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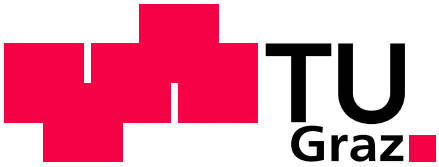
Validation of microRNAs targeting human adipogenesis

Bachelor thesis
to achieve the degree of a
Bachelor of Science in Health Studies (BSc)



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Graz, May 2009

Acknowledgements

This bachelor thesis would not have been possible without the support of many people.

I´d like to thank my supervisor Univ. Ass. Dipl.-Chem. Dr. Marcel Scheideler who was always helpful and gave me a lot of imput, support and guidance.
For his patience and his time deserves Dipl.-Ing. Michael Karbiener Bsc great thanks and I am sure it was not always easy with me.
The next two people I would thank have sweetened the work in the Diplomandenraum with several daily coffee breaks and good and motivating talks, Christoph Fischer and Bettina Halwachs.
I also would thank Claudia, Markus, Lissi and Florian for their support and help. They build the heart of the laboratory.
Many thanks to the head of the Institute, Univ.-Prof. Dipl.-Ing. Dr.techn. Zlatko Trajanoski for the possibility to write this bachelor thesis at the Institute of Genomics and Bioinformatics.

Next, I would like thank my parents for their support. Without their support this work would never have been begun and finished.

Michi for your irrepressible optimism and help, no matter how my mood was, you always supported me in any situation.

Abstract

Objective:

MicroRNAs are short non-coding RNAs, which play an important functional role in gene regulation predicted to be able to regulate around 200 target genes on average. They open up novel oppurtunities in therapy, potentially also with positive effects on obesity and diabetes. Numerous microRNAs are known to play a regulatory function in murine adipogenesis, whereas only one human microRNA has been validated in human adipogenesis so far, with the potential for many more. Therefore, the aim of this thesis is the functional characterization of two microRNAs, miR-222 and miR-27b, in human adipocyte differentiation.

Background:

For the functional characterization of microRNAs in adipocyte differentiation, the two candidate microRNAs were overexpressed, using HiPerFect as transfection agent and microRNA mimics, in human multipotent adipose-derived stem (hMADS) cells during adipogenesis. Alterations in adipocyte differentiation were measured qualitatively by Oil Red O staining and quantitatively by the triglyceride assay. These overexpression experiments were performed in hMADS cells of two donors with biological replicates in order to elucidate the donor-independent function of the miRNAs.

Results:

Overexpression of miR-222 only showed a donor-specific effect in hMADS-2 cells, whereas adipogenesis in hMADS-3 cells was neither inhibited nor promoted.

Overexpression of miR-27b showed a donor-independent effect on adipogenesis in hMADS-2 and hMADS-3 cells. Triglyceride accumulation was significantly inhibited. Interestingly, triglyceride accumulation was inhibited when miR-27b was overexpressed before differentiation induction, but not if overexpressed with differentiation start indicating a very early regulatory function of miR-27b in human adipocyte differentiation.

Zusammenfassung

Einleitung:

MicroRNAs sind kurze nicht-kodierende RNAs, die eine wichtige funktionelle Rolle in der Genregulation spielen, und im Durchschnitt werden pro microRNA rund 200 Targetgene vorhergesagt. Sie eröffnen neue, therapeutische Ansätze, möglicherweise auch mit positiven Auswirkungen auf Übergewicht und Diabetes. Zahlreiche microRNAs sind bekannt dafür, dass sie eine regulatorische Funktion in der murinen Adipogenese haben. Dagegen ist bisher nur eine einzige humane microRNA in der humanen Adipogenese validiert worden, mit dem Potenzial für viele mehr. Daher ist das Ziel dieser Arbeit, die funktionelle Charakterisierung von zwei microRNAs, miR-222 und miR-27b, in der humanen Adipozytendifferenzierung.

Ziel:

Für die funktionelle Charakterisierung von microRNAs in der Adipozytendifferenzierung wurden die beiden ausgewählten microRNAs überexprimiert. Dafür wurden microRNA mimics mit HiPerFect als Transfektionsreagenz in human mesenchymalen Stammzellen (hMADS-Zellen) angewandt. Die Änderungen in der Adipozytendifferenzierung wurden qualitativ durch Oil Red O-Färbung und quantitativ durch den Triglyceridassay gemessen. Die Überexpressionsexperimente wurden mit hMADS-Zellen von zwei Donoren in biologischen Replikaten durchgeführt, um donor-unabhängige Funktionen der microRNAs in der Adipogenese zu identifizieren.

Resultate:

Die Überexpression von miR-222 zeigte einen donor-spezifischen Effekt in der hMADS-2-Adipogenese, wohingegen die Überexpression in hMADS-3-Zellen keinen Effekt auf die Adipogenese hatte. Die Überexpression von miR-27b zeigte donor-unabhängige Effekte auf die Adipogenese in hMADS-2- und hMADS-3-Zellen. Die Triglyceridakkumulation wurde signifikant inhibiert. Interessanterweise wurde die Triglyceridakkumulation nur inhibiert, wenn miR-27b vor dem Start der Differenzierung überexprimiert wurde, aber nicht, wenn die Überexpression am gleichen Tag stattfand wie der Differenzierungsstart. Dies deutet auf eine sehr frühe regulatorische Funktion von miR-27b in der humanen Adipozytendifferenzierung hin.

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1 Introduction

1.1 Obesity

The WHO indicated that 1.6 billion adults were overweight, 400 million adults were obese and the number will highly increase till 2015. Not only adults are affected, in 2005 20 millions children under the age of 5 years are overweight globally. This problem is not only a problem in the western countries due to the higher life-style and income as believed before but also in the low- and middle-income countries, particularly in urban settings. So what are overweight and obesity? Overweight and obesity are defined as abnormal or excessive fat accumulation that may impair health. This will be expressed in the body mass index (BMI). This is an index of weight-for-height that is commonly used in classifying overweight and obesity in adult population and individuals. It is defined as the weight in kilograms divided by the square of the height in meters (kg/m^2). BMI is the most useful population-level measure of overweight and obesity and valid for people both sexes and all ages of adults but it do not correspond to the same degree of fatness in different individuals. A BMI equal to or more than 25 is defined as overweight, more than 30 are deemed as obesity. The cause of obesity and overweight is an energy imbalance between the ingested calories and the expended calories. The diseases related to obesity and overweight are hypertension, type II diabetes, cardiovascular disease and metabolic syndrome (http://www.who.int/mediacentre/factsheets/fs311/en/index.html, May 24th 2009)

1.2 Adipogenesis

The process of adipogenesis, from the mesenchymal precursor to the mature adipocyte is divided in six stages (Lefterova and Lazar, 2009). The characteristic of the first stage, the mesenchymal precursor is the proliferation of stem cells of mesodermal origin and have therefore the ability to differentiate into multiple lineages. The proliferation and the commitment to differentiation along adipocyte lineage are the main features of the second stage, named committed preadipocyte.

The morphology of the cells is fibroblast-like. The third step of the adipogenesis is the growth-arrested preadipocyte because of the lack of proliferation due to contact inhibition. Stage four is the mitotic clonal expansion. Based on the induction by hormonal stimulation the cells reentered into the cell cycle. It comes to several rounds of cell division and to the induction of C/EBPα und C/EBPβ expression and activity. The second last stage is the terminal differentiation. The cell-cycle arrested and the expression of PPARγ and C/EBPα induced. Before the adipocytes got mature the adipocyte marker genes (lipid and carbohydrate metabolism genes, adipokines) would be activated transcriptionally. The last stage is named mature adipocyte. The adipocyte marker genes would be highly expressed and become their signet-ring morphology. The majority of the cell volume is full of large lipid droplets. And last for the requirement for the transcriptional control of adipogenesis, it needs the sequential activation of a transcription factor cascade. These are mainly PPARγ, C/EBPα and C/EBPβ (Lefterova and Lazar, 2009), (Sethi and Vidal-Puig, 2007).

1.2.1 Cascade of transcription factors

The central players in adipogenesis are the nuclear receptor PPARγ (peroxisome proliferatior-activated receptor γ) and the members of the C/EBP (CCAAT / enhancer binding protein) family. PPARγ and C/EBP are responsible for the coordination of the adipogenic gene expression. Figure 1 shows the transient expression of C/EBPβ and C/EBPδ which induce C/EBPα and leads in turn to the activation of PPARγ2. These two main regulators are switching on the broad program of adipogenesis. There is an endeavor for a positive feedback of PPARγ to C/EBPα and acts synergistically to keep the differentiate state stable. The regulation by further transcriptional factors, such as sterol-regulatory element binding protein 1c (SREBP1c) are also required for adipocyte differentiation which is regulated by insulin and lipids. PPARγ can be activatd by SREBP1c on the one hand by inducing its expression, on the other hand by promoting the production of an endogenous PPAR ligand. The adipocyte machinery which includes many genes of the lipogenic program is also activated by SREBP1c. Together, these factors contribute to the expression of genes that characterize the terminally differentiated phenotype. (Sethi and Vidal-Puig, 2007).

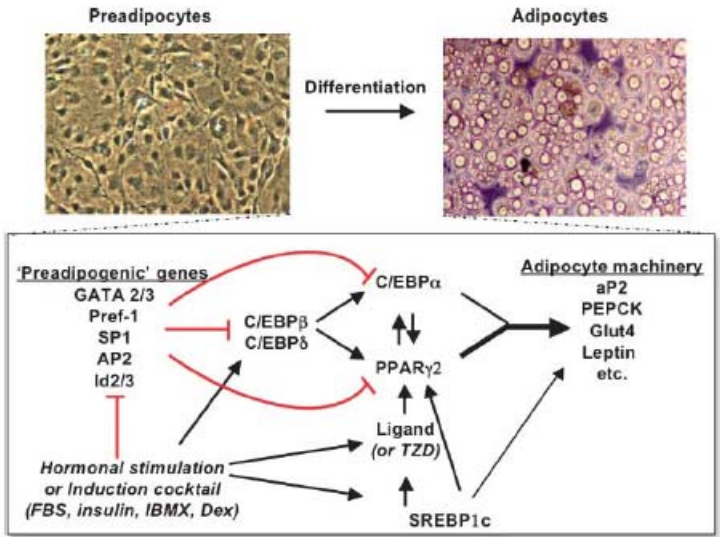


Figure 1: Transcription factor cascade (Sethi et al., 2007)

1.2.2 hMADS cells

Human multipotent adipose-derived stem (hMADS) cells are a novel source of multipotent stem cells isolated from adipose tissue. A multipotent stem cell is by definition a special kind of cell that has the unique capacity to self-renew for indefinite periods. That signifies that the stem cells can be expanded ex vivo and through the right signals this multipotent stem cell can differentiate into many different cell types, e.g. adipogenic, osteogenic, myogenic and chondrogenic cells (Rodriguez et al., 2005a), (Zuk et al., 2001). hMADS cells which derived from young donors, were isolated and characterized from adipose tissue. These cells can be maintained ex vivo using an original procedure (Rodriguez et al., 2005a; Rodriguez et al., 2005b). hMADS cells have the advantage that repeated freezing/thawing causes no loss of multipotential properties. These are attributes that makes hMADS cells unique as alternative stem cell source for studies of fat tissue development and metabolism or as tool for cell-mediated therapies (Rodriguez et al., 2005a)

1.3 microRNAs

miRNAs are a class of small non-coding RNAs, approximately 22 nucleotides in length, which regulate the gene expression post-transcriptionally (Orom et al., 2006) by binding to complementary sequences within the 3' untranslated region (3'UTR) of target mRNAs (Lewis et al., 2005) and finally leads either to transcriptional degradation or translational repression (Bartel, 2004).

Victor Ambros and his colleagues discovered more than 15 years ago a novel gene-regulatory system. The first miRNA lin-4 was found in *C. elegans*, which is elementary for the normal temporal control of diverse postembryonic developmental stages. From the first to the second larval stage of development, lin-4 acts by negatively regulating of lin-14-level. This miRNA produces a 21 nucleotide RNA that identifies complementary sites in the 3' untranslated region (3'UTR) of the lin-14 messenger (Lee et al., 1993). Seven years later a furter miRNA was identified by using forward genetics. Let-7 encodes a temporally regulated 21-nucleotide small RNA that promotes the transition from the fourth stage into the adult stage as the same way that the lin-4 acts earlier in larval development (Reinhart et al., 2000) The breadth and importance of miRNA-directed gene regulation are coming into focus as more miRNAs and their regulatory targets and functions are discovered. These miRNA functions are involved in many biological mechanisms e.g. cellular development and differentiation, control of cell proliferation, cell death and metabolism (Brennecke et al., 2003; Xu et al., 2003).

1.3.1 miRNA involvement in metabolism

Recent studies revealed that miRNAs play a crucial role in metabolism and could represent a novel group of therapeutic targets for metabolic and cardiovascular diseases. One of the first clues that miRNAs may have a functional role in metabolism was from the miR-14 mutant Drosophila. The lipid droplets were enlarged in the fat body, the primary fat storage site in flies (Xu et al., 2003). A further miRNA, which is a liver-specific, is miR-122. It is suggested that this miRNA play an important role in cholesterol, fatty acid and lipid metabolism (Esau et al., 2006; Krutzfeldt et al., 2005). The inhibition of miR-122 with antisense-

oligonucleotides resulted in a reduction of plasma cholesterol levels and an increased hepatic fatty-acid oxidation. It was also shown that the inhibition caused a decreased hepatic fatty-acid and cholesterol synthesis rate. miRNA-375 is a further miRNA involved in metabolism and may have potential to treat diabetes mellitus. Poy et al. showed that overexpression of miR-375 suppressed glucose-induced insulin secretion. In contrast, an inhibition of endogenous miR-375 function amplified insulin secretion. Changes in glucose metabolism or intracellular Ca21-signalling do not influence the mechanism of secretion which is modified by miR-375. A target of miR-375 is Myotrophin (Mtpn) that is already predicted and validated for this microRNA. The effect of miR-375 was mimicked on glucosestimulated insulin secretion and exocytosis by inhibition of Mtpn with small interfering RNA (siRNA). Mir-375 is a further regulator of metabolism especially for insulin secretion and may constitute as a novel pharmacological target for the treatment of diabetes (Poy et al., 2007).

