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Delivery of miRNAs to the adipose organ for metabolic health

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Abstract

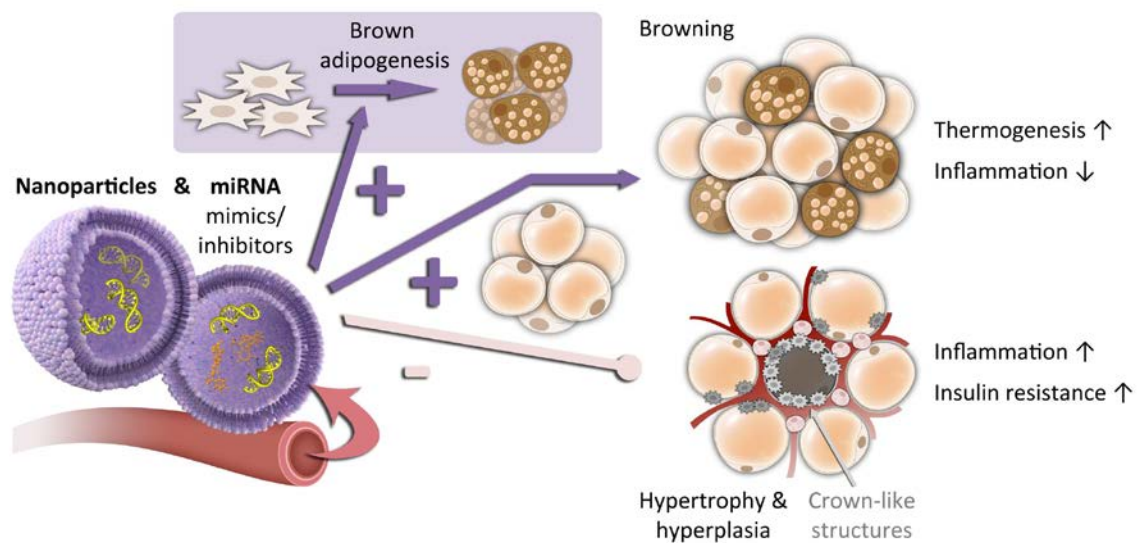
Despite the increasing prevalence of obesity and diabetes, there is no efficient treatment to combat these epidemics. The adipose organ is the main site for energy storage and plays a pivotal role in whole body lipid metabolism and energy homeostasis, including remodeling and dysfunction of adipocytes and adipose tissues in obesity and diabetes.

Thus, restoring and balancing metabolic functions in the adipose organ is in demand. MiRNAs represent a novel class of drugs and drug targets, as they are heavily involved in the regulation of many cellular and metabolic processes and diseases, likewise in adipocytes.

In this review, we summarize key regulatory activities of miRNAs in the adipose organ, discuss various miRNA replacement and inhibition strategies, promising delivery systems for miRNA and reflect the future of novel miRNA-based therapeutics to target adipose tissues with the ultimate goal to combat metabolic disorders.

Keywords: obesity, adipose tissues, miRNA therapeutics, delivery systems

Graphical Abstract



Abbreviations: (alphabetic order)

ABCA1: ATP-binding cassette transporter; ADAM 17: ADAM metalloprotease domain 17; AdipoR2: adiponectin receptor 2; ALK2: activin receptor-like kinase 2; AMOs: antisense miRNA oligonucleotides; APMAP: Adipocyte Plasma Membrane Associated Protein; ASOs: antisense oligonucleotides; AT: adipose tissue; ATP: adenosine triphosphate; BAT: brown adipose tissue; BMI: body mass index; BMP2: bone morphogenic protein 2; C/EBP α : CCAAT/enhancer binding protein alpha; CPP: cell penetrating peptides; CREB: cAMP Response Element-Binding Protein; CtBP2: C-terminal-binding protein 2; D-8Asp: octa-aspartate; DOPE: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine; DOPC: 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine; DOTAP: dioleoyl-3-trimethylammonium propane; DPP-4: dipeptidyl peptidase 4; DSPC:1,2- distearoyl-sn-glycero-phosphatidylcholine; DSDAP: 1,2-distearoyl-3- dimethylammonium-propane; ECM: extracellular matrix; ERK5: Extracellular Signal Regulating Kinase 5; FDA: U.S. Food and Drug Administration; FGF21: fibroblast growth factor 21; FGFR1: fibroblast growth factor receptor 1; GalNAc: N-acetyl-D-galactosamine; GC: glucocorticoid; GLP-1 R: glucagon-like peptide-1 receptor; GR: glucocorticoid receptor; HCV: hepatitis C virus; hMSCs: human adipose tissue-derived mesenchymal stem cells; iNOP: interfering nanoparticle; LNAs: locked nucleic acids; LPL: lipoprotein lipase; miRNA: microRNA; MPS: mononuclear phagocyte system; NASH: non-alcoholic steatohepatitis; ncRNA: noncoding RNA; NWO: normal weight obesity; PAI-1: plasminogen activator inhibitor 1; PAMAM: polyamidoamine; PEG: polyethyleneglycol; PEI: polyethyleneimine; PGC-1 α/β : PPAR-gamma coactivator-1alpha/beta; PLGA: poly(lactide-co-glycolic acid); PPAR α : peroxisome proliferator-activated receptor alpha; PPAR γ : peroxisome proliferator-activated receptor gamma; PRDM16: PR domain containing 16; siRNA: silencing RNA; SAT: subcutaneous adipose tissue; SIRT-1: sirtuin-1; SGLT2: sodium glucose co-transporter; SNALPs: stable nucleic acid lipid particles; T2DM: type 2 diabetes mellitus; UCP1: uncoupled protein; VAT: visceral adipose tissue; VCAM1: vascular cell adhesion molecule 1; VEGF-B: vascular endothelial growth factor-B; VSMC: vascular smooth muscle cell; WAT: white adipose tissue; WHO: World Health Organization.

