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Journal of Cellular Biochemistry 109:737–746 (2010)

Journal of Cellular Biochemistry

Keratinocyte Growth Factor Promotes Preadipocyte Proliferation via an Autocrine Mechanism

Ting Zhang, Haiyan Guan, and Kaiping Yang*

Children's Health Research Institute & Lawson Health Research Institute, Departments of Obstetrics & Gynaecology and Physiology & Pharmacology, University of Western Ontario, 800 Commissioners Rd. E., London, Ontario, Canada N6A 4G5

ABSTRACT

Keratinocyte growth factor (KGF; also known as FGF-7) is a well-characterized paracrine growth factor for tissue growth and regeneration. However, its role in adipose tissue, which is known to undergo tremendous expansion in obesity, is virtually unknown. Given that we previously identified KGF as one of the up-regulated growth factors in adipose tissue of an early-life programmed rat model of visceral obesity, the present study was undertaken to examine the hypothesis that KGF promotes adipogenesis. Using 3T3-L1 and rat primary preadipocytes as in vitro model systems, we demonstrated that (1) KGF stimulated preadipocyte proliferation in a concentration-dependent manner with a maximal effect at 2.5 ng/ml (~2-fold increase); (2) KGF mRNA was highly expressed in rat adipocytes and preadipocytes as well as 3T3-L1 cells; (3) treatment of preadipocytes with a neutralizing antibody against KGF and siRNA-mediated knockdown of KGF led to a 50% reduction in their proliferative capacity; (4) KGF activated the protein kinase Akt, and the PI3 kinase inhibitor LY294002 blocked KGF stimulation of preadipocyte proliferation; and (5) KGF did not promote differentiation of preadipocytes to mature adipocytes. Together, these results reveal adipocytes and their precursor cells as novel sites of KGF production. Importantly, they also demonstrate that KGF promotes preadipocyte proliferation by an autocrine mechanism that involves activation of the PI3K/Akt signaling pathway. Aberrant KGF expression may have consequences not only for normal adipose tissue growth but also for the pathogenesis of obesity. J. Cell. Biochem. 109: 737–746, 2010.

KEY WORDS: ADIPOGENESIS; KGF; FGF-7; PI3 KINASE; SIGNAL TRANSDUCTION

Obesity is becoming a leading health problem not only because of its high prevalence but also because of its substantially impairs quality of life and increases the risk of cardiovascular and metabolic diseases as well as certain cancers [Mokdad et al., 2003; Brown et al., 2009]. Obesity is characterized by excess adipose tissue expansion in all or certain fat depots in the body. Visceral obesity (excess adipose tissue accumulation within the abdomen) is the best predictor of obesity-associated morbidity and mortality [Montague and O'Rahilly, 2000].

Although the etiology of visceral obesity remains largely unknown, accumulating epidemiological evidence indicates that low-birth weight is associated with increased visceral adiposity in adult life, suggesting an early-life origin for this disorder [Hales and Barker, 1992; Ong and Dunger, 2002]. Recently, we have demonstrated that poor early nutrition, evoked by maternal protein restriction (MPR) during pregnancy and lactation, leads to low-birth weight and subsequent development of increased visceral adiposity in adult male rat offspring [Guan et al., 2005]. Moreover, we have obtained evidence suggesting that increased visceral adiposity in our rat model is characterized by adipocyte hyperplasia. Indeed, adipocyte precursor cells derived from MPR offspring exhibit accelerated rate of proliferation, even a few days after removal from their in vivo environment [Zhang et al., 2007]. This suggests that MPR permanently alters adipocyte development, but the factors and molecular mechanisms that are responsible for programming this aberrant phenotype remain largely unknown.

As a first step in identifying the causal factors involved, we utilized a candidate gene approach by capitalizing on our previously published visceral adipose tissue gene expression profiling database generated with our rat model of increased visceral adiposity.

Ting Zhang and Haiyan Guan contributed equally to this work.

Grant sponsor: Heart & Stroke Foundation of Ontario; Grant number: NA-6049; Grant sponsor: Canadian Institutes of Health Research (Operating Grant); Grant number: MOP-79484.

*Correspondence to: Dr. Kaiping Yang, Children's Health Research Institute, Room A5-132, Victoria Research Laboratories, Westminster Campus, 800 Commissioners Road East, London, Ontario, Canada N6A 4G5.
E-mail: kyang@uwo.ca

Received 26 June 2009; Accepted 12 November 2009 • DOI 10.1002/jcb.22452 • © 2010 Wiley-Liss, Inc.
Published online 12 January 2010 in Wiley InterScience (www.interscience.wiley.com).

Candidate genes were selected on the basis that (a) they are known to stimulate non-adipose cell proliferation and differentiation; and (b) their expression is up-regulated in our rat model. One of such candidates is the gene encoding keratinocyte growth factor (KGF; also known as FGF-7), the expression of which is increased ~2-fold [Guan et al., 2005]. KGF is produced by cells of mesenchymal origin, and functions in a paracrine fashion to stimulate epithelial cell proliferation and differentiation [Rubin et al., 1995; Finch and Rubin, 2004]. Thus, KGF plays an important role in tissue regeneration/repair and wound healing [Finch and Rubin, 2004]. Although KGF mRNA is present in the stromal–vascular fraction of human adipose tissue [Gabrielsson et al., 2002], the specific cell types that express KGF remain to be determined. Importantly, the role of KGF in adipose tissue, a dynamic organ that is known to undergo tremendous growth/expansion in obesity, is virtually unknown. Given that adipogenesis (i.e., preadipocyte proliferation and differentiation) is a fundamental mechanism underlying adipose tissue expansion [Rosen and Spiegelman, 2000], the present study was designed to test the hypothesis that KGF stimulates adipogenesis in vitro.

MATERIALS AND METHODS

CULTURE OF 3T3-L1 PREADIPOCYTES

The murine 3T3-L1 preadipocyte cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in growth medium, consisting of Dulbecco's modified Eagle's medium growth medium (DMEM; Sigma–Aldrich Canada Ltd., Oakville, ON, Canada) and 10% fetal bovine serum (FBS; Sigma). Cultures were maintained in a humidified incubator at 5% CO_2 and 37°C. Medium was replaced every 2–3 days.

