Metabolism:

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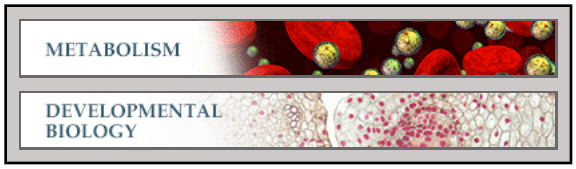

Dinh-Toi Chu, Elzbieta Malinowska, Barbara Gawronska-Kozak and Leslie P. Kozak

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Expression of Adipocyte Biomarkers in a Primary Cell Culture Models Reflects Preweaning Adipobiology*

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Dinh-Toi Chu, Elzbieta Malinowska, Barbara Gawronska-Kozak, and Leslie P. Kozak[1]

From the Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, 10-748 Olsztyn, Poland

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.
Conclusion: Only adipocytes from ear mesenchymal stem cells (EMSCs) failed to express the brown fat biomarkers, and overall gene expression resembled that of neonatal animals.
Significance: Metabolism of fat tissue in the preweaning period is distinct from that of adulthood.

A cohort of genes was selected to characterize the adipogenic phenotype in primary cell cultures from three tissue sources. We compared the quantitative expression of biomarkers in culture relative to their expression *in vivo* because 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 for white adipocytes and brite cells, interscapular brown adipose tissue 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 subphenotypes associated with immune function, the inflammatory response, fat depot blood flow, and insulin sensitivity. 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 that occurs during this period followed by their involution immediately following weaning.

When brown adipocytes are induced in a white fat depot of an adult animal, the type of progenitor cell from which it arises remains controversial. There are two 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 (1). Alternatively, an increase in adrenergic signaling stimulates the PKA pathway that activates mitochondrial biogenesis and *Ucp1* expression in mature white adipocytes (2, 3) and converts them into brown adipocytes. Historically, basic expression data showing that *Pgc1α* and *Ucp1* transcripts are rapidly induced in both interscapular brown adipose tissue (iBAT)[2] 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 (4). 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 Waldén *et al.* (5) named up to 15 types of fat depots that are variable in subtypes of adipocytes. According to their interpretation, some adipocytes are pure brown, some are 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 in brown *versus* white fat. However, if you evaluate genetic strains of mice that are genetically high inducers of brown adipocytes (6), 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. Because 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 may reflect more the

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[1] To whom correspondence should be addressed: Inst. of Animal Reproduction and Food Research, Polish Academy of Sciences, ul. Tuwima 10, 10-748 Olsztyn, Poland. Tel.: 48-89-5234621; Fax: 48-89-5240124; E-mail: l.kozak@pan.olsztyn.pl.

[2] The abbreviations used are: iBAT, interscapular brown adipose tissue; Zic1, zinc finger in the cerebellum 1; Hoxc9, homeobox C9; ING, inguinal fat; WAT, white adipose tissue; BAT, brown adipose tissue; ScaPCs, progenitor cells marked by Sca-1+/CD45−/Mac1−; BMP, bone morphogenic protein; SVF, stromal vascular fraction; EMSC, ear mesenchymal stem cell; T_3, 3,3′,5-triiodo-L-thyronine; qRT-PCR, quantitative RT-PCR; MEST, mesoderm-specific transcript; PPAR, peroxisome proliferator-activated receptor; aP2, adipocyte protein 2; FABP4, fatty acid-binding protein 4; GPDH, glycerol-3-phosphate dehydrogenase; SCD1, stearoyl-CoA desaturase 1; MAF, mature adipocyte fraction.

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 has been selected to characterize adipocyte cell types. These include zinc finger in the cerebellum 1 (Zic1), a transcription factor that is highly expressed in primary BAT (7, 8) but not in brite adipocytes. Adipogenic progenitor cells marked by Sca-1+/CD45−/Mac1− (referred to as ScaPCs), residing in murine brown fat, white fat, and skeletal muscle can differentiate into UCP1-expressing adipocytes *in vitro*, but only ScaPCs isolated from iBAT have a high level of Zic1 (9). *In vivo*, *Zic1* is uniquely expressed in Myf5-derived brown adipocytes (5). PR domain-containing 16 (PRDM16) (10–12) has pivotal activities in mitochondrial biogenesis and function of BAT. It is expressed in both iBAT and brite adipocytes (8), but its expression is higher in BAT. Unlike Zic1, homeobox C9 (Hoxc9) is robustly found on brite adipocytes *in vitro* (8) and is elevated in ScaPCs from white adipose tissue (9) in response to bone morphogenic 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, inguinal WAT, and retroperitoneal WAT (5). 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) (13), 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 "brite" brown adipocyte from a white depot or a "classical" brown adipocyte from a discrete brown fat depot. A recent study suggests that there is no difference in the thermogenic capacity of brite cells and the classical iBAT cells (14). 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 sought 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 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 (15, 16) and the microarray analysis of gene expression during early development of adipose tissue (17). The results suggest that expression of the biomarkers in primary cell culture recapitulates their expression *in vivo*; however, it is not to the expression observed in adult tissues but rather to that observed in preweaning fat of the developing mouse.

MATERIALS 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, ME). Mice were conventionally housed at 23 °C and fed *ad libitum*. Diets were either a high fat diet with 59 kcal % fat (TestDiet number 9G03) or low fat chow diet with 13 kcal % fat (PicoLab Diet 20). Female mice were used in this study. All experimental protocols, which were 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 described previously (18, 19). Complexes were dissolved in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Sigma, D8900) and stored at −20 °C.

Cell Cultures of Stromal Vascular Fractions (SVFs) and Ear Mesenchymal Stem Cells (EMSCs)—Mice (3–8 weeks old) were sacrificed and submerged in 70% ethanol for 2 min. Inguinal fat (ING), iBAT, and ears were removed and placed in 5 ml of sterile Hanks' balanced salt solution (Sigma, H1387) plus 1% penicillin and streptomycin (Sigma, P4333). Ears were resubmerged in 15% H_2O_2 for 1 min to eliminate contamination. Fat tissues and ears were minced and digested with collagenase type I (Invitrogen, 17100-017; 460 units/ml) solution for 1 h at 37 °C in a 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 (RBC) lysing buffer (Sigma, R7757). The cells were suspended in 10 ml of culture medium and seeded in 60-mm Petri dishes (passage 0) with 3 ml of growth medium composed of DMEM/F-12 (Sigma, D8900) supplemented with 15% of inactivated fetal bovine serum (FBS; Invitrogen), 1% penicillin and streptomycin, and with 1× amphotericin (Sigma, A9528) in the case of EMSCs. Media were changed every 2–3 days.

