Suppression of PDGF-induced PI3 kinase activity by imatinib promotes adipogenesis and adiponectin secretion

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Abstract

Improved glucose and lipid metabolism is a unique side effect of imatinib therapy in some chronic myeloid leukaemia (CML) patients. We recently reported that plasma levels of adiponectin, an important regulator of insulin sensitivity, are elevated following imatinib therapy in CML patients, which could account for these improved metabolic outcomes. Adiponectin is secreted exclusively from adipocytes, suggesting that imatinib modulates adiponectin levels directly, by transcriptional upregulation of adiponectin in pre-existing adipocytes, and/or indirectly, by stimulating adipogenesis. In this report, we have demonstrated that imatinib promotes adipogenic differentiation of human mesenchymal stromal cells (MSCs), which in turn secrete high-molecular-weight adiponectin. Conversely, imatinib does not stimulate adiponectin secretion from mature adipocytes. We hypothesise that inhibition of PDGFR α (PDGFRA) and PDGFR β (PDGFRB) is the mechanism by which imatinib promotes adipogenesis. Supporting this, functional blocking antibodies to PDGFR promote adipogenesis and adiponectin secretion in MSC cultures. We have shown that imatinib is a potent inhibitor of PDGF-induced PI3 kinase activation and, using a PI3 kinase p110 α -specific inhibitor (PIK-75), we have demonstrated that suppression of this pathway recapitulates the effects of imatinib on MSC differentiation. Furthermore, using mitogens that activate the PI3 kinase pathway, or MSCs expressing constitutively activated Akt, we have shown that activation of the PI3 kinase pathway negates the pro-adipogenic effects of imatinib. Taken together, our results suggest that imatinib increases plasma adiponectin levels by promoting adipogenesis through the suppression of PI3 kinase signalling downstream of PDGFR.

Journal of Molecular Endocrinology (2012) **48**, 229–240

Introduction

Imatinib mesylate is a rationally designed chemotherapeutic agent that was developed to target the kinase domain of the BCR–ABL fusion protein, the causative molecular abnormality of chronic myeloid leukaemia (CML). Imatinib binds to the inactive ATP-binding pocket of BCR–ABL, preventing ATP binding and, subsequently, catalytic activity (Druker 2004). Imatinib has also been shown to inhibit the function of a number of off-target kinases, resulting in a range of unique side effects that are observed in some imatinib-treated patients (Vandyke *et al.* 2010b). For example, hyperphosphataemia and secondary hyperparathyroidism, observed in over 50% of imatinib-treated CML patients, are thought to relate to changes in normal bone remodelling resulting from inhibition of the macrophage colony-stimulating factor (CSF1) receptor, c-fms (CSF1R) and the platelet-derived growth factor receptor (PDGFR). At therapeutically achievable concentrations, imatinib inhibits c-fms, thereby suppressing osteoclast differentiation and function (Dewar *et al.* 2005, 2006). Conversely,

imatinib is thought to stimulate osteogenesis by inhibiting PDGFR signalling in pluripotent mesenchymal stromal cells (MSCs; Fierro *et al.* 2007, Fitter *et al.* 2008, Jonsson *et al.* 2011). Assessment of skeletal elements in CML patients, before and after imatinib treatment, has revealed an increase in trabecular bone volume and increased bone mineral density consistent with an anti-osteoclastic and/or pro-osteogenic effect (Fitter *et al.* 2008, Jonsson *et al.* 2008, O'Sullivan *et al.* 2009).

Improved glucose metabolism has also emerged as an off-target effect of imatinib therapy in some CML patients. This side effect appears to be limited to CML patients with concurrent type 2 diabetes or those who are insulin resistant (Tsapas *et al.* 2008), as no improvement in fasting glucose levels was observed in non-diabetic CML patients with normoglycaemic profiles at diagnosis (Mariani *et al.* 2010). Typically, responsive patients experience a significant increase in insulin sensitivity within 3 months of commencing imatinib therapy (Breccia *et al.* 2004, 2005, Veneri *et al.* 2005, Gologan *et al.* 2009, Mariani *et al.* 2010). This improvement occurs without significant dietary or

lifestyle changes, suggesting that imatinib modulates systemic insulin sensitivity. In support of this, improved glycaemic control has also been observed following imatinib treatment in animal models of diabetes. In streptozotocin-induced and spontaneous (non-obese diabetic and *db/db*) mouse models of diabetes, imatinib has been shown to preserve islet β -cell function, in part through the suppression of c-abl-mediated islet cell apoptosis (Hagerkvist *et al.* 2007, Han *et al.* 2009). Imatinib and other inhibitors of PDGFR have also been shown to prevent and even reverse diabetes in non-obese diabetic mice through a mechanism that may involve suppression of a PDGF-induced inflammatory response (Louvet *et al.* 2008). More recently, we have demonstrated that plasma levels of an adipokine, adiponectin, are elevated two- to three-fold in CML patients after 3 months of imatinib therapy (Fitter *et al.* 2010). Adiponectin is known to play an important role in glucose metabolism, suggesting that this increase in adiponectin may be a mechanism whereby imatinib causes improvements in insulin sensitivity. In support of this, the increase in plasma adiponectin levels observed following imatinib treatment occurs within a similar time frame as the improvements in insulin sensitivity observed in imatinib-treated CML patients (Breccia *et al.* 2004, 2005, Veneri *et al.* 2005, Gologan *et al.* 2009, Mariani *et al.* 2010).

