

Original Research Report

A Real-Time PCR Approach to Evaluate Adipogenic Potential of Amniotic Fluid-Derived Human Mesenchymal Stem Cells

PAOLA DE GEMMIS,¹ CRISTINA LAPUCCI,¹ MATTEO BERTELLI,¹ ANNA TOGNETTO,¹
ERIKA FANIN,¹ ROBERTO VETTOR,² CLAUDIO PAGANO,²
MASSIMO PANDOLFO,^{1,3} and ANDREA FABBRI¹

ABSTRACT

Regulation of adipocyte differentiation is an important process in the control of adipose tissue development. So far, adipogenesis has been investigated through the use of various experimental models. In this work, we used human mesenchymal stem cells (hMSCs) obtained from amniotic fluid (AF) as an alternative model more representative of what naturally happens in vivo. In our opinion, these hMSCs are still not influenced by differentiation stimuli and could act in a way more correspondent to the physiological process of adipogenesis, representing also an ethically acceptable alternative to totipotent human embryonic stem cells (ES). Adipocyte differentiation was monitored following the expressions of key genes. We measured the expression levels of PPAR γ 2, PPAR γ -C1 α , UCP-1, adipsin, and leptin genes using quantitative real-time PCR. We tested our experimental model with two different media. Understanding in vivo adipogenesis mechanisms will shed light on the pathophysiology of many diseases.

INTRODUCTION

REGULATION OF ADIPOCYTE DIFFERENTIATION is an important process in the control of adipose tissue development and in the understanding of mechanisms involved in obesity and in lipotrophy common to many metabolic diseases (1,2). So far, adipogenesis has been investigated through the use of various experimental models, including immortalized murine cell lines (reviewed in ref. 3), tissue-derived precursors (4), embryonic stem (ES) cells (5,6), whole animals (7), and patients (8). These models actually used to investigate the adipogenic differentiation process at molecular level are not fully representative of what naturally happens in vivo because those cells are already committed.

Amniotic fluid (AF) is composed of normal embryonic or fetal chipping cells derived from the three germ layers (ectoderm, endoderm, and mesoderm). Therefore, it possesses the natural precursors of all differentiative lineages. Recently, AF has been used as a source of human mesenchymal stem cells (hMSCs) (9–11), and it could represent a source of MSCs not yet influenced by differentiation stimuli, opposite to adult stem cells confined in their homing. hMSCs obtained in this way could respond in a way that corresponds more to the physiological process of adipocyte differentiation.

Currently, there is no in vitro model that allows us to reproduce natural adipogenesis; moreover, gene expression data exhibit marked differences, depending on the experimental model used. Therefore, we propose that our

¹BIRD Europe Institute, Vicenza, Italy.

²Endocrine-Metabolic Laboratory, Department of Medical and Surgical Sciences, Biotechnology Centre, University of Padova, Italy.

³Department of Neurology, Erasme Hospital, Brussels Free University, Brussels, Belgium.

model can be helpful in integrating information already acquired, in particular, on adipocyte development starting from embryonic cells. AF-derived hMSCs are similar to hES regarding their totipotency, and, in our opinion, they represent an ethically acceptable alternative.

The various steps of adipocyte differentiation can be traced at the molecular level following the expression trend of key genes. Markers analyzed were chosen using the criterion that each gene must be representative of a different step of adipogenesis to allow us to monitor the differentiation. In fact it is known from the literature that, instead of being expressed at the same time, gene markers appear in a cascade during adipogenesis. We know also from the literature that the program of adipogenesis can be divided into at least four stages: (1) preconfluent proliferation; (2) confluence-growth arrest; (3) hormonal induction-clonal expansion; and (4) permanent growth arrest-terminal differentiation (3,12).

The marker genes we investigated are PPAR γ 2, PPAR γ -C1 α , UCP-1, adipsin, and leptin. The peroxisome proliferator-activated receptors (PPARs) are a nuclear hormone receptor family containing three known receptors, PPAR α , PPAR γ , and PPAR δ . PPAR γ consists of two isoforms, PPAR γ 1 and PPAR γ 2. PPAR γ 2 expression is restricted to adipose tissue. PPAR γ mediates growth arrest during differentiation stage 2 (13), and its expression rapidly increases after hormonal induction during stage 3 (14).

PPAR γ co-activator 1 α (PPAR γ -C1 α) is a protein that interacts with PPAR γ (15). Expression of PPAR γ -C1 α in white fat cells turns on uncoupling protein-1 (UCP-1) gene expression, playing a crucial role in the conversion of white mature adipocytes into brown ones (15,16). Two types of adipose tissue are present in mammals. White adipose tissue stores energy as triglycerides, whereas brown adipose tissue has an opposite physiological function because it allows dissipation of energy, being specialized in adaptive thermogenesis. Understanding the molecular pathways that underlie the white versus brown differentiation is fundamental because an imbalanced ratio of white/brown tissue may lead to several diseases such as obesity.

UCPs are small inner mitochondrial membrane proteins that facilitate proton transport. They generate heat, dissipating the proton gradient linked to metabolic fuel oxidation. Three UCPs have been reported. UCP-1 is specific for brown adipose cells (17–19). Adipsin is the major serine protease secreted by the adipocytes into the bloodstream, and it is a very late marker of adipocyte differentiation appearing at stage 4 (20). Leptin is a hormone and a late adipogenesis marker, being primarily made and secreted by mature adipocytes during stage 4 (21). Its expression is decreased by catecholamines and increased by insulin and glucocorticoids (22–24). White adipocytes express leptin at higher levels than brown ones (25).

