Expression of Adipocyte Biomarkers in a Primary Cell Culture Models Reflects Pre-weaning Adipobiology*

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Background: Obesity depends upon a balance between adipocytes associated with thermogenesis and fat storage.

Results: Molecular biomarkers characterize primary adipocyte cultures from different adipose depots.

Conclusions: Only adipocytes from ear mesenchymal stem cells (EMSC) failed to express the brown fat biomarkers and overall gene expression resembled that of neonatal animals.

Significance: Metabolism of fat tissue in the pre-weaning period is distinct from that of the adulthood.

ABSTRACT

A cohort of genes was selected to characterize the adipogenic phenotype in primary cell cultures from 3 tissue sources. We compared the quantitative expression of biomarkers in culture relative to their expression in vivo, since the mere presence or absence of expression is minimally informative. Although all biomarkers analyzed have biochemical functions in adipocytes, the expression of some of the biomarkers varied enormously in culture relative to their expression in the adult fat tissues in vivo, i.e. inguinal fat (ING) for white adipocytes and brite cells, interscapular brown adipose tissue (iBAT) for brown adipocytes and ear mesenchymal stem cells for white adipocytes from adult mice. We propose that the pattern of expression in vitro does not reflect gene expression in the adult mouse, rather it is predominantly the expression pattern of adipose tissue of the developing mouse between birth and weaning. The variation in gene expression among fat depots in both human and rodent has been an extensively studied phenomenon and as recently reviewed, it is related to sub-phenotypes associated with the immune function, the inflammatory response, fat depot blood flow and insulin sensitivity (1). We suggest that adipose tissue biology in the period from birth to weaning is not just a staging platform for the emergence of adult white fat, but that it has properties to serve the unique needs of energy metabolism in the newborn. A case in point is the differentiation of brite cells which occurs during this period, followed by their involution immediately following weaning (2).

When brown adipocytes are induced in a white fat depot of an adult animal, the type of progenitor cell it arises from remains controversial. There are 2 major thoughts in this area, some believe that the brown adipocyte in adult animals is synthesized de novo from a stem cell within the tissue (3). Alternatively, an increase in adrenergic signaling stimulates the PKA pathway that activates mitochondrial biogenesis and Ucp1 expression in mature white
adipocytes (4,5) and converts them into brown adipocytes. Historically, basic expression data showing that Pgc1α and Ucp1 transcripts are rapidly induced in both iBAT and retroperitoneal fat with similar kinetics, that is, mRNAs are detected within hours of cold exposure, support an epigenetic mechanism consistent with the interconversion of white and brown cells in response to changes in ambient temperature (6). An epigenetic model introduces an interesting problem, that is, are all white adipocytes able to activate a brown adipogenesis program or are there selective white adipocytes that have been epigenetically marked to respond to adrenergic signaling and activate brown adipogenesis? Therefore, a white fat cell, with respect to brown adipogenesis potentiality, may be epigenetically programmed or not. A recent publication by Walden et al (7) named up to 15 types of fat depots variable in sub-types of adipocytes. According to their interpretation some adipocytes are pure brown, some pure white and others are composed of a mixture of brown and white adipocytes. Much of this classification is based upon the expression of biomarkers identified during the microarray analysis of gene expression brown vs white fat. However, if you evaluate genetic strains of mice that are genetically high inducers of brown adipocytes (8), then brown adipocytes can be induced in all white fat tissues. However, it is also possible that some white adipocytes cannot express the brown fat program as evidenced by the absence of Ucp1 expression and other components that represent brown adipocyte character? Since most recently identified biomarkers have neither thermogenic or adipogenic functions, whether they are expressed or not may have little to do with adipocyte structure and function and reflect more the history of the markers in the niches they occupy during early development.

Recently, as part of the process to identify the progenitors of white and brown adipocytes and the pathways that trace their lineages, a set of biomarkers have been selected to characterize adipocyte cell types. These include Zic1 (Zinc finger in the cerebellum 1), a transcription factor that is highly expressed in primary BAT (9,10), but not in brite adipocytes. Adipogenic progenitors marked by Sca-1+/CD45−/Mac1− (referred to as ScaPCs), residing in murine brown fat, white fat, and skeletal muscle can differentiate into UCPI-expressing adipocytes in vitro, but only ScaPCs isolated from iBAT have a high level of Zic1 (11). In vivo, Zic1 is uniquely expressed in Myf5-derived brown adipocytes (7). PR domain containing 16 (PRDM16) (12-14) has pivotal activities in mitochondrial biogenesis and function of BAT, but it is expressed in both iBAT and brite adipocyte (10), despite higher expression in BAT. Unlike Zic1, Hoxc9 (homeo box C9) is robustly found on brite adipocytes in vitro (10), and elevated in ScaPCs from white adipose tissue (11) in response to bone morphogenetic protein 7 (BMP7) treatment. Furthermore, high expression of this marker is observed only in white adipose tissues composed of a mixture of brown and white adipocytes such as cardiac WAT (cWAT), inguinal WAT (iWAT) and retroperitoneal WAT (rWAT) (7). It is unclear whether Hoxc9 expression is determined by the number of white or brown adipocytes present in a specific tissue. Other biomarkers investigated have higher expression in brite adipocytes than BAT, including transmembrane protein 26 (TMEM26), tumor necrosis factor receptor superfamily member 9 (TNFRSF9, 4-1BB or CD137) and T-box 1 (TBX1) (15), but none of them are more specific than Hoxc9 to brite adipocytes.

