# Ceiling culture of mature human adipocytes: use in studies of adipocyte functions

**H H Zhang, S Kumar, A H Barnett** and **M C Eggo**

Department of Medicine, Institute of Medical Sciences, University of Birmingham, Birmingham B15 2TH, UK

(Requests for offprints should be addressed to M C Eggo, Department of Medicine, Queen Elizabeth Hospital, Birmingham B15 2TH, UK; Email: eggomc@novell5.bham.ac.uk)

## Abstract

Adipocytes contain large lipid droplets in their cytoplasm. When cultured, they float on top of the medium, clump together, and do not gain equal and sufficient access to the medium. Morphological changes cannot be observed and the majority of adipocytes undergo cell lysis within 72 h of isolation. We have used a ceiling culture method for human mature adipocytes which uses their buoyant property to allow them to adhere to a floating glass surface, where they remain viable for several weeks. Using confocal immunofluorescence microscopy we showed the cellular expression and subcellular localization of leptin in ceiling-cultured adipocytes. The secretion of leptin was increased from ceiling cultures following tumour necrosis factor-α treatment. Proliferation of mature human adipocytes in serum-containing medium was demonstrated by incorporation of bromodeoxyuridine, 2% of adipocytes showing positive incorporation after 4 h labelling. Proliferation was also evident from the budding of daughter cells. Apoptosis in the ceiling cultures was increased by 48 h serum deprivation (30–35 vs 10–15% in the control) and was assayed by propidium iodide staining and terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick-end labelling. Lipolysis, analysed by liquid scintillation counting, was increased by forskolin (10 μM for 90 min) and lipogenesis, shown by autoradiography, was stimulated by insulin (10 and 100 nM for 4 h). These findings indicate that ceiling-cultured adipocytes maintain adipocyte-specific functions and that ceiling culture, which overcomes the shortcomings of adipocyte suspension culture, can be used to study adipocyte cell biology.
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## Introduction

Owing to their high lipid content, adipocytes in culture clump, float on top of the medium, and therefore do not have equal and sufficient access to nutrients and treatments. Morphological changes cannot be observed. The majority of adipocytes undergo cell lysis within 72 h of incubation. Because of these difficulties, certain murine cell lines, such as 3T3-L1 and 3T3-F442A, which can be induced to differentiate, have been used as cell culture models to study adipocytes. However, differentiated 3T3 cells, characterized by multiple lipid droplets in the cytoplasm, do not resemble mature fat cells, which typically have a single large lipid droplet, and functionally they produce only 1–2% of the leptin of mature adipocytes (Flier 1997). Furthermore, these cell lines are immortalized aneuploid cells whose genetic regulation may be distorted (Moustaid *et al.* 1996). The establishment of a robust human primary cell culture method that can circumvent the unique buoyancy and fragility of mature human adipocytes is therefore important to facilitate the study of adipocyte cell biology.

Ceiling culture is a method that uses the buoyant property of adipocytes by allowing them to adhere to the top inner surface of a culture flask which is filled completely with medium (Sugihara *et al.* 1986). Despite its advantage over suspension culture in providing adipocytes with an attachment surface, the method has received little attention a decade after its publication. We have modified the method by culturing adipocytes underneath a floating glass surface. The modified method is easier to execute and enables adipocyte studies employing (confocal) fluorescence microscopy.

Adipocytes function as energy stores by reserving the body's excess energy in the form of triacylglycerol. The cellular triacylglycerol store, which reflects adipocyte cell size, is determined by the continuous processes of lipogenesis and lipolysis. Lipogenesis and lipolysis are regulated by multiple factors such as insulin, which stimulates triacylglycerol synthesis (Sooranna & Saggerson 1975), and forskolin, an activator of adenylyl cyclase, which increases cAMP accumulation and lipolysis (Litosch *et al.* 1982). To assess the functional responsiveness of ceiling-cultured adipocytes to hormonal treatment, we have examined the


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regulation of lipogenesis by insulin and of lipolysis by forskolin. Adipocytes also function to regulate energy metabolism by producing cytokines such as leptin (Zhang *et al.* 1994) and tumour necrosis factor-α (TNF-α) (Hotamisligil *et al.* 1993, Kern *et al.* 1995), which exert neuroendocrine as well as paracrine/autocrine effects to limit adipose tissue mass. To examine whether ceiling-cultured adipocytes maintain adipocyte-specific functions, we have studied the expression and subcellular localization of leptin, and the regulation of leptin secretion by TNF-α.

Adipocyte cell number in a given adipose depot is regulated by multiple mechanisms such as adipocyte apoptosis and possibly, adipocyte proliferation. Adipocyte apoptosis has been studied in recent years but only with 3T3-L1 murine cell lines (Magun *et al.* 1998) and cultured tissue explants (Prins *et al.* 1997) because culture of mature adipocytes *in vitro* has been difficult. Adipocytes are at the terminal stage of differentiation and have hitherto been assumed to lack proliferative ability (Prins & O'Rahilly 1997). However, research on adipocyte proliferation has produced opposite findings (Klyde & Hirsch 1979*a*,*b*, Miller *et al.* 1984, Sugihara *et al.* 1987), which warrant further investigation. To assess the responsiveness of ceiling-cultured adipocytes to known apoptosis/proliferation inducers, we have examined adipocyte proliferation and apoptosis under serum and serum-deprived conditions.

## Materials and Methods

### *Isolation and culture of adipocytes*

Subcutaneous adipose tissue was obtained from patients undergoing elective abdominal surgery in accordance with the guidelines of the local ethical committee. Adipocytes were isolated by the method of Rodbell (1964) with modifications. Adipose tissue was promptly washed with Hank's balanced salt solution (HBSS; Gibco-BRL Life Technology, Paisley, Strathclyde, UK), and visible blood vessels were removed. Finely cut tissue pieces were digested with type I collagenase (Worthington Biochemical Corporation, Freehold, NJ, USA), 1 mg/ml in HBSS, for 1 h at 37 °C in a water bath, shaken at 100 cycles/min. The disrupted tissue was filtered through a double-layered cotton mesh and cells were washed four times with DMEM:F12 (Gibco-BRL) by centrifugation at 250 **g** for 5 min. The upper floating layer of cells was taken. To culture cells on a plastic surface, 200 μl ($\sim 4 \times 10^5$ cells) of packed adipocytes were cultured in a T25 $cm^2$ flask which was completely filled with DMEM:F12 containing 20% new-born bovine calf serum (NCS). Before the incubation, the flask containing medium was incubated unstoppered in a 5% $CO_2$ incubator at 37 °C for a minimum of 4–6 h to allow equilibration. All gas bubbles were removed from the flask. With the modified ceiling culture method, 50 μl ($\sim 10^5$ cells) of packed mature fat cells were inoculated into 2 ml DMEM:F12 with 20% NCS in a six-well plate (9·6 $cm^2$; Nalge Nunc International, Rochester, NY, USA) and covered with a sterile 20 × 20 mm coverslip. Cells were incubated in a 5% $CO_2$ incubator at 37 °C for 10–14 days depending on their attachment efficiency.

