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Wnt5b stimulates adipogenesis by activating *PPARγ*, and inhibiting the β-catenin dependent Wnt signaling pathway together with *Wnt5a*

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ABSTRACT

Correct Wnt signaling is required for adipogenesis and alterations occur in Type 2 diabetes mellitus (T2DM). Gene expression studies showed that β-catenin independent *Wnt5b* was down-regulated in T2DM preadipocytes, while its paralog *Wnt5a* was unchanged. Our study aimed at defining the expression profile and function of *Wnt5a* and *Wnt5b* during adipogenesis by determining their effect on aP2 and *PPARγ* expression and assessing the level of β-catenin translocation in mouse 3T3-L1 preadipocytes. Additionally, we explored the effect on adipogenic capacity by *Wnt5b* overexpression in combination with stimulation of the β-catenin dependent or β-catenin independent Wnt signaling. Expression of *Wnt5b* was, like *Wnt5a*, down-regulated upon induction of differentiation and both inhibit β-catenin dependent Wnt signaling at the initiation of adipogenesis. *Wnt5b* additionally appears to be a potent enhancer of adipogenic capacity by stimulation of *PPARγ* and *aP2*. Down-regulation of *Wnt5b* could therefore contribute to decreased adipogenesis observed in T2DM diabetic subjects.

Introduction

Gene expression analysis of preadipocytes of Type 2 diabetes mellitus (T2DM) patients displayed decreased expression of a number of genes involved in the formation of new adipocytes, called adipogenesis, when compared to age- and BMI-matched controls. One of the down-regulated genes was *WNT5b*, which is a member of the Wnt family. Expression of *WNT5a*, which is a paralog of *WNT5b,* was unchanged (Van Tienen et al., submitted). As Type 2 diabetes is associated with decreased adipogenesis and altered Wnt expression [1], we decided to study the role *WNT5b* and *WNT5a* during adipogenesis in further detail.

The Wnt family is an evolutionarily conserved family of secreted glycoproteins with well-established roles in cellular proliferation, differentiation, and polarity during embryogenesis as well as control homeostatic self-renewal in a number of adult tissues [2,3]. Wnt membership is based on sequence rather than functional properties. Functionally, the Wnt family can be divided into at least two groups, the β-catenin dependent Wnt pathway, which activates target genes through stabilization of β-catenin, and the β-catenin independent or aspecific Wnt pathway that is independent of β-catenin and activates gene transcription e.g., by stimulation of the intracellular calcium flux leading to activation of Ca^{2+}-dependent effectors, like activation of phospholipase C and protein kinase C (PKC).

The β-catenin dependent Wnt signaling pathway has been reported to inhibit adipogenesis e.g., by *Wnt10b* or *Wnt3a* [4–6]. On the other hand, β-catenin independent Wnt genes like *Wnt4*, *Wnt5a*, *Wnt5b* have been proposed as stimulators of adipogenesis [7]. Expression of β-catenin independent *Wnt5a* has a stimulatory effect in the early phase, since *Wnt5a* gets down-regulated 12 h after induction of differentiation in 3T3-L1 mouse preadipocytes, and knock-down of *Wnt5a* results in decreased adipogenesis and reduced expression of key regulators of adipogenesis, like peroxisome proliferator activated receptor γ (PPARγ) and CCAAT/enhancer binding protein α (CEBPα) [7,8]. In contrast, *Wnt5b*, which is a paralog of *Wnt5a*, is reported to be up-regulated during adipogenesis with the highest expression at day two, and overexpression of *Wnt5b* significantly stimulates adipogenesis in murine preadipocytes [9,10].

Abbreviations: Wnt3a-CM, Wnt3a conditioned medium; Wnt5a-CM, Wnt5a conditioned medium.

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Since *WNT5b*, and not *WNT5a*, was down-regulated in preadipocytes of T2DM patients, our aim was to explore the expression pattern of both *Wnt5b* and *Wnt5a* during adipogenesis, using mouse 3T3-L1 preadipocytes as model for adipogenesis. In addition, the effect of *Wnt5b* on murine adipogenesis was studied under conditions in which either the β-catenin dependent Wnt pathway or the β-catenin independent Wnt pathway was stimulated.

