Molecular and Cellular Endocrinology 339 (2011) 165–171

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

A putative role for endogenous FGF-2 in FGF-1 mediated differentiation of human preadipocytes

Louise J. Hutley[a,b], Felicity S. Newell[a], Yu-Hee Kim[a,b], Xiao Luo[a,b,1], Charlotte H. Widberg[a], Wenda Shurety[a], Johannes B. Prins[a,b], Jonathan P. Whitehead[a,b,*]

[a] Metabolic Program, Mater Medical Research Institute, Mater Health Services, South Brisbane, Queensland 4101, Australia
[b] Diamantina Institute for Cancer, Immunology and Metabolic Medicine, Princess Alexandra Hospital, University of Queensland, Brisbane, Queensland 4102, Australia

ARTICLE INFO

Article history:
Received 24 December 2010
Received in revised form 14 April 2011
Accepted 15 April 2011

Keywords:
Fibroblast growth factor
Adipocyte
Adipogenesis
Obesity

ABSTRACT

The defining characteristic of obesity is increased adipose tissue (AT) mass following chronic positive energy supply. AT mass is determined by adipocyte number and size, which reflect proliferation and differentiation of preadipocytes and hypertrophy of pre-existing adipocytes. The molecular pathways governing AT expansion are incompletely defined. We previously reported that FGF-1 primes proliferating primary human preadipocytes (phPA), thereby increasing adipogenesis. Here we examined whether FGF-1's adipogenic actions were due to modulation of other FGFs. Treatment of phPA with FGF-1 reduced FGF-2 mRNA/protein by 80%. To examine a putative functional role we performed siRNA knockdown studies. Following FGF-2 knockdown preadipocyte proliferation was decreased and expression of adipogenic genes (PPARγ, G3PDH and adiponectin) was increased at day 1 of differentiation. These results suggest that changes in endogenous FGF-2 levels contribute to FGF-1's early adipogenic effects and highlight the complexity of the paracrine interplay between FGFs within human AT.

1. Introduction

The current obesity pandemic is having significant adverse consequences on the health and functionality of both individuals and healthcare systems worldwide. Obesity, characterized by excessive body fat (adipose tissue), occurs in a setting of positive energy balance and is strongly associated with many chronic diseases including type 2 diabetes, cardiovascular disease and many cancers. Given the public health significance of disorders of adipose tissue mass, increased knowledge of the molecular and cellular mechanisms governing adipose tissue expansion is critical to development of effective strategies in prevention and treatment of obesity and its metabolic complications.

Both size and number of adipocytes underpins adipose tissue mass. Initially hypertrophy of existing adipocytes is sufficient to store excess energy in the form of triglycerides but in the face of continuing positive energy balance the system requires generation of new adipocytes. This occurs via the complex process of adipogenesis whereby multi-potent mesenchymal stromal cells within adipose tissue undergo commitment to the adipocyte lineage and proliferate to form a pool of preadipocytes which, upon further stimulation, differentiate into new adipocytes.

Healthy adipose tissue maintains a balance between preadipocyte differentiation and lipid storage within mature adipocytes and impairment of this balance is an important factor linking obesity to its metabolic sequelae (Medina-Gomez et al., 2007; Sethi and Vidal-Puig, 2007; Spalding et al., 2008). Excessive adipocyte hyperplasia and, conversely, excessive adipocyte hypertrophy in the absence of new adipocyte generation have each been implicated in the aetiology of obesity and its complications (Heilbronn et al., 2004; Kim et al., 2007; Spalding et al., 2008; Lebeche et al., 1999; Li et al., 2000; Sherman et al., 1993). It is possible that each of these processes may be important in obesity under different settings, such as individual genetic differences or differences in developmental stages of obesity. Together, this highlights the importance of adipogenesis as a therapeutic target – for either inhibition of excessive adipocyte number or for promotion of the acquisition of new and metabolically healthy adipocytes. Underpinning both approaches is the absolute requirement for a comprehensive understanding of the molecular regulation of adipogenesis.

For the last few years we have investigated human adipogenesis and have identified FGF-1 as a novel and potent adipogenic factor secreted from adipose-derived microvascular endothelial cells

* Corresponding author at: Metabolic Program, Mater Medical Research Institute, Aubigny Place, Raymond Terrace, South Brisbane, Queensland 4101, Australia. Tel.: +61 7 3163 2564; fax: +61 7 3163 2550.
E-mail address: jwhitehead@mmri.mater.org.au (J.P. Whitehead).
[1] Permanent address: Laboratory of Animal Fat Deposition and Muscle Development, College of Animal Science and Technology, Northwest A&F University, People's Republic of China.

0303-7207/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mce.2011.04.012

(Hutley et al., 2004). FGF-1 stimulates all stages of adipogenesis including preadipocyte proliferation, commitment and differentiation (Hutley et al., 2004; Newell et al., 2006). Further we identified FGF receptor 1 (FGFR1) and signalling via the MAPK/ERK pathway as essential components in the adipogenic actions of FGF-1 in human preadipocytes (Widberg et al., 2009). These effects of FGF-1 are specific for this family, in contrast to a range of growth factors, including PDGF-AA, PDGF-BB and IGF-1, which also signal via tyrosine kinase receptors but are not adipogenic (Widberg et al., 2009). Importantly, we also demonstrated that human adipogenesis can be modulated *in vitro* by targeting FGF/FGFR signalling (Hutley et al., 2004; Newell et al., 2006; Widberg et al., 2009). Preadipocytes treated with recombinant FGF-1 during proliferation have a greatly increased capacity for differentiation and adipocytes generated from this model express all examined genetic and metabolic markers of the mature phenotype including insulin sensitivity (Hutley et al., 2004; Newell et al., 2006; Widberg et al., 2009). A paracrine role for the FGF system in the regulation of adipose tissue *in vivo* is strongly suggested by expression of FGF ligands and their receptors in this tissue and by reports demonstrating increased expression of FGF-1 in adipose tissue from obese individuals (Mejhert et al., 2010; Teichert-Kuliszewska et al., 1992). Together this data highlights the FGF-1 model of human adipogenesis as an important platform for further studies of this process particularly of the earliest commitment stage about which little is currently understood.

