Adipobiology
ISSN 1313-3705

REVIEW

ADIPOGENESIS IN FETAL PIG SUBCUTANEOUS ADIPOSE TISSUE: REMARKABLE DEVELOPMENTAL FEATURES BEFORE THE ONSET OF ADIPOGENESIS

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Abstract

The collection of investigations indicating the importance of adipose tissue architecture to vasculogenesis and angiogenesis during adipogenesis is reviewed. Early in development of the architecture vascular structure develops before overt adipocyte differentiation. Adipocyte development and the expanding and elaborating vascularity are closely linked during adipocyte cluster growth. Furthermore, fetal adipose tissue studies show that location-dependent angiogenic potential ranges from more to less in regards to the extent of endothelial cells and developing arterioles present before overt adipogenesis. Fetal adipose tissue cells express and secrete numerous factors that may initiate the development of adipose tissue architecture and associated developmental gradients. Possibly, the neural connection between hypothalamic neurons and adipose tissue represents part of a regulatory pathway between the hypothalamus and adipose tissue development via neural driven patterns of blood vessel development. Finally, small blood vessels in fetal adipose tissue may phagocytose and metabolize circulating lipids in the absence of differentiated adipocytes. Therefore, several aspects of the stromal and vascular components of adipose tissue may play critical roles in the timing and distribution of developing adipose tissue.

Adipobiology 2012; 4: 23-32

Key words: stromal vascular components, adipose tissue differentiation, fetal pig

Received 2 August 2012, accepted 17 August 2012.
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Introduction

Adipocytes have been associated with a variety of metabolic maladies in humans. Elucidating the nature and identity of the adipocyte progenitor is critical to knowledge and insight into the role of adipocytes in physiology and pathophysiology. A number of preadipocyte cell lines and stromal-vascular cell cultures from many species have been used to identify the preadipocyte and examine the differentiation of preadipocytes. For all animals, preadipocytes are primarily derived from mesodermal stem cells during embryogenesis and fetal development. Establishment of the basic adipose architecture allows adipocytes to metabolize and store lipid. Adipocytes are found in various adipose depots postnatally and are distinguished from each other by a number of characteristics (reviewed in 1). However, the cellularity of all depots is a direct function of cells that commit to becoming adipocytes. Therefore, studies of fetal adipose tissue development are essential to understand the genesis of adipocytes and adipose tissue. The fetal pig model is very unique since no

other model utilizes a combined in vivo and in vitro approach before and throughout adipogenesis. (reviewed in 2,3).

Fetal adipose tissue traits before adipogenesis (45-50 days)

Cultures of stromal-vascular (S-V) cells from collagenase digested adipose tissue have served to study the conversion of S-V adipocyte precursors to adipocytes *in vitro*. Fetal S-V cell cultures established before and after the onset of adipogenesis provide a means to supplement in vivo studies of adipogenic and stromal-vascular cells (reviewed in 3). Although primary cultures of adipose tissue S-V cells are used to study adipogenesis, S-V cells, in vivo, may have key regulatory roles in adipose tissue genesis (3). Several other reviews are available that thoroughly describe embryonic/fetal adipose tissue development (4,5), and adipogenesis in a variety of animals (4,5). Several layers of dorsal subcutaneous immature collagen matrix develop early with a sparse vasculature (outer and inner layers) (Table 1). The collagenous matrix develops in advance of adipogenesis and develops in parallel with adipocyte differentiation which begins at 60 days (Table 1; reviewed in 2, 3). Cranial to caudal and ventral to dorsal developmental gradients exist for the thickness of the dorsal subcutaneous layers before adipogenesis. These developmental gradients are maintained throughout the expansion of each layer with adipose tissue development (Figure 1; reviewed in 3). Subcutaneous adipose layer development has not been studied in man, but the adipose layers are recognized as distinct and important compartments of the subcutaneous depot (6).

Small blood vessels in fetal subcutaneous tissue are reactive for major basement membrane proteins and bind lectins that mark capillary endothelium beginning at 50 days of development (Table 1, 7) (2). Additionally, a sparse number of preadipocytes are detected at 50 days (Table 1). Furthermore, TGFβ1 protein and mRNA was detected early on around developing blood vessels in fetal adipose tissue suggesting that TGFβ1 may stimulate fetal angiogenesis (Table 2) (3). *In vivo* and *in vitro* (fetal S-V cultures) studies indicated that IGFs and IGFBPs may regulate preadipocyte and stromal cell proliferation in an autocrine/paracrine manner in fetal adipose tissue prior to adipogenesis (Table 2) (3,8). Expression of PPARγ protein was limited *in vitro* but was easily detectable *in vivo* despite the absence of lipid-accreting preadipocytes (Table 3) (3). Additionally, C/EBPα protein was detected *in vivo* and C/EBPα reactive cells were present *in vitro* (Table 3) (3). In the mouse embryo, PPARγ mRNA was also detected before the onset of adipocyte development (reviewed in 9). Before adipogenesis, fetal adipose tissue cells express and secrete numerous factors that may augment and regulate the onset of adipogenesis.