Materials and methods

Sequence alignment. Nucleotide and protein sequence alignment were performed using the Clustal W multiple sequence alignment program [11]. mRNA sequence alignment of mouse *Wnt5a* mRNA NM_009524.2 and *Wnt5b* NM_009525.2 mRNA, and human *WNT5a* NM_03392.3 and the two isoforms of human *WNT5b* NM_032642.2 and NM_030775.2 were performed. On protein level, mouse Wnt5a NP_033550, human WNT5a NP_003383, rat Wnt5a NP_072153, mouse Wnt5b NP_033551, human WNT5b NP_110402 and rat Wnt5b XP_001057561 were analyzed.

Cell culture. Mouse 3T3-L1 preadipocytes (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 10% FCS (Gibco) and penicillin/streptavidin (Gibco). Differentiation was induced in 2-days post-confluence by changing medium with DMEM containing 0.5 mM 3-isobutyl-1-methylxantine (IBMX) (Sigma), 10 μg/ml insulin (Sigma) and 1 μM dexamethasone (Sigma). After two days, medium was replaced with DMEM containing 10% FCS and 10 μg/ml insulin, and changed every 2 days.

Wnt3a conditioned medium (Wnt3a-CM), Wnt5a conditioned medium (Wnt5a-CM), and L-cell control medium were prepared in DMEM with 2% HS (Invitrogen). Cells were plated on day 0 with a dilution of 1/10. Conditioned medium was collected on days 4 and 7. Both batches were mixed and filtered before use. The media was used at a final concentration of 20% in all experiments. Medium of two days confluent 3T3-L1 cells was replaced with DMEM 10% FCS containing 20% Wnt3a-CM, Wnt5a-CM or L-cell control medium. After 8 h, media was replaced by differentiation medium containing 20% Wnt3a-CM, Wnt5a-CM or L-cell control medium.

Transfection. 3T3-L1 preadipocytes were transfected at 70% confluence with pSPORT-Wnt5b (Openbiosystems) or pcDNA3.1 empty vector with Fugene HD transfection reagents (Roche) according to the manufacturers' protocol.

Quantitative real-time PCR validation. RNA isolation and DNAse treatment were performed with the high pure RNA isolation kit (Roche) according to the manufacturers' protocol. cDNA was generated from 1 μg RNA in a standard reverse transcriptase reaction using M-MulV reverse transcriptase (Finzymes). Primers were designed using Primer Express® software version 3.0 (Applied Biosystems) or derived from literature. *Wnt5b* forward primer 5′-CCCC AGGCCAGAGAAAGC-3′ and reverse primer 5′-CCTCCCCGA TGTAGGACAT-3′; *WNT5a* forward primer 5′-ACTTCGAGAGGCTCCC AGAAC-3′ and reverse primer 5′-CTGGGAAAGGAGTGAAGCAAA-3′; *aP2* 5′-GCGTGGAATTC GATGAAATCA-3′ and reverse primer 5′-CCCGCCATCTAGGGTTATGA-3′ [12]; *Nlk* 'CCCAACCGAAGCATTTC AGT' and 'CCGACCTCTGAGATTGTACCTTT C-3'; *PPARγ* 5′-TGCCAAA AATATCCCTGGTTTC-3′ and reverse primer 5′-GGAGGCCAGCATCGT GTAGA-3′. Quantification of transcripts was carried out using the ABI 7900 HT Real-Time PCR detection system using Eurogentec qPCR Mastermix Plus for SYBR Green® I. The cycling conditions were: an initial step for 2′ at 50 °C, activation of the Hot Goldstar enzyme at 95 °C for 10′, 40 cycles of 15″ at 95 °C followed by 1′ at 60 °C (denaturation, annealing, and elongation). The mRNA levels of each gene were normalized to those of the housekeeping gene encoding Cyclophilin A (*CypA*). Statistic analysis was performed using Student *T*-test.

