Peroxisome-proliferator-activated receptor γ suppresses Wnt/ β -catenin signalling during adipogenesis

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The Wnt/ β -catenin signalling pathway appears to operate to maintain the undifferentiated state of preadipocytes by inhibiting adipogenic gene expression. To define the mechanisms regulating suppression of Wnt/ β -catenin signalling, we analysed the β -catenin expression in response to activation of transcription factors that regulate adipogenesis. The results show an extensive down-regulation of nuclear β -catenin that occurs during the first few days of differentiation of 3T3-L1 preadipocytes and coincides with the induction of the adipogenic transcription factors, C/EBP β (CCAAT-enhancer-binding protein) and PPAR γ (peroxisome-proliferator-activated receptor). To assess the role of each of these factors in this process, we conditionally overexpressed C/EBP β in Swiss mouse fibroblasts using the TET-off system. Abundant expression of C/EBP β alone had minimal effect on β -catenin expression, whereas expression of C/EBP β , in the presence of dexamethasone, induced PPAR γ expression and

caused a measurable decrease in β -catenin. In addition, exposure of cells expressing both C/EBP β and PPAR γ to a potent PPAR γ ligand resulted in an even greater decrease in β -catenin by mechanisms that involve the proteasome. Our studies also suggest a reciprocal relationship between PPAR γ activity and β -catenin expression, since ectopic production of Wnt-1 in preadipocytes blocked the induction of PPAR γ gene expression. Moreover, by suppressing β -catenin expression, ectopic expression of PPAR γ in Wnt-1-expressing preadipocytes rescued the block in adipogenesis after their exposure to the PPAR γ ligand, troglitazone.

Key words: C/EBP β (CCAAT-enhancer-binding protein), dexamethasone, isobutylmethylxanthine, PPAR γ (peroxisome-proliferator-activated receptor) ligand, proteasome.

INTRODUCTION

The differentiation of preadipocytes into adipocytes involves the activation of a cascade of transcription factors that lead to expression of hundreds of adipogenic genes responsible for the mature fat-cell phenotype [1,2]. Initiation of this cascade probably requires suppression of negative effectors responsible for maintaining the preadipocyte phenotype as well as activation of positive factors that induce expression of the adipogenic transcription factors, including the PPARs (peroxisome-proliferator-activated receptors), C/EBPs (CCAAT-enhancer-binding proteins) and SREBP1c (sterol-regulatory-element-binding protein 1c)/ADD-1 (adipocyte differentiation and determination factor 1). For example, C/EBP β and C/EBP δ have been shown to play a role in stimulating the early phase of adipogenesis by inducing expression of PPAR γ and C/EBP α [3–5]. These two factors then act in a co-operative manner to initiate expression of genes associated with terminal differentiation [6]. This process also includes suppression of growth-related events, including induction of the tumour suppressors, p21^{CIP1} and p18^{INK4} as well as inhibition of E2F activity [7,8]. With regard to negative regulation of adipogenesis, Ross et al. [9] showed that Wnt signalling, probably mediated by Wnt10b, is highly active in non-committed preadipocytes, in which it appears to play a role in maintaining the undifferentiated state. Suppression of this Wnt signalling pathway would, therefore, seem to be an important component of the adipogenic programme that facilitates formation of mature fat cells.

Activation of Wnt signalling is mediated by the paracrine and/or autocrine production of members of the Wnt/wg family of secreted polypeptides that bind to select frizzled receptors [10,11].

Frizzleds can signal through pathways that eventually converge on and inhibit the activity of GSK (glycogen synthase kinase)-3 β , which is localized within a multiprotein complex along with APC (adenomatous polyposis coli), axin/conductin and β -catenin [12,13]. GSK-3 β normally phosphorylates β -catenin within this complex and targets it to the proteasome for degradation [14]. Inhibition of GSK-3 β activity through activation of the Wnt pathway leads to accumulation of β -catenin in the cytoplasm and its translocation into the nucleus. Here, in complex with DNA-binding proteins of the LEF (lymphoid enhancer factor)/TCF (T-cell factor) family, it induces expression of a variety of genes, including cyclin D1, *c-myc* and PPAR δ [12,14–18]. In studying the importance of Wnt signalling in adipogenesis, MacDougald and collaborators showed that ectopic expression of Wnt or a GSK-3 β -phosphorylation-defective β -catenin (β catS33Y) or treatment of cells with LiCl that inhibits GSK-3 β activity, all resulted in the inhibition of adipogenesis [9,19]. They showed further that suppression of Wnt signalling in preadipocytes by ectopic expression of either axin or a dominant-negative TCF4 could induce spontaneous differentiation into adipocytes [9].