### *Lipid staining of adipocytes*

Lipid staining was performed using the method described by Culling (1963). Briefly, cells were washed with HBSS, fixed with 10% formaldehyde (Sigma Chemical Co., Poole, Dorset, UK) for 30 min at 4 °C and stained with 2·5% Oil Red O (Gurr Ltd, London, UK) for 15 min at room temperature. After brief treatment with 60% isopropanol (Fisher Scientific UK Ltd, Loughborough, UK) at room temperature, cell nuclei were stained with haematoxylin (Mayer; Raymond A Lamb, London, UK) for 30 s. Cells were washed with distilled water and viewed under a light microscope.

### *The expression of leptin*

Fourteen-day-old adipocytes attached to the culture coverslips were washed with HBSS and incubated in DMEM:F12 for 24 h. Following incubation, the coverslips were air-dried for 1 h. Cells were fixed with cold acetone (Fisher) for 5 min, washed twice with PBS (120 mM NaCl, 2·7 mM KCl, 10 mM sodium phosphate, pH 7·6) for 5 min and blocked with 10% BSA in PBS at room temperature for 30 min. After removing excess blocking solution, cells were incubated for 30 min with anti-leptin IgG (0·3 mg/ml in PBS), which was obtained from sheep injected with a 15 amino acid peptide from the N-terminus of leptin (The Binding Site, Birmingham, UK). The antibody was omitted in negative control samples. Competitive binding experiments in which adipocytes were incubated with an excess of recombinant human leptin (R & D Systems, Abingdon, Oxon, UK) and the antibody were also performed and these showed staining equivalent to that of the negative control samples. Following incubation with or without anti-leptin antibody, adipocytes were washed with PBS twice for 5 min and further incubated with fluorescein isothiocyanate (FITC)-conjugated donkey anti-sheep IgG (1:50 dilution with PBS; The Binding Site) for 30 min. After washing with PBS twice for 5–10 min, cell nuclei were stained with propidium iodide (Sigma) at 0·25 μg/ml in PBS for 30 s and washed with PBS twice for 5 min. After brief drying to remove excess PBS solution, 2·5% 1,4-diazabicyclo[2·2·2]octane (DABCO; Sigma) in 80% glycerol was added to stained cells to inhibit fading of fluorescence (Johnson *et al.* 1982). Staining of leptin was viewed by confocal immunofluorescence microscopy.

Subcellular localization of leptin was determined by confocal laser scanning microscopy that examined a series

of xy optical sections in the z axis at an increment of 1 μm through the full thickness of the adipocyte.

*Regulation of leptin secretion by TNF-α*

To asses the functionality of ceiling-cultured adipocytes, 14-day-old adipocytes attached to the ceiling surface of culture flasks were cultured in DMEM:F12 medium containing 100 nM insulin and 100 nM dexamethasone for 24 h to enhance leptin production. The cells were then treated with/without 100 ng/ml recombinant human TNF-α (PeproTech EC Ltd, London, UK) for 24 h, under which conditions TNF-α has been shown to stimulate leptin secretion from suspension-cultured adipocytes (Zhang *et al.* 1999). Total secreted proteins from equal numbers of cells were precipitated with three volumes of absolute ethanol overnight at −20 °C. After centrifugation, the pellets were dissolved in 100 μl sample buffer and proteins separated on a 15% polyacrylamide gel with a 7·5% stack, using Tris–glycine buffers for SDS-PAGE (Elgin 1975). The separated proteins were transferred to a polyvinylidene difluoride membrane (ICN, Basingstoke, Hampshire, UK). Non-specific binding was blocked by incubating the membrane in 10% non-fat milk in Tris–buffered saline-Tween 20 (TBS-T) for 2 h at room temperature. The blots were incubated with anti-leptin IgG for 1 h at a dilution of 1:500 in TBS-T with 0·1% BSA. After exposure to primary antibody, the blots were washed in TBS-T and exposed to a 1:40 000 dilution (in TBS-T with 3% non-fat milk) of rabbit anti-sheep IgG antibody (Calbiochem, Nottingham, UK) for 1 h. The antigens were detected by the enhanced chemiluminescence system (Amersham Life Sciences, Amersham, Bucks, UK) and analysed using UVP GelBlot software (Gel Analysis Suite for Windows 93). The specificity of anti-leptin antibody was confirmed by Western blotting of leptin secreted by adipocytes and recombinant human leptin. A single 16 kDa band of identical molecular mass to that of recombinant human leptin was detected in conditioned medium from adipocytes.

*Adipocyte apoptosis: terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick-end labelling and propidium iodide staining*

Apoptosis was assessed by changes in nuclear morphology and by the detection of DNA strand breaks, characteristic of apoptosis. Bromodeoxyuridine (BrdU) was catalytically added to the 3′-OH ends of DNA strand breaks by an exogenous terminal deoxynucleotidyl transferase (TdT). The incorporated deoxynucleotide is detected by an FITC-conjugated anti-BrdU antibody, which allows the identification of apoptotic cells (Li *et al.* 1995). Adipocytes attached to the coverslips were maintained in DMEM:F12 for 48 h with/without 20% NCS. Cells were fixed in 1% methanol-free formaldehyde (Polysciences Inc., Washington, PA, USA) for 15 min and 70% ethanol overnight at 4 °C, followed by washing with PBS (pH 7·4) for 5 min. Cells were incubated for 40 min at 37 °C in 50 μl solution containing 10 μl TdT reaction buffer (5 × concentration: 1 M potassium cacodylate, 125 mM Tris–HCl pH 6·6, 1·25 mg/ml BSA; Boehringer-Mannheim, Lewes, East Sussex, UK), 2 l BrdUTP (2 mM; Sigma), 0·5 μl TdT (25 units/μl; Boehringer-Mannheim), 5 μl $CoCl_2$ (25 mM; Boehringer-Mannheim) and 33·5 μl distilled $H_2O$. Cells were washed for 5 min with PBS containing 0·1% Triton X-100 and 0·5% BSA and incubated with FITC-conjugated anti-BrdU monoclonal antibody (Becton Dickinson, Cowley, Oxon, UK) at room temperature for 1 h. A negative control staining was performed by substituting 0·5 μl distilled $H_2O$ for TdT enzyme during the incubation process.