Adiponectin is secreted exclusively from peripheral, omental and bone marrow adipose, where its expression is regulated in response to metabolic effectors. Intramedullary adipose is derived from pluripotent MSCs that reside within the bone marrow microenvironment. Differentiation occurs in response to a variety of extrinsic factors that act to initiate or repress the transcriptional programs that govern lineage determination (Gimble 1998). Examination of the bone marrow cellularity of CML patients before and after imatinib therapy revealed a significant increase in intramedullary adipose after 6 months of therapy (Fitter *et al.* 2010). Furthermore, we and others have observed increased adipocyte numbers in MSC cultures treated with therapeutically relevant doses of imatinib (Fierro *et al.* 2007, Fitter *et al.* 2008). Taken together, these findings suggest that imatinib promotes adipogenic differentiation of human MSCs.

In this study, we have shown that imatinib promotes adipogenic differentiation and adiponectin secretion from human MSCs and have identified the PI3 kinase pathway, downstream of PDGFR, as an important mechanism.

Materials and methods

Reagents

Imatinib mesylate and NVP-BE235 were provided by Novartis International. PI3 kinase isoform-specific inhibitors PIK-75 (p110 α), TGX-221 (p110 β) and

IC87114 (p110 δ) were a kind gift from Prof. Peter Shepherd (Maurice Wilkins Centre for Molecular Biodiscovery, University of Auckland, New Zealand). Pioglitazone was purchased from Cayman (Ann Arbor, MI, USA). Stock solutions of inhibitors were prepared in 100% DMSO and subsequent dilutions made in media. Unless otherwise specified, all reagents were purchased from Sigma.

MSC isolation and differentiation

MSCs were grown from bone chips recovered from posterior iliac crest bone marrow aspirates from haematologically normal human donors as described previously (Fitter *et al.* 2008) and in accordance with procedures approved by the Royal Adelaide Hospital Ethics Committee. To stimulate adipogenesis, cells were cultured in media with reduced foetal bovine serum (5% v/v) and supplemented with 100 nmol/l dexamethasone sodium phosphate (induction media) for 35 days. Conditioned media were collected from control and imatinib-treated cultures 4 days post-media change and stored at -80°C . To identify and enumerate lipid-laden fat cells, formalin-fixed cells in 96-well plates were stained with Nile red (25 ng/ml) and DAPI (300 nmol/l) for 15 min. Nile red-labelled adipocytes and DAPI-stained cell nuclei were visualised using an inverted fluorescence microscope (CKX41; Olympus, Tokyo, Japan), images were captured using a colour CCD camera (4 $\times$ magnification; DP20, Olympus) and adipocytes and cell nuclei were enumerated using Image J software. For mitogen experiments, induction cultures were treated with rhPDGF-BB (10 ng/ml; Prospec, Rehovot, Israel) or rhEGF (20 ng/ml; Prospec) twice weekly for 4 weeks and adipocytes were enumerated as described.

Real-time PCR

Total RNA was isolated using Trizol (Invitrogen) and real-time PCR was performed as described previously (Fitter *et al.* 2008). Changes in gene expression were calculated relative to β -actin using the $2^{-\Delta\Delta C_t}$ method. Primer pairs (forward and reverse) were as follows: β -actin (*ACTB*), 5'-gatcattgctctctctgagc-3' and 5'-gtcatagtcgcctagaagcat-3'; CCAAT/enhancer binding protein, alpha (*CEBPA*), 5'-gggcaaggccaagaagtc-3' and 5'-ttgtcactggtcagctccag-3'; peroxisome proliferator-activated receptor gamma 2 (*PPARG*), 5'-ctcctattgaccagaaagc-3' and 5'-tcaaaggagtgggagtggc-3'; leptin (*LEP*), 5'-ggctttggccctatcttttc-3' and 5'-accggtgactttctgtttgg-3'; complement factor D (*CFD*), 5'-gacaccatcgaccacgac-3' and 5'-ccagtcgcagagagtc-3'; retinoic acid receptor responder (tazarotene induced) 2 (*RARRES2*), 5'-aagcatgcgacggctgctga-3' and 5'-agctgggaaggcgctgtcca-3'; and adiponectin (*ADIPOQ*), 5'-gctgggagctgttctactgc-3' and 5'-cgatgtctcccttagacca-3'.

