Adipocyte Differentiation of Human Bone Marrow-Derived Stromal Cells Is Modulated by MicroRNA-155, MicroRNA-221, and MicroRNA-222

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Human mesenchymal stromal cells (hMSCs) are capable of limited self-renewal and multilineage differentiation in vitro. Several studies have demonstrated that microRNAs (miRNAs), post-transcriptional modifiers of mRNA stability and protein translation, play crucial roles in the regulation of these complex processes. To gain knowledge regarding the role of miRNAs in human adipocyte differentiation, we examined the miRNA expression profile of the immortalized human bone marrow-derived stromal cell line hMSC-Tert20. Such a model system has the advantage of a reproducible and consistent phenotype while maintaining important properties of the primary donor cells, including the potential to differentiate to adipocytes, osteoblasts, and chondrocytes. We identified 12 miRNAs that were differentially expressed during adipogenesis, of which several have been previously shown to play important roles in adipocyte biology. Among these, the expression of miRNA-155, miRNA-221, and miRNA-222 decreased during the adipogenic program of both immortalized and primary hMSCs, suggesting that they act as negative regulators of differentiation. Interestingly, ectopic expression of the miRNAs significantly inhibited adipogenesis and repressed induction of the master regulators peroxisome proliferator-activated receptor gamma and CCAAT/enhancer-binding protein alpha. Our study provides the first experimental evidence that miRNA-155, miRNA-221, and miRNA-222 have an important function in human adipocyte differentiation, and that their downregulation is necessary to relieve the repression of genes crucial for this process.

Introduction

HUMAN BONE MARROW-DERIVED mesenchymal stromal cells (hMSCs) are multipotent cells that, under defined conditions, can differentiate into multiple connective tissue cell types, such as adipocytes, osteoblasts, chondrocytes, and myoblasts [1]. Differentiation of hMSCs into different lineages involves complex regulation and transcriptional activation or repression of a large number of genes, and disruption of this regulation can have severe pathological consequences, such as cancer development [2,3].

Adipocyte differentiation is largely controlled by 2 families of transcription factors, CCAAT/enhancer-binding proteins (C/EBPs) and peroxisome proliferator-activated receptors (PPARs) [4,5], which, following their own induction, subsequently regulate the expression of numerous genes required for terminal differentiation [6,7]. In addition, many cofactors modulate the expression or activity of C/EBPs and PPARs and thereby indirectly regulate adipogenesis. For instance, several cell-cycle regulators exert positive or negative effects on the key transcription factor PPARγ [8–10].

microRNAs (miRNAs or miRs) are short noncoding RNAs that are evolutionarily conserved, and function as negative regulators of gene expression at the post-transcriptional level by targeting mRNAs for degradation, translational inhibition, or both [11]. miRNAs are involved in the regulation of a diverse range of important cellular processes, including proliferation and self-renewal, and aberrant miRNA activity may contribute to carcinogenesis [12,13]. A number of miRNAs are also expressed and regulated during adipogenesis. miR-143 expression increases during differentiation of human white preadipocytes, and its inhibition effectively represses adipogenesis, possibly through the target gene *ERK5* [14]. During adipogenic differentiation of mouse 3T3-L1 cells, it was first described that the expression of 21 miRNAs was either up- or downregulated [15]. Later, it was demonstrated that the miR-17~92 cluster, let-7, and miR-27 can modulate adipogenesis in these cells [16–18]. In human multipotent adipose-derived stem cells, miR-21, miR-27, and miR-138

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have been reported to regulate adipocyte differentiation through the modulation of transforming growth factor (TGF)-β signaling, PPARγ, and EP300 interacting inhibitor of differentiation 1, respectively [19–21]. Finally, in hMSCs it was recently shown that 20 miRNAs are induced during adipogenesis [22]. These studies demonstrate that miRNAs play important roles in the regulation of adipocyte differentiation. However, knowledge of the networks that are active is far from complete.

To better understand the role of miRNAs in human adipocyte differentiation, we examined the miRNA expression profile of the immortalized human bone marrow-derived stromal cell line hMSC-Tert20 [23,24]. hMSC-Tert20 has been previously demonstrated to be capable of differentiation to adipocytes, osteoblasts, and chondroblasts [23]. This article describes the adipogenic differentiation of these cells and the changes in miRNA expression that occur during this process. Further, we demonstrate that miR-155, miR-221, and miR-222 are downregulated during adipocyte differentiation of both immortalized and primary hMSCs. Overexpression of the miRNAs significantly reduces the adipogenic potential of both cell types, suggesting that they are repressors of adipogenesis. Finally, our studies indicate that the levels of C/EBPβ and cyclin-dependent kinase inhibitor 1B (CDKN1B) are modulated by miR-155 alone or miR-221 plus miR-222, respectively, during differentiation.

Materials and Methods

Cell culture

The telomerase-immortalized human bone marrow-derived stromal cell line hMSC-Tert20 was kindly provided by Professor Moustapha Kassem, Department of Endocrinology and Metabolism, University Hospital of Odense, Odense, Denmark. hMSC-Tert20 and HEK-293 cells (ATCC number CRL-1573) were cultured in Dulbecco's modified Eagle's medium (DMEM, cat. no. 10313–039; Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories, Pasching, Austria), GlutaMAX, penicillin (100 U/mL), and streptomycin (100 μg/mL) (all from Life Technologies) at 37°C and 5% CO_2. Primary hMSCs were obtained from the hip of a healthy donor after informed consent. Briefly, mononuclear cells from the bone marrow were isolated using standard density centrifugation and cultured in minimum essential medium (MEM) Alpha medium (cat. no. 22561) supplemented with 20% FBS (PAA Laboratories), GlutaMAX, penicillin (100 U/mL), and streptomycin (100 μg/mL) (all from Life Technologies) at 37°C and 5% CO_2. Non-adherent cells were removed after approximately 24 h by replacement of the medium. Subsequently, the adherent cells were allowed to expand until 70% confluence (5–6 days) before they were harvested and further expanded.