After incubation with FITC-conjugated anti-BrdU antibody, cells were stained with propidium iodide (5 μg/ml) containing DNase-free RNase A (200 μg/ml in PBS) for 30 min at room temperature. Coverslips were washed with PBS and briefly dried. DABCO (2·5%) was added to stained cells. Apoptotic features shown by both propidium iodide and FITC fluorescence of cell nuclei were identified by three independent observers. Apoptotic indices were obtained by counting 200 cells.

*Adipocyte proliferation*

Adipocyte proliferation was studied using a commercially available cell proliferation kit (Amersham). The kit is based on an immunocytochemical system for monitoring cell proliferation using a monoclonal antibody to BrdU incorporated into cellular DNA. Ceiling-cultured adipocytes were incubated at 37 °C for 4 h in serum-containing medium labelled with BrdU and 5-fluoro-2′-deoxyuridine (1:1000 dilution of the supplied labelling reagent). After removing the labelling medium, cells were briefly washed, fixed with acid–ethanol (90% ethanol: 5% glacial acetic acid: 5% water) for 30 min, and washed three times with PBS. The reconstituted nuclease/anti-BrdU solution was added to cells and incubated for 1 h at room temperature. Cells were washed three times with PBS and incubated with peroxidase-conjugated anti-mouse IgG-2α for 30 min at room temperature and stained with diaminobenzidine (0·5 mg/ml in 50 mM phosphate buffer) for 10 min. After washing three times with distilled water, cells were air-dried, and viewed under a light microscope. Adipocyte proliferation was assessed by counting 200 cells.

*May-Grunwald–Giemsa staining*

Giemsa staining was performed on 14-day-old ceiling-cultured adipocytes according to the method previously described with modifications (Bancroft & Cook 1994). Adipocytes were fixed in methanol for 2 min and stained

with May-Grunwald stain (Raymond A Lamb) for 10 min. Cells were washed in de-ionized water for 2 min, followed by Giemsa (Raymond A Lamb) staining for 25 min. After rinsing in tap water for 2 min and in Sorensen's buffer (0·65 M $Na_2HPO_4$, 0·67 M $KH_2PO_4$, pH 6·8) for 3 min, cells were dried and viewed under a light microscope.

### *Regulation of lipolysis by forskolin*

Fourteen-day-old adipocytes cultured on coverslips were cultured for 4 h at 37 °C in DMEM:F12 supplemented with 0·2% fatty-acid-poor BSA (First Link UK Ltd, Brierley Hill, West Midlands, UK), 10 nM insulin, and 5 μM [$^3$H]oleic acid (9 Ci/mmol; Amersham). Cells were then washed with HBSS and incubated in fresh DMEM:F12 with/without 10 μM forskolin (Sigma) for 90 min. For each treatment (with or without forskolin), triplicate samples containing the same number of adipocytes were used. Cellular $^3$H radioactivity was determined by liquid scintillation counting. The amount of $^3$H in forskolin-treated samples was expressed as percentage of the control.

### *Regulation of lipogenesis by insulin*

Triplicate samples of 14-day-old ceiling-cultured adipocytes on coverslips were cultured for 4 h at 37 °C in DMEM:F12 supplemented with 0·2% fatty-acid-poor BSA, 5 μM [$^3$H]oleic acid, and with or without 10 and 100 nM insulin (Sigma). Cells were then washed with HBSS and air-dried for 1 h. Coverslips containing adipocytes were then mounted on plain glass slides using nail vanish. Cellular $^3$H radioactivity was detected by autoradiography as described below.

Slides containing adipocytes were dipped briefly in K5 emulsion gel (Ilford Ltd, Mobberley, Cheshire, UK) which was melted in a 40 °C water bath and diluted with sterile distilled water (1:1). Excess emulsion was drained from the slides, which were then kept in a light-proof box to expose at 4 °C for 10 days. The slides were developed for 3 min with D-19 developer (Kodak, Hemel Hempstead, UK), fixed with thiosulphate (Sigma) for 3 min, and rinsed with tap water for 10 min. Counterstain was performed by staining adipocyte nuclei with propidium iodide (5 μg/ml in PBS) for 30 min. Merged adipocyte images of autoradiography and nuclear staining were captured with the Quips M-FISH image analysis software (Vysis Quips Genetics Workstation, Richmond, Surrey, UK).

### *Statistics*

All the experiments in the study were performed on adipocytes from at least three separate patients ($n \geq 3$). Data from representative preparations are shown. A paired Student's *t*-test was used for data analysis on Western blotting of secreted leptin, and an unpaired Student's *t*-test was used for data analysis on lipolysis. Data are means ± S.E. *P* values less than 0·05 were considered significant.

## Results

### *Ceiling culture of adipocytes*

Isolated adipocytes floated to the top of the medium in culture plates/flasks. During the first 2 days of ceiling culture, they adhered loosely to the ceiling surface. Starting from day 3 or 4, the cytoplasm began to spread on the ceiling surface. While the main cell body remained spherical, the cytoplasmic rim became flattened and tentacle-like. Large adipocytes exhibited attachment, but their cytoplasm did not spread out widely. Figure 1A shows adipocytes that were attached to the ceiling surface after 10 days of incubation. Arrows indicate the tentacle-like appearance of the cytoplasmic rim. Attachment efficiency to the ceiling surface of coverslips varied between 50 and 80%. Cell viability was checked by exclusion of trypan blue and was >85% at the end of 10–14 days of incubation. Adipocytes maintained their unilocular characteristic as shown in Fig. 1A. Multiple small lipid droplets were observed at the periphery of some cells, indicating lipogenic activity. Oil Red O staining (Fig. 1B) showed that adipocytes maintained their lipid stores.

