RNA Biology
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/krnb20

MicroRNA-30c promotes human adipocyte differentiation and co-represses PAI-1 and ALK2

Michael Karbiener, Claudia Neuhold, Peter Opriessnig, Andreas Prokesch, Juliane G. Bogner-Strauss & Marcel Scheideler

Published online: 31 Aug 2011.

To cite this article: Michael Karbiener, Claudia Neuhold, Peter Opriessnig, Andreas Prokesch, Juliane G. Bogner-Strauss & Marcel Scheideler (2011) MicroRNA-30c promotes human adipocyte differentiation and co-represses PAI-1 and ALK2, RNA Biology, 8:5, 850-860, DOI: 10.4161/rna.8.5.16153

To link to this article: http://dx.doi.org/10.4161/rna.8.5.16153

RNA Biology 8:5, 850-860; September/October 2011; © 2011 Landes Bioscience

MicroRNA-30c promotes human adipocyte differentiation and co-represses *PAI-1* and *ALK2*

Michael Karbiener, Claudia Neuhold, Peter Opriessnig, Andreas Prokesch, Juliane G. Bogner-Strauss and Marcel Scheideler*

Institute for Genomics and Bioinformatics; Graz University of Technology; Graz, Austria

Key words: microRNA, miR-30c, adipogenesis, adipocyte, differentiation, obesity, *PAI-1*, ALK2

Abbreviations: 3'UTR, 3' untranslated region; *ALK2*, activin receptor-like kinase 2 (*ACVR1*, *ACTRI*); BMI, body mass index; *BMP*, bone morphogenetic protein; *C/EBP*, CCAAT enhancer binding protein; DM, differentiation medium; DMEM, Dulbecco's Modified Eagle's Medium; *FABP4*, fatty acid binding protein 4; *FASN*, fatty acid synthase; FBS, fetal bovine serum; h*FGF2*, fibroblast growth factor 2; FOP, fibrodysplasia ossificans progressiva; *GLUT4*, glucose transporter 4; HEK293 cells, human embryonic kidney 293 cells; HFD, high-fat diet; hMADS cells, human multipotent adipose-derived stem cells; IBMX, 3- Isobutyl-1-methylxanthine; MEFs, mouse embryonic fibroblasts; miRNA, microRNA; *NFYC*, nuclear transcription factor Y, γ; NTC, non-targeting control; *PAI-1*, plasminogen activator inhibitor 1 (*SERPINE1*); PBS, phosphate buffered saline; PCR, polymerase chain reaction; *PlGF*, placenta growth factor; PM, proliferation medium; *PPARγ*, peroxisome proliferator-activated receptor γ; qRT-PCR, quantitative reverse transcription polymerase chain reaction; TG, triglycerides; *TNFα*, tumor necrosis factor α; *u-PA*, urokinase-type plasminogen activator; *u-PAR*, urokinase plasminogen activator receptor; *VN*, vitronectin; WAT, white adipose tissue

Obesity is characterized by excessive adipose tissue mass and associated with type 2 diabetes and cardiovascular diseases. To fight obesity and its sequels, elucidating molecular events that govern adipocyte differentiation and function is of key importance. MicroRNAs (miRNAs) are a novel class of non-coding RNAs that have been shown to regulate crucial cellular processes, including differentiation. Several studies have already assigned miRNAs to distinct functions in murine adipocyte differentiation but only a few studies did so for humans.

Here, we investigated the function of miR-30c in human adipogenesis. miR-30c expression was increased during adipogenesis of human multipotent adipose-derived stem (hMADS) cells, and miR-30c overexpression enforced adipocyte marker gene induction and triglyceride accumulation. miRNA target prediction revealed two putative direct targets of miR-30c, *PAI-1 (SERPINE1)* and *ALK2 (ACVR1, ACTRI)*, both inversely regulated to miR-30c during adipogenesis and responsive to miR-30c overexpression. Luciferase reporter assays confirmed *PAI-1* and *ALK2* as direct miR-30c targets. Moreover, reciprocal expression between miR-30c and *PAI-1* could also be demonstrated in white adipose tissue of obesity mouse models, suggesting a potential physiological role of miR-30c for *PAI-1* regulation in the obese state. Validating *PAI-1* and *ALK-2* as miR-30c mediators in adipogenesis revealed that not single silencing of *PAI-1* or *ALK2*, but only co-silencing of both phenocopied the pro-adipogenic miR-30c effect. Thus, miR-30c can target two, so far not interconnected genes in distinct pathways, supporting the idea that miRNAs might coordinate larger regulatory networks than previously anticipated.

Introduction

A dysbalance between energy intake and energy expenditure causes obesity that has become a global epidemic with a still increasing prevalence in industrialized as well as developing countries.[1,2]

In addition to its function as energy storage, adipose tissue is also an endocrine organ, secreting various cytokines that influence not only whole-body energy homeostasis, but also inflammation and fibrinolysis.[3] Follow-up complications of the obese state like type 2 diabetes or cardiovascular diseases are not only linked to the exhausted lipid-storage capacity of adipose tissue but also to altered serum levels of adipose tissue-secreted factors.[4] Elucidation of molecular events that govern both adipocyte differentiation and function is therefore of key importance to further dissect the obese state and its pathophysiological consequences.

Recently, microRNAs (miRNAs) have been identified as a group of endogenous RNAs with important gene-regulatory roles. By binding to mRNAs of protein-coding genes to direct their post-transcriptional repression, miRNAs have been shown to regulate crucial cellular processes such as development, differentiation, growth and metabolism.[5] Indeed, interference with the endogenous miRNA processing machinery has profound effects on adipogenesis: silencing of *Drosha*, the endonuclease generating pre-miRNAs from the primary transcript, strongly impaired

*Correspondence to: Marcel Scheideler; Email: marcel.scheideler@tugraz.at
Submitted: 02/21/11; Revised: 04/15/11; Accepted: 04/23/11
DOI: 10.4161/rna.8.5.16153

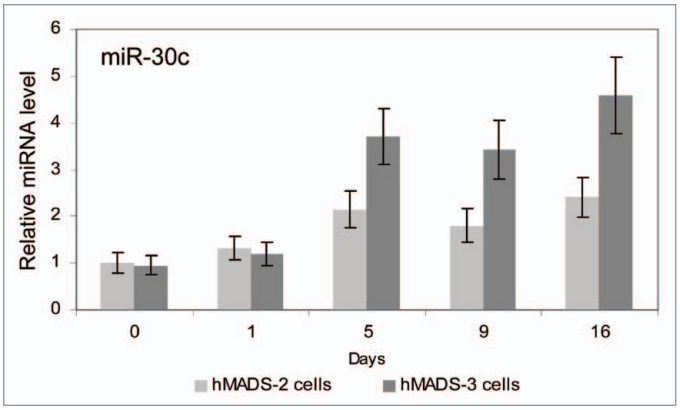

Figure 1. Changes in miR-30c levels during adipogenesis of hMADS cells. hMADS-2 and hMADS-3 cells were stimulated to undergo adipocyte differentiation two days post confluence (day 0). Total RNA was prepared at the indicated time points and subjected to quantitative real-time RT-PCR for miR-30c. miR-30c abundance was normalized to 5S rRNA and is presented relative to day 0.

differentiation of 3T3-L1 cells,[6] whereas silencing of *Dicer*, the endonuclease transforming pre-miRNAs into the mature form, had a strong inhibitory effect on adipogenesis of human mesenchymal stem cells.[7] Mouse embryonic fibroblasts (MEFs) and preadipocytes expressing mutated, non-functional *Dicer* were unable to undergo adipogenesis.[8] Importantly, knockout of *Dicer* in adipose tissue (using *Dicer*-conditional, aP2-Cre transgenic mice) resulted in a severe depletion of white adipose tissue (WAT) in vivo.[9] Collectively, these results suggest a strong involvement of miRNAs in the process of adipogenesis. In line with this, several in vitro studies have already revealed distinct miRNAs to steer murine adipocyte differentiation,[6,10-17] while only a few studies did so for human.[18-21]

We are interested in miRNAs with a regulatory role in adipocyte differentiation and function, particularly in human. In this study, we focused on miR-30c, which is induced during adipocyte differentiation of human multipotent adipose-derived stem (hMADS) cells, a unique human model system for adipogenesis.[22-24] Overexpression of miR-30c in hMADS cells enforced adipocyte marker gene induction and triglyceride accumulation. Target prediction analyses revealed as putative direct miR-30c targets *PAI-1* and *ALK2*, each with a single, conserved miR-30c response element in its 3'UTR. During adipogenesis, *PAI-1* and *ALK2* were inversely regulated to miR-30c and responsive to miR-30c overexpression. Luciferase reporter assays indeed confirmed *PAI-1* and *ALK2* as direct miR-30c targets and validated the predicted miR-30c response elements in the *PAI-1* and *ALK2* 3'UTR. An inverse expression between miR-30c and *PAI-1* could also be demonstrated in WAT of obesity mouse models, suggesting a potential physiological role of miR-30c for *PAI-1* regulation in the obese state. To assess the function of both genes as mediators of miR-30c in adipogenesis, we silenced *PAI-1* and *ALK2* during adipocyte differentiation of hMADS cells. Intriguingly, while single silencing of *PAI-1* or *ALK2* did not affect adipogenesis, co-silencing of both targets phenocopied the pro-adipogenic miR-30c effect. These results suggest that miR-30c functions as enhancer of human adipogenesis, at least partly via direct targeting of *PAI-1* and *ALK2*, thereby indicating an unexpected, cooperative and synergistic function in human adipogenesis.