Adipose tissue traits after the onset of fetal adipogenesis (70-110 days)

Collagen septa surround developing adipocyte clusters forming collagen "tubes" parallel to the long axis of the body (Table 1) (3). The greatest definition of collagen septa is present in the inner subcutaneous layer and by the end of fetal development the densest collagen is in a layer of subcutaneous tissue that is below the inner subcutaneous ie., "innermost" (2,3,10). The dense collagen is associated with reduced adipocyte cluster growth in the "innermost" layer of adipose tissue (3,10). Collagen deposition in the innermost layer may restrict local fat cell cluster growth since collagen deposition is greatly reduced and fat cell cluster development enhanced after removing the fetal pig hypothala-

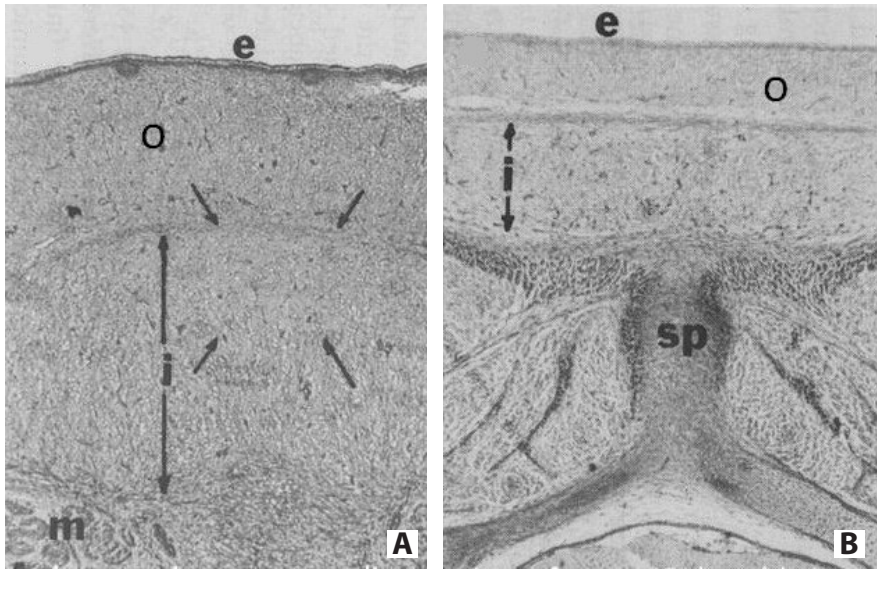

Figure 1. Dorsal most subcutaneous adipose tissue from a 45 days old pig fetus. **A** is from an area cranial and dorsal to the first costa. **B** is from an area over the last costa. I, middle layer of subcutaneous adipose tissue; o, outer layer; e, epidermis; sp, spinal process. Fat cells have not developed yet (adopted from Hausman GJ, Kauffman RG, *J Anim Sci* 1986; 63:642-658).

Table 1. Cytodifferentiation in fetal subcutaneous (SQ) adipose tissue before (45-50 days) and after adipogenesis (70-110 days).

	45-50 day	70-110 day
SQ Layers	Sparse collagen matrix; thickness pattern = that in growing animals; but no lipid accretion	Development of defining collagen septa; adipocyte cluster (capillaries+ adipocytes) development inner > outer
AD-3 reactivity	Strands / cords and isolated cells	Clustered AD-3 reactive capillaries & adipocytes
Vasculature	Sparse; Laminin and type IV collagen reactive; binds lectins	Expanding vasculature binds lectins; Laminin and type IV collagen reactive
Sympathetic nervous system (SNS), adrenergic nerves		Around arterioles and adipocyte associated capillaries

Table 2. Expression / secretion of regulatory factors in fetal subcutaneous (SQ) adipose tissue before (45-50 days) and after adipogenesis (70-110 days)

	45-50 day	70-110 day
TGFβ1	Protein and mRNA in cells around developing blood vessels	Protein and mRNA in adipocytes and other cells around developing blood vessels
IGFBP's	*in vivo* and *in vitro:* primarily IGFBP-2,-3	Secreted *in vitro* and detected *in vivo:* IGFBP-1, -2, -3, -4, -5
IGF-1,-2.	Secreted *in vitro* Microarray studies not done.	Secreted *in vitro*; mRNA detected *in vivo*: small adipocytes, stromal cells including endothelial cells. Microarray studies: > 50 genes expressing secreted regulatory factors detected *in vivo* and *in vitro*.

Table 3. Expression of regulatory factors in fetal SQ adipose tissue before (45-50 days) and after adipogenesis (70-110 days).

	45-50 day	70-110 day
GC receptor	Very low receptor number in vitro (primary fetal S-V cultures)	↑↑ receptor number *in vitro*
C/EBPα	Protein detected *in vivo* & *in vitro* - C/EBPα reactive cells not clustered	Protein↑ – all C/EBPα reactive cells clustered (*in vivo* and *in vitro)*
PPARγ	Protein detected *in vivo*; very few PPARγ reactive cells *in vitro*.	No change in PPARγ protein *in vivo*; ↑ in PPARγ nuclear reactivity in C/EBPα + celis absolutely coupled with lipid accretion *in vitro* Microarray studies: > 150 genes expressing non-secreted regulatory factors detected *in vivo* and *in vitro*.

mus at 45 days (examined at 110 days of fetal life; see 3). Adrenergic innervation around arterioles and adipocyte associated capillaries (Table 1) was absent after removal of the fetal pig hypothalamus at 45 days and after fetal hypophysectomy at 72 days (11). It is important to note that recent studies provided morphological evidence of multisynaptic projections from the hypothalamus to the adipose tissue in the pig (12). In fact, hypothalamic neurons localized in paraventricular nucleus, supraoptic nucleus and arcuate nucleus were transsynaptically connected via the sympathetic nervous system (SNS) to the perirenal and the subcutaneous fat tissue in the pig (12). Possibly, the neural connection between hypothalamic neurons and adipose tissue represents part of a regulatory pathway between the hypothalamus and fat tissue metabolism and development.