ISOLATION AND CULTURE OF RAT PREADIPOCYTES

Epididymal and perirenal fat pads were collected from male Wistar rats (Charles River Laboratories, Wilmington, MA) at 8 weeks of age, and used immediately for isolation of preadipocytes as described below. Preadipocytes were isolated, and their purity verified by greater than 95% conversion to adipocytes as determined by Oil red O staining following an established differentiation protocol, as described previously [Zhang et al., 2007]. Briefly, the epididymal and perirenal fat pads were dissected from visible blood vessels and connective tissue, weighed, finely minced, and digested in digestion buffer (3 ml/g tissue) consisting of DMEM (Invitrogen Life Technologies, Burlington, ON, Canada), 0.5 mg/ml collagenase class IV (Sigma), and 1.5% bovine serum albumin (BSA; Sigma) for 45 min at 37°C, under mild controlled agitation. The resultant digest material was filtered through 250 μm nylon mesh and centrifuged at 600*g* for 5 min to separate the floating adipocytes. The mature adipocytes were washed with phosphate-buffered saline (PBS) and kept at −80°C for RNA isolation. The cell pellet was resuspended, washed with Dulbecco's phosphate-buffered saline (DPBS) containing 10% newborn calf serum (NCS; Invitrogen), filtered through 25 μm nylon mesh, and then centrifuged. The pelleted preadipocytes were either used immediately for total RNA isolation, or resuspended in standard culture medium (DMEM/F-12 medium; Invitrogen) supplemented with 10% FBS (Sigma), 50 U/ml penicillin and 50 μg/ml streptomycin (Invitrogen). Preadipocytes were cultured on 24-well plates in a humidified incubator at 37°C, in the presence of 5% CO_2.

PROLIFERATION ASSAY—[^{3}H]-THYMIDINE INCORPORATION

Proliferation capacity of 3T3-L1 preadipocytes and rat primary preadipocytes was assessed by measuring [^{3}H]-thymidine incorporation, as described previously [Zhang et al., 2007; Yang et al., 2008]. Briefly, 3T3-L1 cells and rat preadipocytes were plated on 24-well plates, and cultured in growth medium until 40–50% confluence. Cells were growth arrested in serum-free growth medium for 24 h, and were then treated in the serum-free medium for 24 h with KGF (PeproTech Canada Inc., Ottawa ON; Cat #100-19), neutralizing antibody against KGF (R&D Systems, Cat #MAB251), normal mouse IgG Isotope Control (R&D Systems, Cat #MAB002), or as indicated otherwise. For studying the signal transduction pathways, 3T3-L1 cells and rat primary preadipocytes were pretreated for 1 h with U0126 (ERK1/2 inhibitor; Sigma), LY294002 (PI3K inhibitor; Sigma), or SB203580 (p38 MAPK inhibitor; Sigma) before the addition of KGF (5 ng/ml). During the last 4 h of treatment, cells were pulsed labeled with [^{3}H]-thymidine (0.5 μCi/well) (75.2 Ci/mmol; PerkinElmer Life and Analytical Sciences, Woodbridge, ON, Canada). Cells were washed twice with ice-cold PBS, once with 5% trichloroacetic acid (TCA), and twice with 95% ethanol. Cells were then solubilized by the addition of 200 μl of 0.5 M NaOH. The solubilized cell lysate (100 μl) was added to 4 ml of scintillation fluid, and the incorporation of [^{3}H]-thymidine into DNA was determined by scintillation counting. Protein concentrations in the cellular lysates were determined by the Bradford technique, and [^{3}H]-thymidine incorporation was normalized by protein content. Results were expressed as a percentage of control.

WESTERN BLOT ANALYSIS

Western blot analysis was used to determine which signal transduction pathways were activated by KGF. Briefly, 3T3-L1 and rat primary preadipocytes were plated on 24-well plates, and cultured under standard condition until they reach 40–50% confluency. Cells were starved for 24 h, and then treated with 5 ng/ml of KGF in serum-free medium. At discrete times thereafter (0–60 min), cells were lysed in SDS sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT, and 0.01% w/v bromophenol blue), and stored at −80°C.

Levels of Akt, ERK1/2, and p38 MAPK proteins in cell lysates were determined by standard Western blot analysis [Yang et al., 2008]. In brief, equal fractions of the cell lysates were subjected to a standard 12% SDS–PAGE. After electrophoresis, proteins were transferred to PVDF transfer membrane (Amersham Hybond^{TM}-P, Cat #RPN303F) using a Bio-Rad Mini Transfer Apparatus. Non-specific antibody binding was blocked with 5% (w/v) milk in TTBS (0.1% v/v Tween-20 in TBS) for 1 h at room temperature. Membranes were then hybridized with primary antibody [the Phospho-Akt Pathway Sampler Kit, Cell Signaling, Cat #9916; human phospho-p38 MAP kinase (Thr180/Tyr182), Cell Signaling, Cat #9211; human p38 MAP kinase, Cell Signaling, Cat #9212; human phospho-p44/42 MAP kinase (Thr202/Tyr204), Cell Signaling, Cat #9101; human

p44/42 MAP kinase, Cell Signaling, Cat #9102] overnight at 4°C. All primary antibodies were used at 1:1,000 dilutions. Membranes were always probed with anti-phospho-protein antibody first. They were then stripped by incubation in stripping buffer (100 mM 2-Mercaptoethanol, 2% SDS, and 62.5 mM Tris–HCl pH 6.7) for 30 min at 55°C, blocked, and re-probed with the corresponding anti-total protein antibody. After 3 × 10 min washes with TTBS, the membrane was incubated with HRP-labelled second antibody (1:2,000 dilution), and developed in ECL detection reagents. The filter was then exposed to X-ray film (Eastman Kodak, Rochester, NY) for 10 s–5 min.

siRNA-MEDIATED KNOCKDOWN OF KGF EXPRESSION

To determine if KGF functions in an autocrine fashion to stimulate preadipocyte proliferation, a siRNA-mediated knockdown approach was utilized [Sharma et al., 2009]. Briefly, 3T3-L1 cells were plated on 24-well plates and cultured under standard conditions for 48 h. Cells were then transfected with 100 nM of the Silencer Pre-designed siRNA targeting mouse KGF (Ambion, Cat #S66011) in Opti-MEM I medium (Invitrogen) containing 1 μl/well of siPORT™ Amine Transfection Agent (Ambion), following the manufacturer's instructions. Cells were also transfected in an identical manner with 100 nM of the Negative Control #1 siRNA (Ambion, Cat #4624) or with the transfection agent alone to serve as controls. At 72-h post-transfection, cells were either collected for determining levels of KGF mRNA with real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR), or pulsed with [^{3}H]-thymidine (0.5 μCi/well) for measuring [^{3}H]-thymidine incorporation, as described above.

ADIPOCYTE DIFFERENTIATION

3T3-L1 and rat primary preadipocytes were cultured and differentiated into adipocytes as described previously [Zhang et al., 2007; Yang et al., 2008]. Briefly, cells were grown in growth medium and allowed to reach confluence. At 2-day post-confluence (referred as day 0), cells were induced to differentiate by the addition of a differentiation cocktail containing 0.5 mM of 3-isobutyl-1-methylxanthine (IBMX; Sigma), 0.25 μM of dexamethasone (Alpharma, Boucherville, QC, Canada), and 1 μg/ml of insulin (Eli Lilly Canada Inc., Toronto, ON, Canada). After 48 h (day 2), the medium was replaced with growth medium supplemented with 1 μg/ml of insulin. Subsequently, medium was changed at days 4 and 6 with fresh growth medium. By day 8, greater than 90% of cells had acquired adipocyte phenotype (i.e., containing lipid droplets). To study the effects of KGF on adipocyte differentiation, 3T3-L1 preadipocytes were subjected to a variety of treatment regimes. They include treatment with KGF: (a) in the standard growth medium without the differentiation cocktail; (b) using charcoal-stripped serum (Invitrogen) without the differentiation cocktail; (c) in the presence of IBMX and dexamethasone; and (d) in the presence of the standard differentiation cocktail. At day 8, differentiation was assessed by Oil red O staining and subsequent quantitation of lipid droplets, and by determining levels of mRNAs encoding the key adipogenic transcription factor peroxisome proliferator-activated receptor γ (PPARγ) and adipocyte marker protein aP2, as described below.

