Xenotransplantation of human fetal adipose tissue: a model of in vivo adipose tissue expansion and adipogenesis

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Abstract  Obesity during childhood and beyond may have its origins during fetal or early postnatal life. At present, there are no suitable in vivo experimental models to study factors that modulate or perturb human fetal white adipose tissue (WAT) expansion, remodeling, development, adipogenesis, angiogenesis, or epigenetics. We have developed such a model. It involves the xenotransplantation of midgestation human WAT into the renal subcapsular space of immunocompromised SCID-beige mice. After an initial latency period of approximately 2 weeks, the tissue begins expanding. The xenografts are healthy and show robust expansion and angiogenesis for at least 2 months following transplantation. Data and cell size and gene expression are consistent with active angiogenesis. The xenografts maintain the expression of genes associated with differentiated adipocyte function. In contrast to the fetal tissue, adult human WAT does not engraft. The long-term viability and phenotypic maintenance of fetal adipose tissue following xenotransplantation may be a function of its autonomous high rates of adipogenesis and angiogenesis. Through the manipulation of the host mice, this model system offers the opportunity to study the mechanisms by which nutrients and other environmental factors that modulate or perturb human adipose tissue development and biology.—Garcia, B., H. Francois-Vaughan, O. Onikoyi, S. Kostadinov, M. E. De Paepe, P. A. Gruppuso, and J. A. Sanders. Xenotransplantation of human fetal adipose tissue: a model of in vivo adipose tissue expansion and adipogenesis. J. Lipid Res. 2014. 55: 2685–2691.

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The increasing prevalence of obesity among children, adolescents, and adults in the United States is a major public health issue. While changes in diet and physical activity are clear contributors to this epidemic, other factors may also play an important role. Among these factors are environmental toxicants (1, 2), genetics (3), and the prenatal fetal environment (4, 5) acting through modulation of the fetal epigenome (6). Of particular concern is childhood obesity, which was recently found to affect >17% of children aged 2 to 19 years old in the United States (7). Childhood obesity is associated with the long-term development of a spectrum of adverse health conditions, which makes it all the more concerning that there is an upward trend in the prevalence and severity of childhood obesity (7).

Our understanding of the process of adipogenesis is largely derived from in vitro studies using a variety of cell line model systems (8, 9), most of which are murine in origin. Most notable among these is the 3T3-L1 mouse preadipocyte cell line (10), which has served as a key model system for the study of adipogetic gene expression (11). Primary cultures derived from white adipose tissue (WAT), human included, are useful not only for discovery of regulatory mechanisms but also for establishing the relevance of findings derived from immortalized cell lines (9). Studies on human WAT are limited to the clinical research realm. Notwithstanding the breadth of model systems used to study adipose tissue biology, the field suffers from a paucity of physiological, human model systems.

One potential approach to the study of human adipose tissue biology is xenotransplantation. While autologous human fat transplantation is an established procedure (12–14), adipose tissue xenotransplantation has proved challenging. High resorption rates are the norm. Though resorption can be attenuated through the administration

Abbreviations:  ADRP, adipose differentiation-related protein; EdU, 5-ethyl-2'-deoxyuridine; FABP4, FA binding protein 4; H&E, hematoxylin and eosin; HIF1α, hypoxia inducible factor 1-α; PLIN, perilipin; qPCR, quantitative PCR; WAT, white adipose tissue.

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Methods
or induced overexpression of proangiogenic, prosurvival agents (15, 16), xenotransplantation has not been utilized as an approach to study adipogenesis or adipose tissue remodeling. With this circumstance in mind, and with a particular interest in studying the fetal epigenetic programming of adipose tissue development, we explored the xenotransplantation of midgestation human fetal adipose tissue into immunocompromised rodents. The result of this work was the establishment of a new model system to investigate human WAT biology and development.

MATERIALS AND METHODS

Human adipose tissue procurement

Human fetal WAT was obtained under sterile conditions from spontaneous fetal stillbirths, gestational weeks 20–22, that were delivered at Women and Infants Hospital (Providence, RI). Fetuses delivered by elective or medical abortion were excluded, as were fetuses with visible congenital anomalies. Macerated fetuses, which were presumed to have had a prolonged period between fetal demise and delivery, were also excluded. The study protocol was approved by the Women and Infants Hospital’s institutional review board. Fully informed written parental consent was obtained in compliance with the approved study protocol.