Results

miR-30c is upregulated during adipocyte differentiation. We identified changes in expression levels of miR-30c in microarray studies of human multipotent adipose-derived stem (hMADS) cells and mouse embryonic fibroblasts (MEF) during adipocyte differentiation (**Fig. S1**). Using qRT-PCR, we confirmed these data in hMADS-2 and hMADS-3 cells, originally established from two different donors (**Fig. 1**). In both cases, miR-30c levels increased 2- to 4-fold during adipogenesis, with a large increase at early stage between day 1 and day 5. Assuming that the increase of miR-30c is associated with adipogenesis, we hypothesized that altering miR-30c levels should modulate adipocyte differentiation.

miR-30c promotes adipocyte differentiation of hMADS cells. To assess our hypothesis, we transiently transfected hMADS-2 and hMADS-3 cells at confluence with miR-30c mimics or non-targeting control (miR-NTC), followed 48 h later by induction of adipocyte differentiation. miR-30c transfected hMADS cells exhibited a 50- to 300-fold increase of mature miR-30c abundance as measured by qRT-PCR 3 and 5 days post-transfection (**Fig. S2**). Notably, for both hMADS cell populations, increasing miR-30c abundance resulted in enhanced induction of adipocyte marker genes compared to miR-NTC transfection (**Fig. 2A**). *PPARγ* and *FABP4* expression already increased at day 1, and *C/EBPα* reached increased levels at day 3 of differentiation. While for hMADS-2 cells elevated marker gene expression was blunted at day 9 (**Fig. 2B,** upper part), the miR-30c effect was still evident in hMADS-3 cells, as indicated by still increased expression levels of *PPARγ*, *C/EBPα*, *FABP4*, *FASN* and *GLUT4* (**Fig. 2B,** lower part).

To further investigate the impact of miR-30c on differentiation, lipid accumulation of miR-30c or miR-NTC transfected hMADS cells was analyzed at day 9 of differentiation by Oil Red O staining and quantification of intracellular triglycerides (TG). In line with elevated mRNA levels of adipocyte marker genes, miR-30c significantly increased TG accumulation of hMADS cells (**Fig. 2C**). Interestingly, although TG levels of control transfected cells differed between hMADS-2 and hMADS-3 cells (**Fig. 2C**), miR-30c enhanced TG levels to a similar value of approximately 1.5 μmol TG/mg protein at day 9. Altogether, marker gene expression as well as TG accumulation indicate that miR-30c promotes adipogenesis of hMADS cells.

Identification and analysis of predicted miR-30c targets. To search for putative direct target mRNAs of miR-30c, we generated an intersection of those genes that were jointly predicted by miRanda,[25] PicTar,[26] TargetScan[27] and ElMMo.[28] We identified *PAI-1* (*SERPINE1, NM_000602*) and *ALK2* (*ACVR1, ACTRI, NM_001150)* as putative direct miRNA targets, each with a single miR-30c binding site that is highly conserved among mammals (**Fig. 3A**). Interestingly, *PAI-1*

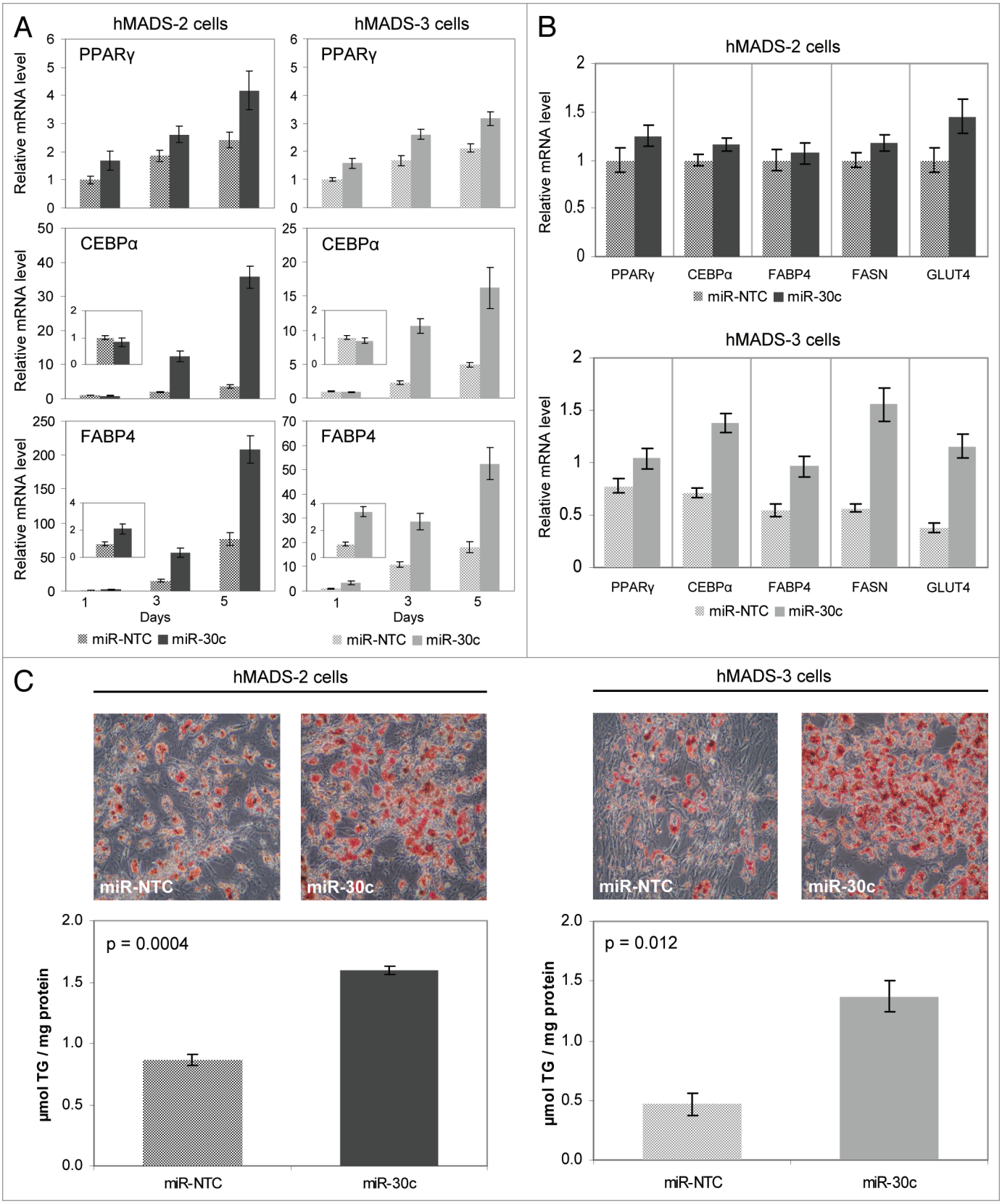

Figure 2. For figure legend, see page 853.

