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Culture effects of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) on cryopreserved human adipose-derived stromal/stem cell proliferation and adipogenesis

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Abstract

Previous studies have demonstrated that EGF and bFGF maintain the stem cell properties of proliferating human adipose-derived stromal/stem cells (hASCs) *in vitro*. While the expansion and cryogenic preservation of isolated hASCs are routine, these manipulations can impact their proliferative and differentiation potential. This study examined cryogenically preserved hASCs (*n = 4* donors), with respect to these functions, after culture with basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) at varying concentrations (0–10 ng/ml). Relative to the control, cells supplemented with EGF and bFGF significantly increased proliferation by up to three-fold over 7–8 days. Furthermore, cryopreserved hASCs expanded in the presence of EGF and bFGF displayed increased oil red O staining following adipogenic induction. This was accompanied by significantly increased levels of several adipogenesis-related mRNAs: *aP2, C/EBPα*, lipoprotein lipase (*LPL*), *PPARγ* and *PPARγ* co-activator-1 (*PGC1*). Adipocytes derived from EGF- and bFGF-cultured hASCs exhibited more robust functionality based on insulin-stimulated glucose uptake and atrial natriuretic peptide (ANP)-stimulated lipolysis. These findings indicate that bFGF and EGF can be used as culture supplements to optimize the proliferative capacity of cryopreserved human ASCs and their adipogenic differentiation potential.

Keywords

adipogenesis; adipose-derived stem cells; edipermal growth factor; basic fibroblast growth factor; cryopreservation; differentiation

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Conflict of interest statements

Dr Gimble consults or collaborates with the following tissue engineering-related companies: Toucan Capital, Cognate Bioservicese, Vesta Therapeutics and Zen-Bio.

1. Introduction

There is a growing interest in adult human adipose-derived stromal/stem cells (hASCs), which are abundant, plastic and useful for exploring the mechanisms of adipose tissue differentiation. The ASCs have been confirmed to differentiate along multiple lineage pathways (adipocyte, chondrocyte, neuronal-like and osteoblast) *in vitro* at the clonal level, consistent with the definition of a 'stem cell' (Guilak *et al*., 2006; Zuk *et al*., 2001, 2002; Halvorsen *et al*., 2000, 2001; Gronthos *et al*., 2001). It is of interest that the adipogenic potential was most difficult to maintain with extensive expansion of the clonal populations (Guilak *et al*., 2006). Since the number of human pre-adipocyte cell lines are limited, our laboratory and others utilize primary cultures of hASCs. Consequently, it is necessary that isolated hASCs be cryopreserved for future experiments; however, current techniques for expanding, preserving and inducing such cells leave room for improvement. While it has been reported that bone marrow-derived mesenchymal stem cells (BMSCs) can be cryopreserved without loss of viability or osteogenic potential (Kotobuki *et al*., 2004), it has been reported that cryopreservation can reduce the viability, proliferation and differentiation ability of $CD34^+$ haematopoietic stem cells (de Boer *et al*., 2002; Keung *et al*., 1996; Stylianou *et al*., 2006). Furthermore, while cryopreservation of intact human adipose tissue has been achieved (Shoshani *et al*., 2001), studies suggest that optimal viability requires the presence of cryoprotectant agents and controlled rate freezing (Moscatello *et al*., 2005). We have noted a decreased viability in the cryopreserved isolated hASCs as a function of cell concentration, although differentiation function was generally maintained (Goh *et al*., 2007). During our efforts to optimize hASC cryopreservation (Devireddy *et al*., 2005; Goh *et al*., 2007; Thirumala *et al*., 2005a, 2005b), we have noted a loss of adipogenic differentiation potentiality in some lots of cryopreserved hASCs post-thaw.

To date, a standard expansion procedure for hASCs after cryopreservation has not been recognized. Multiple laboratories, including our own, maintain the hASCs in a stromal medium consisting of Dulbecco's modified Eagle's medium (DMEM)/F-12 Ham's, supplemented only with 10% fetal bovine serum (FBS) and 1% antibiotics/antimycotics (Mitchell *et al*., 2006]. Some laboratories have supplemented their expansion medium with epidermal growth factor (EGF) and/or basic fibroblast growth factor (bFGF) to accelerate hASC proliferation (Hauner *et al*., 1995; Iwashima *et al*., 2008; Skurk *et al*., 2007), while others have employed commercial media optimized for endothelial cell growth (Miranville *et al*., 2004; Rehman *et al*., 2004; Suga *et al*., 2007). These latter compositions are supplemented with polypeptide growth factors, including EGF and bFGF, that have the potential to increase cell proliferation and modulate differentiation of hASCs. Previous studies have speculated that a synergy exists between EGF and bFGF (Hauner *et al*., 1995; Suga *et al*., 2007) but the dose-dependent effects of these two factors in combination have not been examined closely.