Fetal adipose tissue was harvested from the perirenal or central subcutaneous location. Details of the fetal examination and other pertinent information gathering have been described by De Paeppe et al. (17). Once obtained, tissue was rinsed one time in Hank’s balanced salt solution and then transferred to ice-cold Leibovitz’s L-15 medium (Invitrogen, Grand Island, NY) supplemented with gentamicin (50 μg/ml) and penicillin/streptomycin (50 μg/ml) for transport to Brown University’s Xenotransplant Core Facility. There, the tissue was cut into 1 mm³ pieces under sterile conditions on ice. The remaining tissue was divided and fixed in 10% neutral buffered formalin or flash frozen in liquid nitrogen. Adult intra-abdominal WAT was obtained fresh from the Cooperative Human Tissue Network (National Cancer Institute; http://www.chtn.nci.nih.gov/). Specimens were received and transplanted within 36 h of the time of the surgery at which they were obtained.

Adipose tissue xenotransplantation

Male SCID-beige mice obtained from Charles River Laboratories (Wilmington, MA) were housed under standard conditions and fed standard laboratory chow ad libitum. Xenotransplantation was performed when the mice were 6 to 12 weeks old. Following induction of isoflurane anesthesia, two to four WAT fragments were transplanted underneath the capsule of each kidney (four to eight per animal).

Following surgery, mice were fed ad libitum. They were euthanized by exsanguination under isoflurane anesthesia at times ranging from 2 to 180 days following transplantation. Mice received an intraperitoneal injection of 5-ethyl-2-deoxyuridine (EdU; Life Technologies, Grand Island, NY) at a dose of 50 μg/g body weight 24 h prior to euthanasia. Ninety minutes prior to euthanasia, pimonidazole HCl (Hypoxyprobe-1; Hypoxyprobe Inc., Burlington, MA) was administered at a dose of 60 μg/mg by intraperitoneal injection.

At the end of each experiment, gross images of the explanted kidney and adhered xenografts were taken at 1x, 2x, and 2.5x magnifications. One kidney was fixed in 10% neutral buffered formalin and paraffin-embedded for histological analysis. The xenografts, kidney capsule, kidney, and host fat from the other kidney were dissected, frozen in liquid nitrogen, and stored at −70°C for further analysis. All animal procedures were approved by the Brown University and Rhode Island Hospital Institutional Animal Care and Use Committees and performed in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Histology, immunohistochemistry, and immunofluorescence

Preimplant WAT and formalin-fixed, paraffin-embedded kidneys containing xenografts were stained using hematoxylin and eosin (H&E) and Masson’s trichrome. Immunohistochemistry was performed using antibodies directed toward PPARy (Cell Signaling Technology, Beverly, MA), perilipin (PLIN; Cell Signaling Technology), and CD31 (DakoCytomation Inc., Carpinteria, CA). Paraffin sections (6 μm) were passed through three changes of xylene, 100% ethanol, and 95% ethanol. For PPARy and perilipin, antigen retrieval was performed by subboiling the slides in 1×-diluted Dako Target Retrieval Solution for 20 min (DakoCytomation Inc.). Slides were then quenched in 3% H2O2 and blocked in 2.5% Normal Horse Serum (Vector Laboratories, Burlingame, CA) before overnight incubation in primary antibody at 4°C (1:400 and 1:100 dilutions for PPARy and perilipin, respectively). Slides were incubated with a horseradish-peroxidase-conjugated secondary rabbit antibody (Vector Laboratories) for 1 h prior to staining with 3,3′-diaminobenzidine (Vector Laboratories). Nonspecific staining was assessed by omitting primary antibody.

