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The Effects of Epidermal Growth Factor and Basic Fibroblast Growth Factor on Cryopreserved Human Adipose Derived Stem Cells in Culture: Proliferation, Adipogenesis, and Osteogenesis

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The Effects of Epidermal Growth Factor and Basic Fibroblast Growth Factor on Cryopreserved Human Adipose Derived Stem Cells in Culture: Proliferation, Adipogenesis, and Osteogenesis

by

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TABLE OF CONTENTS

ABBREVIATIONS:

ALP, alkaline phosphatase

aP2, adipocyte P2 protein

bFGF, basic fibroblast growth factor

BMSCs, bone marrow-derived mesenchymal stem cells

C/EBP, CAAT/enhancer binding protein

EGF, epidermal growth factor

GPDH, glycerol phosphate dehydrogenase

hASCs, human adipose-derived stem cells

HGF, hepatocyte growth factor

IBMX, isobutylmethylxanthine

LPL, lipoprotein lipase

PGC1, PPARγ Co-Activator 1

PPARγ, peroxisome proliferator activator receptor γ

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Adipose-derived stem cells (ASCs) are a model tool in the study of animal metabolism, disease, and injury because of their abundance and accessibility. These cells also apply to certain tissue engineering aspects of regenerative medicine. The study and clinical use of ASCs requires both expanding the small number of cells harvested from fat tissue and cryopreserving the cells for future use; however, these manipulations weaken their proliferative capacity and differentiation potential.

Previous studies have demonstrated the abilities of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) to maintain the stem cell properties of human adipose-derived stem cells (hASCs) during proliferation in culture; however, the effects of EGF and bFGF on the in vitro behavior of hASCs has not been investigated extensively. This study examined the changes in proliferation and differentiation of cryogenically preserved hASCs after expansion with bFGF and EGF at varying concentrations.

Relative to the control, cryopreserved cells cultured in the presence of EGF and bFGF demonstrated significantly increased proliferation. Furthermore, hASCs expanded in the presence of EGF and bFGF prior to induction of adipogenesis displayed increased Oil Red O staining and significantly increased levels of several adipogenesis-related mRNAs, such as: adiponectin, aP2, C/EBPα, lipoprotein lipase, PPARγ, and PPARγ Co-activator-1 (PGC1). Following adipogenic induction, EGF and bFGF cultured hASCs gave rise to more functional adipocytes, as demonstrated by insulin stimulated glucose uptake and atrial naturetic peptide (ANP) stimulated lipolysis. In contrast, the absolute activity of the adipogenic enzyme marker, glycerol phosphate dehydrogenase, was not significantly induced.

The effects of EGF and bFGF on osteogenic differentiation were demonstrated by subtle increases in Alizarin Red staining and alteration of several osteogenesis-related mRNAs in EGF and bFGF treated cells when compared to the control, but further experiments will be required to interpret these effects.

These findings indicate that low concentrations of bFGF and EGF in combination can be used as culture supplements to improve the proliferative capacity of cryopreserved human ASCs and their adipogenic differentiation potential. These agents merit consideration for inclusion in ASC expansion media in basic research and clinical protocols.

1. INTRODUCTION & BACKGROUND

1.1 Introduction to Adipose-Derived Stem Cells

Stem cells have the unique ability to maintain a prolonged undifferentiated, or nonspecific, state and then give rise to, or differentiate into, various cell types [Korbling 2003, NIH 2006]. While stem cells found in adult tissue are less flexible in their differentiation potential when compared to embryonic stem cells, adult stem cells bypass ethical concerns and have expanded their status in the field of stem cell research. Adult stem cells are minimally active in mature tissues, but can be stimulated to proliferate and differentiate if they are needed for tissue repair or renewal [Gimble 2003, Korbling 2003]. Adult stem cells have been found in many tissues throughout the body, such as muscle, skin, lung, intestine, CNS, heart, and fat tissue, as well as in bone marrow, peripheral blood, the liver, and the pancreas [Parker 2006].

Adipose-derived stem cells (ASCs) are multipotent stem cells present in the fat tissue of adults. ASCs are considered multipotent because they have the ability to differentiate into various mature cell types when given the correct stimuli, and precise culture conditions can be used to stimulate a specific differentiation in vitro. This study focuses on adipogenesis, the conversion of ASCs into adipocytes (fat cells), and osteogenesis, the conversion of ASCs into osteocytes (bone cells), but ASCs can also undergo chodrogenesis to form chondrocytes (cartilage cells), myogenesis to form myocytes (muscle cells), and have limited capabilities to form myocardiocytes (heart muscle cells), neurons, hepatocytes (liver cells), and endothelial cells [Parker 2006]. This ability of a stem cell derived from one tissue to differentiate into cells of another tissue is referred to as stem cell plasticity [NIH 2006].

Adipose-derived stem cells are isolated from the other components of adipose tissue by a relatively simple process. Fat tissue is washed with a buffer and broken apart using an enzyme

called collagenase, which breaks down the collagen fibers that hold tissue together. Once the tissue is digested, the stem cells, also called the stromal vascular fraction, are separated from mature fat cells via centrifugation. From this point, the stromal cells can be cultured in artificial medium or frozen away for future use [Dubois 2008].

1.2 The Advantages of Studying ASCs and Their Significance to Medicine

As a source for stem cells, adipose tissue has numerous advantages. Fat is abundant in supply, renewable, and easily accessed [Gimble 2007, Parker 2006]. One study shows that adipose-derived stem cells are 500 times more abundant in adipose tissue than bone marrow-derived mesenchymal stem cells are in bone marrow [Fraser 2006]. In contrast to the extraction of bone marrow stromal cells, which requires a relatively painful procedure, adipose-derived stem cells can be harvested from fat tissue without difficulty by the minimally invasive procedures of liposuction, in which fat depots are suctioned out of the body, and abdominoplasty, in which excess fat and skin is removed from the abdomen by incisions [Gomillion 2006]. Liposuction is a common and relatively safe cosmetic procedure. According to the American Society for Aesthetic Plastic Surgery, liposuction is the most common cosmetic surgery in the US—455,489 lipoplasty procedures and 169,314 abdominoplasty procedures were performed in 2005 [ASAPS 2005]. A 2002 national survey reviewing 66,570 liposuction procedures reported no deaths and only 0.68 per 1000 serious adverse events [Housman 2002].