Immunoblotting

Analysis of adiponectin multimers was performed as described previously (Lara-Castro *et al.* 2006, Fitter *et al.* 2010). Briefly, conditioned media were electrophoresed on 8–13% non-denaturing sodium acetate PAGEs (Invitrogen) and protein complexes transferred to PVDF membrane, blocked for 2 h in 2.5% (w/v) blocking buffer (GE Healthcare, Little Chalfont, UK) and probed for 16 h at 4 °C using an adiponectin-specific antibody (BD Biosciences, Franklin Lakes, NJ, USA). Bound antibody was detected using an alkaline phosphatase-conjugated secondary antibody and then visualised on a Typhoon 9410 imager (GE Healthcare) in the presence of an enhanced chemifluorescence substrate. To assess total adiponectin levels, an equivalent amount of culture medium was resolved by denaturing SDS–PAGE (10% v/v), transferred to PVDF membranes and immunoblotted as described earlier. To measure cellular proteins, cell lysates were obtained as described previously (Fitter *et al.* 2008) and equivalent amounts (50 µg) of protein separated on 10% SDS–PAGE gels and transferred to PVDF membranes. Target proteins were detected using antigen-specific antibodies (C/EBP α and PPAR γ 2, Millipore, Billerica, MA, USA; β -actin, Sigma; HSP-90, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

PDGF pulse experiments were carried out as described previously (Fitter *et al.* 2008). Briefly, MSCs were grown to 80–90% confluence in 6 cm dishes and serum deprived for 16 h. Cells were exposed to drugs (at indicated concentrations) for 1 h and then pulse stimulated with rhPDGF-BB (10 ng/ml) for 5 min. Equivalent amounts of protein (50 µg) were separated on 10% SDS–PAGE gels and transferred to PVDF membranes. Target proteins were detected using antigen-specific antibodies (phospho-Akt [Thr 308] and phospho-Erk1/2 [Thr 202/Tyr 204]; Cell Signaling, Danvers, MA, USA) as described earlier. Data are representative of results from three MSC donors.

ELISA

Adiponectin levels in cell culture conditioned media were measured using a commercial ELISA kit (R&D Systems, Minneapolis, MA, USA). Frozen conditioned media were thawed at 4 °C, centrifuged at 16 000 *g* for 15 min at 4 °C and adiponectin levels were determined in triplicate according to the manufacturer's instructions. The inter-plate coefficient of variation was calculated as 2.5–7.5 (%).

Cell proliferation assay

The effect of functional blocking antibodies to PDGFR α (PDGFRA) and PDGFR β (PDGFRB; 10 µg/ml; Millipore),

control IgG (10 µg/ml) and imatinib (3 µmol/l) on cell survival/proliferation was assessed using WST-1 as described previously (Vandyke *et al.* 2010a).

Generation of MSCs expressing myristoylated Akt

A cDNA encoding a 5'-src myristoylation signal in frame with mouse Akt1 (myr-Akt; a gift from Dr Naheed N Ahmed, Fox Chase Cancer Center, Philadelphia, PA, USA) was sub-cloned into pRUF-IRES-eGFP and then introduced into primary human MSCs by retroviral transduction as described previously (Isenmann *et al.* 2009). Infected cells (eGFP⁺) were isolated by preparative flow cytometry and used in differentiation assays. Constitutive Akt activity was confirmed by immunoblotting using a phospho-specific p70S6K antibody (Cell Signaling) and a total p70S6K antibody (Cell Signaling).

Statistical analysis

Statistical analysis was performed using GraphPad Prism (Version 5; GraphPad Software, La Jolla, CA, USA). *t*-Tests or one-way ANOVA with Dunnett's post-tests were applied where indicated. Two-sided *P* values < 0.05 were considered statistically significant.

Results

Imatinib stimulates adipogenesis

Differentiation of human MSCs into adipocytes *in vitro* is typically performed using a drug cocktail containing PPAR γ 2 agonists, phosphodiesterase inhibitors and glucocorticoid, the latter being essential to drive differentiation (Gimble 1998). To examine the effect of imatinib, we chose to use only glucocorticoid (100 nmol/l dexamethasone) to more closely emulate physiological conditions.

At therapeutically relevant doses of imatinib (le Coutre *et al.* 2004), a dose-dependent increase in adipocyte numbers was observed (Fig. 1A and B). Real-time PCR was utilised to investigate transcriptional changes in genes involved in adipogenic differentiation and function. In response to imatinib, the expression of key adipogenic transcription factors *CEBPA* and *PPARG* was elevated at all time points examined (Fig. 1C). Imatinib treatment also stimulated the expression of several adipokines including *LEP*, *CFD* and *RARRES2*. Most profoundly, adiponectin gene expression (*ADIPOQ*) was elevated up to 70-fold in imatinib-treated cultures at day 32, when compared with the vehicle control (Fig. 1B). Consistent with the transcriptional increases seen in response to imatinib, an increase in C/EBP α , PPAR γ 2 and adiponectin protein levels were detected by immunoblotting (Fig. 1D).