Western blots. For Western blot, nuclear and cytoplasmatic protein extracts were isolated using NE-PER reagent according to the manufacturers protocol (Pierce). Protein content was measured using the BCA protein assay (Pierce Biotechnology Inc., Rockford, Ilinois); 20 μg of total protein was denatured by boiling in Laemmli sample buffer (BioRad), separated on a 10% SDS–PAGE gel, and transferred onto a Hybond C nitrocellulose membrane (Amersham Biosciences). After blocking (5% non-fat dry milk (BioRad), 0.1% Tween in TBS) for 1 h, membranes were incubated overnight at 4 °C with primary antibodies directed against β-catenin 1/2000 (BD Biosciences) and β-actin 1/2000 (Sigma). Anti-mouse immunoglobulin G 1/5000 (Vector Labs Inc.) was used as the secondary antibody, and the membranes were developed using the Western blotting luminal reagent for chemiluminescence detection (Santa Cruz), which was detected with the Chemidoc XRS. Analysis of the images was performed independently by two persons with the Quantity 1-D analysis software (Roche).

Results

Wnt5a and Wnt5b homology and expression pattern during 3T3-L1 differentiation

To explore the role of *WNT5b* and its paralog *WNT5a* in normal adipogenesis, we used the well-characterized mouse 3T3-L1 preadipocyte cell line. First we determined if *Wnt5a* and *Wnt5b* mRNA and protein in mouse was comparable to human, using the ClustalW analysis tool. Human *Wnt5b* encodes two different mRNAs which are 97% homologous, but encode the same protein, whereas for mouse only one mRNA is known. In both human and mouse, *Wnt5a* mRNA is about twice the size of *Wnt5b*. Human *WNT5a* mRNA shows resp. 38% and 39% homology to *WNT5b* NM_032642.2 and NM_030775.2 mRNA, and human WNT5a and WNT5b protein are 79.7% homolog. Mouse *Wnt5a* and *Wnt5b* are 42% homologous at the mRNA level and 77.7% at the protein level. Human *WNT5a* mRNA shows 70% mRNA homology and 98.6% on for the protein compared to mouse *Wnt5a*. Mouse Wnt5b shows resp. 73% and 75% mRNA homology with human WNT5b NM_032642.2 and NM_030775.2 and 94.1% for the protein. To study their role in adipogenesis, we first analyzed the normal expression pattern of *Wnt5a* and *Wnt5b* during 3T3-L1 differentiation. At two days post-confluence, day 0, differentiation was induced. RNA was isolated at day 0, 1, 2, 4, 7 and 10. As shown in Fig. 1, the expression of both *Wnt5a* and *Wnt5b* was down-regulated during differentiation. *Wnt5b* was more severely down-regulated and *Wnt5a* expression was higher than *Wnt5b* expression.

The effect of Wnt5b overexpression on adipocyte differentiation

To study the biological role of *Wnt5b* gene expression in adipogenesis, we analyzed the effect of *Wnt5b* overexpression during adipogenesis (experimental design Fig. 2A). In two independent experiments, mouse 3T3-L1 preadipocytes were transfected in duplo with pSPORT-Wnt5b overexpression construct or an empty pcDNA3.1 vector as transfection control. Two days post-confluence, the medium was replaced with differentiation medium and RNA was isolated at day 0, 2, 4, and day 10. *Wnt5b* overexpression resulted in increased expression of adipocyte specific transcription factor *aP2* (Fig. 2B). Additionally, the key transcription factor for adipogenesis, *PPARγ*, was also significantly up-regulated by *Wnt5b* at day 2, 4 and 10 (Fig. 2C). The independent experiments yielded comparable results, although absolute values per culture differed. In Fig. 2B and C, the results of one representative experiment are shown.

