

# Activin A Plays a Critical Role in Proliferation and Differentiation of Human Adipose Progenitors

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**OBJECTIVE**—Growth of white adipose tissue takes place in normal development and in obesity. A pool of adipose progenitors is responsible for the formation of new adipocytes and for the potential of this tissue to expand in response to chronic energy overload. However, factors controlling self-renewal of human adipose progenitors are largely unknown. We investigated the expression profile and the role of activin A in this process.

**RESEARCH DESIGN AND METHODS**—Expression of INHBA/activin A was investigated in three types of human adipose progenitors. We then analyzed at the molecular level the function of activin A during human adipogenesis. We finally investigated the status of activin A in adipose tissues of lean and obese subjects and analyzed macrophage-induced regulation of its expression.

**RESULTS**—INHBA/activin A is expressed by adipose progenitors from various fat depots, and its expression dramatically decreases as progenitors differentiate into adipocytes. Activin A regulates the number of undifferentiated progenitors. Sustained activation or inhibition of the activin A pathway impairs or promotes, respectively, adipocyte differentiation via the C/EBP $\beta$ -LAP and Smad2 pathway in an autocrine/paracrine manner. Activin A is expressed at higher levels in adipose tissue of obese patients compared with the expression levels in lean subjects. Indeed, activin A levels in adipose progenitors are dramatically increased by factors secreted by macrophages derived from obese adipose tissue.

**CONCLUSIONS**—Altogether, our data show that activin A plays a significant role in human adipogenesis. We propose a model in which macrophages that are located in adipose tissue regulate adipose progenitor self-renewal through activin A. *Diabetes* 59: 2513–2521, 2010

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See accompanying commentary, p. 2354.

Growth of white adipose tissue in normal development and in obesity is the result of an increase in size and number of adipocytes. Given that mature adipocytes do not divide *in vivo*, regeneration of adipocytes and the increase in adipocyte number depend on self-renewal of a pool of adipose progenitors that remains present during adult life and that can be recruited to form new fat cells (1,2). Recently, subpopulations of precursor cells have been characterized in the stromal-vascular fraction of rodent and human adipose tissues, such as the CD34<sup>+</sup>/CD31<sup>-</sup> cell population in the human, where they are assumed to self-renew and be responsible for the maintenance and for the potential of this tissue to expand in response to chronic energy excess (3–5).

Obesity is associated with new macrophages that are recruited into adipose tissue and is accompanied by chronic low-grade inflammation in this tissue (6,7). Interestingly, an increase in the proportion of human adipose progenitors exhibiting proliferative potential is observed in obese adipose tissue (8). It has recently been reported that the differentiation potential of human preadipocytes is inversely correlated with obesity, whereas the pool of precursor cells was positively correlated to BMI (9,10), suggesting that the obese microenvironment is capable of inducing proliferation of human preadipocytes while inhibiting their differentiation. Concordantly, human macrophage medium stimulates proliferation of human preadipocytes *in vitro* (11,12). Therefore, immunoinflammatory cells that accumulate within adipose tissue with obesity might contribute to fat mass enlargement through paracrine effects on progenitor cells.

Several factors have been identified as playing a role in proliferation or differentiation of murine preadipose cell lines. However, factors controlling self-renewal, *i.e.*, proliferation and differentiation, of human adipose progenitors *in vitro* and *in vivo* are largely unknown. Identification of these factors is of fundamental importance and could ultimately be translated into clinical interventions.

At the nuclear level, CCAAT/enhancer binding protein (C/EBP) $\beta$  is one of the earliest partners known to play a critical role in adipocyte differentiation in murine models (13). C/EBP $\beta$  exists in two isoforms translated from a single mRNA by using two AUGs within the same reading frame, the liver-enriched transcriptional activator protein (LAP) and the liver-enriched transcriptional inhibitory protein (LIP) (14). C/EBP $\beta$ -LAP homodimer promotes adipogenesis by inducing peroxisome proliferator-activated receptor (PPAR) $\gamma$  and C/EBP $\alpha$  expression.

In contrast, C/EBP $\beta$ -LIP homodimer lacks the transactivation domain but exhibits a higher DNA binding affinity than that of C/EBP $\beta$ -LAP and potently inhibits adipocyte differentiation. Both C/EBP $\beta$  isoforms are expressed during adipogenesis, and changes in the LAP-to-LIP ratio have dramatic consequences on differentiation (15,16). Recently, Krüppel-like factor (KLF)4 has been shown to be an essential transactivator of C/EBP $\beta$  gene transcription in murine 3T3-L1 cells (17). Therefore, C/EBP $\beta$  and KLF4 appear to be key early transcription factors promoting adipogenesis in murine models.

We analyzed the transcriptome of human multipotent adipose-derived stem (hMADS) cells (supplementary Fig. S1, available in an online appendix [http://diabetes.diabetesjournals.org/cgi/content/full/db10-0013/DC1]) to identify factors that control human adipogenesis, and we screened for genes that were differentially expressed in adipocytes compared with their progenitors (18). Among these, we focused our attention on secreted proteins. One of these is activin A. Activin A is a member of the transforming growth factor- $\beta$  superfamily and regulates a wide variety of cellular events, including regulation of growth and metastasis of cancer cells, apoptosis, proliferation, and differentiation of human embryonic stem cells. Activin A is also a critical mediator of inflammation (19). Activin A is a secreted protein composed of two inhibin  $\beta$ A (INHBA) subunits. It has been reported that activin A signaling suppresses differentiation of murine 3T3-L1 cells (20). In humans, activin A has been positioned as a profibrotic factor in adipose tissue of obese patients (12). Altogether, these observations suggest that the activin A pathway could play a role in adipose tissue biology. However, no information is available about the autocrine/paracrine function of activin A on proliferation and differentiation of human adipose progenitors or about activin A regulation.

We show herein that activin A is expressed by human adipose progenitors *ex vivo* and *in vitro*. It is the first demonstration that activin A promotes proliferation of hMADS cells and that activin A-treated cells failed to undergo adipocyte differentiation through activation of Smad2 signaling and downregulation of C/EBP $\beta$ -LAP expression. Activin A is more highly expressed in adipose tissues of obese patients than of lean subjects, and we also found that activin A is upregulated by adipose tissue macrophage-secreted factors, demonstrating a link between adipose progenitor self-renewal and macrophages. Altogether, our data support the following hypotheses: 1) that activin A is a novel crucial regulator of human adipose progenitor's self-renewal and 2) that the expression of activin A is regulated by the microenvironment existing in obese patients where macrophages are present. This pathway could represent a potential target for controlling the size of the adipose precursor pool in adipose tissue.

## RESEARCH DESIGN AND METHODS

Activin A (Preprotech) was prepared as a 100  $\mu$ g/ml stock solution in culture medium supplemented with 0.5% FCS. SB431542 was purchased from Sigma-Aldrich. SB203580 was from CalbioChem. Rosiglitazone (BRL4953) was a gift from Dr. J.F. Dole (GlaxoSmithKline, King of Prussia, PA).