1.3.2 miRNA involvement in lipid metabolism and adipogenesis

miRNAs are numerously involved in metabolism which might be important for future therapeutics in obesity – adipogenesis.

The first and sole miRNA which has been shown so far to play a crucial role in human adipogenesis is miR-143 which is up-regulated during terminal adipocyte differentiation. Esau et al. showed in 2004, that one miRNA, miR-143 normally promotes adipocyte differentiation. It was revealed after inhibition of a number of miRNAs in human white pre-adipocytes by using oligonucleotides and a microarray-based expression analysis of miRNAs in differentiating adipocytes. ERK5, predicted to be a target of miR-143 is up-regulated on protein levels in miR-143 ASO-treated adipocytes. The identification of miR-143 showed a further miRNA as a potential therapeutic target for obesity and metabolic disease (Esau et al., 2004). On the other site, in general, each miRNA is expected to regulate multiple target genes. Hence, it is not known whether the effect of miR-143 inhibition on adipocyte differentiation is mainly mediated through ERK5 or whether other target genes of miR-143 are involved (Esau et al., 2004). It is known that ERK5 promotes cell growth and

proliferation in response to tyrosine kinase signaling (Kato et al., 1998). The role in adipocyte differentiation has not been investigated and requires therefore further experiments.

miR-143 is the only human miRNA but there are many more miRNAs found in mouse with a functional role in adipogenesis. Sun et al. revealed that the well known miRNA let-7 were up-regulated during 3T3-L1 adipogenesis. The transcription factor high mobility group AT-hook 2 (HMGA2) regulates growth and proliferation is encoded by the mRNA. Additionally HMGA2 was inversely correlated with let-7 levels during 3T3-L1 cell adipogenesis and markedly reduced the concentration of this transcription factor. They also showed that a knockdown of HMGA2 caused to an inhibition of 3T3-L1 differentiation. Due to the fact, that HMGA2 is targeted by let-7, by regulating the transition from clonal expansion to terminal differentiation, let-7 is suggested to play an important role in adipocyte differentiation (Sun et al., 2009)

A further miRNA that is found in mouse is miR-335 which is up-regulated in obese mice. Several factors like an elevated body, liver and WAT weight as well as hepatic triglyceride and cholesterol were associated with an increased miR-335 expresssions. Furthermore, miR-335 levels were closely correlated with expression levels of adipocyte differentiation markers such as PPARc, aP2, and FAS in 3T3-L1 adipocyte (Nakanishi et al., 2009)

Wang et al. revealed that miR-17-92, a miRNA cluster that promotes cell proliferation in various cancers was up-regulated at the clonal expansion stage during mouse preadipocyte 3T3-L1 cell differentiation. A transfection of 3T3-L1 cells with miR-17-92 showed in accelerated differentiation and increased triglyceride accumulation after hormonal stimulation. Furthermore it was showed that miR-17-92 directly targeted the 3'UTR region of Rb2/p 130, accounting for subsequently reduced Rb2/p130 mRNA and protein quantities at the stage of clonal expansion. siRNA-mediated knock-down of Rb2/p130 at the same stage of clonal expansion recapitulated the phenotype of overexpression of miR-17-92 in the stably transfected 3T3L1 cells (Wang et al., 2008).

1.3.3 Biogenesis of microRNAs

miRNA genes encode long primary mRNA transcripts which generate mature miRNAs through a series of endonucleolytic maturation steps (Kim, 2005) (Figure 3). It is believed that these steps are essential for the production of functional miRNAs. Some primary miRNA transcripts are made by RNA polymerase II and have 5′ caps and 3′ poly(A) tails (Lee et al., 2004; Cai et al., 2004), whereas others are transcribed by RNA polymerase III (Borchert et al., 2006). The next step is the process of pre-miRNA stem-loops of 60 nucleotides in lengh through transcription of long primary miRNAs. This process occurs by the nuclear-specific "microprocessor" complex, which contains the RNase III enzyme Drosha, and its partner dGCR8 (diGeorge syndrome critical region 8) (Lee et al., 2003; Landthaler et al., 2004; Denli et al., 2004; Han et al., 2004). In *D. melanogaster*, mitrons, some intronic miRNA precursors, are not processed by the Drosh endonuclease. It was revealed that this process passes in the nucleus by the usual RNA splicing machinery (Okamura et al., 2007; Ruby et al., 2007). In either case, the pre- miRNAs are then actively transported to the cytoplasm by exportin-5 in a RAS-related nuclear protein–guanosine triphosphate (RAN–GTP)-dependent manner and are further processed into ~22-nucleotide duplexes by the cytoplasmic RNase III enzyme dicer (Hammond et al., 2000; Bernstein et al., 2001; Lee et al., 2002). After this process the functional miRNA strand is then selectively inserted into RISC (Lingel et al., 2003; Liu et al., 2004). The RISC-loading process is often asymmetric that indicated a miRNA cloning analyses of Griffiths-Jones in 2004 (Griffiths-Jones, 2004). Small RNAs (~22 nucleotides) corresponding to only one side of the miRNA stem-loop precursor are preferentially incorporated. The complementary strand (miR*) may be degraded. The information for the sequential processing, maturation and RISC loading of miRNAs is likely to be encoded in the sequences of the primary and pre- miRNAs. Grounded on this assumption, when expressing a miRNA for experimental aims, it is probably necessary to include the corresponding genomic flanking sequences of that miRNA to ensure proper miRNA processing and maturation (Zeng and Cullen, 2005; Chen et al., 2004). For the regulation of the mature miRNA expression are several stages of biogenesis responsible. Lodish et al. reviewed in 2008 that the pre-miRNA:mature miRNA depends on the tissue, which indicates that regulation occurs

at the post-transcriptional level (Wulczyn et al., 2007; Thomson et al., 2006), although the biological relevance and mechanisms is not completely understood until now.

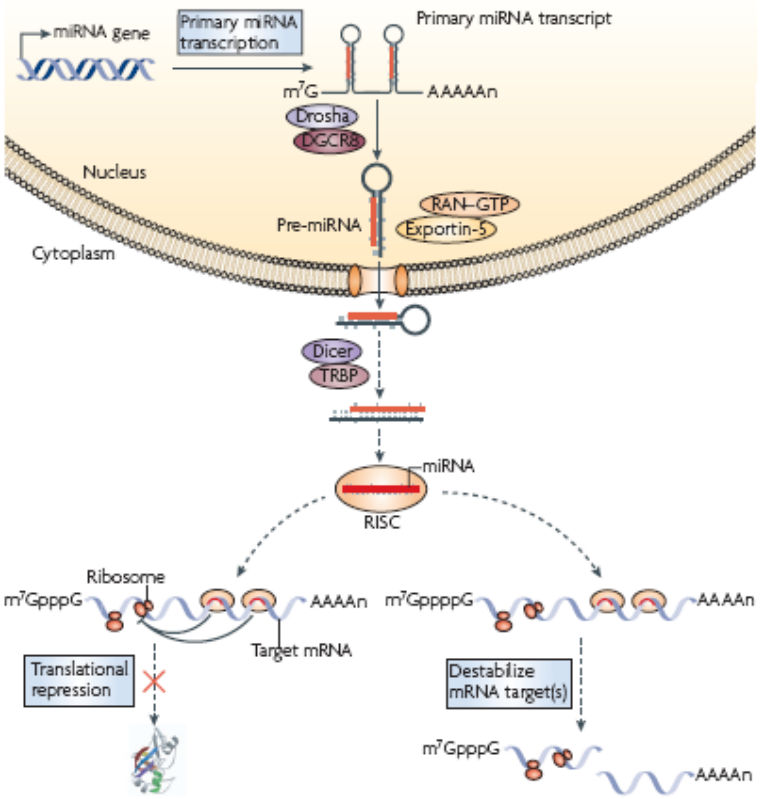


Figure 2: Biogenesis of microRNAs (Lodish et al., 2008)

1.4 Thesis objective

A previous in-house study generated a global miRNA und mRNA profiling of human multipotent adiopose-derived stem cells during adipocyte differentiation. The analysis of these results based on paired miRNA and mRNA profiling and on target prediction revealed several miRNA-mRNA target pairs as interesting candidates with a putative functional role in adipogenesis.

Therefore, the objective of this thesis is the functional characterization of two such miRNA candidates in human adipocyte differentiation: miR-222 and miR-27b.

Therefore, the specific aims of this thesis are:

1. Proliferation and differentiation of hMADS cells
2. miRNA overexpression via tranfection of miRNA mimics using HiPerFect as transfection agent
3. Determination of differentiation changes qualitatively via Oil Red O staining of lipid droplets
4. Determination of differentiation changes quantitatively via a triglyceride assay, containing triglyceride quantification normalized by protein amount
5. Harvesting hMADS cells in Trizol for RNA isolation

2 Materials and Methods

2.1 Materials

2.1.1 Cell culture of hMADS cells

hMADS cells were stored in 1.5 ml tubes in liquid nitrogen boils at -196°C and after thawing, hMADS cells were proliferated in 100mm dishes and differentiated in 12-well plates with the appropriate media. For the proliferation and differentiation three different media were used. Media I contained as main component Dulbecco's Modified Eagle Media (DMEM) and media II and III contained beside DMEM half-full Ham's F12. After 2-4 days the media got changed with fresh prepared media. Following reagents were usually used in cell culture:

Reagent	Manufacturer
Phosphate buffered saline (PBS)	Cambrex
Trypsin-EDTA	Gibco
100mm cell culture dishes	Greiner, Corning
12-well plates	Corning
Ethanol	Merck

Table 1: General reagents

2.1.1.1 Proliferation

During proliferation, media I was used with following reagents:

Reagent	Manufacturer
DMEM	Biowhittaker
Fetal Bovine Serum	PAN Biotech
Penicillin/Streptomycin (P/S)	Cambrex
Hepes	Gibco
L-Glutamine	Gibco
Normocin	InvivoGen
Human Fibroblast Growth Factor 2 (hFGF2)	Sigma

Table 2: Reagents for Proliferation media

2.1.1.2 Differentiation

For the initiation of the differentiation, media II was used with following reagents:

Reagent	Manufacturer
DMEM	Biowhittaker
Ham's F12	Cambrex
Penicillin/Streptomycin	Cambrex
L-Glutamine	Gibco
Human Insulin	Sigma
Apo-Transferrin	Sigma
Triiodthyroin (T3)	Sigma
Rosiglitazone	Sigma
Dexamethasone (DEX)	Sigma
Isobutylmetaxanthin (IBMX)	Sigma

Table 3: Reagents for Differentiation media II

For the differentiation with media III, the media has to be changed 3 days after induction of differentiaton with media II. Media III was used with following reagents:

Reagent	Manufacturer
DMEM	Biowhittaker
Ham's F12	Cambrex
Penicillin/Streptomycin	Cambrex
L-Glutamine	Gibco
Human Insulin	Sigma
Apo-Transferrin	Sigma
Triiodthyroin (T3)	Sigma
Rosiglitazone	Sigma

Table 4: Reagents for Differentiation media III

2.1.1.3 Transfection with HiPerFect

For the optimized transfection of the hMADS cells, HiPerFect was used with following reagent:

Reagent	Manufacturer
HiPerFect	Qiagen
DMEM	Biowhittaker

Table 5: HiPerFect reagents

2.1.2 microRNA overexpression

2.1.2.1 Miridian miRNA mimics stocks

Reagent	Manufacturer
miR-27b	Dharmacon
miR-222	Dharmacon
Scrambled miR	Dharmacon

Table 6: Miridian miRNA mimics overexpression reagents

2.1.2.2 Stock solution

Reagent	Manufacturer
RNase free water	-

Table 7: Reagents for stock solution

2.1.3 Triglyceride quantification

2.1.3.1 Oil-Red O staining

Reagent	Manufacturer
36,5 % Formaldehyde	Sigma
Oil Red O stock	Sigma

Table 8: Reagents for Oil-Red O staining

2.1.3.2 Infinity Triglycerides Reagents

Reagent	Manufacturer
Infinity Triglycerides Reagent	Thermo
4 mmol/l Stock solution	-

Table 9: Triglyceride reagents

2.1.3.3 BCA Reagents

Reagent	Manufacturer
BCA	Thermo Pierce
2 mmol/l Stock solution	-

Table 10: BCA reagents

2.1.4 Instruments

Instrument	Manufacturer
Centrifuge 5415D	Eppendorf
SpectraMax Plus 384	Molecular Devices
Transsonic T420	Elma

Table 11: Instruments

2.2 Methods

2.2.1 Proliferation of hMADS cells

The hMADS cells were thawing in the water bath at 37°C. 1ml of the hMADS cells were pipetted in 10ml proliferation media without hFGF2 and then centrifuged at room temperature (rt) and 3000rpm for 5 minutes. The supernatant was discarded and the cell pellet was resuspended with 10ml proliferation media including hFGF2. The cells were incubated in a 100mm dish at general conditions (37°C and 5% carbon dioxide). A media change was performed every 2-3 days with media preheated in the water bath at 37°C for 10min and hFGF2 was added shortly before usage. Depended from the amount of 12-well plates needed for the application, the dishes were passaged by 70% - 80% confluence in new 100mm dishes or when amount of required dishes was reached the cells were seeded in 12-well plates.

2.2.2 Differentiation of hMADS cells

The differentiation of the cells started two days after reaching 100% confluence by changing the media I to media II. Media II contains as seen in Table DMEM, Ham's F12, HEPES, L-Glut (new added every 4 weeks), Normocin, Insulin, Rosiglitazone, Triiodtyroine, Apo-Transferrin and contrary to media III required reagents, Dexamethasone and 3-Isobutyl-1-metyl-xanthin. The media stayed for 2 days on the cells before it was changed with media III (day: 0). For the optimal differentiation the media was changed every 3 day.

2.2.3 Transfection (using HiPerFect)

One hour before transfection with HiPerFect the media was changed depending on the day the transfection was performed. Next, the working solution was prepared. For the working solution the oligo stock solution miRIDIAN miRNA mimics with 20µM were thawed on ice and diluated with nuclease-free H2O to a 2µM oligo working solution, with caution that for each well 3µl of the working solution was needed. 40

to 45 minutes after the media change, the transfection mixture was prepared because an incubation time of 10 minutes had to be strictly adhered to avoid instability of the transfection mixture. To prepare the transfection mixture, 97µl/well of DMEM with room temperature and 6µl/well of HiPerFect transfection reagent were vortexted after pipetting into the tube contained the 2µM oligo working solution. After the incubation time 106µl of the transfection mixture was added drop by drop to the respective wells while the plate was swayed. Then the 12-well plates were transferred back into the incubator under standard conditions.

