Wnt3a disrupts GR-TEAD4-PPARγ2 positive circuits and cytoskeletal rearrangement in a β-catenin-dependent manner during early adipogenesis

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Abstract

Adipogenesis is a process which induces or represses many genes in a way to drive irreversible changes of cell phenotypes; lipid accumulation, round cell-shape, secreting many adipokines. As a master transcription factor (TF), PPARγ2 induces several target genes to orchestrate these adipogenic changes. Thus induction of Pparg2 gene is tightly regulated by many adipogenic and also anti-adipogenic factors. Four hours after the treatment of adipogenic hormones, more than fifteen TFs including glucocorticoid receptor (GR), C/EBPβ and AP-1 cooperatively bind the promoter of Pparg2 gene covering 400 bps, termed “hotspot”. In this study, we show that TEA domain family transcription factor (TEAD)4 reinforces occupancy of both GR and C/EBPβ on the hotspot of Pparg2 during early adipogenesis. Our findings that TEAD4 requires GR for its expression and for the ability to bind its own promoter and the hotspot region of Pparg2 gene indicate that GR is a common component of two positive circuits, which regulates the expression of both Tead4 and Pparg2. Wnt3a disrupts these mutually related positive circuits by limiting the nuclear location of GR in a β-catenin dependent manner. The antagonistic effects of β-catenin extend to cytoskeletal remodeling during the early phase of adipogenesis. GR is necessary for the rearrangements of both cytoskeleton and chromatin of Pparg2, whereas Wnt3a inhibits both processes in a β-catenin-dependent manner. Our results suggest that hotspot formation during early adipogenesis is related to cytoskeletal remodeling, which is regulated by the antagonistic action of GR and β-catenin, and that Wnt3a reinforces β-catenin function.

Introduction

The mouse 3T3-L1 cells have been widely used as an in vitro model system to investigate molecular mechanisms of adipogenesis1. The 3T3-L1 cells can be differentiated into mature adipocytes by two-day exposure to 3-isobutyl-1-methylxanthine (IBMX/M), dexamethasone (Dex/D), and insulin (I)2–4. Recently, genome wide analyses of DNase I hypersensitive regions revealed that after 4 h MDI treatment, approximately three times more DNA regions became accessible5. ChIP-seq analyses revealed that during such dynamic chromatin remodeling (within 4 h after MDI treatment), hundreds of DNA sequences covering 400 bps, termed “hotspots”, including the promoter of Pparg2, are cooperatively occupied with at least five TFs including C/EBPβ/δ, GR (also known as NR3C1), STAT5A, and other TFs. These early TFs recruit coregulators to induce chromatin remodeling and form early enhanceosomes at the hotspots5,6. Hotspots are enriched with enhancer histone marks, namely, H3K4me1, H3K4me2, and H3K27ac, suggesting that hotspots are key enhancers7–9. Similarly, intracellular and extracellular structures were also remodeled during adipogenesis when
preadipocytes changed to round and lipid-laden adipocytes. Within 24 h, MDI treatment rearranged the actin cytoskeleton from stress fibers to cortical structures in preadipocytes. Thus, MDI dramatically rearranged both chromatin and cytoskeleton in this short period.

Canonical Wnt signaling represses adipogenesis, but enhances osteogenesis of mesenchymal stem cells. 3T3-L1 preadipocytes secrete Wnt10b, which maintains cytoskeleton integrity by interacting with membrane bound cadherins and α-catenin, which are connected to actin fibers.

Previous studies on the anti-adipogenic effects of Wnt/β-catenin signaling focused on the inhibition of PPARY activity by β-catenin. However, how Wnt signaling prevents adipogenic hormones from de-repressing and activating the transcription of Pparg2 during early adipogenesis (within 48 h after MDI treatment) remains unclear. Here, we showed for the first time that canonical Wnt signaling inhibits not only hotspot formation of Pparg, but also cytoskeletal rearrangement in a β-catenin-dependent manner. These two events are regulated by the antagonistic actions of GR and β-catenin, and Wnt3a reinforces β-catenin function.