**Preparation of cell extracts and Western blot analysis.** Cells were rinsed with PBS and solubilized in stop buffer containing 50 mmol/l Hepes, pH 7.2; 150 mmol/l NaCl; 10 mmol/l EDTA; 10 mmol/l Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>; 2 mmol/l Na<sub>3</sub>VO<sub>4</sub>; and 1% Triton X-100 supplemented with Protease Inhibitor Cocktail (Roche). The primary antibodies and dilutions used were as follows: mouse anti-activin A (1/1,000; R&D Systems); C/EBP $\beta$  (C19, 1/200; Santa Cruz); Smad2/3 and phospho-Smad2 (1/1,000; Cell Signaling); extracellular signal-related kinase

(ERK)1/2 and phospho-ERK1/2 (1/2,000 and 1/1,000, respectively; Cell Signaling); enolase 1 (ENO1) (1/1,000; Santa Cruz); KLF4 (1/200; Abcam);  $\beta$ -tubulin-1 (1/5,000; Sigma Aldrich); and fatty acid-binding protein (FABP)4 (1/1,000), which was a gift of Dr. D.A. Bernlohr (Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota-Twin Cities, Minneapolis, Minnesota). Secondary horseradish peroxidase-conjugated antibodies were purchased from Promega.

**Obese and lean adipose tissues.** In the first group, needle biopsies of subcutaneous white adipose tissue from 12 morbidly obese (age 41.5  $\pm$  3.2 years; BMI 45.8  $\pm$  1.8 kg/m<sup>2</sup>) and seven lean (age 43.71  $\pm$  4.51 years; BMI 21.45  $\pm$  0.45 kg/m<sup>2</sup>) women of the same ages were collected. In these groups, we measured gene expression of INHBA. In a second population, subcutaneous and visceral white adipose tissue samples from 11 morbidly obese women who were candidates for bariatric surgery (age 37.7  $\pm$  2.7 years and BMI 49.9  $\pm$  2.6 kg/m<sup>2</sup>) were also collected for expression analysis of the genes described above. Visceral adipose tissue refers to the omental depot. Informed consent was obtained from all subjects. All clinical investigations were performed according to the Declaration of Helsinki and approved by the ethics committees of the Hôtel-Dieu (Paris, France).

**siRNA transfection.** hMADS cells were transfected with small interfering RNA (siRNA) duplexes using HiPerfect reagent (Qiagen) either during the exponential growth cell phase (2.8  $\times$  10<sup>4</sup> cells/well) to investigate the effects of cell proliferation or a day before confluence (7  $\times$  10<sup>4</sup> cells/well) to investigate effects on differentiation. siRNAs for human *Inhba* were as follows: 5' gaaacaguucuuugucagu 3' and siSmad2, 5' uuuuuugucagagccccc 3'; siRNA control duplex was from Eurogentec. Cells were transfected with 8 nmol/l siRNA in medium supplemented with the low concentration of serum described composed of 60% Dulbecco's modified Eagle's medium low glucose, 40% MCDB-201, 10  $\mu$ g/ml insulin, 5  $\mu$ g/ml transferrin, 50 ng/ml selenium, 10<sup>-9</sup> mol/l dexamethasone, 50  $\mu$ g/ml ascorbic sodium acid, and 2.5 ng/ml fibroblast growth factor (FGF)2, supplemented with 0.5% FCS.

**Generation of hMADS3-EcoRec cells.** 293 FT cells (Invitrogen) were transfected with pLenti6/Ubc-Slc7a1 (Addgene plasmid 17224) along with packaging mix using lipofectamine 2000 according to the supplier's instructions (Invitrogen). Forty-eight hours after transfection, the supernatant was collected, filtered through a 0.45-mm pore-size filter, and transferred to dishes containing proliferating hMADS3 cells for 24 h in the presence of 5  $\mu$ g/ml polybrene. Cells expressing the mouse receptor for retrovirus were then selected in the presence of 2.5  $\mu$ g/ml blasticidin. hMADS3-EcoRec cells displayed a similar proliferation rate and the same capacities to undergo differentiation into adipocytes and osteoblasts (not shown).

**Retroviral infection of hMADS3-EcoRec cells.** Ecotopic retrovirus vector expressing C/EBP $\beta$ -LIP (Addgene plasmid 15714), C/EBP $\beta$ -LAP (Addgene plasmid 15712), Klf4 (Addgene plasmid 17219), or green fluorescent protein (GFP) (kindly provided by Dr. Kitamura, University of Tokyo, Tokyo, Japan) were produced in PLAT-E cells (Cell Biolabs). Twenty-four hours after transfection, medium was collected and transferred to hMADS3-EcoRec dishes for 24 h in the presence of 5  $\mu$ g/ml polybrene. Twenty-four hours after cells reached confluence, they were induced to undergo differentiation. With GFP used as a reporter, 80% of hMADS3-EcoRec cells were transduced using this protocol.

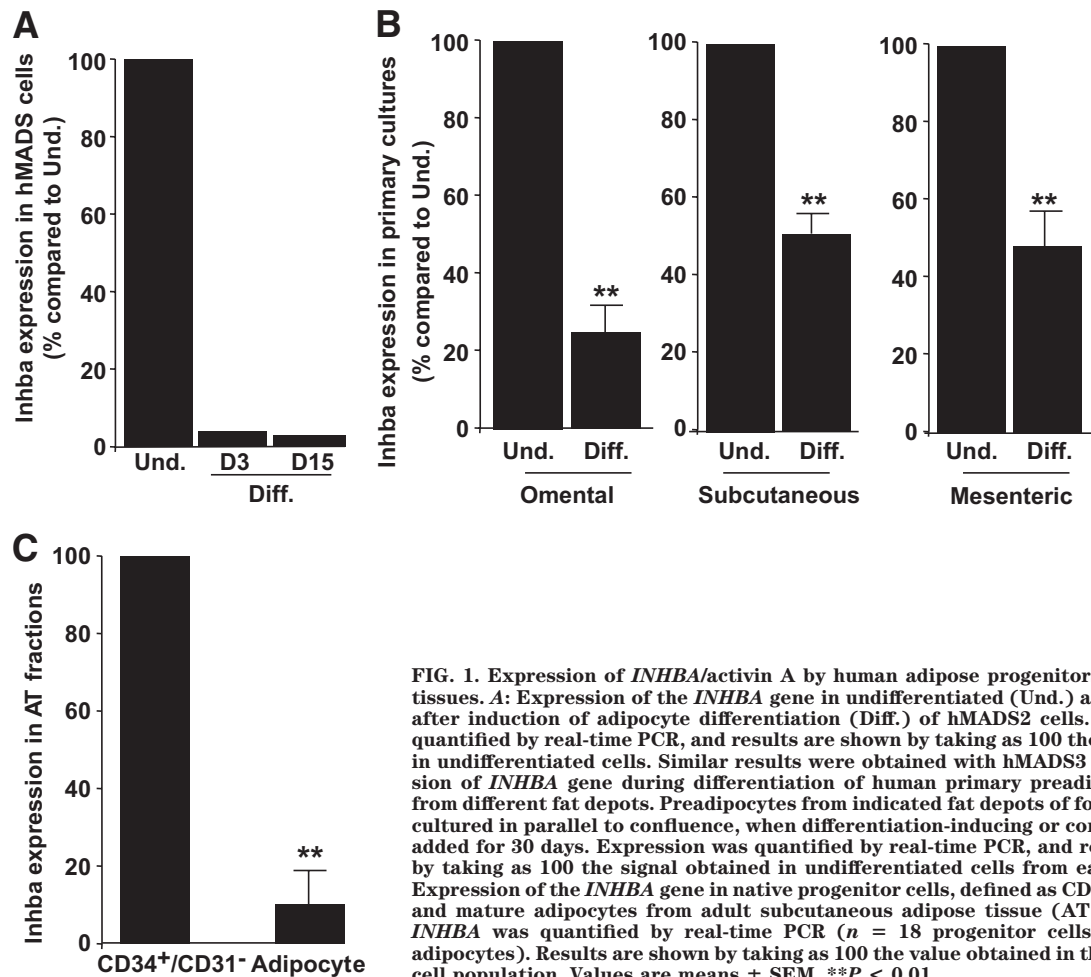
**RT-PCR analysis.** All primer sequences are described in supplemental Table 1. Real-time PCR assays were run on an ABI Prism 7000 real-time PCR machine (PerkinElmer Life Sciences) or in a GeneAmp 7500 detection system (Applied Biosystems, Courtaboeuf, France). Normalization was performed using the geometric averages of the housekeeping genes *G6PDH*, *POLR2A*, and *TBP*. Quantification was performed using the comparative D<sub>CT</sub> method.