2.2.4 RNA isolation (using Trizol)

The cells used for RT-q-PCR were washed twice with PBS. Afterwards the wells were covered with 500µl Trizol and a cell homogenate were generated by scraping the cells with the pipette tips and pipetting up and down. The cell homogenates were transferred into tubes and stored in the -80°C fridge.

2.2.5 Oil Red O staining

Oil Red O (ORO) is a fat soluble dye for staining the lipid droplets of the cells red. For the Oil Red O solution, the Oil Red O stock was diluted at the ratio of 6:4 (6ml ORO stock and 4ml ddH2O). To get rid of the undissolved particles, the solution was filtered in a fresh tube and then incubated for 10 minutes. The wells which were chosen to stain were washed twice with 1ml PBS. Next, the wells were incubated with 1ml 10% Formaldehyd per well (consisting of 36.5% Formaldehyd and PBS) for 1 hour onto the shaker at 50rpm. Afterwards the Formaldehyd was sucked off and washed twice with 1ml PBS. Next, the wells were covered with 1ml of the filtered Oil Red O solution and incubated once more for 1 hour onto shaker at 50rpm. Then the Oil Red O solution was sucked off and washed twice with 1ml PBS. For storage a considerable time, the wells were covered with 1,5ml PBS, sealed with parafilm and kept in the 4°C fridge.

2.2.6 Triacylglyceride assay

2.2.6.1 Triglyceride quantification

Triglycerides are enzymatically hydrolysed by lipase to free fatty acids and glycerol. The glycerol is phosphorylated by adenosine triphosphate (ATP) with glycerol kinase (GK) to produce glycerol-3-phosphate and adenosine diphosphate. Glycerol-3-phosphate is oxidised by dihydroxyacetone phosphate (DAP) by glycerolphosphate oxidase producing hydrogen peroxide (H2O2). In a Trinder5 type colour reaction catalyzed by peroxidise, the H2O2 reacts with 4-aminoantipyrine (4-AAP) and 3,5-dichloro-2-hydroxybenzene sulfonate (DHBS) to produce a red coloured dye. The absorbance of this dye is proportional to the concentration of triglycerides present in the sample.

First the samples were transferred into tubes. For this step 300µl 4°C cold PBS was pipetted into the wells and the cells were scraped from the bottom with pipette tips. After homogenization, the cells were transferred into tubes stored in an ice box and then sonicated twice for 20 seconds therewith the cells were lyzed. Next a 1:2 dilution series for the standard row were made with a 4mM glycerol stock and then the optimal sample volume was tested by comparing the lowest glycerol concentration, the sample with the worst differentiation and with PBS as blank. For the triglyceride test, a 96-well plate was used. After pipetting two or three different volumes in duplicates for a better statistical view, 200µl of the triglyceride reagent were added by using the mulitpipette. After 10 minutes incubation at 37°C, the color differences of the three wells were compared to each other. The right volume is depended of the intensity of the sample with the blank. The intensity of the sample should be visible higher than the blank. Conversely the dilution series should extend for 1 or 2 dilutions. After finding the right volume, the samples were tested similarly as described before. After 10 minutes incubation time the 96-well plate was photometrical measured with SpectraMax Plus 284 at 500nm and the produced data was analyzed by data processing.

2.2.6.2 Protein quantification

The BCA Protein Assay combines the well-known reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline media with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) by bicinchoninic acid. The chelation of copper with protein in an alkaline environment leads to a blue colored complex, which is part of the first step. In this reaction, peptides containing three or more amino acid residues form a colored chelate complex with cupric ions in an alkaline environment containing sodium potassium tartrate. This reaction is also known as biuret reaction. Single amino acids and dipeptides do not give the biuret reaction, but tripeptides and larger polypeptides or proteins will react to produce the light blue to violet complex that absorbs light at 540nm. The intensity of the color produced is proportional to the number of peptide bonds participating in the reaction. In the second step of the color development reaction, BCA, a highly sensitive and selective colorimetric detection reagent reacts with the cuprous cation (Cu^{1+}) that was formed in step 1. The purple-colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion. The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 562nm with increasing protein concentrations. The reaction that leads to BCA color formation as a result of the reduction of Cu^{2+} is also strongly influenced by the presence of any of four amino acid residues (cysteine or cystine, tyrosine, and tryptophan) in the amino acid sequence of the protein.

The BCA test was preformed similarly to the triglyceride test. A dilution series with the 2mg/ml BSA stock solution and BCA protein assay reagent were prepared. The reagent consisting of reagent A and reagent B of the BCA protein assay kit were diluted 1:50, for each well 200µl was needed. After pipetting the samples and the BCA reagent the wells changed their color based on the reaction with the cupreous cations. To amplify this effect, the 96-well plate got incubated for 30 minutes at 37°C. After incubation, the plate was photometric measured with the SpectraMax 384 at a wavelength of 562nm and the produced data was analyzed by data processing.

3 Results

3.1 Adipocyte differentiation of hMADS cells

As a novel source of multipotent stem cells, human multipotent adipose-derived stem (hMADS) cells have been established from fat tissue and have the ability to differentiate into adipocytes in serum-free media (compare with chapter 2.1.1). The growth of the hMADS cells is divided in three parts, the proliferation till confluence of the cells, also defined as day-2 (Figure 3) (A) the start with differentiation on day0 for 3 days (B)and the differentiation on day 3 till harvesting (C)(D).

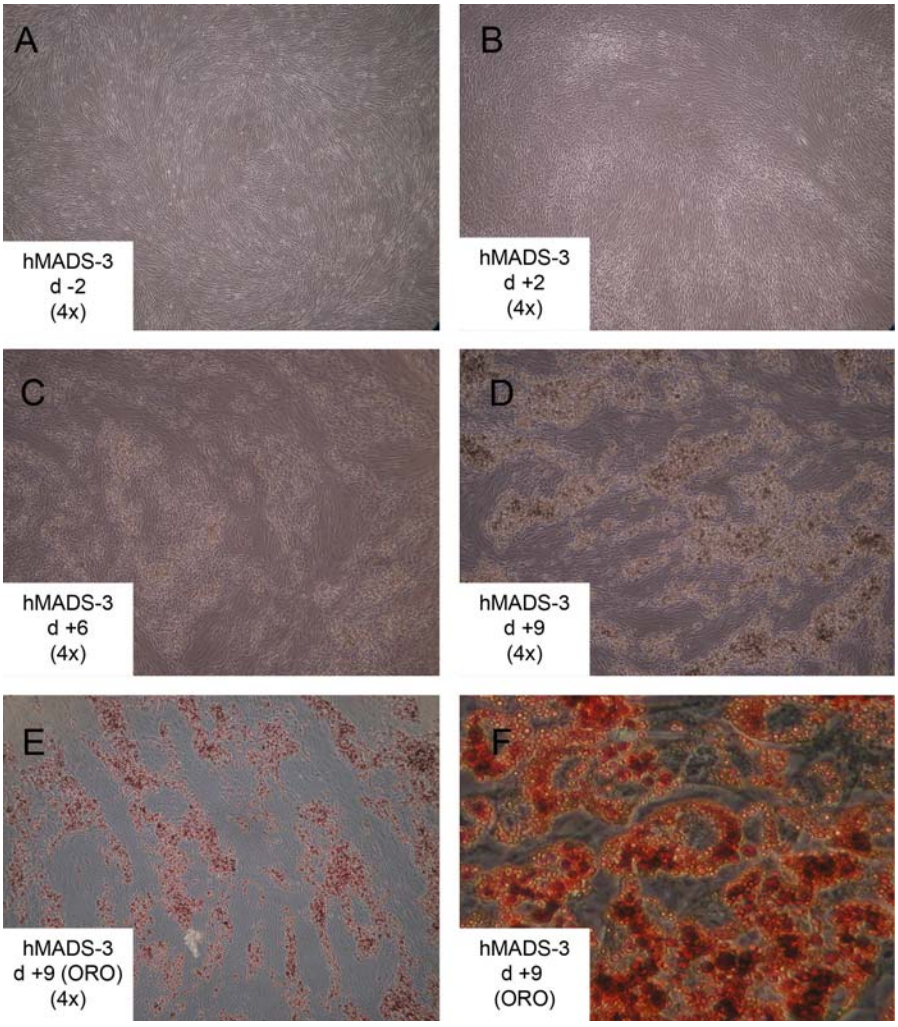


Figure 3: Differentiation of non-treated hMADS cells from day -2 till day 9. (A) shows cells with 100% confluency. (B)(C)(D) show the process of differentiation from induction till harvesting. For a better contrast the cells were stained with Oil Red O (E)(F).

3.1.1 miR-222 and miR-27b as selected candidates

A miRNA microarray profiling, performed in-house, showed that two candidates might be an important regulator of adipogenesis. The microarray revealed a downregulation of miR-27b and miR-222 (seen Figure 4 and Table 12). Hence, these microRNAs are suggested to be a crucial regulator that can inhibit the adipogenesis through overexpressing.

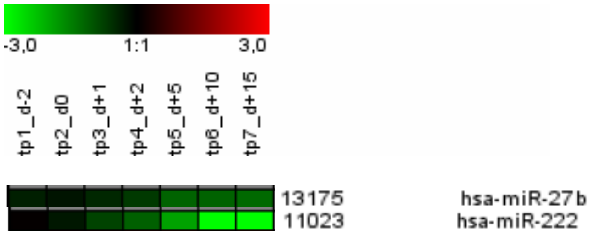


Figure 4: Expression profile of miR-27b and miR-222. green = downregulated, red = upregulated

Table 12: The quantitative valuation of the expression profile of miR-27b and miR-222

UniqueID	Name	tp1_d-2	tp2_d-0	tp3_d+1	tp4_d+2	tp5_d+5	tp6_d+10	tp7_d+15
13175	hsa-miR-27b	-0.26	-0.17	-0.32	-0.46	-0.79	-0.78	-0.84
11023	hsa-miR-222	0.04	-0.19	-0.53	-0.76	-1.29	-2.05	-2.73

3.2 Transfection optimization

3.2.1 Application frequency

For optimizing transfection, the overexpressions were performed on different time points. The miRIDIAN miRNA mimics for miR-222 were applied once on day -2 only or twice on day -2 and +3 in hMADS-3 cells (Figure 5). The results of the triglyceride assay showed that two applications did not lead to a significant better difference contrary to the single application within the same concentration.

3.2.2 miRNA mimic concentration

Beside the application frequency, the concentration of the miRIDIAN miRNA mimics played a crucial role in the overexpression trials. Two different

concentrations were chosen, 5nM and 20nM. The concentration of the transfection reagent HiPerFect was always constant. The results, after a triglyceride test was performed showed that the overexpression of miR-222 revealed no significant difference between the two concentrations. On the contrary, the level of miR-scrambled used as control decreased steadily. This data suggested that the cells are possibly stressed the higher the concentration and application frequency is.

Resumed, miRNA overexpression of miR-222 in hMADS-3 cells at higher concentration and/or two application time points showed that on the one hand the difference between miR-222 and NTC are obvious but on the other hand it turned out that not triglyceride accumulation increased upon miR-222 transfection but the NTC decreased in comparison to CO.

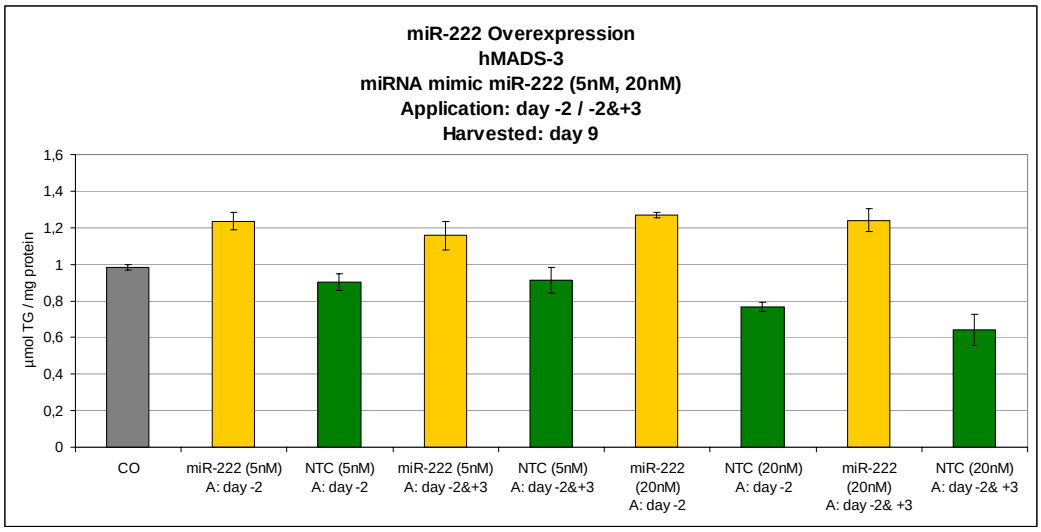


Figure 5: Triglyceride accumulation in hMADS-3 cells upon miR-222 overexpression in experiment 1 (1 or 2 applications). miR-222 mimics were transfected at 5 or 20nM, using HiPerFect as transfection agent, once, 2 days before, or twice, 2 days before and 3 days after induction of differentiation, and harvested 9 days after induction.

3.3 miR-222 overexpression

3.3.1 hMADS cell differentiation into adipocytes

Figure 6 presents the differentiation of the cells treated with miRNA mimic and HiPerFect as transfection agent with the final concentration of 5nM (A,B).