Although β -catenin, acting as a cofactor for LEF/TCF activity, appears to be an inhibitor of adipogenesis, little is known about the factors regulating its expression or activity during adipogenesis. Consequently, we analysed its expression in response to transcription factors that regulate adipogenesis. Our results show that differentiation of 3T3-L1 preadipocytes is accompanied by an extensive down-regulation of β -catenin, which coincides with terminal differentiation, and involves a PPAR γ -associated targeting of β -catenin to the proteasome.

Abbreviations used: APC, adenomatous polyposis coli; C/EBP, CCAAT-enhancer-binding protein; DEX, dexamethasone; DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; GSK, glycogen synthase kinase; LEF, lymphoid enhancer factor; MIX, 3-isobutyl-1-methylxanthine; PPAR γ , peroxisome-proliferator-activated receptor γ ; RT, reverse transcriptase; TCF, T-cell factor.

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MATERIALS AND METHODS

Plasmids and stable cell lines

The TET-off vector expressing the LAP (liver activated protein) isoform of C/EBP β was constructed by subcloning LAP cDNA into the pBI-G vector (ClonTech, Palo Alto, CA, U.S.A.). This involved blunt-ending a *Bam*HI–*Eco*RI fragment of the pBabe-LAP expression plasmid [20] and subcloning it into a blunt-ended pBI-G vector after digestion with *Not*I. The resulting LAP-pBI-G plasmid was transfected into Swiss mouse 3T3 fibroblasts constitutively expressing the TET-off activator protein (ClonTech) along with a puromycin selection plasmid (pBabe-puro). Colonies of cells resistant to 2 μ g/ml puromycin were selected and analysed for expression of the pBI-G vector on the basis of tetracycline-responsive β -galactosidase production. The initial selection gave rise to several non-homogeneous colonies since only 10–20% of the cells expressed β -galactosidase activity. Therefore one of these colonies was subjected to serial dilution single-cell cloning. A colony (LAP-A cells) was selected in which almost the entire population of cells expressed β -galactosidase activity in a tetracycline-responsive manner. The Bosc23 packaging cells, pBabe-puro and pBabe-PPAR γ retroviral expression vectors were kindly provided by Dr B. Spiegelman (Dana Farber Institute, Harvard Medical School, Boston, MA, U.S.A.). The pBabe-Wnt1 expression vector was a gift from Dr D. Seldin (Boston Medical Center, Boston, MA, U.S.A.). Transfection of Bosc23 and subsequent infection of target cells were performed as described in [21,22]. Target cells infected for 6–10 days were selected in a medium containing 1.5–2 μ g/ml puromycin or 50–150 μ g/ml hygromycin B.

Cell culture

Stocks of 3T3-L1 preadipocytes [23] were maintained in DMEM (Dulbecco's modified Eagle's medium) containing 25 mM D-glucose and supplemented with 10% (v/v) donor calf serum. For experiments, cells were grown in DMEM containing 10% (v/v) foetal bovine serum (FBS). Induction of differentiation was achieved by treatment of 2 days post-confluent cells with DEX (dexamethasone, 1 μ M), MIX (3-isobutyl-1-methylxanthine, 0.5 mM) and insulin (1.67 μ M). The Swiss-LAP-A cells and a variety of engineered 3T3-L1 cells were differentiated by the same method as for 3T3-L1 preadipocytes and maintained in the presence or absence of 5 μ M troglitazone (Parke Davis/Warner Lambert, Ann Arbor, MI, U.S.A.) or 1 μ M specific PPAR γ agonist (GW7845) obtained from GlaxoSmithKline (Research Triangle Park, NC, U.S.A.). To block PPAR γ activity, cells were exposed to a selective PPAR γ antagonist (T0070907) as described previously by Lee et al. [24] and obtained from AdipoGenix (Boston, MA, U.S.A.). Inhibition of the proteasomal degradation of proteins was achieved by treating cells with 12.5 μ M MG132 (Calbiochem). Oil Red O staining was performed following the procedure described previously [4]. The cells were then photographed using phase-contrast microscopy.