Adipogenic Stimulation—Subconfluent cultures (passage 0) were trypsinized (0.05% trypsin, EDTA; Invitrogen, 25200056) and subcultured at a density of 0.05×10^6 cells/ml/well in 24-well culture plates in growth medium (passage 1). After 2–3 days, adipogenic I medium (A-I) composed of DMEM/F-12, 5% FBS, 1% penicillin and streptomycin, 0.5 mM 3-isobutyl-1-methylxanthine (Sigma, I5879), 1.7 μM insulin (Sigma, I5523), and 1 μM dexamethasone (Sigma, D2915) was added (20) for 2–3 days followed by adipogenic II medium (A-II) composed of DMEM/F-12, 5% FBS, 1% penicillin and streptomycin, 17 nM insulin, 2 μM troglitazone (Sigma, T2573) or 1 μM rosiglitazone (Sigma, R2408), and 1 nM 3,3′,5-triiodo-L-thyronine (T_3) (Sigma, T6297) for the next 3–5 days. The dose of T_3 was based on published articles (9, 21, 22).

In independent experiments, cells were stimulated with 1 μM norepinephrine (Sigma, arterenol bitartrate, A0937) for 24 h before harvesting. To test the effect of fatty acids on adipogenesis, cells were treated with fatty acids during two different periods: fatty acid-BSA complex with a 7 mM concentration 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 added to growth medium 48 h before adipogenic stimulation with various doses (0.1, 0.5, and 1 μM/ml) to test for the involvement of DNA methylation in *Sfrp5* expression. Seven or 9 days after adipogenic stimulation, cells were stained with oil red O and collected for RNA by TRI Reagent (Molecular Research Center, TR118) and for protein by radioimmune precipitation assay reagent.

Oil Red O Staining—At the time of harvesting cells (day 7 or 9), oil red O staining was performed as described (20). 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, ING tissue, and iBAT was isolated with TRI Reagent (Molecular Research Center, TR118) as described (23, 24). RNA concentration was determined with a NanoDrop spectrometer. Alternatively, total RNA isolated from cultures using an RNeasy Mini kit (Qiagen, 74106) gave similar results.

qRT-PCR—qRT-PCR was performed as described (23, 24). 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 kit (Invitrogen, 4313803). Sequences of primers and TaqMan probes for biomarkers are taken from published studies (23–25) and are available upon request. Relative gene expression was normalized to cyclophilin and calculated per 60 ng of input RNA.

Western Blotting—Cell lysates were prepared with ice-cold radioimmune precipitation assay buffer. Radioimmune precipitation assay buffer contained 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Nonidet P-40, and 0.25% sodium azide with 10 μl of protease inhibitor mixture (Sigma, P8340), 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma, P7626), and 1 ml of PhosSTOP solution (Roche Applied Science, 4906837001)/10 ml of radioimmune precipitation assay buffer. Protein concentration was measured with the Bradford reagent (Sigma, B6916).

Western blot analysis was performed as described (20, 26). 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 Biotechnology, sc-47778 or Abcam, Ab6276) (1:5000). Specific antibody-antigen complexes were detected using fluorescently 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, LI-COR Biosciences, 926-32214; and donkey anti-mouse IRDye 700, Rockland, 610-730-124). Bands were visualized and quantified using the Odyssey imaging system (LI-COR Biosciences). β-Actin was used as an 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 (17). Each microarray contained ~34,000 features with a set of about 1000 controls. Signal intensities across microarrays were normalized using the quantile-quantile method (Bioconductor). Microarray experiments, described according to minimum information about a microarray experiment (MIAME) guidelines, have been deposited in the Gene Expression Omnibus repository with the accession number GSE 19809.

Statistical Analysis—All data are expressed as mean ± S.D. Analyses were performed using GraphPad Prism 5.0. Student's *t* test was used for single comparisons, and one-way analysis of variance (repeated measurement) for multiple comparisons. Unless otherwise specified, *, +, and $ indicate $p \leq 0.05$; **, ++, and $$ indicate $p \leq 0.01$; ***, +++, and $$$ indicate $p \leq 0.001$; and not significant indicates $p > 0.05$.

RESULTS

Identification of Functional Markers—Optimally, when attempting to assess brown and white adipocyte character, it would be useful to utilize biomarkers that have specific biological functions in white or brown adipocytes. Toward this end, we established four categories of functional biomarkers from microarray gene expression data (Fig. 1). The first category of biomarkers is highly expressed in both brown and white adipocytes, consistent with their common adipocyte character. They include peroxisome proliferator-activated receptor γ (PPARγ), adipocyte protein 2 (aP2) or fatty acid-binding protein 4 (FABP4), cytoplasmic glycerol-3-phosphate dehydrogenase (GPDH), and stearoyl-CoA desaturase 1 (SCD1) (Fig. 1*A*). The second category contains 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. 1*B*). The third category contains biomarkers characteristic of brown adipocytes. They include UCP1, PPARα, and PGC1α (Fig. 1*C*). The fourth category contains biomarkers that serve a lipogenic function and are regulated by high fat diets (17) (Fig. 1*D*). 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 (23, 27, 28).

These biomarkers have distinctive developmental patterns and responses to diet that reflect different adipocyte functions. The "pan-adipogenesis" biomarkers were 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. 1*A*). The "adipose tissue expansion" markers were highly expressed during the preweaning period with the exception of *Sfrp5* (Fig. 1*B*). The expression of *Sfrp5* was very low in adult mice on a chow diet and was induced in an obesogenic environment that leads to adipose tissue expansion in adult mice. The "brown adipogenesis" markers for white fat depots showed 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 was further suppressed (Fig. 1*C*). The "lipogenesis" markers were expressed at low levels in the preweaning mouse, but expression was increased during postweaning development in 56-day-old mice fed a chow diet. In adult mice, expression of lipogenic biomark-