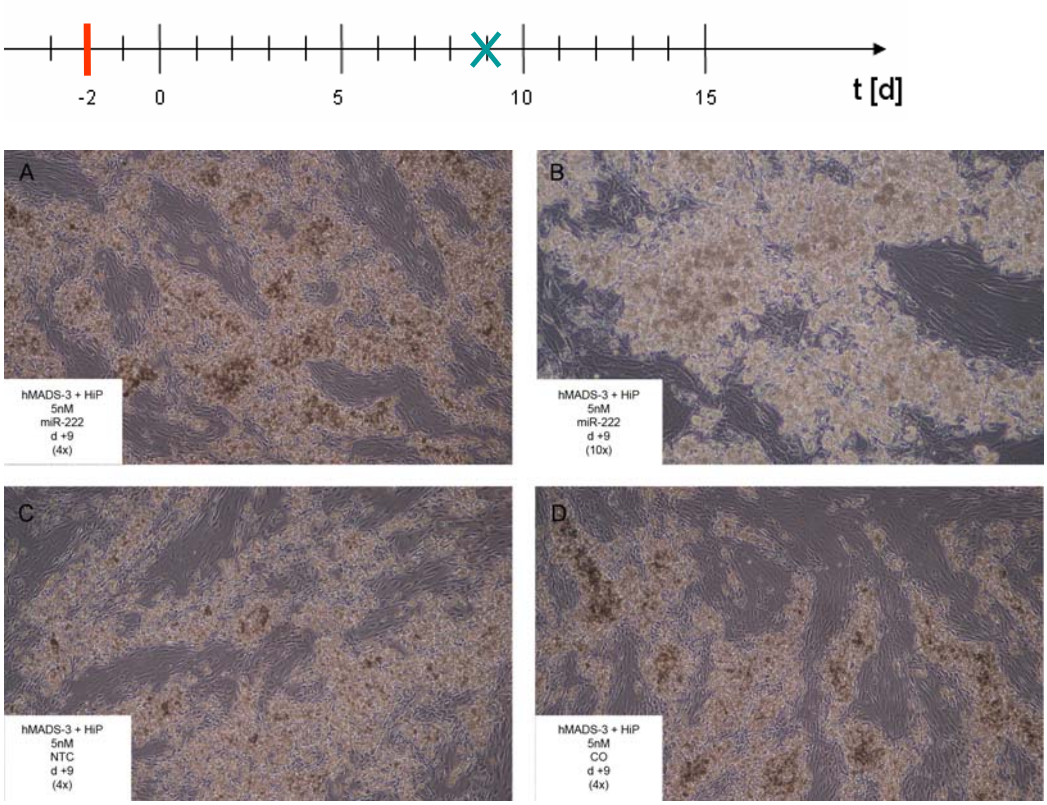


Figure 6: hMADS-3 cell differentiation: (A,B) upon transfection of 5nM miR-222 mimics, (C) upon transfection of 5nM NTC, and (D) upon no treatment. The four pictures show the endpoint after 9 days.

The NTC (C) was treated with a scrambled control oligo with a concentration of 5nM. Cell only (CO) was differentiated as a second control without any treatment. A lower difference between miR-222 (A) (B) and the NTC (C) was detected. This figure represent the qualitative result, the quantitative results are shown in chapter 3.3.3, where a triglyceride assay was performed.

Figure 7 presents the differentiation of the cells treated with miRNA mimic and HiPerFect as transfection agent with the final concentration of 5nM (A) (B).

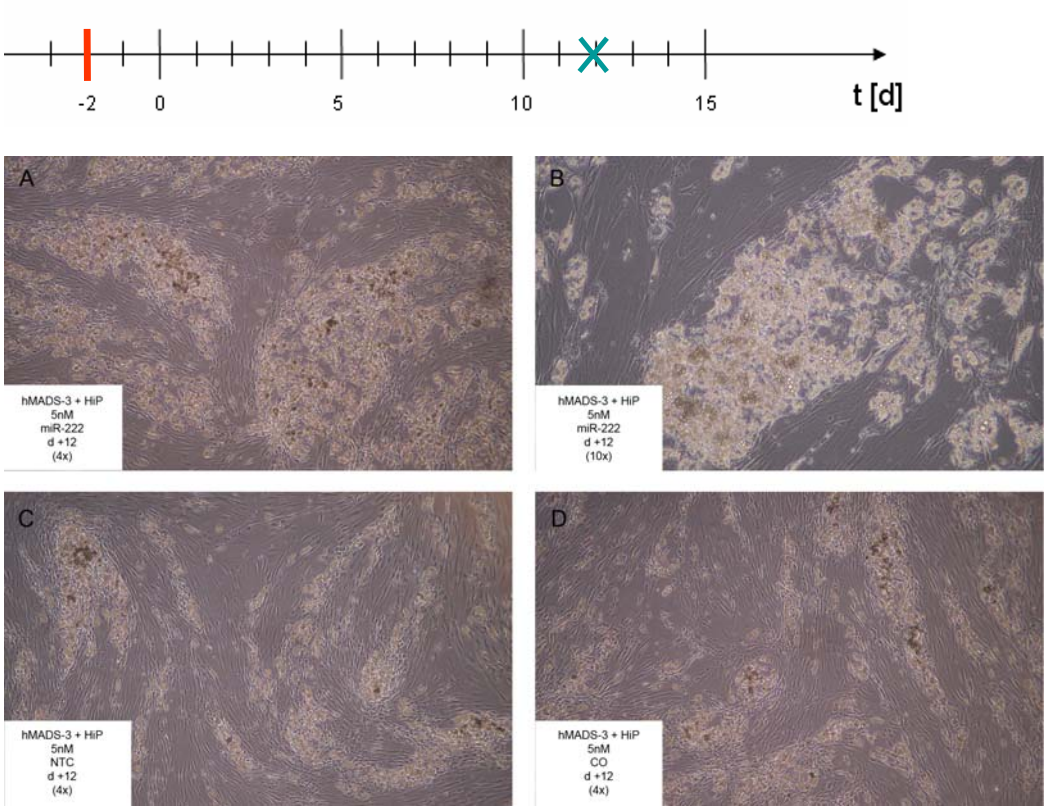


Figure 7: hMADS-3 cell differentiation: (A,B) upon transfection of 5nM miR-222 mimics, (C) upon transfection of 5nM NTC, and (D) upon no treatment (CO). The four pictures show the endpoint after 12 days.

The NTC (C) was treated with a scrambled control with a concentration of 5nM. Cell only (CO) was carried as a second control without any treatment. There is a sharp distinction between miR-222 (A) (B) and the NTC (C) and CO (D). For a quantitative detection, the triglyceride assay has been performed (see chapter 3.3.3).

Figure 8 presents the differentiation of hMADS-2 cells. These are stem cells from another donor but treated in the uniform manner as hMADS-3 cells. It should proof whether the regulation of miR-222 differs due to more possible donor-specificity. The cells were treated with miRNA mimic and HiPerFect as transfection agent with the final concentration of 5nM (A) (B).

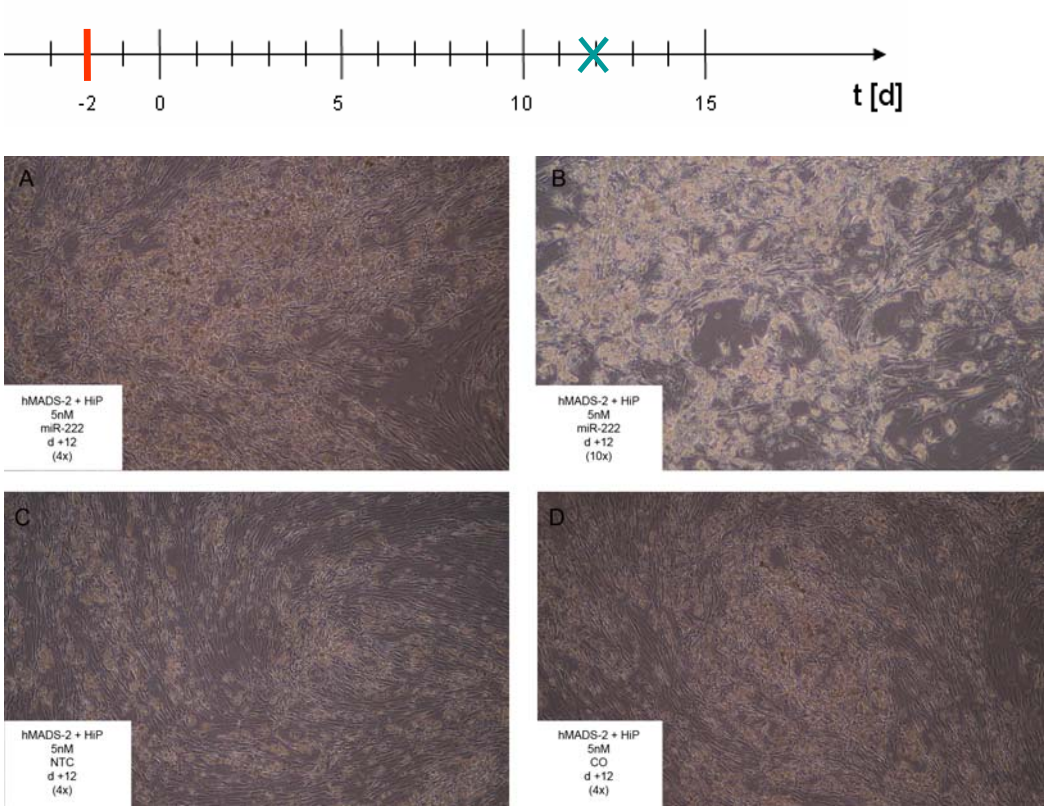


Figure 8: hMADS-2 cell differentiation: (A,B) upon transfection of 5nM miR-222 mimics, (C) upon transfection of 5nM NTC, and (D) upon no treatment (CO).. The four pictures show the endpoint after 12 days.

The NTC (C) was treated with a scrambled control with a concentration of 5nM. Cell only (CO) was differentiated as a second control without any treatment. There is a sharp distinction between miR-222 (A) (B) and the NTC (C) and CO (D). For a quantitative detection, the triglyceride assay has been performed (see chapter 3.3.3).

The experiment with hMADS-2 cells has been performed in duplicate in order to confirm the results from the previous experiment (Figure 9).

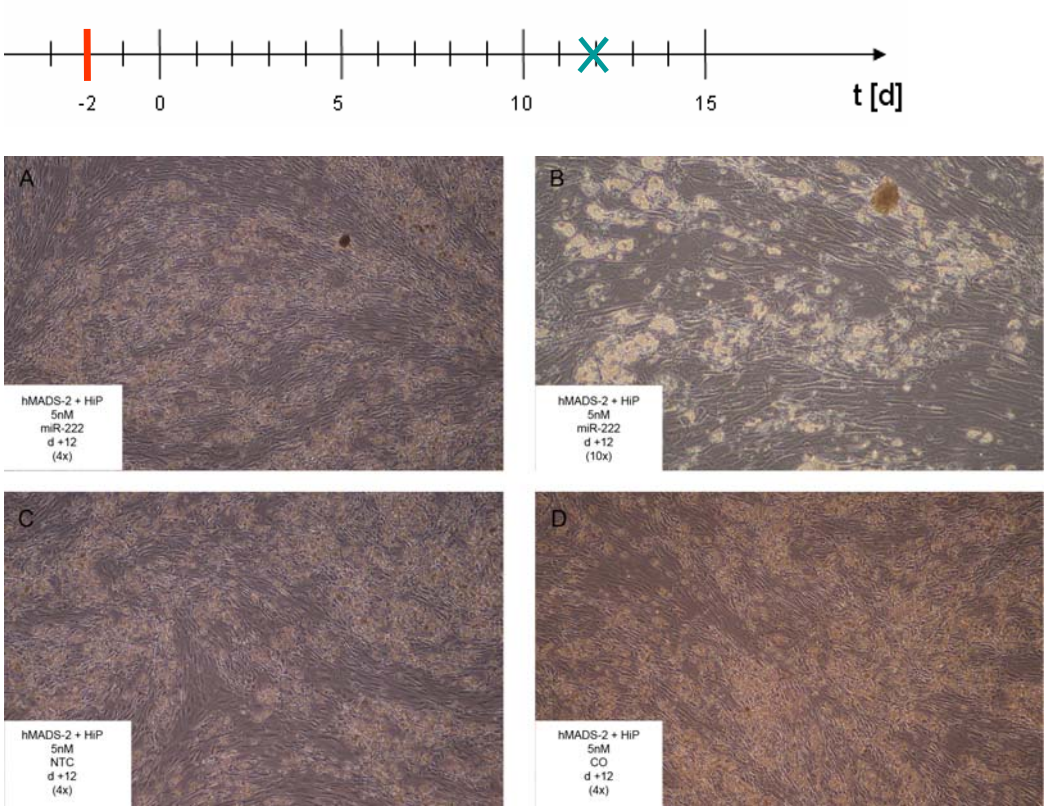


Figure 9: hMADS-2 cell differentiation: (A,B) upon transfection of 5nM miR-222 mimics, (C) upon transfection of 5nM NTC, and (D) upon no treatment (CO).. The four pictures show the endpoint after 12 days.

The cells were treated with miRNA mimic and HiPerFect as transfection agent with the final concentration of 5nM (A) (B). The NTC (C) was treated with a scrambled control with a concentration of 5nM. Cell only (CO) was differentiated as a second control without any treatment. As a qualitative result, there is no significant difference to recognize between miR-222 (A) (B) and the NTC (C). Instead it seems that CO (D) are higher differentiated that NTC (C). For a better evaluation the triglyceride assay showed it more detailed.

3.3.2 Oil Red O staining

For a better contrast the cells were stained with Oil Red O (described in chapter 2.2.5). On this dye the lipid droplets around the cell are stained and are recognizable as red spots. This dye makes the differentiation changes qualitatively visible. In

Figure 10, hMADS-3 cells transfected with miR-222 mimic (A) showed a better differentiation than the negative controls NTC (B) and CO (C).

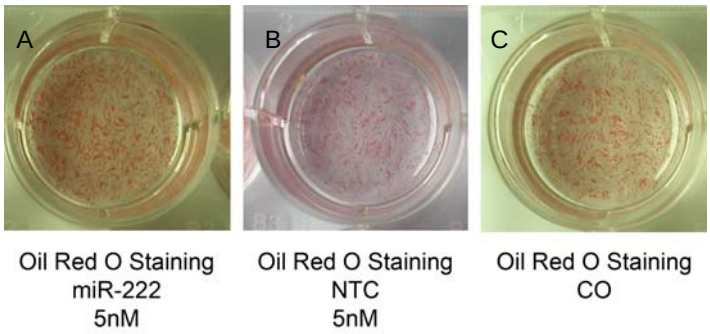


Figure 10: Oil Red O staining of differentiated hMADS-3 cells (A) upon transfection of 5nM miR-222 mimic, (B) transfection of a non-targeting scrambled oligo, and (C) without any treatment.

