

Supplemental Data

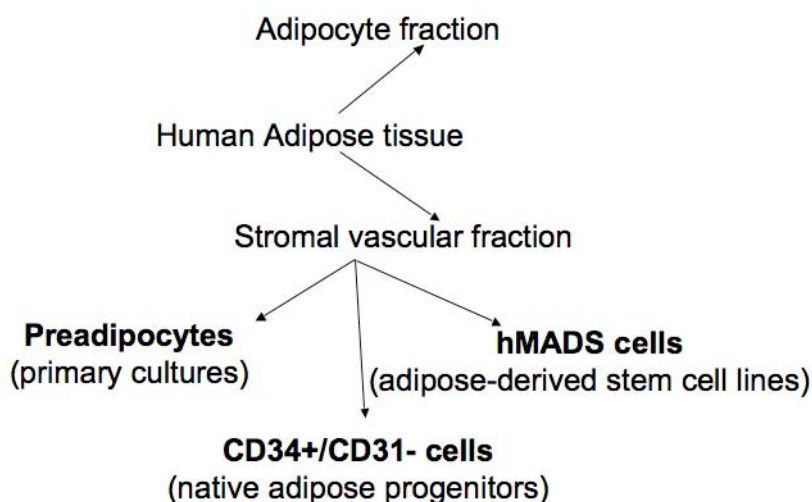


Figure S1: Protocols for isolation and differentiation of human adipose progenitors

Primary preadipocytes. Abdominal subcutaneous (external to the *fascia superficialis*), mesenteric (colonic epiploics), and greater omental fat tissue was biopsied in parallel from 4 subjects during gastric bypass surgery for obesity (2 female, 2 male; age 48.0 ± 3.1 years [mean \pm SEM]; BMI 56.6 ± 4.5 kg/m²). Subjects gave informed consent and the protocol was approved by the Boston University Institutional Review Board. None had diabetes or cancer and none were taking glucocorticoids or thiazolidinediones. Preadipocytes were isolated by mincing the fat biopsies, collagenase digestion, centrifugation, erythrocyte lysis buffer treatment, and differential plating as described previously (1; 2). Adipogenesis was induced as described previously (2). Briefly, confluent undifferentiated preadipocytes were exposed to the proliferation medium but without serum and enriched with dexamethasone, insulin, triiodothyronine, ciglitazone, antibiotics, and isobutyl-methylxanthine (removed after 2 days). After 30 days, RNA was isolated from preadipocytes by the Trizol method (3).

CD34⁺/CD31⁻ cell population containing native progenitors. Human subcutaneous AT was obtained from healthy women undergoing elective surgical procedures to remove fat for aesthetic purposes. Their body mass indices (BMI) ranged from 20 to 38 kg/m². Fat collection protocols were approved by the Institutional Research Board of INSERM and Toulouse University Hospital. The native distinct CD34⁺/CD31⁻ cell populations from the adipose tissue stromal-vascular fractions were isolated using the immunoselection/depletion protocol as previously described (4).

Multipotent Adipose Derived Stem cells. hMADS cells were obtained from the stromal vascular fraction of human adipose tissue as described previously (5). Discarded adipose tissue was collected during surgery with the informed consent of the parents, as approved by the Centre Hospitalier Universitaire de Nice Review Board. The cell populations that were studied in this work were isolated from the pubic fat pad of a 4-month old (hMADS3) and 2-year old (hMADS7) male donors. Proliferation medium for routine maintenance of hMADS cells is supplemented with FGF2 as previously reported (6). Adipogenesis of hMADS cells can take place in serum-free chemically-defined conditions. Adipocyte differentiation was performed as described previously (7; 8).