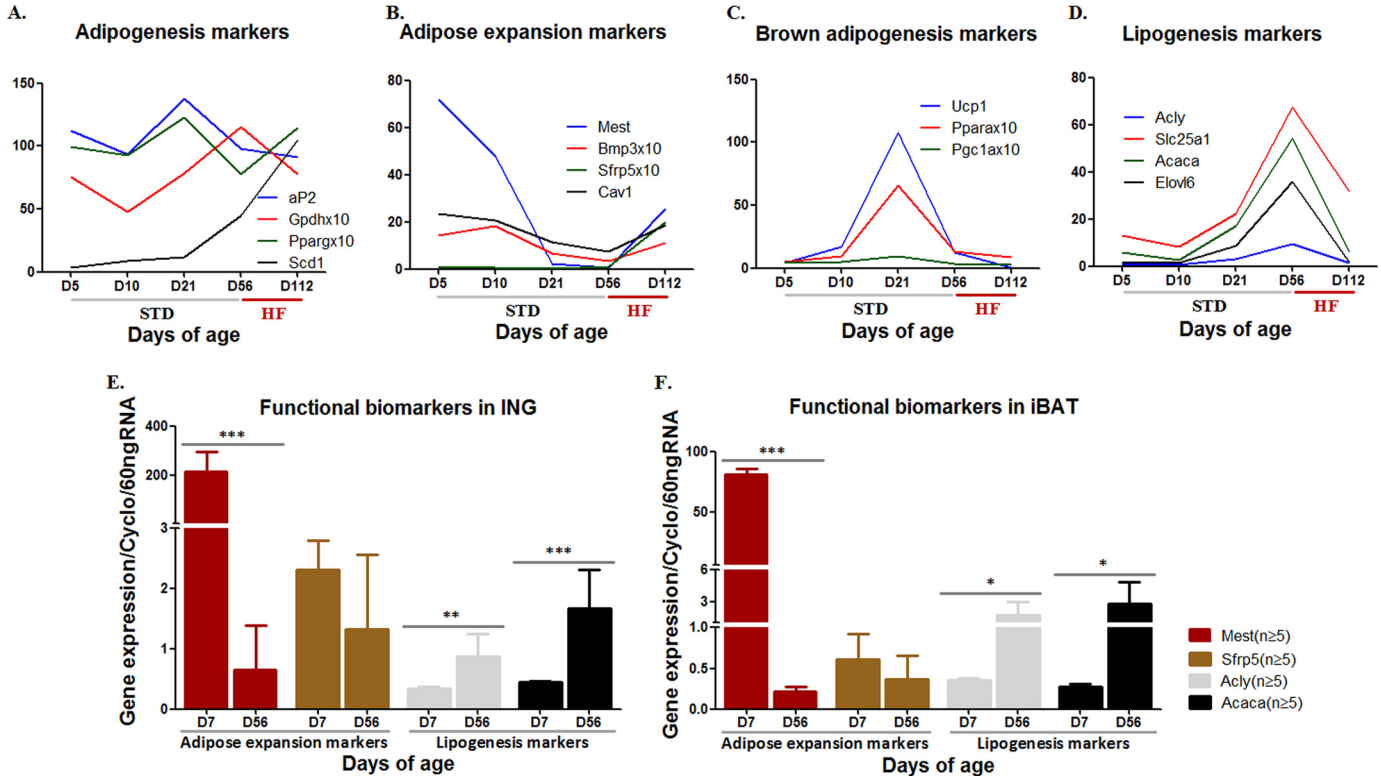


FIGURE 1. **The effects of nutrition and development on functional biomarkers of adipobiology.** Microarray gene expression data were obtained from ING fat tissues of C57BL/6J mice at 5, 10, 21, 56, and 112 days of age. From birth to weaning (21 days of age), mice were kept with mothers who were fed a standard diet (control), a high fat diet (overnutrition), or a standard diet with restricted intake (undernutrition) as described in Kozak *et al.* (17). Only the standard chow diet group is shown. From 21 to 56 days of age, all offspring were fed a standard chow diet (*STD*), and then from 56 to 112 days of age, they were fed a high fat diet (*HF*). There were 15 experimental groups (3 diets × 5 ages), and each contained a pool of RNA from 12 mice. Each pool of RNA was analyzed by microarrays in triplicate. Expression profiles from the microarray 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- or 56-day-old mice fed a standard chow diet were measured by qRT-PCR. Data are the mean ± S.D. (*error bars*) 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: *, $p \leq 0.05$; **, $p \leq 0.01$; and ***, $p \leq 0.001$. *D*, day; *Cyclo*, cyclophilin.

ers was suppressed by a high fat diet fed from 56 to 112 days of age (Fig. 1*D*).

Microarray data of functional biomarkers were further confirmed by qRT-PCR analysis of the expression levels of selected important genes in ING (Fig. 1*E*) and iBAT (Fig. 1*F*) from 7-day-old *versus* 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* mRNA in day 7 mice of both ING and iBAT compared with expression in day 56 mice (*i.e.* ~400-fold difference). These levels are slightly diminished in undernourished preweaning mice (17); 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 fat, interscapular brown fat, and external ears were cultured under conditions utilized by several groups as described under "Materials and 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) *versus* EMSCs. The cells from the adipose tissues are generally more fibroblastic than EMSCs, which have a more epithelial shape (Fig. 2, *A* and *B*). All of the cultures accumulated similar amounts of lipid in their vesicles, although the vesicles in the EMSC cultures appeared larger.

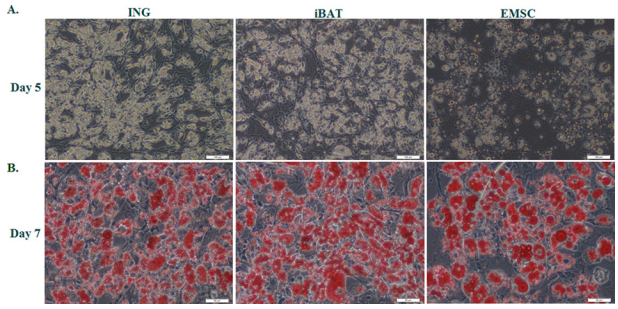


FIGURE 2. **Morphological comparison of ING, iBAT, and EMSC cultures differentiated into the adipocyte lineage.** *A*, morphology of cells at day 5 of adipogenic differentiation (*magnification*, ×100). *B*, oil red O-stained cultures at day 7 of adipogenic differentiation (*magnification*, ×400). The *white scale bar* in the *lower right corner* of each panel represents 100 (*A*) and 400 nm (*B*).

