Fibroblast growth factor receptor 1 is a key regulator of early adipogenic events in human preadipocytes

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Widberg CH, Newell FS, Bachmann AW, Ramnoruth SN, Spelta MC, Whitehead JP, Hutley LJ, Prins JB. Fibroblast growth factor receptor 1 is a key regulator of early adipogenic events in human preadipocytes. Am J Physiol Endocrinol Metab 296: E121–E131, 2009. First published October 21, 2008; doi:10.1152/ajpendo.90602.2008.—Cell number is an important determinant of adipose tissue mass, and the coordinated proliferation and differentiation of preadipocytes into mature lipid-laden adipocytes underpins the increased adipose tissue mass associated with obesity. Despite this, the molecular cues governing such adipose tissue expansion are poorly understood. We previously reported that fibroblast growth factor-1 (FGF-1) promotes both proliferation and differentiation of human preadipocytes and that the major adipogenic effect of FGF-1 occurs during proliferation, priming the cells for adipose conversion. In the current study, we examined whether this effect was linked to the mitogenic action of FGF-1 by investigating the mitogenic and adipogenic potential of other growth factors, platelet-derived growth factor (PDGF; AA and BB) and vascular endothelial growth factor. Although PDGF-AA and PDGF-BB showed comparable mitogenic potential to FGF-1, only FGF-1 treatment resulted in priming and subsequent differentiation. A combination of biochemical and genetic approaches revealed an important role for FGFR1. Knock down of FGFR1 expression by small-interfering RNA reduced FGF-1-stimulated signaling events, proliferation, and priming. Together these data highlight the unique nature of the role of FGF-1 during the earliest stages of adipogenesis and establish a role for FGFR1 in human adipogenesis, identifying FGFR1 as a potential therapeutic target to reduce obesity.

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Because receptor tyrosine kinase growth factors other than FGFs have not been investigated in the context of priming human PA for differentiation, we assessed the effect of several of these factors on PA proliferation and priming in phPA and SGBS PA. Growth factors examined in this study included members of the platelet-derived growth factor (PDGF) family, which have been reported to act as inducers of PA proliferation (6, 7) and vascular endothelial growth factor (VEGF), which is expressed throughout adipogenesis (8). Here we demonstrate that, while PDGF-AA and -BB efficiently stimulated human PA proliferation, only FGF-1 primed the cells for differentiation. VEGF did not influence either PA proliferation or differentiation. Because priming was specific to FGF-mediated proliferation, we examined the role of FGFR in these effects and determined that FGFR activity is required for FGF-1-stimulated proliferation and priming and that FGFR1 plays a key role in these early adipogenic events.

MATERIALS AND METHODS

Materials. Reagents were obtained from Sigma-Aldrich (Castle Hill, Victoria, Australia), unless otherwise stated. Tissue culture reagents were obtained from Invitrogen (Mount Waverley, Victoria, Australia).

Cell isolation and culture. To obtain phPA, subcutaneous adipose tissue biopsies were obtained from 4 male [average age 42 yr (range 21–79)] patients undergoing elective open-abdominal surgery. One of the patients had diabetes, severe systemic illness, or were taking medications known to affect adipose tissue mass or metabolism. The protocol was approved by the Research Ethics Committees of the Princess Alexandra Hospital and the University of Queensland. phPA were isolated, cultured, and differentiated as previously described (14, 25) and used for experiments at passage 2. The SGBS PA strain (36) was cultured as described (25) for ≈50 generations or 6 wk.

FGFR1-4-overexpressing HEK-293 cells were generated using the Flp-In T-Rex tetracycline-inducible cell system according to the manufacturer’s instructions (Invitrogen). The cells were passaged in DMEM supplemented with 100 µg/ml hygromycin and 5 µg/ml blasticidin. To induce expression of FGFR, cells were treated overnight with 1 µg/ml tetracycline.

FGF-1 treatment. Recombinant human FGF-1 was produced as described (37). Unless otherwise stated, 1 ng/ml FGF-1 in the presence of 90 µg/ml heparin was used to treat SGBS PA and phPA.