After the onset of adipogenesis, the expanding vasculature in clustered preadipocytes remains reactive for major basement membrane proteins and bind lectins throughout development (Table 1) (2,7). Furthermore, TGFβ1 protein and mRNA were detected in adipocytes and around developing blood vessels (Table 2) (3). Evidence at the gene and protein level obtained from in vivo and in vitro studies further substantiated the local role of IGFs and IGFBPs in preadipocyte and stromal cell development after the onset of adipogenesis (Table 2). Expression of C/EBPα protein was increased and, unlike before adipogenesis, C/EBPα reactive cells are clustered *in vivo* and *in vitro* (Table 3) (3). No change in PPARγ protein was evident *in vivo* but there was an increase in PPARγ reactive cells *in vitro* (Table 3). Microarray studies indicated that late term fetal adipose tissue and S-V cultures from 90 day fetuses expressed over 50 genes encoding for secreted factors and expressed 150 genes encoding non- secreted regulatory proteins (Table 3; unpublished data). Clearly, fetal adipose tissue cells expressed and secreted numerous factors that may be involved in the maintenance of subcutaneous adipose tissue developmental gradients.

Basement membrane traits of fetal adipocytes *per se*

Consideration of the development of the adipocyte basement membrane shows that laminin reactivity of adipocytes is not age dependent but type IV collagen reactivity is and all adipocytes react strongly for type IV collagen and laminin by 110 days (2). Lectin binding by developing fetal pig adipocytes increases between 70 and 110 days of fetal life (2). In particular, CON-A lectin binding increases indicating an increase in insulin-like activity (13) in developing fetal adipocytes. It is noteworthy that insulin driven lipogenesis in fetal adipose tissue increases between 70 and 110 days of fetal life (4).

Cell to cell interactions during development of adipocyte clusters in the fetus and in other animal models

Adipocyte development and the expanding and elaborating vascularity are closely linked during adipocyte cluster growth (3,14) Extensive studies with anti-adipocyte monoclonal antibodies demonstrated a functional and lineage relationship between preadipocytes /adipocytes, and associated capillaries and perivascular cells throughout development (Table 4) (3). Animal model studies identified developmental relationships between adipose tissue progenitor cells and perivascular and endothelial cells (Table 4). Additionally, adipogenesis and angiogenesis were reciprocally regulated following implantation of 3T3-L1 preadipocytes into a mouse dorsal skin-fold chamber (15).

We reviewed the physical relationships and interactions between preadipocytes /adipocytes, endothelial and perivascular cells evident in vitro and in vivo at the electron microscopic level (Fig. 2) (3). Gap junctions, junctional complexes, intercellular contacts or peg-in-socket morphologies characterized the relationships and interactions between these cells in fetal adipose tissue. Furthermore, studies of human adipose tissue-derived stromal cells (ADSC) and pig S-V cell culture studies described long contiguous preadipocyte cell processes that interacted with either blood vessels or adipocytes (3). Perivascular cells around or near larger capillaries seemed to provide mechanical support for sections of capillary wall (Fig. 2). Pig preadipocytes and human ADSC may form a supporting perivascular network much like perivascular reticuloendothelial cells do (16,17).

Functionality of the fetal pig adipose vasculature

As we have discussed, blood vessel patterns and the development of basement membranes around small blood vessels clearly precede adipocyte development in fetal pig adipose tissue but it is not known if these pre-adipocyte blood vessels are functional (3). Therefore, we examined the capability of these blood vessels to clear lipid from the blood by injecting a bolus of liposyn into the umbilical veins of 70, 90 and 110 day old fetuses followed by adipose tissue sampling one or two hours later for quantifying linoleic acid (predominant liposyn fatty acid) with gas chromatography analysis (18). Additional adipose tissue samples were obtained for electron and light microscopy (Trusty and Hausman, unpublished observations, 1988). We determined that liposyn clearance from the blood was linear during one hour after an injection of 10 ml of liposyn in 110 day old fetuses (18). Furthermore, liposyn was quantitatively deposited into subcutaneous adipose tissue indicating normal metabolism of the injected liposyn bolus (18). Light microscopy of adipose