OIL RED O STAINING

Oil red O staining was performed, as described previously [Zhang et al., 2007]. Briefly, differentiated adipocyte monolayers were washed with DPBS, fixed for 1 h with 4% paraformaldehyde at room temperature and incubated in 60% isopropanol for 5 min. Oil red O (3 g/l; Sigma) in 99% isopropanol was diluted with water, filtered, and added to the fixed cell monolayers for 5 min, and then nuclei were stained with hematoxylin for 30 s. Cell monolayers were then washed with water and the stained triglyceride droplets were visualized and photographed. The extent of adipocyte differentiation was quantitated by determining the amount of extracted dye, as measured by the optimal absorbance at 510 nM following elution of Oil red O with isopropanol [Ramirez-Zacarias et al., 1992].

ANALYSIS OF KGF AND KGF RECEPTOR EXPRESSION—RT-PCR

Expression of KGF and its receptors was analyzed by standard RT-PCR [Yang et al., 2008]. Briefly, total RNA was isolated from freshly isolated rat mature adipocytes and preadipocytes as well as 3T3-L1 cells using TRIZol Reagent (Invitrogen), and subsequently purified by RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada) coupled with on-column DNase digestion with the RNase-Free DNase Set (Qiagen) according to the manufacturer's instructions [Guan and Yang, 2008]. One microgram of total RNA was reverse transcribed in a volume of 20 μl with the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA), following the manufacturer's instructions. For every RT reaction, one RNA sample was set up without reverse transcriptase enzyme to provide a negative control against possible genomic DNA contamination. PCR reactions were carried out in a total volume of 50 μl containing 1 μl of RT and 1.5 U of Platium Taq DNA polymerase (Invitrogen). The primers specific for rat (which were conserved between the rat and mouse) KGF and FGFR2 as well as GAPDH and 28S rRNA (internal controls) are shown in Table I. PCR reactions were performed for various cycles (28 for KGF; 35 for FGFR2; 26 for GAPDH, and 26 for 28S rRNA using 1:500 diluted cDNA) with denaturing at 95°C, annealing at 58°C, and extension at 72°C. PCR products were confirmed with standard restriction enzyme digestions and sequencing analysis. PCR products for FGFR2 were also subjected to restriction endonuclease digestion with either *Ava* I or *Eco*R V (Invotrogen) according to the manufacturer's recommendations. Analysis of undigested and digested PCR products was done by electrophoresis on 8% polyacrylamide non-denaturing gels.

TABLE I. Primers for RT-PCR and qRT-PCR

Gene	Primer sequence
KGF	Forward: 5′-CTGCTCTATAATGCGCAAATGG Reverse: 5′-GAGGTGGAAGCACGGTCTGT
GAPDH	Forward: 5′-ACCACAGTCCATGCCATCAC Reverse: 5′-TCCACCACCCTGTTGCTGTA
FGFR2	Forward: 5′-CCCGGGTCTAGATTTATAGTGATGCCCAGCCC Reverse: 5′-CCCGGGGAATTCACCACCATGCAGGCGATTAA
aP2	Forward: 5′-CTTGGCCATATTTATAGCTGTCATTATT Reverse: 5′-TGTCCTCGATGGGCTTCAC
PPARγ	Forward: 5′-CCCCAGTCGCAAGATCCTT Reverse: 5′-GGGCGTGCAGAGGATTCA
28S rRNA	Forward: 5′-GAATCCGCTAGGAGTGTGTAACAA Reverse: 5′-GCTCCAGCGCCATCCAT

REAL-TIME QUANTITATIVE RT-PCR (qRT-PCR)

Expression of KGF and the key adipocyte markers adipocyte fatty acid binding protein (aP2) and PPARγ was analyzed by a two-step real-time qRT-PCR, as described previously [Yang et al., 2008]. Briefly, 1 μg of total RNA was reverse transcribed in a volume of 20 μl with the High Capacity cDNA Archive Kit (Applied Biosystems), following the manufacturer's instructions. For every RT reaction, one RNA sample was set up without reverse transcriptase enzyme to provide a negative control against possible genomic DNA contamination. Gene-specific primers were designed by using Primer Express software (Applied Biosystems), and the optimal concentrations for each gene were determined empirically. The primers used are listed in Table I. The SYBR Green I assay was performed with the SYBP Green PCR Master Mix (Applied Biosystems) and a modified universal thermal cycling condition (2 min at 50°C and 10 min at 95°C, following by 40 cycles of 10 s each at 95, 60, and 72°C) with the standard disassociation/melting parameters (15 s each at 95, 60, and 95°C) on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The specificity of the SYBR Green I assay was verified by performing a melting curve analysis and by subsequent sequencing of the PCR products.

Levels of 28S rRNA (housekeeping gene) and target mRNAs in each RNA sample were quantified by the relative standard curve method (Applied Biosystems). Briefly, standard curves for 28S rRNA and each target gene were generated by performing a dilution series of a mixed cDNA pool. For each RNA sample, the amount of target mRNA relative to that of 28S rRNA was obtained. For each target gene, fold changes in the treatment groups compared with the control were then calculated, and expressed as mean ± SEM.

STATISTICAL ANALYZES

Results are presented as mean ± SEM of four to six independent experiments. Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test, or Student's *t*-test when two sets of data were compared. Significance was set at $P < 0.05$. Calculations were performed using Prism 3.0 GraphPad software (San Diego, CA).

RESULTS

KGF STIMULATED PREADIPOCYTE PROLIFERATION

Given that enhanced adipogenesis is a hallmark of increased adiposity and KGF is known to stimulate epithelial cell proliferation and differentiation in a variety of organ systems, we first determined if KGF stimulated preadipocyte proliferation. As shown in Figure 1A, treatment of 3T3-L1 cells with recombinant KGF led to a concentration-dependent increase in [^{3}H]-thymidine incorporation with a maximal effect at 2.5 ng/ml (198 ± 9% of control; $P < 0.01$). To validate the physiological relevance of these observations, the ability of KGF to stimulate proliferation in rat primary preadipocytes was examined, and virtually identical results were obtained (Fig. 1B).