Induction of adipocyte differentiation

Adipocyte differentiation was performed as previously described [25]. The hMSC-Tert20 cells and the primary hMSCs (passage 5–7) were seeded at a density of 22,000 cells/cm^2 or 4,500 cells/cm^2, respectively. The following day, adipogenesis was initiated by replacing the normal medium with adipocyte induction medium containing 1 μM dexamethasone (Decadron, Haarlem, The Netherlands), 0.5 mM

isobutylmethylxanthine, and 50 μM indomethacin (both from Sigma-Aldrich, St. Louis, MO). This adipocyte induction medium was replaced twice a week for up to 21 days. To estimate the degree of differentiation, the wells were washed with phosphate-buffered saline (PBS) (Lonza, Basel, Switzerland), fixed with 300 μL 4% formalin, washed with PBS, and subsequently stained with 300 μL of 0.3% Oil Red O (Sigma-Aldrich) in 60% isopropanol. To quantify the Oil Red O staining, the wells were washed with water and the bound dye was extracted with 300 μL of 96% ethanol and measured at 490 nm using a Victor^2 1420 Multilabel Counter (Perkin Elmer, Waltham, MA). To correct for the number of cells, the formalin-fixed cells were incubated with 500 μL of 10% trichloroacetic acid for 1 h, washed with water, and subsequently stained with 300 μL of 0.4% sulforhodamine B (Sigma-Aldrich) in 1% acetic acid. Unbound dye was removed with 1% acetic acid. The bound dye was solubilized with 300 μL of 10 mM Tris and measured at 540 nm using a Victor^2 1420 Multilabel Counter (Perkin Elmer).

Quantitative real-time reverse transcription–polymerase chain reaction

Quantitative real-time reverse transcription–polymerase chain reaction (qPCR) was performed using the ABI PRISM 7500 DNA Sequence Detection System (Life Technologies). The relative expression levels were determined using the comparative threshold cycle ($2^{-\Delta\Delta CT}$) method as described by the manufacturer. Total RNA containing miRNAs was isolated using TRIzol Solution (Life Technologies) and standard conditions. For mRNA quantification, the total RNA was first treated with amplification-grade DNase I (Life Technologies) to avoid amplification of contaminating genomic DNA. The High Capacity cDNA Archive Kit (Life Technologies) was used to synthesize cDNA. In addition, the TaqMan® Gene Expression Cells-to-CT Kit (Life Technologies) was used for direct reverse transcription of cultured cell lysates from the primary hMSCs, essentially as described by the manufacturer. FastStart SYBR Green Master with Rox (Roche Applied Science, Indianapolis, IN) or TaqMan Gene Expression Assays (Life Technologies) was used to perform the quantitative PCR (qPCR) (Supplementary Tables S1 and S2; Supplementary Data are available online at www.liebertonline.com/scd). The average of TATA-binding protein (*TBP*) and human acidic ribosomal phosphoprotein PO (*RPLPO*), or of *TBP* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), was used to normalize the data for the SYBR Green or TaqMan assays, respectively. For miRNA quantification, the TaqMan MicroRNA RT Kit and TaqMan MiRNA Assays (Supplementary Table S2) (Life Technologies) were used to generate cDNA and to quantitatively detect mature miRNAs, respectively. In addition, the TaqMan MicroRNA Cells-to-CT Kit (Life Technologies) was used to quantify the levels of mature miRNAs in the primary hMSCs. The average of the endogenous controls RNU6B (U6 small nuclear RNA 2) and RNU24 (small nucleolar RNA, C/D box 24) was used for normalization.

Isolation, labeling, and hybridization of miRNAs

Total RNA was extracted with TRIzol Solution from cells at various time points during adipogenesis (0, 8, 24, 32, 48, and 72 h and 7, 14, and 21 days). Small RNAs (<40

nucleotides), including mature miRNAs, were enriched from the total RNA samples using the flashPAGE Fractionator System and subsequently purified using the flashPAGE Reaction Clean-Up Kit according to the manufacturer's instructions (Life Technologies). The integrity of the enriched RNA was assessed by an Agilent BioAnalyzer 2100 (Agilent, Palo Alto, CA). The Array900 miRNA Direct Kit (Genisphere Inc., Hatfield, PA) was used to label and hybridize enriched miRNAs (150 ng) as described by the manufacturer. The miRNA microarray experiments were performed using in-house spotted amino silane-coated CMT UltraGAPS slides (Corning Life Sciences, Corning, NY) produced at the Norwegian Microarray Consortium (www.microarray.no), University of Oslo, supported by the Functional Genomics (FUGE) program of the Research Council of Norway. Each slide contained 2 independent arrays printed using the mirVana Probe Set v1, consisting of probes corresponding to 384 human, rat, and mouse miRNAs (Life Technologies). Each probe was printed in triplicate, and the slides were UV-crosslinked to immobilize the double-stranded probes. All hybridizations, except for 0, 8, and 32 h, were performed twice using LifterSlips (Thermo Fisher Scientific) and manual hybridization cassettes (Arrayit, Sunnyvale, CA) submerged in water.

Analysis of miRNA expression and target predictions

The arrays were scanned using an Agilent Technology G2505B scanner (Agilent Technologies, Santa Clara, CA), and the data were processed and filtered as previously described [26]. The expression of each miRNA at a given time point was calculated as a ratio relative to its level on day 0. To avoid zero or negative values in the ratio calculations, spots with background-subtracted intensity less than the standard deviation of the local background in one of the channels were assigned the value of the standard deviation. The spot was retained if the new value was less than the value in the other channel. Spots with background-subtracted intensity < 1.5 times the standard deviation of the local background in both channels were excluded. Log_2-transformed ratios were normalized using a global median ratio algorithm. The data were filtered to only include miRNAs with a value above threshold in at least 5 of the 9 time points, and the data for replicated time points were merged by calculating the mean of the ratio for each of the miRNAs. miRNA expression levels were visualized by heatmaps generated by TIGR MultiExperiment Viewer v4.4 (www.tm4.org/mev.html). TargetScan 5.1 (www.targetscan.org) was used to search for predicted miRNA targets [27]. Functional analysis of the targets was performed in GeneGO (St. Joseph, MI), a manually curated database for the identification of relevant pathways, networks, and cellular processes. The top significant enriched pathways and networks are presented, given a significance level based on the false discovery rate, corrected with the Simes' modification of the Bonferroni procedure. Further, GeneGO and the algorithm auto expand was used to generate a protein network of predicted target genes.

Protein extracts, western blots, and antibodies

Preparation of whole-cell lysates, protein electrophoresis, and western blotting were performed according to standard protocols. The protein content of the supernatant was determined using the BCA Protein Assay Kit (Thermo Fisher Scientific). Blots were probed with antibodies against C/EBPβ (C-19) and stearoyl coenzyme A desaturase 1 (SCD) (N-20) (both from Santa Cruz Biotechnology, Santa Cruz, CA); C/EBPα (p42) and PPARγ (D69) (both from Cell Signaling Technology, Danvers, MA); and cyclin-dependent kinase inhibitor p27Kip1 (CDKN1B) (cat no. 610241; BD Transduction Laboratories, San Jose, CA). Anti-α1a-tubulin (CP06) (Calbiochem, La Jolla, CA) or anti-β-actin (A5316) (Sigma-Aldrich) was used as loading control. All secondary antibodies were obtained from Dako (Glostrup, Denmark). The proteins were visualized using SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific).