Materials and methods

Materials and antibodies

Insulin, dexamethasone, 3-isobutyl-1-methylxanthine, Hoechst33342, Oil Red-O and RU486 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant mouse Wnt3a was purchased from R&D Systems (Minneapolis, MN, USA). Wnt3a-conditioned medium was obtained from confluent Wnt3a-expressing L929 cells. Anti-PPARY (E-8), C/EBPα (14AA), C/EBPβ (H-7), GR (M-20, G-5), p300 (C-20), CBP (A-22), KLF4 (H-180), c-Jun (H-79), and β-catenin (H-102) antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-TAZ (M2-616) antibody was purchased from BD Biosciences (San Jose, CA, USA), anti-lamin A/C and anti-STAT5 antibodies from Cell Signaling Technology (Danvers, MA, USA), anti-FLAG (5A8E5), anti-Myc (2G8D5), and anti-HA (5E11D8) antibodies from GenScript (Piscataway, NJ, USA), anti-TEAD4 and anti-H3 antibodies from Abcam (Cambridge, MA, USA), anti-14-3-3-CG31-2B6) from Millipore (Billerica, MA, USA), and anti-β-actin (AC-15) from Sigma-Aldrich, fluorescent phalloidin conjugate from Invitrogen (Waltham, MA, USA). cDNAs of HA-GR or Myc-Tead4 were subcloned into the pLenti-vector. pBABE-3× FLAG-C/EBPβ and pCMV-3× FLAG vector have been described previously. Primer sequences for qRT-PCR and ChIP-qPCR are summarized in Supplementary Table S1 and S2, respectively.

Cells and adipocyte differentiation

3T3-L1 mouse preadipocytes (ATCC, Rockville, MD, USA) and mouse NIH-3T3 cells were maintained in DMEM containing 10% (v/v) bovine serum as described. AmphoPack™ 293 cells (BD Biosciences, San Jose, CA, USA), HEK-293T cells, C3H10T1/2 cells (ATCC, Rockville, MD, USA) and Wnt3a-L929 cells were maintained in DMEM containing 10% (v/v) FBS. For adipogenesis, post-confluent 3T3-L1 cells and C3H10T1/2 cells were exposed to the adipogenic cocktail (MDI) containing 2 μM D, 0.5 mM M, and 5 μg/ml 1 in DMEM supplemented with 10% FBS as described previously. Lipid droplets were stained using Oil Red-O as described previously.

Generation of stable cell lines using retrovirus or lentivirus infection

C/β-NIH cells and EV-NIH cells were generated by infecting mouse NIH-3T3 cells with retroviruses encoding FLAG-tagged C/EBPβ and empty vector, respectively, using the pBABE retroviral vector system and HEK293-based packaging cells (AmphoPack™ 293 cell line). GR-L1, Cβ-L1, or Tead4-L1 cells were generated by infecting mouse 3T3-L1 preadipocytes with lentiviruses expressing HA-tagged GR, FLAG-tagged C/EBPβ, or Myc-tagged TEAD4, respectively. GR/Cβ-L1 or EV-EV-L1 cells were generated by infecting Cβ-L1 and EV-L1 cells with lentiviruses encoding HA-tagged GR or empty vector, respectively, using the pWZL retroviral vector system. shTead4-L1, shβ-CAT-L1, shGR-L1, and shCtrl-L1 cells were generated by infecting mouse 3T3-L1 cells with lentivirus encoding shRNA against mouse Tead4 (5′-GCT GAAACACTTACCCGAGAA-3′), mouse Ctnnb1 (5′-CCTTCTTCCAGGAA-3′), mouse Ctnnb1 (5′-CCTGCTTCCCTACCATGAA-3′), mouse Nr3c1 (encoding GR) (5′-TGGACTTCTGAGCTTGGTAA-3′), and control (5′-AGGATCTGAGCTTGGTAA-3′), respectively, using the pLKO.1 lentiviral vector system (Addgene, Cambridge, MA, USA).

Chromatin immunoprecipitation (ChIP) and formaldehyde-assisted isolation of regulatory elements (FAIRE)

ChIP analyses were performed as described previously. FAIRE analyses were performed using ChIP..
lysates (30 μg chromatin) as described previously. Briefly, sonicated chromatin lysates were phase-separated by two rounds of phenol/chloroform extraction. Nucleosome-free DNA in the upper aqueous phase was obtained using ethanol precipitation. DNA was further treated with 10 μg RNase A and 20 μg protease K, and extracted using the QIAquick PCR purification kit (QIAGEN, Chatsworth, CA, USA). The isolated genomic DNA was used for FAIRE-qPCR. The Ct value of a target gene in the isolated DNA sample of a ChIP or FAIRE experiment was normalized to the Ct value of the target gene in the input DNA. The percentage of input indicates the value of 100 × 2^−ΔCt.