Avidin-biotin immunoperoxidase staining for CD31 (platelet endothelial cell adhesion molecule, PECAM-1) was performed as previously described (17). The antibody used for these analyses is specific for human CD31; it does not react with mouse CD31. The pimonidazole HCl administered prior to euthanasia is reductively activated in hypoxic cells, resulting in the formation of stable adducts with thiol groups within these cells. Staining for the activated product was performed according to the manufacturer’s protocol. A slide containing perigonadal adipose tissue from a mouse that had been intentionally made hypoxic prior to euthanasia was used as a positive control.

Histological and immunohistochemical slides were scanned using the Aperio Scanscope. Images were acquired using ImageScope software (Leica Biosystems, Richmond, IL). Adipocyte size was determined using H&E-stained slides and National Institutes of Health ImageJ software (18). RGB image channels were split, and the green channel was used to threshold positive staining to the adipocyte membrane. The areas of at least 450 total adipocytes from four to five randomly selected 20× fields were measured.

Immunofluorescence for EdU (Life Technologies), an indicator of DNA synthesis, was carried out as described previously (19). Slides were then incubated in Sudan Black B for 10 min to block background. Slides were mounted using Vectashield with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Images were captured using a Nikon Eclipse E800 microscope and Nikon DS-Fi2 camera (Nikon Instruments Inc., Tokyo, Japan).

Gene expression analysis

Total RNA was isolated from explanted fetal xenografts using the RNeasy Lipid Tissue Mini kit (Qiagen, Valencia, CA). The level of gene expression was assessed using real-time quantitative PCR (qPCR). Primers used for analysis are listed in Table 1. The relative abundance of specific genes was calculated by the comparative Ct method using the expression level of 18S rRNA as the reference.
RESULTS

Fetal WAT xenografts were performed using tissue derived from three separate fetal donors. Their characteristics and the analyses carried out on each are described in Table 2. The gestational ages of the three fetuses were 20.0, 20.6, and 21.0 weeks. All were male. In two cases, the adipose tissue obtained was perirenal. In the third case, subcutaneous adipose tissue from the trunk was utilized for transplantation. Parallel studies were carried out using intra-abdominal WAT from two adult donors, the characteristics of whom are also provided in Table 2.

Histologic and immunohistochemical analysis of fetal (Fig. 1A) and adult (Fig. 1B) preimplant samples consistently demonstrated the appearance of healthy WAT. However, the fetal WAT contained lobules of tightly packed, small adipocytes surrounded by stroma, while the adult tissue was mainly composed of large adipocytes and minimal stroma. Perilipin, the product of the PLIN gene, is present on the surface of lipid droplets in healthy adipocytes (20, 21). Positive staining for perilipin was seen throughout the preimplant fetal and adult samples. PPARγ, a nuclear receptor that is activated in preadipocytes (22), was also detected throughout the fetal and adult tissue, indicating the presence of preadipocytes in both. However, staining was far more abundant and intense in the fetal samples. CD31, an indicator of the tissue’s vascular component, was readily detected in both fetal and adult samples. These findings were interpreted as a post hoc indication that the fetal and adult WAT samples were suitable for xenotransplantation.

Gross images of the mouse kidneys containing human WAT xenografts (Fig. 2A) were acquired at the time of explantation. All samples for transplantation were approximately the same size when placed beneath the kidney capsule. The fetal xenografts first showed focal areas consistent with newly generated adipose tissue at 14 days. Over the subsequent 1 to 2 months, we consistently observed a continual increase in the size of the xenografts. Based on notes that were made at the time of xenotransplantation and direct observations when experiments were terminated, it appeared that all or nearly all of the transplanted fetal fragments engrafted. In contrast, by 2 weeks the adult xenografts showed the presence of what appeared to be lipid droplets. At no point did the adult xenografts acquire the appearance of healthy WAT.

H&E staining of the xenografts (Fig. 2B) was consistent with their gross appearance. After 14 days, the fetal implants showed some hemorrhage as indicated by the presence of red blood cells in the intercellular regions. Many fewer red blood cells were present at longer time points. By 60 days, the fetal xenografts contained adipocytes that were heterogeneous in size, consistent with the appearance of healthy, expanding adipose tissue. In contrast, the adult xenografts showed a marked loss of tissue integrity and hemorrhage at all time points posttransplant. There was a transition from heterogeneous adipocyte size at 14 days, with some surviving adipocytes, to a dominance of large lipid droplets at 2 months. Trichrome staining (Fig. 2C) showed a consistent level of stromal staining in the fetal xenografts. In contrast, the adult xenografts showed a progressive increase in fibrosis.