### *The expression and subcellular localization of leptin*

Adipocytes cultured with the modified ceiling culture method were stained with anti-leptin IgG antibody and FITC-conjugated secondary antibody. Figure 1C represents the negative staining of adipocytes treated only with the FITC-conjugated secondary antibody. Figure 1D shows the green fluorescence staining of leptin in adipocytes. Individual cells were identified by the presence of cell nuclei indicated by the red fluorescence staining of propidium iodide. The yellow fluorescence around and within the nucleus indicates the high intensity of leptin fluorescence. The lipid droplet, a cytoplasmic feature unique to adipocytes, was indicated by the absence of fluorescence in the centre of the cytoplasm.

Subcellular distribution of leptin was examined by confocal laser scanning microscopy, which examined a series of xy optical sections in the z axis at an increment of 1 μm through the full thickness of the adipocyte. This revealed the localization of leptin in the region of plasma membrane, cytoplasm, nuclear periphery and the nucleus. The cytoplasmic and nuclear localization of leptin was found in some but not all the adipocytes. Figure 1E shows propidium iodide staining of adipocyte nuclei. Figure 1F shows nuclear staining of leptin in one optical section of

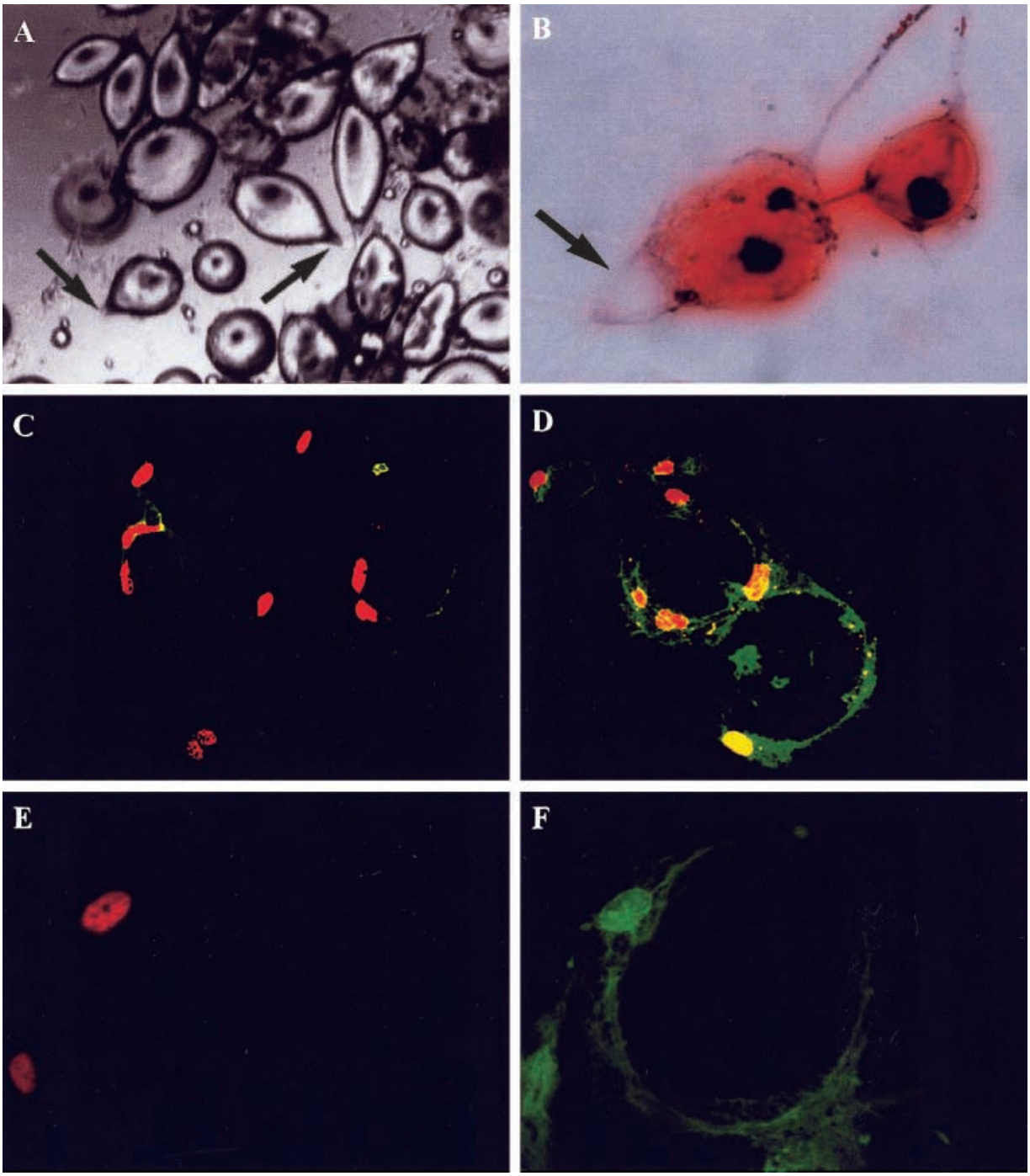


**Figure 1** The expression of leptin in ceiling-cultured adipocytes. (A) Ten-day-old adipocytes in ceiling culture. Magnification 200 × . Arrows indicate the tentacle-like appearance of the cytoplasmic rim. (B) Oil Red O staining of adipocyte cellular lipid. Magnification 400 × . Arrow indicates a budding daughter cell. (C) Negative leptin immunofluorescence staining. Magnification 400 × . (D) Leptin immunofluorescence staining. Magnification 400 × . (E) Propidium iodide staining of adipocyte nuclei. (F) The nuclear staining of leptin in one optical section of the same adipocytes (as in (E)) taken by confocal laser scanning microscopy.

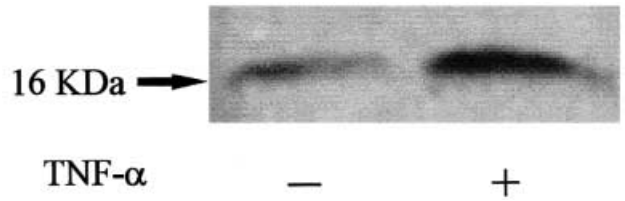


**Figure 2** Effect of TNF-α on leptin secretion by adipocytes. Ceiling-cultured adipocytes were treated with/without TNF-α (5 nM) for 24 h. Leptin secretion (from the same number of cells) was detected by Western blotting using conditioned medium.

the same adipocytes (as in Fig. 1E) taken by confocal laser scanning microscopy.