**Cell proliferation assays.** Cells were plated onto 12-well plates (2,900 cells/cm<sup>2</sup>), and after the appropriate time cells were trypsinized and counted with a Coulter counter. For each experiment, three wells per condition were counted.

**Statistical analysis.** Statistical significance was determined by *t* tests or ANOVA with post hoc comparisons by Duncan's multiple-range test. Probability values <0.05 were considered statistically significant and are indicated with a single asterisk; <0.01 is indicated by double asterisks. Nonparametric tests were used to compare adipose tissue gene expression between lean and obese subjects (i.e., Wilcoxon test) and to compare subcutaneous versus visceral adipose tissue in obese subjects (i.e., paired Wilcoxon ranked test).

## RESULTS

**INHBA/activin A expression is downregulated during human adipogenesis *in vitro* and is preferentially expressed by undifferentiated adipose progenitors *in vivo*.** According to the results of a transcriptome analysis that we previously reported (20), we noticed that the *INHBA* gene was downregulated during adipogenic differ-



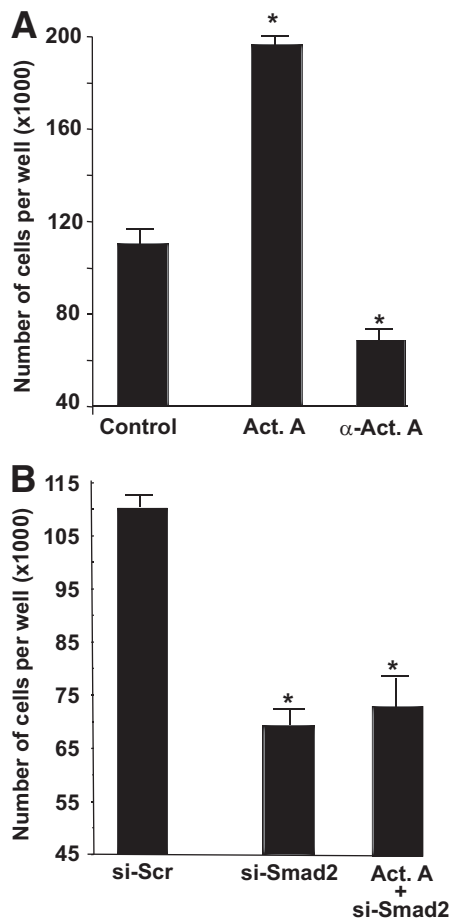
**FIG. 1.** Expression of *INHBA*/activin A by human adipose progenitors and in adipose tissues. **A:** Expression of the *INHBA* gene in undifferentiated (Und.) and 3 and 15 days after induction of adipocyte differentiation (Diff.) of hMADS2 cells. Expression was quantified by real-time PCR, and results are shown by taking as 100 the signal obtained in undifferentiated cells. Similar results were obtained with hMADS3 cells. **B:** Expression of *INHBA* gene during differentiation of human primary preadipocytes isolated from different fat depots. Preadipocytes from indicated fat depots of four subjects were cultured in parallel to confluence, when differentiation-inducing or control media were added for 30 days. Expression was quantified by real-time PCR, and results are shown by taking as 100 the signal obtained in undifferentiated cells from each fat depot. **C:** Expression of the *INHBA* gene in native progenitor cells, defined as CD34<sup>+</sup>/CD31<sup>-</sup> cells, and mature adipocytes from adult subcutaneous adipose tissue (AT). Expression of *INHBA* was quantified by real-time PCR ( $n = 18$  progenitor cells;  $n = 5$  mature adipocytes). Results are shown by taking as 100 the value obtained in the CD34<sup>+</sup>/CD31<sup>-</sup> cell population. Values are means  $\pm$  SEM. \*\* $P < 0.01$ .

entiation of hMADS cells. To ascertain that our conclusions are not limited to one specific system, we used three types of human adipose progenitors, i.e., hMADS cells, primary culture of preadipocytes, and native CD34<sup>+</sup>/CD31<sup>-</sup> cell populations (see supplementary Fig. S1 for the isolation procedure), to confirm the expression of *INHBA*/activin A during adipogenesis. As shown in Fig. 1A, the *INHBA* gene was expressed by undifferentiated hMADS cells and its level of expression decreased dramatically early upon induction of adipocyte differentiation. Protein levels of activin A secreted by undifferentiated and differentiated cells were in agreement with RNA levels (supplementary Fig. S2). Primary preadipocytes that were isolated from subcutaneous, mesenteric, and omental fat depots of adult subjects expressed the *INHBA* gene and also displayed a downregulation of this gene expression when induced to undergo differentiation (Fig. 1B). These data indicate that activin A is expressed in adipose progenitors from various human fat depots independently of the age of the donors and is downregulated as progenitors undergo adipogenesis.