Table 4. Animal models that identify adipose tissue progenitor cells *in vivo**

Study Topic	Results
Anti-adipocyte monoclonal antibody staining in fetal and postnatal pig adipose tissue (3).	Expression of adipocyte surface antigen indicates common lineage for preadipocytes, capillary endothelial cells and perivascular cells.
CD29+; CD34+ ;Sca1+; CD24+ cells isolated from the adipose stromal vascular fraction of mice followed by injection of these cells into residual fat pads of A-Zip lipodystrophic mice. (3)	Reconstitution of adipose tissue in A-Zip lipodystrophic mice with CD24+ cells which are localized in perivascular location.
Adipocyte progenitors studied in SV particulates from PPARγ-GFP mouse adipose tissue and in PPARγ-GFP adipose ; Adipogenic potential of adipose tissue SV cells from PPARγ-GFP mice evaluated. (3).	Adipocyte progenitors are located in the mural cell (pericytes and smooth muscle cells) compartment of the adipose tissue vasculature.
α-SMA -GFP transgenic mice: adipose tissue staining and harvest of adipose tissue stromal cells and sorted into GFP-positive (pericytes) and GFP-negative cells (3)	GFP (+) cells had multilineage differentiation ability, congregated around blood vessels and promoted vascularization in vivo and in vitro,
Living tissue imaging: confocal microscopy- based three-dimensional visualization in vivo in db/db mice (3)	Adipogenic/ angiogenic clusters contain EC's and stromal cells and are sites of active angiogenesis. Anti-VEGF inhibited angiogenesis and formation of adipogenic/ angiogenic clusters.

* From Hausman and Dodson (3), with the permission of *J Genomics*.

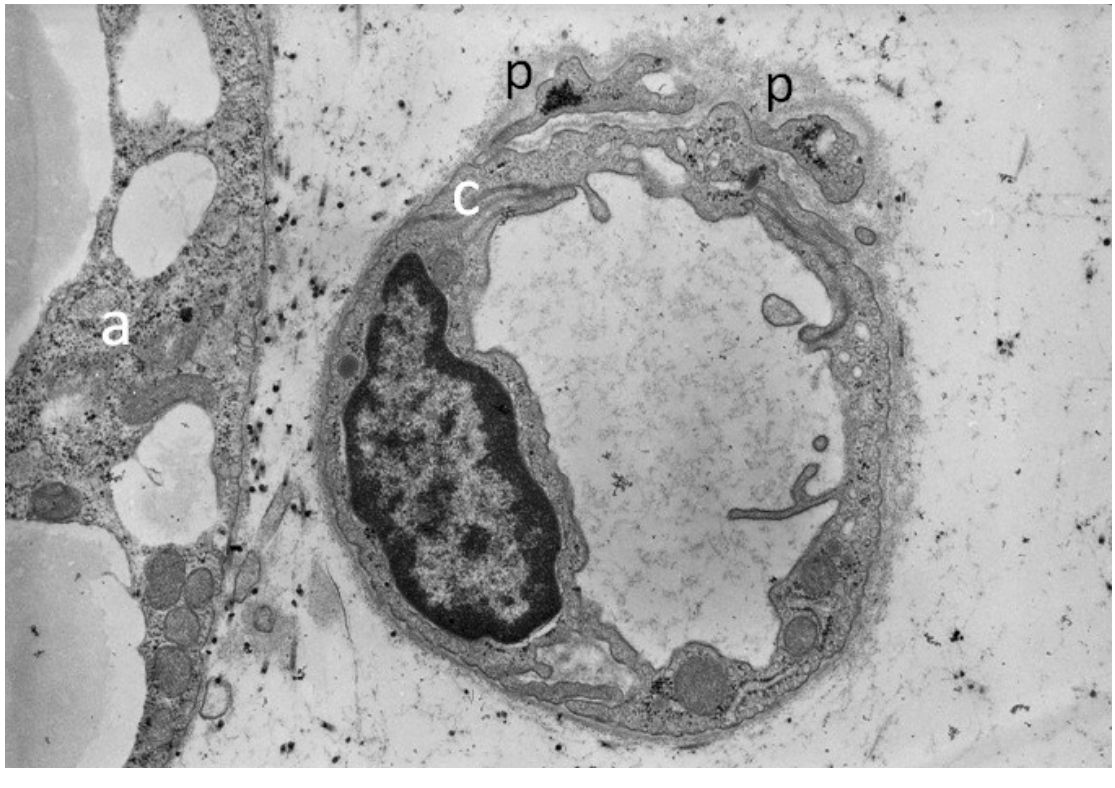

Figure 2. Electron micrograph of adipose tissue from a 110 day fetus after 2 hours of liposyn (10 µl of liposyn). Capillary wall (c), adipocyte (a) and perivascular cells (p) are indicated. Perivascular cells provide physical support for a portion of the capillary wall.

tissue obtained from 70 day fetuses one or two hours after a 2 ml liposyn injection revealed small blood vessels associated with perivascular cells decorated with lipid droplets whereas lipid was not evident in small vessels associated with obvious adipocytes (Fig. 3). Blood vessels within or close to adipocyte clusters and within muscle were devoid of lipid droplets (Fig. 3, 4). Electron microscopy revealed lipid droplets in endothelium, perivascular cells and in the lumens of blood vessels in liposyn (2 ml) injected 70 day fetuses (Fig. 5-7). In some instances, lipid accumulated to such an extent that lipid droplets were lodged between endothelium and adjacent perivascular cells and outside endothelium (Fig. 7). Perivascular cells had thick cell processes and were tightly adhered to the associated endothelium (Fig. 5-7). Furthermore, lipid droplets were often lodged in deep recesses of the endothelium (Fig. 7). Considerable glycogen deposits surrounded lipid droplets in endothelial and perivascu-

