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Prostaglandin F2α Inhibits Adipogenesis Via an Autocrine-Mediated Interleukin-11/Glycoprotein 130/STAT1-Dependent Signaling Cascade

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ABSTRACT

Prostaglandin F2α (PGF2α) is a potent inhibitor of adipocyte differentiation in vitro, that has also recently been implicated in the regulation of the adipogenic process in vivo, by opposing adipose tissue accretion and the subsequent development of obesity and its attendant metabolic consequences. In previous studies, we have demonstrated that PGF2α inhibits adipocyte differentiation by means of a calcium-dependent signaling pathway that is critically dependent upon the activity of the calcineurin phosphatase. In the current study, we have now extended these findings to further elucidate the mechanism by which the PGF2α/calcineurin-pathway inhibits the adipogenic process. We now report that the IL-11 cytokine, a member of the gp130 cytokine co-receptor-related family, is a downstream transcriptional target of this pathway in 3T3-L1 preadipocytes and is actively secreted in differentiating cells in response to PGF2α stimulation. Using a combined shRNA and dominant-negative receptor mutant approach, we provide evidence that IL-11/gp130-signaling is required to mediate the inhibitory effects of PGF2α on adipogenesis. Moreover, by taking advantage of a well-characterized panel of chimeric gp130 mutant receptors, we demonstrate that gp130 signaling is sufficient to inhibit adipocyte differentiation and specifically requires the activation of the STAT1 transcription factor. Conversely, we find that depleting endogenous STAT1 levels rescues adipogenesis in the presence of both IL-11/gp130 signaling and PGF2α. Collectively, our findings support a model in which PGF2α inhibits adipocyte differentiation by means of an IL-11 mediated autocrine negative feedback loop, that acts via gp130 to block adipogenesis through the essential actions of the STAT1 transcription factor. J. Cell. Biochem. 115: 1308–1321, 2014.

KEY WORDS: PGF2α; CALCINEURIN; IL-11; gp130; STAT1; ADIPOGENESIS

White adipocytes are highly specialized cells found in adipose tissue that play a critical role in both energy homeostasis and the regulation of systemic metabolic responses [Rosen and Spiegelman, 2006]. Despite these clearly beneficial physiological functions, the excessive accumulation of adipose tissue can have significant detrimental health consequences, resulting in the development of obesity and its attendant disease sequelae such as type-2 diabetes [Pi-Sunyer, 2009]. At the cellular level adipocytes can contribute to adipose tissue mass and subsequent obesity via two distinct mechanisms: (a) an increase in total adipocyte cell number (hyperplasia) due to de novo adipocyte differentiation by a process known as adipogenesis and (b) an increase in intrinsic adipocyte cell size (hypertrophy) due to increased lipid storage [Hirsch and Batchelor, 1976; Spalding et al., 2008]. However, the role that these two processes play in the development of obesity and its associated co-morbidities is complex. Adipocyte hyperplasia primarily occurs initially early during development, where it plays a role in establishing the overall degree of adipose tissue cellularity, which is a critical determinant for the propensity towards future obesity [Hirsch and Batchelor, 1976; Knittle et al., 1979; Prins and O'Rahilly, 1997]. Adipocyte hypertrophy then largely predominates in adulthood, where, under conditions of a sustained positive nutritional energy balance, pre-existing adipocytes are progressively filled with lipid leading

Abbreviations: Akr1b7, aldo-keto reductase 1B7; aP2, adipocyte protein 2; C/EBP, CCAAT/enhancer-binding protein; CsA, cyclosporin A; DN, dominant-negative; Epo, erythropoietin; EpoR, erythropoietin receptor; gp130, glycoprotein 130; IL-11Rα, interleukin-11 receptor α; MDI, methylisobutylxanthine dexamethasone and insulin; ORO, Oil Red O; PGF2α, prostaglandin F2 alpha; PPARγ, peroxisome proliferators-activated receptor gamma.

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ultimately to the generation of the large, lipid-engorged, dysfunctional, hypertrophic adipocytes that are most closely associated with the pathologic consequences of obesity [Weyer et al., 2000]. However, it is now clear that adipocyte hyerplasia also occurs throughout adulthood, where it is responsible for replacing the estimated 10% of aged adipocytes that are turned over each year [Spalding et al., 2008]. In fact, it is now widely believed that this continued ability of adipose tissue to replenish itself due to de novo adipocyte differentiation likely plays an important protective role against the development of metabolic diseases [Virtue and Vidal-Puig, 2010]. Indeed, there is now considerable evidence to suggest that the impaired adipogenesis of adipocyte precursors within the adipose tissue of obese individuals may play a causal role in triggering the onset of type-2 diabetes by favoring the development of hypertrophic obesity and its associated pathological consequences [Gustafson et al., 2009; Arner et al., 2011]. Consequently, given these important functions of adipocyte differentiation in vivo, there is considerable interest in deciphering the physiologically relevant molecular pathways that serve to regulate adipogenesis.

Adipocyte differentiation is a highly regulated process that is orchestrated by an hormonally induced, sequential transcription factor cascade involving members of the CCAAT/enhancer-binding protein (C/EBP) family and peroxisome proliferators-activated receptor γ (PPARγ), the acknowledged master adipogenic transcriptional regulator [Rosen and MacDougald, 2006]. It is now recognized that the efficiency of this differentiation process can be greatly influenced both positively and negatively by the presence of a number of extrinsic factors, such as hormones, cytokines, growth factors and lipid mediators [MacDougald and Mandrup, 2002]. One such factor that has recently garnered considerable attention is prostaglandin F2α (PGF2α), a cyclooxygenase-derived inflammatory mediator secreted by a number of cell types that is known to be a potent inhibitor of adipocyte differentiation in vitro [Serrero et al., 1992; Casimir et al., 1996]. In fact, recent studies indicate that PGF2α is also produced by preadipocytes themselves, where it appears to act in an endogenous autocrine/paracrine negative feedback loop that serves to restrict the overall adipogenic capacity of these cells [Fujimori et al., 2010; Silvestri et al., 2013]. Importantly, an in vivo role for PGF2α in the regulation of adipocyte differentiation and the development of obesity has recently been revealed from studies analyzing mice genetically deficient in aldo-keto reductase 1B7 (Akr1b7), the critical final enzyme in the biosynthesis of PGF2α in preadipocytes [Volat et al., 2012]. *Akr1b7*-null mice exhibit significantly decreased levels of PGF2α in their adipose tissue and a corresponding increase in basal adiposity that is reversed upon in vivo administration of a PGF2α synthetic agonist. These mice are also more susceptible to diet-induced obesity and the development of insulin-resistance [Volat et al., 2012]. Hence, in addition to being a potent inhibitor of adipogenesis in vitro, PGF2α also appears to represent a critical endogenous negative regulator of adipogenesis in vivo, involved in controlling the expansion of adipose tissue and the subsequent development of obesity and its associated disease sequelae.

Interleukin (IL)-11 is a multi-functional cytokine belonging to the IL-6 cytokine family [Taga and Kishimoto, 1997]. While IL-11 has long been known to be a potent negative regulator of adipogenesis [Kawashima et al., 1991; Keller et al., 1993], the underlying mechanisms and signaling pathways involved have not yet been determined. IL-11 signals via a heteromeric receptor complex comprising the IL-11Rα chain ligand-binding subunit and the gp130 co-receptor β-chain signaling subunit that is a common signaling receptor subunit for all IL-6 cytokine family members [Barton et al., 2000]. The IL-11Rα subunit lacks intrinsic signaling capacity, however, binding of IL-11 to IL-11Rα promotes formation of a complex with gp130 and subsequent gp130 heterodimerization, thereby allowing the activation of the gp130-associated JAK kinases and phosphorylation of critical conserved tyrosine residues within the gp130 cytoplasmic domain that are responsible for the recruitment and activation of specific downstream signaling molecules [Taga and Kishimoto, 1997; Heinrich et al., 2003]. Specifically, phosphorylation of Y759 within the gp130 cytoplasmic domain forms a docking site for the SHP2 adaptor protein that is responsible for triggering the activation of the Ras-Raf-mitogen-activated protein kinase (MAPK) signaling pathway, whereas phosphorylation of Y767, Y814, Y905, and Y915 results in the recruitment and subsequent activation of the signal transducer and activator of transcription (STAT) latent transcription factors, STAT1 and STAT3 [Heinrich et al., 2003].

Previous work from our laboratory has demonstrated that PGF2α inhibits adipocyte differentiation by means of a Gαq-calcium-dependent signaling pathway that is critically dependent upon the activity of the calcium/calmodulin-regulated serine/threonine phosphatase, calcineurin [Liu and Clipstone, 2007]. In the current study we have extended this observation to further delineate this pathway and identify the critical downstream effectors of calcineurin. Our findings now provide evidence that the PGF2α/calcineurin-signaling pathway inhibits adipocyte differentiation, at least in part, by initially inducing the expression and secretion of IL-11, which then acts in an autocrine/paracrine fashion through a gp130 cytokine receptor signaling subunit-dependent signaling mechanism to inhibit adipogenesis via the activation of the STAT1 transcription factor.