**Western blot analyses, nuclear extraction, and reporter assay**

Western blot analyses were performed as described previously. To obtain nuclear extracts, the cells were washed twice with ice-cold phosphate buffered saline (PBS), harvested, and then lysed with hypotonic buffer (20 mM Tris- HCl (pH 8.0), 10 mM NaCl, 0.2% NP-40, 10 mM β-glycerophosphate, 10 mM NaF, 1 mM Na3VO4, and protease inhibitors) and incubated for 10 min on ice. The supernatant (cytosol extracts) was removed and the nuclear pellet was washed with hypotonic buffer and lysed with NETN buffer (20 mM Tris-HCl (pH 8.0), 140 mM NaCl, 0.5% NP-40, 10 mM β-glycerophosphate, 10 mM NaF, 1 mM Na3VO4, and protease inhibitors), followed by 10 cycles of sonication (1 cycle; 30 s on, 30 s off). Nuclear extracts (supernatant) were obtained by centrifugation at 13,000 × g for 10 min at 4°C. The reporter plasmids, C/EBP-Luc and GRE-Luc, contained the luciferase gene under the regulation of three copies of C/EBP binding sequences (GGTGCAGCAAG) and one copy of glucocorticoid responsive element (GRE) (AGAAA-CACTGTGGTCTC), respectively. Reporter assays were performed using Lipofectamine reagent as described previously. The pRL-TK plasmid encoding renilla luciferase was cotransfected for normalizing transfection efficiency.

**Immunofluorescence of F-actin**

The cellular F-actin was stained with fluorescent phaloidin conjugates (25 μM) for 40 min at room temperature prior to Hoechst staining. The stained cells were observed under a Zeiss LSM510 inverted confocal microscope according to the manufacturer’s instructions. F-actin structures in individual cells were categorized into three groups: S (stress) fiber, where F-actin stress fibers were observed both in the nucleus and cytoplasm; T (transition state) fiber, where F-actin stress fibers were observed in the cytoplasm but not in the nucleus; C (cortical structure), where F-actin stress fibers were observed neither in the nucleus nor in the cytoplasm, but were observed as cortical structures near the cellular membrane. Cells (13–48) in each treatment were observed and categorized into three groups.

**Statistical analysis**

All quantitative measurements were performed in at least three independent experiments. Two-tailed unpaired Student’s t-tests were used to compare the data between controls and indicated experimental groups. *p-values < 0.05; **p-values < 0.01; ***p-values < 0.001 were considered statistically significant.

**Results**

**Wnt3a inhibits early induction of Pparg2**

We treated 3T3-L1 preadipocytes with adipogenic hormones (MDI) in the presence of recombinant Wnt3a (Fig. 1a). MDI induced the protein levels of both PPARγ and C/EBPα, but reduced β-catenin proteins. Two ng/ml of Wnt3a was sufficient to block the induction of both PPARγ and C/EBPα proteins and lipid accumulation but increased β-catenin proteins (Fig. 1b, c, and S1A). Although Wnt3a repressed both PPARγ1 and PPARγ2 proteins, PPARγ2 is major target for C/EBPβ and GR in response to MDI (Fig. S1C). Early temporal treatment of Wnt3a (5 ng/ml, for 0–2 days) was sufficient to block the early and later processes of adipogenesis, whereas more Wnt3a (>25 ng/ml) was required to block adipogenesis during the later processes (4–6 days time points) (Fig. 1d–g and S1B). Although late temporal treatment (for 4–6 days) of Wnt3a (25 ng/ml) increased the mRNA levels of Axin2, a Wnt target gene, it did not effectively reduce the mRNA and protein levels of Pparg2 and Cebpa (Fig. 1e, f). These results confirmed the previous findings that the early period (within 0–2 days) is more sensitive to Wnt3a inhibition than the late period.

Wnt3a significantly reduced the nuclear protein level of GR, but not C/EBPβ during early adipogenesis, although Wnt3a did not reduce the total protein levels of either GR or C/EBPβ (Fig. 1h, i and S2A). Interestingly, Wnt3a did not reduce GR protein level in the nuclei of the 3T3-L1 cells treated with only Dex, suggesting that Wnt3a specifically reduced nuclear GR only during early adipogenesis (Fig. 1j). Consistently, Wnt3a reduced GR binding at two major hotspots (−0.3 or +2.6 kb) located near the Pparg2 transcription start site (TSS) (Fig. 1k). Although Wnt3a did not reduce C/EBPβ protein level, it inhibited the binding of C/EBPβ on the hotspots in Pparg2 (Fig. 1l).

We selected seven genes (Acsl1, Hp, Tpce2, Sclt10a6, Krt13, Tsc22d3 and Megf9), the promoters (−5 to +1 kb from TSS) of which contain GR binding peaks, as identified in the ChIP-seq analyses. We found that Wnt3a reduced GR occupancy in the promoter of all seven genes, which are also occupied by C/EBPβ; interestingly, Wnt3a also reduced C/EBPβ binding to these promoters.
Fig. 1 (See legend on next page.)
Wnt3a disrupts cooperation between C/EBPβ and GR on the Pparg2 promoter

To investigate whether the reduction in nuclear GR destabilized C/EBPβ binding to the Pparg2 promoter, we generated C/β-NIH cells that ectopically express FLAG-tagged C/EBPβ (Fig. 2a). Dex alone (without IBMX) can induce Pparg2 expression in C/β-NIH cells, as IBMX is required for the induction of C/EBPβ (Fig. 2b). Furthermore, the constitutively expressed C/EBPβ can bind the Pparg2 promoter only when GR binds it in response to Dex treatment (Fig. 2c), and vice versa, GR binds to the Pparg2 promoter only in C/β-NIH cells but not in EV-NIH cells. These results confirmed that C/EBPβ and GR interdependently bind the Pparg2 promoter. Similar to Wnt3a, a GR antagonist, RU486, prevented not only GR but also C/EBPβ from binding to the Pparg2 promoter (Fig. 2c).