Adipocytes in imatinib-treated cultures secrete high-molecular-weight adiponectin

To investigate whether adiponectin was secreted from imatinib-treated MSCs, an adiponectin-specific ELISA was used. Consistent with the gene expression studies, increased levels of secreted adiponectin were detectable in the conditioned media of MSCs cultured in the presence of imatinib for 21 days or longer (Fig. 2A).

To determine the nature of adiponectin complexes secreted from adipocytes in imatinib-treated MSC cultures, conditioned media were analysed by non-denaturing PAGE and western blotting. The predominant secreted form is the high-molecular-weight (HMW) complex that was evident from 17 to 21 days of culture (Fig. 2B).

To determine whether imatinib promotes adiponectin secretion from mature adipocytes, MSCs were cultured under induction conditions in the presence of imatinib for 28 days to promote adipogenesis. The induction medium was then replaced with normal growth medium for 4 days after which time differentiated adipocyte cultures were stimulated with imatinib or the known PPAR γ 2 agonists pioglitazone or indomethacin for 4 days in induction media. Conditioned media were collected and adiponectin levels were determined by ELISA. No significant increase in adiponectin secretion was observed in differentiated cultures treated with imatinib, whereas both pioglitazone and indomethacin strongly promoted adiponectin secretion when compared with the vehicle control (Fig. 2C).

Blocking antibodies to PDGFR promote adipogenesis and adiponectin secretion

Imatinib inhibits a number of non-receptor and receptor tyrosine kinases. These include PDGFR, which is highly expressed on mesenchymal cells. PDGF acts as a potent MSC mitogen and an inhibitor of osteogenic and adipogenic differentiation (Kratchmarova *et al.* 2005, Tokunaga *et al.* 2008). In light of this,

Figure 1 Imatinib stimulates adipogenesis. (A) Primary human MSCs were treated with imatinib (as indicated) or vehicle (PBS) in induction media for 35 days and adipocytes visualised using Nile red. (B) The number of adipocytes was enumerated by counting Nile red-labelled cells and DAPI-labelled cell nuclei (mean $\pm$ s.d. of triplicate measurements; * P < 0.001. One-way ANOVA with Dunnett's post-test). (C) Temporal gene expression analysis of adipocyte-specific genes in imatinib-treated (3 $\mu\text{mol/l}$) (solid lines) and vehicle-treated (dashed lines) primary human MSC cultures. RNA was harvested at the indicated time points, reverse transcribed into cDNA and comparative gene expression studies performed using real-time PCR. Line graphs show temporal changes in gene expression for indicated genes (mean $\pm$ s.d. of triplicate measurements). (D) Protein lysates from imatinib-treated (+) and vehicle-treated (–) cultures were isolated at the indicated time points and subjected to immunoblotting using antigen-specific antibodies as indicated. Bar = 500 μm .

Figure 2 Adipocytes formed in imatinib-treated cultures secrete adiponectin. (A) MSCs were cultured under induction conditions in the presence of vehicle (PBS, white bars) or imatinib (3 μ mol/l, black bars). Conditioned media were collected from cultures, at the indicated time intervals, and secreted adiponectin levels measured by ELISA (mean $\pm$ s.d. of triplicate measurements; * P < 0.005, t -test, relative to PBS control). (B) Secreted adiponectin was analysed by non-denaturing PAGE, to identify complexes, and denaturing PAGE, to detect total adiponectin, followed by immunoblotting. Molecular weights: HMW, ~360 kDa; LMW, ~180 kDa; total, 30 kDa. (C) MSC cultures were treated with 3 μ mol/l imatinib for 28 days, then stimulated with vehicle (0.1% DMSO), imatinib (3 μ mol/l), pioglitazone (2 μ mol/l) or indomethacin (60 μ mol/l) and secreted adiponectin measured by ELISA (mean $\pm$ s.d. of triplicate measurements; * P < 0.001. One-way ANOVA with Dunnett's post-test).

we hypothesised that imatinib mediates its effects through inhibition of PDGFR function. To test this, functional blocking antibodies to PDGFR α and PDGFR β were included in MSC cultures under induction conditions. An antibody concentration (10 μ g/ml) that inhibited cell proliferation and PDGF-induced activation of Akt equivalent to that observed following treatment with 3 μ M imatinib was selected (Fig. 3A and B). Consistent with a role for PDGF as an inhibitor of differentiation, blocking antibodies to PDGFR promoted the formation of adipocytes in MSC cultures (Fig. 3C and D), and this was associated with an up-regulation of PPAR γ 2 and adiponectin gene expression (Fig. 3E). Adipocytes formed in the presence of functional blocking antibodies to PDGFR α and PDGFR β were shown to secrete the HMW form of adiponectin, consistent with the effects of imatinib (Fig. 3F and G).