Adipogenic Differentiation of Cells from iBAT, ING, and EMSCs—Adipogenic differentiation of cells isolated from ING and iBAT fat tissues as well as EMSCs showed similar levels of fat accumulation as indicated by oil red O staining (Fig. 3*A*). Each of the pan-adipogenesis biomarkers accumulated mRNA in the cells in adipogenic differentiation medium to levels sim-

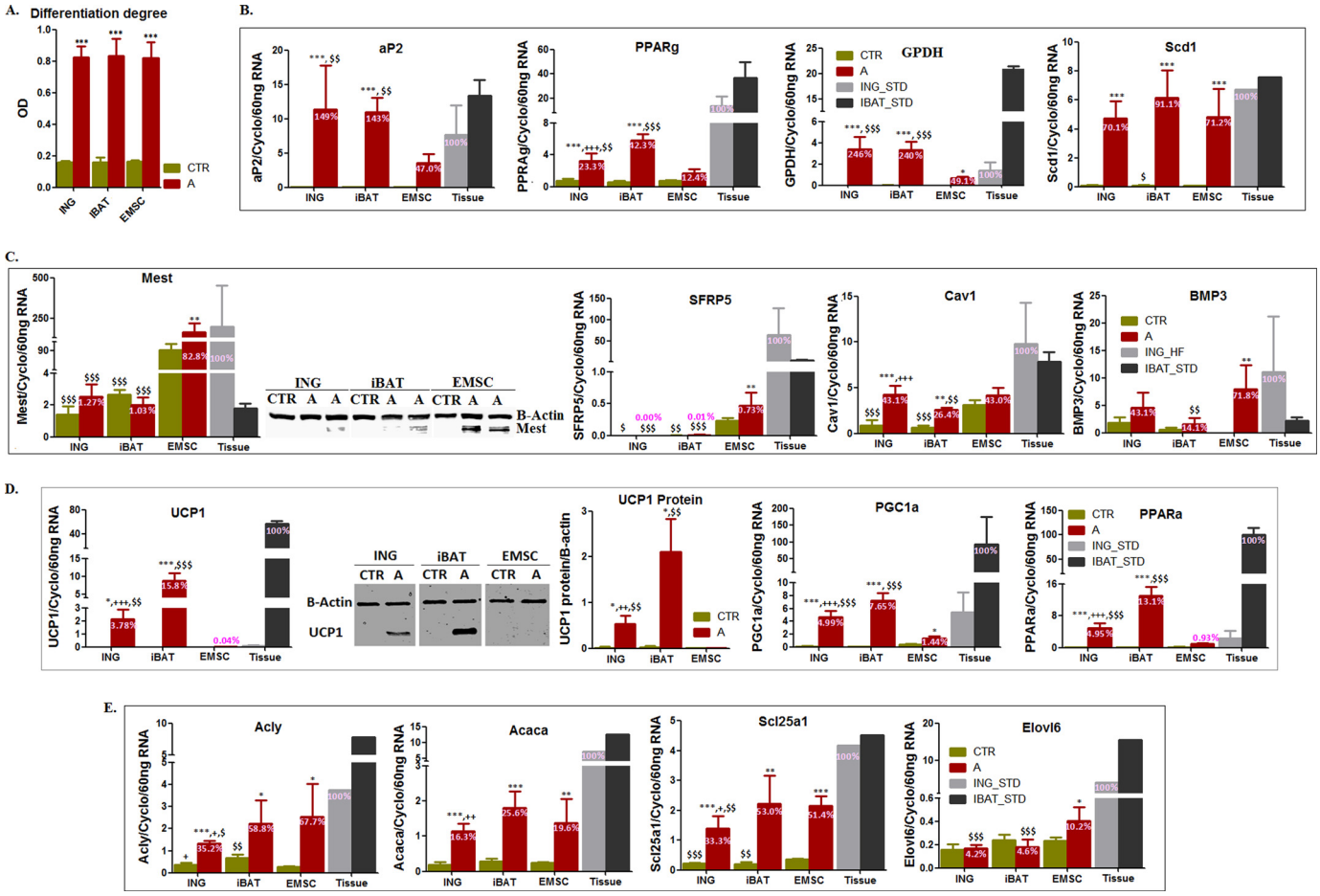
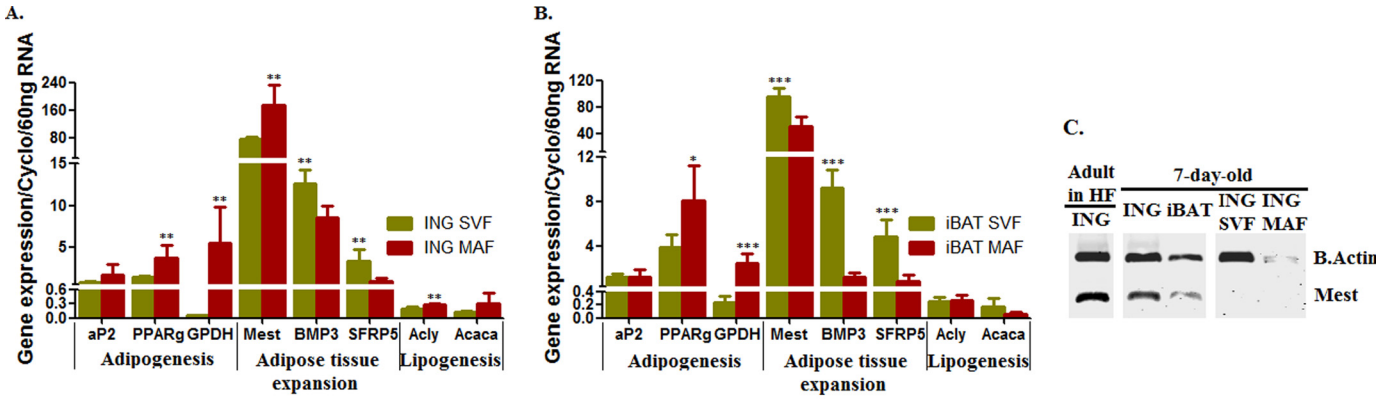


FIGURE 3. **The expression of functional biomarkers in ING, iBAT, and EMSC cultures differentiated into adipocytes.** Primary cell cultures were established from AXB8 (*B*, *C*, and *D*) and C57BL/6J (*A* and *E*) mice. Confluent cultures were differentiated with adipogenic mixture (*A*) or maintained 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 of pan-adipogenesis markers *Fabp4* (*aP2*), *Pparα*, *Gpdh*, and *Scd1* (*B*); adipose tissue expansion markers *Mest*, *Sfrp5*, *Cav1*, and *Bmp3* and MEST protein (*C*); brown adipocyte biomarkers *Ucp1*, *Pgc1α*, and *Pparα* and UCP1 protein (*D*); and lipid homeostasis markers *Acly*, *Acaca*, *Scl25a*, and *Elovl6* (*E*). Data are the mean ± S.D. (*error bars*) with $n = 4$ for *B*, *C*, and *D* (AXB8 mice) and with $n = 8$ from three independent experiments for *A* and *E* (C57BL/6J mice). Significant levels are indicated by * (adipogenic mixture relative to control medium, + (ING cultures relative to iBAT cultures), and $ (between SVF cultures and EMSC cultures in the same group). Differences between groups were analyzed for statistical significance by one-way analysis of variance: *, +, and $, $p \leq 0.05$; **, ++, and $$, $p \leq 0.01$; and ***, +++, and $$$, $p \leq 0.001$. *Cyclo*, cyclophilin; *STD*, standard chow diet; *HF*, high fat diet.

ilar to (aP2 and Scd1) or comparable (PPARγ and GPDH) with that of inguinal fat *in vivo* (Fig. 3*B*). *Pparγ* 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 EMSCs 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 EMSCs 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 three types of culture. Also the levels of MEST protein were much lower than expected from the levels of mRNA (Fig. 3*C*). *Sfrp5*, which is another gene in which expression is strongly associated with adipocyte size (29), showed no expression in ING and iBAT cell cultures and very low expression in EMSCs. In addition, expression patterns of *Cav1* and *Bmp3* resembled that of *Mest*.