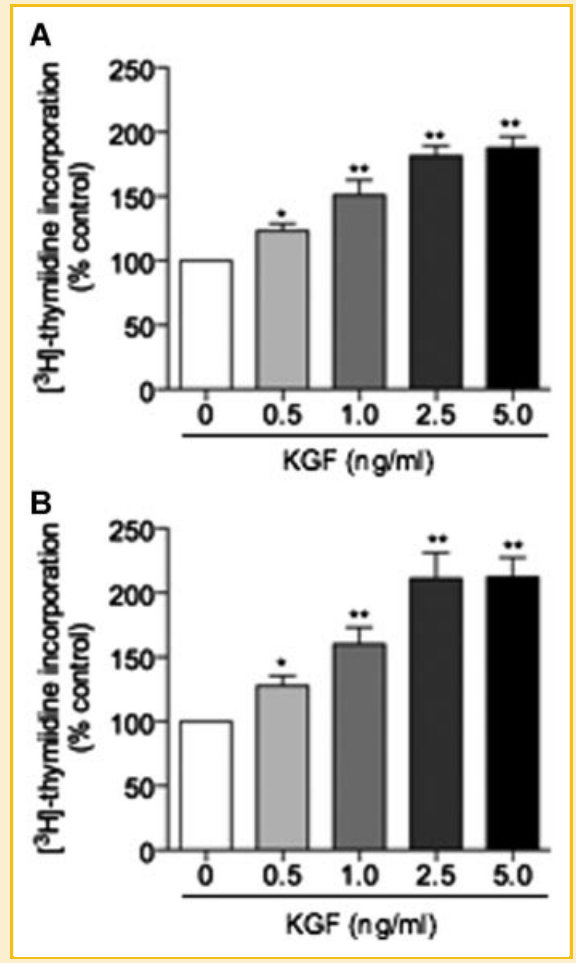

Fig. 1. KGF stimulation of preadipocyte proliferation. 3T3-L1 cells (A) and rat preadipocytes (B) were treated with increasing concentrations of KGF (0.1–5 ng/ml) for 24 h. During the last 4 h of treatment, cells were pulsed with [^{3}H]-thymidine (0.5 μCi/well), and the rate of [^{3}H]-thymidine incorporation was determined. Data are presented as means ± SEM of five independent experiments each performed in triplicate ($^*P < 0.05$; $^{**}P < 0.01$ vs. control).

KGF STIMULATED PREADIPOCYTE PROLIFERATION VIA AN AUTOCRINE MECHANISM

KGF is produced in mesenchymal cells and acts in a paracrine fashion to stimulate epithelial cell proliferation [Finch and Rubin, 2004]. To determine if KGF promoted preadipocyte proliferation via a similar mechanism, we first studied the expression of KGF in freshly isolated rat mature adipocytes and preadipocytes as well as cultured rat preadipocytes and 3T3-L1 cells. Robust expression of KGF mRNA was demonstrated in all the cell types examined (Fig. 2A), suggesting that KGF stimulated preadipocyte proliferation through an autocrine and/or paracrine mechanism.

To ascertain if KGF stimulated preadipocyte proliferation in an autocrine fashion, 3T3-L1 preadipocytes were incubated with a neutralizing antibody against KGF at various concentrations (0.01–1 μg/ml). As shown in Figure 2B, the anti-KGF antibody decreased [^{3}H]-thymidine incorporation in a concentration-dependent manner under basal (i.e., non-stimulating) conditions, such that at 1 μg/ml, the neutralizing antibody reduced [^{3}H]-thymidine incorporation to 54% of the control. In contract, a normal mouse IgG had no effect (Fig. 2B). Similar results were obtained in rat primary preadipocytes (data not shown).

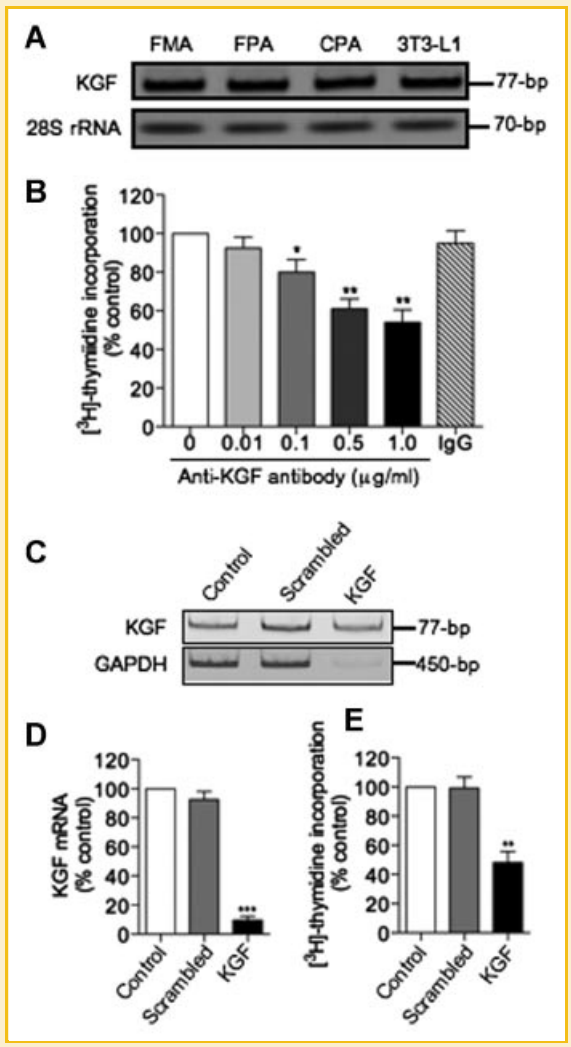

Fig. 2. A: KGF mRNA expression in adipose cells. Total RNA was isolated from freshly isolated rat mature adipocytes (FMA), freshly isolated and cultured rat preadipocytes (FPA and CPA), as well as 3T3-L1 cells, and KGF mRNA and GAPDH mRNA (served as an internal control) were assessed by standard RT-PCR. B: Anti-KGF antibody inhibition of preadipocyte proliferation. 3T3-L1 cells were treated for 24 h with increasing concentrations of an anti-KGF antibody (0.01–1 μg/ml) or with 1 μg/ml of a normal mouse IgG (served as a control) in serum-free medium. During the last 4 h of treatment, cells were pulsed with [^{3}H]-thymidine (0.5 μCi/well), and the rate of [^{3}H]-thymidine incorporation was determined. C–E: Effects of siRNA-mediated knockdown of KGF expression on preadipocyte proliferation. 3T3-L1 cells were transfected with 100 nM of KGF siRNA, or 100 nM of a negative control (i.e., scrambled) siRNA. Seventy-two hours after transfection, levels of KGF mRNA were determined by standard RT-PCR (C: GAPDH mRNA was used as a control to show the specificity of siRNA-mediated knockdown of KGF) and qRT-PCR (D), and the rate of proliferation (E) was assessed by standard [^{3}H]-thymidine incorporation assays. Data are presented as means ± SEM of five independent experiments each performed in triplicate ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. control).