Cloning of miRNA genes, construction of recombinant adenoviruses, transduction of hMSC-Tert20 cells, and transfection of primary hMSCs

Genomic DNA was isolated from hMSC-Tert20 cells using the High Pure PCR Template Preparation Kit according to the manufacturer's recommendations (Roche Applied Science). The miR-155 and miR-221/222 genes with flanking sequences were amplified using standard PCR and cloning procedures (Supplementary Table S3). A more detailed description of the cloning procedures can be found in Supplementary Methods. Briefly, PCR products were T/A-cloned into the CMV-driven expression vector pMaHygro/TO and sequenced on a MegaBACE 1000 using the DYEnamic™ ET dye Terminator Cycle Sequencing Kit as described by the manufacturer (GE Healthcare, Piscataway, NJ). Expression of mature miRNAs was confirmed by transfection of HEK-293 cells with Lipofectamine 2000 (Life Technologies) (Supplementary Fig. S1A) followed by qPCR using specific TaqMan MiRNA Assays (Life Technologies) (Supplementary Table S2). The same miRNA sequences as described above were used to generate recombinant adenoviruses. Briefly, miR-155 and miR-221/222 were T/A-cloned into the pShuttle-CMV vector (Agilent Technologies) and designated Ad-miR-155 and Ad-miR-221/222, respectively. The recombinant adenoviruses were generated essentially as described in Ref. [28]. Before transduction, the different virus stock solutions were diluted in DMEM containing 2% serum to give a multiplicity of infection of 5 when added to the cells. The cells were incubated with the viruses for 1 h, washed once with PBS, and replenished with normal medium. The empty vector Ad-pShuttle-CMV, termed Ad-control, was used as control. The primary hMSCs were transfected with hsa-miR-155 and hsa-miR-222 Pre-miR miRNA Precursors or Negative Control #1 Pre-miR (designated Pre-miR-155, Pre-miR-222, and Pre-control, respectively) using Lipofectamine 2000 (all from Life Technologies). Briefly, the precursors were complexed with 0.25 μL of Lipofectamine 2000 diluted using OptiMEM I Medium (cat. no. 31985-062; Life Technologies) in a total volume of 50 μL for 20 min, before being added to the cells at a final concentration of 25 nM in a total volume of 300 μL. Adipocyte differentiation was induced 24 h post-transduction/transfection.

Statistical analyses

All statistical analyses of the qPCR data were performed using a paired 2-tailed Student's t-test. Statistics were based

on $\Delta\Delta C_T$ values at a given time point at which cells with ectopic expression of either miR-155 or miR-221/222 were compared with control cells. Results are expressed as means ± standard error of the mean. A *P* value of <0.05 was considered to be statistically significant.

Results

Adipocyte differentiation

The ability of the immortalized hMSC-Tert20 cells to differentiate to adipocytes was demonstrated by an increase in triglyceride accumulation using Oil Red O staining (data not shown) and by qPCR analysis of the adipocyte markers *CEBPB*, *CEBPD*, *PPARG*, *CEBPA*, adipsin [complement factor D (*CFD*)], and adiponectin (*ADIPOQ*) (Supplementary Fig. S2). The transcription factors *CEBPB* and *CEBPD* were induced within the first 8 h of differentiation, followed by *PPARG* after 24 h and finally by *CEBPA*, *CFD*, and *ADIPOQ*, whose transcription levels increased dramatically from day 3. Adipogenesis of the primary hMSCs was demonstrated by Oil Red O staining of accumulating triglycerides and by qPCR analysis of *CEBPB*, *PPARG*, *CEBPA*, and *ADIPOQ*, which all increased during differentiation (Supplementary Fig. S3).

Identification of miRNAs regulated during adipocyte differentiation

To study the pattern of miRNA expression during adipocyte differentiation and to identify potential novel regulators, hMSC-Tert20 cells were differentiated for 21 days. Total RNA was isolated at multiple time points (0, 8, 24, 32, 48, and 72 h and 7, 14, and 21 days), and preliminary microarray-based global profiling indicated that 66 miRNAs were expressed (Supplementary Fig. S4). We used qPCR to validate and identify 20 of the miRNAs that showed a change in expression following the induction of adipogenesis (Supplementary Fig. S4). Two more biological replicates were performed, and 12 of the miRNAs showed consistent expression patterns in all 3 experiments; let-7a, miR-21, miR-26a, miR-30d, miR-34a, and

miR-92 were upregulated, whereas miR-18a, miR-146a, miR-155, miR-214, miR-221, and miR-222 showed decreased expression levels during adipocyte differentiation (Fig. 1A).

Identification of targets with predicted relevance to adipogenesis

To determine whether the observed changes in miRNA expression were important for adipogenesis, we focused on miR-155, miR-221, and miR-222 (miR-221 and miR-222 have the same seed sequence and will be referred to as miR-221/222). The expression of these miRNAs decreased during adipogenesis of hMSC-Tert20 cells (Fig. 1A, B), suggesting that they act as negative regulators of this process. miR-155 and miR-221/222 were also found to be downregulated during adipocyte differentiation of primary hMSCs (Fig. 1C). We then searched for targets of these miRNAs known to be relevant to adipocyte differentiation using TargetScan 5.1 [27], which is considered one of the best databases for target prediction [29]. Two interesting candidates were the key transcription factor gene *CEBPB* and the cyclin-dependent kinase inhibitor gene *CDKN1B*, predicted to be regulated by miR-155 and miR-221/222, respectively. An increase of their corresponding proteins at an early stage of differentiation is important for adipogenesis [30–32]. In agreement with this, the levels of C/EBPβ and CDKN1B increased during adipogenesis of both hMSC-Tert20 and primary hMSCs (Fig. 2A), consistent with regulation by miR-155 or miR-221/222, respectively. The transcript levels of *CEBPB* and *CDKN1B* are indicated in Fig. 2B.