These phenotypes for *Mest* and *Sfrp5* are puzzling because 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.* (30)). Furthermore, *Sfrp5* was not expressed at all in ING or iBAT cell cultures (Fig. 3*C*). 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 looks striking similar to expression of these genes *in vivo* between birth and weaning (compare Figs. 1*B* and 3*C*): that is, very high expression of *Mest* between birth and 10 days of age and almost undetectable levels of *Sfrp5* (Fig. 1*B*). To test this further, we analyzed expression of *Mest* in the SVF and mature adipocyte fraction (MAF) isolated from ING tissue of 3–5-day-old mice (Fig. 4). Whereas *Mest* and *Sfrp5* expression in adipose tissue of obese adult mice was ~90% in the MAF (30), in the 5-day-old mouse, *Mest* expression in the SVF was similar to the amount in the MAF (Fig. 4*A*). Accordingly, the expression of *Mest* and *Sfrp5* in EMSC cultures looks very much like expression in a 5-day-old mouse (Figs. 1*B*, 3*C*, and 4). It is note-



D.

Mest mRNA & Protein	Adult in high fat diet		5-7 day old-mice							
	ING Tissue		ING tissue		iBAT tissue		ING SVF		ING MAF	
	n	Mean±SD	n	Mean±SD	n	Mean±SD	n	Mean±SD	n	Mean±SD
Mest mRNA(x10^2)/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(x10^3)/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 4. **The expression of functional biomarkers in SVF and 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 were prepared, and total mRNA was isolated. mRNA expression of pan-adipogenesis genes *Fabp4* (*aP2*), *Pparγ*, and *Gpdh*), adipose tissue expansion markers (*Mest*, *Bmp3*, and *Sfrp5*), and lipogenesis genes (*Acly* and *Acaca*) were measured by qRT-PCR. MEST was determined by Western blotting (*C* and *D*). Data shown in the graphs are the mean ± S.D. (*error bars*) with $n = 6$. Significant differences between SVF and MAF were analyzed by Student's *t* test; significant levels are given by * for $p \leq 0.05$, ** for $p \leq 0.01$, and *** for $p \leq 0.001$. *HF*, high fat diet; *Cyclo*, cyclophilin.

worthy that *Gpdh* is an adipogenesis marker in which expression was 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. 3*B*).

The brown adipogenesis markers *Ucp1*, *Pparα*, and *Pgc1α* were modestly expressed in differentiated ING and iBAT cultures in the presence of thiazolidinedione in the medium, but the expression in EMSCs was less than 0.004% of that in iBAT *in vivo*, essentially below the levels of reliable detection by qRT-PCR in our laboratory (Fig. 3*D*). As cultures from the gonadal fat of AXB8 mice also expressed *Ucp1*,[3] only EMSCs 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. 3*E*).

It has been shown that ScaPCs from subcutaneous fat of 129S1 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 129S1 mice (9). 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 two strains beginning with morphology (Fig. 5, *A* and *E*); however, *Ucp1* mRNA expression in ING cell cultures is about 5 times higher in 129 mice than B6 mice; this is about 30% of the level of expression measured in iBAT tissue (Fig. 5*C*). Higher expression was also seen for *Ucp1* and *Pparα* mRNA in EMSC cultures (Fig. 5*G*). 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. 5*C*). A heightened response in 129 mice was also observed for *Pparγ* in both ING and EMSC cultures in adipogenic but not control medium (Fig. 5, *A* 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 and 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 EMSCs of 129S mice were isolated and then differentiated to adipocytes with the standard adipogenic medium supplemented with three fatty acids at two 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 (18, 31). In the fatty acid-enriched environment, ING and EMSC cultures accumulated more lipid and had larger lipid vesicles (Fig. 6, *A* 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. Because 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 Fig. 6, *F* and *G*, show that fatty acids neither

[3] D. T. Chu, E. Malinowska, B. Gawronska-Kozak, and L. P. Kozak, unpublished results.