To provide further evidence for an autocrine mode of actions of KGF in preadipocytes, we studied the effect of siRNA-mediated knockdown of KGF expression on preadipocyte proliferation. As shown in Figure 2C,D, transient transfection of 3T3-L1 cells with KGF-specific siRNA resulted in over 90% reduction in the level of KGF mRNA without altering levels of GAPDH mRNA. Furthermore, transfection with a negative control siRNA (i.e., scrambled siRNA) did not affect levels of either KGF mRNA or GAPDH mRNA, demonstrating the specificity of the siRNA-mediated down-regulation of KGF expression in 3T3-L1 cells. Concomitant with the diminished expression of KGF, there was a ~50% reduction in the rate of [^{3}H]-thymidine incorporation in cells transfected with KGF siRNA. In contrast, transfection with the negative control siRNA had no effect on [^{3}H]-thymidine incorporation (Fig. 2E).

KGF RECEPTOR WAS NOT EXPRESSED IN ADIPOSE CELLS

To determine if the mitogenic effects of KGF were mediated through the KGF receptor (KGFR; also known as FGFR2-IIIb), the relative expression of mRNAs encoding FGFR2-IIIb and FGFR2-IIIc in adipose cells (freshly isolated rat mature adipocytes and preadipocytes as well as cultured rat preadipocytes and 3T3-L1 cells) and lung (served as a positive control) was investigated by RT-PCR using primers specific for the exons upstream and downstream of the alternative exons IIIb and IIIc, as shown in Figure 3A. Following PCR, these samples were subjected to restriction endonuclease digestion with either *Ava* I or *Eco*R V. As shown in Figure 3A, there is a single *Ava* I site in exon IIIb but none in exon IIIc, whereas IIIc contains a single *Eco*R V site that is not present in IIIb. Thus, PCR products containing IIIb are 343 bp in length, which is not digested by *Eco*R V but yields two fragments of 239 and 104 bp upon digestion with *Ava* I. The IIIc containing PCR products are 349 bp in length that is not cut by *Ava* I but gives rise to 211- and 138-bp fragments following digestion with *Eco*R V.

Using this strategy, we noted that the rat lung, which is known to express both FGFR2-IIIb and FGFR2-IIIc [Shiratori et al., 1996], expressed predominantly the IIIb isoform, because most of the PCR products were cut by *Ava* I and a small portion was digested by *Eco*R V (Fig. 3C,D). However, none of the PCR products from adipose cells were cut by *Ava* I, while all of them were completely digested by *Eco*R V (Fig. 3C,D), suggesting that FGFR2-IIIc, but not FGFR2-IIIb (i.e., KGFR), and mRNA was expressed in adipose cells.

THE PI3 KINASE SIGNALING PATHWAY MEDIATED KGF STIMULATION OF PREADIPOCYTE PROLIFERATION

Given that KGF stimulates epithelial cell proliferation through the activation of ERK1/2 [Liang et al., 1998; Gillis et al., 1999; Zeigler et al., 1999; Sharma et al., 2003; Taniguchi et al., 2003], p38 MAPK [Mehta et al., 2001; Sharma et al., 2003], and/or PI3K pathways [Chandrasekher et al., 2001; Ray et al., 2003; Portnoy et al., 2004; Bao et al., 2005], we investigated the signal transduction pathway involved in mediating the mitogenic effects of KGF on preadipocytes with the use of specific and well-established pharmacological inhibitors. The compounds were U0126 (ERK1/2 inhibitor), LY294002 (PI3-kinase inhibitor), and SB203580 (p38 MAPK inhibitor). Various concentrations of each inhibitor were tested, and results revealed that U0126 at 2 μM, LY294002 at 0.5 μM, and SB203580 at 10 μM did not alter 3T3-L1 cell and rat preadipocyte viability (data not shown). Consequently, these concentrations were used in subsequent experiments. As shown in Figure 4A, pretreatment of 3T3-L1 cells with LY294002, but not U0126 or SB203580, prevented KGF-induced increases in [^{3}H]-thymidine incorporation,