An important question is whether functional differences exist in the thermogenic capacity of brown adipocytes depending on their location, that is, a brown adipocyte “brite” from a white depot or a “classical” brown adipocyte from a discrete brown fat depot. A recent study suggest that there is no difference in the thermogenic capacity of brite cells and the classical iBAT cells (16). So, the potentiality for thermogenesis is likely to depend more on the number of brown adipocytes that can be induced in white fat depots. From this perspective it is important to know the characteristics of a progenitor adipocyte population that differentiates to a white adipocyte, but not to a brown adipocyte. This study seeks to determine the expression of biomarkers with a biochemical function during the in vitro differentiation of stromal vascular
cells from white and brown fat tissues to assess the acquisition of physiological phenotypes of the adipocyte. For this purpose we have collected a set of functional adipocyte biomarkers, largely based on enzymes with functions in lipid and energy metabolism, and transcription factors identified during the analysis of adipogenesis (17,18), and the microarray analysis of gene expression during early development of adipose tissue (19). The results suggest that expression of the biomarkers in primary cell culture recapitulate their expression in vivo; however, it is not to the expression observed in adult tissues, but rather to that observed in pre-weaning fat of the developing mouse.

MATERIAL AND METHODS

Mice-Breeding colonies of C57BL/6J, 129S1/SvImJ and AXB8/PgnJ mice were established with breeders purchased from the Jackson Laboratory (Bar Harbor, Maine USA). Mice were conventionally housed at 23°C and fed ad libitum. Diets were either a high fat diet with 59 kcal % fat (TestDiet #9G03) or low fat chow diet with 13 kcal % (PicoLab Diet 20). Female mice were used in this study. All experimental protocols, conducted at the Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Olsztyn, Poland, were approved by the Local Committee for the Ethical Treatment of Experimental Animals of Warmia-Mazury University (NR 38/2011).

Fatty acid preparation-Bovine serum albumin/fatty acid complexes were prepared as previously described (20,21). Complexes were dissolved in Dulbecco’s modified Eagle’s medium (DMEM-F12, Sigma D8900) and stored at -20°C.

Cell cultures of SVFs and EMSC- Mice, 3–8 weeks old, were sacrificed and submerged in 70% ethanol for 2 min. Inguinal fat (ING), interscapular brown adipose tissue (iBAT), and ears were removed and placed in 5 ml of sterile HBSS (Sigma, H1387) plus 1% penicillin and streptomycin (P/S) (Sigma, P4333). Ears were re-submerged in 15% H2O2 for 1 min to eliminate contamination. Fat tissues and ears were minced and digested with collagenase type I (Life Technologies, #17100-017 460 Units/ml) solution for 1 h at 37°C in shaking bath. The cell suspension was filtered through a 100 μm cell strainer (BD Biosciences # 352350) followed by centrifugation (1350 rpm for 9 min). The cell pellet was treated with red blood cell lysing buffer (RBC lysing buffer; Sigma, R7757); The cells were suspended in 10 ml of culture medium and seeded in 60 mm Petri dishes (passage 0; p=0) with 3 ml of growth medium composed of Dulbecco’s modified Eagle’s medium (DMEM-F12, Sigma D8900) supplemented with 15% of inactivated fetal bovine serum (FBS; Life Technologies), 1% P/S and with 1X Amphotericin (Sigma, A9528) in the case of EMSC. Media were changed every 2-3 days.

Adipogenic stimulation-Sub-confluent cultures (p=0) were trypsinized (0.05% trypsin-EDTA, Life Technologies # 25200056) and sub-cultured at density of 0.05x10^6 cells/ml/well in 24-well culture plates in growth medium (passage=1). After 2-3 days adipogenic I medium was added (22), adipogenic I medium (A-I) contains DMEM/F12, 5%FBS, 1%P/S, 0.5mM isobutyl-1 methylxanthine (IBMX) (Sigma, I5879), 1.7 μM insulin (INS) (Sigma, I5523), and 1 μM dexamethasone (DEX) (Sigma, D2915), for 2-3 days, then followed with adipogenic II medium (A-II) composed of DMEM/F12, 5%FBS, 1%P/S, 17 nM Insulin, 2 μM Troglitazone (Tro) (Sigma, T2573) or 1 μM Rosiglitazone (Ros) (Sigma, R2408), and 1 nM 3,3’,5-triiodo-L-thyronine (T3) (Sigma, T6297) for next 3-5 days. Dose of T3 was based on published articles (11,23,24).

In independent experiments, cells were stimulated with 1 μM norepinephrine (NE) (Sigma, arterenol bitartrate A0937) for 24 hr before harvesting. In order to test the effect of fatty acids (FAs) on adipogenesis, cells were treated with FAs during 2 different periods, fatty acid/BSA complex with 7 mM of each fatty acid added during A-I (named FA1) or during A-II (named FA2). Furthermore, 5-aza-2′-
deoxycytidine (Sigma-Aldrich, A3656) was supplemented to growth medium 48 h before adipogenic stimulation with various doses (0.1 µM, 0.5 µM and 1 µM/ml) to test for the involvement of DNA methylation on Sfrp5 expression. 7 or 9 days after adipogenic stimulation cells were stained with oil red “O”, collected for RNA by Tri-reagent (Molecular Research Center, TR118) and for protein by RIPA reagent.

Oil red “O” staining-At the time of harvesting cells (day 7 or 9), oil red “O” staining was performed as described (22). The degree of adipogenic differentiation was determined by extracting the dye from stained cells with 750 µl of isopropanol and then measuring the absorbance at 500 nm.

RNA isolation-Total RNA from cells, subcellular fractions and ING and iBAT tissue was isolated with Tri-reagent (Molecular Research Center, TR 118) as described (25,26). RNA concentration was determined with the NanoDrop spectrometer. Alternatively, total RNA isolated from cultures using RNeasy Mini Kit (Qiagen, 74106) gave similar results.