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1. INTRODUCTION

The prevalence of obesity is one of the major health burdens approaching pandemic proportions worldwide [1, 2]. In 2016, the World Health Organization (WHO) has estimated around 2 billion adults overweight and about 650 million of these people are considered to be affected by obesity with a body mass index (BMI) ≥ 30 kg/m². If the current trend continues, it is suggested that by 2025, 33 out of 53 European countries will have a prevalence of obesity over 20% of the population [3].

Obesity is a highly complex and heterogeneous disorder that is associated with an increased risk for the pathogenesis of type 2 diabetes, and the premature development of related metabolic, cardiovascular or chronic inflammatory diseases and certain cancers. Among others, these diseases include dyslipidaemia, non-alcoholic fatty liver disease, chronic kidney disease, hypertension, coronary heart disease or stroke [5]. Type 2 diabetes (T2DM) is a progressive chronic metabolic disease characterized by hyperglycemia, often caused by insulin resistance in liver, adipose tissues and skeletal muscle, combined with insufficient secretion of insulin by pancreatic β cells to compensate this resistance [5].

Currently, about 422 million people worldwide have diabetes and 1.6 million deaths are directly attributed to diabetes each year. Both the number of cases and the prevalence of diabetes have been steadily increasing over the past few decades as recently outlined by the WHO (<https://www.who.int/news-room/fact-sheets/detail/diabetes>; April 2021). Furthermore, patients with normal BMI and having high body fat mass belong to the normal weight obesity (NWO) category and appear to represent a new class of patients developing risk for cardio-metabolic morbidity and mortality [4]. Indeed, the concept of NWO has its importance mainly as the measure of BMI is not sufficient to detect obesity. Waist circumference, lipid profiles and fat measures are needed as patients with normal BMI and central obesity are at high mortality risks [6, 7].

Today, lifestyle changes, diet, physical activity and multiple combination therapies are the most common interventions available to address obesity related diseases [8]. Regarding pharmacological treatment of obesity/diabetes only a few U.S. Food and Drug Administration (FDA) approved drugs are currently available with limited success [9, 10].

For the therapy of T2DM most of them are glucose lowering medications with metformin as initial therapy, often administered in combination with basal insulin, sulfonylurea, thiazolidinedione, dipeptidyl peptidase 4 (DPP-4) inhibitor, sodium glucose co-transporter (SGLT2) inhibitor or glucagon-like peptide-1 receptor (GLP-1 R) agonists [11]. Although highly effective, long term safety aspects and the frequent occurrence of multiple comorbidities limit the enthusiasm for available drugs and require the search for new therapeutic options. Regarding anti-obesity therapy four drugs are currently approved by the FDA, and all of them are to promote body weight loss in obese patients [12]. Among them are controlled-release phentermine/topiramate (sympathomimetic amine/anticonvulsant) or naltrexone/bupropion (antagonist of opioid receptors/inhibitor of noradrenaline and dopamine transporters) combination therapeutics, orlistat (inhibitor of gastric, pancreatic and diacylglycerol lipases and $\alpha\beta$ -hydrolase 12), and liraglutide (GLP-1 receptor agonist). The drugs differ in their molecular mechanisms, efficacy and safety profiles and according to the administration guidelines a reduction of the body weight of at least 5% from the baseline level is aimed [13]. Although the medications are carefully tailored to the needs of the individual subjects, the drugs are known to exert severe adverse side effects and are insufficient in a long-term clinical perspective. In this context, it is fair to say that obesity treatment is so far not satisfactory, even if bariatric surgery is proven to be effective for patients with morbid obesity. To overcome these limitations new strategies and innovative approaches are needed to combat obesity/diabetes more efficiently. One ongoing approach in research is the discovery of novel drugs, basically biomolecule-based agents, in particular protein, lipids or RNA related drugs turned out to be very promising. Another important point

concerns the exploration of new therapeutic targets. One of those targets is the adipose organ that is becoming increasingly interesting for pharmaceutical research.

Therefore, the overall aim of this review is to outline the metabolic role of adipose tissues with special emphasis on key regulatory activities of miRNAs. A brief summary of promising delivery platforms for miRNA therapeutics is presented to provide an overview of different nanoparticle classes. Finally, we discuss recent progress in the development of miRNA therapeutics and critically reflect the future of novel miRNA based therapies to address adipose tissue dysfunction.