To check that higher *INHBA*/activin A expression was indeed correlated with the undifferentiated state of adipose progenitors in vivo, we monitored the *INHBA* gene expression in fractionated human adipose tissue. Human stromal-vascular fraction (SVF) is known to be a heterogeneous cell population containing adipose progenitors and other cell types, which can be isolated based on expression of cell-surface markers (4). Isolation of differ-

ent cell subpopulations from the SVF and quantitative RT-PCR revealed that *INHBA* was expressed at a higher level in native CD34<sup>+</sup>/CD31<sup>-</sup> adipose progenitors than in the adipocyte fraction (Fig. 1C). This result did not exclude the possibility that *INHBA* could also be expressed by other cell types resident in the SVF (see DISCUSSION). Altogether, these data show that activin A is expressed by undifferentiated adipose progenitors and that this expression is inversely correlated with adipogenic differentiation. To reveal the potential relevance of this expression profile, we then investigated the functional role of activin A in human adipogenesis.

**Activin A increases the number of human undifferentiated adipose-derived stem cells via autocrine/paracrine activation of the Smad2 signaling pathway.** As shown in Fig. 1 and supplementary Fig. S2, activin A was secreted by undifferentiated cells and its level decreased dramatically upon induction of adipocyte differentiation, suggesting its role in regulating the number of undifferentiated hMADS cells. Thus, we analyzed the effect of activin A addition in serum-reduced culture conditions that were previously set up to sustain hMADS cell proliferation. As displayed in Fig. 2A, activin A supplementation enhanced cell number in proliferating hMADS cell cultures. Enhancement of adipocyte progenitor proliferation by activin A did not impact on their subsequent capacity to undergo adipocyte differentiation; undifferentiated activin A-treated cells displayed a potential to undergo adipocyte differentiation similar to that of untreated cells (not shown).



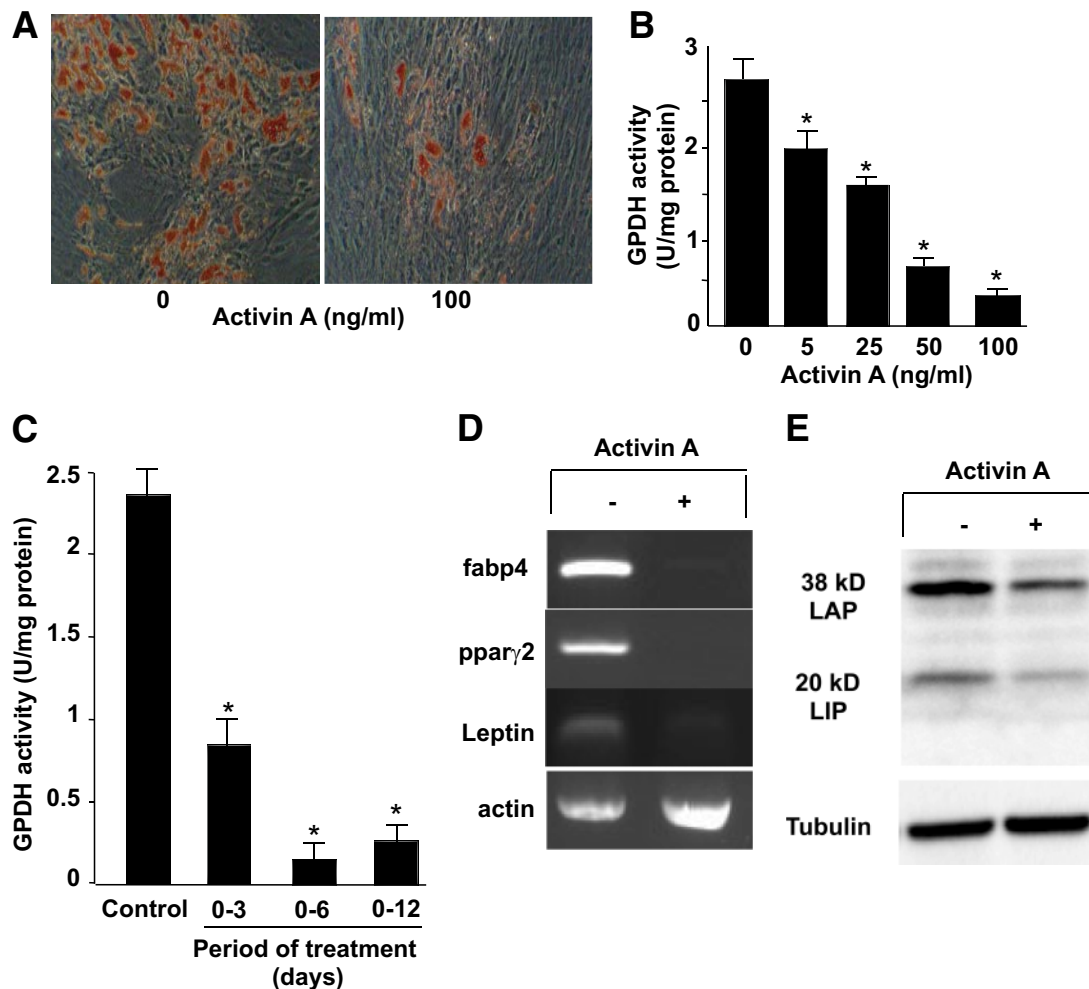
**FIG. 2.** Effects of activin A (Act. A) supplementation and of activin A signaling pathway inhibition on hMADS cell number. **A:** hMADS3 cell number in 0.5% FCS medium in the absence or presence of 100 ng/ml activin A or 1  $\mu$ g/ml neutralizing activin A antibodies ( $\alpha$ -Act.A) for 5 days. Results are the average of counting of three culture wells (12-well plates). **B:** Proliferating hMADS3 cells were transfected with siSmad2 and treated or not with 100 ng/ml activin A 24 h later. Impact on cell number was analyzed 5 days later. Results are the average of three culture wells (12-well plates). Data are means  $\pm$  SEM ( $n = 3$ ). \*Cell numbers significantly different in treated cells vs. controls.

Cell number was decreased when cells were treated with an activin A-neutralizing antibody, showing the existence of a basal activin A autocrine/paracrine signaling pathway involved in undifferentiated hMADS cell number. This conclusion was supported by a gene-silencing approach in which hMADS cells were transfected with *Inhba* siRNA (supplementary Fig. S3). Smad2 became phosphorylated in hMADS cells upon activin A treatment (supplementary Fig. S4A), strongly suggesting that, as in other cell types, Smad2 mediates the biological effects of activin A (21). Therefore, undifferentiated hMADS cells were transfected with siRNA duplex directed against Smad2 to investigate the role of the activin A/Smad2 pathway in regulating number of adipose progenitors (see supplementary Fig. S4B for siRNA effect on Smad2 protein levels). Twenty-four hours after transfection, cells were or were not stimulated with activin A, and cell number was quantified 5 days later. Fig. 2B shows that Smad2 silencing decreased cell number and inhibited the effects of activin A. Collectively, these data show that activin A regulates the number of undifferentiated hMADS cells through autocrine/paracrine pathways and that this effect employed Smad2-dependent mechanisms.