QRT-PCR-QRT-PCR was performed as described (25,26). TaqMan probes (5’-6FAM and 3’-TAMRA for each probe) were used for quantification of target genes using TaqMan® One-Step RT-PCR Master Mix Reagents Kits (Life Technologies, 4313803). Sequences of primers and Taqman probes for biomarkers are taken from published papers (25-27) and are available upon request. Relative gene expression was normalized to Cyclophilin (Cyclo) and calculated per 60 ng input RNA.

Western blot-Cell lysates were prepared with ice-cold RIPA buffer. RIPA buffer contained 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1mM EDTA, 0.1% SDS, 1% NP40, 0.25% sodium azide; with 10 µl protease inhibitor cocktail (Sigma, P8340), 1 mM phenylmethylsulfonyl fluoride (PMSF – Sigma, P7626), and 1 ml PhosStop solution/10 ml RIPA (PhosStop - Roche, 4906837001). Protein concentration was measured with the Bradford reagent (Sigma, B6916).

Western blot analysis was performed as described (22,28). Blots were incubated with antibodies against UCP1 (rabbit anti UCP1 - Abcam, Ab23841) (1:1000), MEST (goat anti-MEST - Abcam, Ab95453) (1:1000) and β actin (mouse anti β actin – Santa Cruz Biotech, Sc-47778 or Abcam, Ab6276) (1:5000). Specific antibody-antigen complexes were detected using fluorescent-labeled secondary antibodies (goat anti-rabbit IRDye 800, Rockland, 611-132-122; goat anti-mouse IRDye 800, Rockland, 610-730-124; Donkey anti-Goat IRDye 700, Licor, 926-32214 and Donkey anti-mouse IRDye 700, Rockland, 610-730-124). Bands were visualized and quantified using the Odyssey imaging system (Licor Bioscience). β-actin was used as internal control to evaluate the uniformity of protein loading and transfer.

Microarray analysis-For this experiment we used data from a previous analysis of mouse inguinal fat as a function of developmental age and diet with the Applied Biosystems Mouse Genome Survey Microarray (19). Each microarray contained approximately 34,000 features with a set of about 1,000 controls. Signal intensities across micro-arrays were normalized using the quartile-quartile method (www.bioconductor.org). Microarray experiments, described according to MIAME guidelines, have been deposited in the GEO repository with the accession number GSE 19809.

Statistical analysis- All data are expressed as mean ± SD. Analyses were performed using GraphPad Prism 5.0. Student’s t-test was used for single comparisons and one-way ANOVA (repeated measurement) for multiple. Unless otherwise specified, *, +, $ P≤0.05; **, ++, $$$ P≤0.01 and $$$*, ++++, $$$$, $$$ P≤0.001; and not significant (n.s) p > 0.05.

RESULTS

Identification of Functional Markers

Optimally, when attempting to assess brown and white adipocyte character, it would be useful to
employ biomarkers that have specific biological functions in white or brown adipocytes. Towards this end we have established 4 categories of functional biomarkers from microarray gene expression data (Fig. 1). The first category of biomarkers are highly expressed in both brown and white adipocytes, consistent with their common adipocyte character; they include peroxisome proliferator-activated receptor gamma (PPARγ), adipocyte protein 2 (aP2) or fatty acid binding protein 4 (FABP4) cytoplasmic glycerol-3-phosphate dehydrogenase (GPDH) and stearoyl-CoA desaturase (SCD1) (Fig. 1A). The second category are biomarkers that are strongly linked to adipose tissue expansion in adult mice in an obesogenic environment, either by the genetic background or by diet; these genes are Mest, Sfrp5, Bmp3 and Cav1 (Fig. 1B). Third, are biomarkers characteristic of brown adipocytes, these are UCP1, PPARα and PGC1α (Fig. 1C). Fourth, are biomarkers that serve a lipogenic function and are regulated by high fat diets (48) (Fig. 1D). Although Ucp1 is specific for brown adipocytes, a marker with comparable specificity for white adipocytes has yet to be established. The Mest gene, which is highly associated with adipose tissue expansion in obese models, is a promising candidate (25,29,30).

These biomarkers have distinction developmental patterns and responses to diet that reflect different adipocyte functions. The “pan-adipogenesis” biomarkers are highly expressed at all stages of development, in all fat depots in both white and brown adipocytes and with no striking inductions or repressions by a high fat diet (Fig. 1A). The “adipose tissue expansion” markers are highly expressed during the pre-weaning period with the exception of Sfrp5 (Fig. 1B). The expression of Sfrp5 is very low in adult mice on a chow diet and induced in an obesogenic environment that leads to adipose tissue expansion in adult mice. The “brown adipogenesis” markers for white fat depots show maximal expression at 21 days of age with their expression suppressed in adult mice during the course of development from weaning at 21 days of age until 56 days of age when fed a standard low fat chow diet. When fed a high fat diet for 8 weeks from 56 to 122 days of age, expression is further suppressed (Fig. 1C). Expression of the “lipogenesis” markers are expressed at low levels in the pre-weaning mouse, but expression is increased during post-weaning development in 56 day-old mice fed a chow diet. In adult mice expression of lipogenic biomarkers is suppressed by a high fat diet fed from 56 to 112 days of age (Fig. 1D).

Microarray data of functional biomarkers were further confirmed by qRT-PCR analysis of the expression levels of selected important genes in ING (Fig. 1E) and iBAT (Fig 1F) from 7 day vs 56 day old-mice fed a standard diet. There is a strong similarity in the profile for the expression of these four genes of lipid metabolism in ING and iBAT. Most impressive are the extraordinarily high levels of Mest in D7 mice of both ING and iBAT compared to expression in D56 mice i.e. ~400 fold difference. These levels are slightly diminished in under-nourished pre-weaning mice (19); however, additional genetic regulatory mechanisms must act in both ING and iBAT, to determine such developmental variation in the expression of these genes. The expression profiles of Sfrp5, Acly and Acaca are very similar for ING and IBAT and quite low.