2. ADIPOSE ORGAN

The adipose organ comprises about 15-20% in males and about 20-30% in females of total body weight in healthy individuals [14, 15], and based on its location fat tissues can be divided into subcutaneous (SAT) and visceral (VAT) adipose tissues. Lipids are stored within two different types of adipocytes classified as white and brown adipocytes. Interestingly, metabolically active brown adipose tissue (BAT) was only recognized more than one decade ago in healthy adult humans, but has gained increasing attention over the last few years. A third type of adipocytes are brown-like adipocytes that emerge within white adipose tissue (WAT) upon cold exposure, long term β -adrenergic receptor or PPAR γ activation. These adipocytes are termed either brite, brown-in-white, beige or inducible thermogenic adipocytes [16, 17]. Brite adipocytes have a high capacity for glucose and lipid uptake making them an excellent target for lowering plasma levels of glucose and fatty acids. In view of this, the formation and activation of brite adipocytes induced by browning of white adipocytes is considered as a novel therapeutic concept in the therapy of obesity [18, 19] and T2DM [20]. A schematic presentation of the three distinct types of adipocytes identified so far in humans is provided in Fig.1.

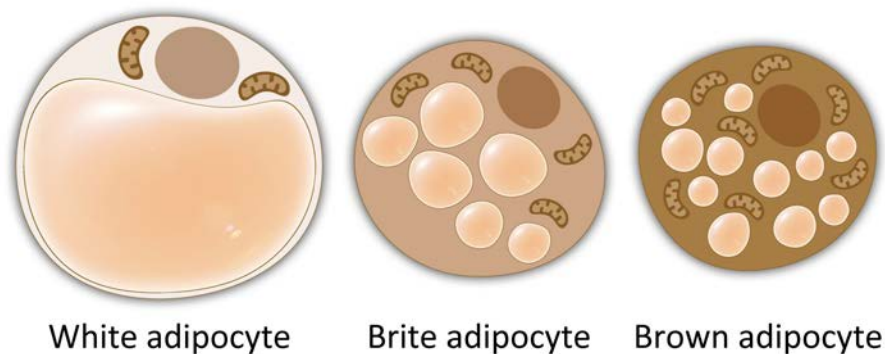


Fig.1: Schematics of the three distinct types of adipocytes.

WAT is the main fat storage of human body and plays a pivotal role in whole body energy homeostasis, thermoregulation and lipid metabolism [21]. In addition to lipid and energy storage functions, WAT has endocrine functions by secreting bioactive adipokines, cytokines, chemokines and lipokines [22]. Healthy WAT expansion (hyperplasia) is characterized by an elevated number of small adipocytes formed during adipogenesis, the formation of functional adipocytes from progenitor cells. In contrast, pathologic WAT expansion is characterized by adipocyte hypertrophy leading to lipid-laden larger dysfunctional adipocytes [23]. Some representative metabolic functions of adipocytes are illustrated in Fig. 2.

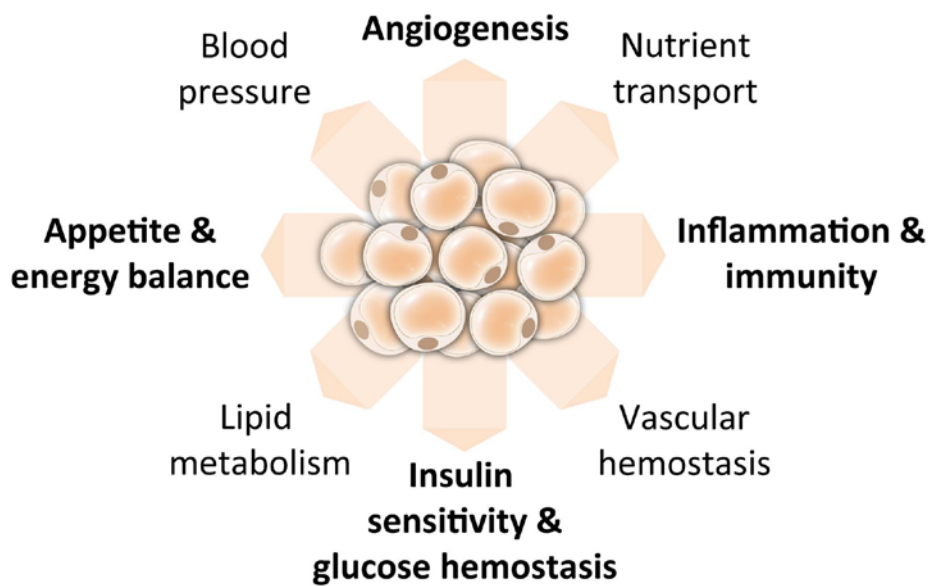


Fig.2 Schematic presentation of the metabolic roles of adipose tissue. Modified according to [24].