Preparation of whole and nuclear cell extracts

Cultured cells were rinsed with phosphate-buffered saline (140 mM NaCl/2.7 mM KCl/1.5 mM KH₂PO₄/8.1 mM Na₂HPO₄, pH 7.4). For total extracts, cells were harvested in ice-cold buffer containing 50 mM Tris (pH 7.4), 100 mM NaCl, 1% (w/v) sodium deoxycholate, 4% (v/v) Nonidet P40, 0.4% (w/v) SDS, 5 μ M aprotinin and 50 μ M leupeptin. Lysates were incubated on ice for 15 min and then centrifuged at 13 793 g. For nuclear

protein extracts, cells were lysed in lysis buffer [10 mM Tris (pH 7.6)/10 mM NaCl/3 mM MgCl₂/0.5% Nonidet P40]. After a low-speed centrifugation, nuclei were resuspended in nuclear extraction buffer [20 mM Hepes, pH 7.9/350 mM NaCl/1.5 mM MgCl₂/0.2 mM EDTA, pH 8.0/25% (v/v) glycerol], then incubated on ice for 20 min and centrifuged at full speed (13 793 g) at 4 °C. The resulting supernatants were stored at –80 °C and protein concentrations were determined using the Bicinchoninic acid kit (Pierce, Rockford, IL, U.S.A.).

Gel electrophoresis and immunoblotting

Proteins were analysed by SDS/PAGE (10% gel) and transferred on to PVDF membranes (NEN Life Sciences, Boston, MA, U.S.A.) in 25 mM Tris, 192 mM glycine and 10% (v/v) methanol. After transfer, membranes were blocked with 5% (w/v) non-fat-dry milk in PBS/0.1% Tween 20 and probed with the antibodies specified below. Horseradish-peroxidase-conjugated secondary antibodies (Sigma) and enhanced chemiluminescence (ECL[®]) substrate kit (PerkinElmer Life Science) were used for detection of specific proteins.

Antibodies

Monoclonal antibodies against PPAR γ , β -catenin and cyclin D1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), BD Transduction Laboratories (San Diego, CA, U.S.A.) and BD Pharmingen (San Diego, CA, U.S.A.) respectively. Polyclonal anti-C/EBP β and -C/EBP α antibodies were purchased from Santa Cruz. Anti-aP2 serum was kindly provided by Dr D. Bernlohr (University of Minnesota, MN, U.S.A.). The perilipin antibody was a gift from Dr A. Greenberg (New England Medical Center, Tufts University, Boston, MA, U.S.A.).

Analysis of RNA

Total RNA was extracted with TRIzol[®] (Invitrogen) according to the manufacturer's instructions. After quantification, mRNA was assayed in equivalent amounts of total RNA by reverse transcriptase (RT)-PCR as described previously [25]. Primers corresponding to β -catenin (5'-GGATTCTGGAATCC-ATTCTGG-3', 5'-TCTGAGCCCTAGTCATTGCAT-3'), PPAR γ (5'-GAGCATGGTGCCTTCGCTGAT-3', 5'-CAACCATTGGGTCAGCTCTTG-3'), aP2 (5'-TACATGAAAGAAGTGGGAGTG-3', 5'-GGTGATTTCATCGAATTCCAC-3') and glyceraldehyde-3-phosphate dehydrogenase (5'-ATCACCATTCTCCAGGAGCGAG-3', 5'-GTTGTTCATGGATGACCTTGCC-3') mRNAs were synthesized by Integrated DNA Technologies (Coralville, IA, U.S.A.), based on the published sequences in the gene banks. All PCRs were performed in the linear range of cycle number for each set of primers, and the corresponding products were analysed by 1.5% (w/v) agarose gel electrophoresis and revealed with ethidium bromide.