Morphology of differentiated adipocytes. Cells isolated from inguinal, interscapular brown fat and external ears were cultured under conditions utilized by several groups, as described in the methods. Photomicrographs by phase contrast illumination and of cells stained by oil red O show a very robust adipogenesis, (Fig. 2). Some differences in morphology can be noted between cells isolated from the fat depots (ING and iBAT) vs EMSC. The cells from the adipose tissues are generally more fibroblastic than those from EMSC, which have a more epithelial shape (Fig. 2A and B). All of the cultures accumulate similar amounts of lipid in their vesicles, although the vesicles in the EMSC cultures appear larger.

Adipogenic differentiation of cells from iBAT, ING and EMSC
Adipogenic differentiation of cells isolated from ING and iBAT fat tissues as well as EMSC
showed similar levels of fat accumulation as indicated by oil red O staining (Fig. 3A). Each of the pan-adipogenesis biomarkers accumulated mRNA in the cells in adipogenic differentiation medium to levels similar (aP2 and Scd1) or comparable (PPARγ and GPDH) to that of inguinal fat in vivo (Fig. 3B). Pparaγ expression was not as robust as occurred with Fabp4, Gpdh and Scd1. The expression of these markers indicates a facile differentiation of cells isolated from ING and iBAT and EMSC to the pan-adipogenic phenotype of adipocytes.

In contrast to the similarity among the pan-adipogenesis biomarkers, the adipose tissue expansion markers behaved very different in culture. Mest was expressed at very low levels in ING and iBAT cells (~1% of in vivo levels in inguinal fat), whereas expression in EMSC was equal to the levels observed in neonatal mice in vivo. Surprisingly, this expression of Mest mRNA occurred with both the control and adipogenic medium in the 3 types of culture. Also the levels of MEST protein was much lower than expected from the levels of mRNA (Fig. 3C). Sfrp5, which is another gene in which expression is strongly associated with adipocyte size (31), showed no expression in ING and iBAT cell cultures and very low expression in EMSC. In addition, Cav1 and Bmp3 resembled Mest in expression patterns.

These phenotypes for Mest and Sfrp5 are puzzling, since expression of these genes in adipocytes is more strongly correlated to the expansion of the adipocyte in adult tissues in vivo than any other known gene (see microarray gene expression data in Koza et al. (32). Furthermore, Sfrp5 is not expressed at all in ING or iBAT cell cultures (Fig 3C). One would expect that the capacity for adipose tissue expansion is a phenotype readily expressed by all adipocytes. The expression of Mest and Sfrp5 in cell culture look striking similar to expression of these genes in vivo, between birth and weaning (compare Fig. 1B and 3C), that is, very high expression of Mest between birth and 10 days of age and almost undetectable levels of Sfrp5 (Fig 1B). To test this further we analyzed expression of Mest in the SVF (stroma-vascular fraction) and MAF (mature adipocytes fraction) isolated from ING tissue of 3 to 5 day old mice (Fig. 4). Whereas, Mest and Sfrp5 expression in adipose tissue of obese adult mice was approximately 90% in the MAF (32), in the 5 day-old mouse Mest expression in the SVF was similar to the amount in the MAF (Fig. 4A). Accordingly, the expression of Mest and Sfrp5 in EMSC cultures looks very much like expression in a 5 day-old mouse (Fig. 1B, 3C and 4). It is noteworthy that Gpdh is an adipogenesis marker in which expression is almost exclusively localized to the MAF of the fat depot of a 5 day old mouse (Fig. 4). Its expression in culture is similar to in vivo expression and there was no detectible expression of Gpdh in cultures not treated with adipogenic medium (Fig. 3B).

The brown adipogenesis markers, Ucp1, Ppara and Pgc1α, were modestly expressed in differentiated ING and iBAT cultures in the presence of TZD in the medium, but the expression in EMSC was less than 0.004% of iBAT in vivo, essentially below the levels of reliable detection by qRT-PCR in our laboratory (Fig. 3D). Since cultures from the gonadal fat of AXB8 mice also expressed Ucp1 (unpublished results), only EMSC cells do not have the ability to express Ucp1, despite their ability to undergo robust adipogenic differentiation. Except for Elovl6, the lipogenic biomarkers all showed robust expression close to in vivo levels in the three types of cultures in a manner dependent on adipogenic medium (Fig. 3E).

It has been shown that ScaPCs progenitor cells from subcutaneous fat of 129-S1 mice have higher levels of Ucp1 induced by BMP7 than B6 mice. This has been interpreted as a factor leading to the higher resistance to diet-induced obesity of the 129-S1 mice (11). We have compared the morphological and biomarker gene expression phenotypes of ING and EMSC cultures from B6 and 129 strains. The similarities are striking between the 2 strains, beginning with morphology (Fig. 5A and E); however, Ucp1 mRNA expression in ING cell cultures is about 5 times higher in 129 mice than B6 mice, which is about 30% of the level of expression measured in iBAT tissue (Fig. 5C). Higher expression was also seen for Ucp1 and
Ppara mRNA in EMSC cultures (Fig. 5G). This increase in Ucp1 expression corresponded to less than 2% of the levels found in iBAT tissue and was much lower than the expression in ING 129 cultures (Fig. 5C). A heightened response in 129 mice is also observed for Pparγ in both ING and EMSC cultures in adipogenic, but not control medium (Fig. 5A and E). Other biomarkers were unaffected by the genetic background.