Dysfunction of adipose tissues related to obesity is a source for chronic low-grade inflammation in which elevated secretion and production of adipokines leads to various systemic metabolic disorders including cardiovascular and chronic liver or kidney diseases [23, 25, 26]. Moreover, obesity induces a complex remodeling of adipose tissues and markedly changes its structure and cellular composition [23]. To accommodate surplus energy, WAT expands by increasing adipose cell size (hypertrophy) through lipid accumulation. Thus, excess fat accumulation in WAT further promotes the release of free fatty acids into the circulation, which may be a critical factor in diminishing insulin sensitivity by generation of lipid metabolites and thus lipotoxicity, pro-inflammatory cytokines and oxidative stress [27]. Obesity-induced WAT remodeling occurs through changes in the composition of the extracellular matrix (ECM) and an increase in the level of pro-inflammatory adipokine secretion [28]. The enlargement of the adipose tissues results in an infiltration of circulating monocytes. Further recruitment of macrophages and immune cells to

the adipose tissues induces a change in the polarization state of macrophages from anti-inflammatory M2 to pro-inflammatory M1 phenotypes, whereas a more complex and heterogeneous profile of activated macrophages is observed in WAT in case of obesity [29]. The accumulation of M1 polarized macrophages in WAT further promotes the secretion of pro-inflammatory cytokines and thus contributes to obesity-induced insulin resistance. M2 macrophages in turn participate in adipose tissue remodeling by removing dead adipocytes and assisting in the recruitment of new adipocytes from progenitor cells. Indeed, active macrophages surrounding the remnant lipid droplet of death adipocytes form the so-called crown-like structures [30, 31]. In the course of obesity, the crosstalk between pre-adipocytes and immune or parenchymal cells of the adipose tissue may critically regulate the differentiation of pre-adipocytes into white adipocytes, brite adipocytes, or myofibroblasts, thereby influencing adipose tissue expansion and dysfunction, including the development of fibrosis [32]. Fibrosis is a pathological consequence of ECM dysregulation caused by an enrichment of ECM proteins, predominantly collagens. Due to elevated levels of collagen, the ECM becomes more rigid and the embedded adipocytes cannot expand to store surplus lipids, which in turn promotes lipotoxicity when lipids become deposited in non-adipose organs like muscle, liver or pancreas [33]. Structural and functional differences between lean and obese adipocytes are schematically depicted in Fig. 3.

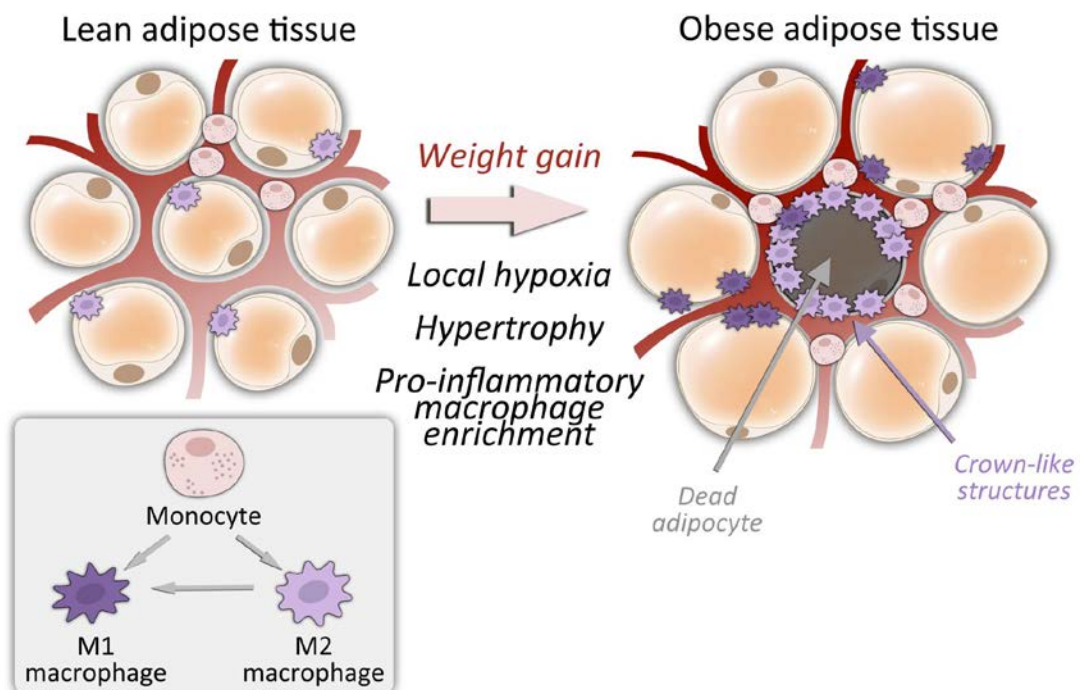


Fig.3 Morphological and functional differences between lean and obese adipose tissues. A crown-like structure is shown in the central part of obese adipose tissue.