One potential mechanism by which FGF-1 primes cells for differentiation is by inducing alterations in gene expression. We demonstrated that FGF-1 treatment of proliferating human preadipocytes (hPA) mediates altered expression of genes regulating adipogenesis, including the key adipogenic transcription factor, PPARγ (Newell et al., 2006; Widberg et al., 2009). In embryonic development FGF ligands are known to act in a paracrine fashion to modulate the expression of other FGF family members and this co-regulation is an important mechanism in cell fate decisions and developmental processes (Lebeche et al., 1999; Li et al., 2000; Sherman et al., 1993). Currently little is known concerning the role of endogenous FGFs in adult physiology and, more specifically, in human adipogenesis. The present work aims to identify effects of FGF-1 on preadipocyte expression of endogenous FGFs, specifically FGF-2 and FGF-10 which have each been implicated in development of white adipose tissue (Hutley et al., 2004; Krieger-Brauer and Kather, 1995; Neubauer et al., 2004; Prusty et al., 2002; Yamasaki et al., 1999). Further, we aim to determine if modulation of expression of these growth factors is a component of the molecular regulation governing the complex process of adipogenesis which involves proliferation, commitment and differentiation of preadipocytes.

2. Materials and methods

2.1. Cell culture and assessment of differentiation

2.1.1. Primary human preadipocytes (phPA)

Subcutaneous adipose tissue biopsies were obtained from 5 male (average age 50 years [range 21–76], average BMI 25.8 kg/m^2 [range 19–33]) and 12 female (average age 43 years [range 25–71], average BMI 30.1 kg/m^2 [range 19–55]) patients undergoing elective open-abdominal surgery. None of the patients had diabetes or severe systemic illness and none were taking medications known to affect adipose tissue mass or metabolism. The protocol was approved by the Research Ethics Committees of the University of Queensland and the Princess Alexandra Hospital and all patients gave their written informed consent. phPA were isolated from subcutaneous adipose tissue and cultured as previously described (Hutley et al., 2001b; Widberg et al., 2009). Cells were used for experiments at passage 2 after 8–12 weeks culture in serum-containing medium. Upon reaching confluence the cells were differentiated in a chemically defined serum-free medium as previously described (Hutley et al., 2004, 2001a). During the culture period phPA were treated ±1 ng/ml FGF-1 (R&D systems, Minneapolis, MN, USA) and 90 μg/ml heparin (Sigma–Aldrich, Castle Hill, Victoria, Australia).

2.1.2. SGBS preadipocytes

Simpson Golabi Behmel Syndrome (SGBS) preadipocytes (PA), a human subcutaneous PA cell strain, were isolated and characterized in 2001 (Wabitsch et al., 2001). These cells were cultured and differentiated as previously described (Newell et al., 2006; Widberg et al., 2009). As for the phPA, these cells were also treated ±1 ng/ml FGF-1 (R&D systems, Minneapolis, MN, USA) and 90 μg/ml heparin (Sigma–Aldrich, Castle Hill, Victoria, Australia) during the culture period.

2.2. Real-time RT-PCR

Total RNA was obtained using RNeasy kits (Qiagen, Doncaster, Victoria, Australia) and concentration and quality determined using a spectrophotometer (Nanodrop ND-1000, Biolab, Clayton, Victoria, Australia). Total RNA (500 ng–1 μg) was reverse transcribed using random hexamers (Promega, Annandale, NSW, Australia) and Superscript III reverse transcriptase according to manufacturer's instructions (Invitrogen). The primers used in this study are available on request and were designed using Primer 3 software and purchased from Sigma–Aldrich (Castle Hill, Australia). Real time RT-PCR was performed as previously described (Widberg et al., 2009).

2.3. Western blot analysis

Western blot analysis was carried out as previously described (Su et al., 2009). All blots were visualised using the LICOR Odyssey system. The following primary antibodies were used: FGF-10 (C17), FGF-2 (H131), Lamin A/C (636) from Santa Cruz Biotechnology and β-tubulin (Sigma–Aldrich).

2.4. FGF-2 immunoassay

hPA were grown to confluence in the presence of 90 μg/ml heparin and in the presence or the absence of 1 ng/ml FGF-1 in six well plates. Cells were incubated in 1 ml of serum-containing medium (10% FBS) for 24 h and the medium collected, supplemented with protease inhibitors (1 μg/ml aprotinin, 1 μg/ml antipain, 1 μg/ml pepstatin, 1.5 μg/ml leupeptin, 0.5 mM AEBSF), and stored at −80 °C until required. Cells were harvested in buffer containing 20 mM HEPES, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM Na_4PO_7, 10 mM NaF and protease inhibitors (Complete Mini, Roche Diagnostics, Germany) and total protein for each sample determined by BCA assay. FGF-2 secretion into the medium was determined using the Quantikine human FGF basic immunoassay kit (R&D systems) according to manufacturer's instructions. As a background control for the medium, a sample supplemented with 10% FBS was also measured. The concentration of FGF-2 in each media sample was determined by comparison with a standard curve and corrected for cell number by normalising to the total protein of the whole cell lysate for each sample.

2.5. Subcellular fractionation

Cells were subjected to subcellular fractionation using established methods, essentially as described previously (Behrmann et al., 2004; Whitehead et al., 2001).

2.6. Immunofluorescence microscopy

Confluent hPA were grown in the presence or the absence of 90 μg/ml heparin and 1 ng/ml FGF-1 on glass coverslips and immunofluorescence analysis carried out as previously described (Widberg et al., 2009).

2.7. FGF-2 knockdown

Small-interfering RNA (siRNA) designed and validated to target FGF-2 and scrambled control (AllStars Negative control siRNA) was from Qiagen (sequences available on request). In preliminary experiments similar results were obtained with three independent siRNAs. The siRNA yielding the most effective FGF-2 knockdown was used for subsequent studies. SGBS PA were seeded on six-well plates at 50–80% confluence in serum-containing medium without antibiotics. Transfections were carried out 24 h later using NanoJuice Transfection Reagents according to the manufacturer's instructions (Novagen). Knock-down was assessed 72 h post-transfection by real-time RT-PCR for FGF-2 mRNA. Transfected cells were then either plated for SYTO 60 (cell number) assay or cultured in serum-containing medium for an additional 3 days prior to induction of differentiation. At differentiation day 1, 7 and 14 cells were harvested for subsequent experiments.

2.8. SYTO 60 assay

Cell number was assessed using SYTO 60 (Invitrogen) assay as previously described (Widberg et al., 2009; Su et al., 2009).