Figure 2 (See opposite page). miR-30c promotes adipocyte differentiation of hMADS cells. hMADS-2 and hMADS-3 cells were transfected at confluence (day -2) with 5 nM miR-30c mimic or non-targeting control mimic (miR-NTC). Adipocyte differentiation was induced 48 h later (day 0). (A) RNA of cells at day 1, 3 and 5 after induction of adipocyte differentiation was analyzed by quantitative real-time RT-PCR for expression of *PPARγ*, *C/EBPα* and *FABP4*. mRNA levels were normalized to *TBP* and are presented relative to miR-NTC transfected cells at day 1. For *C/EBPα* and *FABP4*, framed inserts depict expression levels at day 1. (B) RNA of cells at day 9 of adipocyte differentiation was analyzed by quantitative real-time RT-PCR for expression levels of *PPARγ*, *C/EBPα*, *FABP4*, *FASN* and *GLUT4*. mRNA levels were normalized to *TBP* and are presented relative to miR-NTC transfected hMADS-2 cells. (C) Analysis of triglyceride accumulation at day 9 of adipocyte differentiation. Representative pictures of hMADS-2 and hMADS-3 cells stained with Oil Red O for visualization of intracellular triglycerides are shown in the upper part. Quantification of triglyceride accumulation, relative to total protein, is depicted in the lower part (n = 3).

and *ALK2* are downregulated during adipocyte differentiation of hMADS cells as indicated by gene expression analysis (**Fig. 3B**). To investigate whether *PAI-1* and *ALK2* are responsive to miR-30c, we transfected miR-30c and monitored the expression levels of *PAI-1* and *ALK2* afterwards. Indeed, *PAI-1* and *ALK2* mRNA levels were decreased in miR-30c transfected cells compared with miR-NTC transfected cells (**Fig. 3C**). Furthermore, inhibition of endogenous miR-30c by transfection of antisense oligonucleotides resulted in upregulation of *PAI-1* and *ALK2* (**Fig. S3**).

miR-30c directly targets human *PAI-1* and *ALK2*. To validate the predicted interaction of miR-30c with the *PAI-1* and *ALK2* mRNAs, the 3'UTRs of human *PAI-1* and *ALK2* were cloned into the psiCHECK-2 vector downstream the Renilla

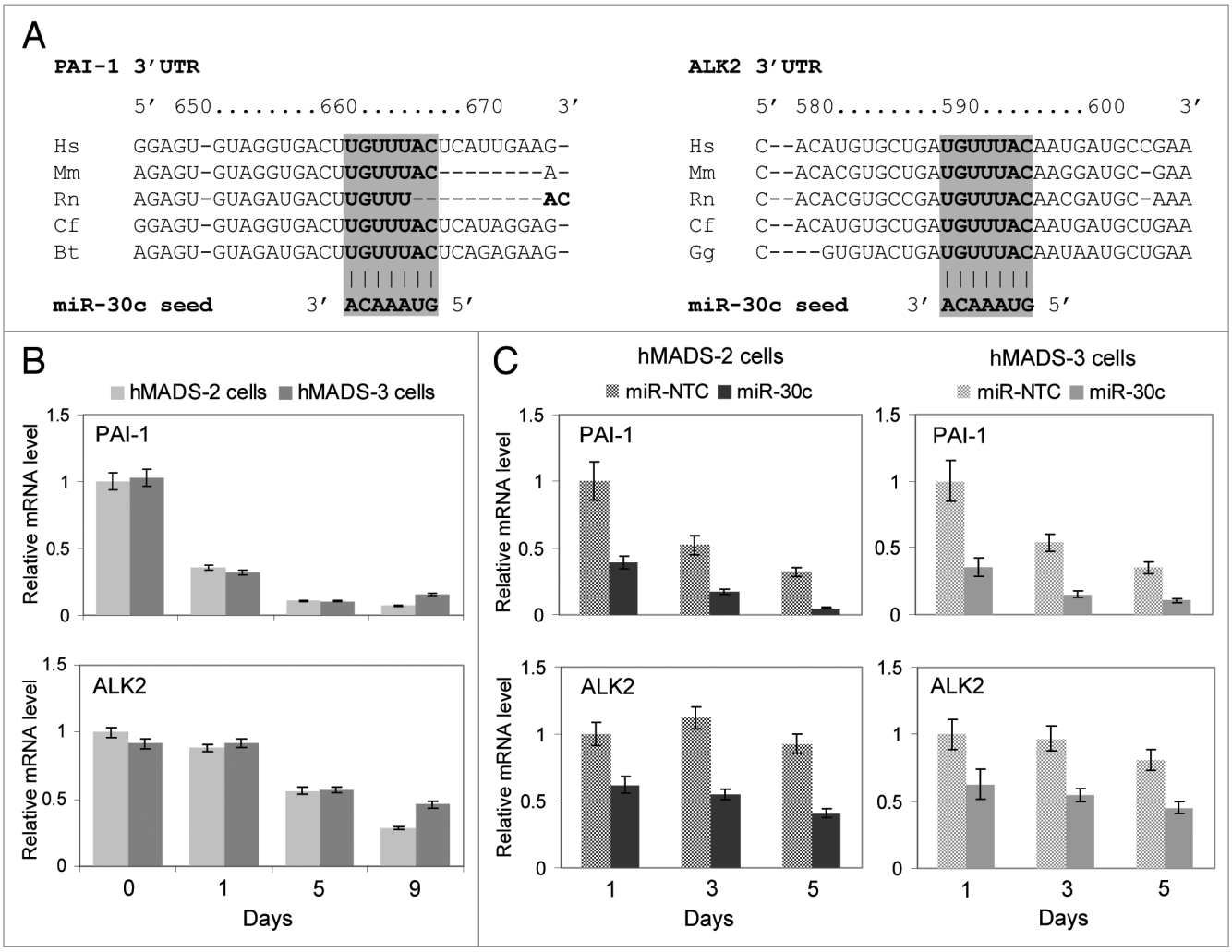

Figure 3. *PAI-1* and *ALK2* are predicted miR-30c targets with inverse expression to miR-30c. (A) Sequence conservation of the predicted miR-30c binding sites in the 3'UTRs of *PAI-1* and *ALK2*, with the miR-30c seed and seed matches highlighted in grey. (B) hMADS-2 and hMADS-3 cells were analyzed by quantitative real-time RT-PCR for *PAI-1* and *ALK2* mRNA levels at day 0, 1, 5 and 9 of adipocyte differentiation. mRNA levels were normalized to *TBP* and are presented relative to day 0. (C) hMADS-2 and hMADS-3 cells were transfected at confluence (day -2) with 5 nM miR-30c mimic or non-targeting control mimic (miR-NTC). Adipocyte differentiation was induced 48 h later (day 0). *PAI-1* and *ALK2* mRNA levels were analyzed by quantitative real-time RT-PCR at day 1, 3 and 5. mRNA levels were normalized to *TBP* and are presented relative to miR-NTC transfected cells at day 1.

luciferase coding sequence and co-transfected with miRNA mimics into human embryonic kidney 293 (HEK293) cells. Indeed, co-transfections of the *PAI-1* and *ALK2* reporters with miR-30c resulted in 40% and 30% lower relative luciferase activity compared to co-transfections with miR-NTC, respectively (**Fig. 4A and B**). Thus, miR-30c directly binds to the 3'UTRs of *PAI-1* and *ALK2*.

To investigate whether the predicted miR-30c binding sites mediate the repressive effect on *PAI-1* and *ALK2*, we performed site-directed mutagenesis of the putative seed matches. Indeed, mutation of the predicted miR-30c seed match derepressed relative luciferase activity for both *PAI-1* and *ALK2* reporters, thus abolishing the inhibitory effect of miR-30c (**Fig. 4A and B**). These results demonstrate that *PAI-1* and *ALK2* are both regulated by miR-30c, each via a single miR-30c binding site in its 3'UTR.

Reciprocal changes of miR-30c and *PAI-1* levels in the obese state. Plasma *PAI-1* levels have been shown to rise with increasing body mass index (BMI),[29,30] presumably contributing to the increased risk of obese subjects for type 2 diabetes and atherothrombotic events.[31] In line with these findings, pro-inflammatory cytokines which are increased in the obese state, e.g., tumor necrosis factor α (*TNF*α), are known to induce *PAI-1* expression;[32,33] however, *PAI-1* regulation is not yet completely understood. We thus aimed to investigate a potential role of miR-30c as mediator of the *PAI-1* induction observed in the obese state. Therefore, we analyzed the levels of miR-30c and *PAI-1* in WAT in mouse models of nutritionally or genetically induced obesity. Indeed, WAT of ob/ob mice not only showed the expected increases in *PAI-1* mRNA levels compared to wild type mice, but also lower levels of miR-30c (**Fig. 5A**). A similar result was obtained for WAT samples of wild type mice fed a high-fat diet (HFD) compared to littermates on a chow diet (**Fig. 5B**). Thus, the reciprocal expression of miR-30c and *PAI-1*, which was already observed during adipocyte differentiation in vitro, appears to be existent also in vivo in WAT. In contrast, *ALK2* mRNA levels did not show any differential expression between obese and normal weight states (**Fig. 5A and B**).

Single silencing of *PAI-1* or *ALK2* does not reproduce the miR-30c effect on adipogenesis. As *PAI-1* and *ALK2* are direct targets of miR-30c, we aimed at analyzing whether either of them mediates the pro-adipogenic miR-30c effect. *PAI-1* is a protein secreted by adipose tissue[34] which has been implicated in various complications of the obese state,[31] though its role in adipogenesis appears controversial.[35,36] *ALK2* is a bone morphogenetic protein (BMP) type I receptor known to mediate *BMP7* signalling[37] and has been shown to regulate *BMP9*-induced osteogenic differentiation[38] as well as chondrogenesis.[39] Thus, a regulatory function of *ALK2* might be hypothesized also for adipogenesis.