Growth factor treatment of PA. Based on preliminary dose-response studies, SGBS PA or phPA were treated with equimolar (60 pM) concentrations of FGF-1 (90 µg/ml heparin), PDGF-AA, PDGF-BB (R&D Biosystems, Minneapolis, MN), or VEGF (10 µg/ml heparin) (Upstate Biotechnology, Lake Placid, NY) in 10% FCS DMEM-F-12. Proliferation was assessed using SYTO 60 assay. SGBS PA or phPA were plated in 48-well plates at 1,500 or 2,500 cells/well, respectively. Cells were allowed to adhere overnight before treatment for 4–5 days. Cells were fixed in 3.7% formaldehyde-PBS and permeabilized in 0.1% Triton X-100-PBS before blocking in Odyssey blocking buffer (OBB; LI-COR Biosciences, Lincoln, NE) and incubation in SYTO 60 (1:10,000) in a 1:1 solution of OBB-PBS. Cells were washed in 0.1% Tween 20-PBS, and the plate was subsequently scanned on the LI-COR Odyssey Infrared Imaging System. The relative intensities of the wells were calculated using Odyssey image analysis software (version 1.2), and results were expressed as the change in fluorescence units (above or below) relevant controls. The SYTO 60 assay was validated against direct cell counts, and results showed a strong positive correlation with cell number in human PA (r² = 0.9948).

Assessment of differentiation. Lipid accumulation was assessed morphologically using phase-contrast microscopy (×100 magnification) (Nikon Eclipse TE300). Differentiation was also assessed by enzymatic assay of glycerol 3-phosphate dehydrogenase (G3PDH) activity as previously described (16, 25) using a Fluostar Optima spectrophotometer (BMG Labtech; Mornington, Victoria, Australia). Results are expressed as milliunits per square centimeter of culture area.

Real-time RT-PCR. Total RNA was obtained using RNeasy kits (Qiagen, Doncaster, Victoria, Australia) and concentration and quality were determined using a spectrophotometer (Nanodrop ND-1000; Biolab, Clayton, Victoria, Australia). Total RNA (500 ng-1 µg) was reverse transcribed using random hexamers (Promega, Amannlade, NSW, Australia) and Superscript III reverse transcriptase according to the manufacturer’s instructions (Invitrogen). The primers used in this study, listed in Table 1, were designed using Primer 3 software (29) and purchased from Geneworks (Hindmarsh, SA, Australia). Real-time RT-PCR was performed using the SyBr Green detection protocol (reaction mix: SyBR Premix Ex Taq from Takara Bio USA; Madison, WI, USA) as per the manufacturer’s instructions on the RotorGene 3000 instrument (Corbett Research, Sydney, Australia). The absence of contaminating genomic DNA was verified by no reverse transcriptase control samples. 2⁻ΔCt values were calculated relative to the expression of cyclophilin and further calibrated to the expression of a control sample.

Tunicamycin treatment and subcellular fractionation. Proliferating SGBS PA or phPA were treated with 10 µg/ml tunicamycin for 48 h and subjected to the same treatments as SGBS PA with the exception of longer growth periods.

Assessment of differentiation. Lipid accumulation was assessed morphologically using phase-contrast microscopy (×100 magnification) (Nikon Eclipse TE300). Differentiation was also assessed by enzymatic assay of glycerol 3-phosphate dehydrogenase (G3PDH) activity as previously described (16, 25) using a Fluostar Optima spectrophotometer (BMG Labtech; Mornington, Victoria, Australia). Results are expressed as milliunits per square centimeter of culture area.

Table 1. Primer sequences for real-time RT-PCR in Simpson Golabi Behmel syndrome preadipocytes and primary human preadipocytes

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer Sequences (5’ to 3’)</th>
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<tbody>
<tr>
<td>PPARγ</td>
<td>Forward: GAAACCTTCAAGAGTACAGTTGCAA&lt;br&gt;Reverse: AGGCTTATTGTCAGTGCGTCTCTC</td>
</tr>
<tr>
<td>FGF1</td>
<td>Forward: CCTTGTTGACAGAGGCAATG&lt;br&gt;Reverse: AGATCCGCTCAAAATCTGGCC</td>
</tr>
<tr>
<td>FGF2</td>
<td>Forward: AAAGGAGAGGGTTTAAAGCA&lt;br&gt;Reverse: CCTTGCCAGATGGSACCACAC</td>
</tr>
<tr>
<td>FGF3</td>
<td>Forward: AGATCCGCTCAAAATCTGGCC&lt;br&gt;Reverse: GCTGTGCTCTTTCAGCATCCTC</td>
</tr>
<tr>
<td>FGF4</td>
<td>Forward: GGCTCCACACACATTGACT&lt;br&gt;Reverse: GTGGTGTACACCGGCTCAAAC</td>
</tr>
<tr>
<td>Cyclophilin</td>
<td>Forward: CGGGCTCCTTTGTAGCGTT&lt;br&gt;Reverse: TCCCTAGTCGCACAGGCG</td>
</tr>
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PPAR, peroxisome proliferator-activated receptor; FGFR, fibroblast growth factor receptor.
before partial fractionation. Cells were washed with 20 mM HEPES, 0.25 M sucrose, and 1 mM EDTA (HE buffer), scraped in HES supplemented with 1 mM Na$_2$VO$_4$ and protease inhibitors (1 μg/ml aprotinin, 1 μg/ml antipain, 1 μg/ml pepstatin, 1.5 μg/ml leupeptin, and 0.5 mM 4-(2-aminomethyl)benzenesulfonfonyl fluoride hydrochloride), and homogenized using a 22-gauge needle. Nuclei and other cellular debris were pelleted by centrifugation at 1,600 g, and the nuclear pellet was purified as described (2). Plasma membrane and other cellular membranes were isolated by centrifugation at 170,000 g for 75 min. This high-speed pellet was resuspended in HES supplemented with protease inhibitors and 1% Triton.