The effects of EGF and bFGF vary greatly among species; however, numerous studies demonstrate that EGF and bFGF increase proliferation and modulate the differentiation potential of hASCs (Butterwith *et al*., 1993; Hauner *et al*., 1995; Tamama *et al*., 2006; Zaragosi *et al*., 2006). EGF is a single-chain polypeptide noted for having proliferative and differentiating effects on many mammalian tissues (Hauner *et al*., 1995; Kurachi *et al*., 1993). EGF is known to enhance migration and cell proliferation of bone marrow-derived mesenchymal stem cells while maintaining differentiation potential (Tamama *et al*., 2006), and the involvement of EGF in adipose tissue development has been postulated based on the correlation between increased levels of EGF and obesity onset in ovariectomized mice (Kurachi *et al*., 1993). Also, EGF has significant effects on *in vitro* adipocyte development and function at concentrations comparable to those found in human serum (Hauner *et al*., 1995). Likewise, bFGF has been found to act as a mitogen and chemoattractant that enhances angiogenesis, migration and adipogenic differentiation of both ASCs and BMSCs (Hauner *et al*., 1995; Locklin *et al*., 1999; Schmidt *et al*., 2006; Vashi *et al*., 2006). Zarogosi *et al*. (2006), by

inhibiting bFGF receptor signalling, have demonstrated that hASCs express bFGF as an autocrine factor. In similar studies, Rider *et al*. (2008) found that inhibition of endogenous bFGF reduced the rate of hASC proliferation. Thus, bFGF appears to be necessary for stem cell self-renewal, proliferation and the maintenance of hASC pluripotency. In contrast, the presence of bFGF in osteogenic medium has been noted to inhibit murine ASC extracellular matrix mineralization and alkaline phosphatase enzyme activity (Quarto and Longaker, 2006). The addition of bFGF inhibited the murine ASC expression of the BMP 1B receptor in response to retinoic acid; however, this mechanism appears to be species-specific, since bFGF addition was not observed to inhibit human ASC osteogenesis *in vitro* (Quarto *et al*., 2008).

Recently, we observed that EGF and bFGF induced hASC secretion of the hepatic growth factor (HGF) (Kilroy *et al*., 2007). This cytokine has the potential to function in an autocrine manner, since hASCs express its receptor (c-Met) mRNA (Kilroy *et al*., 2007). A related study has determined that HGF expression by human umbilical cord blood-derived MSCs correlated with both increased proliferative capacity and adipogenic potential *in vitro* (Markov *et al*., 2007). Based on these observations, we hypothesized that the presence of EGF and bFGF would enhance the proliferation and differentiation potential of cryopreserved hASCs. The current study examines the effect of EGF and bFGF with respect to hASC cell proliferation and adipogenesis.

2. Materials and methods

2.1. Human subjects

Adult human lipoaspirates were obtained with consent from nine female patients undergoing elective liposuction procedures [mean age 36.9 (range 27–62) years; mean BMI 25.6 (range 21.6–29.4); demographic data for one of the nine subjects was not obtained and is missing from these mean values; Table 1]. The hASCs were isolated from the stromal vascular fraction of adipose tissue and cryopreserved in liquid nitrogen after the initial passage at a concentration of 0.5×10^6 cells/ml in 10% dimethylsulphoxide, 10% DMEM/F-12 Ham's (DMEM/F-12), and 80% FBS according to published methods (Goh *et al*., 2007). All protocols were reviewed and approved by the Pennington Biomedical Research Center Institutional Review Board.

2.2. Cell culture

All cells were cryopreserved for a minimum period of >1 month in liquid nitrogen prior to plating (Goh *et al*., 2007). Vials of hASCs were thawed rapidly with vigorous agitation in a 37 °C waterbath washed once with stromal medium (DMEM/F-12, 10% FBS (Hyclone, Logan, UT, USA), 1% antibiotic/antimycotic (MP Biomedicals, Solon, OH, USA) and cultured at 37 °C, 5% CO_2. Following a 48 h expansion, the hASCs were harvested by trypsin digestion and replated at a density of 5000 cells/cm^2 in stromal medium. After 24 h to allow for adherence, the stromal medium was converted to DMEM/F-12 containing 3% FBS and 1% antibiotic/antimycotic and supplemented with EGF (0, 0.1, 1.0 or 10 ng/ml) and/or bFGF (0, 0.1, 1.0 or 10 ng/ml). Cells used in proliferation assays were maintained under these conditions for 7–8 days.

2.2.1. Adipogenic conditions—Cells for GPDH assays, oil red O staining and qRT–PCR were induced for adipogenesis at day 8 of culture with adipogenic medium (DMEM/F-12, 3% FBS, 1% antibiotic/antimycotic, 33 μM biotin, 17 μM pantothenate, 1 μM insulin, 1 μM dexamethasone, 0.5 mM IBMX, 5 μM Rosiglitazone (AK Scientific, Mountain View, CA, USA). After 3 days media was converted to adipogenesis maintenance medium (DMEM/F-12, 3% FBS, 1% antibiotic/antimycotic, 33 μM biotin, 17 μM pantothenate, 1 μM insulin, 1 μM dexamethasone) and cells were fed three times/week (Halvorsen *et al*., 2001; Mitchell *et al*., 2006).

2.3. Cell proliferation assay (*n* = 5 donors)

Cell proliferation was determined on passage 1 hASCs after 7–8 days of conditioning with varying concentrations of EGF and bFGF. Cells from individual wells of a 24-well plate were harvested using 0.05% trypsin in EDTA. An aliquot of cells was stained with Trypan blue, and total number cells per well was determined using a haematocytometer.