**Sustained activin A levels inhibit adipose commitment with a C/EBP $\beta$ -LAP-dependant mechanism.** As shown in Fig. 1, activin A is downregulated upon induction of differentiation. To identify whether activin A downregulation is necessary for commitment toward the adipocyte lineage, hMADS cells were induced to undergo adipocyte differentiation in the absence or presence of exogenous activin A. Chronic exposure of cells to activin A inhibited formation of lipid-containing cells and lowered the triglyceride-synthesizing glycerol-3-phosphate dehydrogenase (GPDH) activity in a dose-dependent manner (Fig. 3A and B). hMADS cells were then treated with 100 ng/ml activin A for defined time intervals, and adipogenesis was scored at day 12. As shown in Fig. 3C, exposure of hMADS cells to activin A for the first 3 days of the differentiation process led to a dramatic inhibition of differentiation. The highest level of GPDH inhibition was obtained when cells were exposed to activin A for the first 6 days. Expression of adipogenic genes, such as *fabp4*, *ppary2*, and *leptin*, was inhibited when cells were exposed to activin A for the first 6 days (Fig. 3D). These data indicate that activin A played a crucial role during the initial stages of the adipocyte differentiation program.

Expression of most of the adipogenic genes studied was downregulated in activin A-treated cells compared with untreated cells (supplementary Fig. S5B). KLF4 protein was expressed at low levels and was not altered by activin A treatment (supplementary Fig. S5C), suggesting that activin A acts either downstream, or independently, of KLF4. In contrast, C/EBP $\beta$  transcript (kinetics of expression during hMADS cell differentiation described in Fig. S6) and C/EBP $\beta$ -LAP/LIP isoform levels were downregulated in response to activin A (Fig. 3E and supplementary Fig. S6). Therefore, we hypothesized that antiadipogenic effects of activin A occur through inhibition of C/EBP $\beta$ -LAP. If this hypothesis is correct, maintenance of C/EBP $\beta$ -LAP should abolish the inhibitory effect of activin A on adipogenesis. To test this hypothesis, we generated an hMADS-EcoRec cell line permissive to infection with murine retrovirus, and cells were transduced with a retroviral vector expressing LAP or LIP isoforms or GFP as control. We ascertained that overexpression of LAP promoted adipogenesis of hMADS cells and that overexpression of LIP inhibited this process (supplementary Fig. S7). Then, we investigated the consequences of this forced expression on activin A effects. Whereas inhibition of adipocyte differentiation by activin A was not affected in GFP- or LIP-hMADS cells, as assessed by GPDH activities and FABP4 protein levels, activin A-mediated inhibition of adipogenesis was abolished in LAP-hMADS cells (Fig. 4). These data are consistent with the hypothesis that the inhibitory effect of activin A on adipocyte differentiation is mediated by regulating C/EBP $\beta$ -LAP levels.

**Inhibition of activin A/Smad2 pathway promotes human adipogenesis.** We showed that activin A is secreted by hMADS cells, while these cells expressed receptors for activin A, ACVR1A, ACVR1B, and ACVR2A (data not shown). Thus, we postulated the existence of a basal autocrine/paracrine signaling pathway that is involved in hMADS cell differentiation. We used complementary approaches to investigate the role of endogenous activin A. First, we used activin A antibodies to neutralize secreted activin A and SB431542, a potent and selective inhibitor of the ALK5 receptor, which binds activin A. hMADS cells were maintained in adipogenic medium in the absence or presence of 1  $\mu$ g/ml anti-activin A antibodies or 5  $\mu$ mol/l

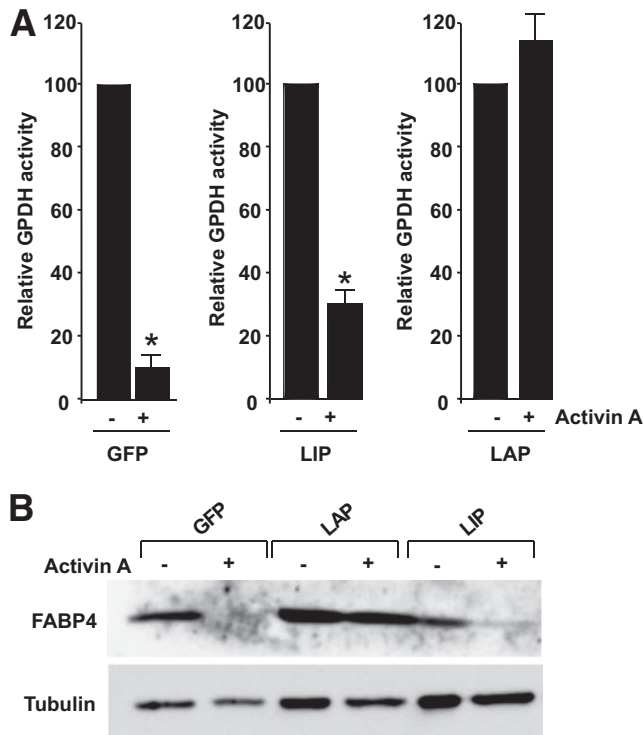


**FIG. 3.** Inhibition of hMADS cells adipogenesis by activin A. **A** and **B**: hMADS3 cells were induced to undergo adipocyte differentiation in the absence or presence of the indicated concentrations of activin A. Twelve days later, adipogenesis was assessed by Oil red O for lipid droplets staining (34) and by GPDH activity (35). **C**: hMADS3 cells were induced to undergo adipocyte differentiation and treated with 100 ng/ml activin A for the indicated time intervals. GPDH activity was determined at day 12. Results are means of three culture wells (24-well plates). Values are means  $\pm$  SEM ( $N = 3$ ). \*Significant differences in GPDH activities in treated cells vs. controls. Similar results were obtained with hMADS2 cells and with hMADS7-B7 and -B9 clones. **D**: Effects of activin A on the expression of adipogenic genes. hMADS3 cells were induced to undergo differentiation in the absence or presence of 100 ng/ml activin A. RNAs were prepared 6 days after induction of differentiation and expression was investigated by semiquantitative PCR. **E**: Effects of activin A on the expression of C/EBP $\beta$ -LAP and -LIP isoforms. hMADS3 cells were induced to undergo differentiation in the absence or presence of 100 ng/ml activin A. Proteins were prepared 6 days after induction of differentiation. LAP and LIP isoforms were examined by Western blot analysis using 25  $\mu$ g total proteins per lane and anti-C/EBP $\beta$  antibodies. Similar results were obtained when proteins were prepared 3 days after induction of differentiation (supplementary Fig. S6). The approximate molecular weight of C/EBP $\beta$  isoforms is indicated. The 14 kDa C/EBP $\beta$  proteolytic degradation product was not detected. Tubulin was used as a loading control. Samples were run on the same gel. (A high-quality digital representation of this figure is available in the online issue.)