Up to now, preadipocyte clonal lines from rodents have been used to gain insight into cellular and molecular mechanisms of adipogenesis (9). Much less is known about the molecular regulation of human adipogenesis, partly due to the lack of appropriate human cellular

models. Primary cultures of preadipocytes derived from stromal-vascular fraction (SVF) of adipose tissue, although being able to differentiate into adipocytes *in vitro*, undergo a dramatic decrease in their ability to differentiate before growth arrest and replicative senescence with serial subculturing, making it difficult to investigate molecular mechanisms in a fully reproducible manner. Multipotent stem cells have been isolated from the SVF of infant adipose tissues and termed human Multipotent Adipose-Derived Stem (hMADS) cells. They exhibit the characteristics of mesenchymal stem cells, i.e., the capacity to self-renew, as cells can be expanded *in vitro* for more than 160 population doublings (i.e., around 30 passages) while maintaining a normal diploid karyotype and the potential to undergo differentiation into adipocytes, osteoblasts, and chondrocytes at the single cell level (5; 6). *In vitro*, these cells enter the adipose lineage at a high rate and differentiate into cells that display a unique combination of properties similar, if not identical, to those of native human adipocytes (10; 11), making them a powerful cellular model to investigate human adipogenesis.

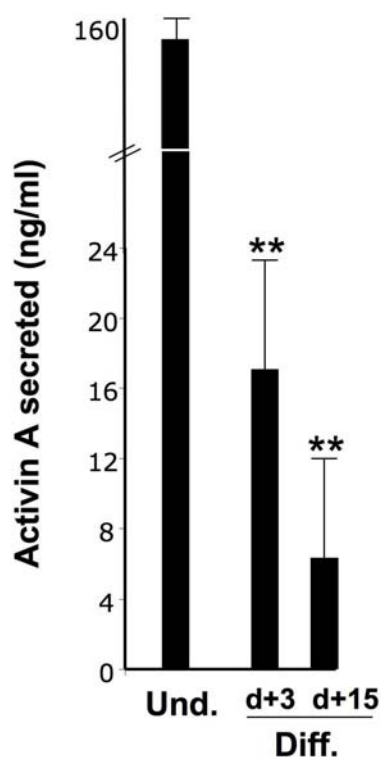


Figure S2: Activin A is secreted by undifferentiated hMADS cells and its level decreased dramatically early upon induction of adipocyte differentiation

Amounts of activin A secreted by undifferentiated hMADS3 cells (Und.) and early and late differentiated cells (3 days and 15 after induction) were quantified by ELISA. Similar results were obtained with hMADS2 cells. Secretion of activin A was measured with the human Activin A ELISA Assay Kit (AbD Serotec, France). Supernatants were stored at -80°C until assay and were applied undiluted.

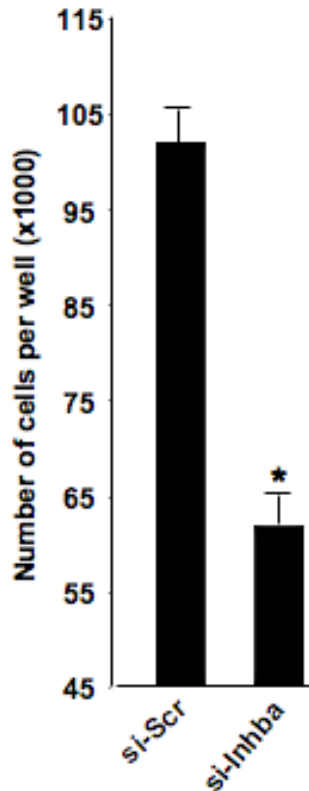


Figure S3: Effects of *inhba* knockdown on hMADS cell number.

Proliferating hMADS3 cells were transfected with siRNAs against *Inhba* or with scrambled siRNA (Si-Scr) and consequences on cell number were analyzed 5 days later. Results are the average of counting of 3 culture wells (12-well plates). Bars: mean \pm SE (n=3). Efficient inhibition of *inhba* expression was obtained as *inhba* RNA levels were reduced by more than 90% in hMADS cells transfected with siRNA duplexes (not shown).