In total, miR-155 and miR-221/222 were predicted to have 281 and 306 conserved targets, respectively (data not shown), of which 32 were common (Supplementary Table S4). Using GeneGO, a graphical network consisting of 22 of the 32 common targets could be generated, which indicated that most of the proteins were highly interconnected (Supplementary Fig. S5). A functional enrichment analysis of the common targets was also performed in GeneGO, and the 10 most significant findings for each of the function categories are presented in Supplementary Table S5. Interestingly, there was
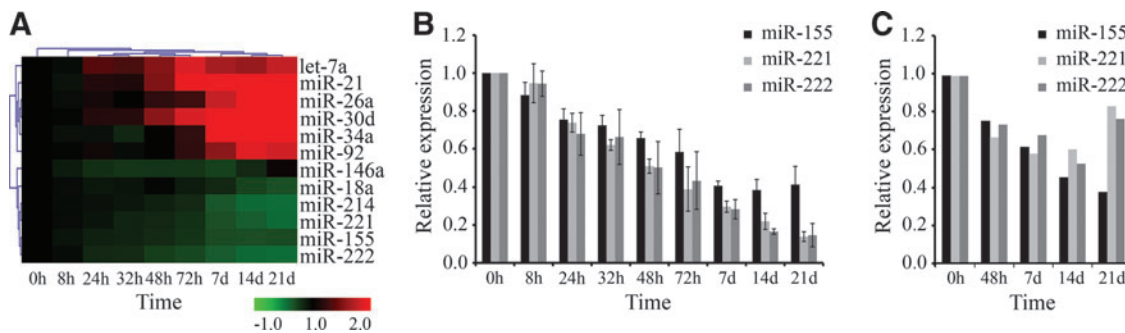

FIG. 1. Expression patterns of differentially regulated miRNAs during adipocyte differentiation. hMSC-Tert20 and primary hMSC cells were differentiated for 21 days, and total RNA was prepared at the indicated time points. **(A)** Validation of miRNAs with altered expression during adipocyte differentiation of hMSC-Tert20 (identified by global microarray analysis) using qPCR. Data are presented as the means of 3 biological replicates relative to unstimulated cells at day 0 (0 h), using a heatmap. The brightest red boxes and the lightest green boxes represent the most upregulated and downregulated miRNAs, respectively. The color key below the heatmap corresponds to the fold-change values. The average of RNU6B and RNU24 was used for normalization. **(B)** Expression levels of miR-155, miR-221, and miR-222 during adipogenesis of hMSC-Tert20 in more details. Data are expressed as mean ± standard error of the mean (*n* = 3). **(C)** Expression levels of miR-155, miR-221, and 222 during adipogenesis of primary hMSCs were analyzed using qPCR. The average of RNU6B and RNU24 was used for normalization. hMSC, human bone marrow-derived mesenchymal stromal cell; miRNA, microRNA; qPCR, quantitative polymerase chain reaction; d and h, days and hours after induction of differentiation, respectively.
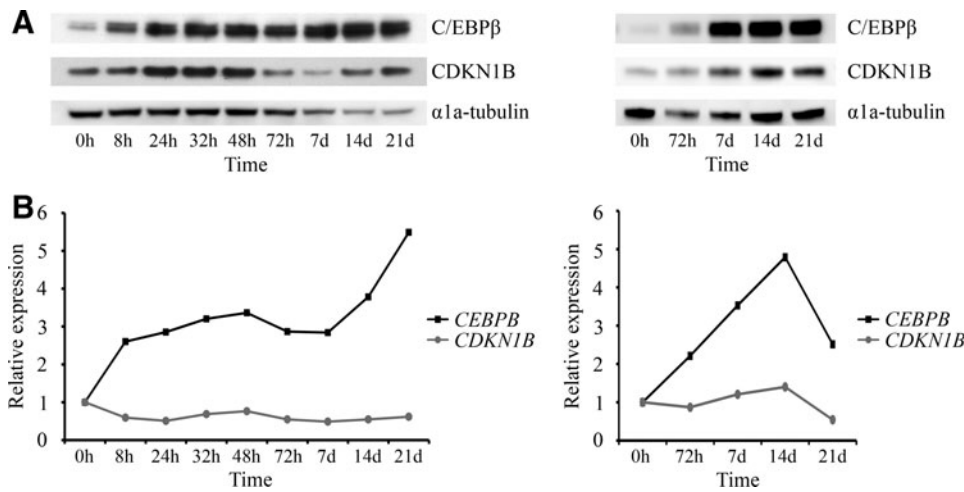
FIG. 2. Expression patterns of *CEBPB* and *CDKN1B* during adipocyte differentiation of hMSC-Tert20 and primary hMSCs. **(A)** The kinetics of CCAAT/enhancer-binding protein beta (C/EBPβ) and cyclin-dependent kinase inhibitor 1B (CDKN1B) protein levels at the indicated time points during adipogenesis were determined by western blot analysis. α1a-tubulin was used as loading control. Data are representative of 2 independent experiments. d and h: days and hours after induction of differentiation, respectively. **(B)** Analysis of the transcript levels of *CEBPB* and *CDKN1B* at the indicated time points during adipogenesis using qPCR. The average of TATA-binding protein (*TBP*) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used to normalize the data. d and h, days and hours after induction of differentiation, respectively.

a significant overrepresentation of genes involved in cell cycle process networks and developmental pathways, including differentiation of hematopoietic stem cells. Furthermore, we identified several interesting common candidate targets whose expression levels inversely correlated with the levels of miR-155 and miR-221/222 (data not shown). Two examples are the phosphoinositide-3-kinase (PI3 kinase) regulatory subunit 1 (*PIK3R1*) and a small subunit of serine palmitoyl-transferase A (*C14orf147*), both of which have been shown to be important in processes related to adipogenesis.

Ectopic expression of miR-155 or miR-221/222 inhibits adipocyte differentiation

To assess the roles of miR-155 and miR-221/222 during adipocyte differentiation of the immortalized hMSC-Tert20 cells, we developed an adenovirus-based expression system that efficiently generates mature miRNAs (Fig. 3A). Cells were transduced 24 h before induction of adipogenesis either with viruses expressing miR-155 (Ad-miR-155) or miR-221/222 (Ad-miR-221/222) or with the empty vector alone (Ad-

control). Increased levels of all 3 miRNAs were observed for at least 14 days after transduction (Fig. 3A).

Interestingly, cells transduced with Ad-miR-155 or Ad-miR-221/222 showed a significant decrease in triglyceride accumulation compared with Ad-control and untransduced cells, as seen by Oil Red O staining on day 14 of differentiation (Fig. 3B, C). To investigate the underlying mechanisms responsible for this inhibitory effect, we used qPCR to determine the transcript levels of 6 key proteins involved in adipogenesis, namely *CEBPB*, *PPARG*, *CEBPA*, *SCD*, *CFD*, and *ADIPOQ*, on days 1, 2, 3, 7, and 14 after induction of differentiation (Fig. 4A). We also included *CDKN1B* because we wanted to determine whether this gene is targeted by miR-221/222 during differentiation. In addition, the protein levels of C/EBPβ, PPARγ, C/EBPα, SCD, and CDKN1B were examined on days 1, 3, and 7 of differentiation (Fig. 4B).