Another advantage of studying ASCs is that the ethical concerns surrounding stem cell research are limited in connection with stem cells isolated from adult fat tissue. Unlike embryonic stem cell research, ASC research does not require embryos to be compromised or gametes to be manipulated.

The abundance and plasticity of adult human adipose-derived stem cells also permits their use in cell-based therapy and regenerative medicine, in which stem cells are differentiated into a specific cell type and used to repair damaged tissue in vivo [Gimble 2007, NIH 2006]. Advances in stem cell research have demonstrated the potential application of hASCs for autologous fat transplants, in which the donor is also the recipient, in reconstructive surgery and repair of various tissue defects caused by disease or injury [Gimble 2007, Gimble 2003, Vashi 2006]. It is also speculated that hASCs can be used to repair damaged cartilage [Estes 2008]. Because adipose tissue renews itself, it can be said that each person has their own reservoir of stem cells, which will be particularly useful in the future of regenerative medicine.

In addition, exploring the in vitro properties of ASCs provides insight into the in vivo roles of ASCs involved in animal metabolism, disease, and injury. For example, studies of hASCs have contributed to the current knowledge on circadian rhythm, diabetes, and osteoporosis [Gimble 2005, Nuttall 200, Zvonic 2007].

1.3 Cryopreservation of ASCs

The standard use of hASCs in clinical practice is imminent; however, techniques for expanding, preserving, and inducing such cells must be optimized before adipose-derived stem cell therapies become an established and economical practice. In basic research and clinical protocols, it is necessary to cryopreserve hASCs in liquid nitrogen for future use; however, cryopreservation creates an obstacle. Unlike bone marrow-derived mesenchymal stem cells, which can be cryopreserved without compromising cell viability or osteogenic potential [Kotobuki 2004], ASCs exhibit reductions in viability, proliferation, and differentiation following cryopreservation [De Boer 2002, Keung 1996, Stylianou 2006].

Efforts have been made to optimize cryopreservation by altering the chemicals in which ASCs are frozen or the concentration at which ASCs are frozen [Devireddy 2005, Goh 2007, Thirumala 2005, Thirumala 2005], yet adipogenic differentiation potential depreciates in some lots of hASCs after they have been cryopreserved. There is currently no standard procedure for expanding hASCs following cryopreservation. Many laboratories maintain hASCs in Stromal Medium (Dulbecco's Modified Eagles Medium/F-12 Hams supplemented with 10% Fetal Bovine Serum and 1% antibiotics/antimycotics) [Mitchell 2006], but other laboratories have successfully utilized commercial medias containing polypeptide growth factors, such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), to improve the post-thaw performance of hASCs [Rehman 2004, Miranville 2004, Suga 2007].

1.4 The Promising Effects of bFGF and EGF are Demonstrated by Previous Studies

Polypeptide growth factors, such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), have the potential to optimize cell proliferation and improve differentiation in hASCs that have been cryopreserved. Previous studies have speculated a synergy between EGF and bFGF [Hauner 1995, Suga 2007], and many growth mediums contain either one or both of the growth factors, but the dose-dependent effects of EGF and bFGF in combination have not been examined closely.

The role of polypeptide growth factors in adipose tissue function is not extensively understood, and the effects of EGF and bFGF are controversial and vary greatly among species; however, numerous studies demonstrate that EGF and bFGF increase cell proliferation and alter differentiation potential [Butterwith 1993, Hauner 1995, Tamama 2006, Zarogosi 2006]. EGF is a single-chain polypeptide noted for having proliferative and differentiating effects on many mammalian tissues [Hauner 1995, Kurachi 1993]. EGF enhances migration and cell

proliferation and maintains differentiation potential of bone marrow-derived mesenchymal stem cells [Tamama 2006]. EFG has been shown to significantly affect in vitro adipocyte development at concentrations comparable to the physiological concentration of EGF in human serum [Hauner 1995]. Also, a correlation has been established between increasing levels of EGF and the induction of obesity in ovariectomized mice [Kurachi 1993].

Basic FGF, or FGF2, is a mitogen and chemoattractant that enhances angiogenesis, the formation of blood vessels, and migration of bone marrow-derived mesenchymal stem cells [Hauner 1995, Schmidt 2006, Vashi 2006]. By inhibiting FGF2 receptor signaling, Zarogosi et. al. have deomonstrated that hASCs express FGF2 in an autocrine loop that is necessary for maintaining differentiation potential [Zarogosi 2006]. FGF has also been demonstrated to inhibit osteogenesis of hASCs and reduce mineralization and alkaline phosphatase activity during osteogenic induction [Quarto 2006].

1.5 Aim and Expectations

A recent study demonstrated that EGF and bFGF induced secretion of the cytokine hepatic growth factor (HGF), which can function in an autocrine manner due to the expression of the HGF receptor (c-Met) on the surface of hASCs [Kilroy 2007]. A related study found that the expression of HGF in mesenchymal stem cells derived from human umbilical cord blood correlated to enhanced proliferation and adipogenic differentiation potential in vitro [Markov 2007]. This observed ability of EGF and bFGF to increase cell proliferation and preserve differentiation potential, in conjunction with the additive effects of the growth factors observed in previous experiments, led to the exploration of their effects on hASCs in further detail. It is hypothesized that the addition of EGF and bFGF to stromal medium during expansion will enhance proliferation and differentiation potential of cryopreserved hASCs. The objective of

this is study is to identify EGF/bFGF concentrations that optimize cell proliferation, and to characterize the effects of EGF and bFGF on adipogenesis and osteogenesis. Hence, this study examines the additive effects of EGF and bFGF with respect to cell proliferation, adipogenesis, and osteogenesis of hASCs.

2. MATERIALS AND METHODS

2.1 Human Subjects

Adult human lipoaspirates were obtained with consent from 6 female patients (mean age: 39.2 years, range 32-62 years, mean BMI 25.4, range 21.6-28.7) (Table 1). The hASCs were isolated from the stromal vascular fraction of adipose tissue and cryopreserved after the initial passage at a concentration of 0.5 X 10^6 cells per ml in 10% dimethylsulfoxide, 10% Dulbecco's Modified Eagles Medium/F-12 Hams (DMEM/F-12), and 80% fetal bovine serum according to published methods [Goh 2007]. All protocols were reviewed and approved by the Pennington Biomedical Research Center Institutional Review Board.

