotrowska-Nitsche K & Zernicka-Goetz M 2005 Spatial arrangement of individual 4-cell stage blastomeres and the order in which they are generated correlate with blastocyst pattern in the mouse embryo. *Mech Dev* 122 487-500.


Figure Legends

Figure 1: Leptin expression in preimplantation embryos. Monocolor images of immunofluorescently stained embryos were pseudocolored green to resemble their appearance on the microscope (columns I, III), or using a heat map scale to visualize differences in staining intensity (columns III, IV), scale bar in IV a. Representative pictures of ova (a) and embryos examined at the 2-cell (b), 4c-cell (c), 8-cell (d), morula (e) and early blastocyst (f) stages. For each embryo examined, the staining pattern was characterized as either “polarized” (columns I, II) or “non-polarized” (columns III, D). Examples shown are CF1, natural ovulation (I-II c,d,e, III-IV d), CF1 superovulation (I-II a, III-IV a,c,e) and CD1 superovulation (I-II b,f, III-IVb,f).

Figure 2: Immunostaining for leptin during blastocyst expansion in CF1 embryos. In two experiments (a, b and c, d) blastocysts were fixed over several hours during blastocyst expansion in vitro. Leptin staining (green) became fainter and punctuate (a,c) in the late afternoon day 3.5 (a) – early morning (c) of day 4. Larger spots became visible as the morning progressed (b, d). In fully expanded blastocysts, leptin staining was only visible in a single large “spot” (arrowhead) that appeared just to the interior of a few trophoblast nuclei (e, f). Cdx2 staining was confined to trophectoderm nuclei (red) at this stage (e). Hatching blastocyst stained with both leptin (green and LRP2 (red) (f). LRP2 was brightest at the periphery of the blastocyst, but was also visible in the “spot” (arrowhead), which appears yellow due to the overlap of green and red staining (f). Scale bar in (a) for images a-d.

Figure 3: Leptin expression in swiss webster embryos from natural ovulation that developed in vivo, without any embryo culture. The staining pattern is similar to that observed for cultured embryos, with leptin concentrated at the periphery of the embryo, and absent from regions of cell-cell contact. Polarized staining is apparent in some embryos.
Figure 4: The effect of zona pellucida removal on leptin polarity. CF1 embryos from superovulation in which the zona pellucida was removed at day 0.5 (c, d) and zona intact controls (a, b) were cultured through day 3.5 and then fixed. The zona was then removed from the control embryos and all were immunostained for leptin. Monocolor images of immunofluorescence stained embryos were pseudocolored green to resemble their appearance on the microscope (a, c), or using a heat map scale to visualize differences in staining intensity (columns b, d).

Figure 5: Co-localization of leptin (I, III) and LRP2 (II, IV) by immunostaining of CF1 embryos from superovulation. Single confocal sections of a 2-cell and 8-cell (a), a morula and two zygotes (b) and a 4-cell (c) are shown. Staining intensity is indicated by the scale bar in (Ia). The brightness of images was adjusted so that a similar dynamic range is shown for leptin and LRP2 channels. Both leptin and LRP2 staining were most intense at the periphery of embryos, and the regions of most intense staining are similar for leptin and LRP2. For example, in the 8-cell embryo, the same three blastomeres (arrowheads) stained most intensely (red) for LRP2 and leptin.

Figure 6: Leptin patterning as evidence for proposed patterns of cell divisions and cell fates in the preimplantation mammalian embryo. Results of leptin staining in CD1 and superovulated CF1 embryos appear consistent with the model of “biased” development summarized by Zernicka-Goetz. (1) In the biased model, the most common pattern observed from the 2-cell stage is a meridional (M) division of the leading (first to divide) blastomere, shown in red, followed by an equatorial division (E), resulting in a tetrahedral shaped 4-cell embryo. The lagging blastomere contributes mainly to the embryonic pole of the blastocyst. Leptin (yellow) would be divided equally into daughter blastomeres by an M division, but would be partitioned unevenly by an E division. Thus, the leptin-rich blastomere would be a product of the lagging cell, and would give rise to the abembryonic pole. This is consistent with our observations and those of Antczak and Van Blerkom in CD1 mice. (2) A less commonly observed pattern in the
“biased” model is an E followed by an M division, again resulting in a tetrahedral 4-cell embryo with one leptin-rich, one leptin poor, and two intermediate leptin-containing blastomeres. However, when this order of cell divisions was observed by Zernicka-Goetz and others, the leading blastomere was equally likely to contribute to the embryonic and abembryonic pole. Our observations of leptin staining in CF1 embryos from superovulated dams are consistent with this model, with leptin staining observed at either pole. (3) Our observations of leptin staining in CF1 embryos obtained from naturally ovulated dams are not consistent with the “biased” model. In the majority of these embryos, leptin was absent from sites of cell-cell contact, but was otherwise distributed equally along the periphery of all 2-cell and 4-cell stage blastomeres, and among all outer blastomeres at later stages.

