Wnt4 and Wnt5a promote adipocyte differentiation

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Abstract The roles of the non-canonical Wnt pathway during adipogenesis are not well known, though Wnt10b is known to function as a negative regulator for adipogenesis by activating the canonical Wnt pathway. We focused on the roles of Wnt4, Wnt5a and Wnt6, which are thought to be part of the non-canonical Wnt pathway. The expression of these genes changed dramatically at the initial stage of adipogenesis. Furthermore, the inhibition of Wnt4 or Wnt5a expression prevented the accumulation of triacylglycerol and decreased the expression of adipogenesis-related genes. Wnt4 and Wnt5a have crucial roles in adipogenesis as positive regulators.

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1. Introduction

Peroxisome proliferator-activated receptor γ (PPARγ), the CCAAT/enhancer-binding proteins (C/EBPs) and sterol regulatory element-binding protein 1 (SREBP1) were known to be master regulators of adipogenesis [1]. On the other hand, several inhibitors are also known. For example, preadipocyte factor-1 (Pref-1) suppresses adipogenesis through the activation of MEK/extracellular signal-regulated kinase (ERK) signaling [2] and transformation growth factor-β (TGF-β) inhibits adipogenesis via SMAD3’s interaction with C/EBP family [3]. In addition to these factors, recent reports have demonstrated that Wnt10b is also a potent inhibitory factor for adipocyte differentiation [4].

The Wnt family consists of at least 19 cysteine-rich, secreted glycoproteins, which participate in a variety of developmental processes such as cell proliferation, cell polarity, cell migration and cell differentiation [5]. Members of the vertebrate Wnt family can be divided into at least two functional classes according to their biological activities: those of the Wnt/β-catenin pathway (canonical Wnt pathway), which leads to the stabilization of β-catenin and activates target genes in the nucleus, and those of the non-canonical Wnt pathway, which is independent of β-catenin signaling, and involves the stimulation of intracellular Ca2+ release and the activation of phospholipase C and protein kinase C (PKC) [6]. In the adipocyte differentiation process, Wnt10b activates the Wnt/β-catenin pathway and maintains preadipocytes in an undifferentiation state by inhibiting expression of C/EBPα and PPARγ [4]. These findings strongly indicate that the Wnt/β-catenin pathway activated by the canonical Wnt family is crucial for inhibiting adipocyte differentiation. However, the roles of the non-canonical Wnt family during adipogenesis are not well understood, even though non-canonical Wnts also have essential organizing roles in development.

In this study, we focused on the roles of Wnt4, Wnt5a and Wnt6 during adipocyte differentiation. Since the ectopic expression of Wnt4, Wnt5a and Wnt6 in C57 mammary epithelial cells did not induce morphological transformation or the accumulation of β-catenin, these proteins were thought to be part of the non-canonical Wnt family [7,8]. In addition, even though the functions of Wnt4, Wnt5a and Wnt6 were well characterized in various cells, little was known about the biological roles of these proteins at the beginning of adipocyte differentiation. We found that the expression of Wnt4, Wnt5a and Wnt6 changed dramatically in the early stages of adipogenesis. The expression profiles of Wnt4 and Wnt5a were closely related to adipocyte differentiation. Furthermore, the inhibition of Wnt4 and Wnt5a expression in 3T3-L1 cells prevented the cytoplasmic accumulation of triacylglycerol and decreased the expression of adipogenesis-related genes. These results strongly suggest that Wnt4 and Wnt5a have crucial roles to promote adipocyte differentiation.