*Regulation of leptin secretion by TNF-α*

To determine whether ceiling-cultured adipocytes secrete leptin and whether the secretion is regulated by TNF-α as described previously for suspension-cultured human adipocytes (Zhang *et al.* 1999), Western blotting of leptin in conditioned medium was performed. In the absence of insulin and dexamethasone, leptin secretion was not detected. To quantitate the differences in leptin secretion, adipocytes were incubated with insulin and dexamethasone, which have been shown to increase leptin production and secretion (Kolacynski *et al.* 1996, 1997, Slieker *et al.* 1996). Figure 2 shows leptin secretion by ceiling-cultured adipocytes that were treated with/without 100 ng/ml TNF-α. A single band of 16 kDa, corresponding to recombinant leptin, was detected. Densitometric analysis of three independent Western blots ($n=3$) showed that TNF-α increased leptin secretion by 2·5- to 4-fold.

*Adipocyte apoptosis*

Following 48 h serum deprivation and no hormone replacement, abnormal nuclear changes including clumping of chromatin beneath the nuclear envelope, convolution of the nuclear outline, and disintegration of the propidium iodide-stained nucleus were observed (Fig. 3A). These apoptotic features were verified by the specific 3′-OH end labelling of DNA strands in abnormal nuclei and the negative labelling of normal nuclei (Fig. 3B). Apoptosis, shown by both propidium iodide staining and 3′-OH end labelling, was observed in 30–35% of serum-deprived adipocytes. In the control (non-serum-deprived) samples, 10–15% of adipocytes were also shown to contain apoptotic features.

*Adipocyte proliferation*

Proliferation of unilocular adipocytes was evident from the incorporation of BrdU into replicating DNA as indicated

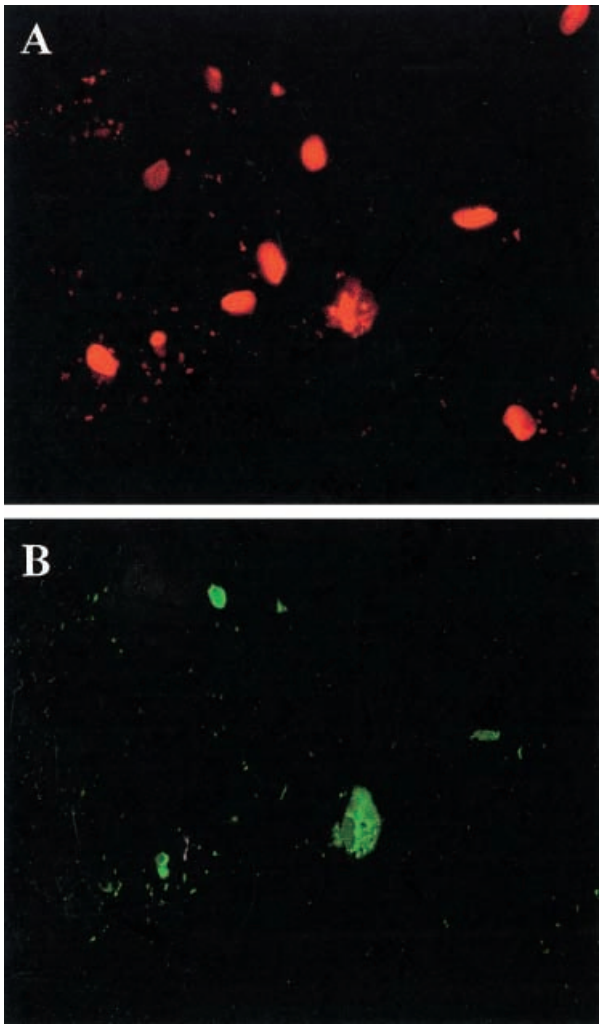


**Figure 3** (A) Propidium iodide staining of adipocyte nuclei. Magnification 400 ×. (B) FITC staining of apoptotic nuclei labelled with BrdU. The same field as in (A).

by the black and blue staining of the nucleus (Fig. 4A). The incorporation occurred in 2% of adipocytes during 4 h incubation in serum-containing medium labelled with BrdU. Proliferation was also evident from the budding of daughter cells from ceiling-cultured adipocytes indicated by arrows in Fig. 1B (Oil Red O staining) and Fig. 4B (May-Grunwald–Giemsa staining).

*Regulation of lipolysis by forskolin*

Lipolysis in ceiling-cultured adipocytes was measured by liquid scintillation counting. Treatment with 10 μM forskolin for 90 min reduced intracellular $^3H$ to ∼50% of the control ($2{\cdot}3 \pm 0{\cdot}4 \times 10^6$ d.p.m. in the control vs

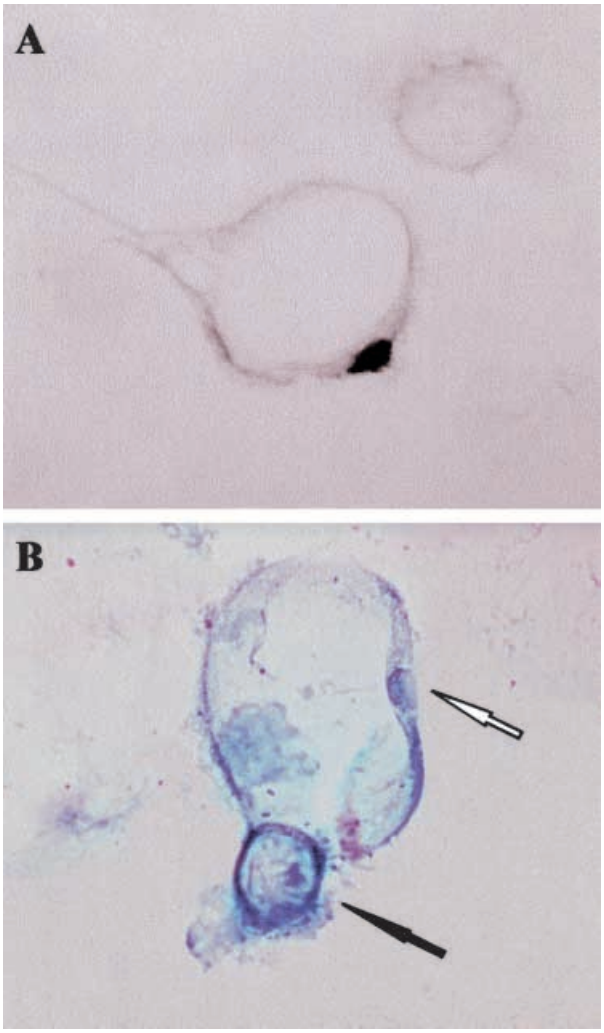


**Figure 4** (A) Incorporation of BrdU into nuclear DNA of adipocytes. Magnification 400 ×. (B) The budding of a daughter cell from a mature adipocyte (Giemsa staining, the white arrow indicates the nucleus of the mature adipocyte, the black arrow shows the budding of a daughter cell).