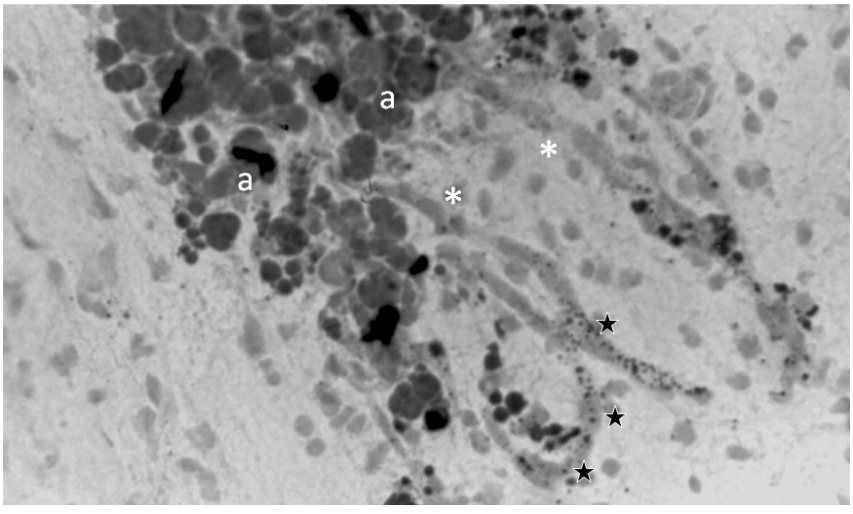

Figure 3. Adipose tissue section stained for lipid from a 70 day fetus after 2 hours of liposyn (2 µl) infusion. Adipocytes are indicated (a) and blood vessels decorated with lipid droplets are indicated (stars). Note the absence of lipid (asterisk) as the blood vessels approach the adipocyte (a) cluster.

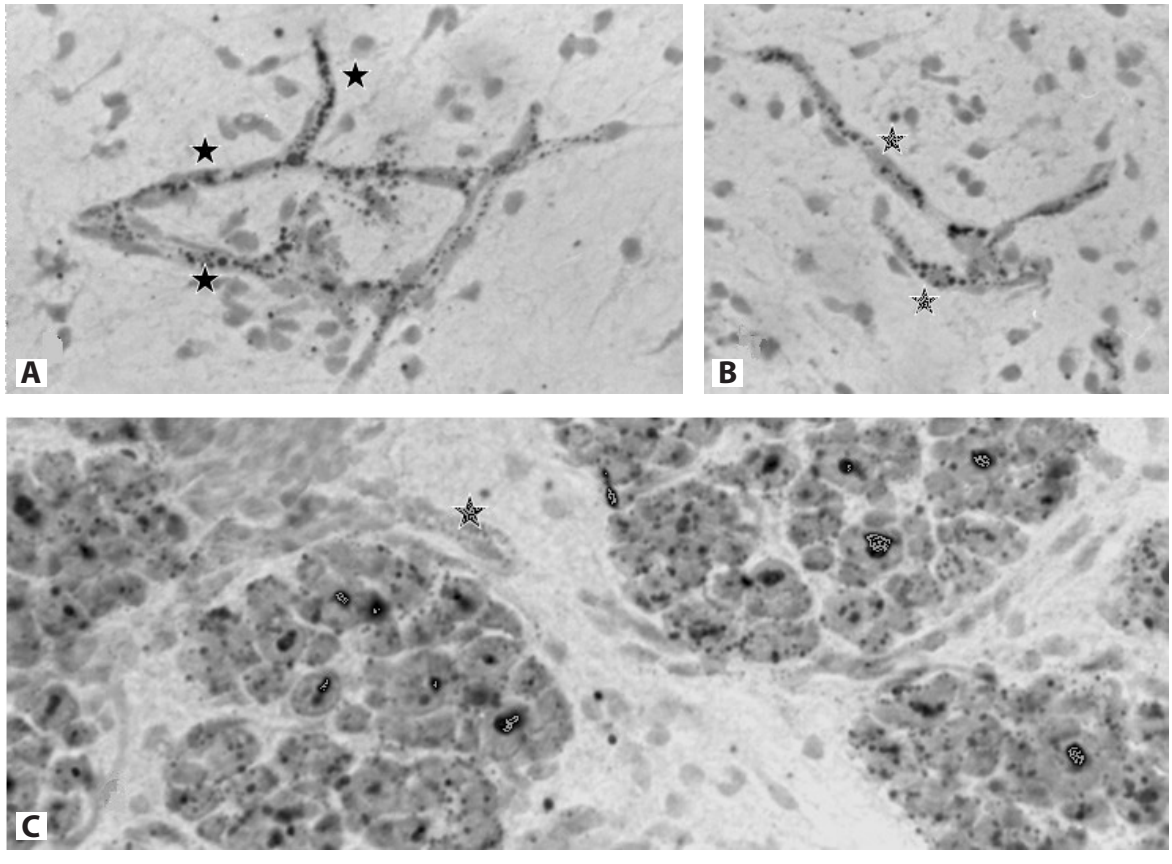

Figure 4. Adipose tissue (a, b) and muscle section (c) stained for lipid from a 70 day fetus 2 hours of liposyn (2 µl) infusion. Blood vessels decorated with lipid droplets (stars) are obvious in adipose tissue but not in muscle.