We followed the expression of these five main adipogenesis markers during adipogenesis in AF-derived

hMSCs by the real-time (RT) PCR technique. We evaluated the expression of these key genes in adipogenesis in our experimental model, starting from AF cells, to make a comparison with existing data regarding other adipogenesis models. Because it is known that adipogenesis steps are influenced not only by the specific experimental model used but also by culture conditions, we performed the experiment using two different culture media.

MATERIALS AND METHODS

hMSC isolation

AF samples were collected from amniocentesis. Karyotypes were normal for all the probands. Amniotic fluid samples of 2 ml, without visible blood contamination, were centrifuged for 10 min at 1,600 rpm. Isolation of hMSCs from the AF was based on mechanical separation and natural selection by the culture medium (26,27). Pellets were plated in 12-well culture dishes. Cells were fed daily with MesenCult medium (Stem Cell Technologies) plus 100 U/ml streptomycin and 100 U/ml penicillin, and placed in a 95% humidified, 5% CO₂ incubator at 37°C. After 24 h, dishes were inspected for cell adhesion. Cells with a predominantly ‘typical mesenchymal morphology’ were trypsinized and plated in 25-mm flasks with MesenCult medium. Confluent cells were split into 40-mm Petri dishes for further expansion.

hMSC markers PCR

Characteristic hMSC markers were investigated using RT-PCR reactions. Petri dishes were washed twice with 10% phosphate-buffered saline (PBS) before proceeding with extraction. RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer’s specifications, and treatment with DNase followed the reaction. cDNA was synthesized using 1 μ g of total RNA in a 60- μ l reaction containing random examers and 200 U of M-MuLV Reverse Transcriptase (Fermentas). Conditions used were 25°C for 25 min, 42°C for 60 min, and 70°C for 10 min. PCR reactions were performed in 25 μ l with Platinum Taq Polymerase (Invitrogen). Primers were used at 300 nM final concentration, and their sequences are shown in Table 1. PCR was performed using following conditions: 95°C for 2 min, 45 cycles at 95°C for 30 sec, 55°C for 15 sec, 72°C for 30 sec.

Cell differentiation

MesenCult PLUS protocol: After confluence, hMSCs were plated at a density of 3×10^3 /cm² in 40-mm Petri dishes and fed with MesenCult PLUS. This day corresponds to day 0 in our time-course experiment. We changed

ADIPOGENESIS IN hMSCs

TABLE 1. OLIGONUCLEOTIDE SEQUENCES USED TO CHARACTERIZE hMSCs, AND ANTIGEN SURFACE MARKERS EXPRESSION

Antigen	# CD	Primers	Expression
Thy-1	CD90	Forward, 5'-TGCTCTTTGGCACTGTGG-3' Reverse, 5'-AGAGGGAGAGCAGGAGCAG-3'	+
Endoglin, SH2	CD105	Forward, 5'-GGGGTCAACACCACAGAG-3' Reverse, 5'-CAGGACCCTCAGGATGTG-3'	+
SH3, SH4	CD73	Forward, 5'-ATGGTGTGGAAGGACTGATC-3' Reverse, 5'-CCTCACTTTCTGAGCGATG-3'	+
SB10/ALCAM	CD166	Forward, 5'-AGGAAATGGACCCAGTGAC-3' Reverse, 5'-CCCCTTCTTTGATGGCA-3'	+
Gp105-120	CD34	Forward, 5'-TGAAGCCTAGCCTGTAC-3' Reverse, 5'-CGCACAGCTGGAGGTCTTAT-3'	-

the medium every 3 days. MesenCult PLUS medium was a cocktail consisting of MesenCult basal medium for hMSCs (StemCell Technologies), 10% hMSCs adipogenic stimulatory supplements (StemCell Technologies), streptomycin 100 U/ml, and penicillin 100 U/ml. We extracted RNA from cells cultured along 20 days and harvested at days 0, 4, 6, 10, 13, 16, and 20 (see Fig. 2, below).

3 + 4 hormonal cycles protocol: After confluence, hMSCs were plated at a density of $3 \times 10^3/\text{cm}^2$ in 40-mm Petri dishes in growth medium GM (GM, minimal essential medium M199) supplemented with 10% fetal bovine serum (FBS). At confluence, GM was replaced by maintenance medium (MM), which has an identical composition to GM except for the presence of 5% FBS instead of 10% FBS. Cells were cultured in this medium for 5 days without splitting them. The last of these 5 days corresponds to day 0 in our time-course experiment. Cells were then incubated in adipogenesis-inducing medium (AIM) (M199, 10% FBS, 1 μM dexamethasone, 0.2 mM indomethacin, 1.7 μM insulin, 0.5 mM 3-isobutyl-1-methylxanthine, 100 U/ml streptomycin, and 100 U/ml penicillin) for 3 days, and then cultured for further 3 days in adipogenesis-maintenance medium (AMM) (M199, 10% FBS, 1.7 μM insulin, 100 U/ml streptomycin and 100 U/ml penicillin). Three cycles consisting of 3 days of hormonal stimulation (AIM) followed by 4 days of rest (AMM). In the last cycle, days of rest were prolonged to 5 (see Fig. 2, below). All reagents were from Sigma Aldrich.

Real-time PCR

The total RNA from undifferentiated hMSCs (control cells) and from induced-hMSCs was extracted as specified for hMSCs extraction and was treated with DNase. RNA from human mature adipose tissue was extracted and used as positive control for the expression of adipogenesis markers. cDNA previously described was purified by GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences).