2. Materials and methods

2.1. Cell culture and differentiation

The mouse 3T3-L1 preadipocytes and mouse NIH-3T3 fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% calf serum. The differentiation experiment was performed as described previously [9]. In brief, the medium was changed to DMEM supplemented with 0.5 mM 3-isobutyl-1-methylxantine, 10 μg/ml of insulin, 1 μM dexamethasone, and 10% fetal bovine serum (FBS) at 2 days postconfluence. After 2 days, the cells were transferred to DMEM containing 5 μg/ml insulin and 10% FBS. For Oil red O staining, 3T3-L1 adipocytes were fixed with 4% paraformaldehyde, then stained with 0.3% Oil red O (Amresco) solution for 60 min. The amounts of triacylglycerol were measured using LIPI DOS LIQUID (Ono) according to the manufacturer’s instructions.
2.2. Real-time quantitative PCR (Q-PCR)

An ABI PRISM 7000 sequence detection system (Applied Biosystems) was used to perform Q-PCR. Pre-designed primers and probe sets for Wnt4, Wnt5a, Wnt6, PPARγ, C/EBPα, adipocyte fatty acid-binding protein 2 (aP2) and 18S rRNA were obtained from Applied Biosystems. The reaction mixture was prepared using a TaqMan Universal PCR Master mix according to the manufacturer’s instructions. Relative standard curves were generated in each experiment to calculate the inputted amounts of the unknown samples.

2.3. RNAi experiment

For the RNAi experiment, the two target regions (1: 374–394 bp and 2: 651–671 bp) for Wnt4 and the four target regions (1: 283–303 bp, 2: 570–599 bp, 3: 602–622 bp and 4: 670–690 bp) for Wnt5a were selected using the QIAGEN siRNA online design tool (http://sirna.quagen.com/). A 19-nt short hairpin RNA (shRNA)-coding fragment with a 5′-TCTAGAGAG-3′ loop was subcloned into the ApaI/EcoRI site of pSilencer 1.0-U6 (Ambion). As a negative control, the scrambled fragment 5′-AAGAGGAGCATATTGGGAAGA-3′, which does not have similarity with any mRNA listed in Genbank, was generated.

Transfection of shRNA-expressing plasmids into 3T3-L1 cells was performed with Nucleofector using Cell Line Nucleofector Kit V (Amamax) according to the manufacturer’s instructions. The transfected cells were subjected to differentiation experiments at 2 days post-confluence.

2.4. Western blot analyses

Proteins were separated using SDS/PAGE and transferred to the polyvinylidene difluoride membrane. Rabbit polyclonal antibodies against Wnt4 or Wnt5a (Santa Cruz) were used as primary antibody.

3. Results

3.1. Time course of Wnt4, Wnt5a and Wnt6 mRNA expression during adipocyte differentiation

We first tested whether Wnt4, Wnt5a and Wnt6 were expressed during adipocyte differentiation of mouse 3T3-L1 cells. At 2 days post-confluence, growth-arrested 3T3-L1 cells were induced into adipocytes. The expression levels of these three genes were investigated by Q-PCR (Fig. 1). The level of Wnt4 mRNA increased after the treatment with inducers and the peak of expression was at 6–12 h. The expression of Wnt5a was slightly increased at 1 h and then rapidly decreased. The expression of Wnt6 mRNA showed a biphasic pattern. While the expression was decreased until 6 h after induction, it was increased at 12 h and decreased at the middle and late stages of adipocyte differentiation. These results indicated that the levels of Wnt4, Wnt5a and Wnt6 changed dramatically during adipocyte differentiation, especially early on.

3.2. Expression profiles of Wnt4, Wnt5a and Wnt6 in the adipocyte differentiable state and the non-differentiable state

Next we examined whether the expression of Wnt4, Wnt5a and Wnt6 at the beginning of adipocyte differentiation was specific to adipogenesis. For the differentiation into adipocytes, 3T3-L1 cells must first be grown to confluence and then kept for 2 days. After that, the inducers are added to the medium. Differentiation into adipocytes occurred only under the growth-arrested condition. On the other hand, proliferating 3T3-L1 cells did not differentiate into adipocytes even in the presence of inducers. Another mouse fibroblast cell line, NIH-3T3, does not differentiate into adipocytes in the growth-arrested and proliferating states. Since both NIH-3T3 and 3T3-L1 cells were fibroblastic cell lines derived from mouse embryo, we compared the expression profiles of these three genes in the two cell lines under two conditions: growth-arrested and proliferation (Fig. 2).