Western blot analysis. Cell lysates were prepared in 20 mM HEPES, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, and phosphatase inhibitors (1 mM Na$_2$VO$_4$, 1 mM Na$_3$P$_2$O$_7$, and 10 mM NaF), and protein concentrations were determined using BCA protein assay (Pierce Biotechnology, Rockford, IL). Laemmli sample buffer (+500 mM dithiothreitol (DTT)) was added, and the samples were resolved by SDS-PAGE and transferred to Immobilon-P or Immobilon-FL polyvinylidene difluoride membrane (Millipore, Bedford, MA). Immobilon-FL membrane was probed with phospho (p)-extracellular signal-regulated kinase (ERK) 1/2 (Thr202/Tyr204), ERK1/2 (Cell Signaling Technology, Danvers, MA), or α-tubulin (Sigma-Aldrich) antibodies and visualized using LI-COR Odyssey as described (25). Enhanced chemiluminescence (Supersignal West Dura Extended Duration Substrate) was used for detection of FGFR1 (C15) (Santa Cruz Biotechnology, Santa Cruz, CA), p-FGFR (Tyro356/354) (R&D Systems), and p-FGFR receptor substrate (FRS) 2αTyro196 (Cell Signaling Technology) on Immobilon-P membrane.

In-cell Western. SGBS PA were seeded on a 48-well plate at a density of 1 × 10$^4$ cells/well and incubated for 72 h. The cells were serum-starved before treatment with FGF-1 or PDGF-BB (both at 3 nM), FGFR kinase inhibitors (0–250 nM PD-173074 or 0–10 μM SU-5402), or the mitogen-activated protein kinase kinase inhibitor U-0126 (25 μM) for 75 min at 37°C. Cells were washed with PBS (containing phosphatase inhibitors), fixed in 0.5% paraformaldehyde (PFA)-PBS (phosphatase inhibitors), and permeabilized with 0.1% Triton X-100-PBS. Cells were blocked and incubated with primary antibodies, p-ERK1/2 (1:100) and α-tubulin (1:1,500), in OBB-PBS. Cells were washed with 0.1% Tween 20-PBS and incubated with secondary antibodies, goat anti-rabbit IgG (1:600; Rockland Immunochemicals, Gilbertsville, PA) and goat anti-mouse IgG (1:500; Invitrogen), in OBB-PBS. Cells were washed a further three times. The data were analyzed using a grid-sheet in Odyssey version 1.2 software. The U-O126 value (background) was subtracted, and data were normalized to tubulin (in the same well) and expressed as fluorescence units.

Immunofluorescence. SGBS PA or phPA grown on cover slips were fixed in 2% PFA, washed three times in PBS, and quenched in 50 mM NH$_4$Cl-PBS. The cover slips were further washed with PBS, blocked in 2% BSA-0.1% saponin, and incubated with FGFR1 antibody (1:100) in blocking solution. Cells were washed three times in PBS, covered, and incubated with Alexa-488 or -594 secondary antibodies (1:200 in blocking solution; Invitrogen) and 4',6-diamidino-2-phenylindole. Finally, cells were washed (as above) and mounted in Vectashield mounting media (Vector Laboratories, Burlingame, CA) for viewing in a Zeiss (Axioskop 2 plus) light microscope with ×63 oil objective.