Quantitative RT-PCR analysis was performed in a 96-well optical reaction plate using an ABI Prism 7700 sequence detector (Applied Biosystems). Oligonucleotide primers were designed with Primer Express (Applied Biosystems). Expression of 18S ribosomal RNA (rRNA) was used as reference. 18S represents a large amount of total cellular RNAs and, because it is generated by a distinct polymerase, its level is less likely to vary under conditions that affect the expression of mRNAs, like adipogenesis (28).

PCR primers for PPAR γ 2, PPAR γ -C1 α , UCP-1, adipsin, leptin, and 18S are shown in Table 2. Reactions were performed in 25 μl with 1.5 μl of cDNA, 2 \times Platinum SYBR Green qPCR SuperMix UDG (Invitrogen), and 300 nM primers. PCR conditions were: 2 min at 50°C, 2 min at 95°C, and 50 cycles of 15 sec at 95°C and 30 sec at 60°C. The target and reference genes were amplified in separate wells. All reactions were performed in duplicate. Reaction mixture, without the cDNA, was used as negative control in each run. A standard curve was performed as follows: PPAR γ 2, $y = -3.3x + 28.4$, $r^2 = 0.98$; PPAR γ -C1 α , $y = -3.5x + 22.3$, $r^2 = 0.99$; UCP-1, $y = -3.4x + 26.3$, $r^2 = 0.99$; adipsin, $y = -3.1x + 20.1$, $r^2 = 0.99$; leptin, $y = -3.3x + 24.2$, $r^2 = 0.99$; 18S, $y = -3.2x + 13.5$, $r^2 = 0.99$. The $2^{-\text{DDCt}}$ method was used to quantify gene expressions compared with 18S rRNA.

RESULTS

Characterization of hMSCs

hMSCs from AF selected as described in Materials and Methods expressed CD90, CD105, CD73, CD166, and typical MSC markers, but were negative for CD34, a hematopoietic marker (Fig. 1). Hematopoietic cells derived from bone marrow were used as negative control for mesenchymal markers and as positive control for CD34.

TABLE 2. OLIGONUCLEOTIDE SEQUENCES USED FOR RT-PCR EXPERIMENTS

Marker	Primers
PPAR γ 2	Forward, 5'-CAGTGTGAATTACAGCAAACC-3' Reverse, 5'-ACAGTGTATCAGTGAAGGAAT-3'
PPAR γ -C1 α	Forward, 5'-GTTCCCGATCACCATATTCCA-3' Reverse, 5'-GCGGTGTCTGTAGTGGCTTGA-3'
UCP-1	Forward, 5'-CTGGAATAGCGCGTGCTT-3' Reverse, 5'-AATAACACTGGACGTCGGGC-3'
Leptin	Forward, 5'-ATGACACAAAACCCCTCATCAA-3' Reverse, 5'-GAAGTCCAAACCGGTGACTTT-3'
Adipsin	Forward, 5'-CCAAGCGCTGTACGACGT-3' Reverse, 5'-GGCCTTCTCCGACAGCTGT-3'
ribosomal 18S	Forward, 5'-AGGAATTCCCAGTAAGTGCG-3' Reverse, 5'-GCCTCACTAAACCATCCAA-3'

These results confirmed that we isolated a hMSC population. To evaluate cell stability, the cells were frozen and thawed many times, and their morphology was maintained (such as marker expression pattern).

Real-time results

Real-time analysis was executed on two induction experiments performed with two different cell growth protocols: MesenCult PLUS protocol and "3 + 4 hormonal cycles protocol" (Fig. 2). MesenCult was a manufactured medium, commonly used to grow stem cells, and was added with appropriate adipogenic supplements. The second protocol is based on a minimal essential medium enriched by hormonal adipogenic cocktail recently optimized (29).

MesenCult PLUS protocol

For each time point, we analyzed cells treated with MesenCult PLUS and control cells cultured in MesenCult basal medium. Control cells analyzed for all investigated adipogenic markers resulted in similar expression levels at each examined time point. For this reason, we summarized all of the results obtained for control cells with a value representing the average value, with its standard deviation (Fig. 3).

As expected, expression of adipogenic markers at day 0 was similar to that of noninduced hMSCs and was higher for human mature adipocytes. PPAR γ 2 expression at days 4, 13, and 20 was similar to that of nontreated cells. The expression dramatically decreased at days 6, 10, and 16. Human mature adipocytes expressed PPAR γ 2 about 12-fold more than the control. PPAR γ -C1 α showed fluctuating expression: Expression increased up to 55-fold (compared to control) at day 13, and then it decreased

during the next week. Human mature adipocytes did not have expression as high as PPAR γ -C1 α (it is eight-fold higher than hMSCs). UCP-1 had increasing expression until day 20, when induced cells presented mRNA levels three times higher than the control. Human mature adipocytes expressed this adipogenic marker at levels seven-fold higher than control. Adipsin showed fluctuating expression levels: It reached spikes of expression at days 4 and 13. In particular, maximum expression was detected at day 13. The positive control showed a huge expression of this marker (about 1.5×10^3 -fold higher than nontreated hMSCs). The leptin increment was remarkable at days 10, 13, 16, and 20, with day 13 being its maximum value. Compared to the hMSC control, the increase of expression had a 10^5 order of magni-