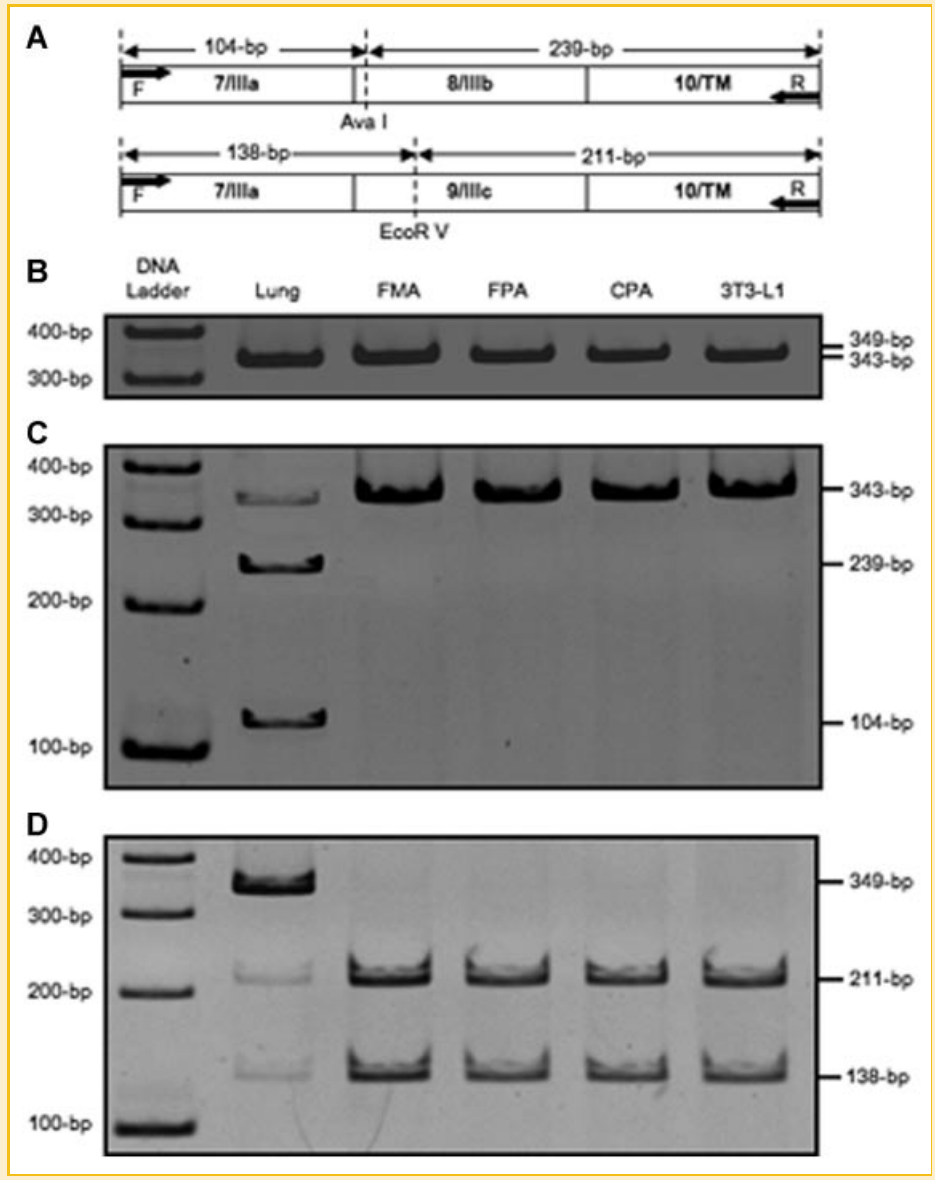

Fig. 3. FGF receptor type 2 (FGFR2) mRNA expression in adipose cells. A: The maps of the RT-PCR products using primers (solid arrows; F, forward primer; R, reverse primer) for both IIIb and IIIc containing isoforms of FGFR2. Locations of *Ava* I and *Eco*R V restriction sites are indicated, as are the sizes of the resulting fragments following digestion. Total RNA was prepared from rat lung (served as a positive control), freshly isolated rat mature adipocytes (FMA), freshly isolated and cultured rat preadipocytes (FPA and CPA) as well as 3T3-L1 cells. One microgram of total RNA was subjected to standard RT-PCR, and the resulting products were resolved using polyacylamide gel electrophoresis. B: Undigested FGFR2 RT-PCR products; C: *Ava* I-digested FGFR2 RT-PCR products to show the expression of FGFR2-IIIb by the presence of 239- and 104-bp fragments; and D: *Eco*R V-digested FGFR2 RT-PCR products to show the expression of FGFR2-IIIc by the presence of 211- and 138-bp fragments.

suggesting that the PI3 kinase signaling pathway mediated KGF stimulation of preadipocyte proliferation.

To provide direct evidence for this contention, we determined whether KGF activated the protein kinase Akt, a downstream target of PI3K. As shown in Figure 4B, KGF induced robust activation of the protein kinase Akt, as evidence by elevated levels of phosphorylated Akt protein in 3T3-L1 cells, while it did not alter levels of total Akt protein. In contrast, KGF did not alter levels of phosphorylated ERK1/2 proteins or p38 MAPK protein (Fig. 4B). A similar strategy was used to examine the signal transduction pathway involved in mediating KGF stimulation of rat primary preadipocyte proliferation, and similar results were obtained (data not shown).

KGF DID NOT ACCELERATE PREADIPOCYTE DIFFERENTIATION

Having established a role for KGF in promoting preadipocyte proliferation, we then studied its effects on adipocyte differentiation. To ascertain if exogenous KGF accelerated preadipocyte differentiation, 3T3-L1 cells were exposed to various concentrations of KGF (known to stimulate preadipocyte proliferation as shown in Fig. 1) under a variety of conditions. KGF was added to the medium from the start of differentiation (day 0) to day 4. At day 8,

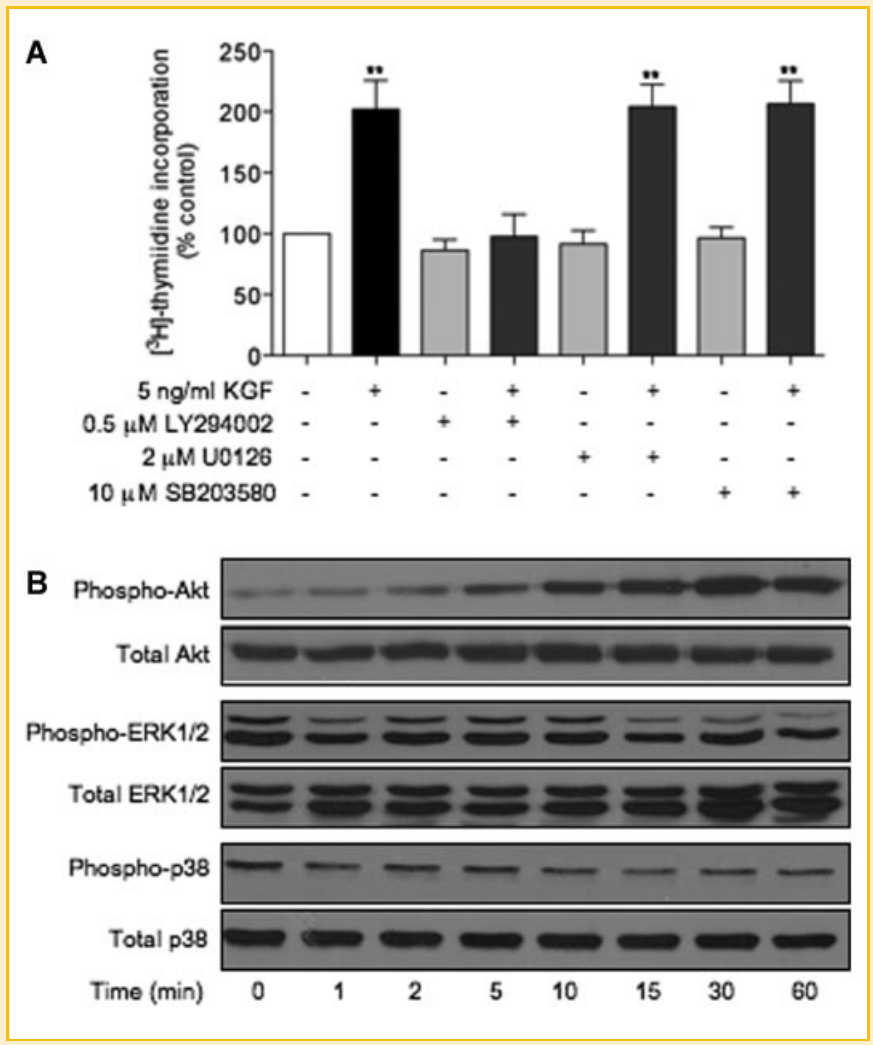

Fig. 4. A: Effects of PI3K, ERK1/2, and p38 MAPK inhibitors on KGF-induced preadipocyte proliferation. 3T3-L1 cells were pre-treated for 1 h with LY294002 (PI3K inhibitor; 0.5 μM), U1026 (ERK1/2 inhibitor; 0.2 μM), or SB203580 (p38 MAPK inhibitor; 10 μM), followed by the addition of KGF (5 ng/ml) for 28 h in serum-free medium. During the last 4 h of treatment, cells were pulsed with [^{3}H]-thymidine (0.5 μCi/well), and the rate of [^{3}H]-thymidine incorporation was determined. Data are presented as means ± SEM of five independent experiments each performed in triplicate ($^*P < 0.05$; $^{**}P < 0.01$ vs. control). B: KGF activation of the protein kinase Akt but not ERK1/2 or p38 MAPK. 3T3-L1 cells were treated with 5 ng/ml KGF in serum-free medium. At indicated times after the addition of KGF, cell lysates were prepared and subjected to standard Western blot analysis with antibodies specific for phosphorylated Akt and total Akt, phosphorylated ERK1/2 and total ERK1/2, and phosphorylated p38 MAPK and total p38 MAPK proteins, respectively. Results of a representative Western blotting are shown.