MATERIALS AND METHODS

CELL CULTURE AND ADIPOCYTE DIFFERENTIATION

3T3-L1 preadipocytes were cultured and induced to differentiate into mature adipocytes as described previously [Neal and Clipstone, 2002]. Briefly, 8×10^4 cells per well of a 6-well plate were grown to confluence, and after 2 days were treated for 48 h with growth media plus the adipogenic cocktail, MDI (0.5 mM methylisobutylxanthine, 1 μM dexamethasone, and 10 μg/ml insulin; all from Sigma). Cells were re-fed with growth media containing 10 μg/ml insulin at day 2 and every 2 days thereafter with growth media alone. Where indicated, cells were also treated for the first 48 h of the induction period with the indicated concentrations of PGF2α (Calbiochem), 1 μg/ml cyclosporin A (Calbiochem), recombinant murine IL-11 (50 ng/ml; Peprotech) and recombinant mouse erythropoietin (40 ng/ml; BD Pharmingen). In some experiments conditioned media collected from 3T3-L1 cells ectopically expressing cDNAs encoding either murine IL-11 or murine erythropoietin were used

instead of recombinant cytokines with identical results. To assess the extent of adipocyte differentiation, cells were fixed on day 8 post-stimulation with 10% formalin for 60 min at 4°C, then after two washes with phosphate buffered saline (PBS), were stained with filtered Oil Red O (ORO) solution (Sigma) for 2 h at room temperature. After staining, the cells were washed with PBS to remove unbound dye and the plates were scanned. Cells were then counter stained with hematoxylin for 4 min and, after washing with PBS, were visualized by light microscopy and photographed (40×).

PLASMID CONSTRUCTS

The dominant-negative version of gp130 (DN-gp130) was generated by using a murine gp130 cDNA (Open Biosystems; clone I.D. 6834623) as a template in a polymerase chain reaction (PCR) using PfuTurbo (Stratagene) together with gene-specific primers designed to delete the C-terminal 213 amino acids containing conserved amino acid residues known to be essential for gp130-mediated signaling events [Taga and Kishimoto, 1997], and replacing them with a FLAG epitope tag followed by a stop codon. The resulting DN-gp130 construct was then subsequently introduced into the MSCV-GFP retroviral expression vector. The wild-type EpoR/gp130 chimera and corresponding mutant chimeras containing tyrosine-to-phenylalanine substitutions in conserved residues of the gp130 cytoplasmic domain were kind gifts of Dr. Fred Schaper, RWTH Aachen, Germany [Schaper et al., 1998; Schmitz et al., 2000]. The wild type EpoR/gp130 chimera was PCR-amplified using gene-specific primers and the resulting PCR product was digested with *Sal*I and *Mfe*I, and introduced into the *Xho*I/*Eco*RI site of the MSCV-GFP retroviral vector, giving rise to MSCV-EG-WT. To generate MSCV-EG-1F and MSCV-EG-4F, *Nsi* I/*Bam* HI fragments corresponding to the C-terminal region of murine gp130 containing either a Y759F single substitution (1F), or Y767F/Y814F/Y905F/Y915F quadruple substitutions (4F) were introduced into Nsi I/Bam HI-digested MSCV-EG-WT backbone. The EpoR/gp130-4F "add-back" mutants were generated using PCR cloning techniques to fuse peptide sequences previously reported to selectively activate either STAT1 (PTPSFGYDKPHV), or STAT3 (PTPSFGYFKQHV) [Gerhartz et al., 1996], in-frame with the extreme C-terminus of EG-4F. Briefly, EG-4F was used as a template in a PCR reaction with the forward primer 5′-CTTCA TGCAT GTCAT CTTCT AGGC-3′ together with either 5′-AGGAT CCTAC ACGTG TGGCT TGTCG TATCC GAAGG ATGTT GGCGT CTGTG GTAAG AAACT TTTAG GC-3′, or 5′-GAGGA TCCTA CACGT GCTGC TTGAA GTATC CGAAG GATGT TGGCG TCTGT GGTAA GAAAC TTTTA GGC-3′ as reverse primers. The resulting PCR products were digested with *Nsi* I/*Bam* HI and introduced into the corresponding sites of MSCV-EG-4F giving rise to EG-4F-STAT1 and EG-4F-STAT3, respectively. To generate retroviral constructs capable of expressing shRNAs specific for STAT1, plasmids encoding STAT1-specific shRNAs in the lentiviral vector pLKO.1 were purchased from Open Biosystems (Clone I.D. TRCN0000054924 and TRCN0000054927) and used as templates in a PCR reaction together with the forward primer 5′-CTCTC AATTG TATCG ATCAC GAGAC TAGCC TC-3′ and the reverse primer 5′-CCTCA AGCTT GGATG AATAC TGCCA TTTGT CTCG-3′. The resulting PCR fragments were digested with *Mfe* I and *Hin*d III and cloned into the pSR-GFP/Neo vector (OligoEngine) previously digested with *Eco* RI and *Hin*d III to generate pSR-STAT1-924 and pSR-STAT1-927, respectively. An shRNA retroviral construct specific for murine IL-11 (Open Biosystems; Clone I.D. TRCN0000066459) was generated using an identical approach. The control shRNA retroviral vector specific for firefly luciferase has been described previously [Liu and Clipstone, 2008]. MSCV-DsRed2-PPARγ2 was generated by isolating a blunted *Afl* III/*Xba* I fragment of the full-length murine PPARγ2 cDNA from pSVSport-PPARγ2 (Addgene) and introducing it into MSCV-DsRed2 at the blunted *Eco*RI site. The integrity of all expression constructs was verified by DNA sequencing.

RETROVIRAL GENERATION AND INFECTION OF 3T3-L1 CELLS

Retroviruses were generated essentially as described previously [Neal and Clipstone, 2002]. For retroviral infections, 5×10^4 3T3-L1 cells were plated per well of a 6-well plate, the next day media was replaced with retroviral particle-containing growth media supplemented with 8 μg/ml polybrene (Sigma) and the plates were centrifuged in a swinging bucket rotor at 1,000 rpm for 90 min at room temperature, then returned to the incubator overnight. After 24 h, cells were subjected to a second round of infection, essentially as described above, then expanded in regular growth medium prior to seeding for subsequent experiments.

IMMUNOBLOT ANALYSIS

Whole cell lysates were prepared by adding boiling SDS sample buffer (120 mM Tris–HCl pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol) directly to cells on the plate. The cell lysates were collected by scraping and boiled for 10 min, then sonicated for 10 s using a Digital sonifier (Branson) at an amplitude of 11% and stored at −80° C. An equal amount of protein from each treatment was resolved by SDS–PAGE gel electrophoresis, transferred to nitrocellulose membrane, and subjected to immunoblot analysis with the following primary antibodies: anti-STAT1 (#9172), anti-phospho-STAT1 (Tyr701; #9171), anti-STAT3 (#9132) and anti-phospho-STAT3 (Tyr705; #9131) from Cell Signaling Technologies; anti-PPARγ (sc-7196) and anti-ERK (sc-93) from Santa Cruz Biotechnology; anti-aP2 (#10004944) from Cayman Chemical Company; anti-mouse EpoR (#AF1390) from R&D systems. Appropriate horseradish peroxidase-conjugated secondary antibodies (anti-rabbit, anti-mouse, anti-goat) were purchased from Vector laboratories, and detected by enhanced chemiluminescence using ECL reagent (Thermo Scientific).

QUANTITATIVE REAL-TIME PCR (qRT-PCR) ANALYSES

Total RNA was isolated from 3T3-L1 cells at 48 h post-differentiation using an RNeasy kit (Qiagen). cDNA was synthesized from 1 μg of mRNA using random primer and reverse transcriptase (Promega). The expression of IL-11 was determined by qRT-PCR using a SYBR Green-based detection system (SA Biosciences) and the HPRT gene as an internal control for normalization. The forward (F) and reverse (R) primers used for qRT-PCR are as follows: IL-11 (F), 5′-CTGTG GGGAC ATGAA CTGTG-3′ IL-11 (R), 5′-CGTCA GCTGG GAATT TGTCT-3′: HPRT (F), 5′-GTTGG ATACA GGCCA GACTT TGTTG-3′ HPRT (R), 5′-GAGGG TAGGC TGGCC TATAG GCT-3′. All qRT-PCR measurements were performed using a iCycler real-time PCR detection system (Bio-Rad) and the fold change in mRNA expression was determined by the ΔΔCT method.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Cell culture supernatants were collected at the indicated time post-induction of adipocyte differentiation and assayed by ELISA for the presence of IL-11 (Cat. No. DY418, R&D Systems) according to the manufacturer's instructions.

STATISTICAL ANALYSIS

The data are expressed as the mean ± SEM. For comparison between groups either one-way ANOVA followed by Bonferroni post hoc test or Student's *t*-test was employed. Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

ACTIVATION OF THE PGF2α/CALCINEURIN-SIGNALING PATHWAY INDUCES THE EXPRESSION OF IL-11 IN DIFFERENTIATING 3T3-L1 PREADIPOCYTES

In previous studies we had demonstrated that PGF2α inhibits the early stages of adipocyte differentiation via the essential actions of the calcineurin phosphatase [Liu and Clipstone, 2007]. Since calcineurin is a well-known positive regulator of a number of transcription factors [Crabtree, 2001], we hypothesized that the PGF2α/calcineurin-signaling pathway was likely to inhibit adipogenesis via the increased expression of a gene(s) capable of inhibiting the differentiation process. In order to test this hypothesis, we initially performed a microarray gene expression profiling experiment to identify candidate anti-adipogenic genes induced by PGF2α in a calcineurin-dependent fashion during the early stages of 3T3-L1 preadipocyte differentiation. Amongst the most highly induced genes identified by this analysis was the multifunctional cytokine IL-11. This observation was of significant interest, as IL-11 had previously been shown to be a potent inhibitor of adipocyte differentiation [Kawashima et al., 1991; Keller et al., 1993].