Table 1. Donor Statistics

DONOR	RACE	GENDER	AGE	BMI	USE
1	Caucasian	Female	32	26.17	Proliferation
2	Caucasian	Female	36	28.17	Proliferation
3	Caucasian	Female	32	28.72	Proliferation, Differentiation, GPDH
4	Caucasian	Female	40	23.32	Proliferation, PCR, GPDH
5	Caucasian	Female	62	24.23	PCR, GPDH
6	Caucasian	Female	33	21.63	PCR, GPDH
Average			39.2	25.4	

2.2 Cell Culture Conditions

All cells were cryopreserved prior to plating. Vials of hASCs were thawed rapidly with vigorous agitation in a 37^{o}C water bath, washed once with Stromal Medium (DMEM/F-12, 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 1% antibiotic/antimycotic (MP Biomedicals, Solon, OH)), and cultured at 37^{o}C, 5% CO_2. Following a 48 hour expansion, the hASCs were harvested by trypsin digestion and re-plated at a density of 5000 cells/cm^2 in Stromal Medium. After 24 hours 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, or 10

ng/ml) and bFGF (0, 0.1, 1, or 10 ng/ml). Cells used in proliferation assays were maintained under these conditions for 7 to 8 days. 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, 33μM Biotin, 17μM Pantothenate, 1μM Insulin, 1μM Dexamethasone, 0.5mM IBMX, 5 μM Rosiglitazone (AK Scientific, Mountain View, CA). After 3 days media was converted to adipogenesis Maintenance Medium (DMEM/F-12, 3% FBS, 1% antibiotic/antimycotic, 33μM Biotin, 17μM Pantothenate, 1μM Insulin, 1μM Dexamethasone) and cells were fed 3 times per week [Mitchell 2006, Halvorsen 2001]. Cells used in Alizarin Red staining and qRT-PCR were induced for osteogenesis on day 8 using Osteogenic Medium (DMEM/F-12, 10% FBS, 1% antibiotic/antimycotic, 10 mM β-Glycerophosphate, 0.1 μM Dexamethasone, 50 ng/ml Ascorbic Acid 2-Phosphate) and fed 3 times per week [Mitchell 2006].

2.3 Cell Proliferation Assay

Cell proliferation was determined 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 hematocytometer.

2.4 Comparing Adipogenic Differentiation

Cells grown in a 24-well plate for 7 days of pre-conditioning with varying concentrations of EGF and bFGF supplemented conditions were induced for adipogenesis for 3 days and maintained for an additional 6 days. Cells were then washed three times with PBS, fixed in 10% formalin for 1 hour at 4°C, and stained using Oil Red O [Halvorsen 2001]. Plates were rinsed

gently three times with distilled water, and photographs were taken of a representative field for each condition.

2.5 Determination of GPDH Activity

The glycerol phosphate dehydrogenase (GPDH) activity of the adipocytes was measured using a modified version of published methods [Kozak 1974, Wise 1979] . Following adipogenesis, cells were washed twice with PBS, extracted in a lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 1 mM 2-mercaptoethanol (2-ME)), scraped from the plate, and passed through a 25 gauge syringe to ensure cell lysis. The lysate was then centrifuged 15 min (4° C, 12,000 x rpm), and the supernatant was isolated. Assay solutions had a reaction volume of 100 µl and a final concentration of 100 mM Tris-HCl, 2.5 mM EDTA, 0.1 mM 2-ME, 0.12 mM NADH, and 0.2 mM dihydroxyacetone phosphate with 10 µl cell extract. The absorbance of individual samples was measured immediately at 340 nm every 10 sec for 5 min using a Beckman coulter DU640 spectrophotometer. Purified α-glycerophosphate dehydrogenase from rabbit muscle (Sigma, St. Louis, MO) was used as a standard. Protein assays were used to normalize samples to protein content (BCA protein assay kit, Pierce, Rockford, IL).

2.6 RNA isolation and Real Time PCR

Quantitative polymerase chain reaction (qPCR) was used to amplify and quantify specific complementary DNA molecules. Total RNA was extracted from cells using TRI-Reagent according to the manufacturer's instructions (Molecular Research Center, Cincinnati, OH). Cells cultured in Adipogenic Medium were harvested 8 days after induction. To study the effects of EGF and bFGF on osteogenesis, cells expanded with EGF/bFGF were harvested prior to osteogenic induction, as well as after 8 days of treatment with either Osteogenic Medium or Stromal Medium. 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). See table below for the specific sequences of primer pairs employed in this study.

Table 2: Primer Sequences

Gene	Accession Number	Forward (F) and Reverse (R) Primer Pairs
Adiponectin	NM_004797	F: GGCCGTGATGGCAGAGAT R: TTTCACCGATGTCTCCCTTAGG
ALP	NM_000478.3	F: AATATGCCCTGGAGCTTCAGAA R: CCATCCCATCTCCCAGGAA
aP2	NM_001442	F: AAAGAAGTAGGAGTGGGCTTTGC R: CCCCATTCACACTGATGATCAT
C/EBPα	NM_004364.2	F: GGGTCTGAGACTCCCTTTCCTT R: CTCATTGGTCCCCCAGGAT
Cyclophilin B	M60857	F: GGAGATGGCACAGGAGGAAA R: CGTAGTGCTTCAGTTTGAAGTTCTCA
LPL	NM_000237.1	F: CAGATGCCCTACAAAGTCTTCCA R: TGATTGGTATGGGTTTCACTCTCA
PGC1a	NM_013261.2	F: CCCAAGGGTTCCCCATTT R: TTAGGCCTGCAGTTCCAGAGA
PPARγ2	NM_015869	F: AGGCGAGGGCGATCTTG R: CCCATCATTAAGGAATTCATGTCATA
Osteocalcin	NM_199173.2	F: GCCCAGCGGTGCAGAGT R: TAGCGCCTGGGTCTCTTCAC
Osteonectin	NM_003118	F: GCGGGACTGGCTCAAGAAC R: GATCTTCTTCACCCGCAGCTT
Osteopontin	NM_001040058.1	F: CAGCCTTCTCAGCCAAACG R: GGCAAAAGCAAATCACTGCAA

2.7 Lipolysis Assay

The hASCs of two donors were seeded in 24 well plates. 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

per 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 PBS, the medium in each well was replaced with 250 µl freshly prepared DMEM/F-12 containing 2% BSA and supplemented with increasing concentrations of isoproterenol (10^{-9} to 10^{-5} M) (Sigma Chemical Co., St. Louis, MO) or human atrial natriuretic peptide 1-28 (10^{-10} to 10^{-6} M) (Bachem, King of Prussia, PA) (29, 30). Following a 3 hour incubation at 37°C and 5% CO_2, the medium was removed and stored at -20 °C for spectrophotometric assay of glycerol release (Sigma Chemical Co., St. Louis, MO) 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). Glycerol release was normalized as µmol/mg protein/3 hrs.