To assess whether *PAI-1* or *ALK2* play a role in human adipogenesis, we transfected hMADS cells with siRNAs to silence each target (siPAI-1, siALK2), and subsequently induced adipocyte differentiation. Neither *PAI-1* nor *ALK2* silencing evoked any changes in TG accumulation (**Fig. 6A**) or in the expression

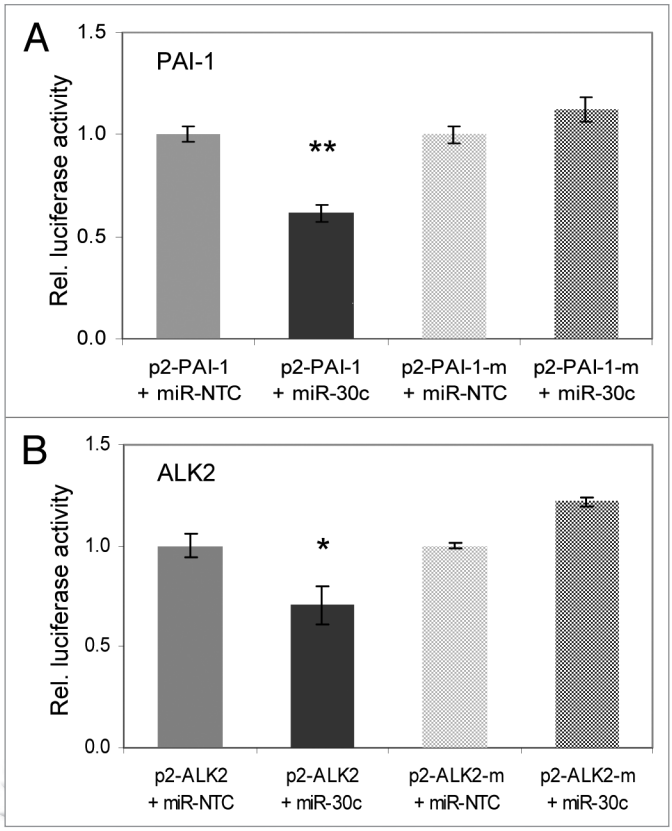

Figure 4. miR-30c directly targets human *PAI-1* and *ALK2*. Luciferase reporter vectors, containing either (A) the wild type 3'UTR of *PAI-1* (p2-PAI-1) or (B) *ALK2* (p2-ALK2), or 3'UTRs with mutated miR-30c seed matches (p2-PAI1-m and p2-ALK2-m, respectively), were co-transfected with 50 nM miR-30c mimic or non-targeting control mimic (miR-NTC) into HEK293 cells. 48 h later, cells were harvested and Luciferase assays were performed. For each sample, Renilla luciferase activity was normalized to firefly luciferase. Data are presented relative to miR-NTC transfections (n = 3). *p < 0.05; **p < 0.01 vs. corresponding co-transfection with miR-NTC.

of adipocyte marker genes (**Fig. 6B**) at day 9 compared to control (siNTC) transfected cells. Thus, the pro-adipogenic miR-30c effect could not be reproduced by single silencing of the validated miR-30c targets *PAI-1* or *ALK2*.

Co-silencing of *PAI-1* and *ALK2* phenocopies the miR-30c effect on human adipogenesis. In light of these results, we aimed to investigate whether both targets have a cooperative impact on adipogenesis. We transfected hMADS cells either with a pool of siRNAs against *PAI-1* and *ALK2*, or with siNTC, and subsequently initiated adipocyte differentiation. Intriguingly, Oil Red O staining at day 9 revealed that co-silencing of *PAI-1* and *ALK2* resulted in elevated lipid accumulation compared to siNTC, and quantification of triglycerides revealed that this increase was significant (**Fig. 6C**). Furthermore, the adipocyte marker genes *PPAR*γ, *C/EBP*α, *FABP4*, *FASN* and *GLUT4* showed elevated mRNA levels upon co-silencing of *PAI-1* and *ALK2* (**Fig. 6D**). We thus demonstrate that co-silencing of *PAI-1* and *ALK2* phenocopies, at least in part, the promoting effect of miR-30c on human adipogenesis.

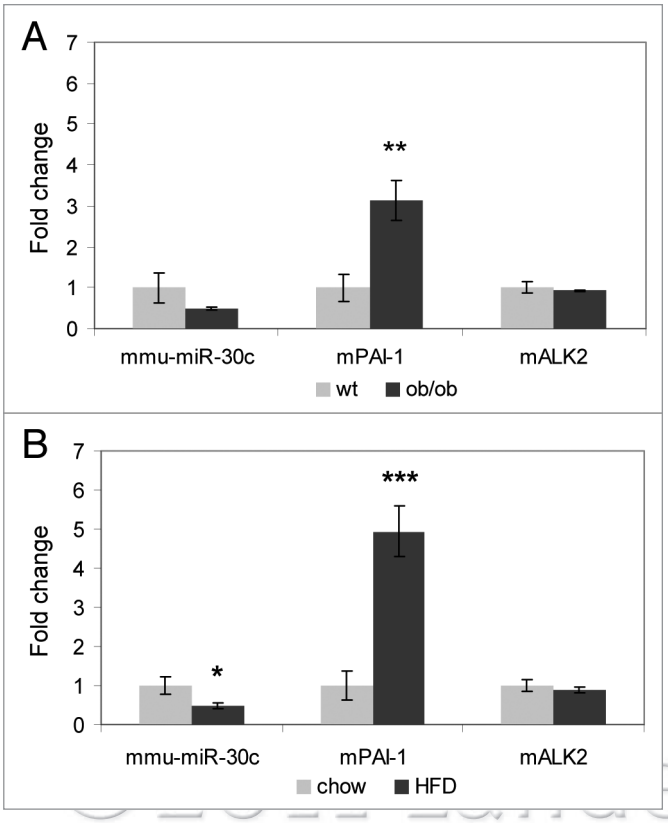

Figure 5. Analysis of miR-30c, *ALK2* and *PAI-1* expression in white adipose tissue of murine obesity models. (A) Ob/ob mice (n = 3) and their wild type littermates (n = 6) were fed a chow diet after weaning. RNA from WAT of 4 months old mice was analyzed by quantitative real-time RT-PCR for expression levels of miR-30c, *mPAI-1* and *mALK2*. 5S rRNA was used as internal reference for miR-30c; *mUxt* mRNA was used as internal reference for *mPAI-1* and *mALK2*. Data is presented relative to wild type mice. **p < 0.01 vs. mice on chow diet. (B) Wild type mice were fed a chow diet (n = 3) or a high fat diet (HFD, n = 5) after weaning. RNA from WAT of 4 months old mice was analyzed by quantitative real-time RT-PCR for expression levels of miR-30c, *mPAI-1* and *mALK2* as described above. *p < 0.05; ***p < 0.001 vs. wild-type mice on chow diet.

Discussion

In this study, we first identified miR-30c to be upregulated during early adipocyte differentiation of murine and human cells (**Fig. 1 and Fig. S1**). Subsequent functional analysis with hMADS cells, a unique model system to study human adipogenesis,[22-24] demonstrated that miR-30c promotes adipocyte differentiation, as evidenced by accelerated upregulation of the adipogenic key transcription factors *PPAR*γ and *C/EBP*α, ultimately resulting in increased expression of adipocyte marker genes and enhanced triglyceride accumulation (**Fig. 2A–C**).

miR-30c belongs to the miR-30 family, which comprises five distinct members (a to e) that are perfectly conserved between mouse and human, and is expressed in a variety of different tissues. In line with this, studies have either proposed or validated functions of miR-30 family members in numerous types of cancer,[40-46] but also in several other biological contexts such as myocardial matrix remodelling,[47] apoptosis[48] and kidney development.[49] A possible involvement of the miR-30 family in adipocyte function was first conceivable after a global miRNA expression analysis during adipogenesis of human preadipocytes showed a potent upregulation of miR-30c expression.[18] This finding was subsequently confirmed for adipogenesis of mouse 3T3-L1 cells.[50] In line with both studies, we identified miR-30c in a miRNA screening during adipogenesis of hMADS cells as one of the miRNAs with the most dramatic upregulation. Moreover, we demonstrate for the first time a function for this member of the miR-30 family in adipocyte differentiation (**Fig. 2**).