To confirm our initial microarray result, we performed quantitative RT-PCR and ELISA experiments to analyze the expression of IL-11 in 3T3-L1 cells induced to undergo adipocyte differentiation in the presence or absence of PGF2α. As shown in Figure 1, while 3T3-L1 preadipocytes induced to undergo adipocyte differentiation by the standard MDI-induced protocol expressed essentially

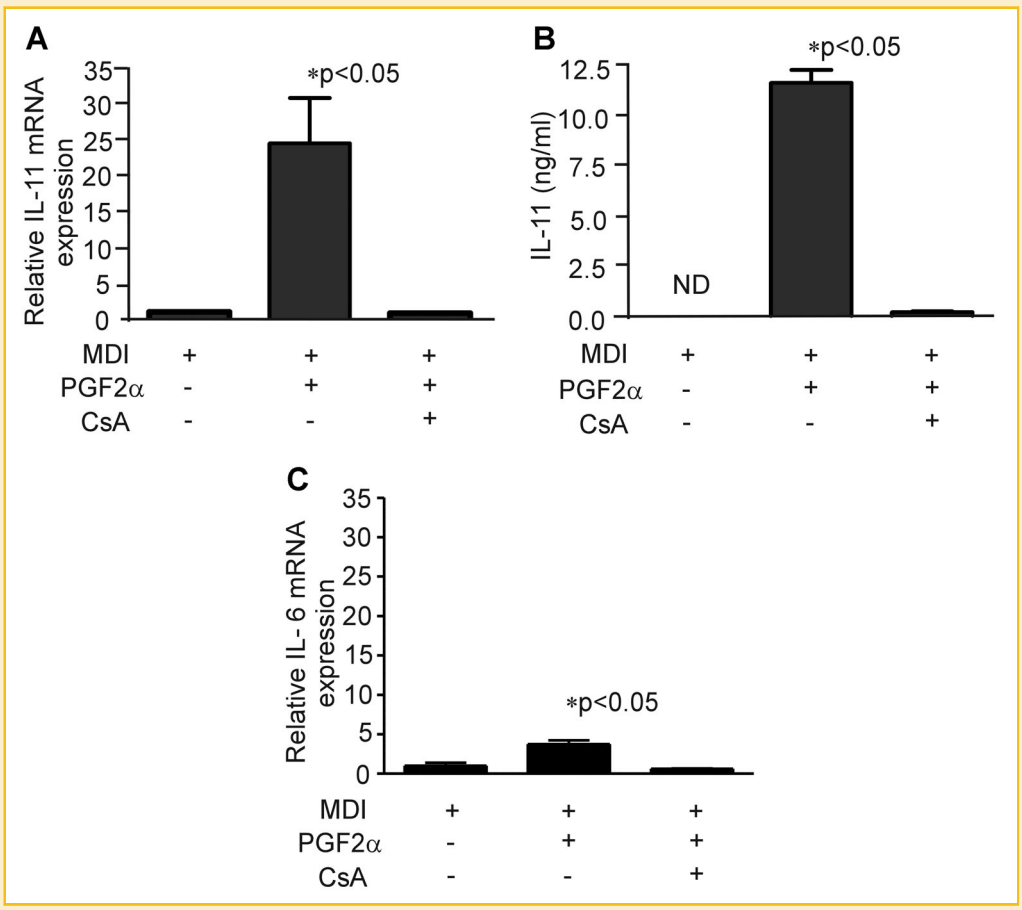

Fig. 1. PGF2α treatment of differentiating 3T3-L1 preadipocytes induces the calcineurin-dependent expression and secretion of IL-11. Two-day post-confluent 3T3-L1 preadipocytes were induced to undergo adipocyte differentiation by treatment with MDI in the presence of either vehicle, PGF2α (25 nM) alone or PGF2α (25 nM) plus Cyclosporin A (CsA) (1 μg/ml). At 48 h post-stimulation, total RNA and cell culture supernatants were collected and the expression of IL-11 was determined by qRT-PCR (A) and ELISA (B), respectively. C: The expression of IL-6 was determined by qRT-PCR. The data are shown as mean ± SEM (either one-way ANOVA followed Bonferroni post hoc test or Student's *t*- test; $P < 0.05$; n = 3). ND; not detectable. Results are representative of at least three independent experiments.

undetectable levels of IL-11 at 48 h post-stimulation, significant levels of both IL-11 mRNA (Fig. 1A) and protein (Fig. 1B) were readily observed in cells induced to differentiate in the presence of PGF2α, an effect that was attenuated in the presence of the calcineurin-specific inhibitor cyclosporin A (CsA). In fact, we observed that treatment of non-differentiated 3T3-L1 cells with PGF2α alone was sufficient to induce IL-11 expression, although in this case the level of induction (~3-fold; data not shown) was significantly less than that observed in the presence of the MDI adipogenic cocktail (23-fold; see Fig. 1A). As IL-11 is a member of the IL-6 family of cytokines [Taga and Kishimoto, 1997], we also investigated whether PGF2α affected the expression of other family members during the differentiation process. In this respect, while our initial microarray data and subsequent qRT-PCR analysis revealed that the expression of IL-6 itself was induced by PGF2α treatment, the level of induction observed (3.5-fold) was considerably lower compared to the effect on IL-11 (Fig. 1C), whereas no substantial effect of PGF2α on the expression of other members of this family was observed. Taken together, therefore, our results indicate that the activation of the PGF2α/calcineurin-signaling pathway synergizes with MDI-induced signals during the early stages of 3T3-L1 preadipocyte differentiation to specifically induce the robust expression and subsequent secretion of the IL-11 cytokine, which given the known anti-adipogenic effects of IL-11 [Kawashima et al., 1991; Keller et al., 1993], raised the possibility that PGF2α might act to inhibit adipogenesis by means of an IL-11 mediated autocrine mechanism.

IL-11 AND GP130 CYTOKINE CO-RECEPTOR-DEPENDENT SIGNALING PLAY A ROLE IN MEDIATING THE INHIBITORY EFFECTS OF PGF2α ON ADIPOCYTE DIFFERENTIATION

Since IL-11 signaling is known to be critically dependent upon the actions of the gp130 cytokine co-receptor signaling subunit [Barton et al., 2000], we initially took advantage of a well-characterized dominant-negative form of gp130 (DN-gp130) [Kumanogoh et al., 1997] to examine the potential role of IL-11 in mediating the inhibitory effects of the PGF2α/calcineurin-signaling pathway on adipocyte differentiation. This dominant-negative gp130 mutant comprises the extracellular domain of gp130 known to be responsible for interacting with IL-11/IL-11Rα complexes, but lacks the cytoplasmic domain containing the conserved residues required for signal transduction. Consequently, ectopic expression of DN-gp130 should act to attenuate gp130-dependent signaling events by sequestering IL-11 in non-functional complexes incapable of signaling. Thus, to initially establish the potential role of gp130 signaling in mediating the inhibitory effects of PGF2α on adipocyte differentiation, 3T3-L1 preadipocytes were transduced with either a control retrovirus or a retrovirus encoding DN-gp130, and then induced to undergo differentiation by the standard MDI-induced adipogenic protocol in the presence of various concentrations of PGF2α. As expected, the graded doses of PGF2α served to produce a potent dose-dependent inhibition of adipocyte differentiation in control cells, as measured by a decrease in cells staining positive for Oil Red O (ORO) (Fig. 2A) and the decreased expression of the adipocyte-specific genes PPARγ and aP2 (Fig. 2B). In contrast, we found that ectopic expression of DN-gp130 was able to greatly attenuate the inhibitory activity of PGF2α, allowing 3T3-L1 preadipocyte cells to differentiate in the presence of concentrations of PGF2α that were able to potently inhibit the differentiation of control cells (Fig. 2A,B). This result demonstrates that inhibiting gp130-dependent signaling in 3T3-L1 preadipocytes is able to partially rescue adipocyte differentiation in cultures containing PGF2α, thereby suggesting a critical role for gp130 signaling in mediating the inhibitory effects of PGF2α on adipocyte differentiation.

While the gp130 cytokine co-receptor signaling subunit is an essential component of the IL-11 receptor [Barton et al., 2000], it is also known to be involved in mediating the signaling of other members of the IL-6 family of cytokines [Taga and Kishimoto, 1997 ; Heinrich et al., 2003]. Consequently, to more directly assess the role of IL-11 in mediating the inhibitory effects of PGF2α on adipocyte differentiation, we adopted an shRNA-mediated knockdown strategy in which 3T3-L1 preadipocytes were transduced with retroviruses encoding either a control shRNA or an shRNA-specific for IL-11, then subsequently examined for their ability to undergo adipocyte differentiation in the presence of an inhibitory concentration of PGF2α (12.5 nM). As expected, the ability of control shRNA-transduced 3T3-L1 preadipocytes to undergo adipocyte differentiation under these conditions was potently inhibited, as determined by both loss of ORO-staining cells and decreased expression of PPARγ and aP2 (Fig. 2C,D). Conversely, in IL-11 specific shRNA-transduced cultures induced to undergo differentiation in the presence of this same inhibitory concentration of PGF2α, we consistently observed the rescue of adipocyte differentiation, as indicated by the presence of substantial patches of ORO-staining cells and the expression of both PPARγ and aP2 (Fig. 2C,D). In these experiments we found that the IL-11 specific shRNA was typically able to reduce the level of IL-11 induced in PGF2α-treated 3T3-L1 preadipocytes by approximately 70% compared to control shRNA-expressing cells (see Fig. 2E). These results therefore provide evidence that IL-11 likely plays an important role in mediating the inhibitory effects of the PGF2α/calcineurin-signaling pathway on adipocyte differentiation and are consistent with a model in which PGF2α-induces the initial expression and subsequent secretion of IL-11, which then acts in an autocrine/paracrine fashion to inhibit adipocyte differentiation by means of a gp130 cytokine co-receptor-dependent pathway.