Supplemental Figure 1: Immunofluorescence staining controls. Embryos stained with secondary antibody only (a, b). Embryos incubated in the presence of excess leptin(c, d). When detergent was omitted, staining was limited to a few clumps on the exterior of the embryo (e). When the embryo was not exposed to acid tyrode’s for a sufficient amount of time, the zona pellucida non-specifically stained (f). Unlike in the embryos in which the zona was removed, the margins of each blastomere of the 4-cell embryo are not visible.

Supplemental Figure 2: Standard curve for detection of leptin by real-time RT-PCR. Concentration of a purified leptin PCR product was determined spectrophotometrically and used to calculate copy number, shown on the x-axis. The number of cycles of real-time PCR required to reach threshold fluorescence (Ct) is shown on the y-axis. Adipose tissue-derived cDNA is included as a positive control.
Table 1: Number of embryos and ova showing polarized leptin expression out of the total number of embryos or ova observed.

<table>
<thead>
<tr>
<th>mouse strain</th>
<th>Embryo stage</th>
<th>natural ovulation</th>
<th>superovulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF1</td>
<td>Ovum</td>
<td>13 of 18 (72.2%)</td>
<td>12.5 of 18 (69.4%)</td>
</tr>
<tr>
<td></td>
<td>2-cell</td>
<td>4 of 12 (33.3%)</td>
<td>10 of 14 (71.4%)</td>
</tr>
<tr>
<td></td>
<td>4-cell</td>
<td>3 of 14 (21.4%)</td>
<td>8 of 10 (80.0%)</td>
</tr>
<tr>
<td></td>
<td>8-cell</td>
<td>2 of 11 (22.2%)</td>
<td>11 of 15 (73.3%)</td>
</tr>
<tr>
<td></td>
<td>morula</td>
<td>2 of 11 (22.2%)</td>
<td>15 of 16 (93.4%)</td>
</tr>
<tr>
<td></td>
<td>early blastocyst</td>
<td>3 of 8 (37.5%)</td>
<td>6 of 10 (60.0%)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>17 of 57 (29.82%)*</td>
<td>50 of 55 (76.9%)</td>
</tr>
<tr>
<td>CD1</td>
<td>2-cell</td>
<td>4 of 6 (66.7%)</td>
<td>8 of 9 (88.9%)</td>
</tr>
<tr>
<td></td>
<td>4-cell</td>
<td>1 of 3 (33.3%)</td>
<td>7 of 8 (87.5%)</td>
</tr>
<tr>
<td></td>
<td>8-cell</td>
<td>7 of 9 (77.8%)</td>
<td>4 of 6 (66.7%)</td>
</tr>
<tr>
<td></td>
<td>morula</td>
<td>4 of 8 (50.0%)</td>
<td>6 of 9 (66.7%)</td>
</tr>
<tr>
<td></td>
<td>early blastocyst</td>
<td>4 of 6 (66.7%)</td>
<td>9 of 11 (81.8%)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>19 of 31 (61.3%)</td>
<td>34 of 43 (79.1%)</td>
</tr>
</tbody>
</table>

* p < 0.0001, Fisher exact test

Table 2: Real-time RT-PCR for leptin and a control gene, β-actin on RNA obtained by pooling 5-8 embryos.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ct leptin†</th>
<th>Ct actin†</th>
</tr>
</thead>
<tbody>
<tr>
<td>no RT control</td>
<td>35.80</td>
<td>37.85</td>
</tr>
<tr>
<td>RNA pool 1 – five morulae</td>
<td>undetectable</td>
<td>24.14</td>
</tr>
<tr>
<td>RNA pool 2 – eight morulae</td>
<td>35.94</td>
<td>22.60</td>
</tr>
<tr>
<td>RNA pool 3 – seven blastocysts</td>
<td>undetectable</td>
<td>24.37</td>
</tr>
<tr>
<td>RNA pool 4 – five blastocysts</td>
<td>35.93</td>
<td>23.25</td>
</tr>
<tr>
<td>RNA pool 5 – six blastocysts</td>
<td>undetectable</td>
<td>23.64</td>
</tr>
<tr>
<td>adipose tissue RNA</td>
<td>23.13</td>
<td>21.85</td>
</tr>
</tbody>
</table>

†Ct is the number of PCR cycles needed to reach fluorescence threshold.
Figure 1. Leptin expression in preimplantation embryos. Monocolor images of immunofluorescently stained embryos were pseudocolored green to resemble their appearance on the microscope (columns I, III), or using a heat map scale to visualize differences in staining intensity (columns III, IV), scale bar in IV a. Representative pictures of ova (a) and embryos examined at the 2-cell (b), 4c-cell (c), 8-cell (d), morula (e) and early blastocyst (f) stages. For each embryo examined, the staining pattern was characterized as either “polarized” (columns I, II) or “non-polarized” (columns III, D). Examples shown are CF1, natural ovulation (I-II c,d,e, III-IV d), CF1 superovulation (I-II a, III-IV a,c,e) and CD1 superovulation (I-II b,f, III-IVb,f).