In addition to the Pparg2 promoter, C/EBPβ and GR can bind to different sets of their own target sequences. Analyses of the luciferase reporter gene show that Wnt3a does not inhibit C/EBPβ to induce the luciferase gene regulated by C/EBPβ binding sites (Fig. 2d). Similarly, RU486, but not Wnt3a, prevented Dex from inducing the luciferase gene, which was under the regulation of the glucocorticoid response element (GRE) (Fig. 2e). These results suggest that Wnt3a blocks neither C/EBPβ nor GR binding to their consensus motifs but disrupts the cooperativity between C/EBPβ and GR on the promoter of Pparg2 by reducing GR level in the nuclei during early adipogenesis. We investigated whether Wnt3a also disrupted the cooperative binding of other hotspot TFs. We found that Wnt3a also reduced binding of STAT5, KLF4, and c-Jun to Pparg2 (Fig. 2f). The cooperative bindings of several TFs significantly increased the recruitment of enhancer-associated coregulators, p300 and CBP, to form enhanceosomes and increased the accessibility of the chromatin structure of Pparg2. As expected, Wnt3a blocked the recruitment of p300 and CBP on Pparg2 in the MDI-treated cells (Fig. 2g). FAIRE (that detects nucleosome-depleted regions in the genome) and H3 ChIP analyses showed that MDI treatment reduced histone occupancy at the Pparg2 promoter, but not in the presence of Wnt3a, suggesting that Wnt3a inhibits MDI-induced chromatin opening of Pparg2 (Fig. 2h, i).

Wnt3a prevents GR from inducing Tead4, a novel hotspot TF of Pparg2

Starick et al. showed that TEA domain transcription factors (TEADs) reinforced GR binding to a subset of GR target genes as a heterodimer binding partner of GR. For the first time, we found that Dex is responsible for inducing the mRNA and protein levels of Tead4 in a GR-dependent manner (Fig. 3a–e and S3A to S3C). Interestingly, Wnt3a completely blocked MDI from inducing the mRNA and protein levels of Tead4, but did not block Dex’s ability to induce Tead4 (Fig. 3f, g). These results are consistent with the findings that Wnt3a inhibits GR function in MDI-treated cells with higher sensitivity than in Dex-treated 3T3-L1 cells (Fig. 1i, j). ChIP revealed that both GR and Tead4 occupied their putative binding sites in Tead4 after MDI treatment (Fig. 3h, i). Interestingly, we found that GR bound to not only GRE (–0.9 kb) but also to TEAD binding elements (TBE) (+0.3 kb) in Tead4 (Fig. 3h, i), and that knockdown of Tead4 reduced GR binding on the TBE of Tead4 (Fig. 3j). These results suggest that GR and Tead4 can cooperatively bind to the TBE of Tead4, and that Tead4 is the target of Tead4 itself as well as GR. Wnt3a prevented both GR and Tead4 from binding to the GRE and the TBE of Tead4 during early adipogenesis (Fig. 3i).

Furthermore, TEAD4 also bound to the Pparg2 promoter during early adipogenesis. We found that Tead4 knockdown reduced Pparg2 induction and adipogenesis (Fig. 4a–d). Interestingly, Tead4 knockdown did not reduce GR and C/EBPβ protein levels but reduced their
binding to the \textit{Pparg2} promoter (Fig. 4e, f), suggesting that TEAD4 reinforced GR binding not only to the \textit{Tead4} promoter, but also to the \textit{Pparg2} promoter, for strong induction of both \textit{Pparg2} and \textit{Tead4}. Although TEADs are major transcription factors that convey the Hippo signal by recruiting their coactivators TAZ/YAP, TEAD4 recruited neither YAP nor TAZ on \textit{Pparg2} (Fig. 4g–j). We also found that TEAD4 binds on the promoter of other
MDI-induced genes, but do not recruit TAZ or YAP (Fig. S4A). Furthermore, TAZ knockdown did not block Wnt3a inhibitory effects suggesting that TAZ is not essential for the anti-adipogenic function of Wnt3a (Fig. 4k and Fig. S4B to S4E). We showed for the first time that TEAD4 and TAZ/YAP oppositely regulate the expression of Pparg2. These findings indicated that TEAD4 and GR form a positive circuit for induction of both Tead4 and Pparg2. Thus, Wnt3a disrupted two mutually related positive circuits by blocking GR binding to the promoters of Tead4 and Pparg2 (Fig. 4l).