2.9. Assessment of differentiation

Lipid accumulation was assessed morphologically using phase contrast microscopy (100× magnification) (Nikon Eclipse TE300). Differentiation was also

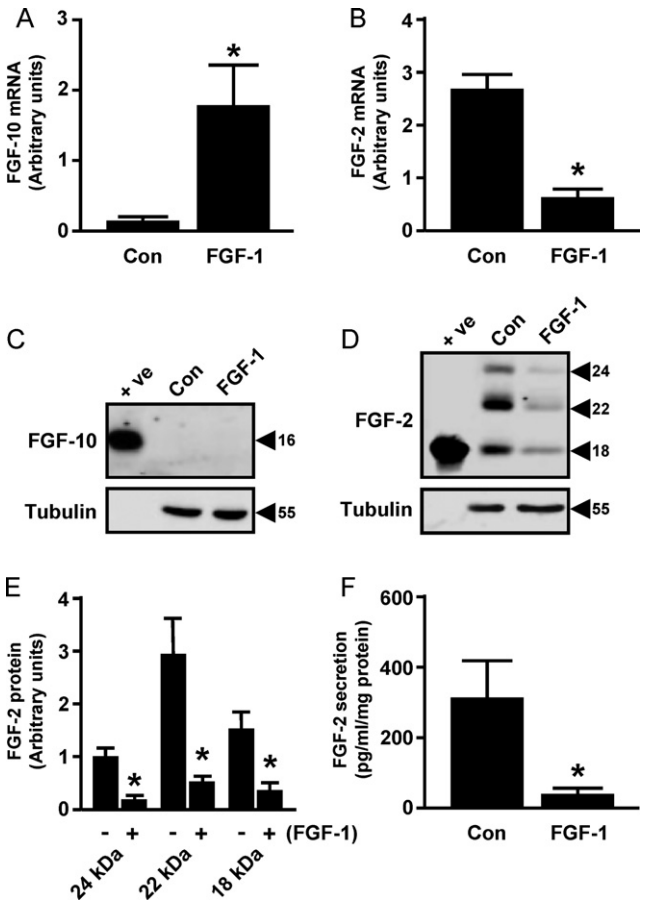

Fig. 1. Effects of FGF-1 on expression of FGF-10 and FGF-2 in primary human preadipocytes. Subcutaneous phPA were grown to confluence in serum-containing medium supplemented with 90 μg/ml heparin and in the presence or the absence of 1 ng/ml FGF-1. (A and B) Total RNA was extracted and expression of FGF-10 (A) and FGF-2 (B) was determined by qRT-PCR. (C and D) Western blot analysis of FGF-10 (C) and FGF-2 (D) protein. Recombinant human protein was used as a positive control and β-tubulin was used as a loading control. (E) Graph showing quantitation of FGF-2 isoform expression. (F) FGF-2 secretion was measured by immunoassay. Results represent the mean ± SEM of samples derived from 3 individuals (A and C) or 5 individuals (B, D, E and F) (*$p < 0.05$).

assessed using real-time RT-PCR for expression of adipogenic markers and enzymatic assay of glycerol 3-phosphate dehydrogenase (G3PDH) activity as previously described (Hutley et al., 2001b; Widberg et al., 2009). Results for enzyme activity are expressed as mU per cm^2 of culture area as described in Newell et al. (2006).

2.10. Statistical analyses

Statistical analyses were performed using the following tests as appropriate: Student's *t*-test in Microsoft Excel 2002 Data Package (this analysis was used unless otherwise stated); one-way ANOVA (repeated measures) for differences across experimental groups in conjunction with Tukey's post hoc test to compare differences between the respective treatment groups in GraphPad Prism 4.02. Data are expressed as means ± SEM. *p*-Values ≤0.05 were considered to be statistically significant.

3. Results

3.1. Effects of FGF-1 on expression of FGF-10 and FGF-2 at confluence

In confluent phPA in response to exogenous FGF-1 treatment, FGF-10 mRNA was increased approximately 20-fold compared to 'no FGF-1' control ($p < 0.05$) (Fig. 1A) and, conversely, FGF-2 mRNA showed a 5-fold decrease ($p < 0.05$) (Fig. 1B). We were unable to establish whether differences in FGF-10 mRNA were recapitulated at the protein level with FGF-10 protein levels being undetectable

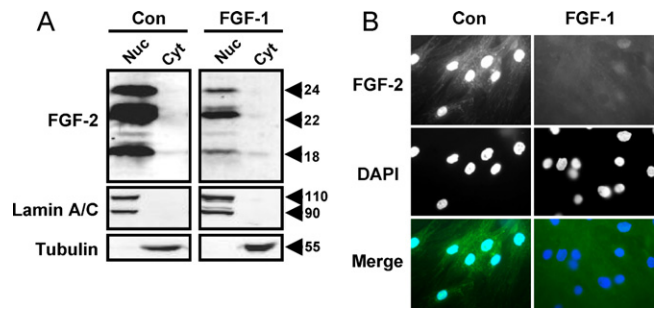

Fig. 2. Subcellular distribution of FGF-2 in primary human preadipocytes. Subcutaneous phPA were grown to confluence in the presence or the absence of 90 μg/ml heparin and 1 ng/ml FGF-1. (A) Western blot showing the distribution of FGF-2 in the Cytosolic (Cyt) and nuclear (N) fractions. Blots for tubulin (cytosolic marker) and lamin A/C (nuclear marker) demonstrate the stringency of the fractionation protocol. (B) Immunofluorescence microscopy showing the subcellular localisation of FGF-2. Cells were counterstained with the nuclear stain DAPI. Images are representative of samples derived from 3 individuals.

by western blot, although recombinant human FGF-10 protein was readily detected (Fig. 1C). In contrast, the FGF-1-induced decrease in FGF-2 mRNA was also observed at the protein level (Fig. 1D). Confluent phPA expressed the 18 kDa low molecular weight isoform as well as two high molecular weight isoforms of 22 kDa (or 22.5 kDa) and 24 kDa (Fig. 1D) which are produced by use of alternative translation start sites (Florkiewicz and Sommer, 1989; Prats et al., 1989). Consistent with mRNA expression, FGF-1-treated phPA exhibited decreased levels of all three FGF-2 isoforms in comparison with non-FGF-1 treated controls (Fig. 1D). Quantitation of the FGF-2 isoforms demonstrated that expression of all three isoforms was reduced by an average 4 to 5-fold in cells exposed to FGF-1 (Fig. 1E).

3.2. Effects of FGF-1 on FGF-2 Secretion

The amount of FGF-2 secreted into the medium by phPA was determined by immunoassay (Fig. 1E). Consistent with the negative effect of FGF-1 exposure on FGF-2 message and protein expression there was also a significant FGF-1-induced reduction in FGF-2 secretion from these cells compared to cells cultured in the absence of FGF-1 (average 47.1 pg/ml/mg versus 315.2 pg/ml/mg protein, respectively; $p < 0.05$) (Fig. 1F). Together, these results demonstrate that treatment with exogenous FGF-1 reduces the expression as well as intracellular and extracellular levels of all three FGF-2 isoforms.