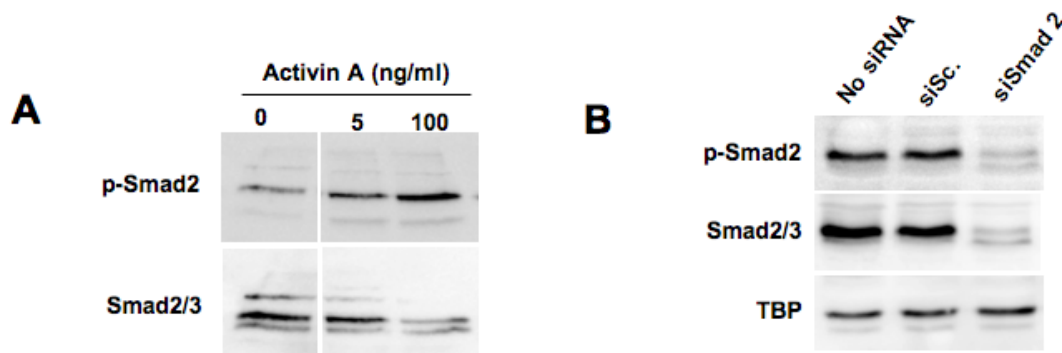


Figure S4: Activation of Smad2 pathway upon activin A addition and effects of siRNA on Smad2

A) Proliferating hMADS3 were treated with 5 or 100 ng/ml activin A for 2 hours and Western blot analyses were performed for total Smad2/3 or phosphorylated Smad2. B) Proliferating hMADS3 cells were transfected with siRNA against Smad2 or with non-relevant siRNA (siScr.) Western-blot analyses were performed 5 days after for total Smad2/3 or phosphorylated Smad2. Lanes were sliced, but samples were run on the same gel and equally exposed

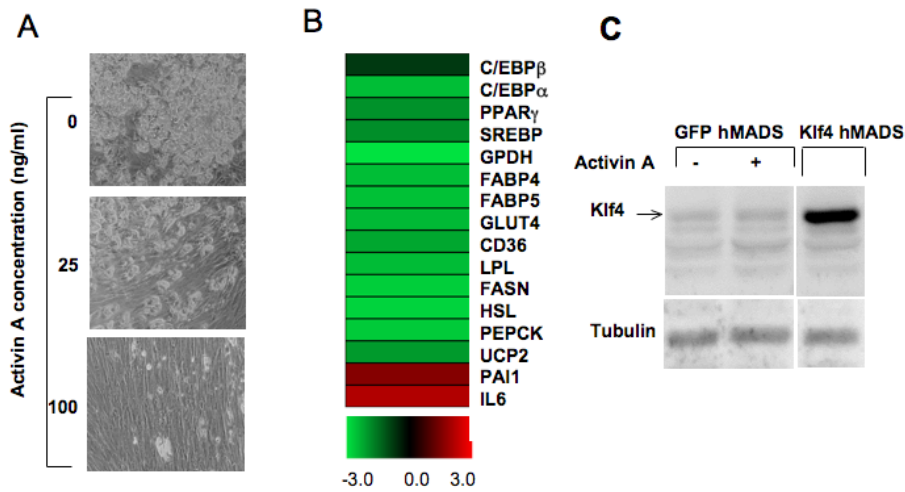


Figure S5: Expression of adipogenic genes in activin A-treated cells

A) Microscopic observation of hMADS3 cells induced to undergo differentiation in the absence or presence of the indicated concentrations of activin A for 12 days. B) hMADS3 cells were induced to undergo differentiation into adipocytes in the absence or presence of 100 ng/ml activin A. RNA was prepared 6 days later and expression of indicated genes was analyzed by real time PCR. Results are log₂ changes in expression of activin A-treated vs. untreated cells. Level of expression was represented using genesis software (12). This software generates expression images using a color code according to expression intensities. Expression data are color-coded as indicated in the scale. C) Expression of Klf4 protein in hMADS cells. hMADS3-EcoRec cells were induced to differentiate into adipocytes in the absence or presence of 100 ng/ml activin A (Act.). Proteins were prepared 6 days later and levels of Klf4 protein were analyzed. Proteins prepared from hMADS3-EcoRec cells transduced with a retrovirus expressing Klf4 were used as positive control. β -tubulin 1 was used as a loading control. Level of expression was represented when indicated using genesis software (12). This software generates expression images using a color code according to expression intensities.