**Overexpression of GR is sufficient for blocking the inhibitory effects of Wnt3a**

We investigated whether overexpression of ectopic GR is sufficient to resume the MDI-mediated induction of
**Fig. 4** TEAD4 as a novel hotspot TF for *Pparg2* induction. a ChiP-qPCR analyses of TEAD4 occupancy on the −0.3 kb region from TSS of *Pparg2* in 3T3-L1 cells. b–f The shCtrl-L1 cells or the shTead4-L1 cells were induced to undergo adipogenesis by treating with adipogenic hormones for the indicated time points as described in Fig. 1a. b Images and optical densities (510 nm) of Oil Red-O stained lipid. Scale bars, 200 μm. c Relative mRNA levels of *Pparg2* to 18S rRNA levels. d, e Western blot analyses using the indicated antibodies. f ChiP-qPCR analyses of GR, C/EBPβ, or TEAD4 occupancy on the −0.3 kb region from TSS of *Pparg2*. g–j 3T3-L1 cells were treated with MDI for the indicated time points. g Western blot analyses of 3T3-L1 cells using the indicated antibodies. h Relative mRNA levels of *Ctgf* to 18S rRNA levels. i, j ChiP-qPCR analyses of TEAD4, TAZ, or YAP occupancy on the −0.3 kb region from TSS of *Pparg2* and the −0.1 kb region from TSS of *Ctgf*. k Western blot analyses using the indicated antibodies in the shCtrl-L1 cells and shTaz-L1 cells. l Schematic diagram showing GR-TEAD4-PPARγ positive circuits during early adipogenesis. Wnt3a disrupted two mutually related positive circuits by limiting the nuclear localization of GR. qPCR data show mean ± SE. All data were repeated at least three independent same or similar experiments. *p < 0.05, **p < 0.01, and ***p < 0.001 by Students’ t-test.

**Tead4** in Wnt3a-treated 3T3-L1 cells. We found that GR overexpression prevented Wnt3a from reducing the mRNA and protein levels of *Tead4*. These results confirmed that Wnt3a reduced the expression of *Tead4* by limiting the GR nuclear protein level during early adipogenesis (Figs. 1i, 5a, b). Overexpression of ectopic TEAD4 further increased the mRNA and protein levels of *Pparg2* in MDI-treated as well as untreated 3T3-L1 cells, confirming that TEAD4 is a positive regulator of *Pparg2* (Fig. 5c, d). However, in TEAD4 overexpressing cells, Wnt3a still limited GR protein level in the nuclei and reduced MDI-mediated induction of *Pparg2*, suggesting that TEAD4 cannot substitute GR for *Pparg2* induction (Fig. 5c–e). Interestingly, overexpression of GR reduced the amount of C/EBPβ protein, which limited the MDI-mediated induction of PPARγ (Fig. 5f, g). This finding is consistent with previous study, which showed that liganded GR inhibits CAMP-activated CREB, an important transcription factor required for the induction of *Cebpβ*. We overexpressed GR together with C/EBPβ in 3T3-L1 cells.
cells (GR/Cβ-L1 cells). Unlike the ectopic expression of only C/EBPβ (Cβ-L1 cells), overexpression of both GR and C/EBPβ resulted in reinitiation of adipogenesis in 3T3-L1 cells even in the presence of Wnt3a (Fig. 5h). Although Wnt3a still reduced early induction of PPARγ, GR/Cβ-L1 cells maintained sufficiently high PPARγ protein level to enable completion of adipogenesis even in the presence of Wnt3a (Fig. 5h, i). ChIP analyses also showed