One strategy to counteract lipotoxicity is to increase energy expenditure either through increased physical activity or by non-shivering thermogenesis by brown adipocytes. BAT contains abundant mitochondria that are uncoupled due to the expression of uncoupled protein 1 (UCP1) within the inner membrane of the mitochondria. UCP1 uncouples oxidative phosphorylation from adenosine triphosphate (ATP) synthesis to produce heat thereby promoting rapid energy consumption without formation of ATP [34, 35]. A therapeutic option to activate BAT is given by the treatment with drugs like β -3 adrenergic agonists, natriuretic peptides, retinoids, capsinoids, thyroid hormones, or glucocorticoids [36]. Another possibility is to induce thermogenesis by external stimuli in order to activate the process of browning in WAT to obtain brite adipocytes. During the browning of white adipocytes process, the upregulation of UCP1 can be promoted by the modulation of transcription factors like PPAR α and PPAR γ , which play an essential role in adipose tissue development and differentiation [16]. Within the last decade browning of WAT by recruiting de novo thermogenic brown-like adipocytes and/or conversion of white adipocytes has emerged as novel therapeutic strategy to increase energy expenditure and to reduce body weight [19, 37, 38]. These therapeutic approaches include PPAR agonists, sirtuin-1 (SIRT-1), β 3-adrenergic receptor stimulation, thyroid hormone, irisin and fibroblast growth factor 21 (FGF21) induction as well as certain lipokines [19, 39-42]. The mentioned cytokines are well recognized activators of BAT and brite adipocytes in humans [43]. Unfortunately, despite extensive research efforts to manage obesity by increasing total energy expenditure via stimulating thermogenesis by browning of WAT [44], an effective negative energy balance associated with measurable loss of body weight could not be achieved in clinical studies so far [45]. These results are rather disappointing, not only because of the minimal effects achieved, but also due to negative adverse side effects, in particular on the cardiovascular system. Thus, the pharmacological implementation of drugs that stimulate adipocyte thermogenesis in humans remains challenging and calls for new strategies including novel classes of drugs, proper delivery systems to improve drug efficacy as well as innovative therapeutic approaches. In this context, nanomaterials and nanoparticles as optimized delivery systems for targeted therapies have emerged over the last two decades providing much hope and new perspectives. Furthermore, specific drug delivery systems targeting AT with the aim to induce browning of WAT have been developed. Among them are clodronate loaded liposomes which eliminate visceral adipose macrophages [46], and rosiglitazone-loaded lipid nanoparticles [47]. These nanoparticles are dual targeted with a peptide sequence targeting prohibitin that is preferentially expressed in adipocytes and an octaarginine cell penetrating peptide. Upon local administration in diet-induced obese mice the authors found a shrinkage of adipocyte size and an inhibition of obesity progression without detectable adverse side effects. As in case of obesity the function of the lymphatic vasculature is impaired, the

subcutaneously injected nanoparticles might not be easily drained from adipose tissue thus preventing undesired side effects, especially in the liver [47]. In another study, dibenzazepine was incorporated into poly(lactide-co-glycolic acid) (PLGA) nanoparticles [48]. Local injection of these polymer nanoparticles into WAT depots of diet-induced obese mice induced browning of adipocytes via inhibition of Notch signaling. These studies highlight the promises for nanoparticles to be leveraged for direct delivery of drugs to adipose tissues.

Taken together, the adipose tissue has been recognized as key player in human metabolism regulating whole body energy homeostasis. Therapeutic targeting of AT would allow to tackle distinct fat mass redistribution and obesity-linked complications e.g. by improving endocrine function based on adipocyte-secreted adipokines [49]; by reducing inflammation of hypertrophic AT [50, 51]; or by increasing energy dissipation via non-shivering thermogenesis [52, 53]. Even though AT has been identified as therapeutic target in obesity, till now there is no effective medication targeting AT approved for the clinics. There is large consensus that understanding adipogenesis and fat cell development at a molecular level is essential to identify new therapeutic targets for the development of effective anti-obesity drugs [19]. In this context, microRNAs (miRNAs) have emerged as a novel class of regulatory determinants and promising targets for therapeutic applications.

3. MiRNAs AND THEIR ROLE IN ADIPOSE TISSUE

MiRNAs play important regulatory roles in many biological processes associated with obesity and T2DM, including adipocyte formation, differentiation, insulin regulation as well as carbohydrate and fat metabolism [54]. During adipogenesis miRNAs can accelerate or inhibit adipocyte differentiation and hence regulate fat cell development. In obese adipose tissue a dysregulation of miRNAs was identified by several reports [55]. Therefore, normalizing miRNA insufficiencies by either inhibiting or restoring miRNA function is a promising therapeutic strategy in the treatment of obesity and related metabolic disorders.

MiRNAs are single-stranded RNAs with 19 to 25 nucleotides in length which regulate gene expression. Today more than 2000 miRNAs are identified in humans, and it is estimated that miRNAs regulate about 60% of all protein-coding genes [56, 57]. MiRNAs are intimately involved in cellular processes such as differentiation, proliferation, apoptosis or autophagy. Especially, the capacity of miRNAs to target multiple mRNAs that are altered in disease conditions makes these molecules highly interesting candidates either as therapeutics or as therapeutic targets. Accordingly, miRNA-based therapeutics are applied either as miRNA mimics or miRNA inhibitors/antagonists. MiRNA mimics are double-stranded short RNAs that are extracellularly introduced into cells to mimic endogenous

miRNA functions [58-60]. Their function is to restore or enhance miRNA abundance. In contrast to miRNA mimics, miRNA inhibitors are single-stranded antisense oligonucleotides, which have a complementary sequence to its targeted miRNA thus preventing miRNA binding to its endogenous target and thereby diminishing miRNA function [61]. In this review we intend to focus on miRNAs which have been identified to play a functional role in adipogenesis.