Immunoprecipitation. FGF-1-pretreated SGBS PA or phPA were incubated in heparin-containing medium for 5 h, serum starved overnight, and stimulated with 10 ng/ml FGF-1 and 90 μg/ml heparin for 5 min at 37°C. Cells were washed with PBS (containing 0.5 mM Na$_2$VO$_4$) and scraped in 25 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% 2-mercaptoethanol, and 1% Nonidet P-40, with phosphatase and protease inhibitors (HEB buffer). Cells were lysed using 22- and 27-gauge needles and solubilized for 30 min, and insoluble material was pelleted by centrifugation at 10,000 g for 10 min at 4°C. The protein lysates were precleared with a 1:1 mix of protein A (ProA)-protein G (ProG) beads, incubated with 3 μg of FGFR1 or phosphotyrosine (Py99; Santa Cruz) antibodies overnight, captured on ProA-ProG beads for 1–2 h before beads were washed, harvested with HEB buffer (10–20× bed volume), and resuspended in an equal volume of 2× Laemmli sample buffer (+200 mM DTT).

FGFR1 knock down. Validated small-interfering RNA (siRNA) against FGFR1 and a scrambled control (AllStars Negative control siRNA) were used (Qiagen). SGBS PA or phPA were seeded on six-well plates at 7.5 × 10$^4$ cells/well in serum-containing medium. Transfections were carried out 24 h later using Oligofectamine-OptiMEM, Oligofectamine [0.6% (vol/vol)], and FGFR1 or negative control siRNA (50 nM) according to the manufacturer’s instructions (Invitrogen). Cells were transfected a second time 24 h after the initial transfection, and knock down was assessed 48 h posttransfection by real-time RT-PCR of FGFR1 mRNA and Western blot analysis of FGFR1 protein. Transfected cells were trypsORIZED 48 h posttransfection and reseeded for subsequent experiments essentially as described above.

Statistics. 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 ± SE. P values <0.05 were considered to be statistically significant.

RESULTS

Priming for differentiation is FGF specific. To determine whether proliferation-priming in human PA was specific to FGF or common to other mitogenic growth factors that act via tyrosine kinase receptors, we performed experiments to examine proliferation of human PA in response to several mitogens, including PDGF-AA and -BB and VEGF at concentrations spanning 30 pM-2 nM. These concentrations were based on previous reports in other models of adipogenesis (11, 13) and aimed to determine a concentration of PDGF and VEGF that gave a similar mitogenic response as FGF-1 at 60 pM. This is a previously optimized and published concentration of FGF-1 for use on human PA to increase adipogenesis (15, 25). PDGF-AA and -BB both increased PA proliferation in a dose-dependent manner, whereas VEGF was not mitogenic at any concentration tested (data not shown). Similar results on proliferation were achieved in both human PA models, with Fig. 1A demonstrating the mitogenic effect of FGF-1, PDGF-AA, and PDGF-BB in SGBS PA at equimolar growth factor concentrations. VEGF and FGF-1 are both heparin-binding growth factors, and, when heparin is added alone (or together with VEGF), there is a nonsignificant reduction in PA proliferation (Fig. 1A).

We next aimed to determine if the increased proliferation of human PA in response to PDGF-AA and -BB also resulted in priming of the cells for differentiation as observed in response to FGF. All growth factors were used at the concentration previously determined to have equal proliferative effects (60 pM). VEGF (60 pM) was included to determine effects of a nonproliferative growth factor in these experiments. The expression of PPARγ mRNA, at confluence, was used as a marker for cell priming (25). In agreement with our published data (25), FGF-1–treated human PA exhibited a two- to three-fold increase in PPARγ expression compared with cells treated with heparin alone (Fig. 1B). In contrast, PDGF-AA and -BB and VEGF treatment had no effect on PPARγ expression in

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either the SGBS PA model (Fig. 1B) or in the phPA model (data not shown). Priming was further assessed in both SGBS and phPA (Fig. 1, C–F) by determining differentiation capacity of the cells following growth factor (60 pM) treatment during the proliferation stage only. As expected, FGF-1 treatment primed human PA for subsequent differentiation, as judged by increased lipid accumulation (Fig. 1, C and E) and increased activity of the differentiation marker G3PDH (Fig. 1, D and F) following exposure to a differentiation induction medium. In these experiments, neither the mitogenic PDGF-AA and -BB nor nonmitogenic VEGF (in the SGBS PA model) had an effect on PA differentiation (Fig. 1, C–E). It is notable that
primary PAs, in contrast to the SGBS PA model, do not differentiate in the absence of FGF-1 (compare lipid accumulation in Fig. 1, C and E, respectively). Thus, despite a similar mitogenic effect to FGF-1, PDGF-AA and -BB had no effect on lipid accumulation and differentiation in the SGBS or phPA model of adipogenesis.