RESULTS

To determine whether β -catenin was a valid marker of the dramatic changes in Wnt signalling during adipogenesis, we measured its abundance at intervals after exposure of 3T3-L1 preadipocytes to MIX, DEX and insulin in the presence of FBS. The distribution of β -catenin present in different cellular fractions was first analysed. The Western-blot analysis presented in Figure 1(A) revealed abundant expression of β -catenin in confluent

Figure 1 Decrease in β -catenin expression during the differentiation of 3T3-L1 preadipocytes

3T3-L1 preadipocytes were induced to differentiate as described in the Materials and methods section. At the indicated times, total cellular (A) or nuclear extracts (B) were collected and subjected to Western-blot analysis for expression of β -catenin, cyclin D1 and PPAR γ . (C) Total RNA was harvested and analysed by RT-PCR for β -catenin, PPAR γ , aP2 and glyceraldehyde-3-phosphate dehydrogenase mRNA expression as described in the Materials and methods section.

preadipocytes, which decreased extensively after induction of terminal differentiation. In fact, the down-regulation process appeared to be initiated rapidly, since there was a measurable decrease in total cellular β -catenin levels during the first 1–2 days, a time when the preadipocytes were progressing through the cell cycle (clonal expansion), as indicated by induction of cyclin D1 expression. This decrease in total cellular β -catenin levels also occurred in both the membranous and cytoplasmic fractions of the differentiating preadipocytes (results not shown). Since activation of the canonical Wnt pathway involves translocation of β -catenin into the nucleus, we measured the accumulation of β -catenin in the nucleus at different times during adipogenesis. The results shown in Figure 1(B) demonstrate detectable amounts of β -catenin in the nucleus of confluent preadipocytes. It is interesting that there was a transient increase in the translocation of β -catenin into the nucleus 8 h after induction of clonal expansion in these confluent preadipocytes, which preceded expression of cyclin D1 occurring at 16 h (Figure 1B). As the preadipocytes started to enter the terminal phase of adipogenesis, there was an extensive decrease in the abundance of β -catenin and cyclin D1 coinciding

Figure 2 Down-regulation of β -catenin expression induced by MIX in a manner associated with the ability of C/EBP β to induce differentiation

(A) 3T3-L1 preadipocytes were exposed at confluence to different combinations of the adipogenic inducers MIX (M), DEX (D) and insulin (I) for 2 days in a medium containing 10% FBS. Total extracts were prepared from the cells on day 2 and subjected to Western-blot analysis for β -catenin expression. (B) 3T3-L1-vector cells were induced to differentiate in the presence of insulin and DEX with MIX (MID) or without MIX (ID) for 2 days and then maintained in the medium containing only insulin and FBS for an additional 6 days. 3T3-L1 cells ectopically expressing a dominant-negative isoform of C/EBP β (LIP cells) were also induced to differentiate with MID for 6 days as described for the 3T3-L1-vector cells. Nuclear extracts were prepared from each set of cells harvested at the indicated times. Samples were subjected to Western-blot analysis of β -catenin and PPAR γ expression.

with accumulation of PPAR γ in the nucleus. Furthermore, the down-regulation of β -catenin appears to be regulated primarily at a post-transcriptional step, since the level of expression of the corresponding mRNA measured by semi-quantitative RT-PCR (Figure 1C) and Northern-blot analysis (results not shown) remained essentially constant throughout the differentiation process.

Recent studies by Bennett et al. [19] suggested that exposure to MIX or increase in cAMP levels was the principal agent regulating the suppression of Wnt 10b mRNA expression observed during adipogenesis in 3T3-L1 cells. We questioned, therefore, whether this MIX-associated decrease in Wnt10b mRNA also resulted in the extensive down-regulation of β -catenin observed in the studies presented in Figure 1. To address this question, we exposed confluent 3T3-L1 preadipocytes to various combinations of the adipogenic inducers, DEX, MIX and insulin for 2 days and analysed the abundance of β -catenin in these cells. The Western blot shown in Figure 2(A) demonstrates that MIX, whether added alone or in combination with DEX and insulin, stimulated the down-regulation of β -catenin during the first 2 days of the differentiation process. In fact, the extensive down-regulation of β -catenin during terminal adipogenesis requires MIX, since exposure of 3T3-L1 preadipocytes to insulin, DEX and FBS was not sufficient to inhibit β -catenin expression or induce differentiation as shown by the low levels in PPAR γ expression (Figure 2B). Since one of the effects of MIX is to induce C/EBP β expression during the first few hours of differentiation [26], we questioned whether C/EBP β might be involved in regulating the inhibition of β -catenin expression. To address this question, we retrovirally overexpressed a dominant-negative isoform of C/EBP β (referred to as LIP) in 3T3-L1 preadipocytes. Our previous studies [20] have shown that LIP