2.4. Oil red O staining (*n* = 5 donors)

Cells grown in a 24-well plate for 7 days of preconditioning with varying concentrations of EGF- and bFGF-supplemented conditions were induced for adipogenesis and maintained for up to 9 days. The cells were then washed three times with PBS, fixed in 10% formalin (1 h, 4° C) and stained using oil red O (Halvorsen *et al*., 2001). The plates were rinsed three times with distilled water and photographs were taken of a representative field for each condition at ×100 magnification. In a subset of studies performed on three independent donors, the oil red O stain retained by the hASCs following adipogenesis was eluted by incubation of the well in the presence of isopropanol. Parallel wells incubated under identical conditions were left unstained, harvested and used to determine total cell counts per well. The optical density at 510 nm (OD_{510}) was determined for the eluted oil red O stain per well using a SpectraMax Plux 384 plate reader from Molecular Devices and the values were normalized relative to the total number of cells per well under each culture condition.

2.5. RT–PCR (*n* = 4 donors)

Total RNA was extracted from cells using TRI-Reagent according to the manufacturer's instructions (Molecular Research Center, Cincinnati, OH, USA). Cells cultured in adipogenic medium were harvested 8 days after induction. Real-time PCR was performed in a final reaction volume of 10 μl, including forward and reverse primers (0.1 mM), 1.5 μg reverse-transcribed RNA and 5 μl SYBR green master mix (Applied Biosystems, Warrington, UK), using an ABI Prism 7900 instrument (Applied Biosystems, Foster City, CA, USA). The following forward (F) and reverse (R) primer pairs (Accession Nos given) were employed: *aP2* (NM 001 442), (F) AAAGAAGTAGGAGTGGGCTTTGC; (R) CCCCATTCACACTGATGATCAT; *C/EBPα* (NM 004 364.2), (F) GGGTCTGAGACTCCCTTTCCTT; (R) CTCATTGGTCCCCCAGGAT; *Cyclophilin B* (M60857), (F) GGAGATGGCACAGGAGGAAA; (R) CGTAGTGCTTCAGTTTGAAGTTCTCA; *LPL* (NM 000 237.1), (F) CAGATGCCCTACAAAGTCTTCCA; (R) TGATTGGTATGGGTTTCACTCTCA; *PGC1a* (NM 013 261.2), (F) CCCAAGGGTTCCCCATTT; (R) TTAGGCCTGCAGTTCCAGAGA; *PPARγ* 2 (NM 015 869), (F) AGGCGAGGGCGATCTTG; (R) CCCATCATTAAGGAATTCATGTCATA. The expression levels of each mRNA were normalized to cyclophilin B, which has been used successfully as a housekeeping gene for comparative purposes in both *in vitro* and *in vivo* studies by our laboratory (Wu *et al*., 2007, 2008; Zvonic *et al*., 2006).

2.6. Lipolysis assay (*n* = 2 donors)

The hASCs were seeded in 24-well plates and equal number of wells were preconditioned in the absence or presence of EGF (10 ng/ml) and bFGF (10 ng/ml) for a period of 6 days. At that time, all hASCs were induced with adipogenic medium for 3 days without any EGF or bFGF supplementation and then fed with adipogenic maintenance medium 3 times/week. Twelve days following adipogenic induction, the hASCs were washed with DMEM/F-12 and left overnight in DMEM/F-12 supplemented with 0.1% bovine serum albumin (BSA). The following day, the adipocyte-differentiated hASCs were washed with phosphate-buffered saline, the medium in each well was replaced with 150 μl freshly prepared DMEM/F-12 containing 2% BSA and supplemented with increasing concentrations of isoproterenol (10^{-9}–10^{-5} M; Sigma Chemical Co., St. Louis, MO, USA) or human atrial natriuretic peptide

1–28 (10^{-10}–10^{-6} M; Bachem, King of Prussia, PA, USA) (Moro *et al*., 2004, 2005). Following a 3 h incubation at 37 °C and 5% CO_2, the medium was removed and stored at −20 °C for spectrophotometric assay of glycerol release (Catalogue No. F6428, Sigma-Aldrich, St. Louis, MO, USA) (Moro *et al*., 2005), while the adherent cells were harvested in lysis buffer (150 mM NaCl, 20 mM Tris–HCl, pH 8.0, 1 mM EDTA, 2% SDS, 1% Igepal CA-630) for protein determination (BCA Protein Assay Kit, Pierce, Rockford, IL, USA). Glycerol release was normalized as μM/mg protein/3 h.