In cells transduced with Ad-miR-155, the C/EBPβ protein level was reduced at day 3 after an initial increase (Fig. 4B). Four days later, the C/EBPβ level was comparable to that of the control. However, the transcript level of *CEBPB* was not changed (Fig. 4A). A strong repression of the PPARγ protein
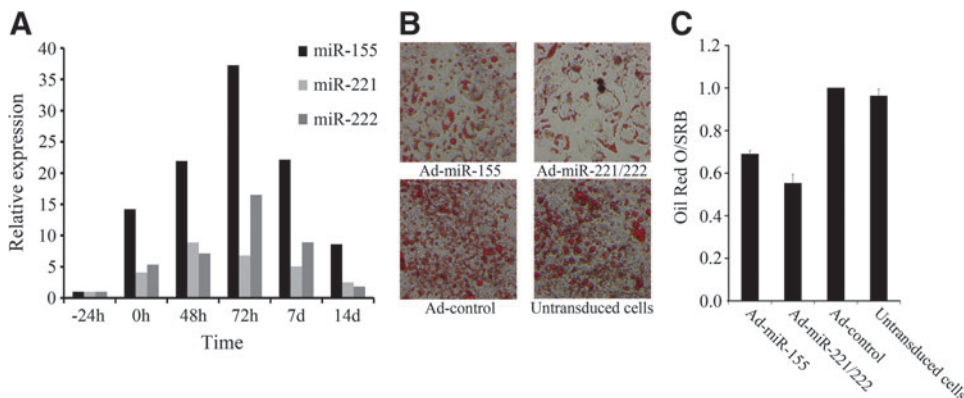

FIG. 3. Ectopic expression of either miR-155 or miR-221/222 inhibits adipocyte differentiation of hMSC-Tert20 cells. Cells were transduced with Ad-miR-155, Ad-miR-221/222, or Ad-control at 24 h before induction to adipocytes, and cultured for 14 days before the experiment was terminated. **(A)** Increased levels of miR-155, miR-221, and miR-222 at the indicated time points were confirmed by qPCR. The average of RNU6B and RNU24 was used for normalization. d and h: days and hours, respectively. **(B)** The cells were fixed and stained with Oil Red O at day 14. Representative images of triglyceride formation are shown (10×magnification). **(C)** Oil Red O was extracted at day 14, quantified, normalized to total protein using sulforhodamine B (SRB) staining, and plotted relative to the level of control cells expressing vector alone (Ad-control). Data shown are the mean value±standard error of the mean (*n* = 3).
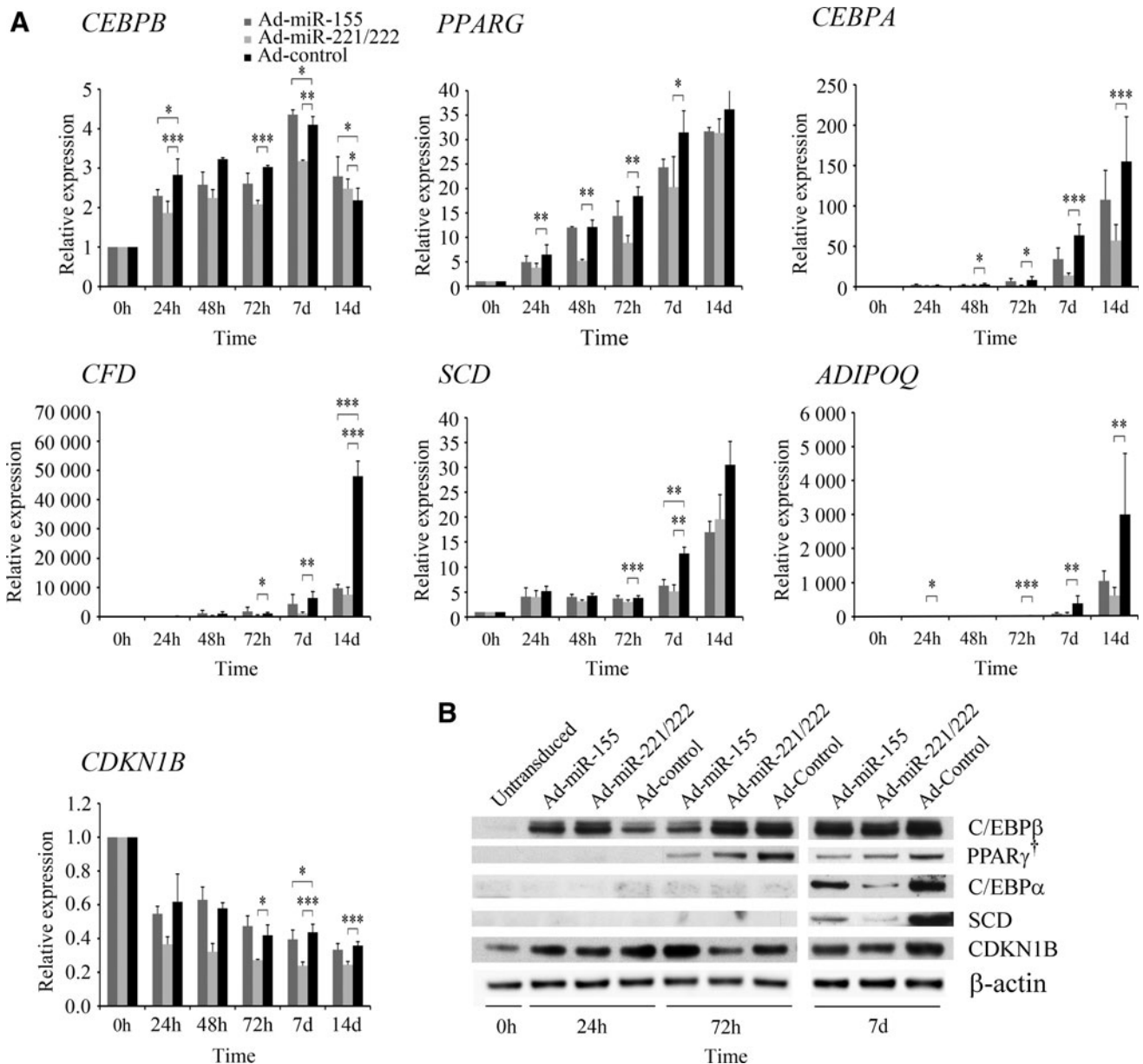
FIG. 4. Overexpression of either miR-155 or miR-221/222 during adipogenesis of hMSC-Tert20 represses the induction of key regulators at the mRNA and protein levels. **(A)** The expression levels of CCAAT/enhancerbinding protein beta *(CEBPB)*, peroxisome proliferator-activated receptor gamma *(PPARG)*, CCAAT/enhancerbinding protein alpha *(CEBPA)*, stearoyl coenzyme A desaturase 1 *(SCD)*, complement factor D *(CFD)*[1], *ADIPOQ*,[1] and cyclin-dependent kinase inhibitor 1B *(CDKN1B)* were analyzed at the indicated time points using qPCR. The average of TATA-binding protein *(TBP)*[1] and human acidic ribosomal phosphoprotein *(RPLPO)*[1], or of *TBP* and glyceraldehyde 3-phosphate dehydrogenase *(GAPDH)*, was used to normalize the data for the SYBR Green or TaqMan Gene Expression Assays, respectively. [1]SYBR Green assays. Quantitative data are expressed as mean ± standard error of the mean *(n = 3)* relative to unstimulated cells at day 0 (0 h). The expression levels in cells transduced with either miR-155 or miR-221/222 were compared with control cells using a 2-tailed Student's *t*-test (***$P < 0.01$; **$P < 0.05$; *$P < 0.1$). Statistics were based on $\Delta\Delta C_T$ values and a *P* value of < 0.05 was considered statistically significant. **(B)** The protein levels of C/EBPβ, PPARγ, C/EBPα, SCD, and CDKN1B were assessed at the indicated time points during adipogenesis by western blot analysis. β-actin was used as loading control. Data shown are representative of 2 biological experiments. [†]The level of PPARγ increased dramatically from day 3 to 7, but the exposure time at the latter day was reduced to show the differences between cells transduced with Ad-miR-155, Ad-miR-221/222, or Ad-control. d and h: days and hours after induction of adipogenesis, respectively.