2.8 Glucose Uptake Assay

Glucose uptake in hASCs of one donor was determined as described by Klip et al [Klip 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 medium 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. 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, Co., Boston, MA) in KRPH for 7 min at 37 ° C, 5% CO_2. In control wells to monitor for nonspecific 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. Cells were then washed three times with PBS and lysed in 500 µl of 0.2 N NaOH per well. Aliquot of 400 µl cell lysate was transferred to scintillation vials and radioactivity was counted. An aliquot of 50 µl cell lysate was used to determine protein concentration. Results were normalized by protein concentration and glucose uptake was expressed as nM/mg protein/min. All assays were performed in triplicate.

3. RESULTS

3.1 Effects of EGF and bFGF on Cell Proliferation

The addition of EGF and bFGF to cell culture medium significantly increased the proliferation of cryopreserved hASCs in a dose-dependent manner (Figure 1, Table 3). The two growth factors acted in an additive manner. While hASCs grown in 10ng/ml bFGF or 10ng/ml EGF alone increased by 1.31-fold and 2.95-fold , respectively, relative to controls, hASCs preconditioned in the presence of both 1ng/ml EGF and 1ng/ml bFGF increased by a factor of 3.42-fold relative to controls.

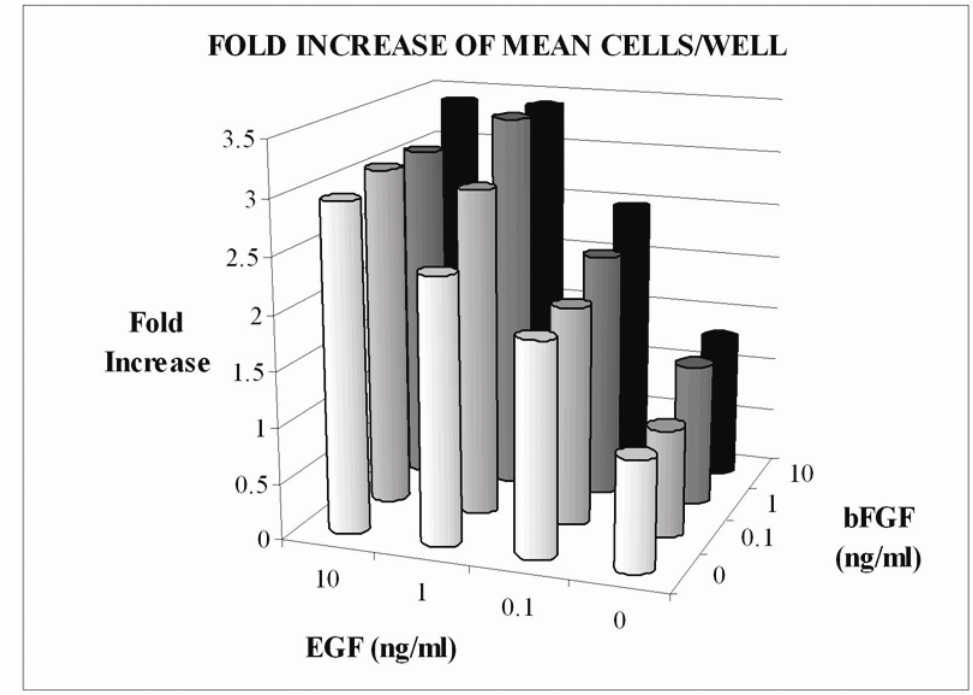


Figure 1: Effect of growth factors on cell proliferation. The graph depicts the fold increase from control of the mean number of cells per well for each growth medium condition for n = 4 donors.

Table 3: hASC proliferation in response to EGF and bFGF supplementation

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

3.2 Effects of EGF and bFGF on Adipogenesis

Preconditioning hASCs with EGF and bFGF led to subsequent dose-dependent increases in Oil Red O staining of neutral lipids with adipogenic differentiation (Figure 2). Oil Red O stain incorporates into lipid droplets accumulated by mature adipocytes, thus the presence of red stain indicates mature adipocytes. The addition of EGF alone was more effective than equal concentrations of bFGF alone with respect to lipid accumulation. The effects of the two growth factors appear to be additive, as hASCs preconditioned in 10ng/ml of EGF or bFGF alone had less Oil Red O staining than hASCs preconditioned with 10ng/ml of both growth factors.