Although the expression of Wnt4 mRNA was slightly increased in the proliferating state, that of Wnt4 was dramatically induced in the growth-arrested state in 3T3-L1 cells 12 h after induction. In the NIH-3T3 cells, significant expression of Wnt4 was not observed in proliferating or growth-arrested cells even after the induction. Although the Wnt5a mRNA decreased in all cases, the expression of Wnt5a in growth-arrested 3T3-L1 cells was drastically decreased. On the other hand, the expression of Wnt 6 also decreased in all four conditions, and the rates of decrease were almost the same. These results indicate that the expression of Wnt4 and Wnt5a, but not Wnt6, was drastically changed under differentiable condition, suggesting that Wnt4 and Wnt5a are closely related adipocyte differentiation.

3.3. The effect of knocking down the expression of Wnt4 or Wnt5a on adipocyte conversion

3.3.1. Expression profiles of Wnt4, Wnt5a and Wnt6 in the adipocyte differentiable state and the non-differentiable state

To characterize the biological functions of Wnt4 and Wnt5a, we blocked their expression at the early stage of adipocyte differentiation using RNAi. Two shRNA expression plasmids for Wnt4 and four plasmids for Wnt5a with different target regions were introduced into 3T3-L1 cells. After that, the levels of Wnt4 and Wnt5a were determined by semi-quantitative PCR, Region 2 for Wnt4 and region 4 for Wnt5a showed the greatest reduction (data not shown). For further analyses, we therefore used the plasmids including region 2 and region 4 for the knockdown of Wnt4 and Wnt5a, respectively.

We first determined the expression levels of Wnt4 and Wnt5a in 3T3-L1 cells transfected with each shRNA expression plasmid by Q-PCR and Western blot analyses. In shRNA expression plasmid-transfected cells, the mRNA and protein levels of Wnt4 (12 h after induction) or Wnt5a (before induction) was reduced compared with that in the control cells (Fig. 3A). Using these transfected cells, we performed a differentiation experiment. After 8 days, these cells were stained with Oil Red O to detect oil droplets and the amounts of triacylglycerol were measured (Fig. 3B). The cell morphology of the control cells altered from fibroblastic preadipocytes to round adipocytes, and the cells stored the oil droplets well.

In contrast, the accumulation of oil droplets in the cells transfected with the shRNA expression plasmids for Wnt4 was clearly inhibited and the knockdown of Wnt5a expression slightly decreased the accumulation of oil droplets. Furthermore, the Wnt4 RNAi-treatment more effectively blocked the accumulation of triacylglycerol than the Wnt5a RNAi-treatment. We next determined the level of aP2, an adipogenic marker. The expression of aP2 was also inhibited by knockdown of the expression of Wnt4 and Wnt5a (Fig. 3C). These results suggest that the reduction in Wnt4 and Wnt5a expression during adipogenesis impaired the adipocyte differentiation.

Finally, we examined the expression levels of PPARγ and C/EBPα during adipocyte differentiation (Fig. 4). The expression of PPARγ and C/EBPα was also found to be inhibited by both the knockdown of Wnt4 and Wnt5a, although the rates of inhibition were relatively low compared with those observed for triacylglycerol accumulation and aP2 expression.
4. Discussion

Wnt10b seems to be a potent inhibitor for adipocyte differentiation [4]. Kanazawa et al. indicated that Wnt5b promotes adipogenesis by inhibiting the canonical Wnt/β-catenin signaling pathway [10]. These reports strongly suggest that the Wnt/β-catenin pathway plays a crucial role in the inhibition of adipogenesis. On the other hand, non-canonical Wnt5a regulates the growth, differentiation, apoptosis and insulin sensitivity of the differentiating adipocytes, and induces osteoblastogenesis by attenuating PPARγ-induced adipogenesis in mesenchymal stem cells of bone marrow [11,12]. However, little is known about the roles of some Wnt family members involved in the non-canonical Wnt signaling pathway at the early stage of adipocyte differentiation. In the present study, we demonstrated that Wnt4 and Wnt5a have an important role in the promotion of adipocyte differentiation at the initial stage.