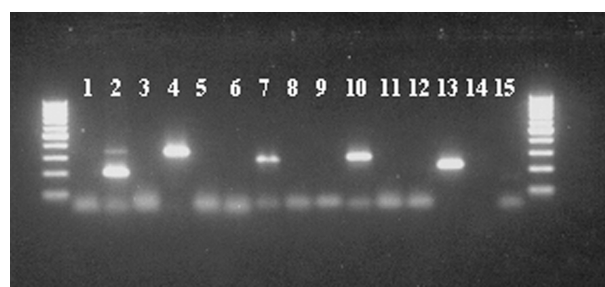


FIG. 1. Expression of markers CD34, CD73, CD90, CD105, and CD166. Lanes 1–3, CD34 expression of AF-derived stem cells (lane 1), hematopoietic cells (lane 2), and reagents control (lane 3); lanes 4–6, CD73 expression of AF-derived stem cells (lane 4), hematopoietic cells (lane 5), and reagents control (lane 6); lanes 7–9, CD90 expression of AF-derived stem cells (lane 7), hematopoietic cells (lane 8), reagents control (lane 9); lanes 10–12, CD 105 expression of AF-derived stem cells (lane 10), hematopoietic cells (lane 11), and reagents control (lane 12).

ADIPOGENESIS IN hMSCs

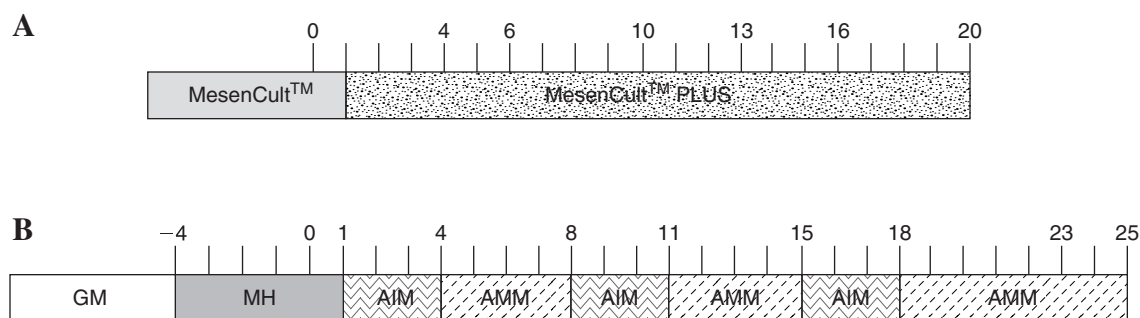


FIG. 2. Schematic representation of time-course experiments. **(A)** Check points for MesenCult PLUS protocol: days 0, 4, 6, 10, 13, 16, and 20 along a 3-week time line. Cells were grown in MesenCult medium until confluence and then induced by MesenCult PLUS. **(B)** Check points for the "3 + 4 hormonal cycles protocol": days 0, 4, 8, 11, 15, 18, and 23 along a 3-week line. Cells were grown in GM until confluence, and then GM was replaced by MM. Cells were cultured in this medium for 5 days. Cells were then incubated in AIM for 3 days, then cultured for a further 3 days in AMM. Three cycles consisting of 3 days of AIM followed by 4 days of AMM were performed. The check point corresponds to the end of the AIM or AMM period. In particular, day 4 represents the end of the first 3-day cycle in AIM medium; day 8 represents the last day of the first 4-day cycle in AMM; day 11 represents the end of the second 3-day cycle in AIM medium; day 15 represents the last day of the second 4-day cycle in AMM; day 18 represents the end of the third 3-day cycle in AIM medium; day 23 represents the last day of the third 5-day cycle in AMM. At the end of the experiment at day 25 of culture in AMM, cells were stained with Oil Red O.

tude. Moreover, expression of leptin in human mature adipocytes had a 10^8 order of magnitude.

3 + 4 hormonal cycles protocol: This time-course experiment was scheduled following the adipogenic differentiation protocol optimized by Janderová (29) and slightly modified during the maintenance period (4 days instead of 3 days) (Fig. 2). Each check point corresponds to the end of a hormonal induction cycle or a maintenance cycle. During this time-course experiment, our negative control of induction (hMSCs) was cultured in MM, which was refreshed on the same days we changed medium for induced cells.

Expression of all investigated adipogenic markers was similar in cells collected on day 0 and in the negative control collected at each check point. Therefore, we summarized all results for control cells with a value representing the average value of each time point with its standard deviation (Fig. 4). Mature adipocytes were used as a positive control, and they expressed each investigated adipogenic marker at levels higher than those detected in our analyzed cells except for PPAR γ -C1 α .

PPAR γ 2 expression showed no significant changes during the time-course experiment in treated and nontreated hMSCs. Otherwise PPAR γ -C1 α showed increased expression at the end of each induction period compared with the following maintenance period, and in particular reached a maximal level (20-fold higher than nontreated cells) during the second cycle in AIM. UCP-1 expression did not vary significantly from that of control cells: mRNA levels were so low that it was difficult to detect them. Only day 11 shows a slight increase compared to all the other time points (less than two-fold). Adipsin showed increased expression during all of the

first part of the experiment, reaching a maximum level at the end of the second cycle of hormonal induction (at day 15, its expression was four-fold higher than in the control), and then the amount of adipsin cDNA rapidly decreased. Leptin was poorly expressed by induced cells, and no significant difference was detected compared to hMSC control cells.

Cell morphology

During both experiments, cells in the control group maintained a fusiform mesenchymal appearance, whereas all cells exposed to inducing agents underwent morphological changes and appeared as round cells with cytosolic droplets that were positive for Oil Red O staining (data not shown). In particular, at the end of the treatment period, it was possible to appreciate 5% of round cells in the experiment performed with MesenCult PLUS, and a 20% in the "3 + 4 hormonal cycles" one. Cytosolic droplets appeared on day 5 of MesenCult PLUS treatment and in the middle of the first hormonal cycle in the case of the "3 + 4 hormonal cycles" protocol.