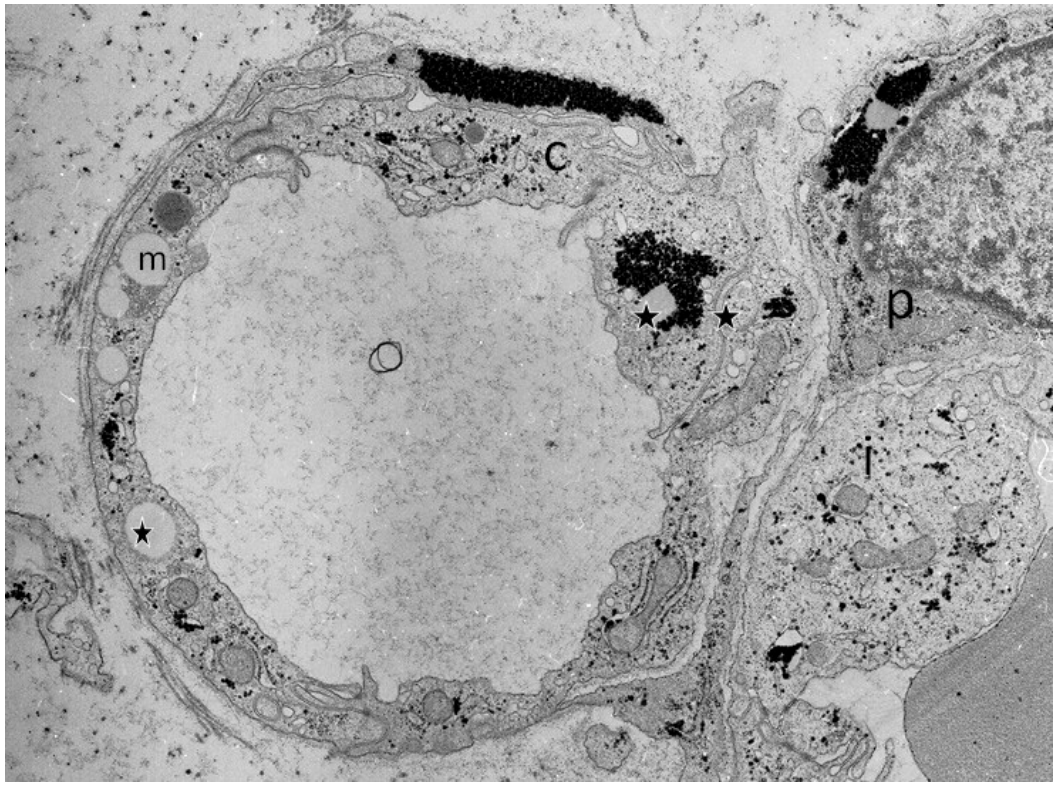

Figure 5. Electron micrograph of adipose tissue from a 70 day fetus after 2 hours of liposyn infusion. Lipid droplets (stars), multivesicular bodies (m), capillary wall (c) endothelial cell of an immature capillary (i) and perivascular cell (p) are indicated.