Stimulation of lipid accumulation with fatty acids: the effects on gene expression -
Cultures were treated with fatty acids to ascertain their possible effects on adipocyte differentiation, the induction of Mest and Sfrp5 expression by enlarged adipocytes and to evaluate their effects on suppression of lipogenic gene expression by fat administration. The ING SVF and EMSC of 129S mice were isolated and then differentiated to adipocytes with the standard adipogenic medium supplemented with 3 fatty acids at 2 different periods during adipocyte differentiation named FA1 and FA2. These fatty acids, C14:0 - myristic acid, C16:0 - palmitic acid, and C16:1 - palmitoleic acid, take part in fatty acid transport, handling and oxidation (20, 33). In the fatty acid-enriched environment, ING and EMSC cultures accumulated more lipid and had larger lipid vesicles (Fig. 6A and B). Under these conditions the expression of the biomarkers of brown adipogenesis was not affected (Fig. 6 C, D and E). Based upon the in vivo gene expression profiles, as illustrated in Fig. 1, the lack of an effect of a fatty acid supplemented medium was not unexpected for the biomarkers of brown adipogenesis. Since increased expression of Mest and Sfrp5 is highly correlated with fat mass expansion and adipocyte size we expected that both Mest and Sfrp5 would have elevated expression in cultures treated with fatty acids. The data in Figure 6F and G shows that fatty acids neither stimulated Mest expression nor activated Sfrp5 expression in ING cultures. Lipogenic gene expression in liver is strongly suppressed by a high fat diet in adult animals, therefore fatty acids were expected to reduced expression of these genes (34); however, none of these genes had alter expression as a result of fatty acid supplementation (Fig. 6H-K). Their expression in cell culture more closely resembled expression in pre-weaning mice in vivo rather than adult mice (Fig. 1).

Regulation of Ucp1 Expression -
The composition of the culture medium, which includes IBMX and TZDs, is able to support the expression of Ucp1 in cultures from the inguinal and iBAT. The only apparent difference is that levels of Ucp1, Ppara and Pgc1α are 2-3 times higher in iBAT cultures than in ING cultures (Figure 7A, F and G). The expression pattern of Pparγ indicates that it is more strongly related to the expression of white adipogenic markers than brown adipogenic markers, although there is no question regarding the importance of Pparγ for brown adipocyte differentiation (35). No significant expression of Ucp1 could be detected in cultures from EMSC. To evaluate the apparent refractory behavior of EMSC we attempted to induce expression by treating cells with norepinephrine and/or T3 (Fig. 7). No increased expression was observed and the levels of both Ppara and Pgc1α were approximately 10-fold lower in EMSC cultures than in cultures from ING and iBAT, suggesting that EMSC have a deficiency in the transcription machinery, which is required to express the brown adipogenesis program.

Comparison of quantitative expression of biomarkers in cell culture to expression in vivo -
In order to accommodate comparisons of expression patterns in vitro and in vivo among the 3 tissue types, the data in Figures 3-6 has been converted to the percent of inguinal fat expression in mice fed a standard low fat diet (a) or a high fat diet (b) or to expression in iBAT (Table 1). The striking observation is that both the pan-adipogenesis genes and the lipid homeostasis genes come very close to achieving the levels of gene expression observed in vivo. Furthermore, for both the pan-adipogenesis and the lipid homeostasis genes the levels of expression in the 3 types of cultures approach that found in vivo of adult mice. Expression of both Gpdh and Fabp4 is similar to expression in pre-weaning and adult mice, whereas expression of the lipogenic genes is more similar to the levels in adult mice fed a low fat diet. The expression of the brown genes in ING and iBAT
cultures is much less than that of adult iBAT tissue, but much greater than the levels found in adult ING tissue. In fact the pattern of expression is most similar to that of Ucp1 mRNA in ING tissue of the 10 to 21 day old mouse (Fig. 1C). The expression of the adipose tissue expansion genes provides a special insight into an explanation of differentiated phenotypes in the 3 types of culture. Mest is expressed at the highest levels just after birth in ING fat and in obese adult mice (Fig. 1B) and at the lowest levels in white adipocytes of lean adults (at 56 days of age, Fig. 1B and brown adipocytes both in vivo and in cell cultures (Table 1 and ref (39)). Mest looks like an in vivo biomarker for adipocytes in adult obese animals (a similar conclusion also applies to Sfrp5). But if Mest is a biomarker for adipocyte hypertrophy, how does one account for the fact that the highest levels of Mest expression occur in the ING fat of the neonatal mouse when the level of adiposity is very low? This expression in the neonatal mouse resembles the high expression of Mest in EMSC cultured in both control and differentiation medium (Fig. 3C). This high expression of Mest and the other adipose tissue expansion genes in EMSC cultures in both control medium and adipogenic medium resembles the high expression of these genes in both the SVF and MAF fractions isolated from the ING fat depot of a 5 day-old mouse (Fig. 4). That is, there is a striking similarity of the biomarker gene expression in culture to the in vivo expression of the pre-weaning period. This suggests that expression in culture reflects adipose tissue phenotypes unique to the pre-weaning mouse.