In Figure 11, hMADS-3 cells transfected with miR-222 mimic (A) showed a better differentiation than the negative controls NTC (B) and CO (C). This figure confirmed the result of the first experiment.

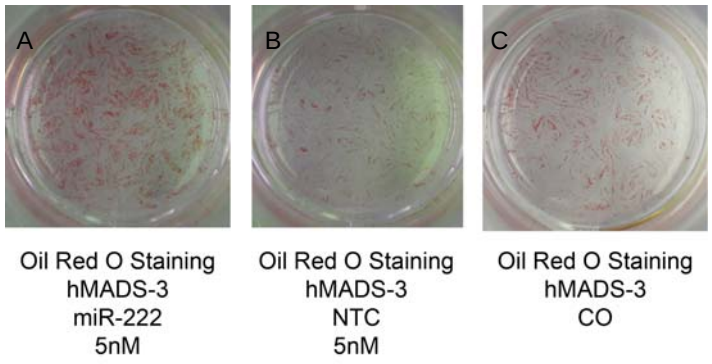


Figure 11: Oil Red O staining of differentiated hMADS-3 cells (A) upon transfection of 5nM miR-222 mimic, (B) transfection of a non-targeting scrambled oligo, and (C) without any treatment.

In order to verify if the miR-222 effect in hMADS-3 cells is donor-specific or not, the same experiment has been performed with hMADS-2 cells. Changes in adipocyte differentiation are not detectable qualitatively, as the differentiations with miR-222 mimic (A), NTC (B) and CO (C) are similarly stained (Figure 12).

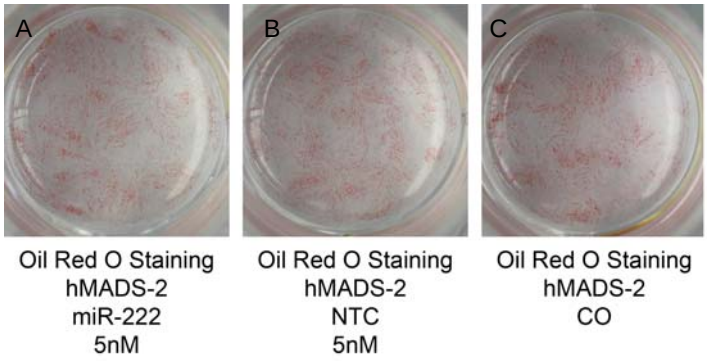


Figure 12: Oil Red O staining of differentiated hMADS-2 cells (A) upon transfection of 5nM miR-222 mimic, (B) transfection of a non-targeting scrambled oligo, and (C) without any treatment.

This experiment in hMADS-2 cells has also been performed in duplicate. And again, changes in adipocyte differentiation are not detectable qualitatively, as the differentiations with miR-222 mimic (A), NTC (B) and CO (C) are similarly stained (Figure 13). This indicates that miR-222 has not functional role in hMADS-2 cells, but needs to be confirmed by the quantitative triglyceride assay.

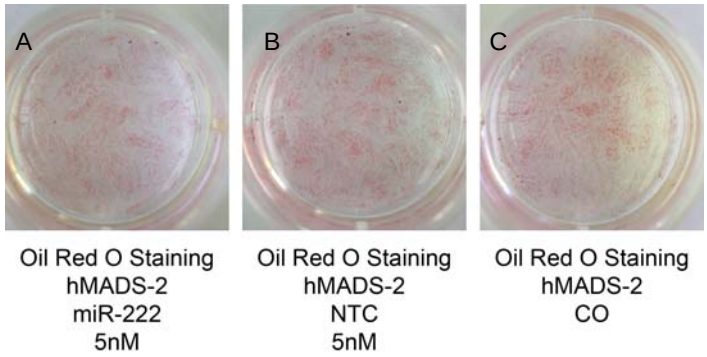


Figure 13: Oil Red O staining of differentiated hMADS-2 cells (A) upon transfection of 5nM miR-222 mimic, (B) transfection of a non-targeting scrambled oligo, and (C) without any treatment.

3.3.3 Triglyceride Test

Based on this optimized transfection procedure, transient overexpression of 5nM miR-222 in hMADS-3 cells at day-2 has been performed in triplicate and resulted in a significant increase in triglyceride accumulation 15 days post transfection compared to NTC and CO, despite the slight decrease of NTC in comparison with CO (Figure 14).

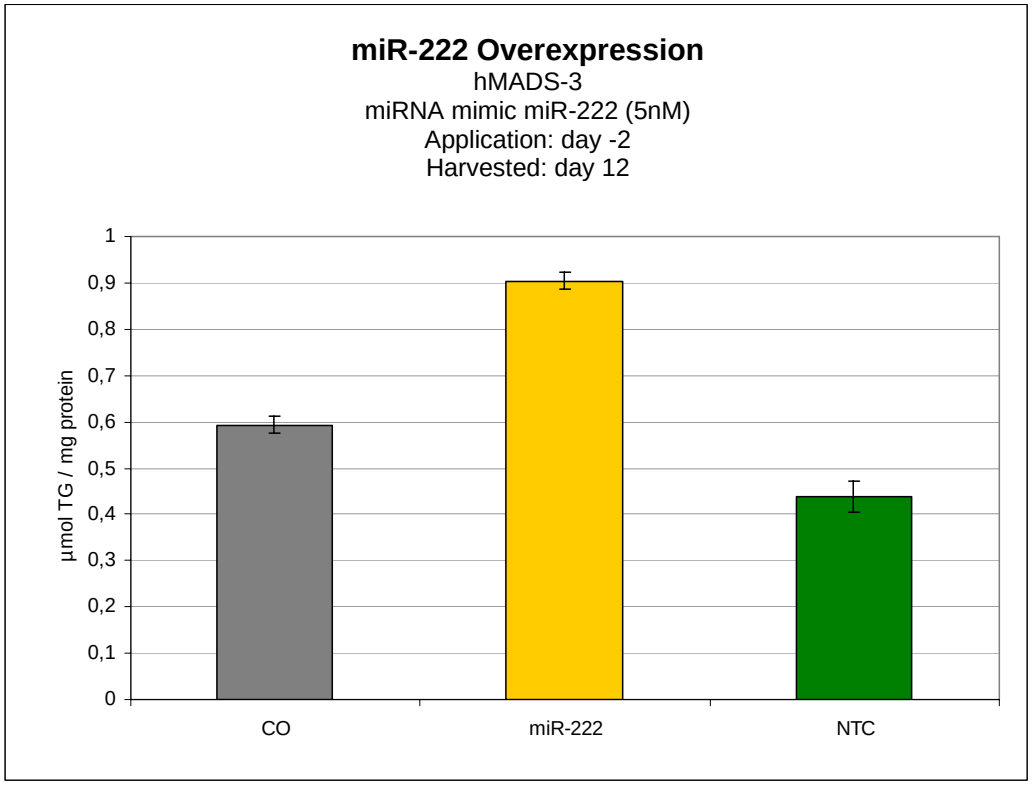


Figure 14: Triglyceride accumulation in hMADS-3 cells upon miR-222 overexpression in experiment 2 (1 application). miR-222 mimics were transfected at 5nM using HiPerFect as transfection agent 2 days before induction of differentiation and harvested 12 days after induction (n=3, s.e.m.).

In order to verify if this effect is donor-specific, this experiment has also been performed in hMADS-2 cells from another donor. The results show either no change in triglyceride accumulation compared to NTC (Figure 15) or a slight but not significant decrease in triglyceride accumulation in hMADS-2 cells (Figure 16). Thus, miR-222 seems to have no effect on hMADS-2 cells, and its miR-222 effect in hMADS-3 cells seems to be donor-specific.

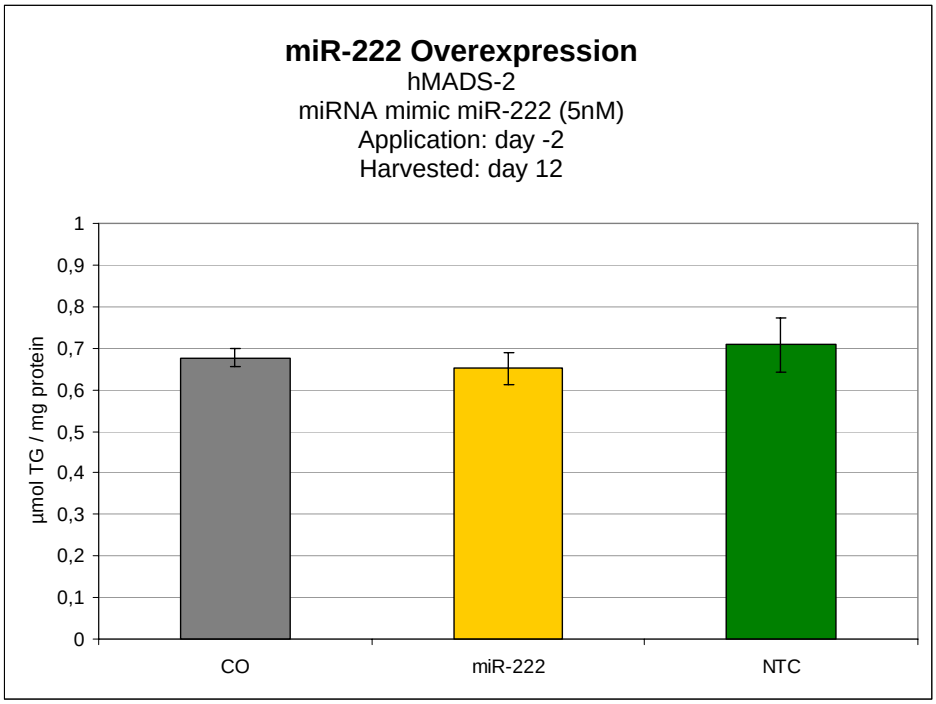


Figure 15: Triglyceride accumulation in hMADS-2 cells upon miR-222 overexpression in experiment 3 (1 application). miR-222 mimics were transfected at 5nM using HiPerFect as transfection agent 2 days before induction of differentiation and harvested 12 days after induction (n=3, s.e.m.).

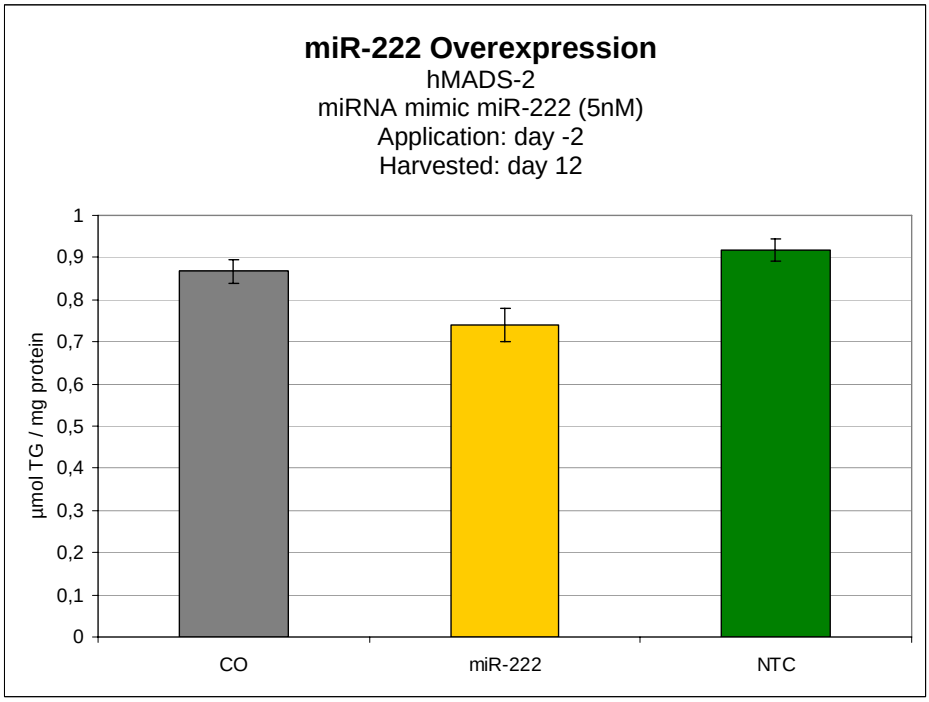


Figure 16: Triglyceride accumulation in hMADS-2 cells upon miR-222 overexpression in experiment 4 (1 application). miR-222 mimics were transfected at 5nM using HiPerFect as transfection agent 2 days before induction of differentiation and harvested 12 days after induction (n=3, s.e.m.).

3.4 miR-27b overexpression

3.4.1 hMADS cell differentiation into adipocytes

Figure 17 presents the differentiation of the cells treated with miRNA mimic and HiPerFect as transfection agent with the final concentration of 5nM (A, B). The NTC (C) was treated with a scrambled control with a concentration of 5nM. Cell only (CO) was carried as a second control without any treatment. The final concentration was assumed from the first experiment with miR-222. A lower difference between miR-27b (A) (B) and the NTC (C) was detected. This figure represents the qualitative result, the quantitative results are shown in chapter 3.4.3, where a triglyceride assay was performed.