103x157mm (300 x 300 DPI)
Figure 2. Immunostaining for leptin during blastocyst expansion in CF1 embryos. In two experiments (a, b and c, d) blastocysts were fixed over several hours during blastocyst expansion in vitro. Leptin staining (green) became fainter and punctuate (a,c) in the late afternoon day 3.5 (a) – early morning (c) of day 4. Larger spots became visible as the morning progressed (b, d). In fully expanded blastocysts, leptin staining was only visible in a single large “spot” (arrowhead) that appeared just to the interior of a few trophoblast nuclei (e, f). Cdx2 staining was confined to trophectoderm nuclei (red) at this stage (e). Hatching blastocyst stained with both leptin (green and LRP2 (red) (f). LRP2 was brightest at the periphery of the blastocyst, but was also visible in the “spot” (arrowhead), which appears yellow due to the overlap of green and red staining (f). Scale bar in (a) for images a-d.

88x133mm (300 x 300 DPI)
Figure 3. Leptin expression in swiss webster embryos from natural ovulation that developed in vivo, without any embryo culture. The staining pattern is similar to that observed for cultured embryos, with leptin concentrated at the periphery of the embryo, and absent from regions of cell-cell contact. Polarized staining is apparent in some embryos.

74x36mm (300 x 300 DPI)
Figure 4. The effect of zona pellucida removal on leptin polarity. CF1 embryos from superovulation in which the zona pellucida was removed at day 0.5 (c, d) and zona intact controls (a, b) were cultured through day 3.5 and then fixed. The zona was then removed from the control embryos and all were immunostained for leptin. Monocolor images of immunofluorescence stained embryos were pseudocolored green to resemble their appearance on the microscope (a, c), or using a heat map scale to visualize differences in staining intensity (columns b, d).
Figure 5. Co-localization of leptin (I, III) and LRP2 (II, IV) by immunostaining of CF1 embryos from superovulation. Single confocal sections of a 2-cell and 8-cell (a), a morula and two zygotes (b) and a 4-cell (c) are shown. Staining intensity is indicated by the scale bar in (Ia). The brightness of images was adjusted so that a similar dynamic range is shown for leptin and LRP2 channels. Both leptin and LRP2 staining were most intense at the periphery of embryos, and the regions of most intense staining are similar for leptin and LRP2. For example, in the 8-cell embryo, the same three blastomeres (arrowheads) stained most intensely (red) for LRP2 and leptin.
Figure 6. Leptin patterning as evidence for proposed patterns of cell divisions and cell fates in the preimplantation mammalian embryo. Results of leptin staining in CD1 and superovulated CF1 embryos appear consistent with the model of “biased” development summarized by Zernicka-Goetz.

(1) In the biased model, the most common pattern observed from the 2-cell stage is a meridional (M) division of the leading (first to divide) blastomere, shown in red, followed by an equatorial division (E), resulting in a tetrahedral shaped 4-cell embryo. The lagging blastomere contributes mainly to the embryonic pole of the blastocyst. Leptin (yellow) would be divided equally into daughter blastomeres by an M division, but would be partitioned unevenly by an E division. Thus, the leptin-rich blastomere would be a product of the lagging cell, and would give rise to the abembryonic pole. This is consistent with our observations and those of Antczak and Van Blerkom in CD1 mice. (2) A less commonly observed pattern in the “biased” model is an E followed by an M division, again resulting in a tetrahedral 4-cell embryo with one leptin-rich, one leptin poor, and two intermediate leptin-containing blastomeres. However, when this order of cell divisions was observed by Zernicka-Goetz and others, the leading blastomere was equally likely to contribute to the embryonic and abembryonic pole. Our observations of leptin staining in CF1 embryos from superovulated dams are consistent with this model, with leptin staining observed at either pole. (3) Our observations of leptin staining in CF1 embryos obtained from naturally ovulated dams are not consistent with the “biased” model. In the majority of these embryos, leptin was absent from sites of cell-cell contact, but was otherwise distributed equally along the periphery of all 2-cell and 4-cell stage blastomeres, and among all outer blastomeres at later stages.