2.7. Glucose uptake assay (*n* = 1 donor)

Glucose uptake in hASCs was determined as described by Klip *et al*(1984). Briefly, hASCs were differentiated in 24-well plates in a manner identical to that used for the lipolysis assay. Twelve days following the induction of adipocyte differentiation, the hASCs were incubated overnight in serum-free low-glucose (1000 mg/l) DMEM medium (Catalogue No. SH30021.01, Hyclone, Logan, UT, USA) containing 1% BSA. The following day, the cultures were fed with serum-free medium with or without 100 nM insulin for 10 min at 37 °C, 5% CO_2. The cells were then rinsed twice in KRPH buffer (5 mM Na_2HPO_4, 20 mM HEPES, pH 7.4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 137 mM NaCl and 4.7 mM KCl), and glucose uptake was assessed with 100 μM 2-deoxy-D-glucose [5 μCi/ml (1,2-^{3}H)-2-deoxy-D-glucose from Perkin-Elmer Life Sciences, Boston, MA, USA) in KRPH for 7 min at 37 °C, 5% CO_2. In control wells to monitor for non-specific uptake, 10 μM cytochalasin B was added at the same time as the glucose; subsequently, the non-specific CPM values were subtracted from the experimental points. The cells were then washed three times with PBS and lysed in 500 μl 0.2 N NaOH/well. Aliquots of 400 μl cell lysate were transferred to scintillation vials and radioactivity was counted. An aliquot of 50 μl cell lysate was used to determine protein concentration. The results were normalized by protein concentration and glucose uptake was expressed as ng/mg protein/min. All assays were performed in triplicate.

2.8. Statistics

Data are reported as the mean ± standard deviation (SD). Comparison between culture conditions was determined using Student's *t*-test, where $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of EGF and bFGF on cell proliferation

The addition of EGF and bFGF to the cell culture medium significantly increased the proliferation of cryopreserved hASCs in a dose-dependent manner (Table 2). The two growth factors acted in an additive manner. While hASCs grown in 10 ng/ml bFGF or 10 ng/ml EGF alone increased proliferation by 31% and 195%, respectively, relative to controls without growth factors, hASCs preconditioned in the presence of both 1 ng/ml EGF and 1 ng/ml bFGF increased by a factor of 242% relative to controls. In the absence of growth factors, the cultures achieved near confluency, with a mean of 33 281 hASCs/cm^2, while in the presence of 10 ng/ml bFGF and EGF the cultures reached a mean density of 75 192 hASCs/cm^2.

3.2. Effects of EGF and bFGF on adipogenesis

Preconditioning of hASCs with EGF and bFGF led to subsequent dose-dependent increases in oil red O staining of neutral lipids with adipogenic differentiation (Figure 1). The addition of EGF alone was more effective than equal concentrations of bFGF alone with respect to lipid accumulation (Figure 1, representative of *n* = 4 donors). The effects of the two growth factors were additive, as hASCs preconditioned in 10 ng/ml EGF or bFGF alone had less oil red O staining than hASCs preconditioned with 10 ng/ml of both growth factors. The combined EGF

and bFGF induction of oil red O staining was independent of the effect of the growth factors on cell proliferation (Figure 2). When normalized for cell number, the extracted oil red O following adipogenesis increased by a mean factor of 2.4-, 5.6- and 5.3-fold in cells pre-conditioned in the presence of 0.1, 1.0 or 10 ng/ml of both growth factors relative to unconditioned cells, respectively (n = 3 donors; Figure 2).

Preconditioning with EGF and bFGF induced dose-dependent increases in adipogenesis-related mRNAs (Figure 3). Significant increases occurred in the expression of mRNAs encoding aP2, C/EBPα, lipoprotein lipase (LPL), PPARγ and PPARγ co-activator-1 (PGC1) as a function of preconditioning and adipogenic induction. In the presence of 10 ng/ml EGF and bFGF, the maximum fold increase in mRNA levels relative to hASCs preconditioned in the absence of EGF and bFGF ranged from a low of ~6.6-fold (C/EBPα) to a high of 19-fold (LPL) (Figure 3). In the absence of adipogenic induction, the presence or absence of EGF and bFGF did not significantly increase any of the mRNA expression levels. The mRNA levels of *aP2, C/EBPα, LPL* and *PGC1* in undifferentiated hASCs were expressed at levels three or more orders of magnitude less than that of the adipogenic-induced hASCs, independent of the EGF and bFGF preconditioning (data not shown). While the mRNA levels of *PPARγ* in undifferentiated hASCs showed a similar lack of response to EGF and bFGF preconditioning, the baseline level of the mRNA was higher than that of other adipogenic biomarkers. The *PPARγ* mRNA levels in undifferentiated hASCs were in the range 0.2–0.5-fold of that observed in the adipogenic induced hASCs preconditioned in the absence of EGF and bFGF. Together, these data document that EGF and bFGF preconditioning significantly increased adipogenesis following cryopreservation.

The mature adipocytes generated from hASCs preconditioned with maximal concentrations of EGF and bFGF (10 ng/ml) exhibited functionality based on lipolytic and glucose uptake assays. Atrial natriuretic peptide (ANP) increased lipolysis in a dose-dependent manner, based on glycerol release assay, by a factor of ca. four-fold from hASC adipocytes preconditioned with EGF and bFGF; however, ANP had little inductive effect on lipolysis in hASC adipocytes differentiated without EGF and bFGF preconditioning (Figure 4 Right Panel). In contrast, both populations of adipocytes increased lipolysis in response to the β-adrenergic agonist isoproterenol (Figure 4 Left Panel). Mature hASC adipocytes preconditioned with or without EGF and bFGF displayed comparable baseline levels of 2-deoxyglucose uptake (0.290 ± 0.031 and 0.299 ± 0.022 ng/mg/min, respectively); however, the hASCs preconditioned with EGF and bFGF exhibited a significant induction of glucose uptake (0.415 ± 0.077 ng/mg/min, +43%, p = 0.006) in the presence of insulin, while the unconditioned hASCs did not (0.315 ± 0.055, +5%, p = 0.275).