SB431542. As shown in Fig. 5A, GPDH activity levels were increased in the presence of anti-activin A antibodies or SB431542. Enhanced expression of PPAR $\gamma$  and of adiponectin mRNAs, two markers of adipocytes, confirmed at the molecular level that inhibition of the activin receptor-like kinase (ALK) 5 induced adipogenesis (not shown). Because ALK5 can also bind transforming growth factor- $\beta$ , which was expressed by hMADS cells (not shown), we specifically blocked the activin A signaling pathway using siRNA-mediated gene-silencing approaches. GPDH activity was increased in early differentiated hMADS cells transfected with *Inhba*-siRNA or with *Smad2*-siRNA compared with GPDH activity in cells transfected with a scrambled siRNA (Fig. 5B). Inhibition of *Smad2* dramatically promoted formation of lipid-containing cells and levels of the adipogenesis-induced FABP4 protein (supplementary Fig. S8). Together, these data demonstrate the existence of an autocrine/paracrine loop comprising the

activin A/*Smad2* pathway that plays a critical negative role in adipocyte differentiation of hMADS cells.

**Activin A expression is inhibited by dexamethasone and stimulated by factors secreted by adipose tissue-derived macrophages.** We investigated factors regulating activin A expression in human adipose progenitors. First, we analyzed the specific component(s) of the adipogenic cocktail that might be involved in downregulating *INHBA* gene expression. To address this, hMADS cells maintained in serum-free medium supplemented with transferrin and insulin were exposed to the various components of the adipogenic induction cocktail either alone or in combination. As shown in Fig. 6A, T3, rosiglitazone, and 3-isobutyl-1-methylxanthine (IBMX) did not alter *INHBA* gene expression. In contrast, its expression was selectively downregulated by dexamethasone added alone or combined with the other components. Levels of activin A secreted by dexamethasone-

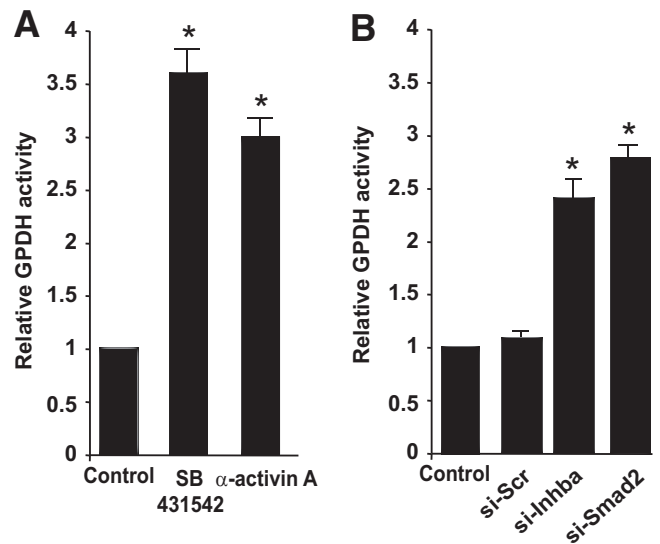


**FIG. 4.** Suppression of antiadipogenic effects of activin A by C/EBP $\beta$ -LAP forced expression. **A:** hMADS3-EcoRec cells were infected with retroviral vectors expressing GFP, C/EBP $\beta$ -LAP, or C/EBP $\beta$ -LIP and induced to differentiate into adipocytes in the absence or presence of 100 ng/ml activin A. Adipogenesis was assessed 6 days later by GPDH activities. GPDH activity obtained in the absence of activin A for each transduced cells was taken as 100%. Results are means of three culture wells (24-well plates). Values are means  $\pm$  SEM ( $n = 3$ ). \*Significant differences between treated versus untreated cells. **B:** Western blot analysis of FABP4 in the absence or presence of activin A. Samples were run on the same gel.

treated cells were in agreement with the inhibition observed at RNA levels (Fig. 6B). We also wanted to evaluate whether the expression of the *INHBA* gene could be regulated by the in vivo microenvironment of adipose progenitors. In this context, we compared the *INHBA* gene expression levels in subcutaneous fat depots of lean and obese individuals. Clinical and biological parameters measured in lean and obese women are shown in Tables S3 and S4.

As shown in Fig. 7A, RNA levels of *INHBA* were significantly increased in subcutaneous adipose tissue of obese subjects compared with lean subjects. Activin A secretion was also investigated in paired subcutaneous and visceral adipose tissue explants from obese subjects. Activin A was similarly secreted by subcutaneous and visceral obese adipose tissue explants (not shown). Expression of activin receptors type 1 receptors ACVR1A and ACVR1B and type 2 receptors ACVR2A and ACVR2B has been investigated by RT-PCR. We observed that gene expression of type 1 (ACVR1B) and type 2 (ACVR2A and ACVR2B) activin receptors was not statistically different in adipose tissue of lean and obese subjects (not shown).

To identify the microenvironment components that might stimulate activin A expression in obese subjects, we tested the potential interaction between macrophages and adipose progenitors. For that purpose, preadipocytes isolated from lean adipose tissue were stimulated with factors secreted by macrophages selected from the stromal

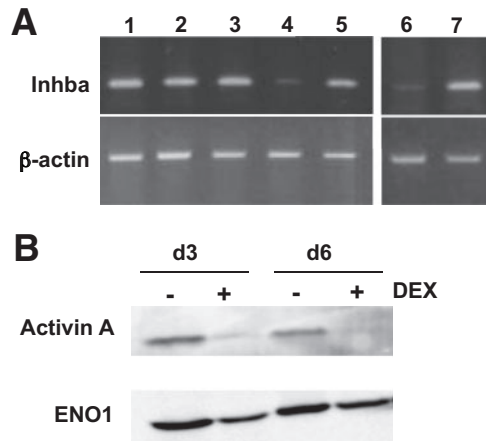


**FIG. 5.** Effects of activin A pathway silencing on adipocyte differentiation of hMADS3 cells. **A:** hMADS3 cells were induced to undergo adipocyte differentiation in the presence of SB431542 (5  $\mu$ M) or neutralizing activin A antibodies (1  $\mu$ g/ml). GPDH activities were quantified after 12 days. Results are the means of three culture wells (24-well plates). Values are means  $\pm$  SEM ( $n = 3$ ). \*Significant differences of GPDH activities in treated cells vs. controls. **B:** hMADS3 cells were transfected with scrambled siRNA (si-Scr.), *Inhba*-siRNA, or *Smad2*-siRNA a day before they reached confluence. The day after, cells were induced to undergo adipocyte differentiation. GPDH activity of cell extracts 6 days after transfection. Results are means of three culture wells (24-well plates). Data are means  $\pm$  SEM ( $n = 3$ ). \*Significant differences in GPDH activities in *siInhba*- and *siSmad2*-treated cells vs. controls.

fraction of obese subcutaneous adipose tissue (adipose tissue macrophages [ATMs]). As shown in Fig. 7B, RNA levels of *INHBA* were significantly increased in human preadipocytes that were maintained for 10 days in media conditioned by ATMs compared with the control condition. As we reported previously, we observed an increase in preadipocyte number compared with the control condition (11). RNA levels of *INHBA* were also increased when native CD34<sup>+</sup>/CD31<sup>-</sup> human adipose progenitors were treated for 24 h with ATM-secreted factors (Fig. 7C). Finally, we investigated levels of activin A secreted by undifferentiated hMADS cells after a 24-h stimulation with ATM-conditioned medium. As shown in Fig. 7D, factors secreted by ATMs selected from three different biopsies dramatically increased levels of activin A secreted by hMADS cells. No activin A was detected in ATM-conditioned media, excluding macrophages as the source of activin A. Levels of activin A secreted by ATM-conditioned media-treated cells were in agreement with the increase observed at the *INHBA* RNA levels (supplementary Fig. S9). Altogether, these data show that human macrophage-secreted factors induce an upregulation of activin A in human adipose progenitors.