DISCUSSION

This study shows that it is possible to isolate hMSCs from AF and then differentiate them into mature adipocytes. To our knowledge, there are few *in vitro* adipocyte differentiation human models, and there are fewer experiments on ES cells. In fact, most of models are adult stem cell-derived cultures, i.e., bone marrow biopsies (30,31), and tissue biopsies, i.e. skin, orbital adipose tissue (32).

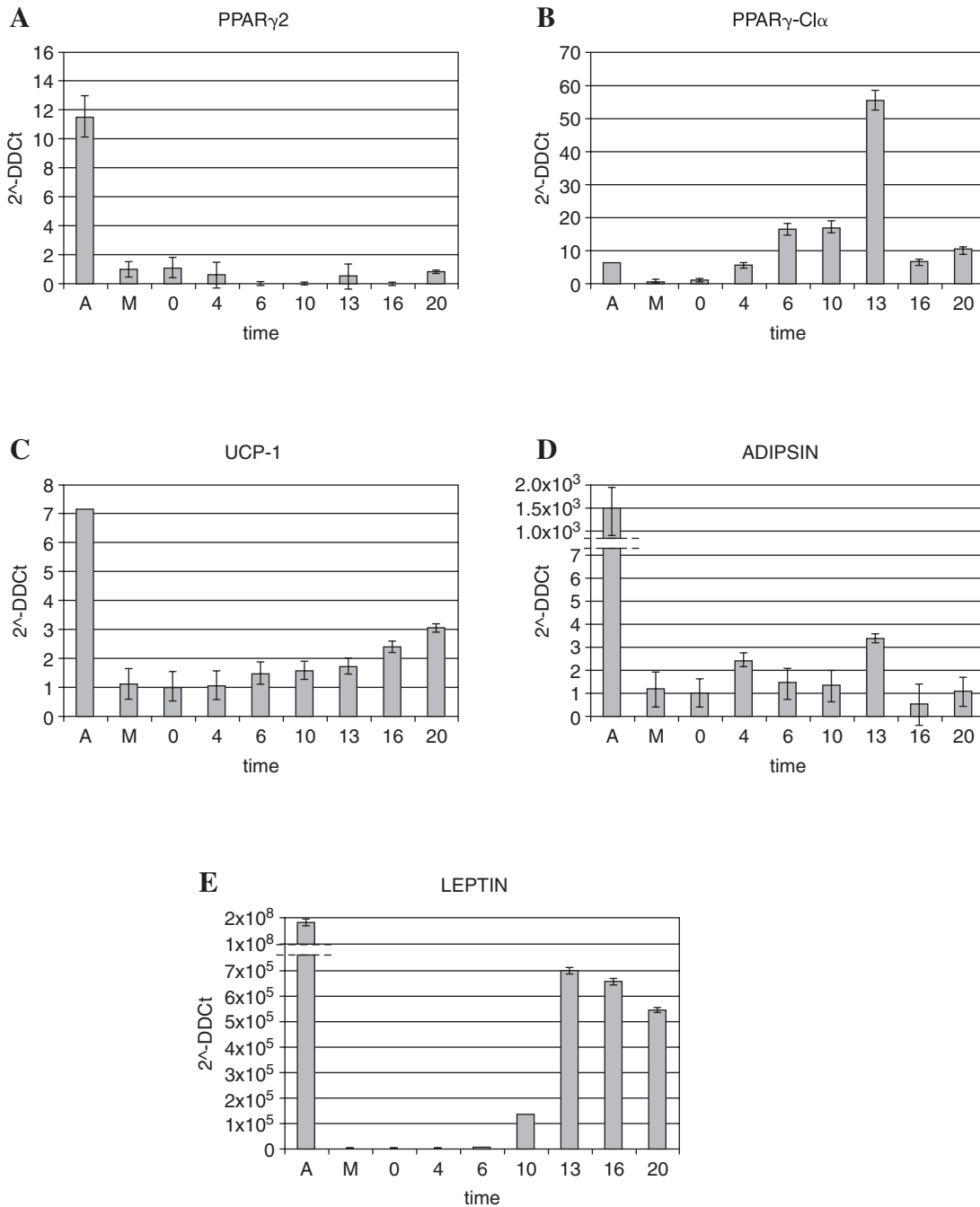


FIG. 3. Real-time expression of investigated adipogenic markers in the MesenCult PLUS time-course experiment. **(A)** PPAR γ 2. **(B)** PPAR γ -C1 α . **(C)** UCP-1. **(D)** Adipsin. **(E)** Leptin. We monitored the expression levels in mature adipocytes (A), in undifferentiated AF-derived hMSCs (M), and in MesenCult PLUS-treated hMSCs collected at check points. Data are combined from three different analyses. Expression of each gene was normalized against ribosomal 18S. Gene expression levels were calculated using the DDCt method.

All of these models contain stem cells already committed to the adipose lineage. Our experiment, instead, was designed on a tissue that has been shown to be similar, regarding differentiation capacity, to embryonic tissue and that, moreover, permits obtaining information about

early adipogenic stages without the ethical limitations related to the use of human embryonic cells. Because our cells are in an earlier developmental stage than the cells used in other cited models, they require more time to differentiate into adipocytes.