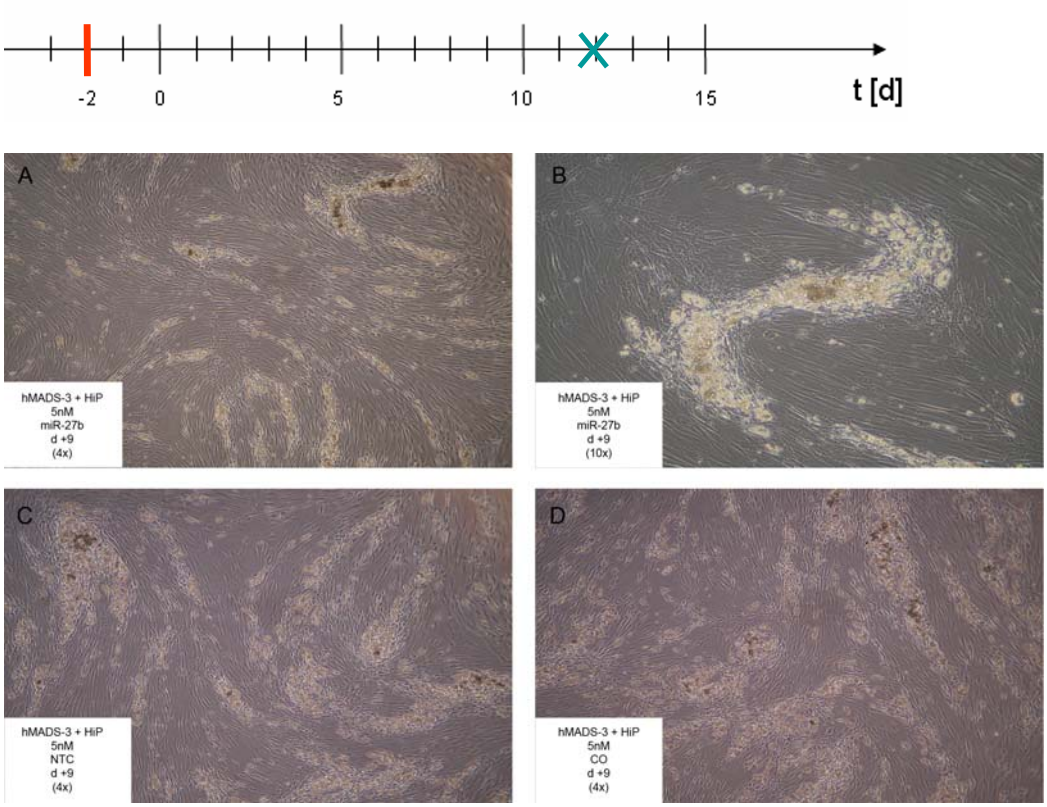


Figure 17: hMADS-3 cell differentiation: (A,B) upon transfection of 5nM miR-27b mimics, (C) upon transfection of 5nM NTC, and (D) upon no treatment (CO). The four pictures show the endpoint after 9 days.

Figure 18 presents the differentiation of hMADS-2 cells, these are stem cells from another donor but treated in the uniform manner as hMADS-3 cells. It should proof whether the regulation of miR-222 differs due to more possible donor-specificity. The cells were treated with miRNA mimic and HiPerFect as transfection agent with the final concentration of 5nM (A) (B). The NTC (C) was treated with a scrambled control with a concentration of 5nM. Cell only (CO) was carried as a second control without any treatment. There is a sharp distinction between miR-27b (A) (B) and the NTC (C) and CO (D). For a better recognisability the triglyceride assay showed it more detailed.

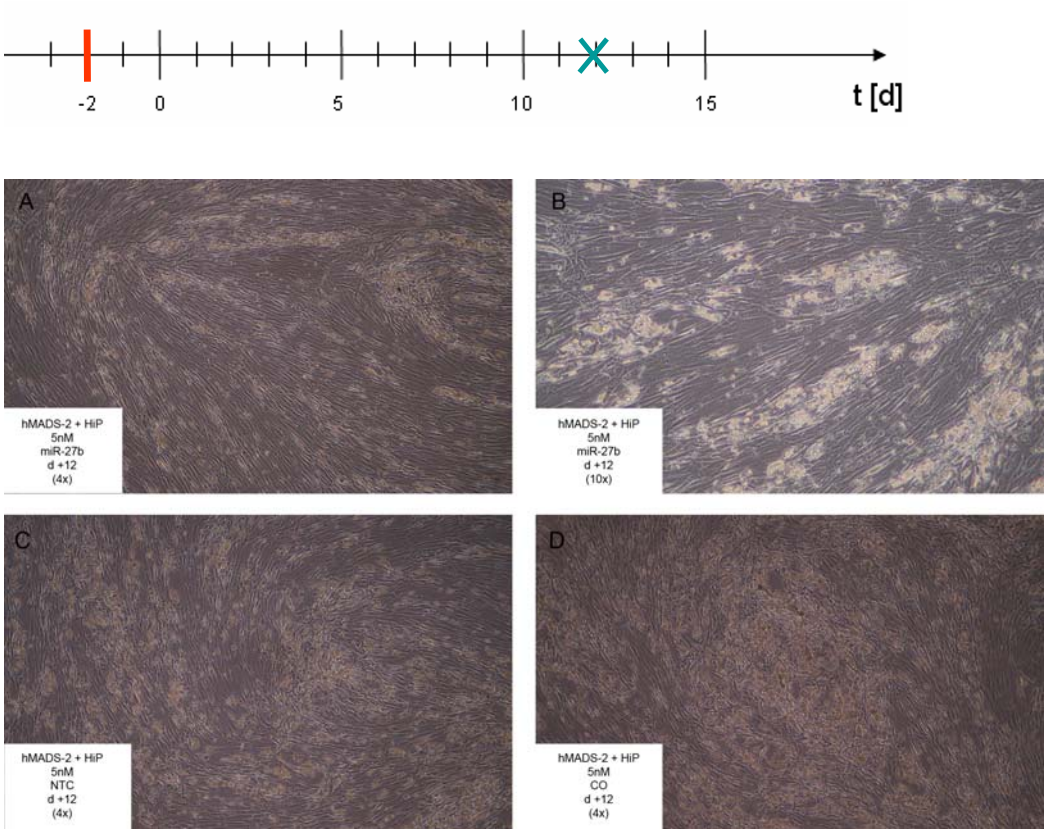


Figure 18: hMADS-2 cell differentiation: (A,B) upon transfection of 5nM miR-27b mimics, (C) upon transfection of 5nM NTC, and (D) upon no treatment (CO). The four pictures show the endpoint after 12 days.

Figure 19 presents the differentiation of the hMADS-2 cells treated with miRNA mimic and HiPerFect as transfection agent with the final concentration of 5nM (A) (B). The NTC (C) was treated with a scrambled control with a concentration of 5nM. Cell only (CO) was carried as a second control without any treatment. A high

difference between miR-27 b (A) (B) and the NTC (C) was to detect. It could be suggested that miR-27b has no donor- specificity.

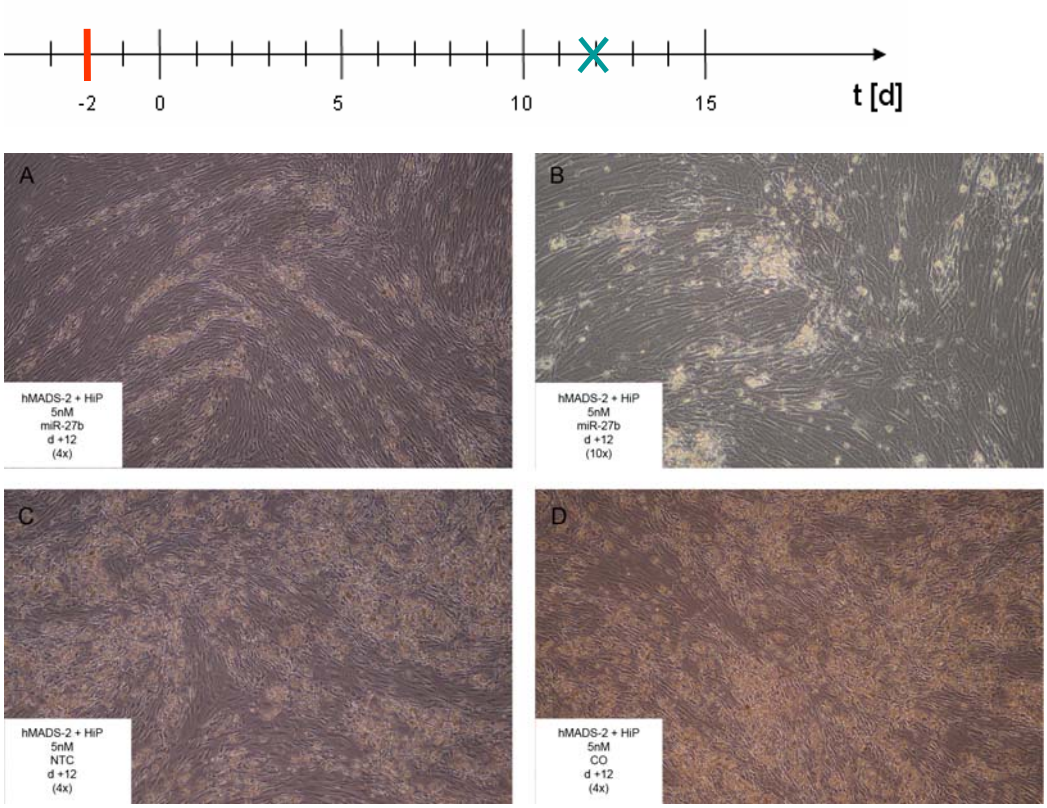


Figure 19: hMADS-2 cell differentiation: (A,B) upon transfection of 5nM miR-27b mimics, (C) upon transfection of 5nM NTC, and (D) upon no treatment (CO). The four pictures show the endpoint after 12 days.

Figure 20 and Figure 21 show the difference between two application time points. The transfection of hMADS cells with HiPerFect and oligonucleotides on day 0 represents a lower effect than the application on day -2. In Figure 21 with the application on day-2, there is a lower differentiation of miR-27b as opposed to the differentiation of miR-27b in Figure 22.

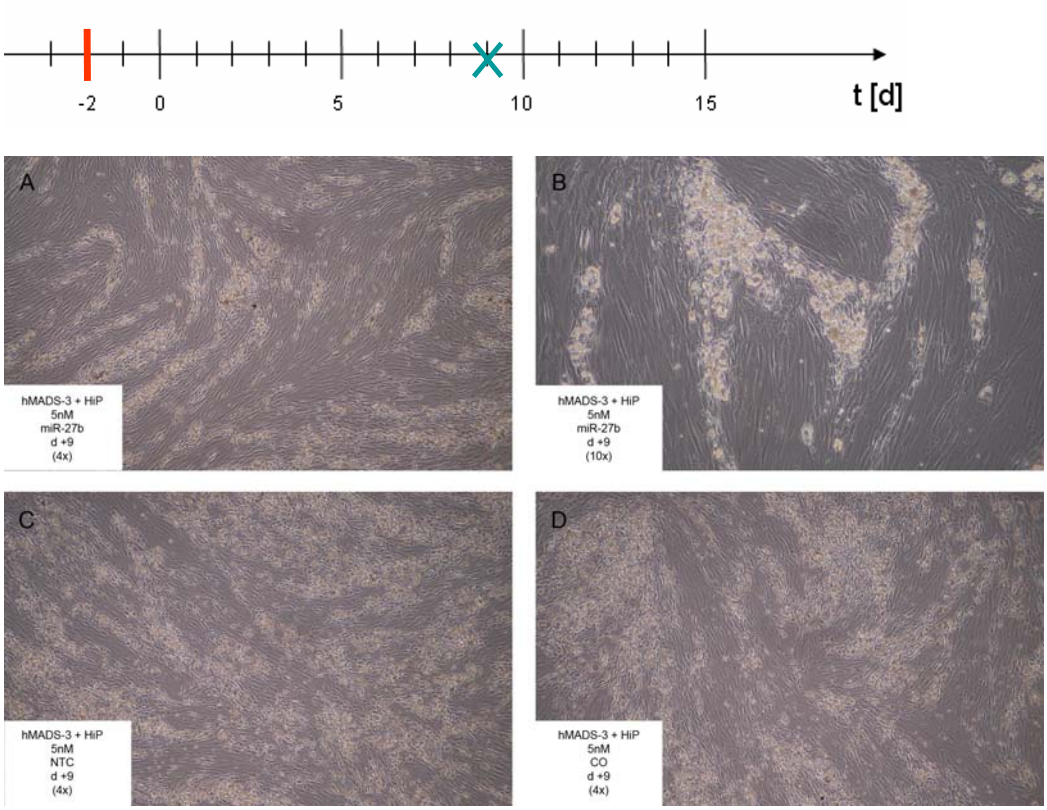
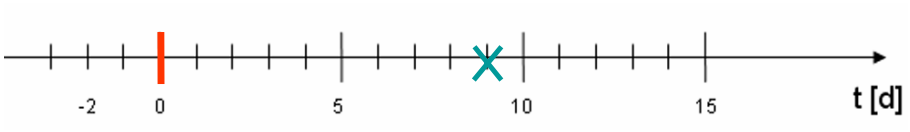


Figure 20: hMADS-3 cell differentiation: (A,B) upon transfection of 5nM miR-27b mimics, (C) upon transfection of 5nM NTC, and (D) upon no treatment (CO). The four pictures show the endpoint after 9 days.



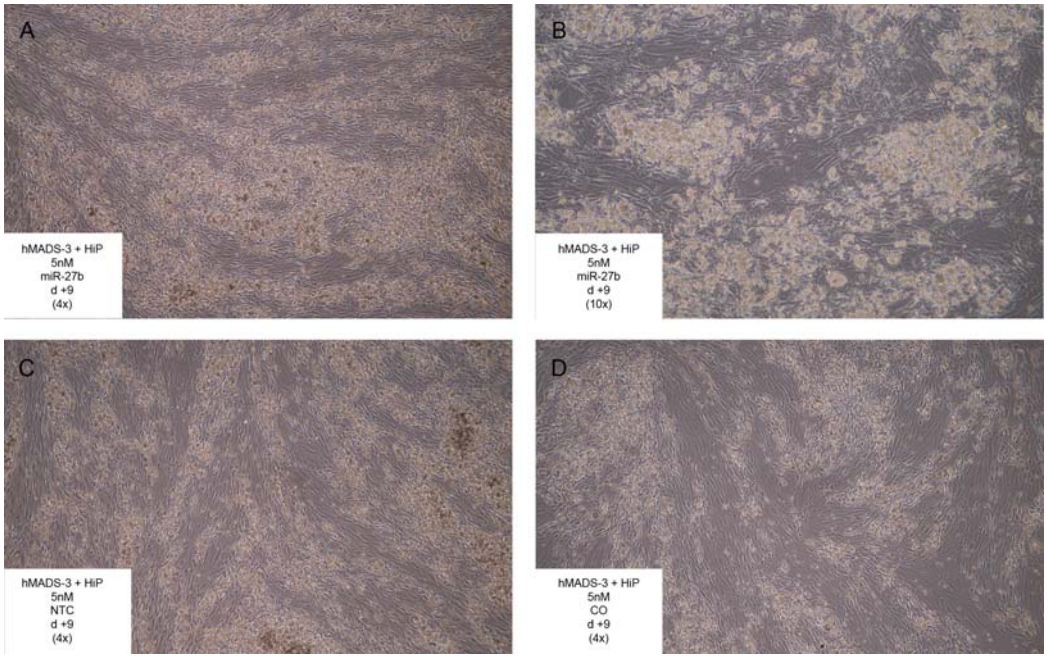


Figure 21: hMADS-3 cell differentiation: (A,B) upon transfection of 5nM miR-27b mimics, (C) upon transfection of 5nM NTC, and (D) upon no treatment (CO). The four pictures show the endpoint after 9 days.