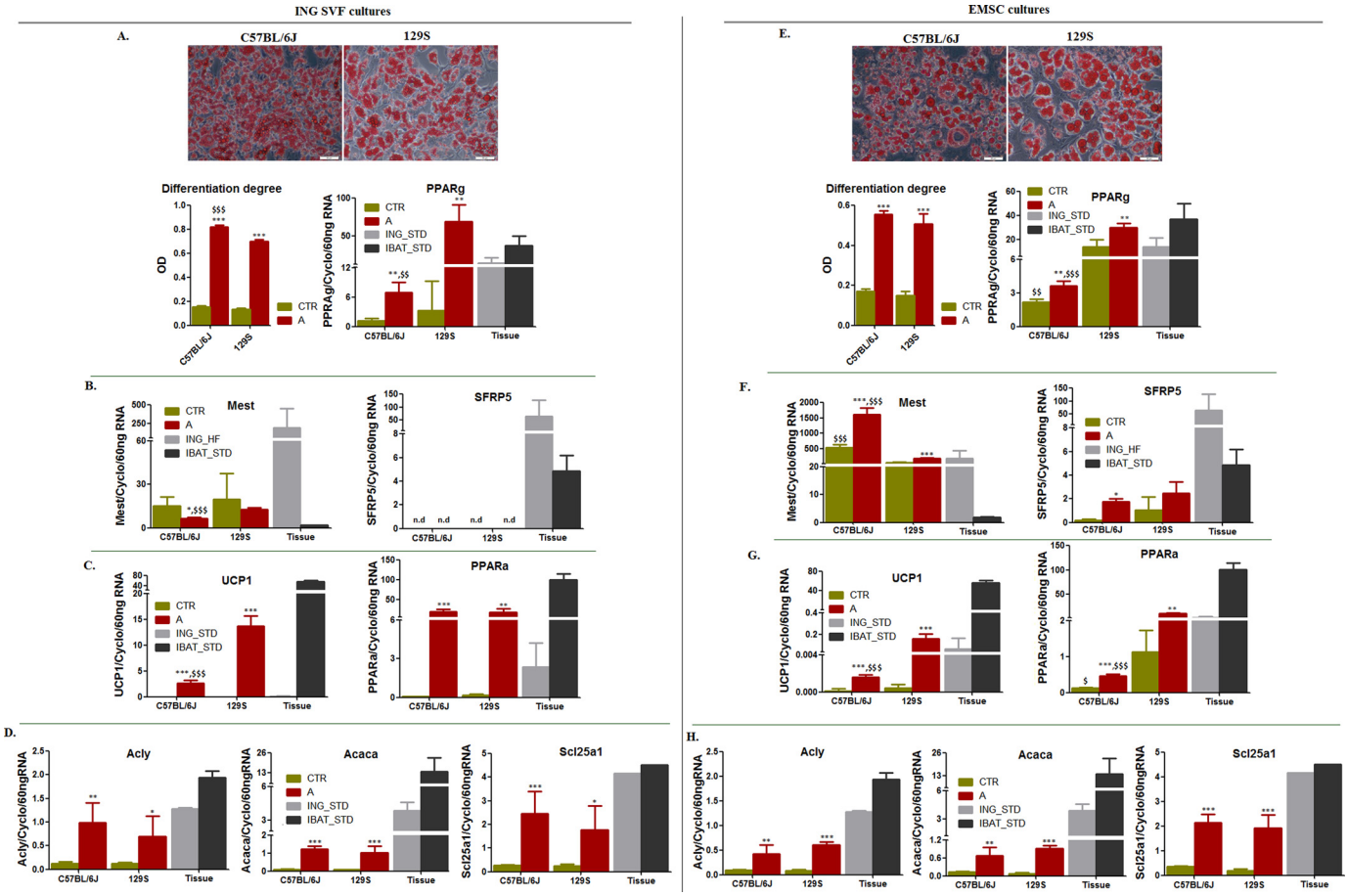


FIGURE 5. **The effect of genetic background on the expression of functional biomarkers in primary adipocytes *in vitro*.** Confluent cell cultures developed from EMSCs and ING SVF of 6–10 C57BL/6J and 129S mice were maintained in control medium (*CTR*) or differentiated with adipogenic mixture (*A*). The morphology (*magnification*, ×40) and degree of differentiation of cells was assessed by oil red O staining (*A* and *E*). mRNA expression of pan-adipogenesis biomarker *Pparγ* (*A* and *E*); adipose expansion markers *Mest* and *Sfrp5* (*B* and *F*); brown adipocyte biomarkers *Ucp1* and *Pparα* (*C* and *G*); and lipogenesis biomarkers *Acly*, *Acaca*, and *Scl25a* (*D* and *H*) was measured. Data compare 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 shown in the graphs are the mean ± S.D. (*error bars*) with $n = 4–10$. Significant levels are given by * (adipogenic mixture relative to control medium) and $ (between C57BL/6J and 129S mice in the same group). Statistical significance between groups was determined by Student's *t* test with significance indicated by * and $ for $p \leq 0.05$, ** and $$ for $p \leq 0.01$, and *** and $$$ for $p \leq 0.001$. *Cyclo*, cyclophilin; *HF*, high fat diet; *STD*, standard chow diet.

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 reduce expression of these genes (32). However, none of these genes had altered expression as a result of fatty acid supplementation (Fig. 6, *H–K*). Their expression in cell culture more closely resembled expression in preweaning mice *in vivo* rather than adult mice (Fig. 1).

Regulation of Ucp1 Expression—The composition of the culture medium, which includes 3-isobutyl-1-methylxanthine and thiazolidinediones, is able to support the expression of *Ucp1* in cultures from the inguinal fat and iBAT. The only apparent difference is that levels of *Ucp1*, *Pparα*, and *Pgc1α* are 2–3 times higher in iBAT cultures than in ING cultures (Fig. 7, *A*, *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 (33). No significant expression of *Ucp1* could be detected in cultures from EMSCs. To evaluate the apparent refractory behavior of EMSCs, we attempted to induce expression by treating cells with norepinephrine and/or T_3 (Fig. 7). No increased expression was observed, and the levels of both *Pparα* and *Pgc1α* were ~10-fold lower in EMSC cultures than in cultures from ING and iBAT, suggesting that EMSCs have a deficiency in the transcription machinery that is required to express the brown adipogenesis program.

Comparison of Quantitative Expression of Biomarkers in Cell Culture to Expression in Vivo—To accommodate comparisons of expression patterns *in vitro* and *in vivo* among the three tissue types, the data in Figs. 3–6 were converted to the percentage of inguinal fat expression in mice fed a standard low fat diet (Footnote a) or a high fat diet (Footnote 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 three types of cultures approach that found *in vivo* in adult mice. Expression of both *Gpdh* and *Fabp4* is similar to expression in preweaning and adult mice, whereas expression of the lipogenic genes is more similar to the levels in adult mice fed a low fat diet. The