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**Fig. 5 Effects of GR and C/EBPβ overexpression.** a-g 3T3-L1 preadipocytes were infected with lentiviruses encoding HA-tagged GR (GR-L1), Myc-tagged TEAD4 (Tead4-L1) or empty vector as a control (EV-L1 cells). These cells were treated with MDI in the presence or absence of W3a for the indicated time points. a) Western analyses of TEAD4 and HA-tagged GR protein. b) Real-time expression of Tead4 mRNA to 18S rRNA. c) Western analyses of PPARγ and Myc-tagged TEAD4. d) Real-time expression of Pparg2 mRNA to 18S rRNA. e) Western analyses of nuclear extracts (NE) of the EV-L1 cells or the Tead4-L1 cells treated with MDI in the presence or absence of W3a for 24 h using the indicated antibodies. Lamin C was used as the loading control for the nuclear proteins. f) Western blot analyses using the indicated antibodies. g) Real-time expression of Pparg2 mRNA to 18S rRNA. h-k 3T3-L1 preadipocytes were infected with lentiviruses encoding FLAG-tagged C/EBPβ (Cβ-L1) or empty vector as a control (EV-L1 cells). Cβ-L1 cells and EV-L1 cells were further infected with retrovirus encoding HA-tagged GR (GR/Cβ-L1) or empty vector (EV/EV-L1 cells), respectively. These cells were treated with MDI in the presence or absence of W3a for the indicated time points. h) Images and optical densities (510 nm) of Oil Red-O stained lipid. Scale bars, 200 μm. i) Western analyses using the indicated antibodies. j) ChIP-qPCR analyses of GR or HA-tagged GR occupancy on the –0.3 kb region from TSS of Pparg2. p = 0.012 for GR and p = 0.085 for HA (GR). k) Western analyses of nuclear extracts (NE) of the EV/EV-L1 cells or GR/Cβ-L1 cells treated with MDI in the presence or absence of W3a. H3 was used as the loading control for nuclear proteins. The relative band intensities of GR and H3 were determined using the ImageJ software from two independent western blot analyses. qPCR data show mean ± S. All data were repeated at least three independent same or similar experiments. *p < 0.05, **p < 0.01, and ***p < 0.001 by Student’s t-test; ns, not significant.
that in GR/Cβ-L1 cells, Wnt3a did not reduce GR bindings to the Pparg2 promoter (Fig. 5j). These results corroborated the observation that GR protein level remained high in the nuclei of GR/Cβ-L1 cells even in the presence of Wnt3a (Fig. 5k) and demonstrated that Wnt3a blocked MDI-mediated induction of both Tead4 and Pparg2 by limiting GR in nuclei during early adipogenesis.

β-Catenin is necessary for conveying the Wnt3a signal to the Pparg2 promoter

Knockdown of β-catenin reduced the mRNA levels of its target genes such as Axin2 and Ccnd1 in 3T3-L1 cells (Fig. 6a, b) and completely blocked the inhibitory effects of Wnt3a on adipogenesis and induction of both Pparg2 and Tead4 (Fig. 6c–g). We found that Wnt3a did not reduce the nuclear amounts of GR proteins in shβ-cat-L1 cells (Fig. 6h). Consequently, in 3T3-L1 cells with β-catenin knockdown, Wnt3a did not block the occupancies of C/EBPβ and GR on the Pparg2 promoter (Fig. 6i) and did not inhibit the opening of chromatin structure (Fig. 6j, k). These results indicate that β-catenin is necessary for conveying the Wnt3a signal to the chromatin of Tead4 and Pparg2 by limiting the nuclear localization of GR.

Wnt3a blocks remodeling of actin cytoskeleton in a β-catenin-dependent manner

β-Catenin is connected to the actin cytoskeleton via interactions with α-catenin and cadherin proteins in cytoplasm26. Upon exposure to adipogenic hormones, 3T3-L1 cells differentiate into round and lipid-laden adipocytes, accompanied by changes in the actin cytoskeleton from stress fibers to cortical structures4. Time course staining with fluorescent phalloidin, a probe specific for filamentous actin (F-actin), revealed that MDI reduced F-actin stress fibers in 50% cells from 4 h, whereas they disappeared in 86% cells within 48 h; instead, F-actin reorganized only at the cortical region. The amount of β-catenin protein decreased during the rearrangement of F-actin from stress fibers to cortical structures. Wnt3a prevented MDI from reducing β-catenin protein and disrupting F-actin stress fibers (Fig. 7a, b). However, this was not observed in shβ-cat-L1 cells, suggesting that β-catenin is required for Wnt3a to prevent F-actin rearrangement (Fig. 7c). However, we observed that active S37A-β-catenin which is constitutively present in the nuclei, is not sufficient to elicit the inhibitory effects of Wnt3a on remodeling of chromatin and cytoskeleton during early adipogenesis although at late phase of adipogenesis, ectopic expression of S37A-β-catenin can reduce the mRNA and protein levels of Pparg2 even in the absence of Wnt3a (Fig. 7d–j and S5A to SSC).

As Wnt3a reduced nuclear GR protein level in a β-catenin-dependent manner (Fig. 6h), we investigated whether GR is necessary for MDI to rearrange F-actin. In shGR-L1 cells with GR knockdown, MDI could neither rearrange F-actin nor induce the mRNA level of Pparg2 (Fig. 8a, b). GR knockdown reduced MDI-induced binding of C/EBPβ to the Pparg2 promoter (Fig. 8c). In contrast, in GR/Cβ-L1 cells without MDI treatment, F-actin stress fibers were observed only around the peripheral region but not in the nuclei of 3T3-L1 cells (Fig. 8d). Wnt3a did not recover F-actin stress fibers in GR/Cβ-L1 cells. Like 3T3-L1 cells, in C3H10T1/2, mouse mesenchymal stem cells, both Dex and MDI increased the mRNA and protein levels of TEAD4 and PPARγ which were also inhibited by Wnt3a (Fig. 8e, f). Furthermore, Wnt3a prevented MDI from rearranging F-actin stress fibers in C3H10T1/2 cells (Fig. 8g). These findings suggest that during early adipogenesis, GR is necessary both for the rearrangement of F-actin and hotspot formation on the Pparg2 promoter, and that Wnt3a blocks these two events by limiting the nuclear level of GR in a β-catenin-dependent manner (Fig. 8h).