3.1 Key regulatory miRNAs in adipose tissues formation and differentiation

The major functional role of miRNAs in adipose tissues is to modulate adipocyte differentiation and to regulate its metabolic functions. So far, numerous different miRNAs have been identified in human adipose tissues whose regulatory (patho-)physiological roles are only partly understood and little is known about the role of miRNAs in the regulation of fat-cell size and number [62]. Moreover, a clear relation between up- and downregulation of individual miRNAs in obesity and diabetes has not been shown. Performing a miRNA expression microarray profiling in human derived SAT, it was found that the expression of 50 out of 799 miRNAs significantly differed between fat cells from obese and non-obese patients [63]. Moreover, miRNAs are differently expressed in SAT and VAT, and it could be shown that upon therapeutic treatment with PPAR γ agonists miRNA expression levels are changed either in the same or in the opposite direction, in both SAT and VAT [64]. Thus, due to the complexity of the miRNA secretion profiles, systematic studies on the distribution of miRNAs in diverse fat depots of healthy and obese patients are largely missing. Nonetheless, it is well established that miRNAs are essential regulators in adipogenesis, adipocyte differentiation, browning, lipid metabolism, glucose homeostasis and insulin resistance [65, 66].

With regard to adipogenesis, numerous pro- and anti-adipogenic miRNA candidates have been identified in humans [67]. Among them are miR-27b and miR-130 that directly target transcription factors and downregulate PPAR γ expression [68, 69]; miR-138 that functionally silences lipoprotein lipase (LPL) in human adipose tissue-derived mesenchymal stem cells (hMSCs) resulting in an inhibition of the expression of CCAAT/enhancer binding protein alpha (C/EBP α) and PPAR γ [70]; and miR-375 that represses adiponectin receptor 2 (AdipoR2), a receptor for globular and full-length adiponectin, which mediates increased PPAR α ligand activities [54].

One of the first miRNAs that were identified to promote human adipogenesis was miR-143 whose direct target is extracellular signal regulating kinase 5 (ERK5) [71]. Other examples are miR-30c, which mediates adipokine regulation, namely by targeting plasminogen activator inhibitor 1 (PAI-1) and activin receptor-like kinase 2 (ALK2) [72]; miR-

17 and miR-106a that target bone morphogenic protein 2 (BMP2), thus regulating the balance between osteogenesis and adipogenesis differentiation of hMSCs towards adipogenesis [73]; miR-26 family, consisting of miR-26a and miR-26b, target the sheddase ADAM metalloprotease domain 17 (ADAM17/TACE), which cleaves Pref-1, an inhibitor of terminal adipocyte differentiation [74], miR-148a promotes adipogenesis via suppressing its direct target Wnt1, an endogenous inhibitor of adipogenesis [75]; miR-342 acts by suppressing C-terminal-binding protein 2 (CtBP2) and releasing C/EBP α [76]; in mouse and human, miR-21 was identified to modulate TGF- β signaling [77]. Some representative miRNA candidates and their roles in adipose tissue formation and regulation are shown in Fig. 4. The presentation of miRNAs is not exhaustive and only a few examples with specific focus on humans are provided. For more details see references [35, 55].

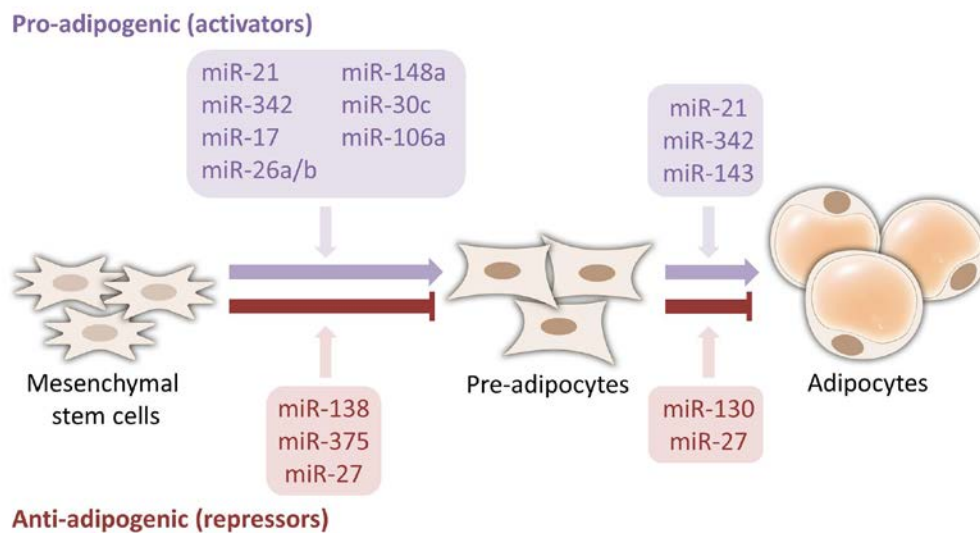


Fig.4. Some representative miRNA candidates that promote or inhibit adipogenesis in humans.

3.2. Key regulatory miRNAs in brown and brite adipose tissue

Several miRNAs preferentially affect the formation and function of brown/brite adipocytes by addressing specific transcriptional factors. Traditionally, the process of browning leads to a remodeling of adipocytes by increasing mitochondrial number and size, altering the morphology of mitochondria. The mitochondrial membrane is more fragmented with a higher uncoupling activity linked to the presence of UCP1 [78]. In addition, each adipocyte contains more lipid droplets of smaller size. So far, various miRNAs have been identified as key regulators in the adipogenesis of brown fat by direct or indirect targeting of transcription factors to promote or inhibit adipocyte differentiation. As selected examples, miR-26a, miR-328, miR-378, miR-30b/c, miR-455, miR-32, and miR-193b-365 were shown

to activate brown adipogenesis, whereas miR-34a, miR-125b, miR-133, miR-155, and miR-27b were identified as inhibitors of brown adipogenesis [35] as summarized in Fig.5.