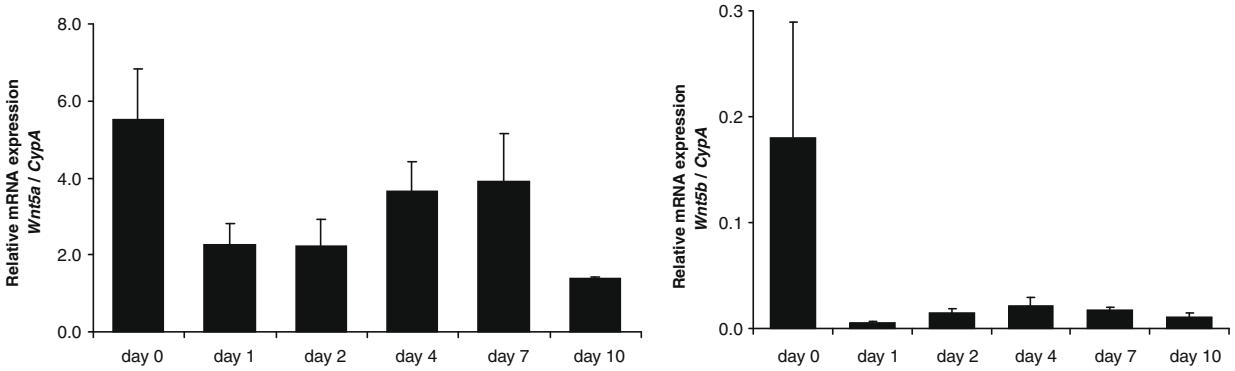

Fig. 1. Both *Wnt5a* and *Wnt5b* mRNA expression is down-regulated during differentiation of 3T3-L1 preadipocytes. Total RNA was isolated at indicated time-points, subjected to qPCR and expression was normalized with Cyclophilin A (CypA) expression. The bars indicate S.E. ($n = 3$).