Discussion

The findings that treatment of Dex followed by IBMX was sufficient for adipogenesis, but that IBMX treatment followed by Dex treatment did not recapitulate this effect suggested that Dex primed preadipocytes to a novel commitment state for adipogenesis44. Adipogenesis of mouse embryonic fibroblasts (MEFs) isolated from GR (Nr3c1) knockout and GR dimerization-defective mutant mice was impaired45. Knockdown of GR or omission of Dex from MDI did not induce Pparg2 in 3T3-L1 cells8,46. These previous studies indicated that GR is essential for Pparg2 induction.

Using extensive genome-wide profiling of 15 TFs at 4 h after MDI treatment, Mandrup and colleagues demonstrated that during early adipogenesis, hotspots co-occupied by more TFs recruited more coactivators such as p300/CBP to constitute super-enhancers4. Enhancers with large numbers of TFs are more sensitive to small changes in TF concentration compared to those with smaller numbers of TFs47–49. This is consistent with our findings that reduction in nuclear GR level by β-catenin sensitively changes super-enhancer formation on Pparg2 and other genes (Fig. S2B). Our findings that GR amplifies MDI signals to Pparg2 by inducing TEAD4, a novel TF for Pparg2, highlighted that changes in GR activity affect Pparg2 expression (Fig. 3). ChIP-exo sequencing using GR antibody in IMR90 cells revealed that TEAD4 and GR co-occupied GR target genes as a heterodimer and that TEAD knockdown decreased the expression of several GR target genes42.
Biochemical CAP-SELEX analyses which identify cooperative interactions between TF pairs and heterodimeric DNA motifs, revealed that TEAD4 is the most common partner that cooperatively binds diverse DNA sequences with 32 TFs among 100 tested TFs. TEAD4 and its partner TFs recognize composite sequences that were considerably different from the individual TF motifs, suggesting that in vivo functions of TEAD4 may
Fig. 7 (See legend on next page.)
encompass diverse biological functions depending on its partner TFs. This is the first study to show that TEAD4 cooperatively binds to Pparg2 together with other hotspot TFs, and that Tead4 knockdown reduced Pparg2 expression. Since GR and TEAD4 cooperatively bind and induce Tead4, GR is a key TF that persistently drives adipogenesis. Primary preadipocytes embedded in stiffer cellular matrices (ECM) is involved in commitment for adipogenesis in the absence of adipogenic hormones, the stiffness of extracellular matrices (ECM) is involved in commitment for adipogenesis. Primary preadipocytes embedded in stiffer matrices show reduced rates of adipogenesis. ECM stiffness increases tissue tension, which leads to increased actin and myosin fiber and cell stretching. Interestingly, mesenchymal stem cells exposed to mechanical strain show increase in β-catenin level and could not differentiate into adipocytes, suggesting that Wnt/β-catenin conveys signals from intracellular tension to the nuclei. In agreement with this result, we found that Wnt signal could not prevent MDI from changing F-actin stress fibers to cortical F-actin structures during adipogenesis in the absence of β-catenin.

Interestingly, Wnt3a reduced GR level in the nuclei during the early phase of adipogenesis, but not in Dex-treated preadipocytes, suggesting that the antagonistic effect of Wnt/β-catenin on GR is specific for adipogenesis (Figs. 1i, j, 6h). Extensive studies have revealed that the chaperone complex and intact cytoskeleton are required for the nuclear transport of GR. Furthermore, GR is connected to actin filaments through HSP90, a main component of GR-chaperone complex suggesting that the nuclear transport of GR and cytoskeletal rearrangement are closely related. Upon ligand binding, the GR-chaperone complex recruits a motor protein dynein to form the liganded GR-HSP90-FKBP52-dynein complex, which is able to move along the microtubules through the nuclear pore complex (NPC) to the nucleus. However, when the cytoskeleton is disrupted like adipogenesis, liganded GR simply diffuses in and out of the nucleus mainly via importins, exportins, and the RanGTPase mechanism. These nuclear and cytoplasmic events should interdependently regulate each other to prevent metabolic and structural catastrophes during adipogenesis. In addition to adipogenic hormones, the stiffness of extracellular matrices (ECM) is involved in commitment for adipogenesis. Primary preadipocytes embedded in stiffer matrices show reduced rates of adipogenesis. ECM stiffness increases tissue tension, which leads to increased actin and myosin fiber and cell stretching. Interestingly, mesenchymal stem cells exposed to mechanical strain show increase in β-catenin level and could not differentiate into adipocytes, suggesting that Wnt/β-catenin conveys signals from intracellular tension to the nuclei. In agreement with this result, we found that Wnt signal could not prevent MDI from changing F-actin stress fibers to cortical F-actin structures during adipogenesis in the absence of β-catenin.