GP130 CYTOKINE CO-RECEPTOR-DEPENDENT SIGNALING IS SUFFICIENT TO INHIBIT ADIPOCYTE DIFFERENTIATION BY BLOCKING EXPRESSION OF PPARΓ

Next, we determined whether gp130 signaling per se was sufficient to inhibit adipocyte differentiation. For this experiment, we took advantage of a previously characterized chimeric form of gp130 in which the cytoplasmic signaling domain of gp130 is fused to the extracellular ligand-binding domain of the erythropoietin receptor (EpoR/gp130; EG-WT; see Fig. 3), which allows gp130-specific signaling events to be induced in response to stimulation with erythropoietin (Epo) [Schaper et al., 1998; Schmitz et al., 2000]. As shown in Figure 4, the presence of Epo in the culture medium had no effect on the ability of control GFP-expressing cells to undergo adipocyte differentiation, but conversely was able to potently inhibit the differentiation of cells ectopically expressing the EG-WT

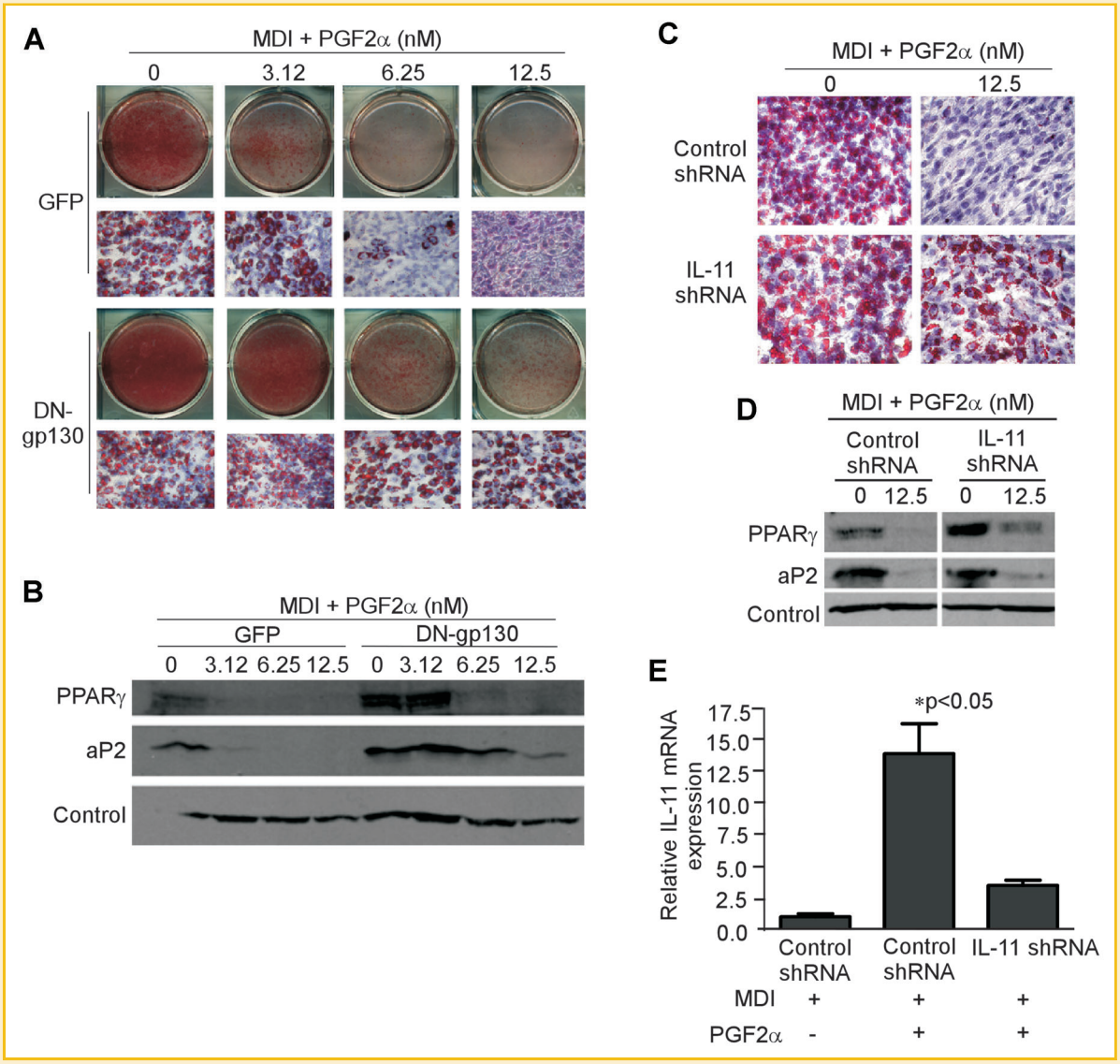

Fig. 2. IL-11 and gp130-dependent signaling play a role in mediating the inhibitory effects of PGF2α on adipocyte differentiation. A,B: 3T3-L1 preadipocytes transduced with either MSCV-GFP or MSCV-DN-gp130 retroviruses were induced to undergo differentiation with MDI in the presence of the indicated concentrations of PGF2α for the initial 48 h and the extent of adipocyte differentiation was determined at day 8 by either ORO staining (A) or immunoblotting for the expression of PPARγ and aP2 (B). Equal protein loading was confirmed by probing with a control antibody directed against ERK. C,D: 3T3-L1 preadipocytes transduced with retroviruses encoding either control or IL-11-specific shRNA were induced to undergo differentiation with MDI in the absence or presence of PGF2α (12.5 nM) for the initial 48 h. After 8 days, the extent of adipocyte differentiation was assessed by ORO staining (C) or immunoblotting for the expression of PPARγ and aP2 (D). Equal protein loading was confirmed by probing with a control antibody directed against ERK. E: The efficiency of knockdown of IL-11 mRNA was determined by qRT-PCR using RNA isolated from PGF2α-treated cells at the 48 h time point. The data are shown as mean ± SEM (one-way ANOVA followed by Bonferroni post hoc test; $P < 0.05$; $n = 3$). Results are representative of at least three independent experiments.

chimeric receptor, as measured by a decrease in both ORO staining and the expression of PPARγ and aP2 (Fig. 4B,C). Furthermore, we found that ectopic expression of PPARγ in cells expressing the EG-WT chimeric receptor was able to bypass the inhibitory effects of Epo on adipocyte differentiation in these cells (Fig. 4B,C). Taken together, these data indicate that gp130 signaling per se is sufficient to inhibit adipocyte differentiation, most likely by acting to block the expression of the pro-adipogenic master regulatory transcription factor, PPAR&t#x03B3.

C-TERMINAL TYROSINE RESIDUES INVOLVED IN THE ACTIVATION OF STAT TRANSCRIPTION FACTORS PLAY A CRITICAL ROLE IN MEDIATING THE INHIBITORY EFFECTS OF GP130 SIGNALING ON ADIPOCYTE DIFFERENTIATION

Signaling via gp130 is critically dependent upon conserved tyrosine residues located within the cytoplasmic domain, which are phosphorylated in response to receptor activation and are responsible for recruiting cytoplasmic signaling molecules involved in the propagation of specific downstream signaling pathways [Taga and

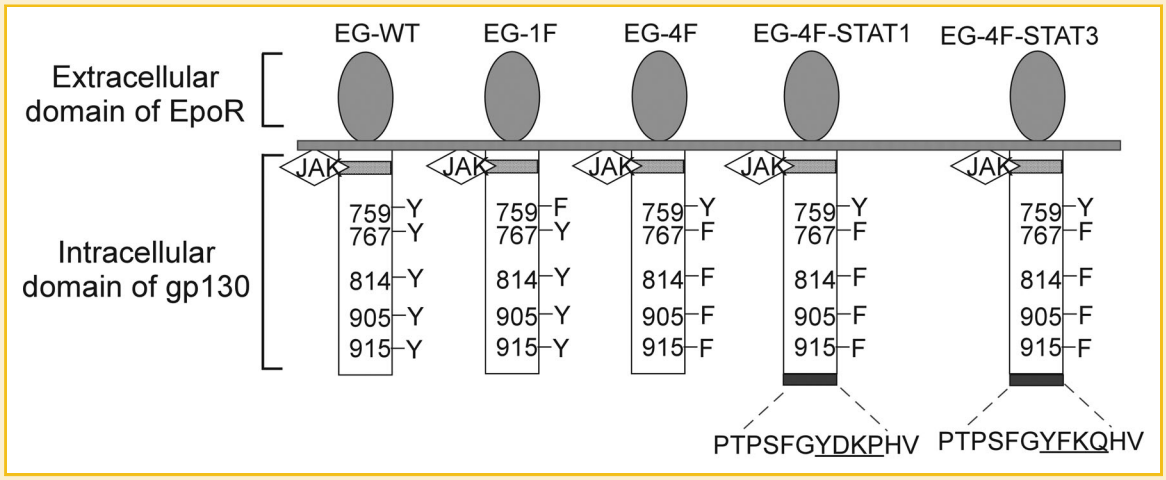

Fig. 3. Schematic model illustrating the chimeric EpoR/gp30 receptors used in this study. The array of chimeric EpoR/gp130 receptors used in this study comprising of the extracellular ligand-binding domain of the EpoR (gray ovals) fused to various amino acid-substituted versions of the intracellular domain of gp130 (open rectangles) are shown. The position of the conserved tyrosine residues in the gp130 cytoplasmic domain and corresponding tyrosine to phenylalanine substitutions are indicated, as are the synthetic peptide sequences used for the selective activation of STAT1 and STAT3 in the EG-4F-STAT1 and EG-4F-STAT3 "add-back" mutants.