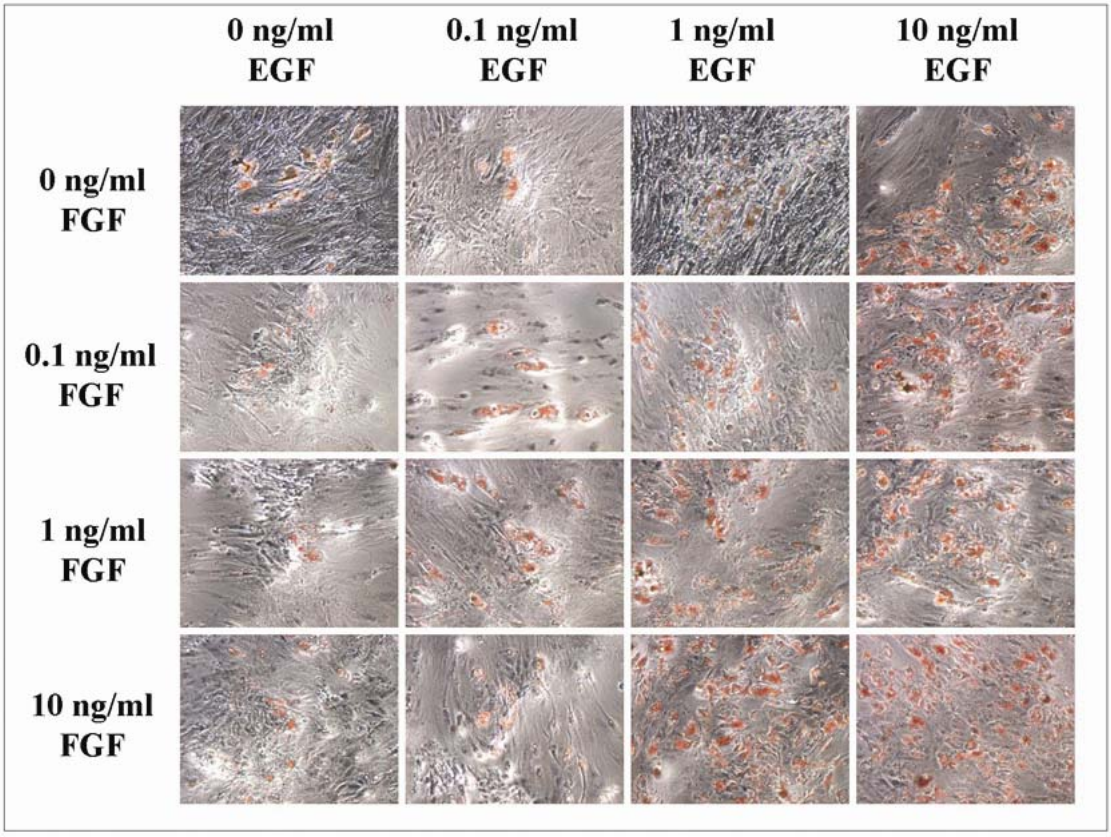


Figure 2: Effects of growth factors on adipogenesis. Photomicrographs of adipocytes from one representative donor grown in 16 different EGF and bFGF supplemented conditions (EGF concentrations on X-axis, bFGF concentrations on Y-axis) prior to induction of adipogenesis. Similar photomicrographs taken for 3 other donors are not shown. Cells are stained with oil red O and microphotographs were taken at a magnification of x100.

Preconditioning with EGF and bFGF induced dose-dependent increases in adipogenesis-related mRNAs (Figure 3). Significant increases occurred in the expression of mRNAs encoding genes correlated to adipogenesis, such as adiponectin, aP2, C/EBPα, lipoprotein lipase (LPL), PPARγ, and PPARγ Co-activator-1 (PGC1); it should be noted that the fold-induction of the PPARγ transcriptional targets, aP2 and LPL, is reported in log scale. In the presence of 10 ng/ml of EGF and bFGF, the maximum –fold increase in mRNA levels ranged from a low of ~3-fold (PPARγ) to a high of ~2-orders of magnitude (aP2); thus, EGF and bFGF preconditioning increased adipogenesis relative to the adipogenic control in the absence of these growth factors.

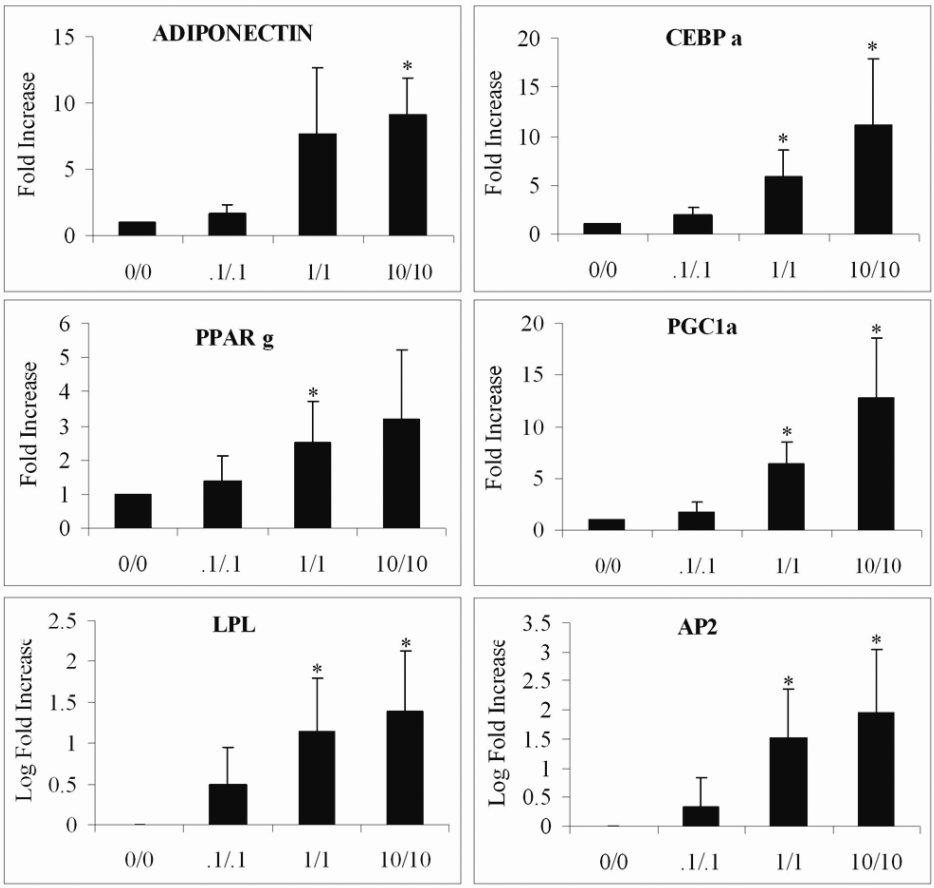


Figure 3. Results of quantitative PCR analysis for cells grown in EGF and bFGF supplemented conditions prior to induction of adipogenesis. Quantities were normalized to Cyclophilin B. Values are the mean ± S.D. of fold increase for each growth medium condition of 4 donors (n=3 for adiponectin). X-axis refers to concentrations of EGF/bFGF in ng/ml. P values are indicated by * ($p<0.05$).

In contrast, preconditioning with EGF and bFGF prior to adipogenic induction did not significantly increase GPDH activity (Table 4). Mean GPDH units/ng protein increased from a mean of 0.20 units in the control to 0.42 units in cells preconditioned in 10ng/ml EGF/bFGF. This increase is not significant according to Student's t-tests (p value = 0.5). A p-value is a measure of the probability that two figures or groups arose from the same data set by chance, and it is generally accepted that a p-value must be below 0.05 for two figures or groups to be significantly different [NCI 2008]. The increases in GPDH observed are, therefore, not significant.