Wnt4 appears to have an important role in activating PKC signaling, as shown by the report that PKCζ is required for Wnt4-stimulated outgrowth of commissural axons [13]. On adipocyte conversion, PKC signaling is very important. Fleming et al. indicated that PKCγ, part of the PKC family, is required for the mitotic clonal expansion, which occurs in the early stages of adipogenesis [14]. Thus, our findings

Fig. 1. Time course of mRNA expression of Wnt4, Wnt5a and Wnt6 during adipocyte differentiation. Total RNA obtained from mouse 3T3-L1 cells at various time points after treatment with inducers was subjected to Q-PCR. The expression level of each gene was normalized with 18S rRNA expression. The bars indicate S.D. (n = 3).

Fig. 2. Expression of Wnt4, Wnt5a and Wnt6 in growth arrested or proliferating 3T3-L1 and NIH-3T3 cells. Total RNA obtained from 3T3-L1 and NIH-3T3 cells at each time point was subjected to Q-PCR. The expression level of each gene was normalized with 18S rRNA expression. Cells were treated with the inducers after growth arrest (growth arrested; the typical condition for adipocyte differentiation), or at the mid-exponential phase of growth (proliferating; not the typical condition for adipocyte differentiation). The bars indicate S.D. (n = 3).
indicate that Wnt4 may promote adipogenesis by regulating PKC signaling and mitotic clonal expansion early on in the differentiation process.

The endogenous expression of Wnt5a was abundant before the induction and rapidly decreased after the induction. Interestingly, even though this expression pattern was similar to that of Wnt10b, the knockdown of Wnt5a expression led to inhibition of adipogenesis. Wnt5a stimulates intracellular calcium release and activation of PKC and calcium/calmodulin-dependent kinase II [15]. On the other hand, Wnt5a has also been suggested to antagonize canonical Wnt activity via inhibition of β-catenin's stabilization [16]. These reports imply that Wnt5a acts in both the Wnt/β-catenin and non-canonical Wnt signaling pathways. Further investigations are required to solve which pathway is a main one at the early stage of adipogenesis.

**Fig. 3.** Effect of the knockdown of Wnt4 or Wnt5a on adipocyte conversion. (A) Knockdown of Wnt4 or Wnt5a gene expression by RNAi during adipocyte differentiation. Total RNA and cell lysate obtained from 3T3-L1 cells transfected with the shRNA expression plasmid for Wnt4 (12 h after induction) or Wnt5a (before induction) were subjected to Q-PCR (left) and Western blot (right). 3T3-L1 cells transfected with a scrambled shRNA expression plasmid was used as a control. On Q-PCR, the expression level of each gene was normalized with 18S rRNA expression. The bars indicate S.D. (n = 3). Cell lysates were separated by SDS–PAGE, and detected by Western blotting with antibodies against Wnt4 and Wnt5a. β-actin was used as a loading control. (B) Differentiation of 3T3-L1 cells transfected with the shRNA expression plasmid for Wnt4 or Wnt5a. The cells after 8 days of treatment were stained with Oil Red O (left). The measurement of triacylglycerol content was done on 24-well plates (right). The bars indicate S.D. (n = 5). *P < 0.01 vs. control. (C) The expression level of the aP2 gene on knockdown of Wnt4 or Wnt5a expression. The bars indicate S.D. (n = 3). *P < 0.01 vs. control.
In Fig. 4, the expression of PPARγ and C/EBPα was only partly inhibited in the knockdown cells. It is possible that the reduction of expression of PPARγ and C/EBPα is caused by either indirect effect or a complementary mechanism. To dissolve the precise mechanisms on the reduction of these genes, further analyses are definitely needed.

In summary, we showed that levels of Wnt4, Wnt5a and Wnt6 changed dramatically at the beginning of adipocyte differentiation. Furthermore, knockdown experiments using RNAi demonstrated that both Wnt4 and Wnt5a function as positive regulators of adipogenesis at the initial stage of the differentiation process. These results may indicate that Wnts family members mainly involved in the non-canonical Wnt signaling pathway also regulates adipocyte differentiation.

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