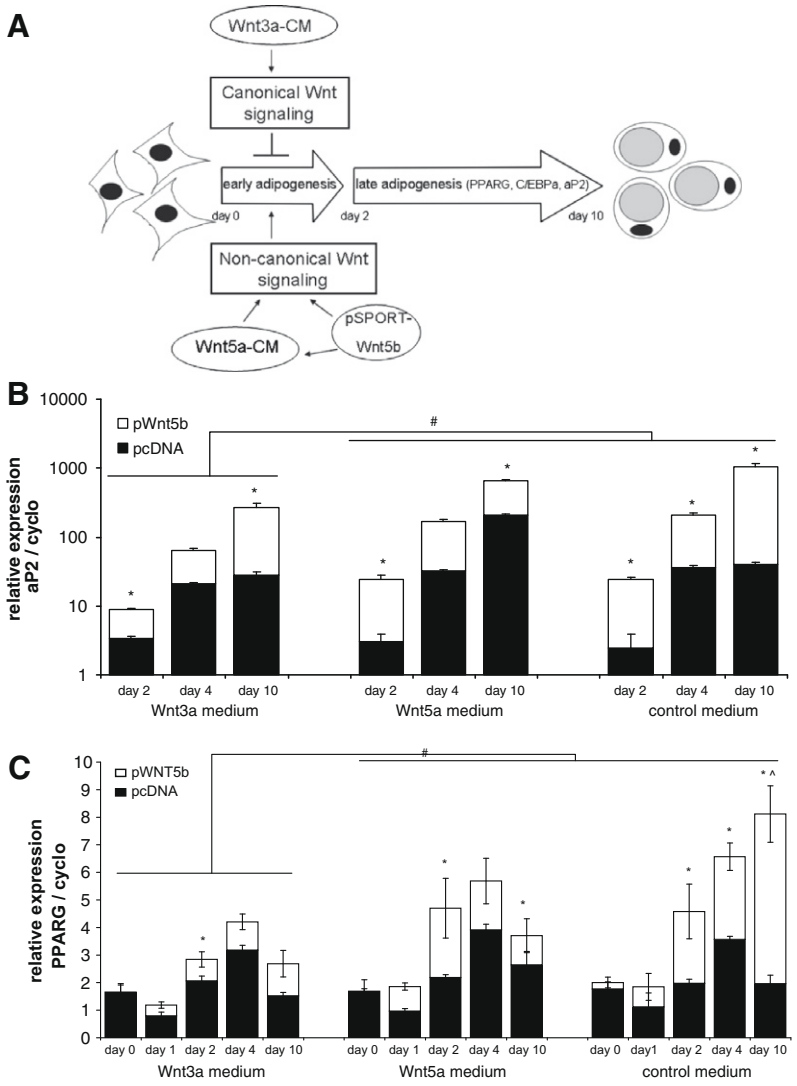

Fig. 2. *Wnt5b* overexpression enhances *aP2* and *PPARγ* expression during 3T3-L1 differentiation irrespective of stimulation of the β-catenin (in) dependent Wnt signaling pathway. 3T3-L1 cells were transfected with pSPORT-Wnt5b or pcDNA3.1 empty vector and differentiated in the presence of resp. 20% L-cell control medium, 20% Wnt5a-CM, or 20% Wnt3a-CM. Total RNA was isolated at indicated time-points, subjected to qPCR and expression was normalized with Cyclophilin A (*CypA*) expression. Error bars indicate SD of triplo RNA measurements for one representative experiment. (A) Illustration of the experimental setup to study the effect of *Wnt5b* overexpression in combination with stimulation of the β-catenin independent Wnt signaling pathway by Wnt5a-CM or by stimulation of the β-catenin dependent Wnt signaling pathway with Wnt3a-CM. (B) *aP2* mRNA expression of *Wnt5b* overexpressed 3T3-L1 cells compared to cells transfected with empty vector/time-point/medium. (C) *PPARγ* mRNA expression of *Wnt5b* overexpressed 3T3-L1 cells compared to cells transfected with empty vector/time-point/medium. $^{*}p < 0.05$ *Wnt5b* overexpressed cells vs. empty vector transfected cells; $^{\#}p < 0.05$ Wnt3a-CM vs. Wnt5a-CM and control medium; $\hat{}p < 0.05$ *Wnt5b* overexpressed cells in control medium vs. *Wnt5b* overexpressed cells in Wnt5a-CM.

The effect of Wnt5b overexpression on adipogenesis with stimulated β-catenin dependent Wnt signaling

The β-catenin dependent Wnt pathway was stimulated by incubation with Wnt3a-CM. which resulted in decreased adipogenesis as assessed by *aP2* and *PPARγ* expression (Fig. 2). *Wnt5b* overexpression resulted in significantly increased *aP2* and *PPARγ* expression. However, *Wnt5b* stimulated adipogenesis in Wnt3a-CM was still significantly less than in control medium without *Wnt5b* overexpression. Additionally, *Wnt5a* expression was reduced in cells incubated with Wnt3a-CM, but overexpression of *Wnt5b* significantly increased *Wnt5a* expression when incubated with Wnt3a-CM (Fig. 3).

To verify at protein level that Wnt3a-CM indeed stimulates β-catenin dependent Wnt signaling and the effect of *Wnt5b* overexpression, β-catenin quantification in cytoplasmatic and nuclear protein extracts was performed a day 0. Wnt3a-CM resulted in a threefold increase in nuclear β-catenin when compared to control medium. On protein level, at day 0, 28% β-catenin was observed in 3T3-L1 cells incubated in Wnt3a-CM and transfected with empty vector. In 3T3-L1 cells overexpressing *Wnt5b* and incubated with Wnt3a-CM, nuclear β-catenin was 17%, while cells incubated with control medium, transfected with Wnt5b overexpression construct or empty vector, nuclear β-catenin was, respectively, 12% and 10% (Fig. 4.). Wnt5b overexpression in cells incubated in WNT3a-CM results in a ~65% reduction in β-catenin translocation, but is still ~35% higher than higher than in cells incubated with control medium.

The effect of Wnt5b overexpression on adipogenesis with stimulated β-catenin independent Wnt signaling

Wnt5a conditioned medium (Wnt5a-CM) yields excessive availability of Wnt5a protein, which activates the β-catenin independent Wnt signaling pathway. We verified on protein level that Wnt5a-CM counteracts the β-catenin dependent Wnt- signaling in 3T3-L1 cells by quantifying nuclear and cytoplasmatic β-catenin from cells incubated with 20% Wnt3a-CM, or with both 20% Wnt3a-CM and 20% Wnt5a-CM. Addition of Wnt5a-CM resulted in a 60% reduction in nuclear β-catenin, indicating that Wnt5a-CM inhibits β-catenin dependent Wnt signaling in 3T3-L1 cells (data not shown).