Table 4: GPDH Assay Results

UNITS GPDH PER ng PROTEIN		
EGF/bFGF	**MEAN** ± S.D.	**P value**
0/0ng/ml	0.199± 0.108	
.1/.1ng/ml	0.173 ± 0.195	Not Significant
1/1ng/ml	0.316 ± 0.176	Not Significant
10/10ng/ml	0.423 ± 0.310	Not Significant

The mature adipocytes generated from hASCs preconditioned with 10ng/ml of EGF and bFGF exhibited functionality based on lipolytic and glucose uptake assays, indicating that cells preconditioned with EGF and bFGF prior to adipogenic induction maintain the ability to lyse or take up glucose when they are stimulated to do so. Atrial naturetic peptide (ANP) increased lipolysis in a dose dependent manner based on glycerol release assay by a factor of ~4-fold from hASC adipocytes preconditioned with EGF and bFGF; however, ANP had little or no effect on lipolysis in hASC adipocytes differentiated without EGF and bFGF preconditioning (Figure 4 A). In contrast, both populations of adipocytes increased lipolysis in a dose-dependent manner in response to the β-adrenergic agonist, isoproterenol (Figure 4B). Mature hASC adipocytes

preconditioned with or without EGF and bFGF displayed comparable baseline levels of 2-deoxy glucose uptake; however, only the hASCs preconditioned with EGF and bFGF exhibited a significant induction of glucose uptake in the presence of insulin (Figure 5).

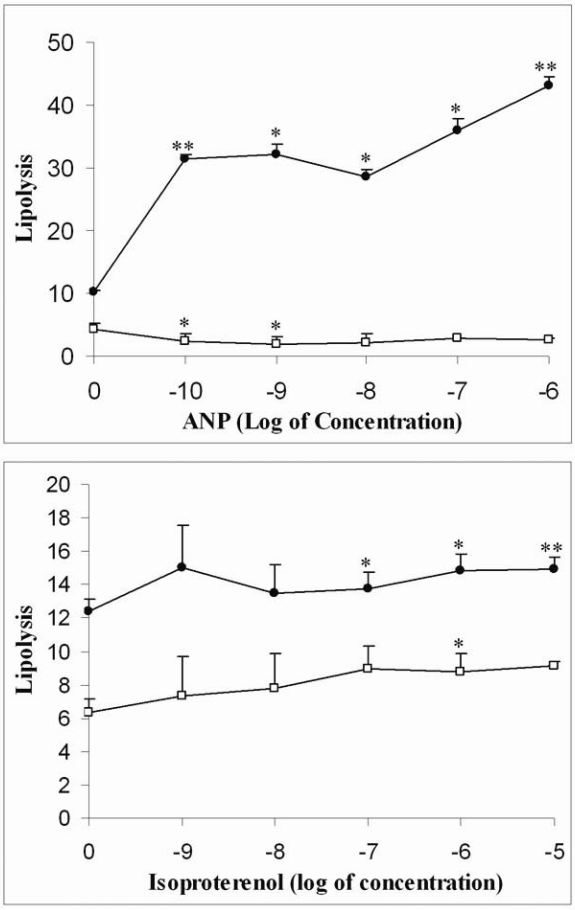
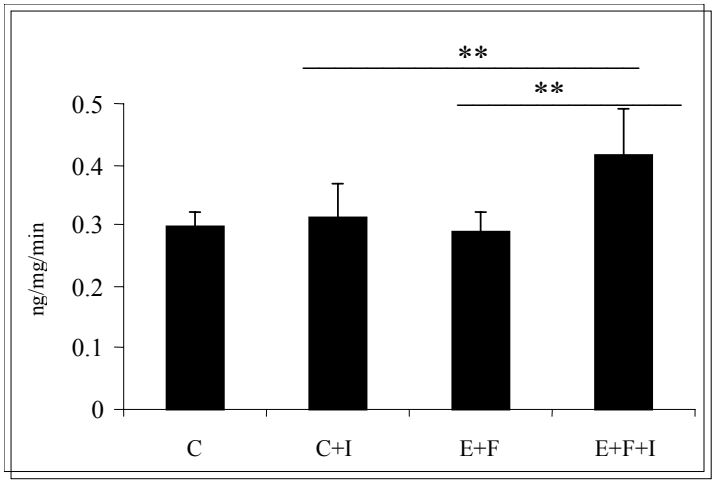


Figure 4. Effects of EGF and FGF on lypolysis. Lipolytic response of adipocytes preconditioned prior to adipogenesis in the absence (open square) or presence (closed circle) of 10 ng/ml EGF and 10 ng/ml FGF were compared in response to increasing concentrations of ANP (A) and isoproterenol (B). Mean lipolysis ± S.D. for n=2 donors is shown. P values are indicated by * ($p<0.05$) and ** ($p<0.01$).

Figure 5. Effects of EGF and bFGF on Glucose Uptake. Glucose uptake analysis was performed on hASC-derived adipocytes preconditioned prior to adipogenesis in the absence (C, Control) or presence of 10 ng/ml EGF and 10 ng/ml FGF (E+F). 2-Deoxy glucose uptake analysis was performed over a ten minute incubation in the absence or presence of insulin (I). Mean uptake of glucose ± S.D. (ng glucose/mg protein/min) is displayed for n = 1 donor. P values are indicated by ** ($p<0.01$).



3.3 Effects of EGF and bFGF on Osteogenesis

Preconditioning of hASCs with EGF and bFGF prior to osteogenic induction led to dose-dependent increases in Alizarin Red staining relative to untreated controls, consistent with increased extracellular matrix mineralization (Figure 6). Only one week after osteogenic induction, stronger Alizarin Red staining was present in preconditioned hASCs in an EGF/bFGF-dose dependent manner as compared to hASCs preconditioned in Stromal Medium alone. Consistent with this observation, the osteogenic-related mRNAs, osteopontin and alkaline phosphate, increased in cells preconditioned with both growth factors prior to osteogenic induction in a dose-dependent manner. The mRNA encoding the late osteogenic marker, osteocalcin, remained stable and osteonectin decreased slightly in an EGF/bFGF dose-dependent manner (Fig. 7). In the hASCs prior to osteogenic differentiation, the mRNAs for osteopontin, osteonectin, and osteocalcin were reduced in a dose-dependent manner by EGF/bFGF. This observation is consistent with reports that bFGF maintains hASC cells in an undifferentiated, stem like state [Zaragosi 2006].