**DISCUSSION**

The initial experimental design was focused upon in vitro differentiation of the 4 classes of biomarkers in ING, iBAT, gonadal and EMSC cultures. With these functional biomarkers we detected significant differences in the pattern of biomarker expression in the differentiated cell cultures; these patterns that did not correspond to differentiation of adult adipose tissue. We reasoned that in vitro differentiation with such cell biomarkers could provide additional perspectives. Because of our earlier studies on adipose expression during early development in vivo, it became apparent that regulation in culture was reflecting to a considerable degree, although not absolutely, the patterns of gene expression observed in the mouse in vivo between birth and weaning. More importantly and more interesting is that the insight from these studies suggest that adipose tissue from baby mice may have unique functions and mechanisms of regulation. We have not found this perspective on adipocyte biology during early development previously expressed in the literature. Given the well-known effects of mal-nutrition during this early period on long-term susceptibility to the metabolic syndrome, the idea that adipose metabolism may be different in the neonatal mouse is an important concept to explore. The adipocyte expansion genes in vivo show a positive correlation with adipose tissue expansion and adipocyte size in an obesogenic environment that was previously shown for leptin (19,30-32). It has been shown that expression of Mest and Sfrp5 is associated with the tissue and adipocyte hypertrophy and not with the high fat diet per se, as occurs with a gene like Scd1 (25,29) or by hyperphagia and obesity induced by leptin deficiency (31). This data indicates that Mest and Sfrp5 are associated with adipose tissue expansion through the control of adipocyte hypertrophy and not hyperplasia. The mechanism by which Mest or Sfrp5 contribute to adipocyte hypertrophy is poorly understood. That both Mest and Sfrp5 KO mice are resistant to DIO suggests that these genes are actually causative factors in controlling adipocyte hypertrophy (25,31). MEST is located in the endoplasmic reticulum, where it has an activity as a glycerol phosphate acyl transferase and SFRP5 is proposed to suppress oxidative metabolism by inhibition of WNT signaling, which leads to fat accumulation in the adipocyte (31). To investigate further the role of these genes in adipocyte hypertrophy we have used a primary adipocyte culture system, in particular
ear mesenchymal stem cells (EMSC), since preliminary results showed robust expression of *Mest* in this culture model. Since *Mest* and *Sfrp5* are broadly expressed in white fat depots as a function of fat cell size, one would expect these genes to have expression patterns similar to the adipogenic or lipogenic genes, since the capacity for fat cell expansion would seem to be a fundamental property of the adipocyte. However, *Mest* is expressed at very low levels in ING and iBAT cultures, but very high levels in EMSC cultures. In contrast, *Sfrp5* is not expressed at all in ING or iBAT cultures and its expression at the mRNA level in EMSC culture is also less than 1% of that occurring in ING tissue in vivo.

Given the putative and important role of adipose tissue expansion in the etiology of insulin resistance (36-38), it behooves us to understand the underlying mechanism controlling adipocyte hypertrophy. In these experiments we have attempted to create conditions that enhance adipocyte hypertrophy to assess the effects on *Mest* and *Sfrp5*. This includes differentiation in adipogenic medium vs control medium and addition of fatty acids and catecholamines to the medium to modulate lipid accumulation, but none of these treatments caused either the induction or suppression of *Mest* or *Sfrp5*. Possibly the induction of adipocyte hypertrophy failed to reach a threshold level that would activate *Mest* or *Sfrp5*. In vivo data indicates that *Sfrp5* and *Mest* are not induced until the adiposity index (fat mass/lean mass; estimated by NMR) in adult mice exceeds 0.2 (31,32). From the in vivo data we think that the induction of adiposity occurs in a mouse with positive energy balance, generally induced by either a high fat diet in B6 mice or a mutant leptin gene (B6.*ob/ob* mice), since a high fat diet per se does induce either *Mest* or *Sfrp5* expression, as it does *Scd1* (25). The mechanism by which adipocytes expand in a mouse with a positive energy is not understood; good evidence suggests a role for matrix metalloproteinases, which allow for changes in the 3-D shape of the adipocyte, but how this collagenase activity is linked to the obesogenic environment is unknown (39-41). From this perspective our cell cultures may not be able to expand via a mechanism resembling that which occurs in vivo so that expression is minimal or even completely absent. In fact a demonstration of controlled induction of adipocyte hypertrophy in response to what we imagine constitutes a positive energy balance in culture has not been described to our knowledge.

Inspection of the data on *Mest* expression showed a huge difference in expression in the adipocyte cultures from the EMSC compared to ING and iBAT cultures and this expression did not depend upon differentiation on adipogenic medium, since it is also expressed in cells cultured in control medium. In contrast to the expression of *Mest*, *Sfrp5* expression is scarcely detectable. This pattern of expression for these 2 genes in culture resembles that observed in vivo from birth to weaning; that is, *Mest* is expressed at the highest levels detectable in vivo shortly after birth, whereas, *Sfrp5* is expressed at low levels (Fig. 1B) (25). The interpretation of the *Mest* data is not obvious, since high expression in adipose tissue of adult mice depends on a high level of adiposity, yet the levels in the 5 day-old mouse when the adiposity index in the mouse is very low (adiposity index = 0.11), greatly exceeds the level of expression observed in adipose tissue of obese adult mice. To compare further the regulation of *Mest* in adult vs neonatal adipose tissue we determined its cell type distribution. Whereas approximately 90% of *Mest* and *Sfrp5* mRNA levels are expressed in the mature adipocyte fraction of epididymal and inguinal fat from adult DIO mice (32), in the inguinal fat from 5 day-old mice *Mest* and *Sfrp5* is almost equally distributed between the mature and stromal vascular fractions. Accordingly, the high expression of *Mest* in EMSC cells cultured in both growth and adipogenic medium suggests that expression in EMSC culture is consistent with expression in the neonatal mouse, that is, it is independent of the adiposity. Expression of the adipogenesis genes, *Gpdh*, *Fabp4* and *Ppary*, which are very similar in the neonatal and adult mice, are also consistent with regulation in culture which is similar to that found in the mouse during the pre-weaning period (Fig. 1A).

In summary, we propose that an explanation for the some of the variability in expression in vitro is that expression in vitro is not simply
replicating or reflecting gene expression characteristic of an adult mouse, rather it is also reflects the pattern of expression observed in the adipose tissue of the developing mouse between birth and weaning, which can differ greatly from that occurring in the adult mouse. The variation in gene expression among fat depots in both human and rodent has been an extensively studied phenomenon and, as recently reviewed, it is associated with such variation in the immune function, the inflammatory response, fat depot blood flow and insulin sensitivity (1). We suggest that adipose tissue biology in the period from birth to weaning is not just a staging platform for the emergence of adult white fat, but that it has properties designed to serve the unique needs of energy metabolism in the newborn. A case in point is the differentiation of brite cells, which occurs during this period, is followed by their involution and eventual disappearance immediately following weaning (2).