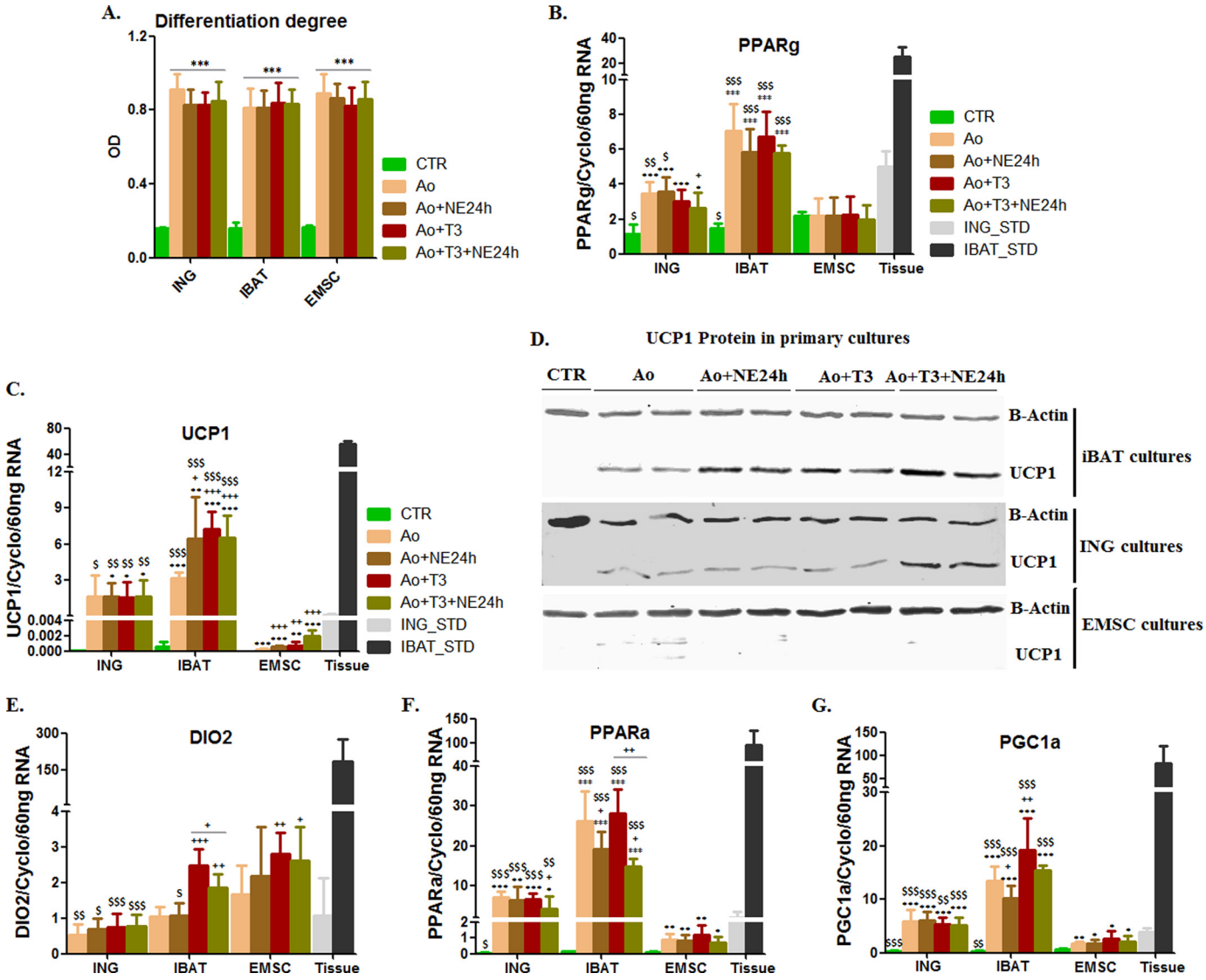


FIGURE 6. **Fatty acids have a modest effect on adipogenesis in primary cell culture models *in vitro*.** Cell cultures of ING and EMSCs were differentiated with an adipogenic mixture (including insulin, 3-isobutyl-1-methylxanthine, dexamethasone, thiazolidinedione, and T_3) (*A*) or the adipogenic mixture plus fatty acids 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 (*magnification*, ×400). *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 shown in the graphs are the mean ± S.D. (*error bars*) with $n = 4–8$. Significant levels are given by * (treated groups relative to CTR), + (FA1 or FA2 relative to adipogenic mixture), and $ (between SVF cultures and EMSC cultures in the same group). Statistical significance between groups was determined by Student's *t* test with significance levels indicated by *, +, and $ for $p \leq 0.05$; **, ++, and $$ for $p \leq 0.01$; and ***, +++, and $$$ for $p \leq 0.001$. *Cyclo*, cyclophilin; *HF*, high fat diet; *STD*, standard chow diet.

expression of the brown genes in ING and iBAT cultures is much lesser 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 10–21-day-old mouse (Fig. 1*C*). The expression of the adipose tissue expansion genes provides special insight into the explanation for differentiated phenotypes in the three types of culture. *Mest* is expressed at the highest levels just after birth in ING fat and in obese adult mice (Fig. 1*B*) and at the lowest levels in white adipocytes of lean adults (at 56 days of age; Fig. 1*B*) and brown adipocytes both *in vivo* and in cell cultures (Table 1 and Ref. 34). *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 EMSCs cultured in both control and differentiation media (Fig. 3*C*). 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 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 preweaning period. This suggests that expression in culture reflects adipose tissue phenotypes unique to the preweaning mouse.