Inhibition of FGFR activation during proliferation decreases FGF-1-priming and differentiation potential of human PA. Although there is some evidence suggesting a role for FGFR activation during the final stages of adipocyte development (28), little is known of the role of FGFR signaling in regulation of human PA proliferation and priming. Therefore, we next assessed the involvement of FGFR in these early adipogenic processes using the ATP-competitive FGFR inhibitors PD-173074 and SU-5402 (22, 23). To confirm the efficacy of the inhibitors, HEK-293 cells stably overexpressing each of the four receptors were treated with PD-173074 or SU-5402, and FGF-1 signaling events were examined by Western blot analysis using p-FGFR (Tyr\textsuperscript{653–654}), p-FRS2αTyr\textsuperscript{106}, and p-ERK1/2 antibodies. Figure 2A shows phosphorylation of a 150-kDa FGFR1 band, as well as phosphorylation of the adaptor protein FRS2 and ERK1/2 in response to acute FGF-1 treatment. In the presence of either PD-173074 or SU-5402, there was a decrease in the phosphorylation of FGFR1, FRS2α, and ERK1/2. Similar results were observed in cells overexpressing FGFR2, FGFR3, or FGFR4 (data not shown), indicating that the inhibitors prevent signaling through all four FGFRs.

The ability of these inhibitors to affect FGFR activation in human PA was assessed by Western blot analysis. General FGFR activation was confirmed in proliferating PA using the p-FGFR antibody (Fig. 2B). Several FGF-1 responsive bands, spanning 90–150 kDa (Fig. 2B) were routinely observed in human PA. Whereas the 90- and 150-kDa p-FGFR bands were the most robustly detected and were completely absent in the presence of PD-173074 or SU-5402 treatment, the

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**Fig. 2.** Characterization and validation of pharmacological FGF receptor (FGFR) inhibitors PD-173074 and SU-5402 in FGFR1-overexpressing cells and human PA. A: HEK-293-FGFR1 cells were stimulated \( \pm \) 50 ng/ml FGF-1 and 10 μg/ml heparin and 250 nM PD-173074 or 20 μM SU-5402. Phosphorylation of FGFR1, FRS2α, and extracellular signal-regulated kinase (ERK) 1/2 was analyzed by Western blot. ERK1/2 and tubulin protein were analyzed and used as expression and loading controls \((n = 3)\). B: proliferating primary human (ph) PA were stimulated \( \pm \) 90 ng/ml FGF-1 and 90 μg/ml heparin and 250 nM PD-173074 or 20 μM SU-5402 and analyzed by Western blot as described in A \((n = 3)\). C: In-cell Western of FGF-1- and PDGF-BB-stimulated ERK1/2 phosphorylation (growth factors at 3 nM) in SGBS PA \( \pm \) FGFR inhibitors \((0–250 nM PD-173074 and 0–10 μM SU-5402)\). Results are expressed as fluorescence units over control cells \((n = 3–4)\).
p-FGFR antibody also recognized 120- and 130-kDa bands that were partially FGF-1 responsive and sensitive to FGFR inhibitor treatment. As expected, FGFR inhibitor treatment also decreased FGF-1-induced phosphorylation of FRS2α and ERK1/2 in PA (Fig. 2B).

To assess the specificity of these FGFR inhibitors for FGF-1 signaling compared with PDGF-BB signaling, we employed an In-Cell Western assay for the detection of ERK1/2 phosphorylation in human PA (Fig. 2C). There was a dose-dependent decrease in FGF-1-stimulated ERK1/2 phosphorylation in the presence of 0–250 nM PD-173074 or 0–10 μM SU-5402; however, there was no effect on PDGF-BB-stimulated ERK1/2 phosphorylation (Fig. 2C). These data confirm the efficacy and relative specificity of PD-173074 and SU-5402 for the FGFRs under the experimental conditions.

Next, we determined the effects of the FGFR inhibitors on human PA proliferation. FGF-1-stimulated proliferation was completely abrogated (100 nM PD-173074) or significantly reduced (10 μM SU-5402) in both SGBS PA (Fig. 3A) and phPA (data not shown). FGF-1-stimulated priming of human PA was also completely abolished (PD-173074) or inhibited (SU-5402) by these compounds, as demonstrated by a reduction in PPARγ mRNA expression at confluence (Fig. 3B). We routinely observed differences in morphology between FGF-1-treated (primed) and non-FGF-1-treated (unprimed) cells. The primed cells are small, uniform in shape, and achieve high cell density at confluence while, in comparison, the unprimed cells are larger, more spread out, and grow in a less-ordered pattern. Treatment with the FGFR kinase inhibitors resulted in either reversal to the unprimed morphology in previously FGF-1-primed cells (Fig. 3C) or failure to achieve the primed morphology when growth factor and inhibitor treatment commenced concurrently in FGF naïve cells.