Inhibition of p110 α promotes adipogenesis and adiponectin secretion

In mesenchymal cells, stimulation with PDGF has been shown to strongly activate the PI3 kinase pathway, as evidenced by the phosphorylation of the regulatory and catalytic subunits of class I PI3 kinase family members and downstream effector molecules such as Akt (Kratchmarova *et al.* 2005). Suppression of PI3 kinase using pharmacological inhibitors has been shown to promote MSC differentiation (Kratchmarova *et al.* 2005, Fitter *et al.* 2008, Martin *et al.* 2010), suggesting that this pathway is important for transducing the inhibitory effects of PDGF on MSC differentiation. Consistent with this hypothesis, imatinib is a potent inhibitor of PDGF-induced PI3 kinase activation, as evidenced by a dose-dependent decrease in the phosphorylation of Akt (Fig. 4A). By contrast, PDGF-induced Erk activation, a measure of MAPK activity, is not affected by imatinib treatment (Fig. 4A).

In light of the inhibitory effect of imatinib on PDGF-induced PI3 kinase activation, we hypothesised that an inhibitor of the catalytic subunit of PI3 kinase would promote adipogenesis. As MSCs express PI3 kinase subunits p110 α , p110 β and p110 δ (data not shown), the isoform-specific inhibitors BEZ235, PIK-75, TGX-221 and IC87114 were tested initially to assess the role of each subunit in PDGF-induced Akt activation. Only PIK-75, an inhibitor of p110 α (Chaussade *et al.* 2007) and BEZ235, a pan class I inhibitor (Serra *et al.* 2008), were found to inhibit PDGF-induced Akt phosphorylation (Fig. 4B). PIK-75 inhibits PDGF-induced Akt phosphorylation dose dependently (Fig. 4C) with an IC₅₀ of 10 nM.

Consistent with the results obtained for imatinib and functional blocking PDGFR antibodies, PIK-75 was shown to dose dependently promote adipocyte formation under induction conditions (Fig. 4D and E). Similarly, high levels of secreted HMW and LMW

adiponectin complexes were detected in cultures treated with PIK-75 for 18 days or more, when compared with vehicle-treated control cultures (Fig. 4F and G).

Mitogens that activate PI3 kinase negate the proadipogenic effects of imatinib but not PIK-75

To further investigate the role of the PI3 kinase pathway in MSC differentiation, we examined the effects of adding

exogenous mitogens to imatinib or PIK-75-treated MSC cultures under induction conditions. Both PDGF and EGF activate the PI3 kinase pathway in human MSCs (Kratchmarova *et al.* 2005). Consistent with previous reports (Artemenko *et al.* 2005, Gagnon *et al.* 2009), we found that addition of growth factors alone completely inhibited glucocorticoid-induced adipogenesis (Fig. 5A and B). In the presence of imatinib, PDGF had no effect on imatinib-mediated adipogenesis whereas EGF inhibited the imatinib response (Fig. 5A and B). In contrast, PIK-75 promoted adipogenesis, albeit to a lesser extent, in the presence of either PDGF or EGF (Fig. 5A and B), suggesting that the PI3 kinase pathway is important for transducing the inhibitory effects of mitogens on MSC differentiation.

Constitutive activation of Akt negates the effects of imatinib on MSC differentiation

Having established a signalling pathway that links imatinib with MSC differentiation, we sought to confirm our findings using a genetic approach. Akt is the primary effector molecule of the PI3 kinase pathway, and its activation is suppressed by imatinib in response to PDGF stimulation. It therefore follows that constitutive activation of Akt would negate the pro-adipogenic effects of imatinib. To test this, MSC cultures that express constitutively activated Akt (myr-Akt) or vector only were treated with imatinib and their ability to differentiate into adipocytes was measured. To confirm activation of Akt, the phosphorylation status of p70S6K, an Akt effector

Figure 3 Functional blocking antibodies to PDGFR promote adipogenesis. (A) MSC cultures were starved overnight, treated with functional blocking anti-PDGFR antibodies or imatinib and then stimulated with rhPDGF-BB (10 ng/ml) for 5 min. Cell proteins were harvested, resolved by SDS-PAGE and then transferred to PVDF membranes. The activation status of Akt was determined using a phospho-specific antibody. (B) MSCs were cultured in normal growth medium supplemented with functional blocking PDGFRα and PDGFRβ antibodies (mAbs, 10 µg/ml, black dashed line), isotype control antibodies (clgG, 10 µg/ml, grey dashed line), imatinib (3 µmol/l, black solid line) or vehicle (PBS, grey solid line) for 6 days. The relative number of viable, metabolically active cells per well was then detected using WST-1 reagent read at 540 nm. (C) MSC cultures were treated with mAbs, clgG, imatinib (3 µmol/l) or vehicle (PBS) under induction conditions for 28 days and adipocytes were visualised using Nile red. (D) The number of adipocytes was enumerated by counting Nile red-labelled cells and DAPI-labelled cell nuclei (mean ± s.d. of triplicate measurements). (E) RNA harvested from treated cultures was reverse transcribed into cDNA and comparative gene expression studies performed using real-time PCR. Bar graphs show changes in gene expression for indicated genes (mean ± s.d. of triplicate measurements). (F) An ELISA was used to determine the amount of adiponectin secreted from MSC cultures treated as indicated. (G) Secreted adiponectin was analysed by non-denaturing PAGE and immunoblotting. Molecular weights: HMW, ~360 kDa; LMW, ~180 kDa (**P* < 0.05, *t*-test, relative to controls). Bar = 500 µm.