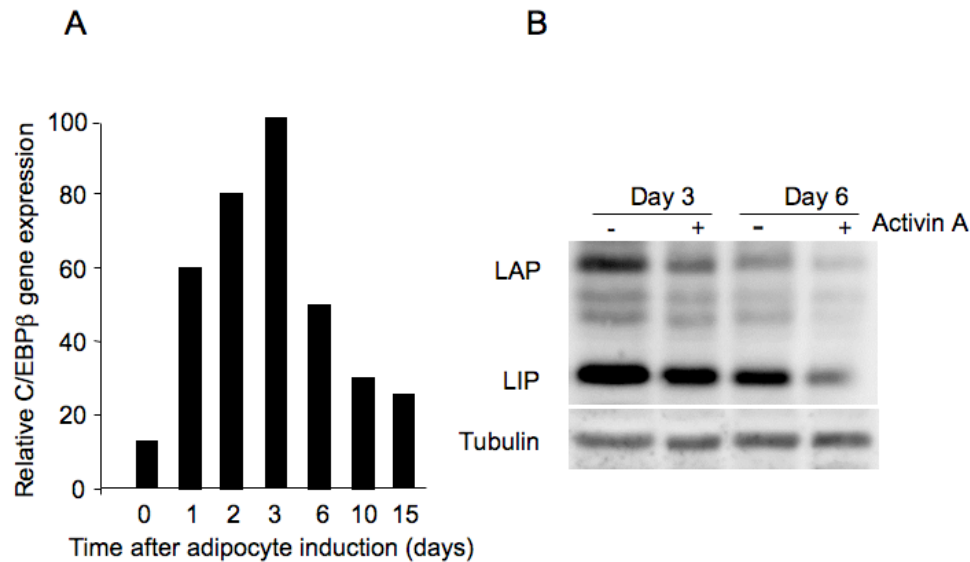


Figure S6: Expression of C/EBPβ during adipocyte differentiation and regulation by activin A.

A) hMADS cells were induced to undergo adipocyte differentiation (day 0) and C/EBPβ gene expression was analyzed at indicated times by real-time PCR. B) Regulation of C/EBPβ-LAP/LIP protein by activin A. hMADS3 cells were induced to differentiate into adipocytes in the absence or presence of 100 ng/ml activin A. Proteins were prepared 3 days or 6 days later and levels of C/EBPβ protein were analyzed. β-tubulin 1 was used as a loading control.

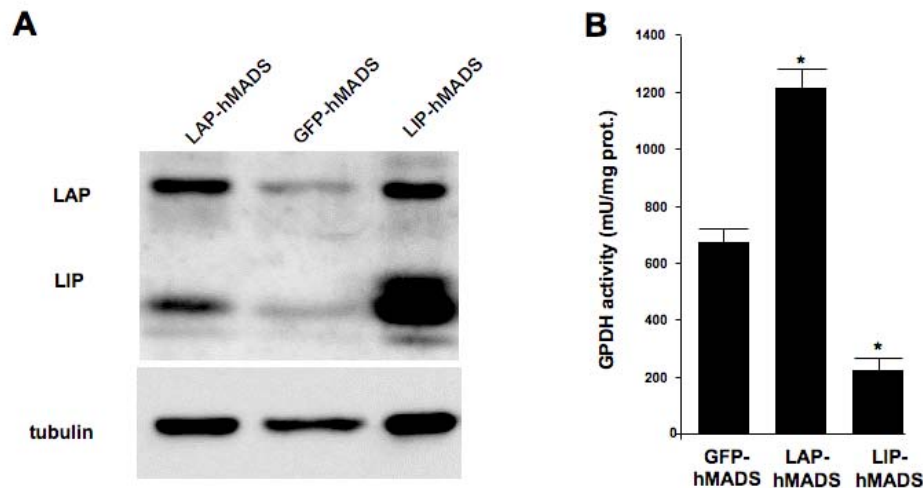
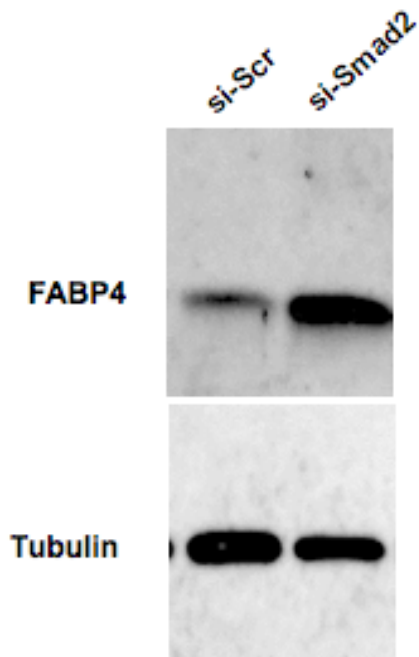