Kishimoto, 1997 ; Heinrich et al., 2003]. IL-11 signaling mediated via the gp130 co-receptor signaling subunit is known to primarily activate two independent downstream effectors pathways: the ERK and JAK/STAT signaling pathways [Heinrich et al., 2003]. In order to ascertain which of these gp130-dependent signaling events were responsible for mediating the inhibition of adipocyte differentiation, we took advantage of previously well-characterized EpoR/gp130 chimeric mutants known to be selectively deficient in the activation of one or another of these signaling pathways [Schaper et al., 1998; Schmitz et al., 2000]. Specifically, we utilized chimeric mutant

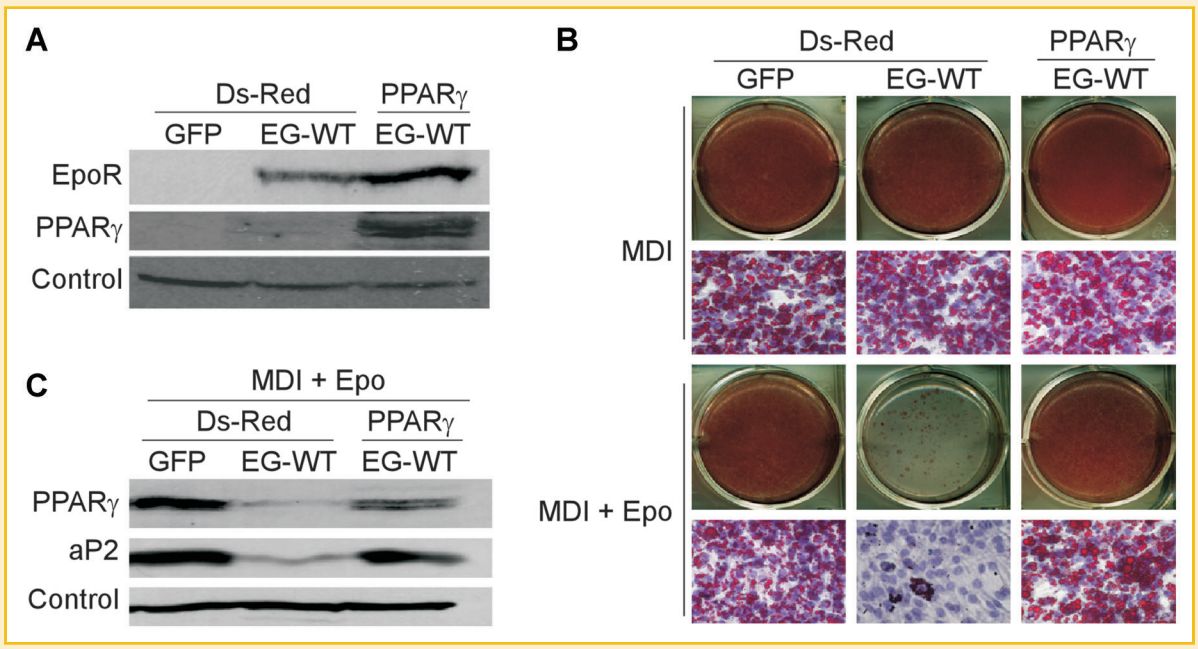

Fig. 4. gp130-dependent signaling is sufficient to inhibit adipocyte differentiation. 3T3-L1 preadipocyte cells were initially infected with either MSCV-GFP or MSCV-EG-WT retroviruses then super-infected with either MSCV-DsRed or MSCV-DsRed-PPARγ retroviruses, as indicated. A: The expression of EG-WT and PPARγ was confirmed by immunoblotting with antibodies specific for EpoR and PPARγ, while equal protein loading was confirmed by immunoblotting with a control antibody directed against ERK. B,C: Two-day post-confluent cells were then induced to differentiate with MDI in the presence or absence of Epo for the initial 48 h and the degree of adipocyte differentiation was assessed at day 8 post-stimulation by ORO staining (B) and immunoblotting for the expression of PPARγ and aP2 (C). Equal protein loading was confirmed by probing with a control antibody directed against ERK. Results are representative of more than three independent experiments.

receptors with either a Y759F substitution (EG-1F) that is known to be selectively deficient in the activation of the downstream ERK pathway, or a quadruple chimeric mutant receptor containing Y767F, Y814F, Y905F and Y915F substitutions (EG-4F) that is selectively deficient in the activation of the STAT1 and STAT3 latent transcription factors (see Fig. 3 for details). Each of these mutant receptors, along with EG-WT, was independently introduced into 3T3-L1 preadipocytes by retroviral transduction, and immunoblot analysis was performed to confirm that each receptor was expressed at comparable levels (Fig. 5A). Subsequently, cells were then induced to undergo adipocyte differentiation by the standard MDI-induced protocol in either the presence or absence of Epo, and the effects on