molecule (Hay & Sonenberg 2004), was assessed. Hyperphosphorylated p70S6K was detected in immunoblots of proteins isolated from cells expressing myr-Akt, when compared with vector-only control cells lysates (Fig. 6A). Under induction conditions, imatinib

promoted adipogenic differentiation of vector control cells but failed to promote adipogenesis in hMSCs expressing myr-Akt (Fig. 6B). By contrast, indomethacin, a PPAR agonist (Lehmann *et al.* 1997), promoted adipogenesis in both control and myr-Akt-expressing cells.

Figure 4 Suppression of p110 α promotes adipogenesis. (A) MSC cultures were starved overnight, treated with imatinib or vehicle (PBS), as indicated, and then stimulated with rhPDGF-BB (10 ng/ml) for 5 min. Cell proteins were harvested, resolved by SDS–PAGE and then transferred to PVDF membranes. The activation status of PDGFR effector molecules was determined using phospho-specific antibodies as indicated. (B) Starved MSC cultures were treated with vehicle (0.1% DMSO), NVP-BEZ235 (100 nmol/l), PIK-75 (50 nmol/l), TGX-221 (200 nmol/l) or IC87114 (2 μ mol/l) for the indicated times, pulsed with rhPDGF-BB and the phosphorylation status of Akt was determined by immunoblotting. (C) Starved MSC cultures were treated with PIK-75 (as indicated) or vehicle (0.1% DMSO) for 1 h, pulsed with rhPDGF-BB and the phosphorylation status of Akt was determined by immunoblotting. (D) MSC cultures were treated with PIK-75 (as indicated) or vehicle (0.1% DMSO) under induction conditions for 32 days and adipocytes visualised using Nile red. (E) The number of adipocytes was enumerated by counting Nile red-labelled cells and DAPI-labelled cell nuclei (mean $\pm$ s.d. of triplicate measurements; * P < 0.001. One-way ANOVA with Dunnett's post test. (F) An ELISA was used to determine the amount of adiponectin secreted from PIK-75 (10 nmol/l)-treated MSC cultures (black bars) and vehicle controls (white bars) at the indicated time points (mean $\pm$ s.d. of triplicate measurements). (G) Secreted adiponectin was analysed by non-denaturing PAGE, to identify complexes, and denaturing PAGE, to detect total adiponectin, followed by immunoblotting. Cellular adiponectin was analysed by denaturing PAGE and immunoblotting. As a loading control, heat-shock protein 90 (HSP90) levels are shown. Molecular weights: HMW, $\sim$ 360 kDa; LMW, $\sim$ 180 kDa; total, 30 kDa. (* P < 0.005. t -test, relative to controls). Bar = 500 μ m.

Figure 5 Mitogens that activate PI3 kinase negate the pro-adipogenic effects of imatinib. MSC cultures were stimulated with rhPDGF-BB (10 µg/ml) or rhEGF (20 ng/ml) in the presence or absence of imatinib (3 µmol/l) or PIK-75 (10 nmol/l) for 28 days. (A) Adipocytes were labelled using Nile red. (B) The number of adipocytes were enumerated by counting Nile red-labelled cells and DAPI-labelled cell nuclei (mean ± s.d. of triplicate measurements; * $P < 0.001$. One-way ANOVA with Dunnett's post-test). Bar = 500 µm.

Discussion

Here, we demonstrate that therapeutically relevant concentrations of imatinib promote the differentiation of human MSCs into adipocytes, which in turn secrete the HMW form of adiponectin. Imatinib failed to promote adiponectin secretion from mature adipocytes, suggesting that imatinib does not promote adiponectin secretion through a direct effect on adiponectin gene expression but rather indirectly through an effect on adipocyte numbers.

MSCs are pluripotent and lineage specificity can be orchestrated *in vitro* using a combination of ligands that act directly, or indirectly, to drive lineage-specific transcription factors (Pittenger *et al.* 1999). Crucial to this response is the addition of glucocorticoid that acts

to prime cells to be responsive to differentiation signals (Gimble 1998). Treatment of MSCs with imatinib alone is sufficient to stimulate adipogenesis in glucocorticoid-treated cells, as evidenced by a temporal increase in key adipogenic transcription factors C/EBP α and PPAR γ 2, the accumulation of lipid-laden vacuoles and secretion of the adipokine adiponectin. Glucocorticoid-treated cells appear highly sensitive to the pro-adipogenic effects of imatinib, resulting in adipogenic differentiation of MSCs at hydrocortisone concentrations well below physiological levels (5 nmol/l; data not shown). As the reductase required to regenerate active cortisone *in vivo*, 11 β -hydroxysteroid dehydrogenase type I (11 β HSD), is highly expressed in MSCs and bone cells (Justesen *et al.* 2004), bone marrow-synthesised steroid and imatinib could act locally on MSC populations to drive adipogenic differentiation.