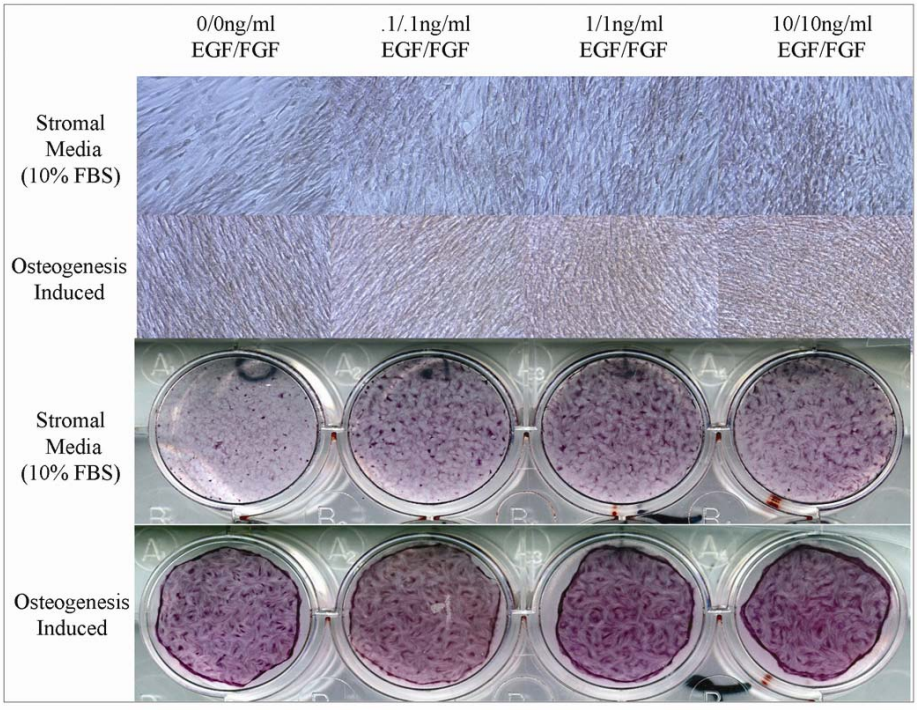


Figure 6. Effects of growth factors on osteogenesis. Cells were cultured for 8 days in either osteogenic induction media or stromal media (10% FBS) following expansion in EGF and FGF supplemented media. Microphotographs taken at x100 (top) and scanned images (bottom) were obtained following alizarin red staining.

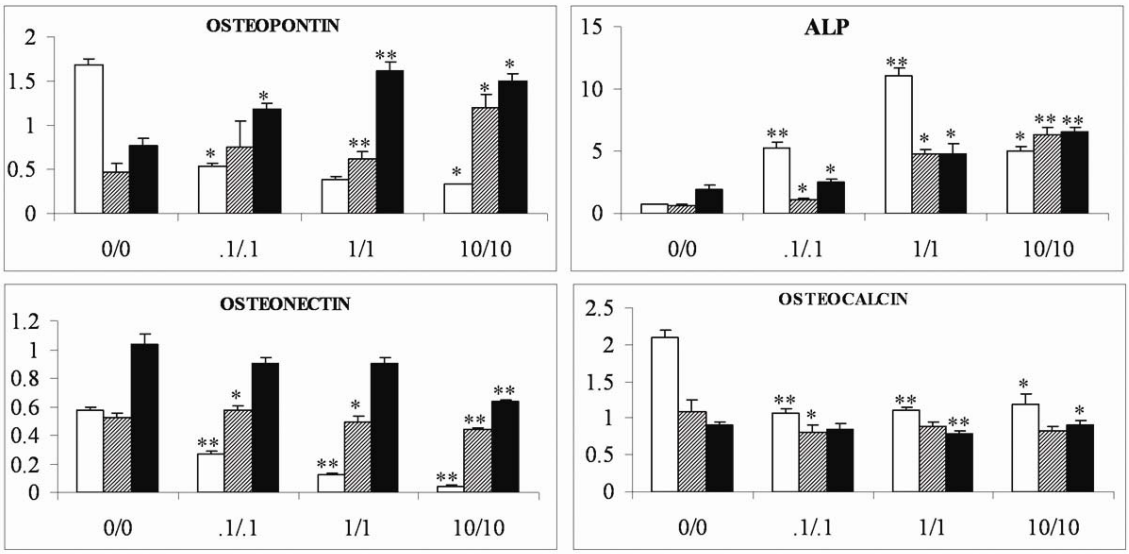


Figure 7. Results of quantitative PCR analysis of cells expanded in EGF and bFGF supplemented conditions. RNA was harvested after expansion before induction (open bars), after 8 days of treatment with osteogenic media (solid bars), and after 8 days of treatment in Stromal Media (10% FBS) (hatched bars). cDNA was pooled from n = 4 donors for each growth condition. Quantities were normalized to Cyclophilin B and displayed as the mean ± S.D. Values on X-axis indicate the preconditioning concentration of EGF/bFGF in ng/ml. P values are indicated by * ($p<0.05$) and ** ($p<0.01$).

4. DISCUSSION

4.1 EGF and bFGF Improve Proliferation and Differentiation of Cryopreserved hASCs

This study demonstrates that the use of EGF and FGF supplementation during the expansion of undifferentiated cryopreserved hASCs improves cell proliferation, adipogenic and osteogenic differentiation, and adipocyte function. The observed additive effects of EGF and bFGF permit the use of a combination of low concentrations of EGF and bFGF to achieve significant results, as opposed to the use of high and more costly concentrations of one growth factor alone. This conclusion is supported by the fact that cells cultured in concentrations of 1ng/ml EGF and 1ng/ml bFGF displayed similar cell proliferation to cells cultured in 10ng/ml EGF and 10ng/ml bFGF. Thus, EGF/bFGF supplementation is an effective and inexpensive method of enhancing proliferation of cryopreserved hASCs.