$1{\cdot}1 \pm 0{\cdot}2 \times 10^6$ d.p.m. in the treated samples, $P<0{\cdot}01$, $n=3$), indicating stimulation of lipolysis by forskolin.

*Regulation of lipogenesis by insulin*

Lipogenesis in ceiling-cultured adipocytes was detected by autoradiography. Figure 5A shows control cells that were incubated with [$^3$H]oleic acid in the absence of insulin. Figure 5B and C show adipocytes that were treated with 10 and 100 nM insulin respectively. The black staining corresponds with $^3$H radioactivity in ceiling-cultured adipocytes, which were identified by propidium iodide staining of nuclear DNA (shown in red). The stronger black staining in Fig. 5B and C compared with that in Fig. 5A indicates that insulin (10 and 100 nM) stimulated lipogenesis in ceiling-cultured adipocytes.

## Discussion

Ceiling culture utilizes the buoyant feature of adipocytes by providing them with an attachment surface (the top inner surface of a culture flask). We have further refined this method by culturing adipocytes on a floating coverslip which is placed in a cell culture plate/dish. The modified method requires less culture medium (12 ml medium for a six-well plate compared with 70 ml for a T25 cm$^2$ flask), no pre-gassing of the culture medium (minimum of 4–6 h for the flask method) and above all, it enables the study of adipocytes using (confocal) fluorescence microscopy. Ceiling-cultured adipocytes can be maintained viable for a long time (>4 weeks). Their lipolytic and lipogenic functions can be observed morphologically by the behaviour of lipid droplets in the cytoplasm (Fig. 1A and B). Adipocyte apoptosis, proliferation, cellular protein expression/localization, lipogenesis, and lipolysis can be studied with ceiling-cultured adipocytes.

Using the modified ceiling culture method, we have demonstrated apoptosis in isolated human mature adipocytes that were serum deprived for 48 h (30–35%). Apoptosis (10–15%) was also detected in control adipocytes cultured with serum-containing medium. This is not a true measurement of the rate of apoptosis but of the accumulation of apoptotic adipocytes. We do not know how long it takes adipocytes to complete apoptosis but we postulate that they may exist in the apoptotic state for some time since ceiling-cultured adipocytes can survive in serum-containing medium for 4 weeks without noticeable cell loss.

The loss of adipocytes by apoptosis may be partially countered by cell division. In our study of adipocyte proliferation, 2% of adipocytes incorporated BrdU into cellular DNA during a short incubation of 4 h in serum-containing medium. The positive labelling is consistent with the findings of Sugihara *et al.* (1987) and compatible with the *in vivo* results observed by Miller *et al.* (1984) and Klyde & Hirsch (1979*a*,*b*), who described the incorporation of [$^3$H]thymidine into mature rat adipose tissue *in vivo*. Adipocyte proliferation is also evident from the budding of daughter cells from ceiling-cultured adipocytes as shown in Figs 1B and 4B, further supporting the notion that mature human adipocytes are capable of proliferating *in vitro* and suggesting an additional mechanism by which increases in adipocyte number and mass may occur.

Preadipocyte contamination is a potential confounding problem for studies using primary cultured adipocytes. Prior to ceiling culture, isolated adipocytes and preadipocytes were washed in medium four times by centrifugation at 250 **g** for 5 min to minimize the presence of preadipocytes in adipocyte preparations. During