Figure S7:

Adipocyte differentiation of hMADS cells over-expressing C/EBPβ-LAP or -LIP isoforms

A) hMADS3-EcoRec cells were transduced with retroviral vectors expressing GFP, LAP, or LIP. Proteins were prepared 3 days later and 5 μg were used to analyze expression of LAP and LIP isoforms. B) Cells were induced to differentiate into adipocytes and GPDH activity was quantified 6 days later. Results are means from counting of 3 culture wells (24-well plates). Bars: means ± SEM (N=3). *significant differences of GPDH activities in LAP and LIP cells vs. GFP cells (p<0.05).



Figures S8 : Effects of Smad2 inhibition on adipocyte differentiation

Cells were transfected with Smad2 siRNA. The day after, cells were induced to undergo adipocyte differentiation and proteins were prepared 6 days later. FABP4 and β tubulin 1 proteins are shown on the same blots.

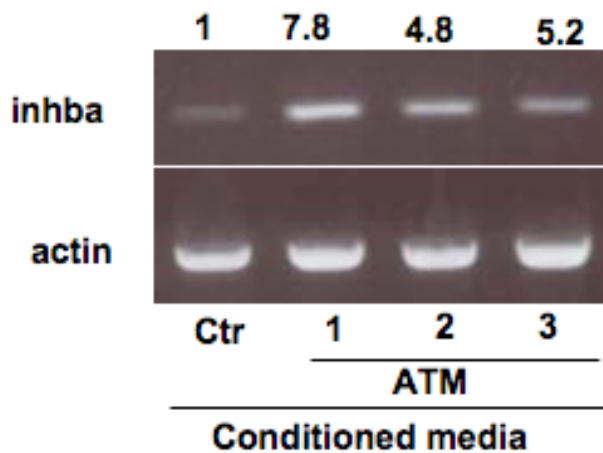


Figure S9: Expression of *inhba* gene in hMADS cells treated with ATM-conditioned media.

Undifferentiated hMADS cells were treated with ATM conditioned media (ATM 1-3) or with control media for 24h. RNAs were prepared and analyzed for the expression of *INHBA* gene by semi-quantitative PCR. Factors of stimulation, indicated on the top of the gel, were determined by quantitative real time PCR.

Isolation of macrophages.

CD34⁻/CD14⁺ cells, defined as adipose tissue macrophages (ATMs), were isolated from AT-SVF using an immunoselection/depletion protocol as previously described (13). Conditioned media (CM) from ATMs were collected after a 24-hour plating of the cells (200 000 cells/cm²) in basal medium (i.e. Endothelial cell basal medium (ECBM, Promocell, Heidelberg, Germany)/0.1%BSA), centrifuged (20 000g, 3 minutes, room temperature), and the media was stored at -20°C until further use. Our previous studies concerning the phenotype of human ATM clearly showed that human ATM are not strictly polarized compared to the mice studies and exhibited a mixed M1 and M2 phenotypes that is a hallmark of cells involved in chronic inflammatory process (4). We did not detect changes in cell number when treatment was performed on the CD34⁺/CD31⁻ cells for 24h.

Table S1: Sequence primers used for semi-qPCR.

Gene	forward	reverse
INHBA	GGGAGAACGGGTATGTGGAGAT	GCTGTTCTGACTCGGCAAA
PPAR γ	AGCCTCATGAAGAGCCTTCCA	TCCGGAAGAAACCCTTGCA
ADIPO.	GCAGTCTGTGGTTCTGATTCCATAC	GCCCTTGAGTCGTGGTTTCC

Table S2: Sequence primers used for real time PCR.