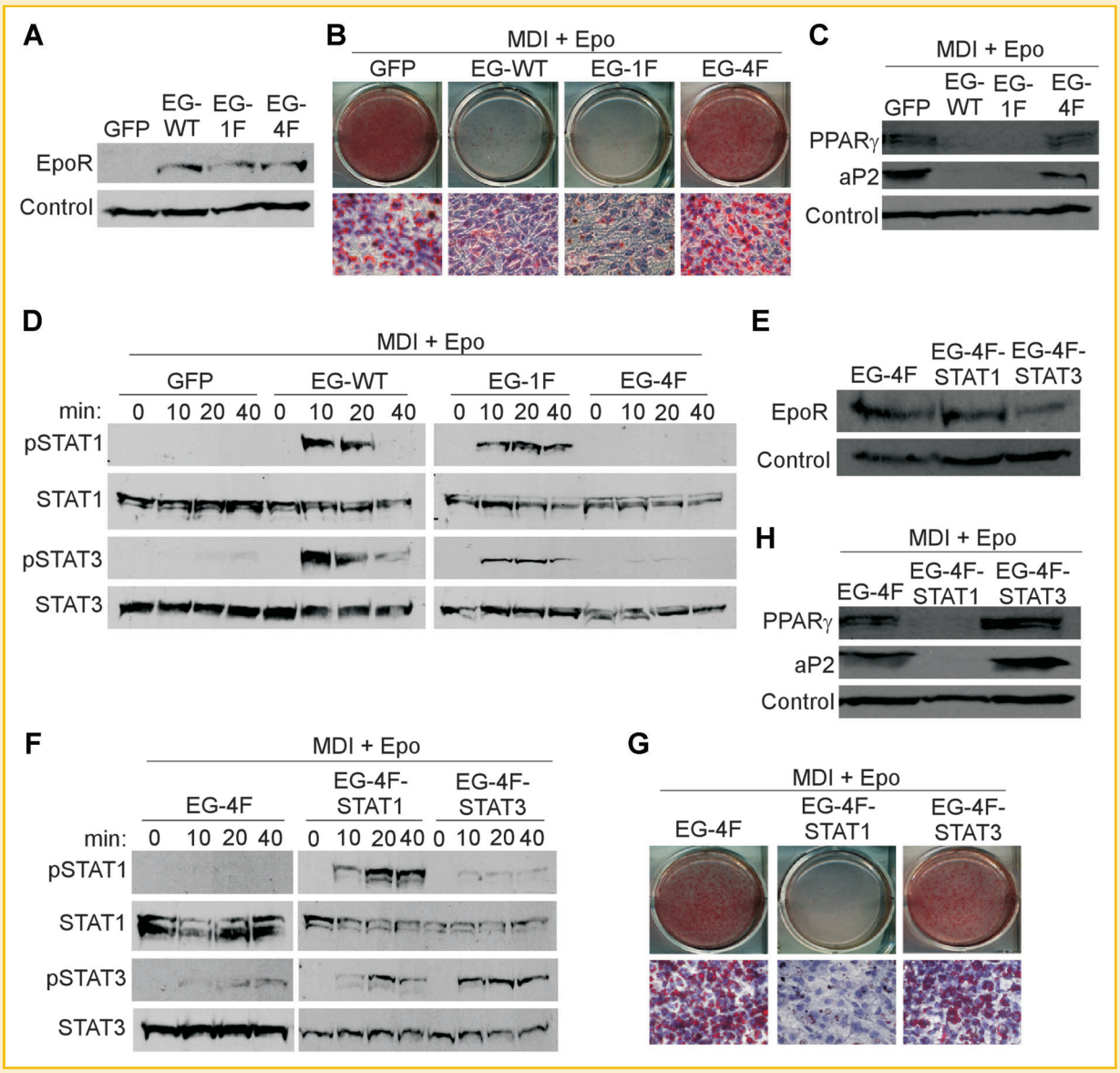

Fig. 5. Tyrosine residues involved in STAT transcription factor activation play a role in mediating the inhibitory effects of gp130-dependent signaling on adipocyte differentiation. A: 3T3-L1 preadipocyte cells were infected with either MSCV-GFP, EG-WT, EG-1F, or EG-4F encoding retroviruses and the expression of each chimeric receptor was determined by immunoblotting with an antibody specific for EpoR, while equal protein loading was confirmed by immunoblotting with a control antibody directed against ERK. B,C: Two-day post-confluent cells were induced to differentiate with MDI in the presence of Epo for the initial 48 h. After 8 days, adipocyte differentiation was assessed by ORO staining (B) and immunoblotting for the expression of PPARγ and aP2 (C). D: To confirm the ability of the chimeric receptors to activate STAT1 and STAT3, whole cell extracts from cells induced to differentiate with MDI in the presence of Epo were analyzed by immunoblotting at the indicated times with antibodies specific for pSTAT1-Y701, STAT1, pSTAT3-Y705, and STAT3. E: Expression of chimeric add-back receptors in 3T3-L1 preadipocyte cells transduced with either EG-4F, EG-4F-STAT1, or EG-4F-STAT3 encoding retroviruses was assessed by immunoblotting whole cell extracts with an antibody specific for EpoR, and ERK as a control for equal protein loading. F: To determine the activation selectivity of the chimeric add-back receptors, cells from (E) were induced to differentiate with MDI in the presence of Epo and whole cell extracts prepared at the indicated times were analyzed by immunoblotting with antibodies specific for pSTAT1-Y701, STAT1, pSTAT3-Y705, and STAT3. G,H: Two-day post-confluent cells from (E) were induced to differentiate with MDI in the presence of Epo and after 8 days, the extent of adipocyte differentiation was assessed by ORO staining (G) and immunoblotting for the expression of PPARγ and aP2 (H). Results are representative of more than three independent experiments.

differentiation were assessed. As shown in Figure 5B,C, the presence of Epo was able to inhibit the differentiation of EG-1F-expressing cells to essentially the same degree as that observed for cells expressing EG-WT. By contrast, the presence of Epo had no effect on the ability of EG-4F expressing cells to differentiate. As shown in Figure 5D, immunoblot analysis confirmed that unlike EG-WT and EG-1F, the EG-4F receptor was deficient in its ability to efficiently activate STAT1 and STAT3 in response to Epo stimulation. These data therefore demonstrate that the four C-terminal tyrosine residues present in the cytoplasmic domain of gp130 are required for the gp130-mediated inhibition of adipocyte differentiation. Moreover, since these latter tyrosine residues are required for the gp130-mediated activation of STAT1 and STAT3, the data further suggests a potentially important role for the activation of STAT transcription factors in mediating the inhibitory effects of gp130 co-receptor signaling on adipocyte differentiation.

A CRITICAL ROLE FOR STAT1 IN MEDIATING THE INHIBITORY EFFECTS OF GP130, IL-11 AND PGF2α ON ADIPOCYTE DIFFERENTIATION

To further examine the potential role of STAT1 and STAT3, we next generated chimeric gp130 "add-back" receptors in which consensus peptide sequences previously reported to selectively activate either STAT1 or STAT3 [Gerhartz et al., 1996] were fused in-frame to the C-terminus of STAT signaling-deficient EG-4F mutant receptor, thereby generating EG-4F-STAT1 and EG-4F-STAT3, respectively (see Fig. 3). Each of these mutant "add-back" receptors, along with the parental EG-4F receptor, was independently expressed in 3T3-L1 preadipocytes (Fig. 5E) and their ability to activate STATs (Fig. 5F) and inhibit adipocyte differentiation (Fig. 5G,H) was assessed. As shown in Figure 5F, Epo-treatment of cells expressing the EG-4F-STAT1 mutant receptor resulted in the preferential activation of STAT1, whereas Epo-stimulation of EG-4F-STAT3-expressing cells led to the preferential activation of STAT3. We note that although the EG-4F receptor is completely deficient in its ability to activate STAT1, it is still able to induce residual levels of STAT3 activation, albeit considerably reduced compared to EG-WT (see Fig. 5D). Based upon analysis of mutant receptors lacking all cytoplasmic tyrosine residues, we believe that this effect is likely due to the ability of gp130-associated JAK2 to very inefficiently activate parallel gp130-independent pathways leading to STAT3 activation (data not shown). Nonetheless, it is clear from our data that EG-4F-STAT1 and EG-4F-STAT3 preferentially activate STAT1 and STAT3, respectively. When 3T3-L1 preadipocytes expressing these mutant receptors are induced to differentiate in the presence of Epo, we find that only those cells expressing EG-4F-STAT1 are blocked from undergoing adipogenesis, as measured by a decrease in both ORO staining (Fig. 5G) and the expression of PPARγ and aP2 (Fig. 5H). These data therefore suggest that it is the activation of the STAT1 transcription factor, rather than STAT3, that is associated with the ability of gp130 to inhibit adipocyte differentiation.

In order to further define the role of STAT1 as a critical anti-adipogenic effector of the gp130-signaling pathway, we next chose to determine whether STAT1 function was necessary for the ability of gp130-induced signaling to inhibit adipocyte differentiation. To accomplish this goal, we adopted an shRNA knockdown strategy using STAT1-specific shRNAs to stably deplete endogenous STAT1 levels in 3T3-L1 preadipocytes. By initially screening a panel of STAT1-specific shRNAs we were able to identify two independent shRNAs capable of efficiently and specifically depleting endogenous levels of STAT1 (Fig. 6A). 3T3-L1 preadipocytes expressing either of these two independent STAT1-specific shRNAs or a control shRNA were then each superinfected with a retrovirus encoding EG-WT and induced to undergo adipocyte differentiation in the presence of Epo. As expected, when control shRNA expressing cells were treated with Epo their differentiation was potently inhibited (Fig. 6B,C). In contrast, we found that the inhibitory effect of Epo was greatly attenuated in cells expressing either of the two STAT1-specific shRNAs (Fig. 6B,C). Similarly, we found that these two STAT1-specific shRNAs also greatly attenuated the ability of exogenous IL-11 to inhibit adipocyte differentiation (Fig. 6D,E). It is noteworthy that in each case the degree of rescue caused by each of the STAT1-specific shRNAs was proportional to their degree of knockdown of endogenous STAT1 levels.

Finally, we wished to examine the requirement for STAT1 as a downstream anti-adipogenic effector of the PGF2α/calcineurin-signaling pathway. As shown in Figure 6F,G, while PGF2α effectively inhibited the differentiation of control shRNA-expressing cells as expected, we found that depletion of endogenous STAT1 levels with either of our STAT1-specific shRNAs allowed for significant rescue of adipogenesis in the presence of inhibitory concentrations of PGF2α capable of potently inhibiting control cells. Taken together, these data identify an important role for STAT1 as a critical downstream effector in mediating the inhibitory effects of PGF2α and IL-11/gp130-signaling on adipocyte differentiation.

DISCUSSION

Recent studies identifying an emerging *in vivo* role for PGF2α as an important endogenous negative regulator of adipocyte differentiation involved in opposing the development of obesity and its contingent pathological metabolic sequelae [Volat et al., 2012], have raised considerable interest in elucidating the molecular pathways and mechanisms by which this important prostaglandin mediates its inhibitory effects on adipogenesis. Building upon our previous identification of calcineurin as a critical downstream anti-adipogeneic effector of PGF2α [Liu and Clipstone, 2007], we have now further delineated the molecular mechanism by which this signaling pathway inhibits adipocyte differentiation. Collectively, our findings support a model in which exposure of differentiating 3T3-L1 preadipocytes to PGF2α results in the initial calcineurin-dependent expression and secretion of the IL-11 cytokine, which then acts in an autocrine/paracrine fashion to inhibit adipocyte differentiation via the essential actions of the gp130 cytokine co-receptor signaling subunit and the subsequent activation of the STAT1 transcription factor.