We observed considerable heterogeneity of adipocyte size in the fetal xenografts. To examine this quantitatively, adipocyte size was assessed for two of the xenotransplant experiments at posttransplant intervals ranging from 14 to 180 days. Results (Fig. 3) did indeed show a broad range of adipocyte surface areas in two dimensions. Because it was not possible to know at what level in the vertical dimension a particular cell was sectioned, we could only assess results relative to one another. An examination of pretransplant fetal and adult adipose tissue showed smaller median adipocyte size in the fetal samples than in the adult samples. The second of our two adult samples, which came from an individual with morbid obesity, showed the highest median and range for adipocyte size. Among the explanted

![Table 1. Primer sequences used for real-time qPCR](image)

| Gene   | 5' Primer Sequence     | 3' Primer Sequence     |
|--------|------------------------|------------------------|
| PPARγ  | GACGCCAAGTTTGAAGTTGC   | CAGGGCTTCATGAGGATTGT   |
| Adiponecin | CCTGCTGAAGGGTGGAGAA   | GTAAAGGATGTGGGATTT   |
| ADRP   | TGGATGGGACAGGAACCGTGG   | GCCATGGCACAATCTGAGT   |
| FABP4  | CAGTGTGATGGGGATGTTGA   | CGTGGAGGATGACCCCGT   |
| Leptin | CACACCCGACTGCTCTTCCT   | AGGTTCCGGGATGTGG   |
| PLI    | CGGGGACGACGAGCAGTT   | GCGAACGGACTGCTACTCC   |
| HIF1α  | GAAGCTGAAAAAGAAATGCTCG | CTATTACAGATGGGACACTCA   |
| 18s    | CGGGGTTCTATTTGTGGT   | GGGCCTTCCCTTAAACTG   |

ADRIP, adipose differentiation-related protein; FABP4, FA binding protein 4; HIF1α, hypoxia inducible factor 1-α.
presence of xenograft endothelial cells in the host tissue was persistent. Xenograft endothelium was present but reduced at 2 months. While the extension of xenograft blood vessels into the host tissue was present in the adult xenograft tissue samples analyzed, we observed considerable size heterogeneity across the full postimplantation time frame. However, adipocyte size tended to be greatest at the longest time points. Comparing the xenotransplants with the pretransplant fetal tissue, it appeared that xenotransplantation was associated with an increase in adipocyte size that occurred at the earliest stages following transplantation.

Immunohistochemistry for perilipin (Fig. 4A) showed consistent membrane staining throughout the fetal xenografts at all time points, consistent with the viability of the transplanted tissue. In contrast, the adult xenografts showed a low level of perilipin staining at 14 days and virtually no specific staining at longer time points. Immunohistochemical analysis of fetal xenografts for PPARγ (Fig. 4B) showed numerous positive nuclei, particularly at time points up to 35 days. There was sparse staining for PPARγ in the adult human xenografts at the earliest time points (Fig. 4B). By 1 month, few positive nuclei were apparent. By 2 months, there was little to no positive staining for PPARγ in the adult xenografts.

Staining of the fetal xenografts for EdU incorporation into DNA (Fig. 4C) showed a greater number of positive cells at 14 days than at 35 days. By 60 days, very few EdU-positive cells were detected. We were unable to determine the identity of the EdU-positive cells, though they were consistently observed to be perivascular.

Staining for CD31 (Fig. 5A) showed abundant human endothelial cells within the fetal xenografts at all time points. Most striking was the extension of staining into the host renal parenchyma by 2 weeks. At 1 month, the
tissue transplants, the degree of staining was considerably less than was seen in the fetal xenotransplants. We interpreted the extension of human endothelium into the host renal parenchyma as indicating active angiogenesis intrinsic to the xenotransplanted tissue. We further concluded that angiogenesis was considerably more active in the fetal than in the adult xenografts.