Previous studies have demonstrated that PDGF is a potent MSC mitogen that has been shown to inhibit MSC differentiation (Hock & Canalis 1994, Kubota *et al.* 2002, Chaudhary *et al.* 2004, Kratchmarova *et al.* 2005). Imatinib is a potent PDGFR inhibitor (Buchdunger *et al.* 2000), suggesting that imatinib mediates its pro-adipogenic effects through inhibition of PDGFR. In support of this, we have shown that functional blocking antibodies to PDGFR promote adipogenesis and adiponectin secretion when used at concentrations that suppress cell proliferation and PDGF-induced Akt activation similar to that achieved with imatinib. We therefore hypothesise that, in cultures treated with physiological levels of glucocorticoid alone, the basal adipogenic response to glucocorticoid is suppressed by PDGF, which is present in bovine serum, and is secreted in an autocrine and/or paracrine fashion by MSCs (Bonner 2010). Addition of imatinib or functional blocking antibodies ameliorates this inhibitory effect allowing differentiation to proceed unabated.

Studies using mouse 3T3-L1 pre-adipocytes and human subcutaneous adipose-derived stem cells have suggested that PDGF-induced activation of PKC α and IKK β mediates the inhibitory effect of PDGF on adipogenesis (Artemenko *et al.* 2005, Gagnon *et al.* 2009). However, we failed to detect activation of these pathways in response to PDGF in bone marrow-derived MSCs (data not shown), which may be due to cell-type differences or the PDGF isoform used. In hMSCs, PDGF strongly activates the PI3 kinase pathway and previous studies have suggested that the anti-differentiative effects of PDGF could be inhibited using PI3 kinase inhibitors such as Wortmannin and LY94002 (Kratchmarova *et al.* 2005, Fitter *et al.* 2008, Martin *et al.* 2010). Consistent with this view, we have demonstrated that imatinib is a potent inhibitor of PDGF-induced Akt phosphorylation, a major substrate of the PI3 kinase pathway. Furthermore, the pro-adipogenic effect of imatinib, but not that of indomethacin, was negated by

Figure 6 Constitutively activated Akt negates the imatinib response. Myristoylated murine Akt (myr-Akt) and empty vector (vector) were introduced into MSCs by retroviral transduction. (A) Cellular proteins from vector control and myr-Akt-infected cells were harvested, resolved by SDS-PAGE and then transferred to PVDF membranes. The activation status of p70S6K was determined by immunoblotting. (B) Vector control and myr-Akt-expressing cells were cultured under induction conditions with vehicle (0.1% DMSO), imatinib (3 $\mu\text{mol/l}$) or indomethacin (60 $\mu\text{mol/l}$) for 28 days. The number of adipocytes was enumerated by counting Nile red-labelled cells and DAPI-labelled cell nuclei (mean $\pm$ s.d. of triplicate measurements; * $P < 0.001$. One-way ANOVA with Dunnett's post-test).

constitutive activation of Akt. Addition of exogenous EGF to MSC cultures also inhibited imatinib-induced adipogenesis. EGF activates the PI3 kinase pathway in MSCs, albeit to a lesser extent than PDGF (Kratchmarova *et al.* 2005) and, unlike PDGFR, EGFR is insensitive to imatinib (Buchdunger *et al.* 2000). The anti-differentiative effects of PI3 kinase signalling on hMSCs was further demonstrated using PIK-75, a selective inhibitor of p110 α , the catalytic subunit of the obligate heterodimer type I PI3 kinase. PIK-75 promotes hMSC adipogenic differentiation and adiponectin secretion even in the presence of exogenous growth factors. Unlike imatinib, PIK-75 inhibits PI3 kinase activity irrespective of the nature of the mitogen. At the concentrations used in this study, PIK-75, imatinib and anti-PDGFR antibodies inhibit proliferation and suppress the PI3 kinase pathway.

While inhibition of proliferation may be important for the pro-adipogenic effects, studies using γ -irradiation show that it is not sufficient to promote differentiation under induction conditions (data not shown). Further analysis of Akt substrates that are suppressed or activated in PIK-75- and imatinib-treated MSCs is required to gain further mechanistic insight.

Our hypothesis that inhibition of PI3 kinase, downstream of PDGFR, promotes adipogenic differentiation of bone marrow-derived hMSCs is in contrast to a large body of data demonstrating an essential role for PI3 kinase signalling in adipogenesis in murine cells. Much of this evidence has been obtained using the well-characterised murine 3T3-L1 cell line (derived from white adipose tissue), which differentiates into adipocytes in response to insulin. This insulin-mediated differentiation process can be blocked using PI3 kinase inhibitors Wortmannin and LY94002 and by over-expression of a dominant-negative p85 (Sakaue *et al.* 1998, Xu & Liao 2004). Furthermore, over-expression of constitutively active forms of PI3 kinase or Akt has been shown to recapitulate the effects of insulin on Glut4 translocation and adipogenic differentiation in these cells (Kohn *et al.* 1996, Magun *et al.* 1996). Insulin and the PI3 kinase pathway has also been demonstrated to be essential for adipogenic differentiation in primary mouse cells, as demonstrated by a complete lack of adipocytes and complete abrogation of PI3 kinase activity in murine embryonic fibroblasts (MEFs) isolated from insulin receptor substrate (*Irs1* and *Irs2*) knockout mice (Miki *et al.* 2001). Furthermore, MEFs isolated from p110 α null mice fail to differentiate into adipocytes in response to insulin (Zhao *et al.* 2006).