differentiation was assessed using both morphological (i.e., Oil red O staining) and biochemical (i.e., expression of key adipocyte markers) methods. As shown in Figure 5A,B, KGF did not influence lipid accumulation under any treatment conditions. This was consistent with the lack of an effect of KGF on levels of mRNAs encoding the key adipocyte marker proteins aP2 (Fig. 5C) and PPARγ (Fig. 5D). We also determined if KGF mRNA expression might be increased during preadipocyte differentiation in vitro. To do so, 3T3-L1 cells were subjected to a standard differentiation protocol, and KGF mRNA was measured at discrete time points (days 0, 2, 5, and 8). As shown in Figure 5E, the level of KGF mRNA remained constant during 3T3-L1 preadipocyte differentiation. The same set of experiments was also performed in rat primary preadipocytes, and virtually identical results were obtained (data not shown).

DISCUSSION

The present study examined the role and the molecular mechanisms of KGF in adipogenesis using 3T3-L1 and rat primary preadipocytes as in vitro model systems. Our results revealed that KGF is produced by rat mature adipocytes and preadipocytes as well as 3T3-L1 cells. Importantly, we demonstrated that KGF stimulates preadipocyte proliferation, but not differentiation, via an autocrine mechanism. This is in marked contrast to the well-established paracrine mode of KGF actions in epithelial cells [Finch and Rubin, 2004]. Furthermore, we provided evidence suggesting that KGF stimulation of preadipocyte proliferation is likely mediated through a non-classical KGFR and involves activation of the PI3K-Akt signaling pathway.

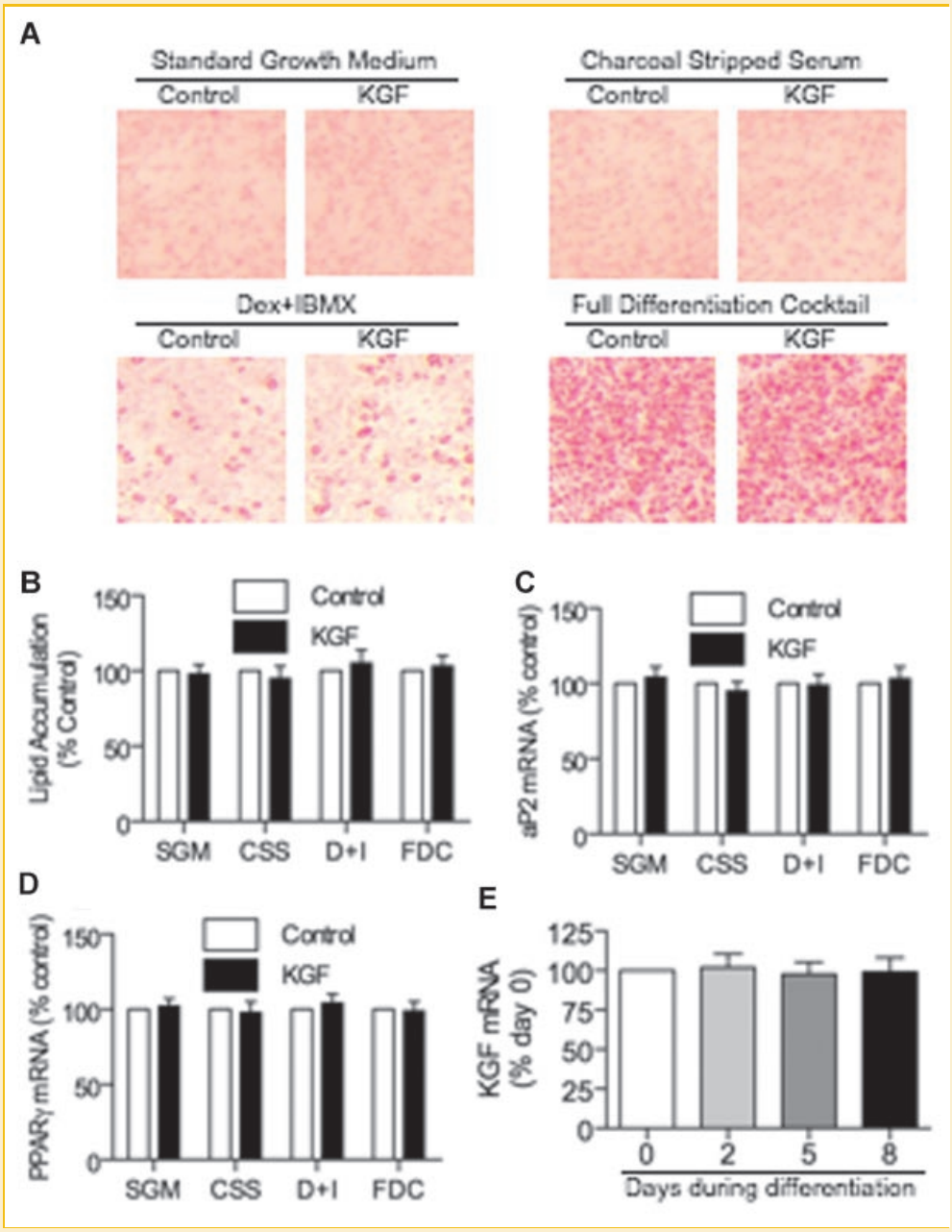

Fig. 5. Effects of KGF on preadipocyte differentiation. 3T3-L1 cells were cultured in standard growth medium (10% FBS). At 2-day post-confluence (day 0), cells were treated for 4 days with or without 5 ng/ml KGF in standard growth medium (SGM), growth medium containing charcoal stripped serum (CSS), in the presence of 0.25 μM dexamethasone and 500 μM IBMX (D + I), or with full differentiation cocktail (FDC) containing 0.25 μM dexamethasone, 500 μM IBMX, and 1 μg/mL insulin. At days 4 and 6, medium was replaced with standard growth medium. At day 8, differentiation was assessed by Oil red O staining (A) followed by quantitation of lipid accumulation as measured by the optimal absorbance at 510 nM (B), and analysis of mRNAs encoding aP2 (C) and PPARγ (D) with qRT-PCR. Data are presented as mean ± SEM of five independent experiments. Temporal profile of KGF mRNA expression during differentiation of preadipocytes to mature adipocytes (E). 3T3-L1 cells were cultured and differentiated under standard conditions. At discrete times during differentiation (days 0, 2, 5, 8), levels of KGF mRNA were determined by qRT-PCR. Data are presented as mean ± SEM of five independent experiments.