4. Discussion

This study demonstrates that the use of EGF and FGF supplementation during the expansion culture of undifferentiated cryopreserved hASCs improves their proliferation rate and subsequent adipogenic differentiation and functionality. These actions may be inter-related. The improved proliferative capacity of the cryopreserved hASCs may allow them to achieve a greater density, thereby improving their subsequent adipogenic differentiation upon exposure to inductive agents. The additive effects of EGF and bFGF permit their use in combination at low concentrations to achieve significant results, as opposed to the more costly use of high concentrations of a single growth factor alone. Our results support the original studies by Hauner *et al*. (1995), which found that EGF had notable effects on the growth and development of hASCs at physiological concentrations (0.5–2 ng/ml). Moreover, our findings confirm and extend a recent report by Suga *et al*. (2007), which demonstrated that the individual components of a commercial endothelial growth medium (EGF and bFGF) elicited less robust hASC proliferation than the endothelial growth medium itself. Likewise, studies in the murine 3T3-

L1 preadipocyte model have demonstrated that EGF supplementation, when added subsequent to induction, enhanced adipogenesis (Adachi *et al*., 1994). Finally, these studies extend those of Shoshani *et al*. (2001) and Moscatello *et al*. (2005), which demonstrated the feasibility of maintaining viable intact human adipose tissue by cryopreservation. Observations made using the human bone marrow MSC model are comparable. Tamama *et al*. (2006) found that EGF supplementation increased the expansion of BMSCs for *in vivo* transplantation. Martin *et al*. (1997) observed that bFGF increased the size of individual BMSC clones by 2.5-fold *in vitro*, consistent with enhanced proliferation. Furthermore, they found that bFGF preconditioning increased the ability of BMSCs to mineralize their extracellular matrix *in vitro* and to form bone *in vivo*, suggesting that bFGF preconditioning enhanced the differentiation potential of the BMSCs (Martin *et al*., 1997). Recently, Stewart *et al*. (2007) have shown that chondrogenesis was enhanced by bFGF in equine BMSCs. Thus, these growth factors have been found to modulate ASC and BMSC proliferation and/or differentiation in multiple species.

Stromal medium supplementation with EGF and bFGF maintained the differentiation potential of cryopreserved hASCs following expansion and enhanced their subsequent adipogenic response. A dose-dependent increase in qualitative oil red O staining was observed in treated hASCs when compared to their control. PCR analysis in these same experiments demonstrated EGF- and bFGF-dependent increases in mRNA levels encoding adipogenic transcription factors (C/EBPα, PPARγ, PGC1) as well as their downstream targets (aP2, LPL). In addition to providing improved adipogenesis, EGF and bFGF supplementation produced adipocytes with equivalent or improved functionality relative to controls. The hASC-differentiated adipocytes preconditioned with EGF and bFGF displayed enhanced insulin sensitivity based on glucose uptake. While the lipolytic response to a β-adrenergic agent was equivalent in the treated and untreated hASCs, the treated hASCs displayed an enhanced lipolytic response to ANP. Recent studies have demonstrated that ANP, like isoproterenol, is a potent lipolytic stimulus (Moro *et al*., 2004, 2005). It remains to be determined whether EGF and bFGF act at the level of the ANP receptor or of downstream elements in its signal transduction pathway. Nevertheless, these findings demonstrate that the inclusion of EGF and bFGF during hASCs expansion improve their adipogenic response and the potential functionality of the differentiated adipocytes *in vitro*.

In conclusion, EGF/bFGF preconditioning at concentrations in the range 1–10 ng/ml prior to, but not during, induction of differentiation led to a rapid expansion of hASCs and improved their subsequent adipogenic differentiation. Further studies will be necessary to fully characterize the *in vitro* and *in vivo* effects of EGF and bFGF prior to using the two growth factors in clinical applications, but the above lines of evidence support the use of EGF and bFGF as media supplements to improve cell proliferation and adipogenesis of thawed, cryopreserved hASCs.