Unlike lipogenesis in other cells such as hepatocytes and myocytes, adipogenesis requires dramatic cytoskeletal remodeling of F-actin stress fibers to cortical actin structures, which is required to hold a large lipid vacuole in the center, relocate the nucleus and other organelles, and attain a round shape. Cytoskeletal remodeling starts within 24 h after adipogenic hormone treatments, suggesting that cytoskeletal remodeling is followed by complete induction of lipogenic genes as a feed-forward mechanism. These nuclear and cytoplasmic events should interdependently regulate each other to prevent metabolic and structural catastrophes during adipogenesis. In addition to adipogenic hormones, the stiffness of extracellular matrices (ECM) is involved in commitment for adipogenesis. Primary preadipocytes embedded in stiffer matrices show reduced rates of adipogenesis. ECM stiffness increases tissue tension, which leads to increased actin and myosin fiber and cell stretching. Interestingly, mesenchymal stem cells exposed to mechanical strain show increase in β-catenin level and could not differentiate into adipocytes, suggesting that Wnt/β-catenin conveys signals from intracellular tension to the nuclei. In agreement with this result, we found that Wnt signal could not prevent MDI from changing F-actin stress fibers to cortical F-actin structures during adipogenesis in the absence of β-catenin.

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Fig. 8 Effects of GR on cytoskeletal rearrangement. The shCtrl-L1 or shGR-L1 cells were induced to undergo adipogenesis by treating with adipogenic hormones for the indicated time points in the presence or absence of W3a (5 ng/ml). a Confocal microscopic images of F-actin stress fibers (left panel). The graph indicates the percentage of cells in each category (S, T, and C described in Fig. 7a) (right panel). b qRT-PCR analyses of Pparg2 mRNA levels to 18S rRNA levels. c ChIP-qPCR analyses of C/EBPβ or GR occupancy on the −0.3 kb region from TSS of Pparg2. d Confocal microscopic images of cellular F-actin stress fibers (left panel) in the EV/EV-L1 or GR/Cβ-L1 cells. The graph indicates the percentage of cells in each category (S, T, and C described in Fig. 7a) (right panel). e-g C3H10T1/2 cells were treated with Dex (2 μM) or MDI for the indicated time points in the presence or absence of W3a (5 ng/ml). e qRT-PCR analyses of Tead4 and Pparg2 mRNA levels to 18S rRNA levels. f Western blot analyses showing TEAD4 and PPARY protein levels. g Confocal microscopic images of cellular F-actin stress fibers (left panel) in C3H10T1/2 cells. The graph indicates the percentage of cells in each category (S, T, and C described in Fig. 7a) (right panel). h Schematic diagram showing the inhibitory effects of Wnt3a/β-catenin on positive circuits of GR-TEAD4-PPARγ2 and cytoskeletal remodeling during early adipogenesis (details in Results). qPCR data show mean ± SE. All data were repeated at least three independent same or similar experiments. ***p < 0.001 by Students' t-tests.
system. The mechanism via which the liganded-GR translocates into the nucleus during adipogenic cytoskeletal rearrangement remains unknown. In contrast, β-catenin harbors armadillo repeats, which are similar to the importin-β HEAT repeats. Therefore, β-catenin can pass through NPC like importin and equilibrates between the nucleus and the cytoplasm by passive diffusion. Therefore subcellular distribution of β-catenin in the nuclei, cytoplasm, and the membrane can be determined from its location and the levels of diverse interacting complexes, namely, the TCF/LEF transcription factors in the nucleus, APC and AXIN in the cytoplasm, and cadherin complex in the membrane. It remains to be investigated whether GR and β-catenin compete with each other for nuclear translocation during adipogenic cytoskeletal rearrangement.

In conclusion, this study provides insights into an intriguing question regarding chromatin remodeling and actin rearrangement, which are interdependently regulated intranuclear translocation during adipogenic cytoskeletal rearrangement.

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Authors contributions
H.P. and B.P. conceived the study, designed the experiments, and analyzed data; B.P. performed most of the experiments; S.C. and G-J.L. performed the immunofluorescence assay. J.K.K. and B.K. analyzed the bioinformatics data; H.P. and B.P. wrote the paper.

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