Our results clearly identify IL-11 as a downstream transcriptional target of the PGF2α/calcineurin-signaling pathway in 3T3-L1 preadipocytes. Interestingly, this same pathway has also recently been shown to induce IL-11 expression in endometrial

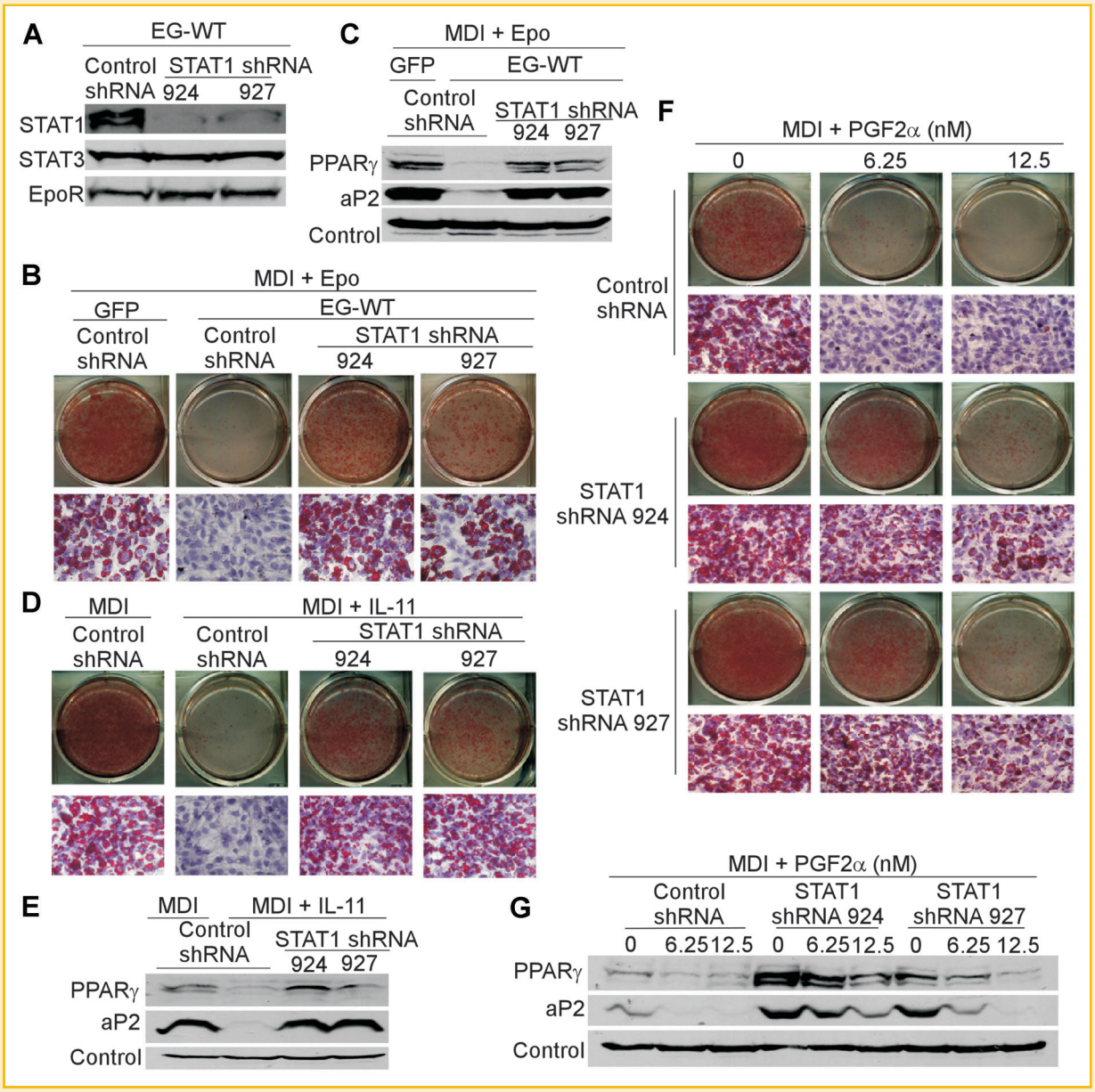

Fig. 6. STAT1-depletion partially rescues adipocyte differentiation in the presence of EpoR/gp130-chimeric receptor signaling, IL-11 and PGF2α. A: The effectiveness of STAT1-specific shRNA knockdown was confirmed by immunoblotting. 3T3-L1 preadipocyte cells transduced with retroviruses encoding either a control shRNA or one of two independent STAT1-specific shRNAs (924 and 927), were super-infected with a MSCV-EG-WT encoding retrovirus and whole cell extracts from these cells were analyzed by immunoblotting with antibodies specific for STAT1, EpoR, and STAT3 as a protein loading control and to show specificity of the shRNA knockdown. B,C: These independent cell populations were then induced to differentiate with MDI in the presence of Epo for the initial 48 h and after 8 days, the extent of adipocyte differentiation was assessed by ORO staining (B) and immunoblotting for the expression of PPARγ and aP2 (C). D,E: 3T3-L1 preadipocyte cells transduced with either control shRNA or STAT1-specific shRNA (924 and 927) encoding retroviruses were induced to differentiate with MDI in the presence of IL-11 for the initial 48 h and the extent of adipocyte differentiation was assessed at day 8 by either ORO staining (D) or immunoblotting for the expression of PPARγ and aP2 (E). F,G: 3T3-L1 preadipocyte cells transduced with either control shRNA or STAT1-specific shRNA (924 and 927) encoding retroviruses were induced to differentiate with MDI in the presence of increasing concentrations of PGF2α for the initial 48 h and the extent of adipocyte differentiation was assessed after 8 days by ORO staining (F) and immunoblotting for the expression of PPARγ and aP2 (G). Results in (B,C) are representative of two independent experiments, whereas results in (F) are representative of at least five independent experiments.

adenocarcinoma cells via the calcineurin-dependent activation of the nuclear factor of activated T cells (NFAT) transcription factor family [Sales et al., 2010]. By analogy with this finding, together with the knowledge that NFAT proteins are expressed in 3T3-L1 preadipocytes [Ho et al., 1998], it is tempting to speculate that NFAT proteins are also likely to play a role in coupling the PGF2α/calcineurin-signaling pathway to the expression of IL-11 in these cells. However, the recent demonstration that IL-11 can be transcriptionally regulated by the hypoxia-inducible factor 1 transcription factor [Onnis et al., 2013], which we have previously

shown is activated in 3T3-L1 cells in response to PGF2α stimulation [Liu and Clipstone, 2008], raises the possibility that this pathway may also play a role.

IL-11 has long been known to be a potent inhibitor of adipocyte differentiation [Kawashima et al., 1991; Keller et al., 1993]. Moreover, 3T3-L1 preadipocytes are known to express functional IL-11 receptors [Tenney et al., 2005]. Hence, our observation that activation of the PGF2α/calcineurin-signaling pathway in 3T3-L1 preadipocytes induces the expression and subsequent secretion of IL-11, strongly suggested a model in which PGF2α inhibits adipocyte differentiation by means of an autocrine IL-11-mediated negative regulatory pathway. Evidence in support of this model is provided by our dual observation that both the shRNA-mediated knockdown of IL-11 and the dominant-negative inhibition of the gp130 cytokine co-receptor signaling subunit, an essential signaling component of the IL-11 receptor, are both able to rescue 3T3-L1 preadipocyte differentiation in the presence of inhibitory concentrations of PGF2α. Together, these results demonstrate that the ability of PGF2α to inhibit adipocyte differentiation is dependent upon both IL-11 and gp130 cytokine co-receptor signaling. In this regard, although the DN-gp130 mutant used in our experiments is capable of inhibiting IL-11 signaling, it can also inhibit signaling via all other members of the IL-6 cytokine family, whose receptors all share the same gp130 cytokine co-receptor signaling subunit [Taga and Kishimoto, 1997; Heinrich et al., 2003]. However, of this family of cytokines, we find that only IL-11 and, to a somewhat lesser extent, IL-6 is significantly induced by the PGF2α/calcineurin-signaling pathway in differentiating 3T3-L1 preadipocytes. Hence, based upon our collective data, we conclude that IL-11, acting in an autocrine fashion via a critical gp130 cytokine co-receptor-dependent mechanism, most likely plays the prominent role in mediating the inhibitory effects of the PGF2α/calcineurin-signaling pathway on adipocyte differentiation, although we do not rule out the possibility that IL-6, and potentially other related family members, may also additionally contribute.

Having identified a clear role for the gp130-signaling axis in mediating the inhibitory effects of the PGF2α/calcineurin-signaling pathway on adipocyte differentiation, and having further showed that the activation of gp130-dependent signaling per se is sufficient to inhibit the adipogenic process, our attention naturally focused towards investigating the underlying gp130-dependent mechanisms involved. In this respect, signaling via the gp130 cytokine co-receptor signaling subunit has been extensively characterized and is known to primarily involve the activation of both the STAT1 and STAT3 latent transcription factors and the MAPK signaling pathway [Heinrich et al., 2003]. By turning to previously characterized chimeric mutants of gp130 known to be selectively deficient in the activation of either one of these latter pathways [Schaper et al., 1998; Schmitz et al., 2000], we were able to interrogate their respective roles in mediating the inhibitory effects of gp130 signaling on adipogenesis. Surprisingly, although the MAPK signaling pathway has previously been shown to inhibit adipocyte differentiation in certain contexts [Font de Mora et al., 1997; Tanabe et al., 2004], we found that tyrosine 759, responsible for coupling gp130 to the activation of MAPK, was completely dispensable for the inhibitory effects of gp130 signaling. Conversely, we found that the ability of gp130 to inhibit adipocyte differentiation was critically dependent upon the conserved tyrosine residues known to be involved in the activation of STAT1 and STAT3, suggesting a potentially important role for STAT transcription factors. By adopting the use of additional gp130 mutant receptors that are preferentially able to activate either STAT1 or STAT3 [Gerhartz et al., 1996], we were able to demonstrate that the gp130-mediated inhibition of adipogenesis correlated most closely with the activation of STAT1, but not STAT3. More significantly, we found that the shRNA-mediated depletion of endogenous STAT1 protein served to largely attenuate the inhibitory effects of either IL-11 or chimeric EpoR/gp130 receptor signaling on 3T3-L1 preadipocyte differentiation, as well as significantly rescue adipogenesis in the presence of PGF2α. Taken together, therefore, our data provides evidence that STAT1 plays an important role in mediating the inhibitory effects of the PGF2α/calcineurin-signaling pathway on adipogenesis, where it appears to serve as a critical negative regulatory effector downstream of gp130 receptor complexes to couple PGF2α/calcineurin-induced autocrine IL-11 cytokine signaling-events to the inhibition of adipocyte differentiation (see Fig. 7).