3.3. Effects of FGF-1 on subcellular localisation of FGF-2 in confluent phPA

In order to determine the subcellular localisation of FGF-2 isoforms in phPA and to determine if FGF-1 treatment resulted in altered localisation, subcellular fractionation was carried out and the cytosolic and nuclear fractions were subjected to western blot analysis. Blotting with other markers for specific subcellular compartments (ß-tubulin for the cytosol and Lamin A/C for the nucleus) confirmed the fidelity of the fractions (Fig. 2A). All three FGF-2 isoforms were primarily localised to the nucleus. As observed previously, FGF-1 treatment decreased expression of all FGF-2 isoforms and this effect was most apparent in the nuclear fraction (Fig. 2A). The subcellular localisation of FGF-2 was also examined by immunofluorescence microscopy. In cells which had not been exposed to FGF-1, there was strong nuclear staining of FGF-2. However, in clear contrast, FGF-2 nuclear staining was dramatically decreased in FGF-1-treated phPA (Fig. 2B) with no obvious shift in localisation detected. Overall, these findings demonstrate that the

majority of intracellular FGF-2 protein is nuclear and that exogenous FGF-1 reduces this nuclear pool of FGF-2.

3.4. FGF-2 expression during differentiation

As phPA differentiate with limited efficiency in the absence of FGF-1 treatment we next characterized FGF-2 expression during differentiation of the widely used SGBS PA. These cells were originally derived from the stromal fraction of subcutaneous adipose tissue of an infant with SGBS (Wabitsch et al., 2001) and unlike other human PA, SGBS cells maintain a high capacity for differentiation over many generations resulting in their widespread use (Bour et al., 2007; Hossain et al., 2010; Newell et al., 2006). We have previously characterized SGBS PA and found them to be metabolically similar to phPA, particularly with regards response to FGF-1 and subsequent regulatory events during adipogenesis (Newell et al., 2006; Widberg et al., 2009). The subcellular distribution of FGF-2 in the SGBS PA was predominantly nuclear (data not shown), consistent with our observations in the phPA. FGF-2 mRNA expression was significantly lower in FGF-1-treated SGBS PA compared to 'no FGF-1' controls (3- to 5-fold decrease *cf* control; $p < 0.01$) during the critical period of proliferation and commitment leading up to confluence (Fig. 3A; day −3 to day 0). Upon induction of differentiation (Diff) at day 0, FGF-2 expression was decreased in both −/+ FGF-1 treatment groups to similar, low levels by Diff day 3 (Fig. 3A). This low level of FGF-2 expression was maintained through the mid-stages of differentiation and began to rise as the cells attained a mature adipocyte phenotype (cells are considered mature adipocytes by day 14). This increase was more pronounced in the FGF-1-treated cells (Fig. 3A). These data suggest that the reduction in FGF-2 mRNA during the early stage of differentiation is a general characteristic of the adipogenic program which prompted us to hypothesise that this reduction may represent an integral part of adipogenesis which is, in turn, enhanced by FGF-1 treatment.

3.5. Knockdown of FGF-2 in SGBS PA

To investigate the above RNA interference techniques were used. FGF2 mRNA and protein levels were determined 72 h after transfection of SGBS PA with scrambled (control) siRNA or FGF-2 siRNA. Results demonstrated significant knockdown of FGF-2 mRNA (Fig. 3B) and protein (Fig. 3C). Interestingly, whilst all three FGF-2 isoforms were decreased by treatment with FGF-2 siRNA the extent of knockdown ranged from 50% (22 kDa) to 90% (18 kDa) (Fig. 3D) which probably reflects differences in the properties of the three isoforms.

3.6. Effect of FGF-2 knockdown on SGBS PA proliferation

An important early stage in the adipogenic process is the proliferation of preadipocytes. We have previously reported that this is a critical period in FGF-1's pro-adipogenic actions (Hutley et al., 2004; Newell et al., 2006; Widberg et al., 2009). The effect of FGF-2 knockdown on SGBS proliferation was determined after four days of proliferation using the SYTO 60 assay. There was a 30% decrease in cell number following knockdown of FGF-2 compared to control cells ($p < 0.05$) demonstrating that reduction of endogenous FGF-2 decreased proliferation.

3.7. Effect of FGF-2 knockdown on SGBS PA differentiation

We previously reported that FGF-1 increased expression of several adipocyte markers (compared to non-FGF-1-treated controls) from early time-points both pre- and post-induction of differentiation (Hutley et al., 2004; Newell et al., 2006; Widberg et al., 2009). In the present study we wished to determine if knockdown

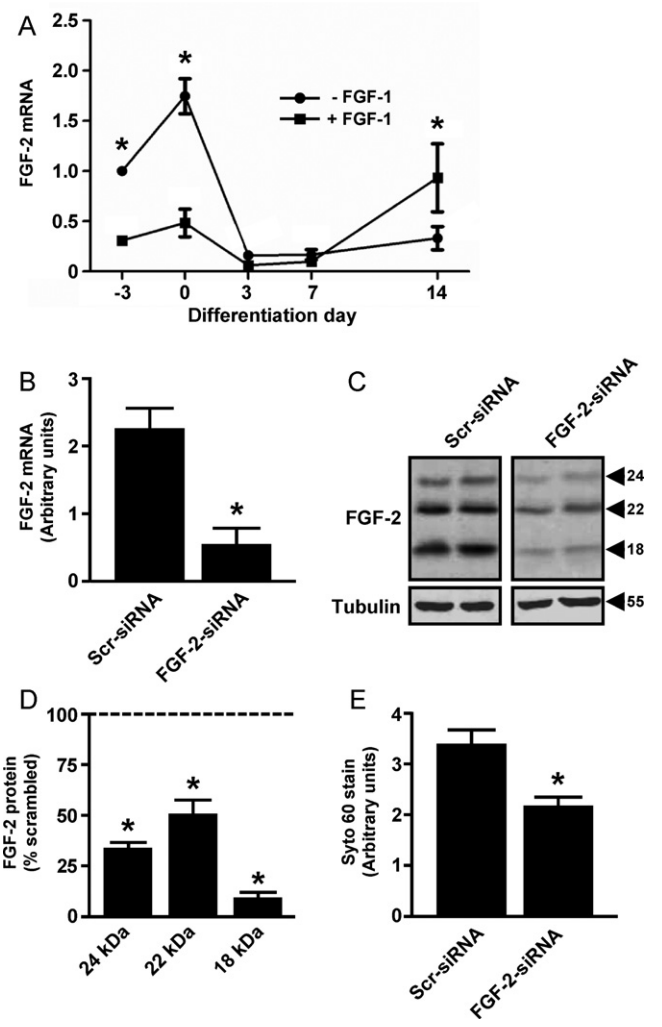

Fig. 3. Effects of FGF-2 knockdown on proliferation of SGBS preadipocytes. SGBS PA were grown to confluence in the presence or the absence of 90 μg/ml heparin and 1 ng/ml FGF-1. (A) Total RNA was extracted from cells at the indicated timepoints and the expression of FGF-2 was determined by qRT-PCR. Results are the mean ± SEM of 4 independent experiments (*$p < 0.05$ comparing −/+ FGF-1 treated cells at the respective timepoint). The levels of FGF-2 mRNA (B) and protein (C and D) were determined by qRT-PCR and western blot 72 h after transfection with scrambled (Scr)-siRNA or FGF-2-siRNA. Data are representative of 3 independent experiments (mean ± SEM) (*$p < 0.05$). (E) Proliferation of cells treated with scrambled-siRNA or FGF-2-siRNA was determined using the Syto60 assay. Results are the mean ± SEM of 3 independent experiments (*$p < 0.05$).