ADIPOGENESIS IN hMSCs

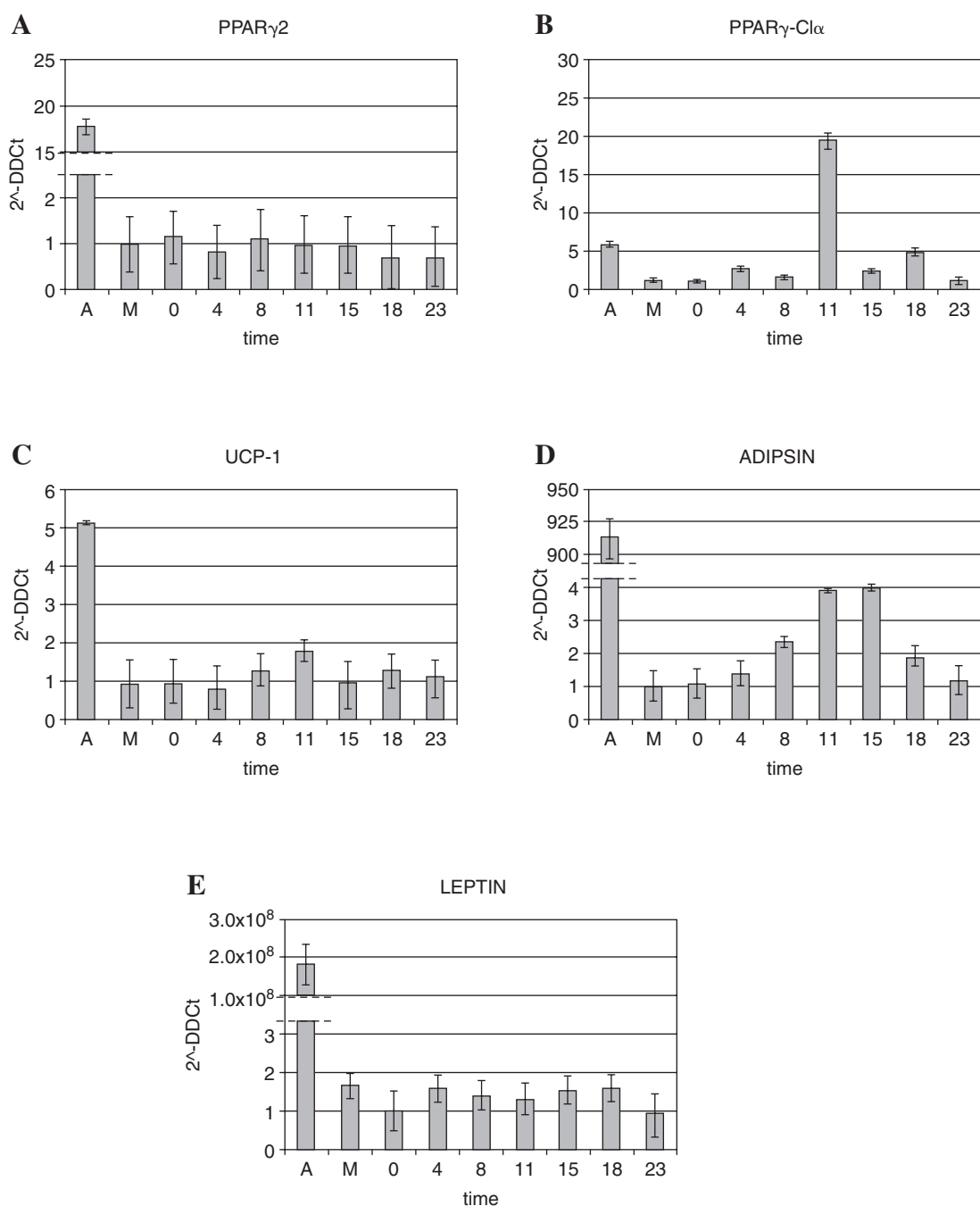


FIG. 4. Real-time expression of investigated adipogenic markers in the “3 + 4 hormonal cycles” time-course experiment. **(A)** PPAR γ 2. **(B)** PPAR γ -C1 α . **(C)** UCP-1. **(D)** Adipsin. **(E)** Leptin. We monitored the expression levels in mature adipocytes (A), in undifferentiated AF-derived hMSCs (M), and in “3 + 4 hormonal cycle”-treated hMSCs collected at check points. Data are combined from three different analyses. Expression of each gene was normalized against ribosomal 18S. Gene expression levels were calculated using the DDCt method.

In all of these models, the differentiation process shows many different gene expression patterns, depending on culture conditions used. Thus, we decided to test two different media for our experiments. MesenCult PLUS is the commonly used medium in adipogenesis experi-

ments; on the other hand, the Janderová (29) protocol seems to be an alternative interesting protocol for obtaining a higher percentage of adipocytes. Our goal in choosing this approach was to replicate results starting from AF instead of bone marrow.

To follow expression changes during the time-course experiments, we used relative RT-PCR. In both time-course experiments, PPAR γ 2 expression showed no significant changes when compared to control cells (Figs. 3 and 4). Preliminary observations suggested that during the following week of culture, PPAR γ 2 levels increase (data not shown), suggesting a delayed differentiation. It is important to underline that the responsiveness to inducing agents varies considerably, depending on the different sources of preadipocytes and on the specific differentiation protocol employed. It is possible to suppose that, in AF-derived hMSCs, PPAR γ 2 has an expression pattern different from that in other adipogenesis cell models (29). Our human mature adipocytes expressed this marker at high levels.