Our identification of STAT1 as a negative regulator of adipocyte differentiation is consistent with prior studies that have suggested a potential role for STAT1 in mediating the anti-adipogenic effects of IFNγ [Waite et al., 2001 ; McGillicuddy et al., 2009]. Interestingly, in contrast to the anti-adipogenic role of STAT1, other members of the STAT transcription factor family, namely STAT3 and STAT5 [Richard and Stephens, 2011], have conversely been reported to play pro-adipogenic roles. In this regard, STAT3 has been proposed to play a role in promoting the expression of the early transcription factor C/EBPβ and is believed to play a critical role in the mitotic clonal expansion phase of adipogenesis [Deng et al., 2000; Zhang et al., 2011], whereas STAT5 has been reported to make a more direct

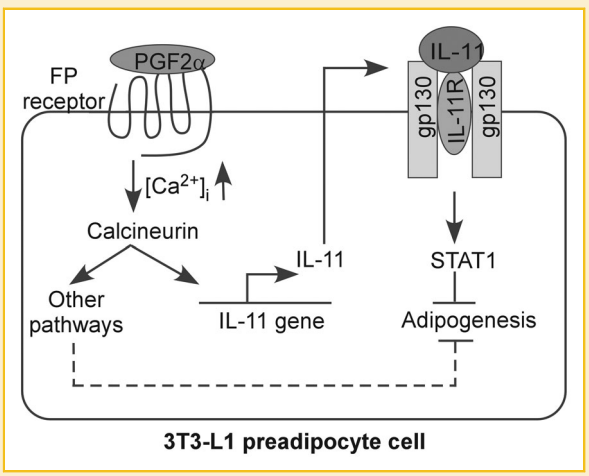

Fig. 7. Schematic model illustrating the proposed mechanism by which PGF2α inhibits adipogenesis. PGF2α induces the calcineurin-dependent expression of IL-11, which in turn acts in an autocrine fashion to stimulate the gp130-dependent activation of the STAT1 transcription factor responsible for inhibiting the adipogenic process. The dashed line represents other alternative parallel calcineurin-dependent pathways that may additionally contribute to the inhibition of adipocyte differentiation downstream of PGF2α.

contribution towards adipocyte differentiation by directly helping promote the expression of PPARγ [Nanbu-Wakao et al., 2002; Kawai et al., 2007]. STAT proteins therefore play complex, diverse and opposing roles in the regulation of adipocyte differentiation. By virtue of their established regulatory roles and specific activation in response to diverse ligand-activated plasma membrane receptors [Levy and Darnell, 2002], STAT proteins offer the opportunity to modulate and fine-tune the efficiency of the adipogenic process in response to changing extracellular cues. However, exactly how the relative activities of these related transcription factors are integrated at the level of the transcriptome to influence the adipogenic cell fate decision remains to be determined.

Mechanistically, it is currently unclear how STAT1 activation results in the inhibition of adipocyte differentiation. However, we believe that STAT1 likely acts at a point proximal to the expression of the PPARγ master adipogenic transcription factor, as our data show that gp130 signaling is sufficient to attenuate the expression of PPARγ in a STAT1-dependent manner, whereas ectopic expression of PPARγ2 is able to readily bypass the inhibitory effects of gp130-mediated signaling (see Fig. 4). While STAT1 is commonly thought to primarily play a role as a positively acting transcription factor, it is evident that it can also act to inhibit transcriptional events in certain biological contexts [Ramana et al., 2000; Liu et al., 2008; Furukawa et al., 2009]. Hence, we envision that STAT1 may contribute towards the inhibition of adipocyte differentiation via either of two non-mutually exclusive mechanisms. On the one hand, the most direct mechanism by which STAT1 may inhibit adipogenesis is via the direct repression of a critical pro-adipogenic gene(s) that is required for adipogenesis. In this regard, it is perhaps noteworthy that a nucleotide region capable of specifically binding STAT1 in vitro has previously been identified in the immediate upstream regulatory region of the murine PPARγ2 promoter and has been proposed to play a negative regulatory role in the expression of this critical pro-adipogenic transcription factor [Hogan and Stephens, 2001], thereby raising the possibility that STAT1 may inhibit adipogenesis by directly blocking the expression of PPARγ itself. Alternatively, STAT1 may contribute to the inhibition of adipocyte differentiation via a more indirect mechanism in which STAT1 is responsible for inducing the expression of a distinct anti-adipogenic gene(s) that is in turn itself responsible for inhibiting the adipogenic process. In this respect, the increased expression of many genes and microRNAs are known to be capable of inhibiting adipogenesis by preventing expression of PPARγ [Rosen and MacDougald, 2006 ; McGregor and Choi, 2011], although whether any of these known inhibitors act as functional downstream anti-adipogenic effectors of STAT1 remains to be determined. Consequently, the respective roles of either of these two non-mutually exclusive pathways in mediating the STAT1-dependent inhibition of adipocyte differentiation and the precise molecular mechanism(s) involved await further investigation.

Interestingly, while PGF2α has long been known to be a potent exogenous inhibitor of adipocyte differentiation in vitro [Serrero et al., 1992; Casimir et al., 1996], more recent data has highlighted an emerging role for PGF2α as an important endogenous negative regulator of adipogenesis that is transiently synthesized by preadipocytes themselves early during the normal differentiation process, where it appears to participate in an autocrine-mediated, negative feedback loop that acts to restrict the overall adipogenic capacity of these cells [Fujimori et al., 2010; Silvestri et al., 2013]. We believe that this latter intrinsic PGF2α-mediated negative feedback loop likely proceeds, at least in part, via the calcineurin/IL-11/gp130/STAT1-mediated pathway that we have described here. In this regard, we find that MDI-induced 3T3-L1 preadipocytes themselves transiently produce low levels of IL-11 early in the differentiation process via a pathway that is dependent upon the activation of calcineurin (Supplemental Fig. 1), which is presumably induced in response to endogenously produced PGF2α. More importantly, we find that inhibition of the endogenous calcineurin/IL-11/gp130/STAT1-signaling pathway in 3T3-L1 preadipocytes with either calcineurin inhibitors [Neal and Clipstone, 2002], or ectopic expression of either DN-gp130 (Fig. 2B), or STAT1 shRNAs (Fig. 6F,G) all consistently result in enhanced levels of MDI-induced adipocyte differentiation, as determined by increased expression of PPARγ and a corresponding increase in ORO-staining cells. Furthermore, we find that abrogation of the calcineurin/IL-11/gp130/STAT1-signaling pathway by either specific calcineurin inhibitors [Neal and Clipstone, 2002], or the shRNA-mediated depletion of endogenous STAT1 proteins (see Supplemental Fig. 2) leads to a markedly enhanced efficiency of 3T3-L1 preadipocyte differentiation induced in response to suboptimal adipogenic conditions. Taken together, these collective results lend support to the notion that the PGF2α/calcineurin/IL-11/gp130/STAT1-signaling pathway represents an intrinsic negative regulatory feedback mechanism that normally serves to restrict the differentiation potential of 3T3-L1 preadipocyte cells and therefore likely plays a role in helping determine the efficiency of the adipogenic process. Given the fact that adipocyte-derived PGF2α production has been implicated as an important negative regulator of adipose tissue expansion in vivo, involved in opposing the development of obesity and its attendant diseases [Volat et al., 2012], it is tempting to speculate that these effects, at least in part, may also be mediated by this same calcineurin/IL-11/gp130/STAT1-signaling module.

Finally, although collectively our results provide strong evidence of a role for the autocrine IL-11/gp130/STAT1-signaling axis in mediating the inhibitory effects of the PGF2α/calcineurin-signaling pathway on adipocyte differentiation, it is noteworthy that ectopic expression of neither the IL-11 shRNA, the gp130 dominant-negative mutant, nor the STAT1-specific shRNAs, were able to fully rescue the differentiation of 3T3-L1 preadipocytes in the presence of PGF2α. These findings contrast with our prior results demonstrating that the inhibition of calcineurin is able to essentially fully abrogate the anti-adipogenic effects of PGF2α [Liu and Clipstone, 2007]. While in part these differences may be explained by incomplete knockdown or inhibition of endogenous IL-11, gp130 and STAT1, as well as potentially the non-cell autonomous actions of the IL-11 cytokine, another possibility is that there are other parallel calcineurin-dependent signaling pathways downstream of PGF2α, in addition to the IL-11/gp130/STAT1 signaling axis, that are capable of independently inhibiting adipogenesis (see Fig. 7). In fact, consistent with this notion, we have previously reported that activation of the PGF2α/calcineurin-signaling pathway in differentiating 3T3-L1 preadipocytes induces the hypoxia-inducible factor 1-dependent expression of DEC1 [Liu and Clipstone, 2008], a

transcriptional repressor that is known to inhibit adipocyte differentiation by preventing the expression of PPARγ2 [Yun et al., 2002]. Additionally, we have recently uncovered evidence of another anti-adipogenic transcription factor induced downstream of the PGF2α/calcineurin-signaling pathway in 3T3-L1 preadipocytes (Annamalai and Clipstone, unpublished observation). Hence, it seems likely that PGF2α triggers the activation of multiple downstream calcineurin-dependent signaling pathways that ultimately collaborate together to effectively inhibit the adipogenic process.

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