of FGF-2 in FGF-1 naïve cells would replicate this effect on adipocyte gene expression. Using the same siRNA techniques as above, FGF-2 was knocked down in SGBS PA and differentiation induced when the cells reached confluence (differentiation day 0), approximately 3–5 days post-transfection. Total RNA was harvested on day 1, 7 and 14 of the differentiation period. At Diff day 1 real-time RT-PCR analysis of adipogenic markers demonstrated a significant increase in expression of PPARγ as well as glycerol-3-phosphate dehydrogenase (G3PDH) and adiponectin (all $p < 0.05$) (Fig. 4A–C, respectively). However, this increased expression was not observed at day 7 or day 14, with a trend toward reduced expression of all three markers at day 14 (Fig. 4A–C). Phase contrast microscopy revealed marked lipid accumulation at day 7 and day 14 which was comparable between cells treated with either scrambled or FGF-2 siRNA (Fig. 4D). Finally, the effect of FGF-2 knockdown on G3PDH activity was assessed in mature adipocytes harvested on day 14. Again, there were no significant differences in the levels of G3PDH activity (Fig. 4E).

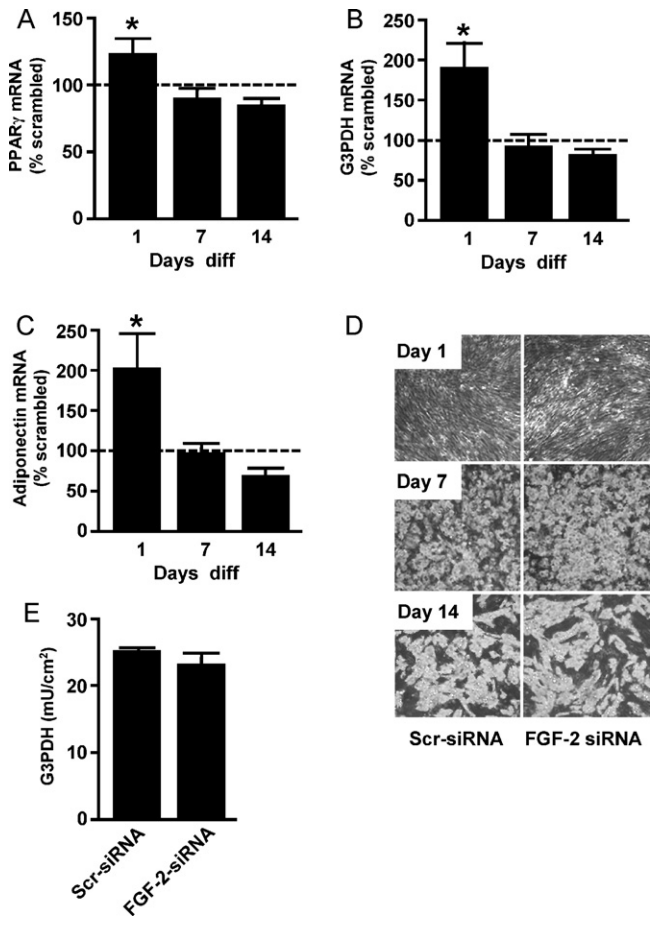

Fig. 4. Effects of FGF-2 knockdown on differentiation of SGBS preadipocytes. SGBS PA were transfected with scrambled or FGF-2-siRNA 4 days prior to induction of differentiation. Total RNA was extracted from cells at the indicated timepoints and the expression of (A) PPARγ, (B) G3PDH and (C) adiponectin were determined by qRT-PCR. Results from 4 independent experiments are presented as the mean (±SEM) percentage of expression observed in cells treated with FGF-2-siRNA *cf* cells treated with scrambled siRNA which were set at 100% (represented by the broken line) (*$p < 0.05$ comparing −/+ FGF-1 treated cells at the respective timepoint). (D) SGBS cells were transfected with siRNA as described above and photomicrographs showing lipid accumulation were taken on day 1, 7 and 14. (E) SGBS cells were transfected with siRNA as described above and G3PDH activity was measured in cells harvested on day 14.

4. Discussion

We previously identified a novel role of FGF-1 as a potent regulator of human adipogenesis (Hutley et al., 2004). This factor is produced in adipose tissue and acts in a paracrine manner to enhance all aspects of the adipogenic program including proliferation and adipose commitment of multi-potent mesenchymal stromal cells (preadipocytes) (Hutley et al., 2004, 2001a; Newell et al., 2006). Proliferation and commitment to the adipocyte lineage of these cells is integral to the generation of new adipocytes, however, little is understood of this developmental phase. We have demonstrated the importance of signalling via FGFR1 and activation of the MAPK/ERK pathway and reported that inhibition of this signalling attenuates human adipocyte development *in vitro* (Newell et al., 2006; Widberg et al., 2009). FGF ligands are known to regulate cellular development in other organs, at least in part, through paracrine interactions leading to co-regulation of expression of other FGF family members (Lebeche et al., 1999; Li et al., 2000; Sherman et al., 1993). In the current paper we aimed to further explore the molecular mechanisms mediating the demonstrated adipogenic effects of FGF-1 and focused on a putative role of other, endogenous FGFs in these effects.

Both FGF-2 and FGF-10 are expressed in white adipose tissue (WAT) and have each been implicated in the adipogenic process with murine knock-out studies demonstrating modulation of WAT development in both models. *In vitro* studies have also determined effects of exogenous application of these factors on adipogenesis. Data demonstrates a role for FGF-10 in preadipocyte proliferation, however, no effect on differentiation in response to exogenous FGF-10 was determined (Yamasaki et al., 1999). Exogenous FGF-2 has also been shown to promote preadipocyte proliferation whilst studies examining differentiation have yielded inconsistent results (Hutley et al., 2004; Krieger-Brauer and Kather, 1995; Neubauer et al., 2004; Prusty et al., 2002). Nothing is yet known of the expression of these factors during human adipocyte development and whether regulation of expression is part of the complex machinery coordinating this process.