Mature miR-30c can be generated from two distinct pre-miRNAs, both residing in intronic regions of distinct host genes. While hsa-miR-30c-1 is located within the open reading frame of the nuclear transcription factor Y, γ (*NFYC*) gene (NM_014223) at chromosome 1, hsa-miR-30c-2 is intronic to *C6orf155* (NR_026807), a processed transcript with no known protein product, at chromosome 6. We found both host genes to be expressed during adipocyte differentiation of hMADS cells (**Fig. S4**). Thus, it is reasonable to propose that both loci contribute to miR-30c expression during human adipogenesis. However, the expression profiles are not congruent with miR-30c, as *NFYC* was only modestly upregulated at late stages (day 9 and day 16), while *C6orf155* expression showed a peak at day 5, followed by a decrease (**Fig. S3**). This could be explained by different half-lives of mRNA and miRNA. Alternatively, miR-30c could be transcribed from transcription start sites other than the host gene promoter, i.e. independently of its host genes, as recently shown for several intronic miRNAs.[51] Future studies addressing mechanisms that regulate miR-30c transcription will be of interest and will also enable the integration of this miRNA in the cascade of regulatory events that promote adipogenesis.

As genome-wide studies revealed that a single miRNA can directly regulate hundreds of target mRNAs,[52-54] for any biological process investigated it is therefore obvious that (1) identification of those targets that mediate (at least predominantly) the miRNA effect is challenging; and that (2) the miRNA likely mediates its effect via more than a single target. In order to decrease the false-positive rate for predicted direct miR-30c target candidates to be further analyzed, we combined several prediction algorithms and in-house generated gene expression data of hMADS cells during adipogenesis. As a result, among the most interesting candidates we identified *PAI-1* and *ALK2*, being not only predicted by at least four distinct algorithms, but also showing inverse expression profiles compared to miR-30c expression, i.e., downregulation during adipocyte differentiation (**Fig. 3B**). Furthermore, both genes were responsive to miR-30c overexpression (**Fig. 3C**) and miR-30c inhibition (**Fig. S3**). Lastly, luciferase reporter assays indeed confirmed the direct interaction of miR-30c with *PAI-1* and *ALK2* via single miRNA binding sites in their 3'UTRs (**Fig. 4A and B**). The luciferase activity is quantified by a bioluminescent signal that directly and very sensitively reflects protein levels. As miR-30c overexpression repressed the reporter activity in the presence of the 3'UTR of *PAI-1* and *ALK2*, but not upon miR-30c binding site mutations in these 3'UTRs, miR-30c was able to repress

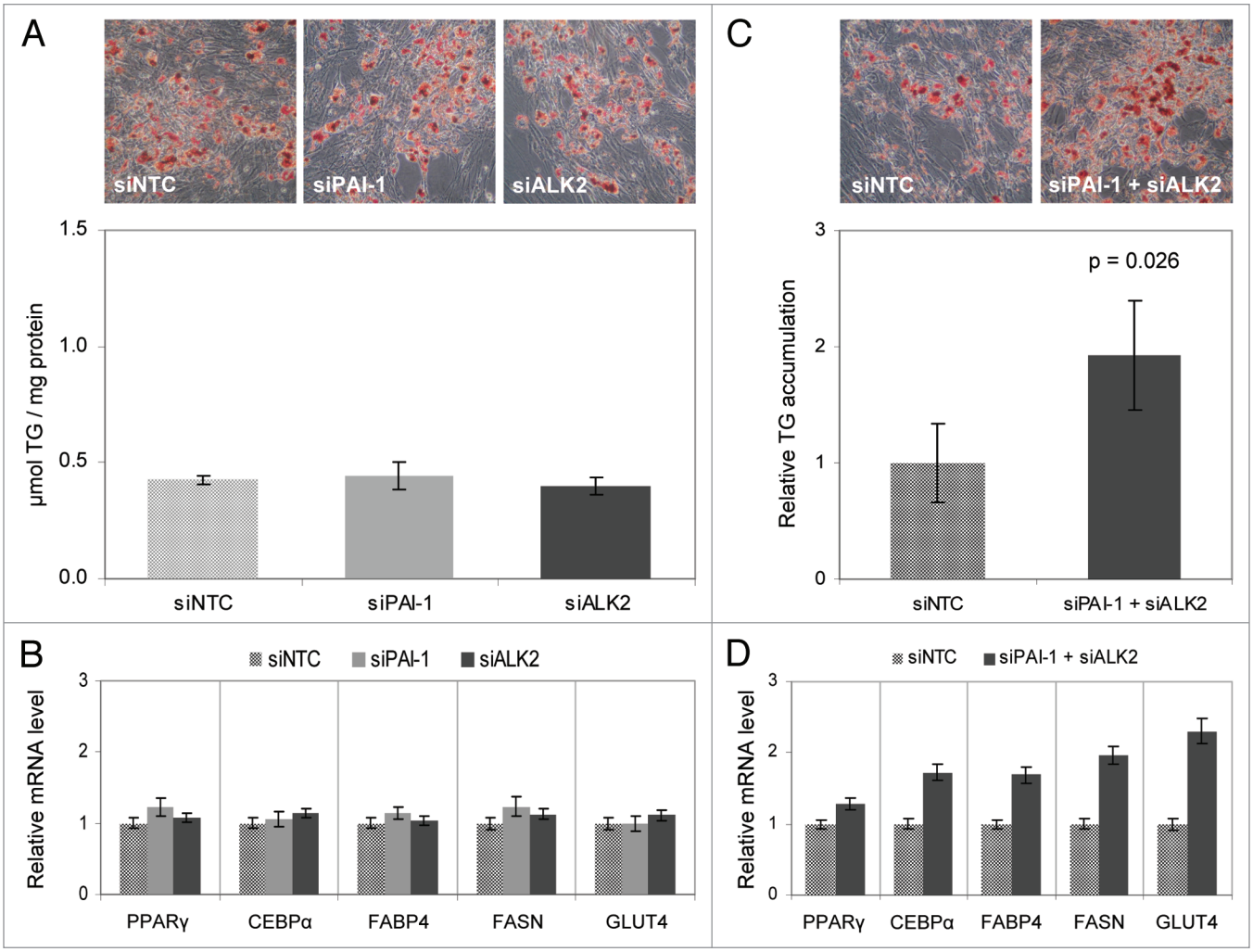

Figure 6. Effects of miR-30c target gene silencing on human adipocyte differentiation. hMADS-3 cells were transfected at confluence (day -2) with 5 nM siRNA against *PAI-1* (siPAI-1) and *ALK2* (siALK2), either separately (A and B) or in combination (C and D), or with equal concentrations of a non-targeting control siRNA (siNTC). Adipocyte differentiation was induced 48 h later (day 0). (A and C) Analysis of triglyceride accumulation at day 9 of adipocyte differentiation. Representative pictures of cells stained with Oil Red O for visualization of intracellular triglycerides are shown in the upper part. Quantification of triglyceride accumulation, relative to total protein, is depicted in the lower part (n = 3). (B and D) Analysis of *PPARγ*, *C/EBPα*, *FABP4*, *FASN* and *GLUT4* mRNA levels by quantitative real-time RT-PCR at day 9 after start of differentiation. mRNA levels were normalized to *TBP* and are presented relative to siNTC transfected cells.

protein levels by direct binding to the 3'UTRs of *PAI-1* and *ALK2*. Moreover, our finding of a direct *PAI-1* regulation by miR-30c was recently confirmed in a different context, as *PAI-1* induction in human pulmonary microvascular endothelial cells by placenta growth factor (*PlGF)* is presumably mediated via downregulation of miR-30c.[55]

It has been demonstrated that several miRNAs are differentially expressed upon genetically induced obesity in mice.[11] In line with this, we demonstrate decreased miR-30c levels in WAT upon genetically as well as diet-induced weight gain (**Fig. 5A and B**). This suggests that miR-30c could be involved in the detrimental effects of obesity. Furthermore, we were interested whether miR-30c and its identified direct targets *PAI-1* and *ALK2* are also reciprocally expressed in this context. While *ALK2* was unaltered, weight gain indeed evoked reciprocal changes in *PAI-1* and miR-30c expression (**Fig. 5A and B**). Among possibilities to explain such difference, distinct expression of the two genes between adipocytes and the cells of the stromal vascular fraction cannot be ruled out. Future experiments should shed some light on that issue.