Gene	forward	reverse
IL-6	GCCCAGCTATGAACTCCTTCT	GAAGGCAGCAGGCAACAC
INHIBIN β A	CTCGGAGATCATCACGTTTG	CCTTGGAAATCTCGAAGTGC
LEPTIN	TTGTCACCAGGATCAATGACA	GTCCAAACCGGTGACTTTCT
CEBP α	CTTGTGCCTTGGAAATGCAA	GCTGTAGCCTCGGGAAGGA
CEBP β	AACCAACCGCACATGCAGAT	GGCAGAGGGAGAAGCAGAGAGT
PPAR γ	AGCCTCATGAAGAGCCTTCCA	TCCGGAAGAAACCCTTGCA
SREBP	AGGCCATCGACTACATTCG	TCCTTCAGAGATTTGCTTTTGTG
GPDH	TTGTGGTGCCCCATCAGTTC	CCCAATCACTTCCGAGATGA
FABP4	TGTGCAGAAATGGGATGGAAA	CAACGTCCCTTGGCTTATGCT
FABP5	ACAGCTGATGGCAGAAAACTCA	ACACTCCACCACTAATTTCCCATCT
GLUT4	CGTCGGGCTTCCAACAGATA	CACCGCAGAGAACACAGCAA
CD36	GGGAAAGTCACTGCGACATGAT	ACGTCGGATTCAAATACAGCATAGA
LPL	TGGAGGTACTTTTCAGCCAGGAT	TCGTGGGAGCACTTCACTAGCT
FASN	TGAACTCCTTGCGGAAGAGA	GTAGGACCCCGTGAATGTCA
HSL	GCACTACAAACGCAACGAGACA	GGTTCTGTGTGATCCGCTCAA
PEPCK	AGGGCCATCAACCCAGAAAA	TGGTCTCGGCCACATTGG
CREB	AGTTTGACGCGGTGTGTTACG	TGCATCTCCACTCTGCTGGTT
PTX3	TATTCCTCAATGCGTTCCAAGAA	GCCATAGGAAAACAGGATGGTT
RXR α	GCCCTCGAGCCAATGAGAA	GGAGTCGGGAGTCTGAAACCA

Table S3: Clinical and biological parameters measured in lean and obese women.

	lean	obese	p
No of subjects	7	12	
Age (yrs)	43.7 ± 4,5	41,5 ± 3,1	Ns
BMI (kg/m ²)	21.5 ± 0,4	45,8 ± 1,7	<0.001
Fat mass (%)	27.0 ± 2.3	47±1	<0.001
Fat free mass (%)	68.8±2.19	49.9±1.13	<0.001
Fasting Glucose (mM)	4.7 ± 0,1	5,2 ± 0,2	0.05
total cholesterol (mM)	5.4 ± 0,5	4,2 ± 0,3	0.1
HDL cholesterol (mM)	1.8 ± 0,2	1,2 ± 0,1	0.03
Triglycerides (mM)	0.9 ± 0,1	1,2 ± 0,3	NS
Adiponectin (µg/ml)	15.8 ± 2,4	7,2 ± 0,9	0.004
Leptin (ng/ml)	7.5 ± 1,2	60,0 ± 5,6	0.005

Mean +/- SEM is presented. A non parametric Wilcoxon ranked test was realized for data comparison between lean and obese subjects. Fat mass and fat free mass was measured by biphotonic absorptiometry (DXA, lunar, SE)

We further performed a correlation analysis between corpulence related parameters, metabolic factors and Inhibin β A gene expression using spearman correlations. No correlation was found between Inhibin β A gene expression and subject's age. We observed that Inhibin β A gene expression correlated positively with BMI, fat mass. A positive trend was observed for circulating leptin while the association between Inhibin β A gene expression and adiponectin appeared negative.