To determine if these observed effects of FGFR inhibition during proliferation resulted in decreased differentiation potential of human PA, the inhibitor-treated cells were induced to differentiate using a standard cocktail in the absence of FGFR inhibitors. Data showed that both SGBS PA and phPA treated
with FGF-1 and either PD-173074 (100 nM SGBS or 50 nM phPA) or SU-5402 (10 μM) during proliferation exhibited significantly decreased differentiation, as measured by G3PDH activity assay (Fig. 3, D and E). We used 50 nM PD-173074 in the phPA cells due to marked growth inhibitory effects of this compound at 100 nM in this model. Overall, these studies demonstrate that the phPA model is more sensitive than the SGBS PA model to the effects of FGFR inhibitors. This is in agreement with our previously published data showing a greater dependence on FGF-1 priming for differentiation (compare Fig. 1, C and E) in the primary cells compared with the SGBS PA strain (25).

**FGFR expression and activation in human PA.** To determine the role of individual FGFRs in this system, the relative expression levels of each receptor were assessed in proliferating cells using real-time RT-PCR (Fig. 4A). FGFR1 was the most abundantly expressed receptor in both SGBS PA and phPA, being 1,000- to 10,000-fold higher than FGFRs 2–4. FGFR1 expression was unchanged by FGF-1 treatment. FGFR2 was more abundant than FGFR3 and FGFR4, and expression of FGFR2 was decreased upon FGF-1 treatment (Fig. 4A, inset). Expression levels of FGFR4 were low but detectable in both SGBS PA and phPA, whereas FGFR3 was only detectable in phPA (data not shown).

Because FGFR1 was the predominant receptor expressed in proliferating PA, the expression of this receptor was investigated further by Western blot and immunofluorescence analysis (Fig. 4, B and C). A number of protein bands, which were similar in size to previously described FGFR1 species (33, 34), were recognized by the FGFR1 antibody in proliferating, confluent, and differentiated human PA (Fig. 4B). Expression of the 90- and 150-kDa FGFR1 species increased throughout differentiation and in response to FGF-1 treatment. N-glycosylation of FGFR1 gives rise to several FGFR species of

![Fig. 4](image-url)
different molecular weight, starting at the predicted unmodified FGFR1 weight of 90 kDa (9, 10). We found that tunicamycin inhibition of N-glycosylation resulted in selective loss of the high-molecular-weight FGFR1 species (120–150 kDa) and an increase in the 90-kDa band (Fig. 4B), further establishing the identity of these bands as FGFR1. It is also notable that the multiple FGFR1 species detected in PA are similar in size to the FGF-1-responsive phosphorylated FGFR species detected by the p-FGFR antibody in Fig. 2B.

Immunofluorescence analysis demonstrated a peripheral cytoplasmic/membrane and strong nuclear localization of FGFR1 in proliferating SGBS PA, in the absence or presence of FGF-1 (Fig. 4C). Upon reaching confluence, there was a marked translocation of FGFR1 from the nucleus only in FGF-1-treated cells. These FGFR1 translocation data were further confirmed biochemically using Western blot of the nuclei enriched fraction from subcellular fractionation experiments (Fig. 4C, bottom). FGFR1 staining was also observed in differentiated SGBS (Fig. 4C) and primary cells (data not shown), with predominantly intracellular membrane and plasma membrane localization.

The ability of FGF-1 to acutely activate FGFR1 in proliferating SGBS PA was further assessed by immunoprecipitation (IP) and Western blot analysis. Figure 4D demonstrates FGF-1-responsive FGFR bands (90, 120, 130, and 150 kDa) and downstream activation of FRS2α and ERK1/2 in the SGBS PA lysate. FGFR1 and general tyrosine phosphorylated proteins were immunoprecipitated from the lysate and subjected to Western blot analysis. FGF-responsive bands of 120, 130, and 150 kDa were observed in the FGFR1 IPs following Western blot with the p-FGFR antibody. In addition, following general phosphotyrosine (Py99) IP, a 120- to 130-kDa FGF-responsive band was subsequently detected by the FGFR1 antibody. Collectively, these data provide evidence for a potential role of FGFR1 in mediating some of the effects of FGF-1 in PA.