at days 3 and 7 of differentiation was also observed in these cells, whereas the transcript level was not decreased. Furthermore, C/EBPα was not detected at day 1 or 3 but was highly expressed after a week in the control cells, an increase that was reduced in cells with enforced expression of miR-155. The transcript level of *CEBPA* was not significantly repressed. An expression pattern similar to that of *CEBPA* was also observed for *SCD* both at the transcript and protein levels. In addition, a significant reduction of the late marker *CFD* was observed in these cells.

In cells transduced with Ad-miR-221/222, a significant reduction of *CEBPB*, *PPARG*, *CEBPA*, *CFD*, *SCD*, and *ADIPOQ* was observed at one or more time points (Fig. 4A). As observed in cells transduced with Ad-miR-155, the C/EBPβ protein level was also higher after 24 h in cells with ectopic expression of miR-221/222, but it was comparable to control cells at later time points (Fig. 4B). The level of PPARγ was reduced at days 3 and 7, although the repression was weaker compared with the control cells and appeared later than when miR-155 was overexpressed. Further, C/EBPα and SCD were reduced to a much greater extent compared to the control in cells with enforced expression of miR-221/222 than in cells transduced with Ad-miR-155. Interestingly, a significant reduction of the transcript level of the predicted target gene *CDKN1B* was observed in cells with ectopic expression of miR-221/222, whereas overexpression of miR-155 had no effect. The CDKN1B protein level was also lower in these cells, with the most pronounced effect at day 3.

To validate our findings in the immortalized hMSC-model system, we extended the functional studies to primary hMSCs. In these experiments we used synthetic miRNA precursors (Pre-miR-155 and Pre-miR-222) (Fig. 5A), because these cells do not tolerate transduction with adenoviruses well (data not shown). A negative control precursor was used as control (Pre-control). As observed in hMSC-Tert20, introduction of either miR-155 or miR-222 (which represents miR-221/222) at 24 h before induction of adipogenesis impaired differentiation of the primary hMSCs. This was observed both as a decrease in triglyceride accumulation (Fig. 5B) and as a significantly reduced expression of *CEBPA*, *PPARG*, and *ADIPOQ* at the transcript levels (Fig. 5C). Further, the protein levels of PPARγ and C/EBPα at days 3 and 7 during differentiation were reduced in these cells (Fig. 5D). In contrast to what was observed in hMSC-Tert20, a significant repression of the transcript levels of *CEBPB* and *CDKN1B* was not observed in the primary hMSCs. However, induction of the C/EBPβ and CDKN1B proteins was attenuated in cells transfected with Pre-miR-155 or Pre-miR-222, respectively, with the most pronounced effect after 72 h.

Discussion

This study aimed to identify miRNAs that might be involved in human adipogenesis, using immortalized multipotent bone marrow-derived stromal cells as a model system. By global microarray analysis we identified 12 miRNAs (let-7a, miR-18a, miR-21, miR-26a, miR-30d, miR-34a, miR-92, miR-146a, miR-155, miR-214, and miR-221/222) with validated changes in expression during adipocyte differentiation. Among these, several have been previously shown to have important roles in adipocyte biology. Increased levels of miR-21 have been reported to enhance adipocyte differentiation of hMSCs derived from human adipose tissue by blocking TGF-β signaling [33]. In 3T3-L1 cells, let-7 regulates adipogenesis, most likely by targeting HMGA2 [17]. Removal of this repression blocks adipocyte differentiation and induces a transformed phenotype [34]. Overexpression of the miR-17~92 cluster accelerates adipocyte differentiation by negatively regulating the tumor-suppressor *RBL2* [18]. Finally, miR-30d seems to be involved in insulin production in murine pancreatic cells [35].