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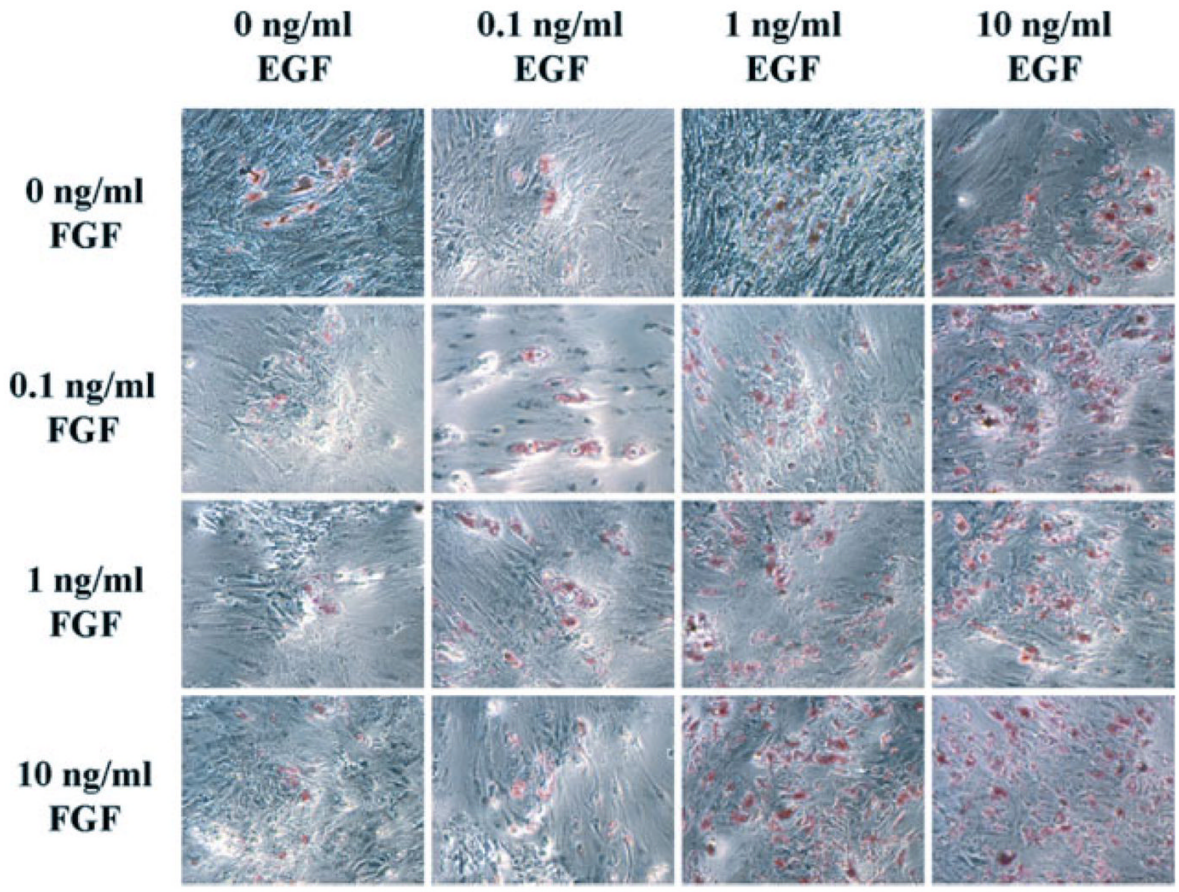

Figure 1.
Effects of growth factors on adipogenesis. Photomicrographs of cells from one representative donor grown in 16 different EGF- and bFGF-supplemented conditions (EGF concentrations on *x* axis, bFGF concentrations on *y* axis) prior to induction of adipogenesis. Similar photomicrographs taken for three other donors are not shown. The cells are stained with oil red O. Magnification, ×100

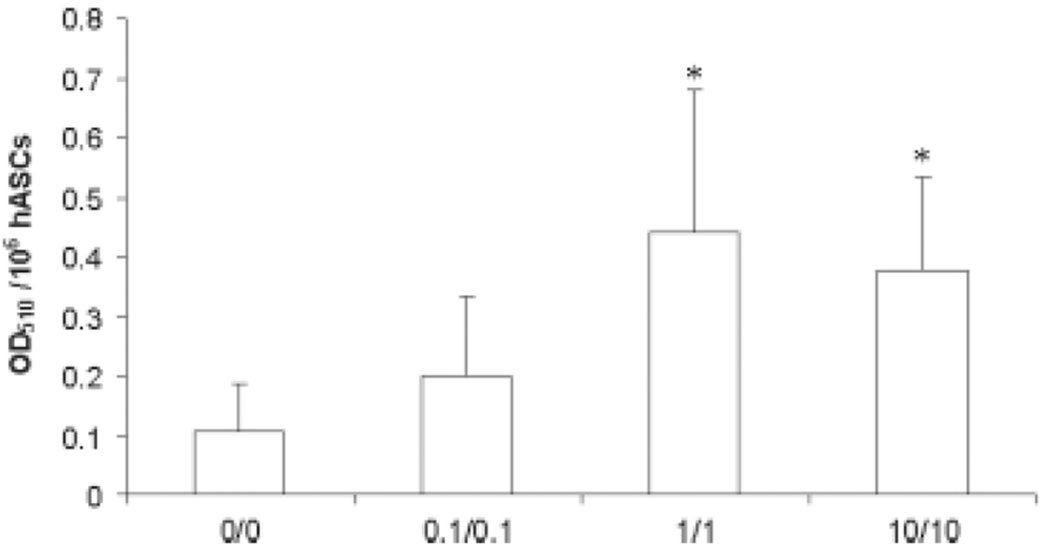

Figure 2.