These data support studies by Hauner et. al., which indicate that EGF has notable effects on the growth and development of adipocytes at physiological serum concentrations (0.5-2ng/ml) [Hauner 1995]. Consistent with enhanced proliferation, Martin et. al. demonstrated that bFGF caused an increase in size of individual bone marrow-derived mesenchymal stem cell clones by 2.5-fold in vitro [Martin 1997]. Tamama et. al. have shown that EGF supplementation enhanced the expansion of bone marrow-derived mesenchymal stem cells for in vivo transplantation [Tamama 2004]. The additive effects shown by the data are also in agreement with experiments preformed by Suga et. al., which demonstrated that, separately, the individual components of commercial endothelial growth medium, including EGF and bFGF, elicit less robust cell proliferation than the sum of growth factors present in the medium [Suga 2007]. Endothelial growth medium, which rapidly expands hASCs while preserving differentiation potential [Suga

2007], is an excellent illustration of how the additive effects of EGF and bFGF are currently being utilized.

In addition, the presence of EGF and bFGF in stromal medium maintains differentiation potential during expansion and enhances the subsequent adipogenesis of cryopreserved hASCs. A dose-dependent increase in oil red O staining is observed; however, it is uncertain whether this effect is contributable to EGF and bFGF directly. Studies have shown that adipogenic conversion is significantly influenced by cell shape and adhesion, which are consequences of cell density [McBeath 2004]; therefore, the increased oil red O staining may be an indirect effect of the increased proliferation in wells supplemented with higher concentrations of EGF and bFGF prior to adipogenic induction. Nevertheless, PCR analysis, which compensates for cell number, support that EGF and bFGF enhance differentiation. Several mRNAs characteristic of adipogenesis was observed in treated cells when compared to the control. PCR analysis in these same experiments demonstrated marked increases in the expression levels of several mRNAs that encode adipogenic transcription factors and their downstream targets (adiponectin, aP2, C/EBPα, lipoprotein lipase, PPARγ, and PPARγ Co-activator-1 (PGC1)). Previous studies also support that EGF and bFGF modulated differentiation of ASCs and bone marrow-derived mesenchymal stem cells in several species. Recent experiments demonstrate that, when compared to the control, hASCs expanded in EGF and bFGF maintain differentiation potential and have a narrower, more spindle-like morphology usually associated with the stem cell phenotype [Estes 2008]. It was also demonstrated that EGF supplementation following adipogenic induction improved adipogenesis of murine 3T3-L1 preadipocytes [Adachi 1994]. It is also suggested that bFGF enhances differentiation potential of bone marrow-derived mesenchymal stem cells. A study demonstrates that bFGF preconditioning increased the ability

of bone marrow-derived mesenchymal stem cells to mineralize extracellular matrix in vitro and form bone in vivo [Martin 1997]. Likewise, Stewart et. al. have shown that bFGF enhanced chondrogenesis in equine bone marrow-derived mesenchymal stem cells [Stewart 2007].

In addition to providing improved adipogenesis, EGF and bFGF supplementation produced adipocytes that were more functional than the control. Exposure of hASCs to EGF and bFGF previous to induction enhanced insulin sensitivity and response to lipolytic stimuli of differentiated adipocytes, as indicated by glucose uptake and triglyceride assays. EGF and bFGF pretreatment may, therefore, be valuable to cell-based therapies which require adipocytes with optimal metabolic capability.

Although the effects of exposure to EGF/bFGF supplementation prior to induction were less profound in osteocytes when compared to adipocytes, a dose-dependent increase in alizarin red staining was observed. Improved osteogenesis was demonstrated by dose-dependent increases in the expression of ALP and osteopontin mRNAs in differentiated osteocytes. Other osteogenic markers (osteopontin, osteocalcin, osteonectin) decreased prior to induction in cells treated with EGF and bFGF, indicating that the growth factors maintain differentiation potential. These alterations are consistent with findings by Quarto et. al., which demonstrate that bFGF inhibits osteogenic differentiation when added to differentiation medium [Quarto 2006].

The capability of EGF and bFGF to maintain differentiation potential in ASCs is supported by several studies that demonstrate that the presence of EGF and bFGF during induction or differentiation reduce hASC adipogenesis and osteogenesis [Adachi 1994, Butterwith 1993, Hauner 1995, Quarto 2006, Zaragosi 2006]. Furthermore, an unpublished study by Kenneth Eilertsen and Heather Kirk-Ballard of the Pennington Biomedical Research Center demonstrates that the expression for several genes related to stemness increased in

undifferentiated hASCs treated with EGF, such as CD34, the gene for a cell surface marker commonly associated with hematopoeitic stem cells.

In conclusion, the data generated is consistent with the hypothesis. EGF and bFGF supplementation at concentrations between 1 and 10ng/ml prior to, but not during, induction of differentiation yields rapid expansion and improved adipogenesis and osteogenesis of hASCs.

4.2 Significance of Results

The above lines of evidence suggests that EGF and bFGF merit inclusion in standard hASC expansion procedures for basic research protocols and should be considered for routine use as media supplements to improve cell proliferation and adipogenesis of cryopreserved and fresh hASCs. An expansion method involving EGF/bFGF supplementation may also prove to be of significant importance to clinical use of hASCs in the near future. In regards to stem cell therapies involving transplantation, EGF/bFGF supplementation has the ability to provide rapid ex vivo expansion of stem cells isolated from adipose tissue, and may enhance differentiation and function of the cells after transplantation.

4.3 Future Directions

The observational data obtained in this study can be further supported by assays that give quantitative values. Efforts to quantify Oil Red O and Alazarin Red stain to better assess the improvement in adipogenesis and osteogenesis in hASCs pretreated with EGF and bFGF are underway. Western blot analysis of adipogenic proteins present in adipocytes pretreated with EGF and bFGF may provide additional evidence to support these conclusions.

Once the effects of EGF and bFGF on proliferation, differentiation potential, and adipocyte functionality are firmly established, tumorigenic potential must be assessed prior to clinical use of the growth factors to insure that EGF and bFGF supplemented hASCs do not

increase the occurrence of cancer following in vivo transplantation. While it is presumed that tumor formation is less likely to occur in mesenchymal stem cells than in embryonic stem cells [Rosenthal 2003], Rubio et. al. report that hASCs can spontaneously transform in vivo following extensive periods of in vitro culture [Rubio 2005]. Further investigation of hASCs will be required to assess the safety of these cells for clinical use; however, hASCs continue to be a valuable basic research tool for the understanding of human biology.

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