## DISCUSSION

The identification of factors regulating self-renewal of adipose progenitor cells could provide a means for better understanding the mechanisms that lead to hyperplasia and excessive development of adipose tissue. To our knowledge, only FGF1 (22) and FGF2, as we have shown previously (23), were shown to stimulate proliferation of human adipose progenitors and subsequently to increase their capacity to undergo differentiation. However, the



**FIG. 6.** Effects of dexamethasone (DEX) on the expression of *INHBA*/activin A in human adipose progenitors. **A:** Individual components of the adipogenic cocktail were added to confluent hMADS3 cells alone or in combination. Cells were harvested 3 days later for semiquantitative PCR analysis. 1, insulin (5  $\mu\text{g/ml}$ ) and 10  $\mu\text{g/ml}$  transferrin; 2, insulin (5  $\mu\text{g/ml}$ ) and 10  $\mu\text{g/ml}$  transferrin plus 1  $\mu\text{mol/l}$  rosiglitazone; 3, insulin (5  $\mu\text{g/ml}$ ) and 10  $\mu\text{g/ml}$  transferrin plus 0.2 nmol/l triiodothyronine; 4, insulin (5  $\mu\text{g/ml}$ ) and 10  $\mu\text{g/ml}$  transferrin plus 1  $\mu\text{mol/l}$  dexamethasone; 5, insulin (5  $\mu\text{g/ml}$ ) and 10  $\mu\text{g/ml}$  transferrin plus 100  $\mu\text{mol/l}$  isobutyl-methylxanthine; 6, insulin (5  $\mu\text{g/ml}$ ) and 10  $\mu\text{g/ml}$  transferrin plus 1  $\mu\text{mol/l}$  rosiglitazone, 0.2 nmol/l triiodothyronine, 100  $\mu\text{mol/l}$  isobutyl-methylxanthine, and 1  $\mu\text{mol/l}$  dexamethasone; 7, insulin (5  $\mu\text{g/ml}$ ) and 10  $\mu\text{g/ml}$  transferrin plus 1  $\mu\text{mol/l}$  rosiglitazone, 0.2 nmol/l triiodothyronine, and 100  $\mu\text{mol/l}$  isobutyl-methylxanthine. **B:** Inhibition of activin A secretion by dexamethasone. hMADS3 cells were maintained in 5  $\mu\text{g/ml}$  insulin and 10  $\mu\text{g/ml}$  transferrin medium in the absence or presence of 1  $\mu\text{mol/l}$  dexamethasone. Culture media were collected 3 or 6 days later, filtered on 0.2- $\mu\text{m}$  membranes, and concentrated with Amicon ultra-15 columns (NMWL, three kDa; Millipore). Levels of activin A were analyzed by Western blot performed under nonreducing conditions because anti-activin A antibody selectively binds to the dimeric form of activin A. Secreted enolase one (ENO1) was used as a loading control (36).

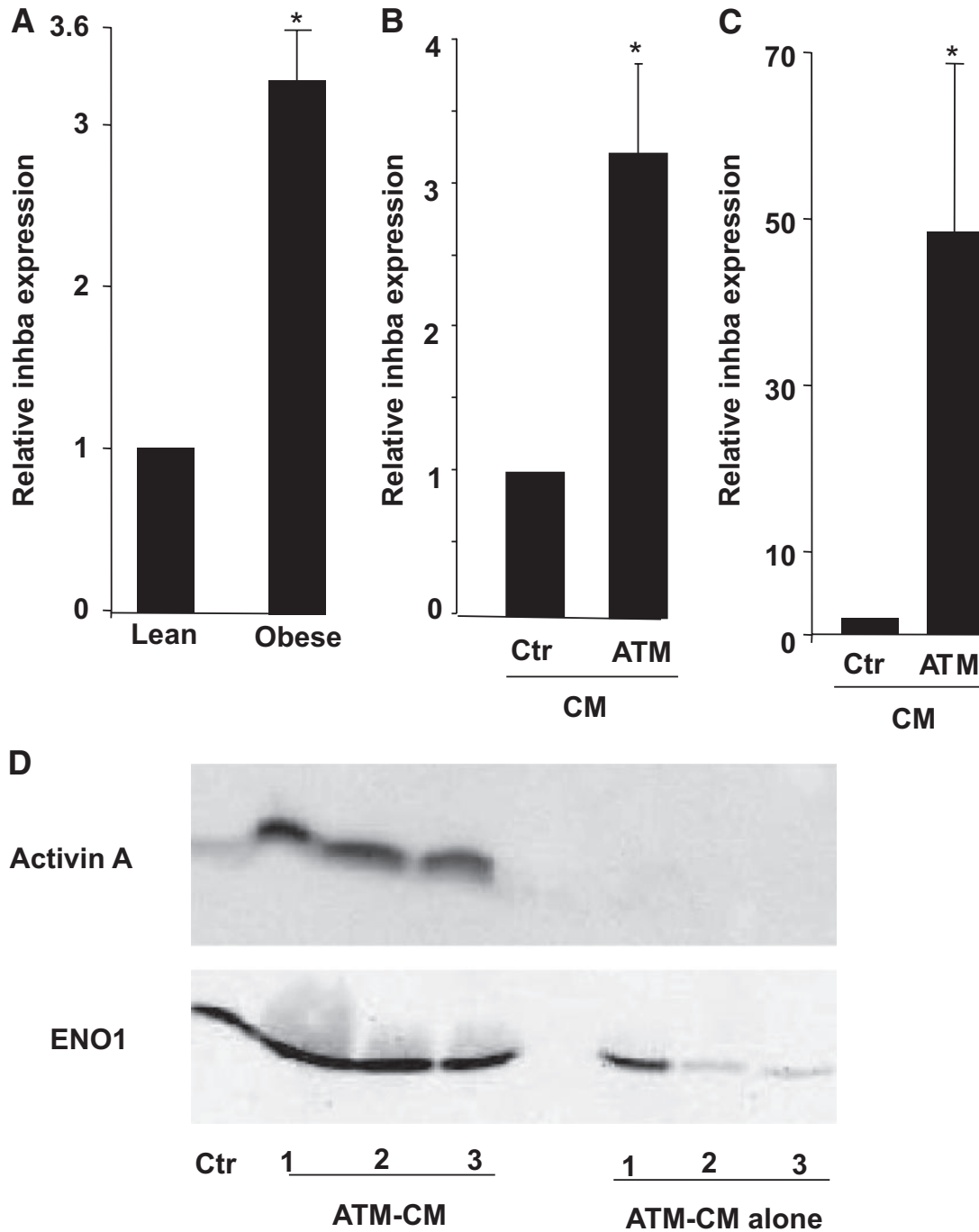
involvement of FGFs in human white adipose tissue growth remains to be investigated. It has been proposed recently that the bone morphogenetic protein pathway, which shares signaling components with the activin pathway, regulates both adipose cell fate determination and differentiation of committed preadipocytes as well as function of mature adipocytes in mouse models (24). The present study specifies the function of activin A in this context and proposes that macrophages are niche components that are, at least in part, responsible for the activation of the activin A pathway in human adipocyte progenitors. *INHBB* subunits are expressed in mouse and human adipose tissues, suggesting that activin B and/or activin AB could also play a role in adipogenesis (25,26). In contrast to what we show for *INHBA*, *INHBB* is preferentially expressed in mature adipocytes, strongly suggesting that activin B/AB are not involved in early steps of adipocyte differentiation. Indeed, the authors propose that *INHBB* may play an autocrine/paracrine role in energy balance or the insulin insensitivity associated with obesity (25–27). Altogether, these data support the hypothesis that activins are regulators of the different steps of adipogenesis.