The function of *PAI-1* in adipogenesis and adipose tissue biology still remains controversial. Liang et al. showed that *PAI-1* overexpression in 3T3-L1 cells inhibits adipocyte differentiation, while preadipocytes from *PAI-1*$^{-/-}$ mice showed stronger differentiation.[35] However, a different study showed no effects of a *PAI-1* neutralizing antibody or *PAI-1* overexpression on adipogenesis of 3T3-F442A preadipocytes, and also comparable adipogenesis of *PAI-1*$^{-/-}$ and wild type MEFs.[56] Investigations of mouse models appear controversial as well: Morange et al. described faster weight gain of PAI1$^{-/-}$ compared to wild type mice on a high-fat diet,[57] and a study of transgenic mice overexpressing *PAI-1* under control of the adipocyte promoter *aP2* showed reduced body

and fat mass compared to wild type mice.[58] In contrast, another study showed that *PAI-1* deficiency protected mice against diet-induced obesity.[59] This apparent divergence of results might be explained by different mouse strains used, by different effects of local or systemic *PAI-1* overexpression or knockout, due to a postulated dose-dependent effect of *PAI-1* on adipogenesis,[36] and lastly also due to different *ALK2* levels. With respect to human adipogenesis, in this study we present evidence that cell-autonomous modulation (via siRNA silencing) of the newly identified miR-30c target *PAI-1* in adipocyte precursors has—on its own—negligible effects on adipocyte differentiation (**Fig. 6A and B**), which is in line with previously published data using murine adipogenesis models.[56]

ALK2 is a receptor tyrosine kinase belonging to the class of BMP type I receptors and has been shown to mediate *BMP7* [37] and *BMP9* signalling.[38] Reports describing a stimulatory role of constitutively active *ALK2* on osteogenic and chondrogenic differentiation[39,60] are in line with the identification of *ALK2* mutations as the cause of fibrodysplasia ossificans progressiva (FOP), a rare autosomal disease characterized by ectopic osteogenesis and chondrogenesis in soft tissues.[61] Concerning adipogenesis, there have been no indications for a direct *ALK2* involvement so far. *BMP7*, though, has been implied in directing mesenchymal stem cell differentiation from white to brown adipogenesis,[62] however, *BMP7* can also signal via type I receptors other than *ALK2*. Similar to *PAI-1*, our study showed no effects of single *ALK2* silencing on adipogenesis of hMADS cells (**Fig. 6A and B**).

Interestingly, combined silencing of both miR-30c targets, *PAI-1* and *ALK2*, enforced adipogenesis (**Fig. 6C and D**), thereby recapitulating the miR-30c effect, at least partly. We thus present, for the first time in adipogenesis, a miRNA that might regulate differentiation via direct targeting of (at least) two genes which operate in distinct signaling pathways. In addition, we have revealed a cooperative, anti-adipogenic action of *PAI-1* and *ALK2*, two proteins that were not known before to be interconnected. This opens up the question how the two corresponding gene regulatory networks might be related. *PAI-1* is known to bind to the extracellular matrix components vitronectin (*VN*) and urokinase-type plasminogen activator (*u-PA*), thereby altering binding of *VN* and *u-PA* to integrins and the urokinase plasminogen activator receptor (*u-PAR, CD87*).[63] Based on our observations, we could envision that subsequent downstream signalling of *PAI-1* via *VN*/*u-PA* and integrins/*u-PAR*, as well as *BMP*-elicited, *ALK2*-mediated signalling might relay two redundant inhibitory signals, each retarding the progression of adipogenesis. Consequently, downregulation of both pathways, which might be mediated by miR-30c via *PAI-1* and *ALK2* targeting, removes the inhibitory signals, thereby promoting adipocyte differentiation. It will be interesting to explore this cross-talk of *PAI-1* and BMP signalling in more detail in the future.

Collectively, our study depicts miR-30c as a promoter of adipocyte differentiation and—via direct targeting of *PAI-1* and *ALK2*—as a possible link between two distinct, so far not interrelated pathways. Thus, our findings support the idea that miRNAs might connect and co-ordinate regulatory networks larger than previously anticipated. Moreover, the identification of direct miRNA targets, combined with the analysis of their cooperative effect on a biological process, can provide novel insights into those larger regulatory networks.

Materials and Methods

Materials. 100 mm cell culture dishes (#664160) and 12-well cell culture plates (#665180) were bought from Greiner. 96-well cell culture plates (#3596) were obtained from Corning. Dulbecco's Modified Eagle's Medium (DMEM, #BE-12-707F) and Ham's F12 Medium (#BE12-615F) were purchased from Lonza. Fetal bovine serum (FBS, #P30-3300) was from Pan Biotech. Normocin (Invivogen, #ant-nr-2) was bought from Eubio. Human fibroblast growth factor 2 (*hFGF2*, #F0291), Insulin (#I9287), apo-Transferrin (#T2252), Triiodothyronin (T3, #T6397), 3-Isobutyl-1-methylxanthine (IBMX, #I7018) Dexamethasone (#D4902), formaldehyde (36.5%, #F8775) and chloroform (≥99%, #C2432) were purchased from Sigma-Aldrich. 2-propanol (#7343.1), Glycerol (≥98%, #7530.1) and nuclease-free water (#T143.3) were obtained from Roth. Rosiglitazone was a product from Cayman Chemicals (#71740). miRIDIAN microRNA mimics, ON-TARGETplus SMARTpool siRNAs and DharmaFECT Duo (#T-2010-02) were produced by Dharmacon and purchased from THP. HiPerFect transfection reagent (#301707) was bought from QIAGEN. Oil Red O (#1155984) reagent was obtained from ICN Biomedicals. Infinity Trigylcerides Reagent (#TR22203) was purchased from Microgenics. BCA Protein Assay Kit (#Pier-23227) was bought from VWR. High Fidelity PCR Enzyme Mix (#K0192) was obtained from Fermentas. RQ1 RNase-Free DNase (#M6101), *XhoI* (#R6165) and *NotI* (#R6435) restriction enzymes, psiCHECK-2 vector (#C8021) and Dual Luciferase Reporter Assay System (#E1980) were bought from Promega. Mutagenesis of luciferase reporters was performed using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, #210519) with primers synthesized by Integrated DNA Technologies. Protease Inhibitor Cocktail (PIC, #1836170) was obtained from Roche. Trypsin (#15400054), Phosphate buffered saline (PBS, #10010015), 1M HEPES (#15630122), L-Glutamine (#25030024), TRIzol reagent (#15596018), SuperScript II Reverse Transcriptase Kit (#18064014), dNTP mix (#10297018), random hexamer primers (#48190011), oligo $(dT)_{12-18}$ primers (#18418012), Platinum SYBR Green qPCR SuperMix-UDG w/ROX (#11744500) and RNase OUT Recombinant Ribonuclease Inhibitor (#10777019) were purchased from Invitrogen. Universal cDNA Synthesis Kit (#203300), SYBR Green master mix (#203400) and primers for detection of miR-30c (#204783) and 5S rRNA (#203906) were purchased from Exiqon.

Cell culture. hMADS-2 and hMADS-3 cells were grown in proliferation medium (PM), consisting of DMEM with 10% FBS, 10 mM HEPES, 2 mM L-Glutamine, 100 μg/ml Normocin and 2.5 ng/ml hFGF-2. For adipocyte differentiation, cells were grown to confluence in PM (designated day -2) and medium was changed to PM without *hFGF2*. After two days (designated day 0), adipocyte differentiation was initiated

by differentiation medium (DM), consisting of DMEM/Ham's F12 Medium, 5 mM HEPES, 2 mM L-Glutamine, 100 μg/ml Normocin, 860 nM Insulin, 10 μg/ml apo-Transferrin, 0.2 nM Triiodothyronin, 100 nM Rosiglitazone, 100 μM 3-Isobutyl-1-methylxanthine (IBMX) and 1 μM Dexamethasone. IBMX and Dexamethasone were omitted after 3 days, and DM was changed every 2–3 days. Mouse embryonic fibroblasts (MEFs) were isolated and differentiated as described recently in reference 64. Human embryonic kidney 293 (HEK293) cells were grown in DMEM with 10% FBS, 4 mM L-Glutamine and 100 μg/ml Normocin.

Transfection of miRNA mimics and siRNAs. hMADS cells were seeded in 12-well plates and transfected at day -2 with 5 nM miRIDIAN microRNA mimics (miR-30c or Negative Control #1), or 5 nM ON-TARGETplus SMARTpool siRNAs (siPAI-1, siALK2 or siGENOME Non-Targeting siRNA Pool #2) using HiPerFect following manufacturer's instructions. After 2 days (day 0), adipocyte differentiation was initiated by DM.

Oil red O staining. Cells were washed with PBS, fixed in 3.7 % formaldehyde (in PBS) for 15 min, washed with PBS, stained by 1 h incubation with Oil Red O (0.5 g Oil Red O in 100 ml isopropanol diluted with water (60:40) and filtrated), washed twice in water and then photographed.

Triglyceride assay. Cells were washed with PBS (4°C) and detached from the plates using a cell scraper and 300 μL PBS per well. Subsequently, samples were homogenized by sonication. Triglyceride quantification was performed with Infinity Triglycerides Reagent according to manufacturer's protocol with a dilution series of glycerol in PBS serving as standard. Triglyceride concentration was normalized to total protein determined by BCA Assay.

qRT-PCR. Total RNA was obtained using TRIzol. 0.5–1 μg of RNA were DNase digested with RQ1 RNase-Free DNase and cDNA synthesis was performed with random hexamer primers and oligo $(dT)_{12-18}$ primers using SuperScript II Reverse Transcriptase following manufacturer's instructions. The qRT-PCR reaction volume was 18 μL, consisting of 4.5 ng reverse transcribed RNA in water, 200 nM forward and reverse primer (sequences in online **Table S1**) and Platinum SYBR Green qPCR SuperMix-UDG with ROX. Assays were run on ABI Prism 7000 with 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 sec at 95°C and 1 min at 60°C. Data evaluation was performed using the QPCR online application.[65]

miRNA qRT-PCR. miR-30c expression levels were analyzed using the miRCURY LNA Universal RT microRNA PCR system (Exiqon) according to the manufacturer's instructions. 5S rRNA was used as endogenous reference RNA. Relative quantification of miRNA expression levels was performed as described above.