The current work determined that FGF-1 treatment of preadipocytes results in modulation of mRNA expression of both FGF-2 and FGF-10. FGF-2 mRNA and protein were significantly decreased by FGF-1 during the pre-confluent and confluent stages and prior to induction of differentiation. This developmental stage is particularly important for cell fate decisions and commitment to specific lineages both of which are known to be regulated by FGF signalling (Patel and McFarlane, 2000; Dusterhoft and Pette, 1999; Harada et al., 2002; Moody, 2004; Dvorak et al., 2005). The finding of decreased FGF-2 expression during this temporal development suggests a putative role for this factor as a regulator of the earliest stages of the adipogenic process. Conversely FGF-10 mRNA increased upon FGF-1 treatment, however, this change in expression was not confirmed at the protein level. Also, FGF-10 signals specifically via the IIIb isoform of FGFR2 (Yeh et al., 2003) and we have previously observed that only the IIIc isoforms of FGF receptors are expressed in human preadipocytes (unpublished observations). This observation is consistent with reports that cells of mesenchymal origin (which includes preadipocytes) express the IIIc isoforms of FGF receptors (Ornitz et al., 1996; Orr-Urtreger et al., 1993). In light of the above, subsequent studies focused on a putative regulatory role for FGF-2 in differentiation of human preadipocytes.

In response to FGF-1, the expression of all FGF-2 isoforms decreased and this correlated with reduced FGF-2 secretion from the cells. This decreased expression in response to treatment with 'adipogenic' FGF-1 is suggestive of a negative role for FGF-2 in adipogenesis. Support for a putative negative role of FGF-2 in this process is provided by the strong correlation between high FGF-2 expression in non-FGF-1-treated primary preadipocytes and their limited capacity to differentiate *in vitro*.

Initially this potential negative role of FGF-2 was an unexpected finding as we, and others, have previously demonstrated a pro-adipogenic role of this factor (Hutley et al., 2004; Prusty et al., 2002). However, such observations were made with exogenously applied recombinant FGF-2 which exists solely as the 18 kDa isoform, and therefore the intracellular action of the FGF-2 isoforms in the nucleus (in particular the 22 and 24 kDa isoforms) may be of particular interest (Quarto et al., 2005).

Further work examined whether inhibition of FGF-2 expression altered aspects of the adipogenic program. We demonstrated that silencing FGF-2 expression resulted in a decrease in phPA proliferation suggesting that FGF-2 may contribute to the regulation of cell cycle in phPA as has been shown in many other cell types (Kisiday et al., 2010; Neary et al., 2005). We did not investigate an effect of FGF-2 knockdown on proliferation following induction of differentiation, termed mitotic clonal expansion (MCE), as we have previously shown that phPAs (Newell et al., 2006) and

SGBS cells (unpublished observations) do not go through MCE during differentiation. The gene silencing studies also demonstrated that decreased FGF-2 expression resulted in significant increases in expression of a number of genes known to be central to the adipogenic process. This increased expression, including that of PPARγ, the central regulator of adipogenesis, signifies increased commitment to the adipocyte lineage and mirrors the affects of FGF-1 treatment (Hutley et al., 2004; Newell et al., 2006; Widberg et al., 2009). Taken together the above findings suggest that the modulation of FGF-2 expression observed in response to FGF-1 may be an important mechanism in the potent adipogenic actions of FGF-1. One possibility is that the FGF-1-induced decrease in FGF-2 is important for cell cycle exit and growth arrest thus allowing the cells to commit to adipose conversion (Ailhaud et al., 1990; Cho and Jefcoate, 2004; Dani et al., 1990; Morrison and Farmer, 1999). Although this appears somewhat paradoxical, evidence indicates that nuclear FGF-2, in combination with FGFR-1, plays an important role in regulating transition from proliferation to differentiation in various cell types (Stachowiak et al., 1997, 2007; Dunham-Ems et al., 2009). We previously demonstrated that one of the effects of FGF-1 was to promote extrusion of FGFR-1 from the nucleus in confluent cells (Widberg et al., 2009). Thus, it is tempting to speculate that the FGF-1-induced decrease in nuclear FGF-2 underpins the translocation of FGFR-1 from the nucleus and that this combination of events contributes to the growth arrest and commitment to the adipocyte lineage, as reflected by increased expression of adipocyte markers. Further studies are required to investigate this possibility.

The above work demonstrates a novel regulatory role for endogenous FGF-2 in the early stages of proliferation and commitment of human preadipocytes. However, these effects were not maintained in the latter stages of the adipogenic program. Indeed, by day 14 there was a trend toward reduced expression of the adipogenic markers and no obvious difference in lipid accumulation or G3PDH activity. Collectively these observations suggest that FGF-2's role in the adipogenic process may be relatively complex, with tight temporal regulation of its expression a prerequisite for efficient differentiation. It is noteworthy that FGF-2 expression increased in the final stages of differentiation (see Fig. 3A) and this elevation was particularly marked in FGF-1 treated cells. Thus, it is tempting to speculate that this late increase in FGF-2 contributes to the final maturation of the adipocyte.

In conclusion, this work identifies a novel role for endogenous FGF-2 in regulation of human adipogenesis. This factor is a positive regulator of human PA proliferation and negative regulator of adipocyte commitment (demonstrated by increased expression of adipogenic markers at the earliest differentiation timepoints in response to FGF-2 knockdown). Further, these effects of FGF-2 knockdown mimic the adipogenic effects of exogenous FGF-1 suggesting that regulation of FGF-2 expression is an important component of the potent adipogenic actions of FGF-1. Further work is required to determine the extent of the role played by this factor in co-ordinating or modulating the temporal conversion of these multi-potent stromal cells into mature adipocytes and how endogenous expression of this factor fits into the complex network of regulatory events regulating adipocyte number in humans.

Acknowledgements

This work was funded by The National Health and Medical Research Council of Australia and The Diabetes Australia Research Trust and The National Heart Foundation. We acknowledge the expertise and technical assistance of Dr. Anthony Bachmann in developing methodologies used in this study.

References

Ailhaud, G., Amri, E., Bardon, S., Barcellini-Couget, S., Bertrand, B., Catalioto, R.M., Dani, C., Djian, P., Doglio, A., Forest, C., et al., 1990. The adipocyte: relationships between proliferation and adipose cell differentiation. Am. Rev. Respir. Dis. 142 (6 Pt 2), S57–59.