Homozygous *inhba* mutant mice display a neonatal lethality phenotype (28). This precludes the study of the role of activin A during the development of white adipose tissue.

The Smad2 pathway mediates proliferative and antiadipogenic effects of activin A on hMADS cells. Even if other

pathways in activin A-regulated self-renewal might be involved, the Smad2 pathway is likely to play a major role. Indeed, we observed a weak activation of ERK1/2 upon addition of activin A and that inhibition of p38 MAPK had no effect on activin A-induced cell proliferation (not shown).

Obese adipose tissue is characterized by accumulation of macrophages (6,7), and we show that human macrophage-secreted factors strongly induce an up-regulation of activin A in human adipose progenitors (demonstrated here and previously [12]). Given that we report here that activin A promotes proliferation and inhibits adipocyte differentiation of adipose progenitors, it is tempting to propose that the pool of adipose progenitors increases in adipose tissue of obese patients compared with that of lean subjects, where macrophages are barely present. In agreement with this, we recently reported an increase of proliferative markers in the  $\text{CD34}^+/\text{CD31}^-$  cell population of obese adipose tissue (8). Moreover, adipose tissue macrophages have been shown to inhibit adipogenesis of the  $\text{CD34}^+/\text{CD31}^-$  cells (29). Altogether, these data support a model of self-renewal of adipose progenitors regulated by the obese microenvironment, where activin A plays a critical role. ATM-secreted factors involved in stimulation of activin A expression remain to be identified. Interleukin-1 $\beta$  and/or tumor necrosis factor- $\alpha$  are potent candidates; previous studies have shown in another cell model that activin A secretion is increased upon treatment with these two cytokines (30). The disappearance of macrophages (31) and reducing activin A levels in adipose tissue, for instance as a consequence of dieting, might be favorable to the formation of additional adipocytes from adipose progenitors upon ending dietary restriction—a situation reminiscent of the “yoyo” phenomenon. Activin A is also a critical mediator of inflammation (rev. in [19]). It is interesting to note that dexamethasone, an anti-inflammatory molecule, regulates activin A levels (Fig. 6). In addition to its stimulating effect on expression of proinflammatory cytokines in macrophages, activin A could promote fibrosis in adipose tissue (12). Therefore, activin A signaling could be a target for therapeutic interventions. At the extracellular level, follistatin can bind to activin A with high affinity and antagonizes binding to the activin A receptor. Recently, follistatin has been shown to reduce inflammatory disease severity in mouse animal models (32). More recently, it has been shown that recombinant follistatin treatment promotes adipogenic differentiation of progenitor cells in vitro (33). Activin A and follistatin can be synthesized by several cell types in the body. Based on expression of *INHBA*, preliminary experiments suggest that activin A is more expressed in adipose tissue than in muscle and less expressed in adipose tissue than in liver. We observed that *Inhba* is highly expressed in the  $\text{CD34}^-/\text{CD14}^+$  cell population isolated from adipose tissue. Activin A might also be expressed by macrophages resident in adipose tissue (not shown). It is interesting to note that activin A was not detected in adipose tissue macrophage-conditioned media excluding macrophages as an abundant source of secreted activin A, whereas adipocyte progenitors secrete high levels of activin A in the presence of macrophage factors (see Fig. 7). Expression of activin A and of follistatin in different cell types has to be taken into



**FIG. 7.** Expression of *INHBA*/activin A in adipose tissues and regulation by factors secreted by adipose tissue-derived macrophages. **A:** Expression of *INHBA* in subcutaneous adipose tissue of lean ( $n = 7$ ) and morbidly obese ( $n = 12$ ) women. Expression was quantified by real-time PCR and  $CD34^+/CD31^-$ , and results are shown by taking as 1 the signal obtained in adipose tissue of lean subjects. **B:** Effects of conditioned medium from  $CD14^+$  adipose tissue macrophages on *INHBA*/activin A expression. *INHBA* expression in human preadipocytes treated for 10 days with ATM-conditioned media (ATM-CM) compared with control medium ( $n = 5$ ). **C:** *INHBA* expression in  $CD34^+/CD31^-$  adipose progenitors treated for 24 h with ATM-conditioned media compared with control medium ( $n = 7$ ). Expression was quantified by real-time PCR. Values are means  $\pm$  SEM. ATMs were isolated from women (mean age  $39.33 \pm 4.37$  years and mean BMI  $26.21 \pm 2.54$  kg/m<sup>2</sup>). \*Differ with  $P < 0.01$ . **D:** Undifferentiated hMADS2 cells were treated with factors secreted by ATM isolated from three biopsies (ATM-CM 1–3) or with control media (Ctr) for 24 h. The same volumes of conditioned media were maintained at 37°C in the absence of hMADS cells (ATM-CM alone). Culture media were collected and analyzed for expression of activin A as described in Fig. 6B. Enolase 1 (ENO1) was used as a loading control. Enolase signals shown on the left part of gel combine enolase levels present in the ATM-CM and levels secreted by hMADS cells. ATMs were isolated from women (mean age  $46.20 \pm 6.80$  years and mean BMI  $25.48 \pm 1.26$  kg/m<sup>2</sup>).

account when studying activin A as a potential therapeutic target. Further studies to determine activin A circulating levels in lean subjects and in obese patients are required to better define the clinical role of activin A

and to validate activin A as a secreted biomarker. Blocking activin A in adipose tissue of obese patients could represent a new therapeutic avenue to modulate the pool of adipose progenitors.



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