Figure 3 Suppression of β -catenin expression under conditions that enhance PPAR γ activity by conditional ectopic expression of C/EBP β in Swiss mouse fibroblasts

The Swiss-C/EBP β TET-off cells (LAP-A cells) were grown to confluence in the absence of tetracycline to induce C/EBP β expression. Some cultures were exposed to 1 μ g/ml tetracycline (+) to prevent the induction of C/EBP β . At 2 days post-confluence, cells were treated with various combinations of DEX (D), MIX (M) and insulin (I) in a medium containing 10% FBS for 2 days. Cells were cultured for an additional 4 days in the presence of FBS and in the absence (A) or presence (B) of a PPAR γ ligand (1 μ M GW7845) and then harvested for preparation of nuclear extracts as described in the Materials and methods section. Aliquots corresponding to equivalent amounts of nuclear proteins were then subjected to Western-blot analysis of β -catenin, PPAR γ , C/EBP β and the adipose-selective fatty acid-binding protein aP2.

blocks the ability of DEX, MIX, insulin and FBS to induce adipogenesis by preventing C/EBP β from activating PPAR γ 2 expression. To determine whether MIX could down-regulate β -catenin in the absence of C/EBP β activity, cells expressing either LIP or a vector control were induced for differentiation following the established method, and the abundance of β -catenin in the nucleus was measured by Western-blot analysis. Figure 2(B) shows an extensive reduction in nuclear β -catenin levels in the control cells, which coincides with the differentiation process as indicated by high expression of PPAR γ . Exposure of the LIP cells to MIX along with DEX and insulin did not induce differentiation; more importantly, it did not facilitate the down-regulation of β -catenin, as observed in the control cells (Figure 2B). These results suggest that the MIX-associated decrease in β -catenin during adipogenesis is related to the ability of C/EBP β to induce differentiation.

To gain further insight into the possible role of C/EBP β in regulating β -catenin expression, we adopted a gain-of-function approach in which we conditionally expressed C/EBP β in Swiss mouse fibroblasts using the TET-off expression system. The resulting cell line, referred to as LAP-A cells, expressed the 34 kDa isoform of C/EBP β (LAP) when cultured in the absence of tetracycline. Our previous studies demonstrated that conditional expression of LAP in NIH-3T3 fibroblasts in the presence of glucocorticoids induced PPAR γ expression and facilitated conversion of these fibroblasts into adipocytes after exposure to PPAR γ ligands [3]. Consequently, we considered it important to assess the role of C/EBP β in regulating β -catenin under conditions that also result in PPAR γ expression and adipogenesis. Culture of the LAP-A cells in the absence of tetracycline resulted in a significant induction of C/EBP β when compared with cells exposed to 1 μ g/ml tetracycline (Figure 3A, cf. lanes 1–8 with lane 9). Inhibition of β -catenin expression, however, only occurred under conditions (exposure to DEX and insulin) that also induced PPAR γ 2 expression (Figure 3A, lanes 6 and 8). Suppression of the ectopic C/EBP β by maintaining the cells in tetracycline prevented the induction of PPAR γ 2 and, consequently, resulted in abundant levels of β -catenin in the nucleus even though the cells were exposed to DEX, MIX and insulin (Figure 3A, lane 9). Enhancement of

Figure 4 Inverse relationship between PPAR γ activity and β -catenin expression

(A) The Swiss-C/EBP β (LAP-A) cells were cultured in the absence of tetracycline for 2 days before reaching confluence. The confluent cells were then exposed to DEX, MIX and insulin for 2 days. At this stage, the medium was changed and the cells were treated in the presence of 10% FBS with increasing concentrations of the PPAR γ ligand, GW7845, as indicated. Two days later, nuclear extracts were prepared and subjected to Western-blot analysis for expression of β -catenin, PPAR γ and C/EBP β . (B) Confluent 3T3-L1 preadipocytes were induced to differentiate as described in the Materials and methods section, and after 1 day they were exposed to the indicated doses of the PPAR γ antagonist, T0070907. On day 5 of differentiation, cells were harvested for preparation of nuclear extracts and equivalent amounts of nuclear proteins were subjected to Western-blot analysis of β -catenin, PPAR γ and C/EBP β expression.