3.4.2 Oil Red O staining

For a better contrast the cells were stained with Oil Red O (described in chapter 2.2.5). On this dye the lipid droplets within the cells are stained and are recognizable as red spots. This dye makes the differentiation changes qualitatively visible. In Figure 22, hMADS-3 cells transfected with 5nM miR-27b mimics (A) showed a lower differentiation than the negative controls NTC (B) and CO (C).

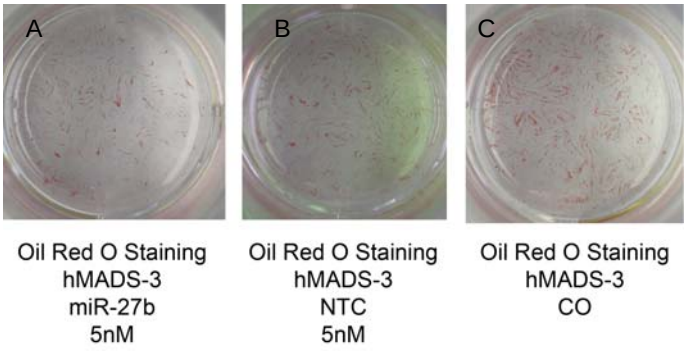


Figure 22: Oil Red O staining of differentiated hMADS-3 cells (A) upon transfection of 5nM miR-27b mimic, (B) transfection of 5nM non-targeting scrambled control oligo (NTC), and (C) cell only without any treatment (CO).

In order to verify if miR-27b has a donor-specific or general effect on human adipocyte differentiation, Oil Red O staining was also performed with hMADS-2 cells (Figure 23). Congruently, a decrease in adipocyte differentiation was detectable if miR-27b was transfected compared to NTC and CO.

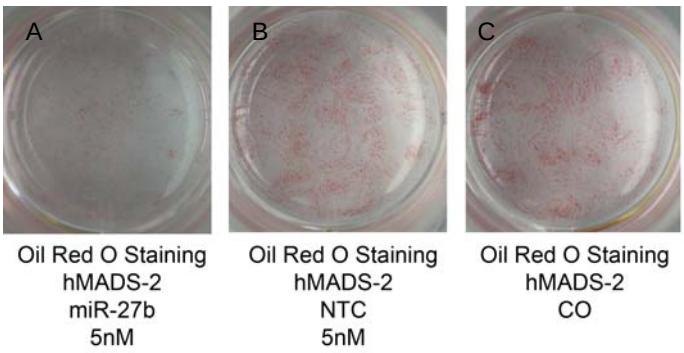


Figure 23: Oil Red O staining of differentiated hMADS-2 cells (A) upon transfection of 5nM miR-27b mimic, (B) transfection of 5nM non-targeting scrambled control oligo (NTC), and (C) cell only without any treatment (CO).

In Figure 24, the Oil red O staining of differentiated hMADS-2 cells show again that miR-27b overexpression leads to inhibition of triglyceride accumulation.

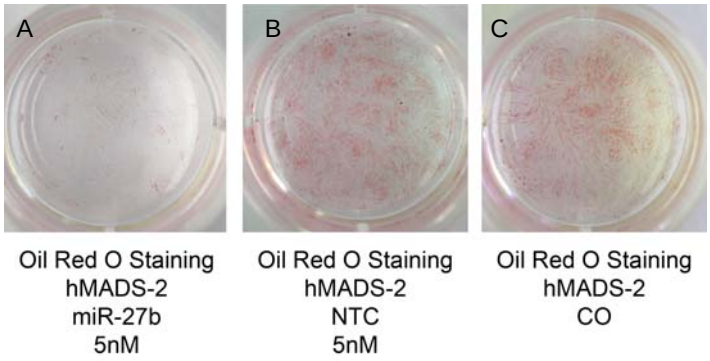


Figure 24: Oil Red O staining of differentiated hMADS-2 cells (A) upon transfection of 5nM miR-27b mimic, (B) transfection of 5nM non-targeting scrambled control oligo (NTC), and (C) cell only without any treatment (CO).

In the next experiment, the transfection was performed on two different application time points to look whether the differentiation was influenced. In Figure 25 where the transfection was done on day -2, a lower differentiation of miR-27b (A) is recognized in comparison to NTC (B) and CO (C). In Figure 26, the application was performed on d0 and showed a more intensive red effect of miR-27b as in Figure 25.

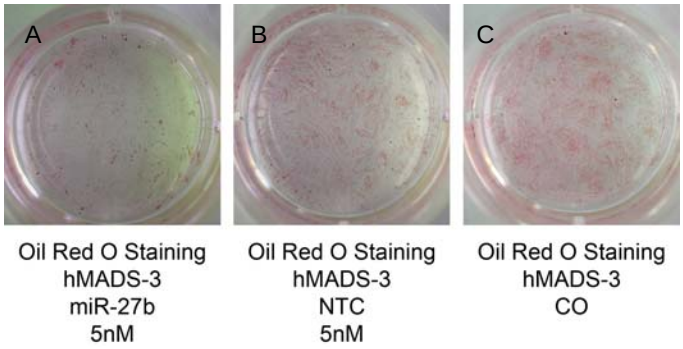


Figure 25: Oil Red O staining of differentiated hMADS-3 cells (A) upon transfection of 5nM miR-27b mimic, (B) transfection of 5nM non-targeting scrambled control oligo (NTC), and (C) cell only without any treatment (CO).

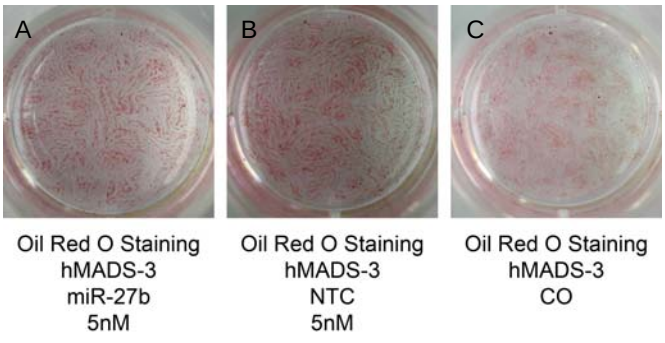


Figure 26: Oil Red O staining of differentiated hMADS-3 cells (A) upon transfection of 5nM miR-27b mimic, (B) transfection of 5nM non-targeting scrambled control oligo (NTC), and (C) cell only without any treatment (CO).

3.4.3 Triglyceride Test

Based on this optimized transfection procedure of experiment 1, transient overexpression of 5nM miR-222 in hMADS-3 cells at day-2, the same procedure was applied in triplicates upon miR-27b and resulted in a significant decrease in triglyceride accumulation 15 days post transfection compared to NTC and CO, despite the slight decrease of NTC in comparison with CO (Figure 27).

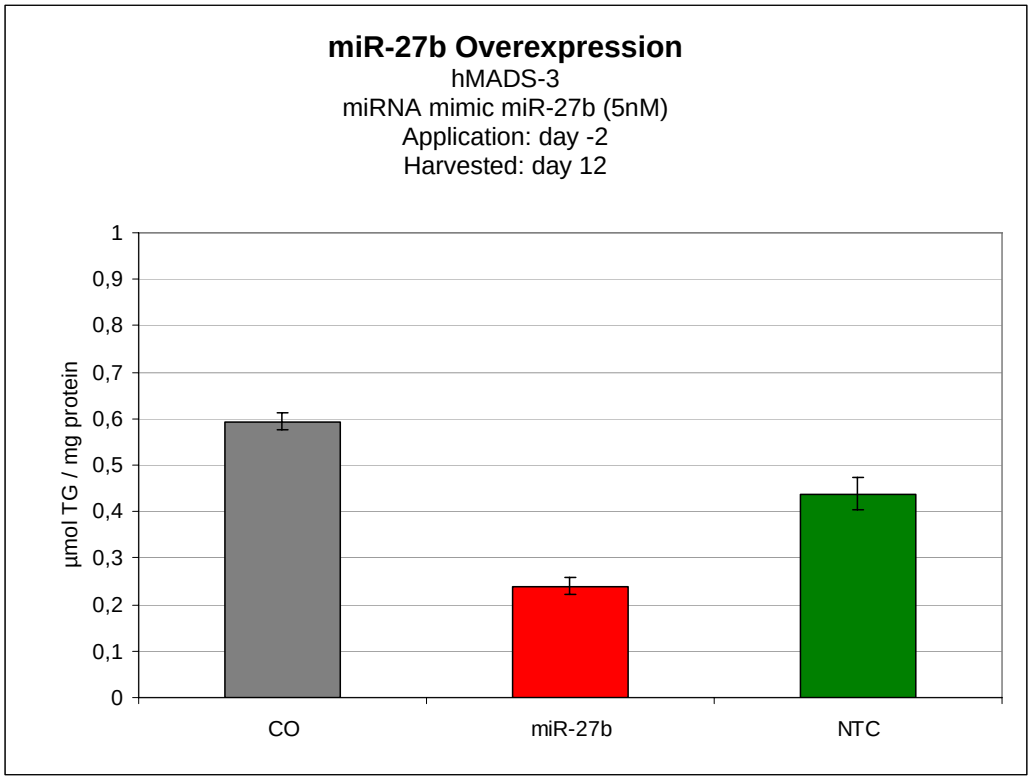


Figure 27: Triglyceride accumulation in hMADS-3 cells upon miR-27b overexpression in experiment 1 (1 application). miR-27b mimics were transfected at 5nM using HiPerFect as transfection agent 2 days before induction of differentiation and harvested 12 days after induction (n=3, s.e.m.).

The overexpression of miR-27b showed a significant inhibition of the triglyceride/protein level. To confirm this effect, the experiment was performed twice with hMADS-3 cells and two times with hMADS-2 cells at the same conditions (Figures 28, 29). The final concentration of 5nM comes from the first experiment with miR-222.

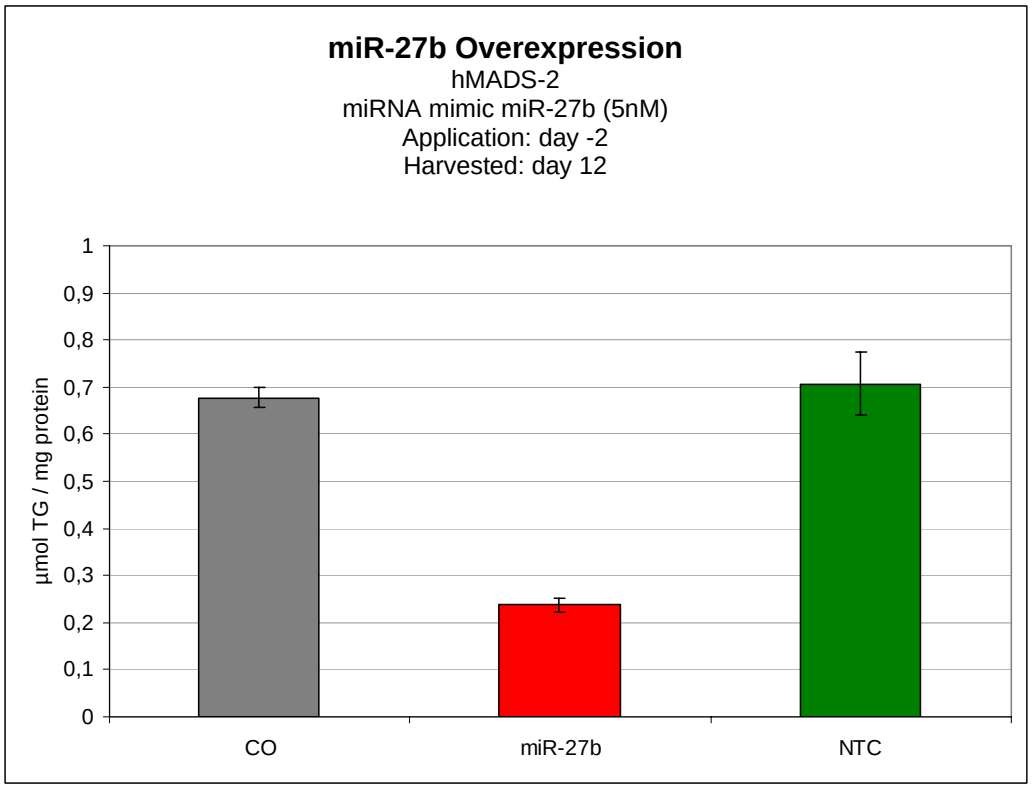


Figure 28: Triglyceride accumulation in hMADS-2 cells upon miR-27b overexpression in experiment 2 (1 application). miR-27b mimics were transfected at 5nM using HiPerFect as transfection agent 2 days before induction of differentiation and harvested 12 days after induction (n=3, s.e.m.).

To figure out whether the effect of the overexpression of miR-27b was donor-specific, the experiment was performed with hMADS-2 cells. The results in Figure 29 showed again a significant inhibition of the adipocyte differentiation. For a confirmation of the result the application with HiPerFect as transfection agent and miRNA mimics was repeated a second time at the same conditions and showed once more a significant inhibition.