During our time-course experiments, we noticed that in MesenCult PLUS, PPAR γ -C1 α had one spike of expression at day 13; on the other hand, in the “3 + 4 hormonal cycles” experiment, we observed a single spike at day 11. Interestingly, MesenCult PLUS increased the cDNA level 55-fold over the nontreated cells, whereas the “3 + 4 hormonal cycles” protocol showed an increase of about 20-fold only. During both time-course experiments, UCP-1 had a low level of expression, even if in the MesenCult PLUS treatment mRNA levels had a small increase at day 20. Taken together, these data are consistent with the knowledge that PPAR γ -C1 α is the main activator of UCP-1: in fact, it is known from the literature that PPAR γ -C1 α is expressed preferentially in brown fat instead of white fat, in which the endogenous levels are very low. Moreover, it is a co-activator of PPAR γ and turns on UCP-1 gene expression, thus playing a crucial role in the conversion of white mature adipocytes into brown ones. On the other hand, UCP-1 is specific for mitochondria of brown adipose cells. Afterwards, seeing that PPAR γ -C1 α showed an increasing expression that reached a spike of 55-fold induction compared to control in MesenCult PLUS treatment and only reached a spike of about 20-fold induction in “3 + 4 hormonal cycles” experiment, we have supposed that the low expression of UCP-1 reflects a PPAR γ -C1 α trend. This is the only examined marker whose expression was higher in treated hMSCs than in human mature adipocytes. This could be explained by the fact that PPAR γ -C1 α is a differentiation marker expressed at low levels in mature adipocytes. In fact, preliminary observations show that in prolonging our experiments PPAR γ -C1 α expression has a decreasing trend until the achievement of a mature adipocyte expression. Interestingly, because PPAR γ -C1 α and UCP-1 are typical brown adipocyte markers, we may argue that, starting from day 13, the MesenCult PLUS experiment leads some of our cells toward the brown phenotype.

Adipsin is a very late marker of adipocyte differentiation. Adipsin expression in the MesenCult PLUS ex-

periment doubled at day 4 compared to that of noninduced hMSCs and tripled at day 13. This fluctuating expression is consistent with the observation that genes are switched on and off several times during cell differentiation. On the other hand, in the “3 + 4 hormonal cycles” experiment, adipsin cDNA levels had spikes at days 11 and 15. These days of maximal expression correspond to the second cycle of hormonal induction.

Compared to results obtained for the other differentiation markers, leptin levels, measured in the MesenCult PLUS experiment, was surprisingly high for days 10, 13, 16, and 20 (10^5 -fold higher than the control). Our positive control still maintained the highest observed expression. Leptin expression in the “3 + 4 hormonal cycles” showed no significant difference among various check points. Also, in this case, the positive control still expressed leptin at the expected high levels.

The results from the two time-course experiments led us to assess that MesenCult PLUS medium caused changes in the expression of the five examined adipogenesis markers that were more remarkable than the other experimental protocol. Otherwise, these differences were not reflected by morphological appearance because, compared with the MesenCult PLUS protocol, a higher percentage of cells cultured according to the “3 + 4 hormonal cycles” protocol presented a typical adipocyte phenotype. By analyzing these markers during time-course experiments, we could monitor their levels of expression over 3–4 weeks; in particular, during the MesenCult PLUS treatment, we recorded increased expression at day 13 or 20 according to delayed adipogenesis differentiation under our conditions. In the case of the “3 + 4 hormonal cycles” experiment, the increased expression levels of some markers appeared to correspond to the second hormonal cycle according to the literature (29), although the expression levels were not as consistent as in the previous protocol.

We conclude that due to species and culture conditions, results from different studies are occasionally quite controversial, so our work provides a reliable model for understanding adipogenesis. Clearly, further studies are required to understand how to reproduce real stimuli that induce adipogenesis *in vivo*. Starting from our observation of a delayed adipogenesis, prolonging the weeks of induction could show intriguing information. Understanding *in vivo* differentiation mechanisms will shed light on the physiology and pathology of many diseases, such as obesity and type II diabetes.

ACKNOWLEDGMENTS

We are grateful to Leonarda Ianzano for helpful discussion and critical revision of the manuscript. This study

was supported by the grant FIRB #RBNE01KCX4_009 of the Italian Ministry of Education, University and Research (MIUR).