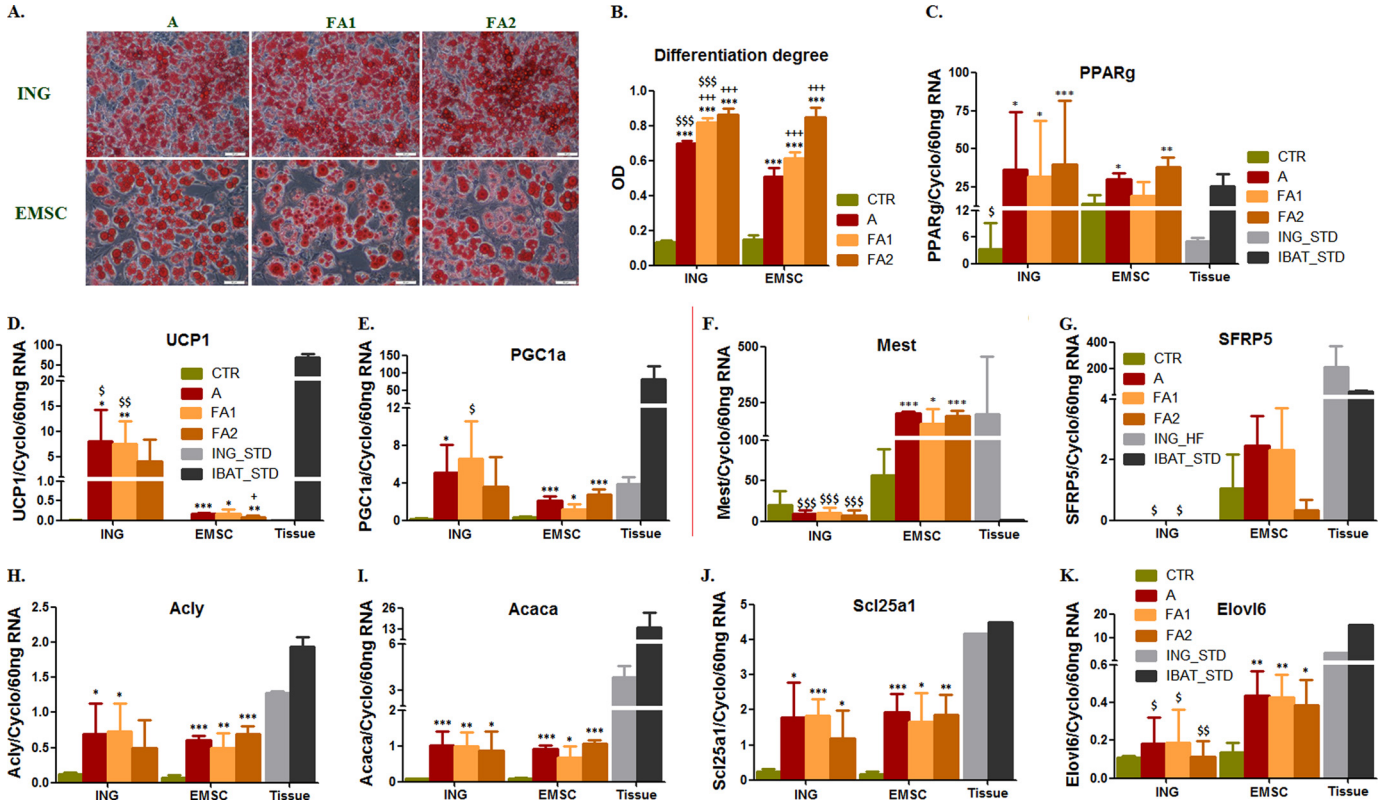


FIGURE 7. **The response of adipogenically differentiated EMSC, ING, and iBAT cultures to norepinephrine and T_3 treatment.** EMSC, iBAT, and ING cultures were differentiated by the standard adipogenic mixture minus T_3 (*Ao*), adipogenic mixture plus norepinephrine for 24 h (*Ao+NE24h*), adipogenic mixture plus T_3 (*Ao+T3*), or adipogenic mixture plus T3 and norepinephrine for 24 h (*Ao+T3+NE24h*) or maintained in control medium (*CTR*). The degree of cell differentiation (*A*), mRNA expression of *Pparγ* (*B*), *Ucp1* (*C*), and other brown adipogenesis-associated genes, including *Dio2* (*E*), *Pparα* (*F*), and *Pgc1α* (*G*) was measured by qRT-PCR. UCP1 protein was determined by Western blotting (*D*). Three independent experiments were performed. Data shown in the graphs are the mean ± S.D. (*error bars*) with $n = 8$. Significant levels are given by * (treated groups relative to control medium), + (other treated groups relative to standard adipogenic mixture minus T_3), and $ (SVF cultures *versus* EMSC cultures). Statistical significance between groups was determined by Student's *t* test with significant levels given by *, +, and $ for $p \leq 0.05$; **, ++, and $$ for $p \leq 0.01$; and ***, +++, and $$$ for $p \leq 0.001$. *Cyclo*, cyclophilin; *STD*, standard chow diet.

TABLE 1

Gene expression percentages of cultures versus positive control (iBAT or ING adipose tissue)

A, adipogenic medium; CTR, control medium.

Genes		ING cultures		iBAT cultures		EMSC cultures		Control tissues	
		CTR	A	CTR	A	CTR	A	ING	iBAT
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 standard chow diet-fed mice.
[b] ING WAT from high fat diet-fed mice and iBAT from standard chow diet-fed mice.

DISCUSSION

The initial experimental design was focused upon *in vitro* differentiation of the four 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 insights 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 malnutrition 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 (17, 28–30). 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* (23, 27) or by hyperphagia and obesity induced by leptin deficiency (29). These data indicate 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* contributes to adipocyte hypertrophy is poorly understood. That both *Mest* and *Sfrp5* KO mice are resistant to diet-induced obesity suggests that these genes are actually causative factors in controlling adipocyte hypertrophy (23, 29). MEST is located in the endoplasmic reticulum where it has an activity as a glycerol-phosphate acyltransferase, and SFRP5 is proposed to suppress oxidative metabolism by inhibition of WNT signaling, which leads to fat accumulation in the adipocyte (29). To investigate further the role of these genes in adipocyte hypertrophy, we used a primary adipocyte culture system, in particular EMSCs, because preliminary results showed robust expression of *Mest* in this culture model. Because *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 because the capacity for fat cell expansion would seem to be a fundamental property of the adipocyte. However, *Mest* was expressed at very low levels in ING and iBAT cultures but very high levels in EMSC cultures. In contrast, *Sfrp5* was not expressed at all in ING or iBAT cultures, and its expression at the mRNA level in EMSC culture was 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 (35–37), it behooves us to understand the underlying mechanism controlling adipocyte hypertrophy. In these experiments, we attempted to create conditions that enhance adipocyte hypertrophy to assess the effects on *Mest* and *Sfrp5*. This includes differentiation in adipogenic medium *versus* 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 indicate that *Sfrp5* and *Mest* are not induced until the adiposity index (fat mass/lean mass; estimated by NMR) in adult mice exceeds 0.2 (29, 30). From the *in vivo* data, we think that the induction of adiposity occurs in a mouse with a positive energy balance, which is generally induced by either a high fat diet in B6 mice or a mutant leptin gene in B6.*ob*/*ob* mice, because a high fat diet *per se* induces either *Mest* or *Sfrp5* expression as it does *Scd1* (23). The mechanism by which adipocytes expand in a mouse with a positive energy balance is not understood. Good evidence suggests a role for matrix metalloproteinases, which allow for changes in the three-dimensional shape of the adipocyte, but how this collagenase activity is linked to the obesogenic environment is unknown (34, 38, 39). 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 EMSCs compared with ING and iBAT cultures, and this expression did not depend upon differentiation on adipogenic medium because *Mest* was also expressed in cells cultured in control medium. In contrast to the expression of *Mest*, *Sfrp5* expression was scarcely detectable. This pattern of expression for these two genes in culture resembles that observed *in vivo* from birth to weaning; that is, *Mest* was expressed at the highest levels detectable *in vivo* shortly after birth, whereas *Sfrp5* was expressed at low levels (Fig. 1*B*) (23). The interpretation of the *Mest* data is not obvious because high expression in adipose tissue of adult mice depends on a high level of adiposity; however, the level in the 5-day-old mouse when the adiposity index in the mouse is very low (adiposity index = 0.11) greatly exceeded the level of expression observed in adipose tissue of obese adult mice. To compare further the regulation of *Mest* in adult *versus* neonatal adipose tissue, we determined its cell type distribution. Whereas ~90% of *Mest* and *Sfrp5* mRNA levels is expressed in the mature adipocyte fraction of epididymal and inguinal fat from adult diet-induced obese mice (30), in the inguinal fat from 5-day-old mice, *Mest* and *Sfrp5* were almost equally distributed between the mature and stromal vascular fractions. Accordingly, the high expression of *Mest* in EMSCs cultured in both growth and adipogenic media 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 *Pparγ*, which are very similar in the neonatal mouse and adult mouse, was also consistent with regulation in culture (Fig. 1*A*).

In summary, we propose that an explanation for 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 also reflects the pattern of expression observed in the adipose tissue of the developing mouse between birth and weaning that 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 immune function, the inflammatory response, fat depot blood flow, and insulin sensitivity (40). 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 that occurs during this period followed by their involution and eventual disappearance immediately following weaning (41).

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ADDITIONS AND CORRECTIONS

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Expression of adipocyte biomarkers in a primary cell culture models reflects preweaning adipobiology.

Dinh-Toi Chu, Elzbieta Malinowska, Barbara Gawronska-Kozak, and Leslie P. Kozak

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

Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.