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FOOTNOTES

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Figure Legends

Figure 1. The effects of nutrition and development on functional biomarkers of adipobiology. Microarray gene expression data was obtained from ING fat tissues of C57BL/6J mice at 5, 10, 21, 56 and 112 days of age. From birth to weaning (21 days of age) mice were kept with mothers who were fed a standard diet (Control – CONT), or a high fat diet (over-nutrition – LON), or a standard diet (STD) with restricted intake (under-nutrition – LUN) as described in Kozak et al. (19). Only the standard chow diet group is shown in Fig. 1. From 21 to 56 days of age all offspring were fed a standard chow diet, then from 56 to 112 days of age they were fed a high fat diet (HF). 15 experimental groups (3 diets x 5 ages) each contained a pooled of RNA from 12 mice. Each pool of RNA was analyzed by microarrays in triplicate. Expression profiles from the microarrays data are presented for selected pan-adipogenesis genes (A), adipose tissue expansion genes (B), brown adipogenesis genes (C), and lipogenesis genes (D). The mRNA levels of selected biomarkers (Mest, SFRP5, Acly and Acaca) in ING (E.) and iBAT (F.) tissues from 7 day or 56 day old mice fed a STD were measured by qRT-PCR. Data is the mean ± SD with n= 5-7. Significant levels are indicated by * 7 day relative to 56 day. Differences between groups were analyzed for statistical significance by student’s t-test; *P≤0.05; **P≤0.01 and ***P≤0.001.

Figure 2. Morphological comparison of ING, iBAT and EMSC cell cultures differentiated into the adipocyte lineage. (A) Morphology of cells at Day 5 of adipogenic differentiation at magnification x100. (B) Oil red “O” stained cultures at the 7th day of adipogenic differentiation (magnification x400). The white bar in the lower right corner of each panel represents 100 nm (A) and 400 nm (B).

Figure 3. The expression of functional biomarkers in ING, iBAT and EMSC cultures differentiated into adipocytes. Primary cells cultures were established from AxB8 mice (panel B, C and D) and C57BL/6J (panel A and E). Confluent cultures were differentiated with adipogenic cocktail – A or maintained in control medium -CTR. (A) Degree of cell differentiation estimated by Oil red O staining. Functional biomarkers were assessed by qRT-PCR and Western blot: (B) pan-adipogenesis markers including: Fabp4(aP2), Ppara, Gpdh and Scd1 (C) adipose tissue expansion markers: Mest, Sfrp5, Cav1, Bmp3, and MEST protein (D) brown adipocyte biomarkers: Ucp1, Pgc1a, Ppara and UCP1 protein. (E) lipid homeostasis: Acly, Acaca, Scl25a, and Elovl6. Data is the mean ± SD with n= 4 for panels B, C, and D (AXB8 mice) and with n=8 from 3 independent experiments for panels A and E (C57BL/6J mice). Significant levels are indicated by * A relative to CTR, * ING cultures relative to iBAT cultures, and $ between SVF cultures and EMSC cultures in a same group. Differences between groups were analyzed for statistical significance by one-way ANOVA; *, +, $ P≤0.05; **, ++, $$ P≤0.01 and $$$, ++++, $$$$ P≤0.001.

Figure 4. The expression of functional biomarkers in stromal vascular fraction (SVF) and mature adipocyte fraction (MAF) collected from ING (A) and iBAT (B) tissues of 3-5-day-old mice. SVF and MAF ING (A.) and iBAT (B.) tissues from 14 C57BL/6J mice at 3-5 days of age were pooled, SVF and MAF prepared and total mRNA was isolated. mRNA expression of pan-adipogenesis genes (aP2) Fabp4, Pparγ and Gpdh), adipose tissue expansion markers (Mest, Bmp3 and Sfrp5) and lipogenesis genes (Acly and Acaca) were measured by qRT-PCR. Mest protein was determined by Western blot (C.
and D). Data shown in graph is the mean ± SD, (n=6). Significant differences between SVF and MAF were analyzed by student’s t-test; significant levels are given by * P≤0.05; ** P≤0.01 and *** P≤0.001.

Figure 5. The effect of genetic background on the expression of functional biomarkers in primary adipocytes in vitro. Confluent cell cultures, developed from EMSC and ING SVF of 6-10 C57BL/6J and 129S mice, were maintained in control medium (CTR) or differentiated with adipogenic cocktail (A). Morphology (magnification 40X) and differentiation degree of cells was assessed by oil red “O” staining (A. and E.); mRNA expression of pan-adipogenesis biomarkers Pparγ (A. and E.); adipose expansion markers (Mest and Sfrp5) (B. and F.); brown adipocyte biomarkers (Ucp1 and Ppara) (C. and G.); and lipogenesis biomarkers (Acly, Acaca and Scl25a) (D. and H.). Data compares expression in cultures from C57BL/6J and 129S mice for both ING SVF cultures (A., B., C. and D.) and EMSC cultures (E., F., G. and H.). Data in graphs is the mean ± SD with n=4 to 10; significant levels are given by * A relative to CTR, and $ between C57BL/6J and 129S mice in a same group. Statistical significance between groups were determined by student’s t-test with significance indicated by*, $ P≤0.05; **, $$ P≤0.01 and ***, $$$ P≤0.001.