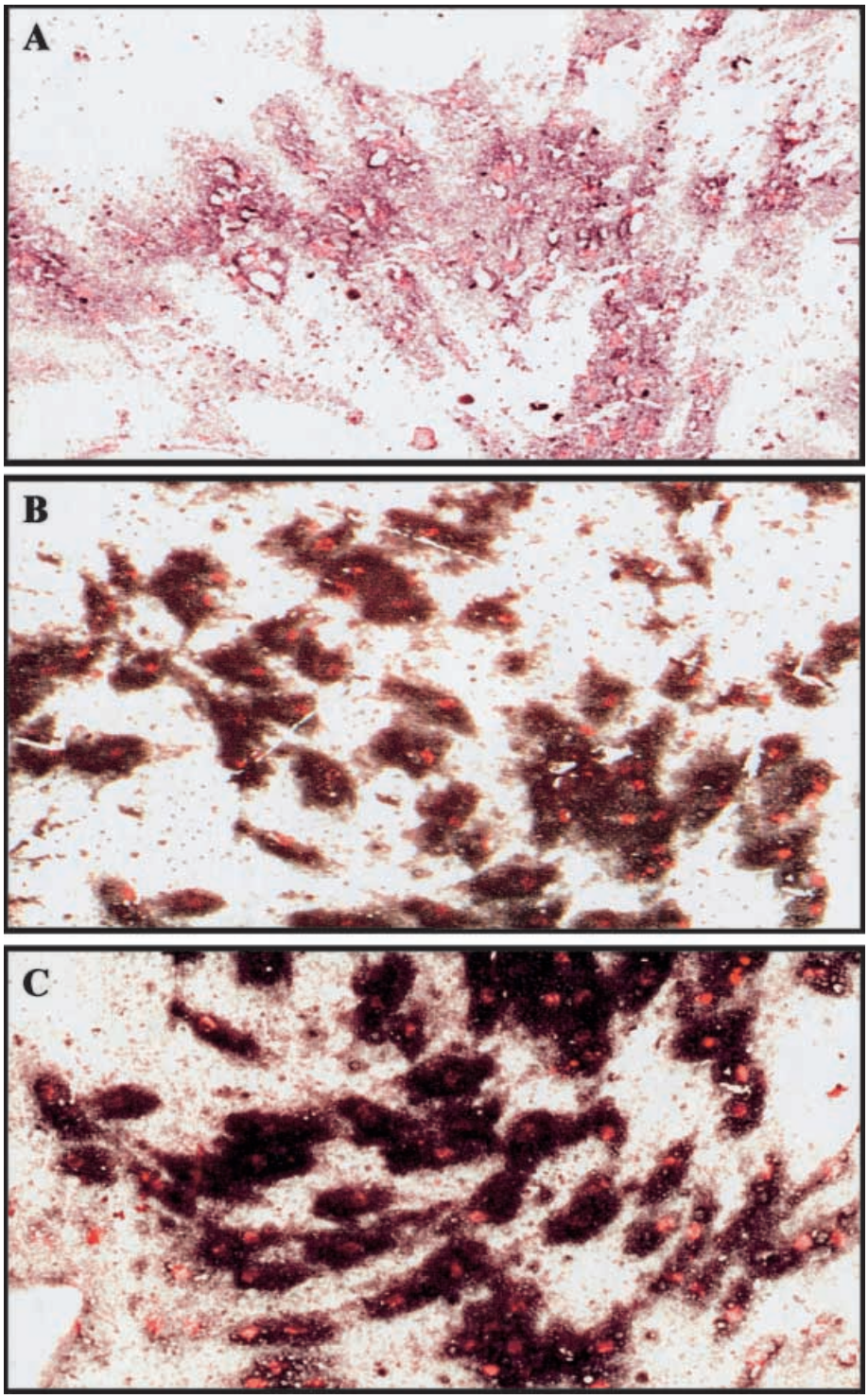


**Figure 5** Autoradiography of ceiling-cultured adipocytes cultured with 5 μM [$^3$H]oleic acid for 4 h. Magnification 100 × . Black staining indicates $^3$H radioactivity. Red staining shows nuclear DNA staining with propidium iodide. (A) Adipocytes cultured without insulin; (B) adipocytes cultured with 10 nM insulin; (C) adipocytes cultured with 100 nM insulin.

10–14 day ceiling culture, most remaining preadipocytes adhere to the bottom surface of the culture flask/plate due to gravity while mature adipocytes attach to the ceiling surface. Preadipocyte contamination in ceiling culture is therefore minimal. When present, preadipocytes attached to the ceiling surface can be distinguished by their fibroblast-like appearance and therefore can be excluded in studies of adipocyte cell biology such as cellular protein expression. Hence compared with suspension culture, ceiling culture enables assessment of preadipocyte contamination and their exclusion in studies involving single-cell analyses.

With the modified ceiling culture system, we have visually observed leptin in mature human adipocytes. Confocal laser scanning microscopy, which scanned serial cross-sections of adipocytes, revealed that the nuclear staining of leptin was due to the nuclear localization of leptin rather than an overlay of cytoplasm around the nucleus. Leptin was also localized in the region of plasma membrane and cytoplasm of adipocytes in some but not all the adipocytes examined. Heterogeneity of leptin staining has also been described in rat adipocytes (Barr *et al.* 1997). By double immunofluorescence staining of leptin and endoplasmic reticulum, Barr *et al.* (1997) demonstrated the localization of leptin in the endoplasmic reticulum encasing the lipid droplet and lining the plasma membrane of rat adipocytes. Cytoplasmic staining of leptin was evident in some of the rat adipocytes but was not discussed. It seems likely that leptin found in the plasma membrane region in our studies is localized in the endoplasmic reticulum. The occurrence of nuclear staining in some but not all the human adipocytes and its absence in rat adipocytes remains to be clarified. Nuclear leptin may act with a nuclear receptor, nuclear factor, and or have direct effects on the genome in target cells. There have been similar findings of protein nuclear localization of secreted cytokines, such as interleukin-1 in mouse brown adipocytes (Burysek & Houstek 1996), and fibroblast growth factor (Rifkin *et al.* 1994) and insulin-like growth factor-binding protein-3 (Michell *et al.* 1997) in other cell types.

TNF-α limits human obesity by various mechanisms such as induction of apoptosis (Prins *et al.* 1997) and inhibition of differentiation (Petruschke & Hauner 1993). We have shown that TNF-α (5 nM) increases leptin secretion from human adipocytes in short-term suspension culture (Zhang *et al.* 1999). The finding that TNF-α (5 nM) also increased leptin secretion from ceiling-cultured adipocytes demonstrates their fidelity of response to cytokine treatment in long-term culture and shows that they can be used in studies of regulation of adipocyte protein expression.

Insulin has been shown to stimulate fatty acid incorporation into intracellular triacylglycerol of adipose cells and the incorporation increased proportionately during the first 3–4 h incubation with radiolabelled oleic acid ($\leq$50 μM) (Maslowska *et al.* 1993). During 4 h incubation with 5 μM [$^3$H]oleic acid, insulin treatment (10 and 100 nM) increased intracellular $^3$H in ceiling-cultured adipocytes as detected by autoradiography in our study (Fig. 5). The increase in intracellular $^3$H reflects the increase in lipogenesis of treated adipocytes, as it has been shown that the uptake and subsequent sequestration of intracellular fatty acids lead to quantitative conversion into triacylglycerol and other cellular lipids (Berk *et al.* 1997). Therefore the stimulation of lipogenesis observed in our study confirms the hormonal responsiveness of ceiling-cultured adipocytes.

Forskolin has been shown to stimulate lipolysis in adipocytes *in vitro* through stimulation of cAMP production (Litosch *et al.* 1982). We similarly found that forskolin (10 μM) treatment of ceiling-cultured adipocytes increased lipolysis, further demonstrating the function of ceiling-cultured adipocytes.

In summary, to assess the validity of the ceiling culture method, we have demonstrated apoptosis and proliferation of ceiling-cultured adipocytes under controlled conditions. We have also demonstrated cellular expression/localization of leptin by confocal immunofluorescence microscopy and the effect of TNF-α on leptin secretion. Lipogenesis was shown to be increased by insulin and lipolysis was stimulated by forskolin treatment. All these findings show that ceiling-cultured adipocytes are functional and can be used to study adipocyte cell biology.

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## References

Bancroft JD & Cook HC 1994 Clinical cytology. In *Manual of Histological Techniques and their Diagnostic Applications*, pp 323–341. London: Churchill Livingstone.

Barr VA, Malide D, Zarnowski MJ, Taylor SI & Cushman SW 1997 Insulin stimulate both leptin secretion and production by rat white adipose tissue. *Endocrinology* **138** 4463–4472.