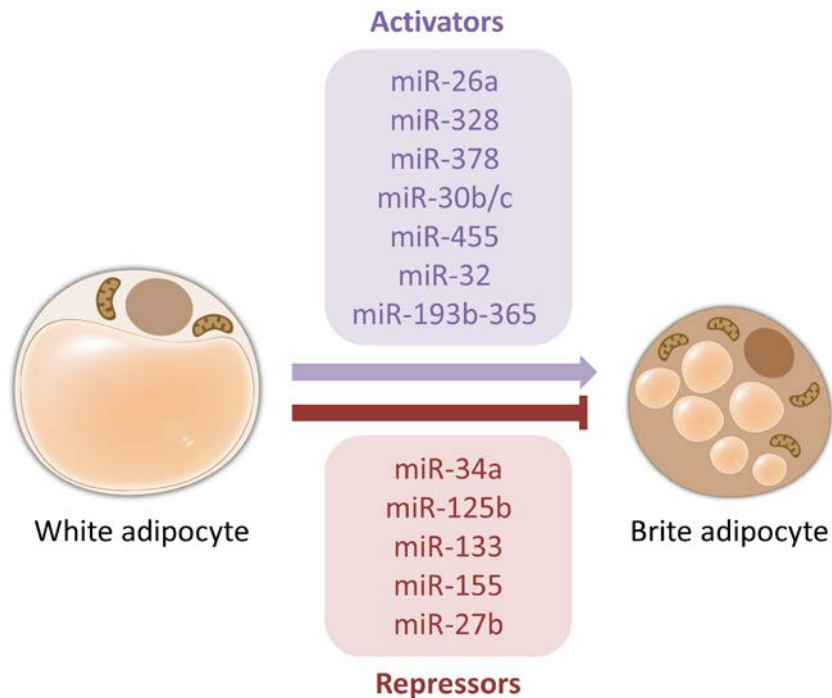


Fig.5. Some representative examples for miRNAs that regulate the browning process.

Modulation of these miRNAs either by selective inhibition or activation has been shown to affect thermogenesis, energy expenditure, mitochondrial function and respiration, glucose tolerance and insulin sensitivity [38]. Accordingly, miRNAs appear as attractive targets for pharmaceutical interventions in the therapy of obesity, diabetes and other metabolic disorders as cardiovascular diseases [79]. Some research efforts have now been focused on the understanding of the molecular pathways and targets that are regulated by miRNAs. For instance, miRNAs that target the gene encoding PR domain containing 16 (Prdm16) and the adipogenic transcription factor CCAAT/enhancer-binding protein beta (C/EBP β) are miR-133 and miR-27. Interestingly, it was shown that *in vivo* downregulation of miR-133 in BAT and subcutaneous WAT in response to cold exposure promotes the differentiation of precursors from BAT and WAT to mature brown adipocytes [80]. Hence, specific inhibition of miR-133 in these tissues leading to an increase of brown fat mass was suggested as potential treatment option for obesity [81]. MiR-27 was identified as central upstream inhibitor of PPAR γ in white adipocyte differentiation and was endogenously downregulated during white to brite differentiation directly targeting and repressing PRDM16,

CREB, PPAR α , PPAR γ and PGC-1 α/β to impair human adipogenesis [82, 83]. MiR-34a, for example, targets the fibroblast growth factor receptor 1 (FGFR1), reduces expression of β klotho and SIRT1, which finally results in a deacetylation of PGC-1 α repressing the browning process. The transcriptional coactivator PGC-1 α is an important regulator in the biogenesis of mitochondria and is intimately involved in the regulation of lipid and carbohydrate metabolism. MiR-155 controls the development of BAT by direct targeting of C/EBP β [84]. It was shown that miR-155 forms a bi-stable feedback loop together with its target. Inhibition of miR-155 enhanced brown adipocyte differentiation and browning of WAT [84]. While on the contrary high expression of miR-155 is correlated with inflammation in obese patients, impaired adipocyte function, hypoxia and chemotherapy resistance [65]. Let-7i is a repressor of brite adipocyte function and inhibition of let-7i promotes the conversion of white in brite adipocytes, while treatment with let-7i mimics inhibits β -adrenergic activation of the browning process [85]. Another example is miR-125b that impairs brite adipocyte formation and function by modulating oxygen consumption and mitochondrial gene expression [86]. miR-22 was shown to modulate thermogenesis through the activation of the glycolytic and mTORC1 signaling pathways and represents a potential therapeutic target for human metabolic disorders [87]. Furthermore, it has been shown that miR-22 attenuates fat accumulation in response to a high-fat diet (HFD) by reducing white adipocyte differentiation and increasing BAT activity [88]. It has been shown that miR-203 inhibits IFN- γ signal pathway and promotes white adipocyte browning and thermogenesis in SAT and improves glucose tolerance in HFD fed mice [89]. The most prominent pathways in adipocyte browning which are known to be regulated by miRNAs have been shown elsewhere [18].