Pimonidazole HCl (Hypoxprobe) staining (Fig. 5B) was negative in all the fetal and adult xenograft sections that were tested. Our positive control, a mouse injected with pimonidazole HCl 10 minutes prior to being euthanized by slow administration of carbon dioxide, did indeed show intense staining of several tissues (data not shown). The periphery of hypoxic cells within perigonadal fat from the control animal (Fig. 5B) showed subtle but clearly visible staining that was not apparent in any of the xenografts.

To assess the changes in xenograft gene expression over time, we explanted tissue at various time points from one of the donors at 35, 60, and 95 days after transplantation. qPCR showed consistent expression of PPARγ, HIF1α, and several genes associated with mature adipocytes: adiponectin, ADRP (PLIN2), FABP4, leptin, and LPL (Fig. 6).

DISCUSSION

There is intense interest in adipose tissue biology that derives in large part from the high incidence of obesity and its consequences. Nonetheless, there are surprisingly few models of human adipose tissue expansion and adipogenesis. Based on our interest in epigenetic programming as it relates to the fetal origins of metabolic disease (23), we initiated xenotransplantation studies using midgestation human fetal adipose tissue. The gestational age window for these studies was limited. Discreet abdominal or subcutaneous fat depots were not visible prior to 18 weeks, and we set our upper limit for gestation at the previability age of 22 weeks. This constraint did not render our intended studies impractical. In fact, our observations indicating the utility of fetal adipose tissue xenotransplantation were consistent across several experiments using both perirenal and subcutaneous samples as well as numerous host mice. The transplantation of fetal tissue reliably and reproducibly resulted in the establishment of xenografts that were viable for at least 3 months.

The results of xenotransplantation experiments using adult adipose tissue were starkly different from those obtained using the fetal tissue. Although early time points indicated an initial involution of the fetal tissue, there was successful engraftment and, subsequently, robust adipose tissue expansion. Given the marked increase in xenograft size over the several months of observation, and the absence of a concurrent increase in cell size, we considered our observations to be a strongly, albeit indirect, indication of ongoing adipogenesis. Given that fully differentiated adipocytes lack the ability to proliferate, we interpreted these results as indicating de novo adipogenesis in the fetal xenotransplants, possibly originating from perivascular
Previous xenotransplantation studies using adult adipose tissue indicate the importance of intrinsic angiogenic capacity to successful engraftment and expansion. They suggest that adult human adipose tissue does not have the capacity for sufficient angiogenesis to support adipocyte survival (26, 27). Successful human adult adipose tissue xenotransplantation has involved supplementation of mice with proangiogenic growth factors, such as neuropeptide Y and erythropoietin (15, 16). One adult adipose tissue transplant study found that vascularization of the implanted fat originated from neighboring host vessels (26). However, even with stimulation of angiogenesis, the degree of adipose tissue expansion in these experiments does not appear to be at the level observed in our fetal adipose tissue xenotransplants. The combination of robust angiogenesis and adipogenesis, both of
which may emanate from a population of perivascular cells (24), may best explain our results.

Our model of human fetal adipose tissue xenotransplantation should be applicable to the study of the molecular mechanisms involved in human adipogenesis and to the investigation of WAT angiogenesis, adipose tissue remodeling, and brown adipogenesis. The ability of the implants to persist and expand for as long as 6 months posttransplant allows for long-term manipulations of the host, such as changes in diet, alterations in the host hormonal milieu, or exposure to environmental toxicants. A critical aspect of the utility of this model is the ability to obtain fetal adipose tissue. While we considered informed consent to be the ideal, it does substantially reduce sample procurement (28). In fact, obtaining tissue derived from spontaneous fetal loss is not considered as constituting human subject research. Thus, human fetal adipose tissue might be readily available with little infrastructure beyond that of a pathology department in an obstetric hospital. Given the straightforward surgical technique used for xenotransplantation at the renal subcapsular site, the experimental approach we describe represents methodology that could be readily applied to human adipose tissue biology research and the pathophysiology of obesity.

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