We explored if high availability of Wnt5a by addition of Wnt5a-CM had the same stimulatory effect on adipogenic potential as *Wnt5b* overexpression, and explored if a combination of *Wnt5b* overexpression and Wnt5a-CM (Fig. 2) would increase adipogenic potential further. As shown in Fig. 2, Wnt5a-CM itself did not enhance *aP2* or *PPARγ* expression compared to control medium, but *Wnt5b* overexpression in combination with Wnt5a-CM resulted in significantly enhanced *aP2* (Fig. 2B) and *PPARγ* (Fig. 2C) expression at day 2 and 10. *Wnt5a* expression was reduced in Wnt5a-CM, which indicates compensation by paracrine signaling.

Discussion

Inhibition of β-catenin dependent Wnt signaling and stimulation of β-catenin independent Wnt signaling has been shown to

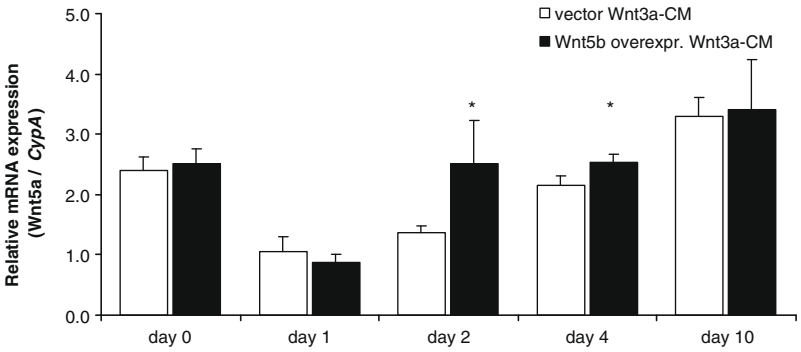

Fig. 3. *Wnt5b* overexpression enhances *Wnt5a* expression during 3T3-L1 differentiation with Wnt3a-CM. 3T3-L1 cells were transfected with pSPORT-Wnt5b or pcDNA3.1 empty vector and differentiated in the presence of 20% Wnt3a-CM. Total RNA was isolated at indicated time-points, subjected to qPCR and *Wnt5a* expression was normalized to the expression of Cyclophilin A (*CypA*). Error bars indicate SE ($n = 2$).

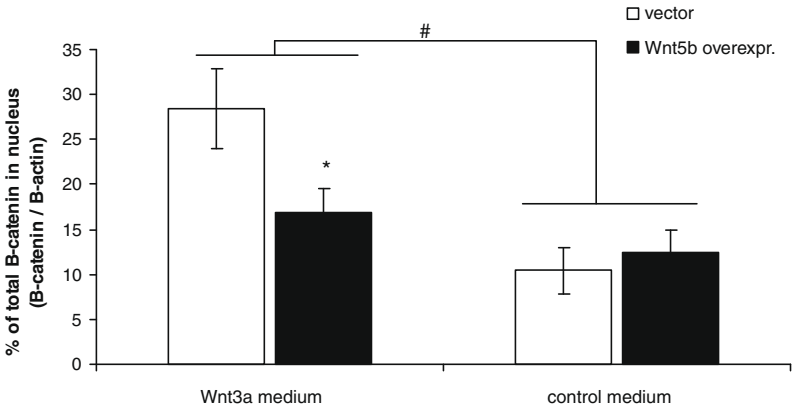

Fig. 4. Wnt5b overexpression reduces translocation of β-catenin to the nucleus. 3T3-L1 cells were transfected with pSPORT-Wnt5b or pcDNA3.1 empty vector and after 16 h incubation with 20% Wnt3a-CM, nuclear and cytoplasmic proteins were isolated, loaded on SDS–PAGE gel, and incubated with anti-β-catenin. β-catenin level was normalized to the level of β-actin for loading. $^{*}p < 0.05$ *Wnt5b* overexpressed cells vs. vector transfected cells; $^{\#}p < 0.05$ Wnt3a-CM vs. control medium. Error bars indicate SD ($n = 2$).