PPAR γ activity by exposure of the cells to a potent PPAR γ ligand (GW7845) in cells that were also treated with DEX and insulin resulted in an extensive reduction in nuclear β -catenin levels (cf. lanes 6 and 8 in Figure 3B with the corresponding lanes in Figure 3A).

To examine further this apparent role of PPAR γ in suppressing β -catenin expression, we adopted two approaches that involved either enhancing or inhibiting PPAR γ activity. In the former approach, LAP-A cells were grown to confluence in the absence of tetracycline to induce C/EBP β expression. At this stage, cells were exposed to DEX, MIX and insulin for 2 days and the medium was then replaced with DMEM containing FBS and increasing concentrations of the PPAR γ ligand, GW7845. Three days later, nuclear extracts were prepared and subjected to Western-blot analysis. The data presented in Figure 4(A) demonstrate that the decrease in β -catenin abundance in the nuclei of these Swiss fibroblasts occurs in a dose-dependent manner in PPAR γ ligand (GW7845). Alternatively, we also chose to attenuate PPAR γ activity by exposing 3T3-L1 preadipocytes to increasing doses of a selective PPAR γ antagonist (T0070907/AGX0104) during the terminal phase of differentiation (days 1–5). On day 5, cells were harvested and nuclear proteins were analysed by Western-blot analysis. Figure 4(B) demonstrates that the PPAR γ antagonist inhibits adipogenesis as revealed by a dose-dependent decrease in C/EBP α expression and, consequently, it also prevents the differentiation-associated decrease in nuclear β -catenin.

Recent studies have shown that ectopic expression of Wnt 1 or Wnt10b in preadipocytes blocks adipogenesis, and that this inhibition can be partially overcome by ectopic expression of

Figure 5 Overexpression of PPAR γ eliminates inhibition of adipogenesis by Wnt-1 signalling

(A) Confluent 3T3-L1 preadipocytes expressing Wnt-1 or a control vector were induced to differentiate with MIX, DEX and insulin in the presence or absence of troglitazone (Trog; 10 μ M). On day 10, cells were fixed and stained for neutral lipids with Oil Red O. (B) Nuclear extracts were prepared from the Wnt-1 (W) and control cells (C) at the indicated times and subjected to Western-blot analysis for PPAR γ , β -catenin and cyclin D1 expressions. (C) 3T3-L1 preadipocytes expressing Wnt-1 alone (Wnt) or in combination with PPAR γ (Wnt-P γ) were exposed to MIX, DEX and insulin in the presence or absence of troglitazone (5 μ M). After an additional 5 days of culture in FBS, and insulin with or without troglitazone, nuclear extracts were prepared and subjected to Western-blot analysis for β -catenin, C/EBP α and aP2 expressions.

PPAR γ . We have, therefore, employed this system to examine the relationship between Wnt/ β -catenin signalling and PPAR γ expression during adipogenesis. In agreement with the results of Ross et al. [9], we found that retroviral expression of Wnt-1 in 3T3-L1 preadipocytes prevented their differentiation into adipocytes, as indicated by the absence of Oil Red O-positive staining of triacylglycerols (Figure 5A). Furthermore, the Wnt-expressing cells (W) failed to induce PPAR γ and did not down-regulate β -catenin or cyclin D1 expression in response to DEX,

Figure 6 Involvement of proteasomes in PPAR γ -associated degradation of β -catenin

Confluent 3T3-L1 preadipocytes were exposed to DEX and insulin for 48 h. On day 3, cells were treated with or without troglitazone (TROG) in the presence or absence of the proteasome inhibitor MG132 (12.5 μ M). Total cell extracts were prepared at the indicated times and subjected to Western-blot analysis for β -catenin expression.