Effects of growth factors on oil red O staining. Human ASCs from $n = 3$ donors were preconditioned for 8 days in the absence or presence of EGF and bFGF at concentrations of 0.1, 1.0 or 10 ng/ml of both reagents. The hASCs under the different conditions were then induced to undergo adipogenesis. Following adipogenic differentiation for 9 days, the hASCs were stained with oil red O. The retained dye was eluted from the adherent cells and quantified by absorbance reading at OD_{510}. Unfixed and unstained representative wells maintained under each of the EGF/bFGF preconditions and under the subsequent adipogenic culture condition were trypsinized and the total number of cells per well determined. The mean ± SD ($n = 3$ donors) oil red O absorbance was normalized relative to 10^6 hASCs, as indicated on the *y* axis. The OD_{510} values are displayed relative to the preconditioning EGF/bFGF concentrations (*x* axis)

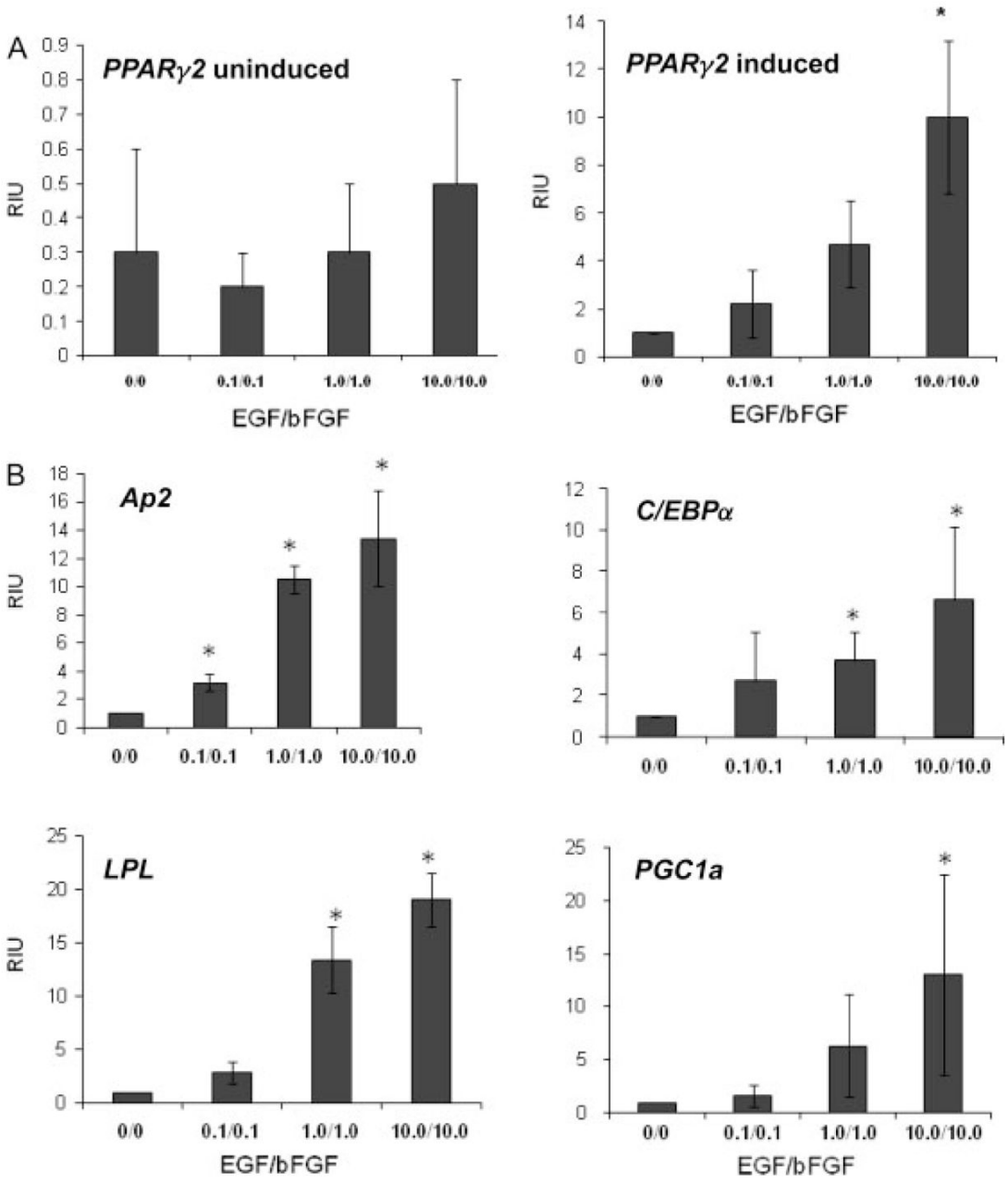

Figure 3.