To identify new aspects of miRNA regulation of human adipocyte differentiation, we focused on miR-155 and miR-221/222. The expression of these miRNAs decreased during adipogenesis of both immortalized hMSC-Tert20 cells and primary hMSCs. A recent study has also validated the downregulation of miR-155 and miR-221/222 during differentiation of primary cultures of human subcutaneous preadipocytes [36], and a decrease in miR-221/222 has been observed during adipocyte differentiation of other primary hMSCs and 3T3-L1 cells [37,38]. These observations strongly suggest that miR-155 and miR-221/222 act as crucial negative regulators of genes that are important for this process. Interestingly, ectopic expression of either miR-155 or miR-221/222 reduced the adipogenic potential of hMSC-Tert20 cells as measured by both a significant reduction in triglyceride accumulation and attenuation of the induction of several important adipocyte proteins, including PPARγ and C/EBPα. Moreover, the genes coding for the adipocyte marker C/EBPβ and the cyclin-dependent kinase inhibitor CDKN1B were 2 of the most interesting candidates for regulation by miR-155 and miR-221/222, respectively.

miR-155 has been demonstrated to block the translation of C/EBPβ in several studies unrelated to adipogenesis [39–41], suggesting that it is in fact a *bona fide* target. Interestingly, the protein level of *CEBPB* was reduced at 72 h after induction of adipogenesis in cells transduced with Ad-miR-155, although no significant change was observed at the transcript level. Thus, here the reduction of C/EBPβ appears to be a result of translational inhibition, rather than mRNA degradation, although indirect effects cannot be excluded. Nevertheless, this decrease could be expected to impair adipocyte differentiation as inhibition of C/EBPβ activity has been demonstrated to reduce the expression of the master regulators PPARγ and C/EBPα [30]. In agreement with this, a reduction of both proteins was observed in cells with a reduced level of C/EBPβ. However, no decrease in *PPARG* transcripts was seen, suggesting that post-transcriptional regulation is involved also here. Further, C/EBPα transactivates several genes, including *SCD*, coding for an enzyme that is important for fatty acid synthesis [42]. Consistent with this, both the mRNA and protein levels of *SCD* were reduced in cells with decreased level of C/EBPα. Two other genes that have been reported to be regulated by C/EBPα are the adipocytokines *CFD* and *ADIPOQ* [43,44]. This regulatory pathway seems to be intact in these cells as well, as their levels were reduced.

Inhibition of several adipocyte genes was also observed in cells transduced with Ad-miR-221/222. Interestingly, the level of *CEBPB* mRNA was significantly reduced in these cells, although it is not a predicted target. This suggests that miR-221/222 may indirectly affect *CEBPB* expression or mRNA turnover in an early stage of differentiation. However, miR-221/222 did not repress the level of C/EBPβ protein, suggesting a more complex mode of regulation. Despite this, a strong reduction of *PPARG* both at the transcript and protein levels was observed in cells with overexpression of miR-221/222. *PPARG* has been suggested to be a direct target of miR-221/222, but this has not been experimentally verified [45]. As observed in cells with ectopic expression of miR-155, C/EBPα and SCD were also repressed in cells with overexpression of miR-221/222. In addition to adipocyte markers, several checkpoint-control proteins are also involved in adipocyte differentiation [46]. The cyclin-dependent inhibitor CDKN1B is essential for the early stages of hMSC-adipogenesis, and its upregulation has
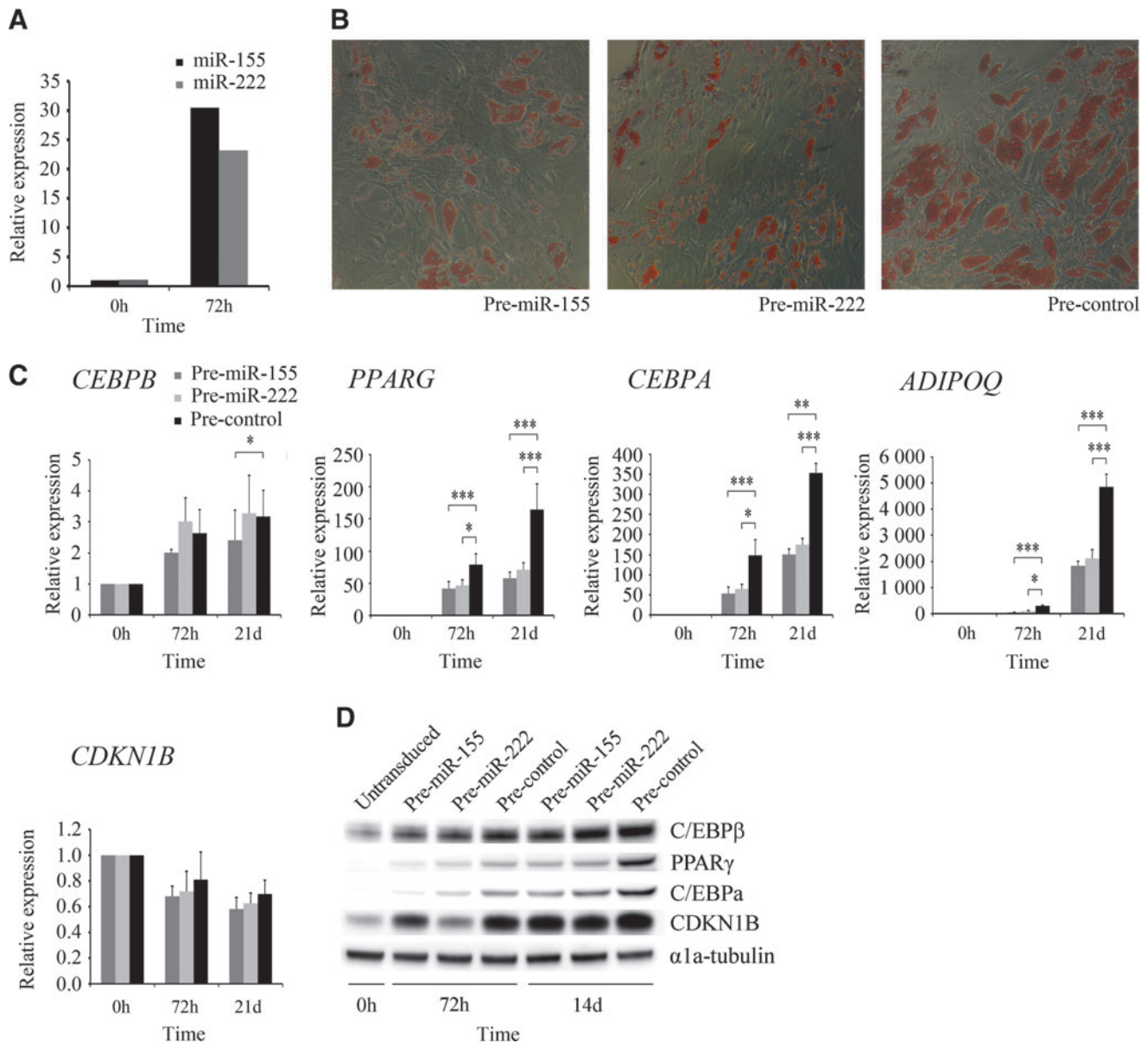
FIG. 5. Overexpression of either miR-155 or miR-222 during adipogenesis of primary hMSCs inhibits accumulation of triglycerides and represses the induction of key regulators at the mRNA and protein levels. Primary hMSCs were transfected with miR-155 or miR-222 (which represents miR-221/222) precursor RNAs (Pre-miR-155 and Pre-miR-222, respectively) or with a negative control precursor (Pre-control). **(A)** Increased levels of miR-155 and miR-222 in transfected cells were confirmed by qPCR. The average of RNU6B and RNU24 was used for normalization. h, hours after transfection. **(B)** The cells were fixed and stained with Oil Red O at day 21. Representative images of triglyceride accumulation are shown (10× magnification). **(C)** The expression levels of CCAAT/enchancerbinding protein beta *(CEBPB)*, peroxisome proliferator-activated receptor gamma *(PPARG)*, CCAAT/enchancerbinding protein alpha *(CEBPA) ADIPOQ*[1], and cyclin-dependent kinase inhibitor 1B *(CDKN1B)* at the indicated time points were determined using qPCR. The average of TATA-binding protein *(TBP)*[1] and human acidic ribosomal phosphoprotein *(RPLPO)*[1], or of *TBP* and glyceraldehyde 3-phosphate dehydrogenase *(GAPDH)*, was used to normalize the data for the SYBR Green or TaqMan Gene Expression Assays, respectively. [1]SYBR Green assays. Quantitative data are expressed as mean ± standard error of the mean ($n = 3$) relative to unstimulated cells at day 0 (0 h). The expression level in cells transfected with either Pre-miR-155 or Pre-miR-222 was compared to control cells (Pre-control) using a 2-tailed Student's *t*-test (***$P < 0.01$; **$P < 0.05$; *$P < 0.1$). Statistics were based on $\Delta\Delta C_T$ values and a P value of < 0.05 was considered statistically significant. d and h: days and hours after induction of differentiation, respectively. **(D)** The protein levels of C/EBPβ, PPARγ, C/EBPα, and CDKN1B were assessed at the indicated time points by western blot analysis. α1a-tubulin was used as loading control.