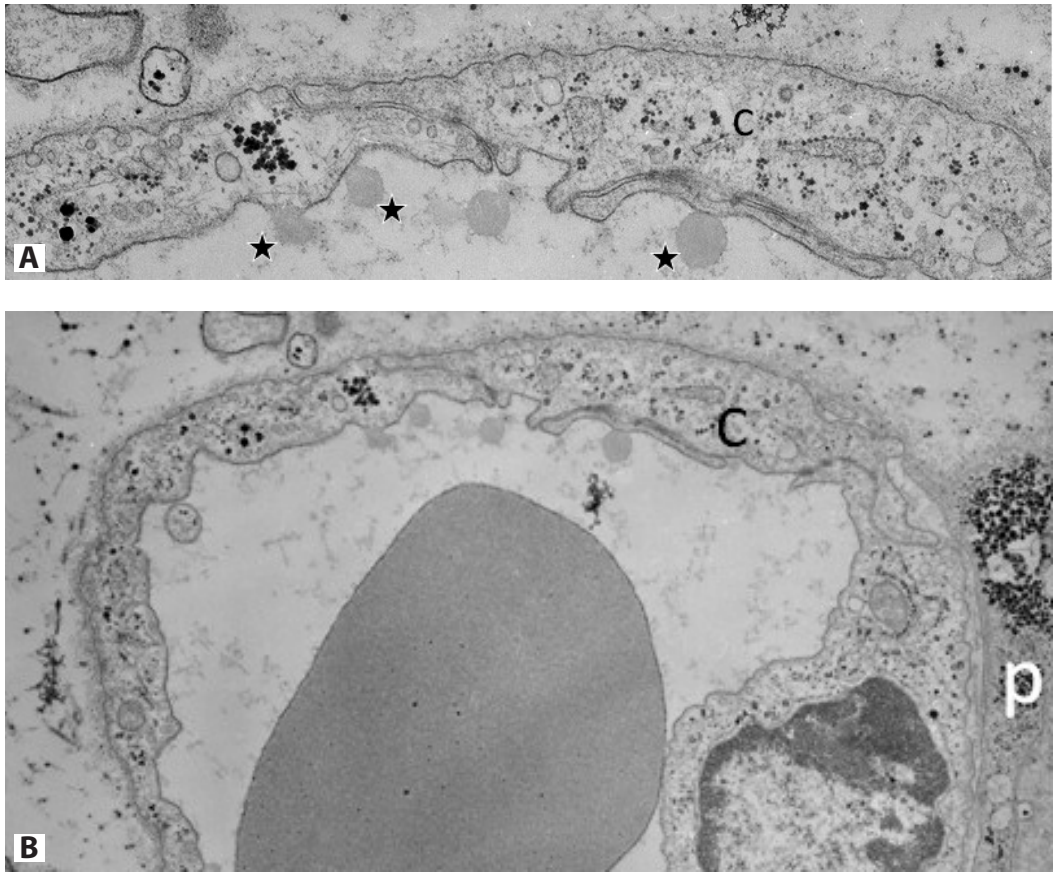

Figure 6. Electron micrograph of adipose tissue from a 70 day fetus after 2 hours of liposyn infusion. Lipid droplets (stars) in various stages of phagocytosis (top panel). The top panel is a higher magnification of the top of the capillary in the bottom panel. Capillary wall (c) and perivascular cell (p) are also indicated

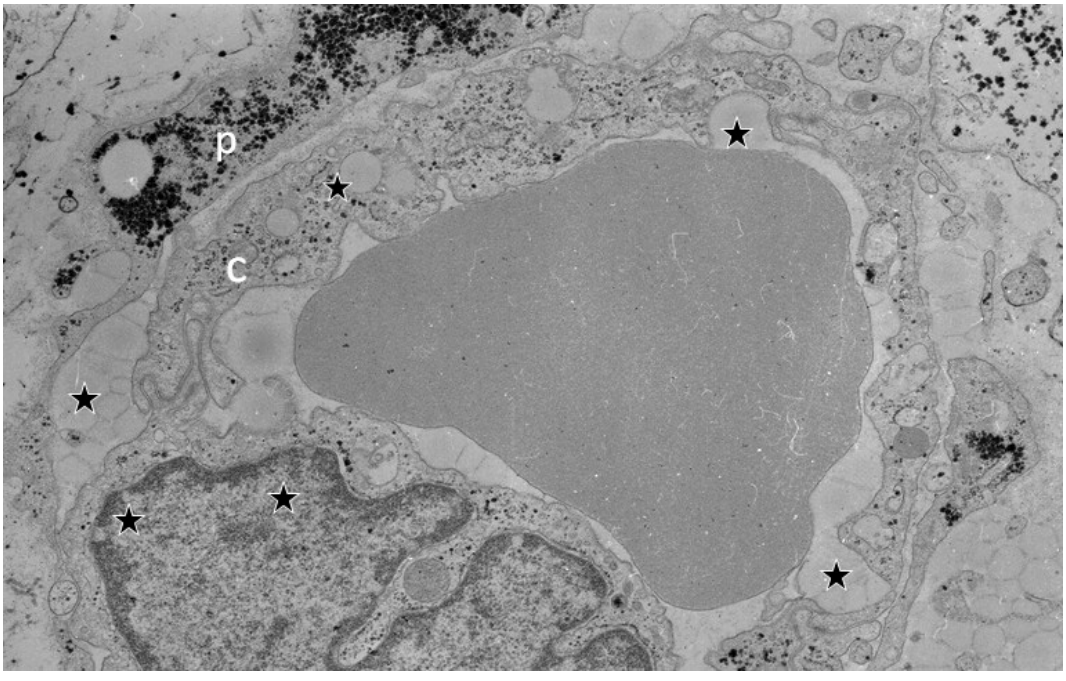

Figure 7. Electron micrograph of adipose tissue from a 70 day fetus after 2 hours of liposyn (2 µl) infusion. Lipid droplets (stars), capillary wall (c) and perivascular cell (p) are indicated. Note the lipid accumulation in a deep recess of the capillary and between the capillary and perivascular cell.

lar cells (Fig. 5-7). Multivesicular bodies with lipid were only observed in endothelium (Fig. 5). Lipid droplet phagocytosis in endothelium was only apparent in capillaries not associated with adipocytes (Fig. 6). Endothelial flaps were evident in endothelium in liposyn injected 70 and 90 day old fetuses (Fig. 8). Capillaries in older fetuses (10 ml) adjacent to larger adipocytes had a remarkable irregular endothelium which contained lipid droplets, endothelial flaps and an extensive arrangement of thin perivascular cell processes surrounding the capillary (Fig. 8). Endothelium adjacent to large adipocytes in older control (no liposyn) fetuses had a typical mature morphology (Fig. 9). In a related study, lipemia was induced in newborn mice by reducing litter size and adipose and other tissues were obtained from 1 to 2 day old mice (19). Electron microscopic studies of subcutaneous adipose tissue from these 1 to 2 day old mice revealed many of the endothelium characteristics described above i.e., endothelium flaps, lipid phagocytosis, multivesicular bodies and lipid within deep endothelium recesses (19). In the mouse study the stage of adipogenesis was such that capillaries were associated with lipid filling small adipocytes (19). In contrast, we identified many capillaries that were very active in clearing liposyn from the blood that were not associated with adipocytes. Our study clearly shows that developing endothelium can metabolize circulating lipid in the absence of differentiated adipocytes. Furthermore, blood vessel patterns and expression of basement membranes and lectin binding may be associated with this functional aspect of these small blood vessels.