Luciferase reporter assay. The 3'UTRs of *PAI-1* (NM_000602) and *ALK2* (NM_001105) were amplified by RT-PCR (primer sequences in online **Table S2**) using HighFidelity PCR Enzyme Mix. The 207 bp and 983 bp products were inserted into the *Xho*I and *Not*I restriction sites of the psiCHECK-2 vector. Correct insertion was validated by sequencing, and generated reporter vectors were termed p2-PAI-1 and p2-ALK2, respectively. Subsequently, reporter vectors with mutated miR-30c binding site were established by site-directed mutagenesis (primer sequences in online **Sup. Table S2**), with mutations at positions 2–5 of the *PAI-1* seed match and positions 3, 5 and 7 of the *ALK2* seed match. Successful mutation was assessed by sequencing and mutated reporter vectors were termed p2-PAI-1-m and p2-ALK2-m. 20 x 10^3 ≥ HEK293 cells were seeded in 96-well plates, and 20 h later, transfections were performed using 0.2 μl DharmaFECT Duo, 100 ng of vector constructs and either 50 nM of miRIDIAN microRNA mimic Negative Control #1 (NTC) or miR-30c mimic per well. Cells were harvested 48 h after transfection and assayed for Renilla and firefly luciferase activity using the Dual Luciferase Reporter Assay System (Promega) and the lumino-meter Orion II (Berthold).

Animal studies. All animal procedures used were approved by the Austrian Federal Ministry for Science and Research. C57/Bl6 mice were put on a chow diet (4.5% fat calories) or on a HFD (40% fat calories, both diets from ssniff Spezialdiaeten GmbH, Soest, Germany) immediately after weaning. Ob/ob mice and their wild type littermates were put on a chow diet immediately after weaning. All mice were kept on a 12 h/12 h light/dark cycle. After 3 months, mice were fasted overnight and refed for one hour before sacrification to harvest epididymal fat pads.

Statistical analysis. Data are presented as means ± SEM. Differences between groups were analyzed by applying Student's two-tailed t-test for independent samples.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Prof. Gérard Ailhaud, Dr. Christian Dani and Dr. Ez-Zoubir Amri for providing hMADS cells. This work was supported by GEN-AU, the Austrian Genome Research (GEN-AU) funding program [Grants "non-coding RNAs" (no. 820982), "GOLD-III" (no. 820979) and by the 'Austrian Agency for International Cooperation in Education & Research', OeAD (FR 14/2010)].

Note

Supplemental materials can be found at:
www.landesbioscience.com/journals/rnabiology/article/16153

References

1. Mokdad AH, Ford ES, Bowman BA, Dietz WH, Vinicor F, Bales VS, et al. Prevalence of obesity, diabetes and obesity-related health risk factors, 2001. JAMA 2003; 289:76-9.
2. Hossain P, Kawar B, El Nahas M. Obesity and diabetes in the developing world—a growing challenge. N Engl J Med 2007; 356:213-5.
3. Lau DCW, Dhillon B, Yan H, Szmitko PE, Verma S. Adipokines: molecular links between obesity and atheroslcerosis. A J Physiol 2005; 288:2031-41.
4. Ahima RS, Flier JS. Adipose tissue as an endocrine organ. Trends Endocrinol Metabol 2000; 11:327-32.
5. Singh SK, Pal Bhadra M, Girschick HJ, Bhadra U. MicroRNAs—micro in size but macro in function. FEBS J 2008; 275:4929-44.
6. Wang Q, Li YC, Wang J, Kong J, Qi Y, Quigg RJ, et al. miR-17-92 cluster accelerates adipocyte differentiation by negatively regulating tumor-suppressor Rb2/p130. Proc Natl Acad Sci USA 2008; 105:2889-94.
7. Oskowitz AZ, Lu J, Penfornis P, Ylostalo J, McBride J, Flemington EK, et al. Human multipotent stromal cells from bone marrow and microRNA: regulation of differentiation and leukemia inhibitory factor expression. Proc Natl Acad Sci USA 2008; 105:18372-7.