Behrmann, I., Smyczek, T., Heinrich, P.C., Schmitz-Van de Leur, H., Komyod, W., Giese, B., Muller-Newen, G., Haan, S., Haan, C., 2004. Janus kinase (Jak) subcellular localization revisited: the exclusive membrane localization of endogenous Janus kinase 1 by cytokine receptor interaction uncovers the Jak.receptor complex to be equivalent to a receptor tyrosine kinase. J. Biol. Chem. 279 (34), 35486–35493.

Bour, S., Daviaud, D., Gres, S., Lefort, C., Prevot, D., Zorzano, A., Wabitsch, M., Saulnier-Blache, J.S., Valet, P., Carpene, C., 2007. Adipogenesis-related increase of semicarbazide-sensitive amine oxidase and monoamine oxidase in human adipocytes. Biochimie 89 (8), 916–925.

Cho, Y.C., Jefcoate, C.R., 2004. PPARgamma1 synthesis and adipogenesis in C3H10T1/2 cells depends on S-phase progression, but does not require mitotic clonal expansion. J. Cell. Biochem. 91 (2), 336–353.

Dani, C., Amri, E.Z., Bertrand, B., Enerback, S., Bjursell, G., Grimaldi, P., Ailhaud, G., 1990. Expression and regulation of pOb24 and lipoprotein liPAe genes during adipose conversion. J. Cell. Biochem. 43 (2), 103–110.

Dunham-Ems, S.M., Lee, Y.W., Stachowiak, E.K., Pudavar, H., Claus, P., Prasad, P.N., Stachowiak, M.K., 2009. Fibroblast growth factor receptor-1 (FGFR1) nuclear dynamics reveal a novel mechanism in transcription control. Mol. Biol. Cell 20, 2401–2412.

Dusterhoft, S., Pette, D., 1999. Evidence that acidic fibroblast growth factor promotes maturation of rat satellite-cell-derived myotubes in vitro. Differentiation 65 (3), 161–169.

Dvorak, P., Dvorakova, D., Koskova, S., Vodinska, M., Najvirtova, M., Krekac, D., Hampl, A., 2005. Expression and potential role of fibroblast growth factor 2 and its receptors in human embryonic stem cells. Stem Cells 23 (8), 1200–1211.

Florkiewicz, R.Z., Sommer, A., 1989. Human basic fibroblast growth factor gene encodes four polypeptides: three initiate translation from non-AUG codons. Proc. Natl. Acad. Sci. U.S.A. 86 (11), 3978–3981.

Harada, H., Toyono, T., Toyoshima, K., Yamasaki, M., Itoh, N., Kato, S., Sekine, K., Ohuchi, H., 2002. FGF10 maintains stem cell compartment in developing mouse incisors. Development 129 (6), 1533–1541.

Heilbronn, L., Smith, S.R., Ravussin, E., 2004. Failure of fat cell proliferation, mitochondrial function and fat oxidation results in ectopic fat storage, insulin resistance and type II diabetes mellitus. Int. J. Obes. Relat. Metab. Disord. 28 (Suppl 4), S12–21.

Hossain, M.G., Iwata, T., Mizusawa, N., Shima, S.W., Okutsu, T., Ishimoto, K., Yoshimoto, K., 2010. Compressive force inhibits adipogenesis through COX-2-mediated down-regulation of PPARgamma2 and C/EBPalpha. J. Biosci. Bioeng. 109 (3), 297–303.

Hutley, L., Shurety, W., Newell, F., McGeary, R., Pelton, N., Grant, J., Herington, A., Cameron, D., Whitehead, J., Prins, J., 2004. Fibroblast growth factor 1: a key regulator of human adipogenesis. Diabetes 53 (12), 3097–3106.

Hutley, L.J., Herington, A.C., Shurety, W., Cheung, C., Vesey, D.A., Cameron, D.P., Prins, J.B., 2001a. Human adipose tissue endothelial cells promote preadipocyte proliferation. Am. J. Physiol. Endocrinol. Metab. 281 (5), E1037–1044.

Hutley, L.J., Newell, F.S., Suchting, S.J., Prins, J.B., 2001b. Adipose tissue. In: Koller, M.R., Palsson, B.O., Masters, J.R.W. (Eds.), Primary Mesenchymal Cells. Kluwer Academic Publishers, Dordrecht, pp. 173–187.

Kim, J.Y., van de Wall, E., Laplante, M., Azzara, A., Trujillo, M.E., Hofmann, S.M., Schraw, T., Durand, J.L., Li, H., Li, G., Jelicks, L.A., Mehler, M.F., Hui, D.Y., Deshaies, Y., Shulman, G.I., Schwartz, G.J., Scherer, P.E., 2007. Obesity-associated improvements in metabolic profile through expansion of adipose tissue. J. Clin. Invest. 117 (9), 2621–2637.

Kisiday, J.D., Hale, B.W., Almodovar, J.L., Lee, C.M., Kipper, M.J., Wayne McIlwraith, C., Frisbie, D.D., 2010. Expansion of mesenchymal stem cells on fibrinogen-rich protein surfaces derived from blood plasma. J. Tissue Eng. Regen. Med..

Krieger-Brauer, H.I., Kather, H., 1995. Antagonistic effects of different members of the fibroblast and platelet-derived growth factor families on adipose conversion and NADPH-dependent H_2O_2 generation in 3T3 L1-cells. Biochem. J. 307 (Part 2), 549–556.

Lebeche, D., Malpel, S., Cardoso, W.V., 1999. Fibroblast growth factor interactions in the developing lung. Mech. Dev. 86 (1–2), 125–136.

Li, C.M., Khosla, J., Pagan, I., Hoyle, P., Sannes, P.L., 2000. TGF-beta1 and fibroblast growth factor-1 modify fibroblast growth factor-2 production in type II cells. Am. J. Physiol. Lung Cell. Mol. Physiol. 279 (6), L1038–1046.

Medina-Gomez, G., Gray, S.L., Yetukuri, L., Shimomura, K., Virtue, S., Campbell, M., Curtis, R.K., Jimenez-Linan, M., Blount, M., Yeo, G.S., Lopez, M., Seppanen-Laakso, T., Ashcroft, F.M., Oresic, M., Vidal-Puig, A., 2007. PPAR gamma 2 prevents lipotoxicity by controlling adipose tissue expandability and peripheral lipid metabolism. PLoS Genet. 3 (4), e64.

Mejhert, N., Galitzky, J., Pettersson, A.T., Bambace, C., Blomqvist, L., Bouloumie, A., Frayn, K.N., Dahlman, I., Arner, P., Ryden, M., 2010. Mapping of the fibroblast growth factors in human white adipose tissue. J. Clin. Endocrinol. Metab. 95 (5), 2451–2457.