KGF is a well-known paracrine growth factor that is produced by mesenchymal cells and stimulates epithelial cell proliferation and differentiation [Rubin et al., 1995; Finch and Rubin, 2004]. Thus, KGF plays an important role in tissue regeneration/repair and wound healing [Finch and Rubin, 2004]. Although KGF mRNA is expressed in human and rat adipose tissue [Gabrielsson et al., 2002; Guan et al., 2005], and its expression is up-regulated in our early-life programmed rat model of increased visceral adiposity [Guan et al., 2005], the role of KGF in adipose tissue remains virtually unknown. Given that increased adipogenesis is a hallmark of obesity but the causal factors and the underlying molecular mechanisms are poorly understood [Gregoire et al., 1998; Otto and Lane, 2005; Rosen and MacDougald, 2006], we hypothesized that KGF stimulated adipogenesis. As a first step in examining this hypothesis, we determined

if KGF increased DNA synthesis, a widely used quantitative proxy for cell proliferation, in both 3T3-L1 and rat primary preadipcytes. Our results revealed that KGF was a novel mitogen for preadipocytes. In addition, the concentrations of KGF used in our study were comparable to those reported in human circulation [Verghese et al., 1998], thus suggesting that KGF may stimulate preadipocyte proliferation in vivo.

There is evidence that KGF mRNA is present in the stromal–vascular fraction of human adipose tissue [Gabrielsson et al., 2002], which contains different cell types, including preadipocytes, endothelial cells, smooth muscle cells, and macrophages. Both microvascular endothelial cells [Smola et al., 1993] and smooth muscle cells [Koji et al., 1994; Winkles et al., 1997] are known to produce KGF, suggesting that KGF may stimulate preadipocyte proliferation in a paracrine fashion. To define the mode of KGF actions in preadipocytes, we first determined if preadipocytes and/or adipocytes produced KGF. Our results showed that KGF mRNA was highly expressed in freshly isolated rat adipocytes and preadipocytes as well as 3T3-L1 cells, suggesting that KGF may stimulate preadipocyte proliferation through an autocrine and/or paracrine mechanism. To ascertain if KGF functioned in an autocrine manner, we studied the effects of a neutralizing antibody against KGF on preadipocyte proliferation. We demonstrated that treatment with the KGF antibody led to reduced DNA synthesis, indicating that endogenous KGF exerted mitogenic effects on preadipocytes. Importantly, siRNA-mediated knockdown of KGF expression resulted in a similar decrease in DNA synthesis. Taken together, our data suggested that KGF functioned in an autocrine fashion to stimulate preadipocyte proliferation.

KGF is a 28 kDa member of the fiborblast growth factor family, which modulate cellular activity via four FGF receptors (FGFR1 to FGFR4) that comprise an extracellular domain, which consists of two or three immunoglobulin-like (Ig) loops, a transmembrane segment and an intracellular domain. Ligand-binding specificity is conferred by the second and third Ig-loop. For FGFR1 to FGFR3, the third Ig loop is encoded by two exons, an invariant exon termed IIIa and an alternatively spliced exon, termed IIIb and IIIc, respectively [Ornitz and Itoh, 2001]. This alternative-splicing event generates two receptor isoforms with quite different ligand-binding specificities. KGF is distinct from most other FGF family members by only signaling through FGFR2-IIIb (also called KGFR) [Emoto et al., 1997], although one study reported that KGF enhanced endothelial cell (derived from small vessels) proliferation through an unknown receptor [Gillis et al., 1999]. To determine if the effects of KGF on preadipocytes were mediated through KGFR/FGFR2-IIIb, the relative expression of mRNA encoding FGFR2-IIIb and FGFR2-IIIc was investigated in freshly isolated rat mature adipocytes and rat preadipocytes as well as 3T3-L1 cells using RT-PCR coupled with restriction digestion. Although FGFR2-IIIb mRNA was readily detectable in rat lung (served as a positive control), it was undetectable in all the adipose cells studied. However, abundant FGFR2-IIIc mRNA was detected in both mature adipocytes and preadipocytes. It is known that besides the four high-affinity tyrosine kinase receptors (FGFR1 to FGFR4), FGFs can also act via a class of low-affinity receptors (i.e., the heparin sulfate proteoglycans) [Roghani and Moscatelli, 1992; Rusnati et al., 1993]. However, the mitogenic effects of KGF have so far been associated only with interactions with high affinity receptors [Gillis et al., 1999]. Therefore, our present results suggested that KGF stimulated preadipocyte proliferation through an unknown receptor rather than KGFR, although it remains possible that FGFR2-IIIc may be involved. Obviously, the receptors responsible for mediating the mitogenic effects of KGF on preadipocytes await future investigation.

KGF stimulates epithelial cell proliferation by activating ERK1/2 [Liang et al., 1998; Gillis et al., 1999; Zeigler et al., 1999; Sharma et al., 2003; Taniguchi et al., 2003], p38 MAPK [Mehta et al., 2001; Sharma et al., 2003], and/or PI3K pathways [Chandrasekher et al., 2001; Ray et al., 2003; Portnoy et al., 2004; Bao et al., 2005]. Therefore, these three signal transduction pathways were targeted in our studies with the use of pharmacological inhibitors. In an attempt to exclude the possibility of a toxic effect of these inhibitors, we first tested different concentrations of each inhibitor on 3T3-L1 cells and rat primary preadipocytes, and chose optimal concentrations at which inhibitors did not affect cell viability. Using the non-toxic concentrations, we showed that LY294002, a PI3K inhibitor, blocked the KGF-induced preadipocyte proliferation. In contrast, both the ERK1/2 inhibitor U0126 and the p38 MAPK inhibitor SB203580 were ineffective. Moreover, we demonstrated that KGF activated the protein kinase Akt, a downstream target of PI3 kinase, but not ERK1/2 or p38 MAPK. Collectively, these results suggested that the mitogenic effect of KGF on preadipocytes was mediated by the PI3K-Akt signaling pathway.

Adipogenesis involves preadipocyte proliferation and differentiation. The second objective of our study was to determine if KGF also stimulated adipocyte differentiation. Using a variety of treatment paradigms, we showed that the addition of KGF did not alter either the amount of lipid accumulation or expression levels of the two key adipocyte marker genes aP2 and PPARγ, indicating that KGF had no effect on adipocyte differentiation. This was consistent with the lack of a change in levels of KGF mRNA expression throughout both 3T3-L1 and rat primary preadipocyte differentiation.

Recently, it has been shown that adipocyte number is a major determinant of fat mass in adult humans [Spalding et al., 2008]. Furthermore, adipocytes have a high turnover rate: approximately 10% new adipocytes per year are continually being generated to replace their dead predecessors. The average age of an adipocyte is about 10 years, and the number of adipocytes stays constant in adulthood in lean and obese individuals, even after weight loss. However, obese individuals have a greater number of adipocytes added per year than lean individuals, suggesting that adipocytes are replenished from a larger pool of preadipocytes in obese state. Thus, our present findings would suggest that increased KGF expression within adipose tissue might contribute to the pathogenesis of obesity by augmenting the number of preadipocytes, and represents a potential therapeutic target to combat the obesity epidemic.

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