8. Mudhasani R, Imbalzano AN, Jones SN. An essential role for Dicer in adipocyte differentiation. J Cell Biochem 2010; 110:812-6.
9. Mudhasani R, Puri V, Hoover K, Czech MP, Imbalzano AN, Jones SN. Dicer is required for the formation of white but not brown adipose tissue. J Cell Physiol 2010; 226:1399-406.
10. Kennell JA, Gerin I, MacDougald OA, Cadigan KM. The microRNA miR-8 is a conserved negative regulator of Wnt signaling. Proc Natl Acad Sci USA 2008; 105:15417-22.
11. Xie H, Lim B, Lodish HF. MicroRNAs induced during adipogenesis that accelerate fat cell development are downregulated in obesity. Diabetes 2009; 58:1050-7.
12. Sun F, Wang J, Pan Q, Yu Y, Zhang Y, Wan Y, et al. Characterization of function and regulation of miR-24-1 and miR-31. Biochem Biophys Res Comm 2009; 380:660-5.
13. Sun T, Fu M, Bookout AL, Kliewer SA, Mangelsdorf DJ. MicroRNA let-7 regulates 3T3-L1 adipogenesis. Mol Endocrinol (Baltimore, Md.) 2009; 23:925-31.
14. Lin Q, Gao Z, Alarcon RM, Ye J, Yun Z. A role of miR-27 in the regulation of adipogenesis. FEBS 2009; 276:2348-58.
15. Kim SY, Kim AY, Lee HW, Son YH, Lee GY, Lee J, et al. miR-27a is a negative regulator of adipocyte differentiation via suppressing PPARgamma expression. Biochem Biophys Res Comm 2010; 392:323-8.
16. Gerin I, Bommer GT, McCoin CS, Sousa KM, Krishnan V, MacDougald OA. Roles for miRNA-378/378* in adipocyte gene expression and lipogenesis. Am J Physiol Endocrinol Metab 2010; 299:198-206.
17. Qin L, Chen Y, Niu Y, Chen W, Wang Q, Xiao S, et al. A deep investigation into the adipogenesis mechanism: profile of microRNAs regulating adipogenesis by modulating the canonical Wnt/beta-catenin signaling pathway. BMC Genomics 2010; 11:320.
18. Esau C, Kang X, Peralta E, Hanson E, Marcusson EG, Ravichandran LV, et al. MicroRNA-143 regulates adipocyte differentiation. J Biol Chem 2004; 279:52361-5.
19. Karbiener M, Fischer C, Nowitsch S, Opriessnig P, Papak C, Ailhaud G, et al. microRNA miR-27b impairs human adipocyte differentiation and targets PPARgamma. Biochem Biophys Res Comm 2009; 390:247-51.
20. Kim YJ, Hwang SJ, Bae YC, Jung JS. miR-21 regulates adipogenic differentiation through the modulation of TGF beta signaling in mesenchymal stem cells derived from human adipose tissue. Stem Cells 2009; 27:3093-102.
21. Lee EK, Lee MJ, Abdelmohsen K, Kim W, Kim MM, Srikantan S, et al. miR-130 suppresses adipogenesis by inhibiting PPAR{gamma} expression. Mol Cell Biol 2010; 31:626-38.
22. Rodriguez A, Elabd C, Delteil F, Astier J, Vernochet C, Saint-Marc P, et al. Adipocyte differentiation of multipotent cells established from human adipose tissue. Biochem Biophys Res Comm 2004; 315:255-63.
23. Rodriguez A, Pisani D, Dechesne CA, Turc-Carel C, Kurzenne J, Wdziekonski B, et al. Transplantation of a multipotent cell population from human adipose tissue induces dystrophin expression in the immunocompetent mdx mouse. J Exper Med 2005; 201:1397-405.
24. Scheideler M, Elabd C, Zaragosi L, Chiellini C, Hackl H, Sanchez-Cabo F, et al. Comparative transcriptomics of human multipotent stem cells during adipogenesis and osteoblastogenesis. BMC Genomics 2008; 9:340.
25. Betel D, Wilson M, Gabow A, Marks DS, Sander C. The microRNA.org resource: targets and expression. Nucleic Acids Res 2008; 36:149-53.
26. Lall S, Grün D, Krek A, Chen K, Wang Y, Dewey CN, et al. A genome-wide map of conserved microRNA targets in *C. elegans*. Curr Biol 2006; 16:460-71.
27. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 2009; 19:92-105.
28. Gaidatzis D, van Nimwegen E, Hausser J, Zavolan M. Inference of miRNA targets using evolutionary conservation and pathway analysis. BMC Bioinform 2007; 8:69.
29. Alessi MC, Bastelica D, Morange P, Berthet B, Leduc I, Verdier M, et al. Plasminogen activator inhibitor 1, transforming growth factor-beta1, and BMI are closely associated in human adipose tissue during morbid obesity. Diabetes 2000; 49:1374-80.
30. Estellés A, Dalmau J, Falcó C, Berbel O, Castelló R, España F, et al. Plasma PAI-1 levels in obese children—effect of weight loss and influence of PAI-1 promoter 4G/5G genotype. Thromb Haemost 2001; 86:647-52.
31. De Taeye B, Smith LH, Vaughan DE. Plasminogen activator inhibitor-1: a common denominator in obesity, diabetes and cardiovascular disease. Curr Opin Pharmacol 2005; 5:149-54.
32. Samad F, Yamamoto K, Loskutoff DJ. Distribution and regulation of plasminogen activator inhibitor-1 in murine adipose tissue in vivo. Induction by tumor necrosis factor-alpha and lipopolysaccharide. J Clin Invest 1996; 97:37-46.
33. Pandey M, Tuncman G, Hotamisligil GS, Samad F. Divergent roles for p55 and p75 TNFalpha receptors in the induction of plasminogen activator inhibitor-1. Am J Pathol 2003; 162:933-41.
34. Morange PE, Alessi MC, Verdier M, Casanova D, Magalon G, Juhan-Vague I. PAI-1 produced ex vivo by human adipose tissue is relevant to PAI-1 blood level. Arterioscler, Thromb and Vasc Biol 1999; 19:1361-5.
35. Liang X, Kanjanabuch T, Mao S, Hao C, Tang Y, Declerck PJ, et al. Plasminogen activator inhibitor-1 modulates adipocyte differentiation. Am J Physiol Endocrinol Metab 2006; 290:103-13.
36. Scroyen I, Jacobs F, Cosemans L, De Geest B, Lijnen HR. Effect of plasminogen activator inhibitor-1 on adipogenesis in vivo. Thromb Haemost 2009; 101:388-93.
37. Macías-Silva M, Hoodless PA, Tang SJ, Buchwald M, Wrana JL. Specific activation of Smad1 signaling pathways by the BMP7 type I receptor, ALK2. J Biol Chem 1998; 273:25628-36.
38. Luo J, Tang M, Huang J, He B, Gao J, Chen L, et al. TGFbeta/BMP type I receptors ALK1 and ALK2 are essential for BMP9-induced osteogenic signaling in mesenchymal stem cells. J Biol Chem 2010; 285:29588-98.
39. Shen Q, Little SC, Xu M, Haupt J, Ast C, Katagiri T, et al. The fibrodysplasia ossificans progressiva R206H ACVR1 mutation activates BMP-independent chondrogenesis and zebrafish embryo ventralization. J Clin Invest 2009; 119:3462-72.
40. Sorrentino A, Liu C, Addario A, Peschle C, Scambia G, Ferlini C. Role of microRNAs in drug-resistant ovarian cancer cells. Gynecol Oncol 2008; 111:478-86.
41. Budhu A, Jia H, Forgues M, Liu C, Goldstein D, Lam A, et al. Identification of metastasis-related microRNAs in hepatocellular carcinoma. Hepatology 2008; 47:897-907.
42. Wang G, Zhang H, He H, Tong W, Wang B, Liao G, et al. Upregulation of microRNA in bladder tumor tissue is not common. Int Urol Nephrol 2010; 42:95-102.
43. Busacca S, Germano S, De Cecco L, Rinaldi M, Comoglio F, Favero F, et al. MicroRNA signature of malignant mesothelioma with potential diagnostic and prognostic implications. A J Resp Cell Mol Biol 2010; 42:312-9.
44. Zhong X, Li N, Liang S, Huang Q, Coukos G, Zhang L. Identification of microRNAs regulating reprogramming factor LIN28 in embryonic stem cells and cancer cells. J Biol Chem 2010; 285:41961-71.
45. Braun J, Hoang-Vu C, Dralle H, Hüttelmaier S. Downregulation of microRNAs directs the EMT and invasive potential of anaplastic thyroid carcinomas. Oncogene 2010; 29:4237-44.
46. Yu F, Deng H, Yao H, Liu Q, Su F, Song E. Mir-30 reduction maintains self-renewal and inhibits apoptosis in breast tumor-initiating cells. Oncogene 2010; 29:4194-204.
47. Duisters RF, Tijsen AJ, Schroen B, Leenders JJ, Lentink V, van der Made I, et al. miR-133 and miR-30 regulate connective tissue growth factor: implications for a role of microRNAs in myocardial matrix remodeling. Circulation Res 2009; 104:170-8.
48. Li J, Donath S, Li Y, Qin D, Prabhakar BS, Li P. miR-30 regulates mitochondrial fission through targeting p53 and the dynamin-related protein-1 pathway. PLoS Genetics 2010; 6:1000795.
49. Agrawal R, Tran U, Wessely O. The miR-30 miRNA family regulates Xenopus pronephros development and targets the transcription factor Xlim1/Lhx1. Development 2009; 136:3927-36.
50. Kajimoto K, Naraba H, Iwai N. MicroRNA and 3T3-L1 pre-adipocyte differentiation. RNA 2006; 12:1626-32.
51. Monteys AM, Spengler RM, Wan J, Tecedor L, Lennox KA, Xing Y, et al. Structure and activity of putative intronic miRNA promoters. RNA 2010; 16:495-505.
52. Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, et al. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature 2005; 433:769-73.
53. Baek D, Villén J, Shin C, Camargo FD, Gygi SP, Bartel DP. The impact of microRNAs on protein output. Nature 2008; 455:64-71.
54. Selbach M, Schwanhäusser B, Thierfelder N, Fang Z, Khanin R, Rajewsky N. Widespread changes in protein synthesis induced by microRNAs. Nature 2008; 455:58-63.
55. Patel N, Tahara SM, Malik P, Kalra VK. Involvement of miR-30c and miR-301a in immediate induction of plasminogen activator inhibitor-1 by placenta growth factor in human pulmonary endothelial cells. Biochem J 2010; 434:473-82.
56. Scroyen I, Christiaens V, Lijnen HR. No functional role of plasminogen activator inhibitor-1 in murine adipogenesis or adipocyte differentiation. J Thromb Haemost 2007; 5:139-45.
57. Morange PE, Lijnen HR, Alessi MC, Kopp F, Collen D, Juhan-Vague I. Influence of PAI-1 on adipose tissue growth and metabolic parameters in a murine model of diet-induced obesity. Arterioscl Thromb Vasc Biol 2000; 20:1150-4.
58. Lijnen HR, Maquoi E, Morange P, Voros G, Van Hoef B, Kopp F, et al. Nutritionally induced obesity is attenuated in transgenic mice overexpressing plasminogen activator inhibitor-1. Arterioscl Thromb Vasc Biol 2003; 23:78-84.
59. Ma L, Mao S, Taylor KL, Kanjanabuch T, Guan Y, Zhang Y, et al. Prevention of obesity and insulin resistance in mice lacking plasminogen activator inhibitor 1. Diabetes 2004; 53:336-46.
60. van Dinther M, Visser N, de Gorter DJJ, Doorn J, Goumans M, de Boer J, et al. ALK2 R206H mutation linked to fibrodysplasia ossificans progressiva confers constitutive activity to the BMP type I receptor and sensitizes mesenchymal cells to BMP-induced osteoblast differentiation and bone formation. J Bone Mineral Res 2010; 25:1208-15.
61. Shore EM, Xu M, Feldman GJ, Fenstermacher DA, Cho T, Choi IH, et al. A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva. Nat Genetics 2006; 38:525-7.
62. Tseng Y, Kokkotou E, Schulz TJ, Huang TL, Winnay JN, Taniguchi CM, et al. New role of bone morphogenetic protein 7 in brown adipogenesis and energy expenditure. Nature 2008; 454:1000-4.
63. Lijnen HR. Pleiotropic functions of plasminogen activator inhibitor-1. J Thromb Haemost 2005; 3:35-45.

64. Prokesch A, Bogner-Strauss JG, Hackl H, Rieder D, Neuhold C, Walenta E, et al. Arxes: retrotransposed genes required for adipogenesis. Nucleic Acids Res 2010; 39:3224-39.
65. Pabinger S, Thallinger GG, Snajder R, Eichhorn H, Rader R, Trajanoski Z. QPCR: Application for real-time PCR data management and analysis. BMC Bioinform 2009; 10:268.