Moody, S.A., 2004. To differentiate or not to differentiate: regulation of cell fate decisions by being in the right place at the right time. Cell Cycle 3 (5), 564–566.

Morrison, R.F., Farmer, S.R., 1999. Insights into the transcriptional control of adipocyte differentiation. J. Cell. Biochem. (32-33), 59–67.

Neary, J.T., Kang, Y., Shi, Y.F., 2005. Cell cycle regulation of astrocytes by extracellular nucleotides and fibroblast growth factor-2. Purinergic Signal 1 (4), 329–336.

Neubauer, M., Fischbach, C., Bauer-Kreisel, P., Lieb, E., Hacker, M., Tessmar, J., Schulz, M.B., Goepferich, A., Blunk, T., 2004. Basic fibroblast growth factor enhances PPARgamma ligand-induced adipogenesis of mesenchymal stem cells. FEBS Lett. 577 (1–2), 277–283.

Newell, F.S., Su, H., Tornqvist, H., Whitehead, J.P., Prins, J.B., Hutley, L.J., 2006. Characterization of the transcriptional and functional effects of fibroblast growth factor-1 on human preadipocyte differentiation. FASEB J. 20 (14), 2615–2617.

Ornitz, D.M., Xu, J., Colvin, J.S., McEwen, D.G., MacArthur, C.A., Coulier, F., Gao, G., Goldfarb, M., 1996. Receptor specificity of the fibroblast growth factor family. J. Biol. Chem. 271 (25), 15292–15297.

Orr-Urtreger, A., Bedford, M.T., Burakova, T., Arman, E., Zimmer, Y., Yayon, A., Givol, D., Lonai, P., 1993. Developmental localization of the splicing alternatives of fibroblast growth factor receptor-2 (FGFR2). Dev. Biol. 158 (2), 475–486.

Patel, A., McFarlane, S., 2000. Overexpression of FGF-2 alters cell fate specification in the developing retina of Xenopus laevis. Dev. Biol. 222 (1), 170–180.

Prats, H., Kaghad, M., Prats, A.C., Klagsbrun, M., Lelias, J.M., Liauzun, P., Chalon, P., Tauber, J.P., Amalric, F., Smith, J.A., et al., 1989. High molecular mass forms of basic fibroblast growth factor are initiated by alternative CUG codons. Proc. Natl. Acad. Sci. U.S.A. 86 (6), 1836–1840.

Prusty, D., Park, B.H., Davis, K.E., Farmer, S.R., 2002. Activation of MEK/ERK signaling promotes adipogenesis by enhancing peroxisome proliferator-activated receptor gamma (PPARgamma) and C/EBPalpha gene expression during the differentiation of 3T3-L1 preadipocytes. J. Biol. Chem. 277 (48), 46226–46232.

Quarto, N., Fong, K.D., Longaker, M.T., 2005. Gene profiling of cells expressing different FGF-2 forms. Gene 356, 49–68.

Sethi, J.K., Vidal-Puig, A.J., 2007. Thematic review series: adipocyte biology. Adipose tissue function and plasticity orchestrate nutritional adaptation. J. Lipid Res. 48 (6), 1253–1262.

Sherman, L., Stocker, K.M., Morrison, R., Ciment, G., 1993. Basic fibroblast growth factor (bFGF) acts intracellularly to cause the transdifferentiation of avian neural crest-derived Schwann cell precursors into melanocytes. Development 118 (4), 1313–1326.

Spalding, K.L., Arner, E., Westermark, P.O., Bernard, S., Buchholz, B.A., Bergmann, O., Blomqvist, L., Hoffstedt, J., Naslund, E., Britton, T., Concha, H., Hassan, M., Ryden, M., Frisen, J., Arner, P., 2008. Dynamics of fat cell turnover in humans. Nature 453 (7196), 783–787.

Stachowiak, M.K., Moffett, J., Maher, P., Tucholski, J., Stachowiak, E.K., 1997. Growth factor regulation of cell growth and proliferation in the nervous system. A new intracrine nuclear mechanism. Mol. Neurobiol. 15, 257–283.

Stachowiak, M.K., Maher, P.A., Stachowiak, E.K., 2007. Integrative nuclear signaling in cell development – a role for FGF receptor-1. DNA Cell Biol. 26, 811–826.

Su, H., Gunter, J.H., de Vries, M., Connor, T., Wanyonyi, S., Newell, F.S., Segal, D., Molero, J.C., Reizes, O., Prins, J.B., Hutley, L.J., Walder, K., Whitehead, J.P., 2009. Inhibition of inosine monophosphate dehydrogenase reduces adipogenesis and diet-induced obesity. Biochem. Biophys. Res. Commun. 386 (2), 351–355.

Teichert-Kuliszewska, K., Hamilton, B.S., Deitel, M., Roncari, A.K., 1992. Augmented production of heparin-binding mitogenic proteins by preadipocytes from massively obese persons. J. Clin. Invest. 90, 1226–1231.

Wabitsch, M., Brenner, R.E., Melzner, I., Braun, M., Moller, P., Heinze, E., Debatin, K.M., Hauner, H., 2001. Characterization of a human preadipocyte cell strain with high capacity for adipose differentiation. Int. J. Obes. Relat. Metab. Disord. 25 (1), 8–15.

Whitehead, J.P., Molero, J.C., Clark, S., Martin, S., Meneilly, G., James, D.E., 2001. The role of Ca2+ in insulin-stimulated glucose transport in 3T3-L1 cells. J. Biol. Chem. 276 (30), 27816–27824.

Widberg, C.H., Newell, F.S., Bachmann, A.W., Ramnoruth, S.N., Spelta, M.C., Whitehead, J.P., Hutley, L.J., Prins, J.B., 2009. Fibroblast growth factor receptor 1 is a key regulator of early adipogenic events in human preadipocytes. Am. J. Physiol. Endocrinol. Metab. 296 (1), E121–131.

Yamasaki, M., Emoto, H., Konishi, M., Mikami, T., Ohuchi, H., Nakao, K., Itoh, N., 1999. FGF-10 is a growth factor for preadipocytes in white adipose tissue. Biochem. Biophys. Res. Commun. 258 (1), 109–112.

Yeh, B.K., Igarashi, M., Eliseenkova, A.V., Plotnikov, A.N., Sher, I., Ron, D., Aaronson, S.A., Mohammadi, M., 2003. Structural basis by which alternative splicing confers specificity in fibroblast growth factor receptors. Proc. Natl. Acad. Sci. U.S.A. 100 (5), 2266–2271.