Important distinctions that may account for our findings relate to the intrinsic responsiveness of hMSCs to insulin signalling and to the nature of the pro-adipogenic stimuli used in our study. Unlike mouse 3T3-L1 cells, hMSCs do not differentiate into adipocytes in response to insulin or insulin-like growth factor 1 (IGF1) alone, instead requiring glucocorticoid to prime the cells to be responsive to pro-adipogenic stimuli (Greenberger 1979, Scavo *et al.* 2004). In light of this, we have used a differentiation media solely consisting of physiological levels of glucocorticoid to enable us to measure adipogenic responses in the absence of other known pro-adipogenic stimuli including insulin and IGF1. It is therefore possible that PI3 kinase may play a lesser role in transducing differentiation signals in the absence of insulin or IGF1 signalling, as seen in our system. Alternatively, the pro-adipogenic effects of imatinib and PIK-75 may be due to dysregulation of Akt as is seen in MSCs isolated from mice lacking the regulatory subunit of PI3 kinase, p85 α . Loss of p85 α results in the down-regulation of p110 α and hyper-activation of Akt, which induces adipogenesis (Wu *et al.* 2011). This result suggests that PIK-75 may promote

adipogenesis by uncoupling the normal regulation of Akt. However, our results indicate that Akt phosphorylation is decreased during PIK-75- or imatinib-mediated differentiation of hMSCs (Supplementary Figure 1, see section on supplementary data given at the end of this article), indicating that Akt signalling is not hyper-activated in response to these drugs under induction conditions. PI3 kinase-independent mechanisms of adipogenic differentiation have also been described. Suppression of MAPK activity in hMSCs has been shown to promote adipogenesis in the absence of insulin and IGF1 signalling (Jaiswal *et al.* 2000). However, we failed to detect a decrease in the activation status of the MAPK substrates Erk1/2 in the presence of imatinib or PIK-75 during differentiation (Supplementary Figure 1), suggesting that suppression of the MAPK pathway was not responsible for the pro-adipogenic effect of these inhibitors. Conversely, p38 MAPK signalling has been proposed as a pro-adipogenic stimuli, as inhibition of p38 has been shown to negate adipogenic differentiation in hMSCs (Aouadi *et al.* 2007). However, imatinib suppresses PDGF-induced p38 activation (Fitter *et al.* 2008), suggesting that activation of p38 MAPK is an unlikely mechanism by which imatinib or PIK-75 promotes adipogenesis.

In cultures treated with PIK-75 or imatinib, the HMW form of adiponectin is the predominant form secreted. Several lines of evidence suggest that the HMW form is the most active with respect to modulating insulin sensitivity (Waki *et al.* 2003, Lara-Castro *et al.* 2006, Wang *et al.* 2006). Consistent with this, analysis of plasma adiponectin complexes in CML patients, before and after 3 months of imatinib therapy, revealed a significant increase in the level of HMW complexes (Fitter *et al.* 2010). In humans, adiponectin levels correlate negatively with insulin resistance and metabolic syndrome, with low adiponectin levels being associated with a higher risk of type 2 diabetes (Li *et al.* 2009). Thiazolidinedione compounds, a class of anti-diabetic drugs that improve systemic insulin sensitivity, are thought to work, at least in part, by promoting the formation of small adipocytes that secrete high levels of HMW adiponectin (Yamauchi *et al.* 2001, Phillips *et al.* 2003, Nawrocki *et al.* 2006).

In summary, we have identified the PI3 kinase pathway downstream of PDGFR as important in mesenchymal cell differentiation. Inhibition of this pathway, using imatinib or PIK-75, promotes adipogenesis and secretion of HMW adiponectin. In light of this, we hypothesise that inhibition of PDGFR signalling in imatinib-treated CML patients results in increased intramedullary adipogenesis and, subsequently, in an increase in circulating adiponectin levels. These increased adiponectin levels may account for the improvements in glucose and lipid metabolism observed

in some imatinib-treated CML patients with concurrent type 2 diabetes.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-12-0003>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was funded by a Translational Research Grant from the Leukaemia and Lymphoma Society (grant number 6040-09) awarded to A C W Z.

Acknowledgements

Imatinib mesylate was kindly provided by Novartis Pharmaceuticals.

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Received in final form 27 March 2012

Accepted 2 April 2012

Made available online as an Accepted Preprint 2 April 2012