Berk PD, Zhou S-L, Kiang C-L, Stump D, Bradbury M & Isola M 1997 Uptake of long chain free fatty acids is selectively up-regulated in adipocytes of Zucker rats with genetic obesity and non-insulin-dependent diabetes mellitus. *Journal of Biological Chemistry* **272** 1143–1146.

Burysek L & Houstek J 1996 Multifactorial induction of gene expression and nuclear localization of mouse IL-1 alpha. *Cytokine* **8** 460–467.

Culling CFA 1963 *Handbook of Histopathological Techniques,* edn 2, pp 298. London: Butterworth.

Elgin SCR 1975 Methods for the isolation and characterization of nonhistone chromosomal protein. *Methods in Enzymology* **40** 144–160.

Flier JS 1997 Leptin expression and action: new experimental paradigms. *Proceedings of the National Academy of Sciences of the USA* **94** 4242–4245.

Hotamisligil GS, Shargill NS & Spigelman BM 1993 Adipose expression of tumor necrosis factor-α: direct role in obesity-linked insulin resistance. *Science* **259** 87–90.

Johnson GD, Davidson RS, McNamee KC, Russell G, Goodwin D & Holborow EJ 1982 Fading of immunofluorescence during microscopy: a study of the phenomenon and its remedy. *Journal of Immunological Methods* **55** 231–242.

Kern PA, Saghizadeh M, Ong JM, Bosch RJ, Deem R & Simsolo RB 1995 The expression of tumor necrosis factor in human adipose tissue. *Journal of Clinical Investigation* **95** 2111–2119.

Klyde BJ & Hirsch J 1979*a* Isotopic labelling of DNA in rat adipose tissue: evidence for proliferating cells associated with mature adipocytes. *Journal of Lipid Research* **20** 691–704.

Klyde BJ & Hirsch J 1979*b* Increased cellular proliferation in adipose tissue of adult rat fed a high-fat diet. *Journal of Lipid Research* **20** 705–715.

Kolacynski JW, Nyce MR, Considine RV, Boden G, Nolan JJ, Henry R, Mudaliar SR, Olefsky J & Caro JF 1996 Acute and chronic effect of insulin on leptin production in humans. *Diabetes* **45** 699–701.

Kolacynski JW, Goldstein BJ & Considine RV 1997 Dexamethasone, OB gene, and leptin in humans: effect of exogenous hyperinsulinemia. *Journal of Clinical Endocrinology and Metabolism* **82** 3895–3897.

Li X, Traganos F, Melamed MR & Darzynkiewicz Z 1995 Single-step procedure for labelling DNA strand breaks with fluorescein- or BODIPY-conjugated deoxynucleotides: detection of apoptosis and bromodeoxyuridine incorporation. *Cytometry* **20** 172–180.

Litosch I, Hudson TH, Mills I, Li SY & Fain JN 1982 Forskolin as an activator of cyclic AMP accumulation and lipolysis in rat adipocytes. *Molecular Pharmacology* **22** 109–115.

Magun R, Boone DL, Tsang BK & Sorisky A 1998 The effect of adipocyte differentiation on the capacity of 3T3-L1 cells to undergo apoptosis in response to growth factor deprivation. *International Journal of Obesity and Related Metabolic Disorders* **22** 567–571.

Maslowska MH, Sniderman AD, Maclean LD & Cianflone K 1993 Regional differences in triacylglycerol synthesis in adipose tissue and in cultured preadipocytes. *Journal of Lipid Research* **34** 219–228.

Michell NP, Dent S, Langman MJS & Eggo MC 1997 Insulin-like growth factor binding proteins as mediators of IGF-1 effects on colon cancer cell proliferation. *Growth Factors* **14** 269–277.

Miller WH, Faust IM & Hirsch J 1984 Demonstration of *de novo* production of adipocytes in adult rats by biochemical and radioautographic techniques. *Journal of Lipid Research* **25** 336–347.

Moustaid N, Jones BH & Taylor JW 1996 Insulin increases lipogenic enzyme activity in human adipocytes in primary culture. *Journal of Nutrition* **126** 865–870.

Petruschke TH & Hauner H 1993 Tumor necrosis factor-α prevents the differentiation of human adipocyte precursor cells and causes delipidation of newly developed fat cells. *Journal of Clinical Endocrinology and Metabolism* **76** 742–747.

Prins JB & O'Rahilly S 1997 The regulation of adipose cell number in man. *Clinical Science* **92** 3–11.

Prins JB, Niesler CU, Winterford CM, Bright NA, Siddle K, O'Rahilly S, Walker NI & Cameron DP 1997 Tumor necrosis factor-α induces apoptosis of human adipose cells. *Diabetes* **46** 1939–1944.

Rifkin DB, Moscatelli D, Roghani M, Nagano Y, Quarto N, Klein S, Bikfalvi A *et al.* 1994 Studies on FGF-2: nuclear localization and function of high molecular weight forms and receptor binding in the absence of heparin. *Molecular Reproduction and Development* **39** 102–104.

Rodbell M 1964 Metabolism of isolated adipocytes. I. Effects of hormones on glucose metabolism and lipolysis. *Journal of Biological Chemistry* **239** 375–380.

Slieker LJ, Sloop KW, Surface PL, Kriauciunas A, LaQuier F, Manetta J, Bue-Valleskey J & Stephens TW 1996 Regulation of expression of *ob* mRNA and protein by glucocorticoids and cAMP. *Journal of Biological Chemistry* **271** 5301–5304.

Sooranna SR & Saggerson ED 1975 Studies on the role of insulin in the regulation of glyceride synthesis in the epidermal adipose tissue. *Biochemical Journal* **150** 441–451.

Sugihara H, Yonemitsu N, Miyabara S & Yun K 1986 Primary culture of unilocular fat cells: characteristics of growth *in vitro* and changes in differentiation properties. *Differentiation* **31** 42–49.

Sugihara H, Yonemitsu N, Miyabara S & Toda S 1987 Proliferation of unilocular fat cells in the primary culture. *Journal of Lipid Research* **28** 1038–1045.

Zhang HH, Kumar S, Barnett AH & Eggo MC 1999 Tumor necrosis factor-α increases leptin release from mature human adipocytes but inhibits differentiation-related leptin production by blocking preadipocyte differentiation. *Journal of Endocrinology* **160** (Suppl) P119.

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L & Friedman JM 1994 Positional cloning of the mouse obese gene and its human homologue. *Nature* **372** 425–431.

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