Figure 6. Fatty acids have a modest effect on adipogenesis in primary cell culture models in vitro. Cell cultures of ING and EMSC were differentiated with an adipogenic cocktail (including INS, IBMX, DEX, TZD and T3) (A); or (A) plus fatty acids (FA) during the first 2 days (FA1) or last 5 days (FA2) of the differentiation process or maintained in undifferentiated/control (CTR) medium. A. Morphology of cells stained by oil red O staining (magnification 400X); B. Concentration of Oil red O taken up by the cultures. mRNA expression of Pparγ (C); Ucp1 (D); Pgc1α (E); Mest (F); Sfrp5 (G); Acly (H); Acaca (I); Scl25a1 (J) and Elovl6 (K) was measured by qRT-PCR. Three independent experiments were performed, Data in graphs is the mean ± SD with n=4 to 8; significant levels are given by * treated groups relative to CTR, + FA1 or FA2 relative to A, and $ between SVF cultures and EMSC cultures in a same group. Statistical significances between groups were determined by student’s t-test with significance levels indicated by *, $ P≤0.05; **, $$ P≤0.01 and ***, $$$ P≤0.001.

Figure 7. The response of adipogenically differentiated EMSC, ING and iBAT cultures to NE and T3 treatment. EMSC, iBAT and ING cultures were differentiated by the standard adipogenic cocktail (Ao is A medium minus T3), adipogenic cocktail plus NE for 24h (Ao+NE24h), Ao + T3 and Ao + T3 + NE24h, or maintained in control medium (CTR). Degree of cell differentiation (A), mRNA expression of Pparγ (B), Ucp1 (C) and other brown adipogenesis associated genes, including Dio2 (E), Ppara (F) and Pgc1α (G) were measured by qRT-PCR. UCP1 protein was determined by Western blot (D.). Three independent experiments were performed. Data shown in graph is the mean ± SD with n=8; * treated groups relative to CTR, + other treated groups relative to Ao and $ SVF cultures versus to EMSC cultures. Statistical significances between groups were determined by student’s t-test; significant levels are given by *, $ P≤0.05; **, $$ P≤0.01 and ***, $$$ P≤0.001,
| Genes                  | ING cultures | IBAT cultures | EMSC cultures | Control tissues |
|------------------------|--------------|---------------|---------------|-----------------|
|                        | CTR | A   | CTR | A   | CTR | A   | A   | A   |                    |
| Adipogenesis genes     |     |     |     |     |     |     |     |     |                    |
| aP2                    | 0.31 | 149.26 | 0.42 | 143.85 | 1.14 | 47.06 | 100(\(^a\)) | 174.9             |
| GPDH                   | 0.04 | 246.19 | 0.40 | 240.40 | 0.05 | 49.12 | 100(\(^a\)) | 1496.2            |
| PPARγ                  | 5.14 | 23.35 | 4.37 | 42.34 | 5.11 | 12.43 | 100(\(^a\)) | 266.5             |
| Scd1                   | 1.33 | 70.11 | 1.29 | 91.11 | 0.88 | 71.21 | 100(\(^a\)) | 112.7             |
| Adipose expansive genes|     |     |     |     |     |     |     |     |                    |
| Mest                   | 0.72 | 1.27 | 1.34 | 1.03 | 47.18 | 82.87 | 100(\(^b\)) | 0.9               |
| SFRP5                  | 0.00 | 0.00 | 0.00 | 0.01 | 0.37 | 0.73 | 100(\(^b\)) | 7.6               |
| BMP3                   | 16.35 | 41.43 | 4.72 | 14.16 | 0.00 | 71.87 | 100(\(^b\)) | 20.3             |
| Cav1                   | 9.30 | 43.15 | 6.61 | 26.44 | 31.79 | 43.03 | 100(\(^b\)) | 80.8             |
| Brown genes            |     |     |     |     |     |     |     |     |                    |
| UCP1                   | 0.00 | 3.78 | 0.00 | 15.83 | 0.00 | 0.04 | 0.1(\(^a\)) | 100              |
| PGC1α                  | 0.10 | 4.99 | 0.07 | 7.65 | 0.43 | 1.44 | 5.8(\(^a\)) | 100              |
| PPARα                  | 0.08 | 4.95 | 0.06 | 13.01 | 0.19 | 0.93 | 2.3(\(^a\)) | 100              |
| Lipogenesis genes      |     |     |     |     |     |     |     |     |                    |
| Acly                   | 9.71 | 35.21 | 17.68 | 58.83 | 7.48 | 67.78 | 100(\(^a\)) | 207.2            |
| Acaca                  | 2.76 | 16.30 | 3.98 | 25.68 | 3.47 | 19.65 | 100(\(^a\)) | 179.9            |
| Scl25a1                | 5.29 | 33.32 | 4.87 | 53.01 | 8.52 | 51.42 | 100(\(^a\)) | 108.2            |
| Elovl6                 | 4.02 | 4.28 | 6.15 | 4.64 | 5.94 | 10.26 | 100(\(^a\)) | 395.1            |

(\(^a\)) - ING WAT from STD fed mice, (\(^b\)) - ING WAT from HF fed mice, and IBAT from STD fed mice
Figure 1.
Figure 2.

A.

Day 5

ING

iBAT

EMSC

B.

Day 7

ING

iBAT

EMSC
Figure 4.

A. Gene expression per mg CytoIDing RNA

B. Gene expression per 10^6 ng total RNA

| Mest mRNA & Protein | Adult in high fat diet | 5.7 day old-mice |
|---------------------|------------------------|------------------|
|                     | n | Mean±SD | n | Mean±SD | n | Mean±SD | n | Mean±SD | n | Mean±SD |
| Mest mRNA(x10^6) μg total RNA | 6 | 56.2±13.3 | 6 | 10.8±3.4 | 5 | 2.9±0.7 | 6 | 38.8±5.6 | 6 | 25.2±16.9 |
| Mest protein(x10^6) μg total Protein | 8 | 51.6±38.7 | 4 | 10.2±1.5 | 4 | 5.4±1.3 | 4 | 3.7±3.5 | 4 | 2.2±2.5 |
Figure 5.
Figure 7.
Expression of adipocyte biomarkers in a primary cell culture model reflects pre-weaning adipobiology

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The images in Figs. 6 and 7 are reversed, but the figures legends are correct.