REFERENCES

- Klaus S (1997). Functional differentiation of white and brown adipocytes. *BioEssays* 19:215–223.
- MacDougald OA and S Mandrup. (2002). Adipogenesis: forces that tip the scales. *Trends Endocrinol Metab* 13: 5–11.
- Gregoire FM, CM Smas and HS Sul (1998). Understanding adipocyte differentiation. *Physiol Rev* 78:783–809.
- Anderson LA, PG McTernan, AH Barnett and S Kumar. (2001). The effects of androgens and estrogens on preadipocyte proliferation in human adipose tissue: influence of gender and site. *J Clin Endocrinol Metab* 86: 5045–5051.
- Dani C. (1999). Embryonic stem cell-derived adipogenesis. *Cells Tissues Organs* 165:173–180.
- Vernochet C, DS Milstone, C Iehlé, N Belmonte, B Phillips, B Wdziekonski, P Villageois, EZ Amri, PE O'Donnell, RM Mortensen, G Ailhaud and C Dani. (2002). PPAR γ -dependent and PPAR γ -independent effects on the development of adipose cells from embryonic stem cells. *FEBS Lett*. 510:94–98.
- Shimomura I, RE Hammer, JA Richardson, S Ikemoto, Y Bashmakov, JL Goldstein and MS Brown. (1998). Insulin resistance and diabetes mellitus in transgenic mice expressing nuclear SREBP-1c in adipose tissue: model for congenital generalized lipodystrophy. *Genes Dev* 12: 3182–3194.
- Morrison RF and SR Farmer. (1999). Role of PPAR γ in regulating a cascade expression of cyclin-dependent kinase inhibitors, p18(INK4c) and p21(Waf1/Cip1), during adipogenesis. *J Biol Chem* 274:17088–17097.
- In't Anker PS, SA Scherjon, C Kleijburg-van der Keur, WA Noort, FH Claas, R Willemze, WE Fibbe and HH Kanhai. (2003). Amniotic fluid as a novel source of mesenchymal stem cells for therapeutic transplantation. *Blood* 102:1548–1549.
- Tsai MS, JL Lee, YJ Chang, SM Hwang. (2004). Isolation of human multipotent mesenchymal stem cells from second-trimester amniotic fluid using a novel two-stage culture protocol. *Hum Reprod* 19:1450–1456.
- In't Anker PS, SA Scherjon, C Kleijburg-van der Keur, GM de Groot-Swings, FH Claas, WE Fibbe and HH Kanhai. (2004). Isolation of mesenchymal stem cells of fetal or maternal origin from human placenta. *Stem Cells* 22:1338–1345.
- Cowherd RM, RE Lyle and RE McGehee Jr. (1999). Molecular regulation of adipocyte differentiation. *Semin Cell Dev Biol* 10:3–10.
- Altiock S, M Xu and BM Spiegelman. (1997). PPAR γ induces cell cycle withdrawal: inhibition of E2F/DP DNA-binding activity via down regulation of PP2A. *Genes Dev* 11:1987–1998.
- Brun RP, JB Kim, E Hu, S Altiock and BM Spiegelman. (1996). Adipocyte differentiation: a transcriptional regulatory cascade. *Curr Opin Cell Biol* 8:826–832.
- Puigserver P, Z Wu, CW Park, R Graves, M Wright and BM Spiegelman.(1998). A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* 92:829–839.
- Tiraby C and D Langin. Conversion from white to brown adipocytes: a strategy for the control of fat mass? (2003). *Trends Endocrinol Metab* 14:439–441.
- Jacobsson A, U Stadler, MA Glotzer and LP Kozak (1985). Mitochondrial uncoupling protein from mouse brown fat. Molecular cloning, genetic mapping, and mRNA expression. *J Biol Chem* 260:16250–16254.
- Bouillaud F, J Weissenbach and D Ricquier. (1986). Complete cDNA-derived amino acid sequence of rat brown fat uncoupling protein. *J Biol Chem* 261:1487–1490.
- Klaus S, M Ely, D Encke and G Heldmaier. (1995). Functional assessment of white and brown adipocyte development and energy metabolism in cell culture. *J Cell Sci* 108:3171–3180.
- Ailhaud G, P Grimaldi and R Negrel (1992). Cellular and molecular aspects of adipose tissue development. *Annu Rev Nutr* 12:207–233.
- Zhang Y, R Proenca, M Mattei, M Barone, L Leopold and JM Friedman (1994). Positional cloning of the mouse obese gene and its human omologue. *Nature* 375:425–432.
- Ahren B, S Mansson, RL Gingerich and PJ Havel. (1997). Regulation of plasma leptin in mice: influence of age, high-fat diet, and fasting. *Am J Physiol* 273:113–120.
- Ricci MR and SK Fried. (1999). Isoproterenol decreases leptin expression in adipose tissue of obese humans. *Obes Res* 7:233–240.
- Russell CD, RN Petersen, SP Rao, MR Ricci, A Prasad, Y Zhang, RE Brolin and SK Fried. (1998). Leptin expression in adipose tissue from obese humans: depot-specific regulation by insulin and dexamethasone. *Am J Physiol* 275:E507–E551.
- Moinat M, C Deng, P Muzzin, F Assimacopoulos-Jeannet, J Seydoux, AG Dulloo and JP Giacobino. (1995). Modulation of obese gene expression in rat brown and white adipose tissues. *FEBS Lett* 373:131–134.
- Kaviani A, TE Perry, A Dzakovic, RW Jennings, MM Ziegler and DO Fauza. (2001). The amniotic fluid as a source of cells for fetal tissue engineering. *J Pediatr Surg* 36:1662–1665.
- Kaviani A, K Guleserian, TE Perry, RW Jennings, MM Ziegler and DO Fauza. (2003). Tissue engineering from amniotic fluid. *J Am Coll Surg* 196:592–597.
- Barbu V and F Dautry. (1989). Northern blot normalization with a 28s rRNA oligonucleotide probe. *Nucleic Acids Res* 17:7115.
- Janderová L, M McNeil, AN Murrel, RL Mynatt and SR Smith. (2003). Human Mesenchymal Stem Cells as an In Vitro Model for Human Adipogenesis. *Obes Res* 11: 65–74.
- Hung SC, CF Chang, HL Ma, TH Chen and L Low-Tone Ho. (2004). Gene expression profiles of early adipogenesis in human mesenchymal stem cells. *Gene* 340:141–150.

31. Nakamura T, S Shiojima, Y Hirai, T Iwama, N Tsuruzoe, A Hirasawa, S Katsuma and G Tsujimoto. (2003). Temporal gene expression changes during adipogenesis in human mesenchymal stem cells. *Biochem Biophys Res Commun* 303:306–312.
32. Kumar S, MJ Coenen, PE Scherer and RS Bahn. (2005). Evidence for enhanced adipogenesis in the orbits of patients with Graves' ophthalmology. *J Clin Endocrinol Metab* 89: 930–935.

Address reprint requests to:

*Dr. Paola de Gemmis
BIRD Europe Institute
via Bartolomeo Bizio. 1
36023 Costozza di Longare (VI)
Italy*

E-mail: paola.degemmis@birdfoundation.org