Effects of growth factors on adipogenic mRNA levels. Results of quantitative PCR analysis for cells grown in EGF- and bFGF-supplemented conditions prior to the induction of adipogenesis. Quantities were normalized to cyclophilin B. Values are the mean ± SD fold increase for $n = 3$ donors. (A) Values for *PPARγ2* mRNA from hASCs preconditioned with the indicated levels of EGF/bFGF in the absence (left panel) and presence (right panel) of adipogenic induction. All levels are expressed as fold increase relative to the level of hASCs preconditioned in the absence of EGF/bFGF and induced for adipogenesis; this value is defined as '1' relative inductive unit (RIU) on the *y* axis. (B) Relative mRNA values for the adipogenic biomarkers *aP2* (fatty acid binding protein 4), *C/EBPα* (CAAT/enhancer binding protein *α*), *LPL* (lipoprotein lipase), and *PGC1* (*PPARγ* co-activator 1) in hASCs induced to undergo

adipogenesis. All mRNA values for these same biomarkers in hASCs without adipogenic induction were two or three orders of magnitude lower (data not shown). Values on the *x* axis refer to concentrations of EGF/bFGF in ng/ml. Statistical significance relative to the hASC preconditioned in the absence of EGF and bFGF and induced to undergo adipogenesis is indicated by $^{*}(p < 0.05)$

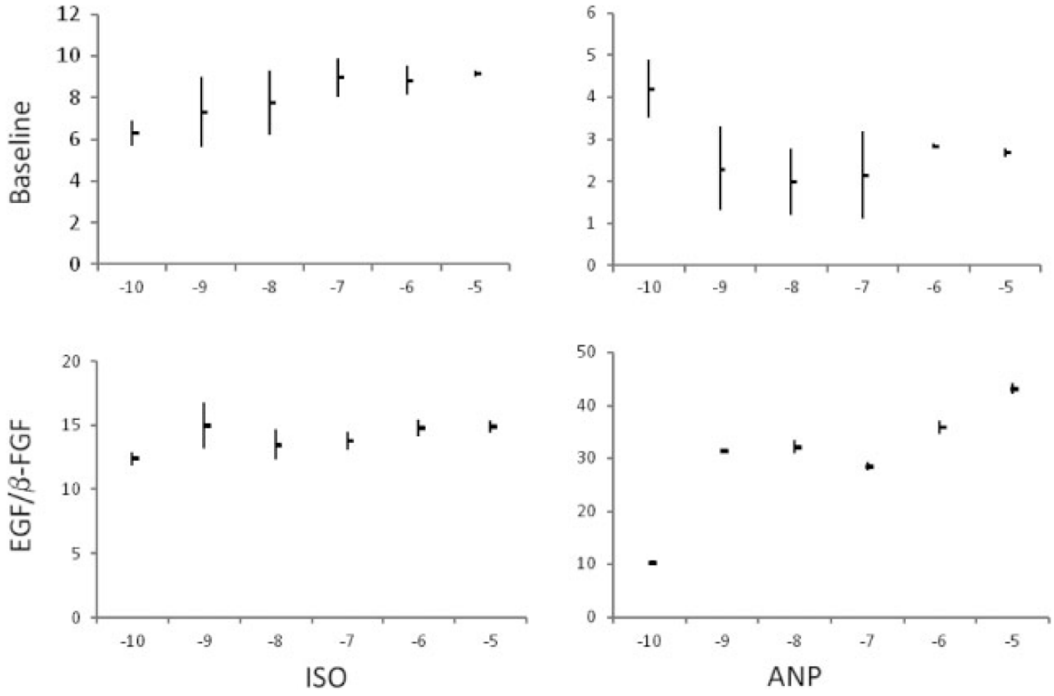

Figure 4.

Effects of EGF and FGF on lipolysis. Lipolytic response of adipocytes preconditioned prior to adipogenesis in the absence (top panel) or presence (bottom panel) of 10 ng/ml EGF and 10 ng/ml FGF were compared in response to increasing concentrations of ANP (right panels) and isoproterenol (left panels). Average lipolysis (with maximum and minimum values) for $n = 2$ donors is shown

Table 1

Donor demographics

Donor	Race	Gender	Age	BMI	Use
1	Caucasian	Female	32	26.17	Cell count
2	Caucasian	Female	36	28.17	Cell count
3	Caucasian	Female	32	28.72	Cell count, PCR, GPDH
4	Caucasian	Female	40	23.32	Cell count, PCR, GPDH
5	Caucasian	Female	62	24.23	PCR, GPDH, lipolysis
6	Caucasian	Female	33	21.63	PCR, GPDH, lipolysis, glucose uptake
7	Caucasian	Female	33	29.4	Cell count, oil red O staining
8	ND	Female	ND	ND	Cell count, oil red O staining
9	Caucasian	Female	27	23.19	Cell count, oil red O staining
Mean ± SD			36.9 ± 10.8	25.6 ± 2.9	

ND, not determined.

Table 2

hASC proliferation in response to EGF and bFGF

bFGF\EGF (ng/ml)	0 ng/ml	0.1 ng/ml	1.0 ng/ml	10 ng/ml
0	1.00 ± 0.72	1.92 ± 0.81	2.39 ± 1.06	2.95 ± 0.77
0.1	0.97 ± 0.56	1.97 ± 0.50	2.94 ± 1.14	3.06 ± 0.64*
1.0	1.29 ± 0.65*	2.22 ± 0.36**	3.42 ± 1.20**	3.07 ± 0.82*
10	1.31 ± 0.72*	2.49 ± 0.92**	3.40 ± 0.92**	3.40 ± 1.04**

Mean values of fold increase ± SD from control of mean number of cells per well are shown (n = 4 donors).

* $p < 0.05$ or

** $p < 0.01$ relative to 0 ng/ml bFGF and 0 ng/ml EGF based on paired, 1-tailed t-test.