	Rs (spearman)	P
BMI	0.56	0.01
Fat mass	0.51	0.02
Fat free mass	-0.50	0.03
Fasting glucose	0.56	0.01
Triglycerides	0.42	0.067
Adiponectin	-0.43	0.06
Leptin	0.42	0.06

REFERENCES

1. Tchkonina T, Lenburg M, Thomou T, Giorgadze N, Frampton G, Pirtskhalava T, Cartwright A, Cartwright M, Flanagan J, Karagiannides I, Gerry N, Forse RA, Tchoukalova Y, Jensen MD, Pothoulakis C, Kirkland JL: Identification of depot-specific human fat cell progenitors through distinct expression profiles and developmental gene patterns. *Am J Physiol Endocrinol Metab* 292:E298-307, 2007
2. Tchkonina T, Giorgadze N, Pirtskhalava T, Tchoukalova Y, Karagiannides I, Forse RA, DePonte M, Stevenson M, Guo W, Han J, Waloga G, Lash TL, Jensen MD, Kirkland JL: Fat depot origin affects adipogenesis in primary cultured and cloned human preadipocytes. *Am J Physiol Regul Integr Comp Physiol* 282:R1286-1296, 2002
3. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156-159, 1987
4. Bourlier V, Zakaroff-Girard A, Miranville A, De Barros S, Maumus M, Sengenès C, Galitzky J, Lafontan M, Karpe F, Frayn KN, Bouloumie A: Remodeling phenotype of human subcutaneous adipose tissue macrophages. *Circulation* 117:806-815, 2008
5. Rodriguez A-M, Pisani D, Dechesne CA, Turc-Carel C, Kurzenne J-Y, Wdziekonski B, Villageois A, Bagnis C, Breittmayer J-P, Groux H, Ailhaud G, Dani C: Transplantation of a multipotent cell population from human adipose tissue induces dystrophin expression in the immunocompetent mdx mouse. *J. Exp. Med.* 201:1397-1405, 2005
6. Zaragosi LE, Ailhaud G, Dani C: Autocrine fibroblast growth factor 2 signaling is critical for self-renewal of human multipotent adipose-derived stem cells. *Stem Cells* 24:2412-2419, 2006
7. Rodriguez A-M, Elabd C, Delteil F, Astier J, Vernochet C, Saint-Marc P, Guesnet J, Guezennec A, Amri E-Z, Dani C, Ailhaud G: Adipocyte differentiation of multipotent cells established from human adipose tissue. *Biochemical and Biophysical Research Communications* 315:255-263, 2004
8. Elabd C, Chiellini C, Massoudi A, Cochet O, Zaragosi LE, Trojani C, Michiels JF, Weiss P, Carle G, Rochet N, Dechesne CA, Ailhaud G, Dani C, Amri EZ: Human adipose tissue-derived multipotent stem cells differentiate in vitro and in vivo into osteocyte-like cells. *Biochem Biophys Res Commun* 361:342-348, 2007
9. Rosen ED, Spiegelman BM: Molecular regulation of adipogenesis. *Annu Rev Cell Dev Biol* 16:145-171., 2000
10. Poitou C, Divoux A, Faty A, Tordjman J, Hugol D, Aissat A, Keophiphath M, Henegar C, Commans S, Clement K: Role of serum amyloid a in adipocyte-macrophage cross talk and adipocyte cholesterol efflux. *J Clin Endocrinol Metab* 94:1810-1817, 2009
11. Bezaire V, Mairal A., Ribet C., Lefort C., Girousse A., Jocken J., Laurencikiene, J., Anesia, R., Rodriguez, A-M., Ryden, M., Stenson, B.M., Dani, C., Ailhaud, G., Arner, P., Langin, D.: Contribution of Adipose Triglyceride Lipase and Hormone-sensitive Lipase to Lypolysis in Human hMADS Adipocytes. *JBC* in press, 2009
12. Sturn A, Mlecnik B, Pieler R, Rainer J, Truskaller T, Trajanoski Z: Client-server environment for high-performance gene expression data analysis. *Bioinformatics* 19:772-773, 2003
13. Miranville A, Heeschen C, Sengenès C, Curat CA, Busse R, Bouloumie A: Improvement of postnatal neovascularization by human adipose tissue-derived stem cells. *Circulation* 110:349-355, 2004