MIX and insulin (Figure 5B). To delineate further the role of PPAR γ in suppressing Wnt signalling during adipogenesis, we ectopically expressed PPAR γ in Wnt-expressing preadipocytes (Figure 5C). The results demonstrate that constitutive expression of PPAR γ induced adipogenesis in the Wnt-1 cells after exposure to the adipogenic inducers, as indicated by an increase in the expression of C/EBP α and aP2. This activity of the ectopic PPAR γ is enhanced manifold by treatment of the Wnt-P γ cells with troglitazone (a PPAR γ ligand), DEX, MIX and insulin. More importantly, the down-regulation of nuclear β -catenin occurred only after exposure of the cells to troglitazone (Figure 5C, lane 6).

The data presented in Figure 1(C) suggest that PPAR γ regulates the abundance of β -catenin by mechanisms that operate predominantly at a post-transcriptional level. The abundance of β -catenin is tightly regulated by the ubiquitin/proteasome system that constitutes a key component of the Wnt signalling pathway. To determine whether the PPAR γ -associated down-regulation of β -catenin involves the proteasome system, we cultured 3T3-L1 preadipocytes with DEX and insulin for 2 days to facilitate the expression of PPAR γ 1 [20]. On day 5, cells were treated with or without troglitazone and/or the proteasome inhibitor MG132. Results presented in Figure 6 show that the down-regulation of β -catenin induced by the activation of PPAR γ with troglitazone is inhibited by exposure of the cells to MG132 (Figure 6, lanes 6–9).

DISCUSSION

Recent studies have suggested that Wnt signalling plays a role in regulating the fate of mesenchymal stem cells [9]. For the adipogenic lineage, Wnt-10b signalling was shown to be active in preadipocytes and down-regulated during their differentiation into adipocytes [19]. To identify the mechanisms responsible for suppressing the canonical Wnt signalling pathway during adipogenesis, we investigated the relationship between PPAR γ activity and the expression and subcellular localization of β -catenin. Our results have shown that adipogenesis in 3T3-L1 preadipocytes is accompanied by an extensive down-regulation of total cellular β -catenin content as well as a decrease in its accumulation in the nuclei of these cells. This process coincides with the induction of adipogenic transcription factors, C/EBP β and PPAR γ , during the switch-over from clonal expansion to terminal differentiation. By employing systems that render 3T3-L1 preadipocytes and Swiss 3T3 fibroblasts dependent on an exogenous PPAR γ ligand for their differentiation into adipocytes, we demonstrated that activation of PPAR γ is involved in

Figure 7 Molecular pathways regulating Wnt/ β -catenin signalling during adipogenesis

Wnt/ β -catenin signalling is attenuated during the early phase of adipogenesis by exposure of 3T3-L1 preadipocytes to DEX, MIX and insulin allowing expression of PPAR γ . Stimulation of PPAR γ activity results in an extensive down-regulation of β -catenin to ensure terminal differentiation into mature adipocytes.

suppressing Wnt signalling by mechanisms that include targeting β -catenin to the proteasome rather than its accumulation in the nucleus. Our studies also suggest a reciprocal relationship between PPAR γ activity and β -catenin expression, since ectopic production of Wnt-1 in preadipocytes blocked the induction of PPAR γ gene expression. Moreover, ectopic expression of PPAR γ in Wnt-1 expressing preadipocytes rescued the block in adipogenesis by suppressing β -catenin expression, after their exposure to troglitazone. On the basis of these observations, we propose a model in which an event initiated in some preadipocytes suppresses Wnt signalling at the early phase of adipogenesis to a level that permits induction of PPAR γ . Once PPAR γ is produced and activated, it can down-regulate β -catenin levels in the nucleus further to ensure complete terminal differentiation into mature adipocytes (see Figure 7).

The mechanisms involved in regulating this balance between nuclear β -catenin activity and PPAR γ expression are, as yet, unknown. It is probable that Wnt signalling contributes to suppressing PPAR γ expression and adipogenesis in mesenchymal stem cells and preadipocytes, as suggested previously [9]. Candidate proteins involved in this process include known targets of the Wnt signalling pathway, such as cyclin D1 and *c-myc* [16,17,28]. Whereas the role of cyclin D1 in adipogenesis is still unknown, the present study has shown a reciprocal relationship between cyclin D1 expression and adipogenesis (Figure 1). Studies with *c-myc* have shown that sustained expression of *c-myc* blocks adipogenesis at least in part by preventing expression of C/EBP α [29]. Future studies will have to address the potential role of cyclin D1 and other targets of the Wnt signalling pathway in the negative regulation of adipogenesis.