**FGFR1 is required for FGF-1-stimulated human PA proliferation and priming.** To investigate a putative role for FGFR1 in FGF-1-mediated proliferation and priming of PA, we performed transient transfections using a validated FGFR1 siRNA in phPA (Fig. 5). Primary PAs were the model of choice for these FGFR1 knock down experiments, since we have previously demonstrated that these cells are more sensitive to the effects of FGFR inhibition (Fig. 3) than SGBS PA.

In phPA, transfection of FGFR1 siRNA resulted in decreased FGFR1 mRNA and protein, assessed at 48 h posttransfection, compared with cells transfected with a scrambled control siRNA (Fig. 5, A and B). Our results did not show any adverse effects using the scrambled control siRNA compared with untransfected phPA (data not shown). Subsequent experiments determined that knock down of FGFR1 in phPA resulted in both decreased FRS2α and ERK1/2 phosphorylation following FGF-1 stimulation (Fig. 5B) as well as a significant decrease in FGF-1-induced proliferation (Fig. 5C). These reductions in FGF-1-mediated signaling events and proliferation correlated with the degree of FGFR1 mRNA knock down at 48 h posttransfection (60%, \( P < 0.05 \)). Furthermore, siRNA

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**Fig. 5. FGFR1 small-interfering RNA (siRNA) knock down inhibits FGF-1-mediated proliferation and priming in phPA.** phPA were transfected with FGFR1 siRNA or a scrambled negative ctrl siRNA, and effects of knock down were assessed on FGFR1 mRNA expression using real-time RT-PCR 48 h posttransfection, fold compared with ctrl siRNA (Fig. 5A). FGFR1 protein expression (nuclei-enriched fraction) and FGFR1-mediated signaling (whole cell lysate) by Western blot analysis. The phosphorylation of FRS2α and ERK1/2 was determined in response to acute FGF-1 stimulation. Lamin A/C, tubulin, and ERK1/2 were used as loading controls. Data are representative of 3 independent experiments. C: cell number by SYTO 60 assay. Results are expressed as arbitrary units over non-FGF-1-treated heparin ctrl cells, set as 0 (\( n = 4, \* P < 0.05 \); A). D: morphology (×100 magnification) (\( n = 3 \)). E: priming by PPARγ mRNA expression at confluence using real-time RT-PCR. Results are fold compared with ctrl siRNA (\( n = 6, \** P < 0.01 \)).
knock down of FGFR1 attenuated FGF-1 priming, as judged by reversal of FGF-1-induced changes in cell morphology (Fig. 5D) and a significant reduction (41%, \( P < 0.01 \)) in FGF-1-mediated PPAR\( \gamma \) mRNA expression at confluence (Fig. 5E). This reduction in FGF-1-stimulated PPAR\( \gamma \) mRNA correlated with the degree of FGFR1 mRNA knock down at this later time point, confluence (42%, \( P < 0.01 \)).

**DISCUSSION**

This study extends our current understanding of the molecular mechanisms underpinning the earliest stages of human adipogenesis and demonstrates the importance of the FGF signaling pathway in regulating these events. Earlier work from our laboratory demonstrated a novel “priming for differentiation” effect of FGF-1 that occurred concurrently with increased proliferation of human PA. It was unknown, however, whether these effects on early adipogenic events were specific to FGF-1 signaling or whether any mitogen might also increase the capacity for human PA adipose conversion. To address this question, we compared the effects of FGF-1 with that of other mitogenic growth factors, in particular PDGF-AA and -BB and VEGF, in two human models of adipogenesis.

Results demonstrated a significant proliferative effect in human PA of both PDGF-AA and -BB that was comparable to that mediated by FGF-1 at similar molar concentrations. However, in contrast to the adipogenic events associated with FGF-1-stimulated proliferation, no increase in commitment or differentiation capacity was observed in cells treated with either of the PDGFs. VEGF had neither a proliferative nor adipogenic effect in human PA. These results demonstrate that early adipogenic events in human PA are not due to \( J \) increased mitogenesis per se, 2) increased cell-cell contact, or 3) signaling through general receptor tyrosine kinase pathways. Although the scope of this study did not allow a more comprehensive analysis of a wider range of growth factors, preliminary experiments also showed no effect of epidermal growth factor, nerve growth factor, and insulin-like growth factor-I on differentiation potential of human PA in this system (unpublished observations). Because FGF-1 and -2 are the only growth factors known to stimulate these early events in human adipogenesis (15, 24), it is tempting to speculate that the molecular mechanisms may be unique to the FGF family. These data, however, do not rule out the possibility that FGF-mediated adipogenesis is not a direct consequence of FGF-stimulated proliferation and could, for example, be associated with specific kinetics of ERK1/2 activation previously reported to be important in adipose conversion (19, 25).