be required for adipogenesis, and altered Wnt signaling has been observed in adipose tissue of T2DM subjects [1]. *WNT5b* was down-regulated in T2DM preadipocytes whereas the expression of its paralog *WNT5a* was unchanged (Van Tienen et al., submitted). The aim of our study was to determine the functional significance of this observation. First we determined the expression of both genes during adipogenesis. Analysis of mRNA and protein sequences showed that Wnt5a and Wnt5b are moderately conserved (±70%) at the mRNA level and highly conserved (±95%) at the protein level between mouse and human. Therefore, we used the mouse preadipocyte cell line 3T3-L1, which allows reproducible differentiation, to analyze expression of these genes during adipogenesis. *Wnt5a* was more abundantly expressed than *Wnt5b*, but both were down-regulated upon induction of differentiation. Overexpression of *Wnt5b* in control medium resulted in increased *PPARγ* and *aP2* expression, which are markers for adipogenesis (Fig. 2B and C). In addition, stimulation of the β-catenin independent Wnt signaling pathway by enhancing Wnt5a protein availability (Wnt5a-CM) combined with *Wnt5b* overexpression did not enhance adipogenesis compared to control medium with *Wnt5b* overexpression. Stimulation of the β-catenin dependent Wnt signaling by Wnt3a has been shown to decrease adipogenic potential [4]. Inhibition of adipogenesis was also observed with Wnt3a-CM, but *Wnt5b* overexpression could partly overcome this inhibition and increase *PPARγ* and *aP2* expression (Fig. 2B and C). Still, *aP2* expression was lower with Wnt3a-CM than control medium. On protein level, nuclear β-catenin translocation was partially inhibited (65%) by *Wnt5b* overexpression. These data indicate that *Wnt5b* is a potent stimulator of adipogenesis, but is in our model not sufficient to completely overcome the inhibition of adipogenesis induced by Wnt3a-CM. Additionally, stimulation of the β-catenin dependent Wnt signaling pathway with Wnt3a-CM resulted in reduced expression of β-catenin independent *Wnt5a*, but *Wnt5b* overexpression was able to enhance *Wnt5a* expression, indicating that *Wnt5b* can enhance adipogenesis by stimulating *Wnt5a* expression and inhibiting β-catenin dependent Wnt signaling. In contrast to the stimulation by *Wnt5b* overexpression, enhancing availability of Wnt5a protein by using Wnt5a-CM inhibited β-catenin translocation to the nucleus, but did not enhance adipogenesis or PPARγ expression. Additionally, decreased *Wnt5a* expression was observed, indicating that paracrine signaling limits the *Wnt5a* availability. Our data suggests that *Wnt5b* is more crucial for stimulation of key regulators of adipogenesis than *Wnt5a*. The down-regulated expression of *Wnt5a* during differentiation is similar to that previously reported for *Wnt5a* by Nishizuka et al. [7], but the *Wnt5b* down-regulation is contradictory to the up-regulation at day 2 observed by Kanazawa et al. [9]. Since both *Wnt5a* and *Wnt5b* function as inhibitors of β-catenin dependent Wnt signaling, and this inhibition of β-catenin dependent Wnt signaling at the initial phase of adipogenesis is required to allow adipogenesis, the observed expression pattern fits their function [13,14]. Additionally, *Wnt5b* can stimulate *PPARγ* expression and subsequently enhance adipogenesis. However, *PPARγ* is expressed from day 2 of differentiation, and not when *Wnt5b* has its highest expression, this stimulation is possibly mediated through upstream activators of *PPARγ*, like *C/EBPβ* or *C/EBPδ*, which are expressed in the same time-frame as *Wnt5b* [15].

The down-regulation of β-catenin independent *Wnt5b* in preadipocytes could contribute to decreased adipogenesis as observed in insulin resistance and Type 2 diabetic subjects by reduced stimulation of *PPARγ* [1]. However, the exact mechanism by which *Wnt5b* stimulates *PPARγ* remains to be elucidated.

Acknowledgments

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