been associated with growth arrest [31]. miR-221/222 are predicted to target *CDKN1B* and have been demonstrated to be regulators of this gene in several systems [47]. Interestingly, both the transcript and protein levels of *CDKN1B* were

significantly decreased in cells with enforced expression of miR-221/222, whereas ectopic expression of miR-155 had no effect. This supports that *CDKN1B* is regulated by miR-221/222 also during adipogenesis of hMSC-Tert20. However, this

inhibition did not result in significant growth stimulation, which could have been because these cells were transformed [3], or because a subpopulation continued to rapidly proliferate during differentiation. The repression of adipogenesis could alternatively be mediated through cell-cycle independent functions, as CDKN1B can repress transcription directly or indirectly by inhibiting cyclin–CDK complexes. It also seems to be involved in the regulation of Rho-signaling and cytoskeletal dynamics [48]. Intriguingly, RhoA activity has been demonstrated to regulate the switch between osteogenic and adipogenic hMSC-fates [49].

To confirm the functional data obtained using the immortalized hMSC-Tert20 cells, we transfected miR-155 and miR-222 (which represents miR-221/222) precursor RNAs into primary hMSCs. Also here adipocyte differentiation was strongly impaired by the miRNAs. Although we did not see any significant changes in the transcript levels of *CEBPB* and *CDKN1B*, as was observed in hMSC-Tert20, the corresponding protein levels were reduced in cells transfected with miR-155 or miR-222, respectively. In agreement with the previous findings using immortalized hMSCs, this strongly suggests that *CEBPB* and *CDKN1B* are regulated by a post-transcriptional mechanism. The repression of differentiation by the 2 miRNAs was also more similar than what was observed in hMSC-Tert20. These discrepancies could reflect intrinsic differences between the immortalized and primary hMSCs, but also be due to technical issues, such as the mode of delivery.

As miRNAs are predicted to interact with a large number of genes, it is likely that miR-155 and miR-221/222 could also regulate differentiation by inhibition of various cofactors that act indirectly on adipogenesis [46]. For instance, the mediator complex subunit 1 is required for PPARγ-stimulated adipogenesis [50]. Interestingly, this gene is predicted to be regulated by miR-221/222. In addition, several miRNAs can act together and repress the same gene [51], and the fact that enforced expression of either miR-155 or miR-221/222 repressed adipogenesis suggests the possibility of common targets. Two of the most interesting common genes proposed to be regulated by all 3 miRNAs were *PIK3R1* and *C14orf147*, which were part of an interconnected network. The transcript levels of these genes increased during adipogenesis, consistent with regulation by either miR-155 or miR-221/222, or both. *PIK3R1* encodes the 85-kDa regulatory subunit of PI3 kinase, which is involved in a wide variety of cellular processes and is an important component of the insulin signaling pathway [52,53]. Moreover, inhibition of PI3 kinase during adipogenesis reduces the upregulation of several adipocyte markers [54]. *C14orf147* encodes a small subunit of serine palmitoyltransferase A [55], and its level has been correlated with the activity of the transferase, which is a key enzyme for the *de novo* synthesis of sphingolipids [56]. Sphingolipids are involved in the uptake and release of long-chain fatty acids, which is one of the major activities of adipocytes [57].

In addition to their roles in adipogenesis, miR-155 and miR-221/222 also participate in the differentiation of other cell types. miR-155 negatively regulates myeloid and erythroid differentiation [45,58], and miR-221/222 block erythropoiesis [45,59]. In addition, miR-221/222 are downregulated during myogenesis [60]. Thus, they may act as more general inhibitors of cell differentiation and not be specific for adipogenesis.

In summary, we provide mechanistic evidence that miR-155 and miR-221/222 inhibit adipocyte differentiation of human bone marrow-derived stromal cells. Introduction of either miRNA into immortalized hMSCs (miR-155 or miR-221/222) or miR-155 or miR-222 into primary hMSCs (miR-155 or miR-222) efficiently repressed adipogenesis. This suggests that downregulation of these specific miRNAs is necessary to relieve the repression of genes important for human adipogenesis. As miR-155 and miR-221/222 are predicted to regulate a large number of genes, their effects on differentiation are likely to be complex. Candidate target genes include *CEBPB* and *CDKN1B*, which protein levels were reduced when miR-155 and miR-221/222, respectively, were overexpressed. We propose that the expression of these genes is modulated by miR-155 and miR-221/222 during adipocyte formation. However, further studies will be needed to better define the role of these miRNAs. Finally, the fact that similar results were obtained using both hMSC-Tert20 and primary hMSCs demonstrates that immortalized hMSCs can be a good model system to study adipocyte differentiation.

Acknowledgments

Author Disclosure Statement

The authors have no conflicts of interest.

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