A defining feature of the FGF family of growth factors is the high-affinity FGF tyrosine kinases. Our data using two structurally distinct FGF receptor inhibitors, PD-173074 and SU-5402, demonstrated that FGF receptor activation is essential for FGF-1-mediated human PA proliferation and priming for differentiation. In accordance with previous reports, we found that PD-173074 was more efficient at blocking FGF-1-mediated effects than SU-5402 (22). Overall, these results demonstrate the importance of FGF receptor activity in adipogenesis, but, as the FGF kinase inhibitors efficiently blocked signaling through all four FGF, further work was needed to determine the role of specific FGFRs.

Based on our findings that FGFR1 mRNA was the most abundantly expressed isoform in proliferating PA and was activated in response to FGF-1 during this developmental stage, a detailed study of the specific role of this receptor in FGF-1 adipogenic effects was undertaken. Using an siRNA knock down approach, we determined a key regulatory role for FGFR1 in human adipogenesis with decreases in FGF-1-mediated proliferation and priming (at the level of both cell morphology and PPAR\( \gamma \) mRNA expression). These inhibitory effects on FGF-1-mediated adipogenesis were closely correlated to the observed decrease in FGFR1 mRNA and were substantiated using two further oligonucleotide sequences targeting different regions of the FGFR1 gene (unpublished observations). Although signaling via FGFR1 accounts for a large proportion of the observed FGF-1 adipogenic actions, further work is required to determine whether other FGFR(s) may also play a role. This may be either individually or as heterodimers, since FGFR1 has previously been shown to form functional heterodimers with other FGFR subtypes (3). Interestingly, FGFR2 expression was reduced in proliferating PA in response to FGF-1 treatment, suggesting that FGFR2 may play an inhibitory role in human PA proliferation or priming.

Another possibility is that priming of human PA by the FGF/FGFR axis may be dependent on specific interactions in the nucleus. FGF-1, FGF-2, and FGFR1 have all been detected in the nucleus (4) and have been reported to play a role in cellular mitogenesis from this locale (1, 17, 18, 37), possibly through formation of functional receptor-ligand complexes (33, 34). In the current study, immunofluorescence analysis clearly demonstrated a nuclear localization for FGFR1 during the proliferative stage in both FGF-treated and control cells. Furthermore, we showed that FGFR1 moved out of the nucleus at confluence in FGF-1-treated cells. Because nuclear localization of FGFR1 may be important for proliferation, one consequence of priming could be the translocation of FGFR1 from the nucleus, resulting in a growth-arrested state permissive for adipocyte differentiation. This hypothesis agrees with previous reports demonstrating the importance of nuclear translocation of FGFRs in regulation of different cellular developmental stages (30, 33).

Ultimately, FGF-1 priming results in altered gene expression in a manner that allows for efficient PA differentiation. This is evidenced by increased expression of the master adipogenic transcription factor PPAR\( \gamma \) in the primed cells. In addition, GATA2, which acts as a negative regulator of PPAR\( \gamma \) expression, is downregulated in PA in response to FGF-1 (unpublished observation). The increased expression of PPAR\( \gamma \) in response to FGFs indicates increased commitment of these mesenchymal stem cells to the adipocyte lineage. FGFRs have previously been reported to play a role in commitment of mesenchymal stem cells to other lineages, including osteoblast and chondrocyte (20, 21, 31). Therefore, further experiments are required to determine specific mechanisms by which this signaling system promotes the adipocyte phenotype in human FGF-treated PA.

In conclusion, this work provides further evidence for the role of FGFRs as key regulators of human adipogenesis and demonstrates that the FGF/FGFR axis regulates early adipogenic events that may be important in maintaining a pool of stem cells responsible for hyperplastic expansion of adipose tissue mass. These effects appear specific to FGFRs relative to a
range of other receptor tyrosine kinase growth factors, and our data establish an important role for FGFR1 in mediating the early stages of human adipogenesis. Understanding the adipose tissue-specific mechanisms underlying FGF/FGFR-mediated adipogenesis may lead to identification of novel targets for intervention in development of obesity.

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DISCLOSURE

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