Conclusion

Before adipogenesis the S-V components dictate or regulate the structure of the adipose depot including the pattern of vascularization for a given location or depot and surrounding tissues. The development of adipocytes and capillary associated cells are closely linked during adipocyte cluster development. Results of recent lineage tracing studies with specific genetic markers support these observations since they demonstrate either a perivascular or endothelial source of preadipocytes in mice (20-22). Secreted and non-secreted factors play key autocrine/ paracrine roles in adipose tissue differentiation. Proteomic and genomic studies are being devised and improved to identify all such factors expanding our understanding of all potential regulators or regulatory loops. Furthermore, considerable evidence is accumulating that the SNS influences adipose since hypothalamic neurons localized in specific hypothalamic nuclei are trans-synaptically connected via the SNS to adipose depots in the pig and other species. Recent studies demonstrate that the nervous system influences blood vessel patterning resulting in very close physical proximity of the developing nerves and blood vessels (23). Possibly, the nervous system indirectly governs the onset and distribution of adipocyte development given the close relationship between developing adipocytes and blood vessels. The brain to adipose tissue connection would then include adipogenesis as well as the production and secretion of adipokines.

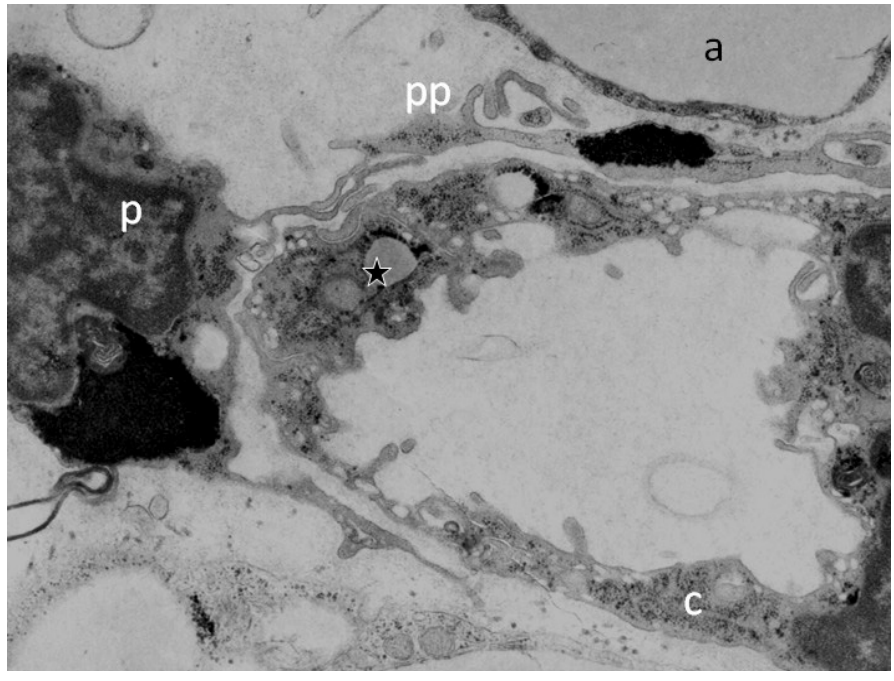

Figure 8. Electron micrograph of adipose tissue from a 90 day fetus after 2 hours of liposyn (6 µl) infusion. Lipid droplets (stars), capillary wall (c), adipocytes (a), perivascular cell (p) and perivascular cell processes (pp) are indicated.

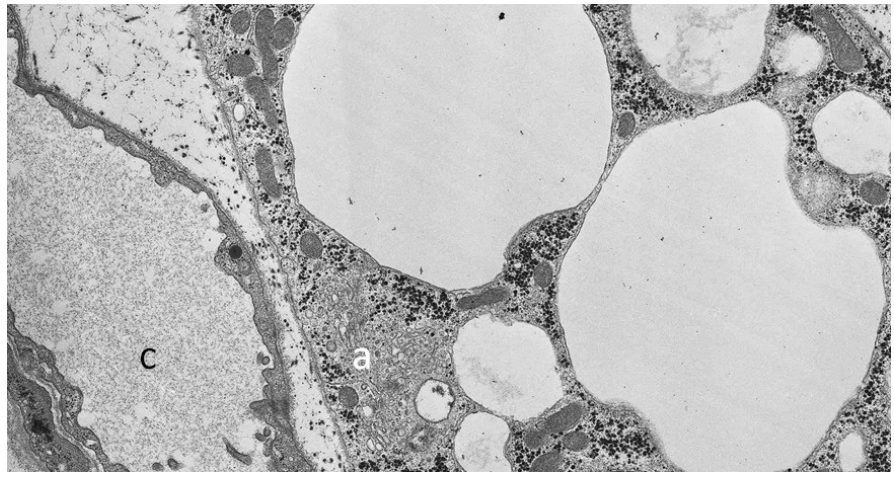

Figure 9. Electron micrograph of adipose tissue a 110 day fetus not receiving liposyn. Capillary (c) and adipocyte (a) are indicated. Note the uniform and regular nature of the capillary wall indicative of a mature capillary.

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