Suppression of Wnt/ β -catenin signalling by the activation of PPAR γ may involve various mechanisms. The most likely scenario is that it induces expression of proteins that lead to a decrease in the abundance of β -catenin in the nucleus. One possibility is that it induces a cascade of events that result in suppression of the Wnt signalling pathway. For example, expression or activity of the Wnt protein itself could be blocked due to suppression of the Wnt-10b gene or by induction of soluble factors that are known to modulate the binding of Wnt to the frizzled receptors [19,30]. Similarly, production of other components in the Wnt signalling pathway (including frizzled) may be down-regulated in response to PPAR γ activity. Additionally, PPAR γ may induce proteins that act at the terminal steps in the Wnt signalling pathway, which involve translocation of β -catenin into the nucleus. The data presented in Figure 6 strongly suggest that the PPAR γ -mediated down-regulation of β -catenin requires a functional proteasome. Consequently, the mechanisms responsible for this effect may involve phosphorylation of β -catenin through kinases such as GSK-3 β or may involve inhibition of phosphatases. In fact, Spiegelman and collaborators [8] have shown that the PPAR γ -

associated arrest of cell growth involves a down-regulation of the protein phosphatase PP2A. Interestingly, other investigators have recently suggested that the regulated degradation of β -catenin by the tumour-suppressor p53 may involve mechanisms that are both dependent on and independent of GSK-3 β activity [31–33]. With regard to the latter, studies have demonstrated a role for Siah-1 in mediating the p53-associated down-regulation of β -catenin through a pathway independent of both GSK-3 β -mediated phosphorylation and β -TrCP proteolysis [32,33]. In fact, expression of Siah-1 appears to be induced by p53 in co-operation with the cyclin-dependent kinase inhibitor, p21^{CIP1} [34,35]. It is conceivable, therefore, that PPAR γ may regulate β -catenin expression during terminal adipogenesis through a similar pathway, which may include a PPAR γ -associated induction of Siah-1 expression. In this regard, it is noteworthy to mention that PPAR γ does induce p21^{CIP1} during the switch-over from growth to differentiation in preadipocytes [7].

What is the role of PPAR γ in down-regulating β -catenin during adipogenesis? As suggested by others [9], the Wnt/ β -catenin pathway appears to be active in preadipocytes to maintain the undifferentiated state. Suppression of this pathway by PPAR γ seems to be crucial for complete differentiation of preadipocytes into mature adipocytes. In addition, it is possible that β -catenin-mediated transcription is also contributing to the proliferation of preadipocytes during the clonal expansion phase of adipogenesis. In fact, Figure 1 shows a transient accumulation of β -catenin in the nucleus following exposure of preadipocytes to DEX, MIX and insulin that precedes the induction of cyclin D1 during clonal expansion. In fact, cyclin D1 is a Wnt/ β -catenin target gene and recent studies have demonstrated a role for β -catenin in cell growth [36]. It is conceivable, therefore, that β -catenin signalling participates in the proliferation of preadipocytes before their differentiation. Whether Wnt is the extracellular signal regulating this process is unlikely, but other effectors such as insulin/IGF-1, which do regulate clonal expansion, have been shown to regulate β -catenin through an Akt-associated modulation of GSK-3 β activity [37].

An additional role for PPAR γ , therefore, is to down-regulate β -catenin as a component of the growth arrest that precedes terminal differentiation. In this regard, it is possible that the tumour-suppressing activity of PPAR γ observed in various carcinomas may also involve inhibition of β -catenin signalling [38]. In this regard, a recent study shows that PPAR γ can suppress β -catenin levels and colon carcinogenesis, but only in colon cancer cells that contain a functional APC molecule [39]. This finding may explain why PPAR γ does not suppress the growth of tumours in the colon of the C57BL/6J-APC^{Min/+} mouse [40,41]. In conclusion, further analysis of the role of PPAR γ in regulating β -catenin signalling should provide insight into the processes controlling growth and differentiation in many cell types.

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