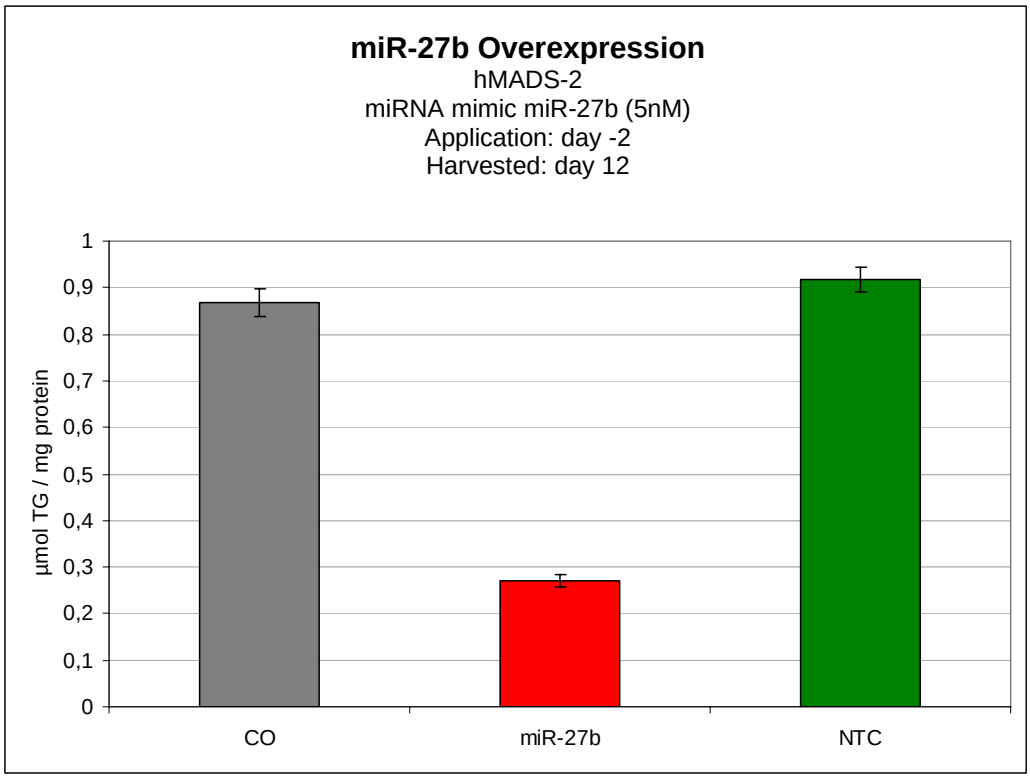


Figure 29: Triglyceride accumulation in hMADS-2 cells upon miR-27b overexpression in experiment 2 (1 application). miR-27b mimics were transfected at 5nM using HiPerFect as transfection agent 2 days before induction of differentiation and harvested 12 days after induction (n=3, s.e.m.).

To reproduce the results of the first experiment (Figure 27), the application was performed a second time with hMADS-3 cells (Figure 30). Additionally a second application time point was tested whether the inhibition effect could changed in any way if transfection of hMADS-3 cells was performed on day 0. The results show that the effect could duplicate when the application time point day -2 was chosen. In comparison the transfection of hMADS-3 cells on day 0 shows no such conspicuous effect as on day -2. In summary, the overexpression of miR-27b performed on day 0 revealed that it appears no effect. Contrary to the application performed on day -2 in hMADS-2 and hMADS-3 cells, it shows a significant tendency to inhibit the adipocyte differentiation.

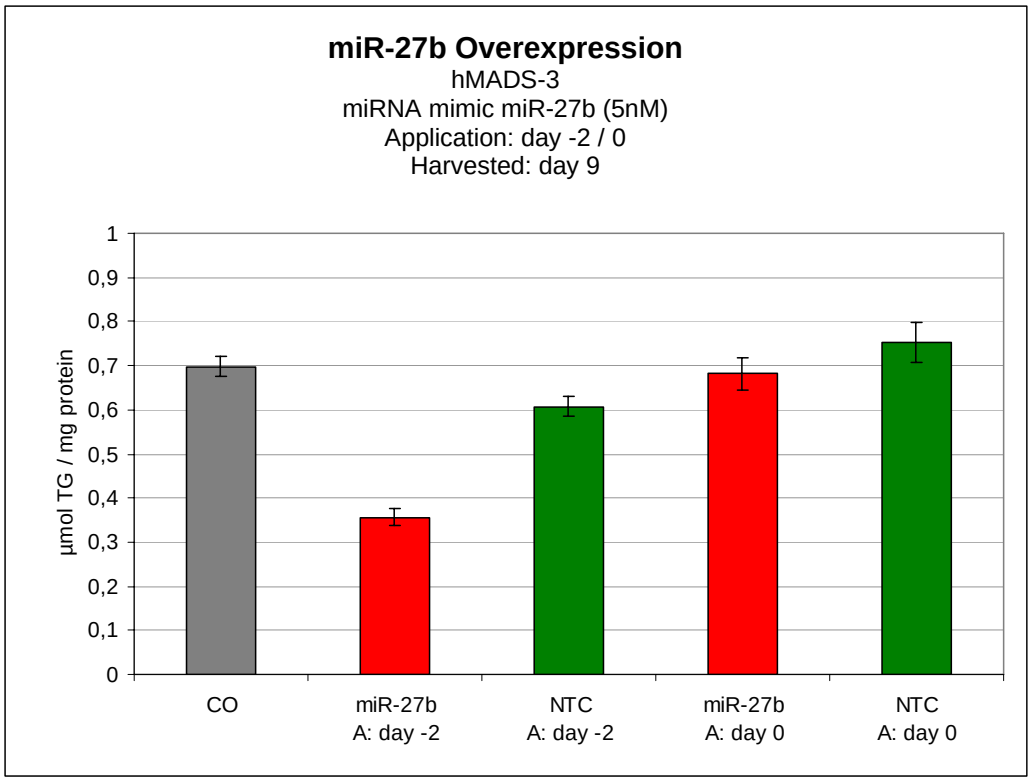


Figure 30: Triglyceride accumulation in hMADS-3 cells upon miR-27b overexpression in experiment 4 (1 application). miR-27b mimics were transfected at 5nM using HiPerFect as transfection agent 2 days before and at the day of induction of differentiation and harvested 9 days after induction (n=3, s.e.m.).

The previous experiments were performed in triplicates, also mentioned as technical triplicates. To reveal whether the results showed in total an inhibition effect, all 4 results of the overexpression experiments with the same procedure protocol were merged, also mentioned as biological replicates and showed again a significant inhibition of the adipocyte differentiation (Figure 31).

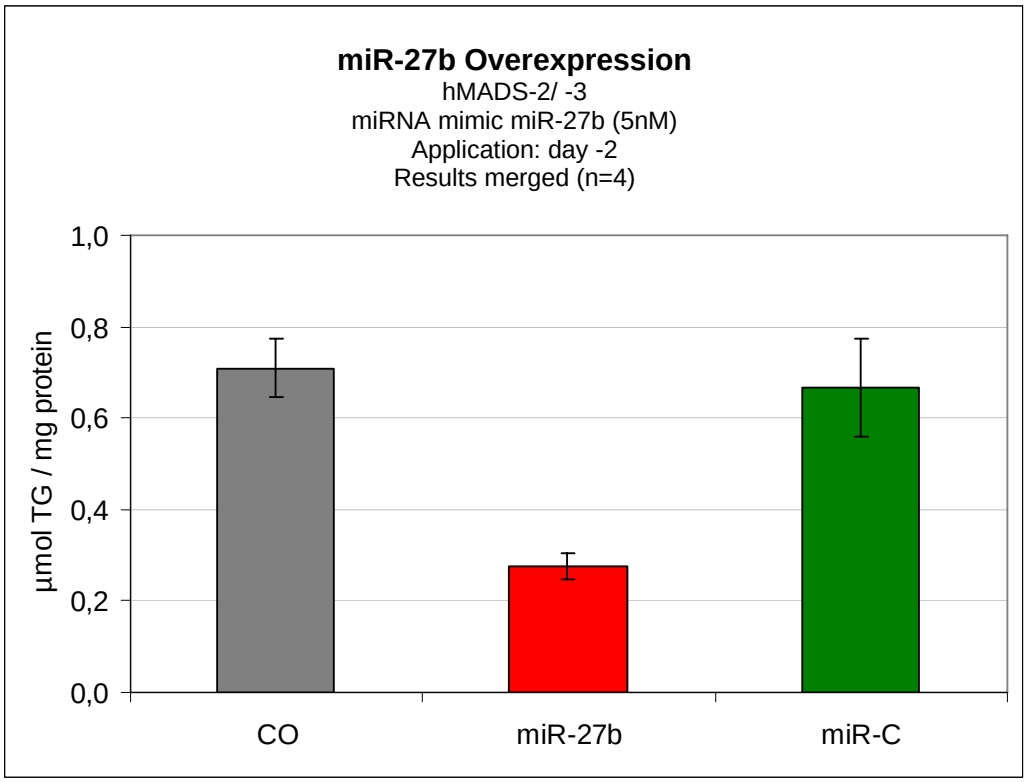


Figure 31 Triglyceride accumulation in hMADS-2 and hMADS-3 cells upon miR-27b overexpression (1 application). miR-27b mimics were transfected at 5nM using HiPerFect as transfection agent 2 days before induction of differentiation (n=4, s.e.m.).

3.4.4 Comparison of results for application day -2 and day 0

The last experiment (Figure 30) shows how the effect changed when the application time point varies. The transfection of hMADS-3 cells with HiPerFect and miRNA mimics was performed on day -2 and on day 0 with a final concentration of 5nM. The second application time point was tested therefore to determine whether the inhibition effect could maybe intensified if the time point will relocated at day 0. The results of the overexpression show a sharp distinction between the different application time points. The cells were less differentiated when the transfection was done on day -2. Unlike the transfection on day 0, the cells are more differentiated and there is no significant difference to reveal. A further point is, that not only the miR-27b increases but also the NTC increases.

In conclusion, the miRNA effect on adipogenesis is much stronger when the HiPerFect-mediated transfection of hMADS cells with miRNA mimics was performed two days before induction of differentiation.

4 Discussion

4.1 Discussion of the methods

hMADS cells: This kind of cells are not easy to handle due to their origin. The growth depended of the passage of the cells and the origin, hMADS-2 or hMADS-3 cells. The 12-well plates in which the cells were seeded might be involved caused by probably uneven coating. This gave rise to a discontinuation of the experiment. The affected experiment had to be repeated.

Triglyceride assay: The triglyceride and BCA test were performed in a 96-well plate. Therefore a definite maximal volume of the samples were allowed to pipette in the plate. The pipetting of the triglyceride und BCA reagent had to be performed carefully in order not to overspill the well.

4.2 Discussion of the results

For the optimized transfection efficiency, the application frequency and the concentration were varied and tested. The result yielded that the lowest concentration of 5nM and one application time point on day-2 showed the best effect without stress for the cells. The higher the concentration and/or more application time points were chosen the lower the differentiation was in hMADS cells treated with the non-targeting control. In order to exclude donor-specific and confirm donor-independent miRNA effects, hMADS-2 und hMADS-3 cells from different donors were used. It could be concluded that the application with a concentration of 5nM and the time point at day -2 were the ideal conditions for the best results and not neglectable for financial reasons.

miR-222 was chosen because the expression profile revealed a down-regulation in adipocytes differentiation. In conjunction with the results of the target prediction tool analysis, that miR-222 might target and therefore block PLAGL1 which might be in turn a regulator of Pref-1, which blocks the terminal adipogenesis and regulates preadipogenesis. Concluded, miR-222 suggested being a perfect candidate for the overexpression experiments to inhibit the human adipocyte differentiation.
miR-222 overexpression in hMADS-2 and 3 cells revealed that miR-222 only had a donor-specific effect on adipocyte differentiation in hMADS-3 cells, but no effect in hMADS-2 cells.

miR-27b was chosen because it is down-regulated during adipogenesis of hMADS cells and more than half of the available target prediction tools indicated PPARγ as a direct target gene of miR-27b.
miR-27b overexpression resulted in an inhibition of triglyceride accumulation in differentiated hMADS adipocytes. This effect was donor-independent because the inhibitory effect was visible in hMADS-2 and hMADS-3 cells. This result is in agreement with data from Lin et al. (2009) in murine OP9 cells. In addition, transfection of hMADS cells not before differentiation induction, but at the start of differentiation showed no impairment of adipocyte differentiation, as also shown in hMADS-3 cells. Interestingly, a couple of target prediction tools showed that PPARγ has miRNA binding site it its 3´UTR and therefore might be a direct target of miR-27b. Lin et al. argued that another target has to be responsible for the miRNA effect, but they did not completely exclude the possibility of a direct miR-27b-PPARγ interaction in mouse by performing a Luciferase reporter assay (Lin et al., 2009). In another recent publication, RXRα was identified as direct miR-27 target (Ji et al., 2009). This might be another possible target by which miR-27b is mediating its inhibitory effect on adipocyte differentiation, because RXRα has been documented to play a central role in adipogenesis, probably as a heterodimeric partner for PPARγ (Imai et al., 2004).

5 Outlook

It has been suggested that miR-27b directly represses PPARγ expression. Further tests are necessary to confirm a connection of miR-27b and PPARγ. qRT-PCR results of the miR-27b-PPARγ pair in an anti-correlated manner should indicate a direct interaction, but finally, a Luciferase reporter assay with the PPARγ 3´UTR is needed to confirm a direct miR-27b-PPARγ interaction.

6 List of Tables

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8 Abbreviations

BCA	Bicinchoninacid
BMI	Body-Mass-Index
CEPB	CAAT enhancer binding protein
CO	Cell only
ddH2O	Double distilled water
dGCR	diGeorge critical region
dGCR8	diGeorge critical region 8
DMEM	Dulbecco's modified eagled media
Gk	Glycerol-kinase
hFGF2	Human fetal growth factor2
hMADS cells	Human multipotent adipose-derived stem cells
miR	microRNA
Mtpn	Mytrophin
nm	nano meter
NTC	Non-targeting control
OP9	osteopetrotic 9
ORO	Oil Red O
PBS	phosphate buffered saline
PPAR	Peroxysome proliferator-activated receptor
qRT-PCR	quantitative reverse-trans
RAN-GTP	RAS-related nuclear protein–guanosine triphosphate
RISC	RNA-induced silencing complex
RNA	ribonucleicacid
rpm	rounds per minute
Rt	Room temperature
RXRα	retinoid X receptor α
UTR	untranslated region
WHO	World Health Organization

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Statutory declaration

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