3.3. Regulatory miRNAs in obesity, diabetes and insulin resistance

Numerous miRNAs are dysregulated in adipose tissues from obese humans and might contribute to the pathology of disease [63]. For example, miR-221 is upregulated in adipose tissues in human obesity contributing to the development of insulin resistance [90], while miR-100 is down-regulated in visceral adipose tissues of obese and diabetic patients [91]. Of note, the miRNA expression profile can be altered by different weight-loss approaches including diet, physical training or bariatric surgery [92]. Moreover, the miRNA expression pattern in VAT and SAT can be changed upon weight loss depending on the type of intervention. As example, the expression profile of miR-146b, which was identified as regulator of human visceral pre-adipocyte proliferation and differentiation, is significantly altered in VAT and SAT in overweight/obese patients compared to lean patients [93]. In a recent study, Hilton et al. [94] were able to correlate differences in the human body fat distribution of miRNAs to adipose tissue ECM composition. The authors have performed an

expression profiling of abdominal subcutaneous and gluteal adipose tissues and have identified miR-196a as regulator of pre-adipocyte proliferation, human body fat distribution and ECM composition.

Some miRNAs are directly involved in the regulation of lipid and glucose metabolism, lipolytic activity and adipokine production in AT. Among them are miR-320, miR-14, miR-278, let-7 or miR-192. Other miRNAs are involved in insulin production/secretion or resistance, for instance miR-9, miR-124a, miR-130, miR152, miR-335, miR-375 or miR-29a. The latter shows anti-adipogenic effects by repressing glucocorticoid receptor (GR) signaling. Other miRNAs play important roles in the direct regulation of lipids in AT. For instance, elevated levels of miR-221-3p were shown to impair adipocyte lipid storage and differentiation and modify adipocyte ceramide, sphingomyelin and diacylglycerol content [95]. In mature adipocytes, miR-107 was shown to induce a triglyceride storage defect by impairing glucose uptake and triglyceride synthesis. [95]. These are only a few examples in relation to AT. The tremendous impact of miRNAs on lipid metabolism concerned with the role of lipids, triglycerides and cholesterol in oxidation or inflammation during the progression of atherosclerosis or coronary artery diseases is discussed elsewhere [96, 97]. Since the focus of this review is on specific delivery and targeting of miRNAs in AT dysfunction, we will not discuss the increasingly important role of circulating miRNAs and the prominent role of extracellular vesicles in miRNA transport. For details, there are several excellent recent reviews addressing these topics [65, 98-102].

Considering the large variety of miRNAs and their manifold complex functions in healthy and pathological settings in which miRNAs regulate multiple genes simultaneously, it remains a big challenge to identify the most promising miRNA for pharmacological therapy. Accordingly, a screening of multiple miRNA candidates in the same experimental setting covering *in vitro* and *in vivo* experiments would be essential and critical for the selection of the best candidates that might be applicable for clinical translation. Moreover, to achieve specific targeting, highly efficient and safe delivery of active miRNAs are key requirements for the application of miRNA-based therapeutics. Although some success has already been achieved in the translation of miRNA-based therapeutics towards the clinic and several pharmacological therapies targeting miRNAs have been developed yet it remains a challenge to address a specific miRNA in a specific tissue or cell type. To overcome these issues nanoparticles have been proposed and explored as carrier systems for miRNAs. Following these lines several highly promising *in vitro* studies using miRNA loaded nanoparticles have been presented, however, specifically controlled, safe and efficient delivery of miRNAs *in vivo* is still a problem as the miRNAs are often degraded, not bioavailable or inactive. To be clinically applicable, it seems to be inevitable that miRNAs are

protected by chemical modifications or delivered by a proper carrier system that enables specific targeting of the desired tissues with only minimal off-target effects.

4. MiRNA BASED THERAPEUTIC STRATEGIES

For the purpose of miRNA-based therapy two different pharmaceutical approaches prevail. The first one is to use miRNA inhibitors, which are single-stranded antisense miRNA oligonucleotides (AMOs) that have a complementary sequence to the targeted miRNA thereby preventing miRNA binding to its endogenous mRNA targets. This approach is typically referred to as antagonist therapy. The second one concerns the use of miRNA mimics which are double-stranded oligonucleotides that have the function to restore or enhance the functional activity of miRNAs, which are downregulated in tissue dysfunction or diseases. This approach is known as miRNA replacement therapy.

4.1. miRNA inhibition strategy

AMOs are frequently used as antagonists for therapeutic targeting and inhibition of miRNAs. To improve stability and the *in vivo* performance of AMOs, the oligonucleotides are often modified by covalent conjugations. As a representative example miR-122 which plays an important role in fatty acid metabolism and cholesterol accumulation was recognized and silenced by miR-122-AMO linked to several conjugates including cholesterol [103-106]. The silencing activity was intimately linked to the length of the oligonucleotides and could be improved by modifications with phosphorothioate groups [107]. The phosphorothioate backbone modifications are essential for *in vivo* applications, as phosphorothioate modified AMOs containing at least 12 phosphorothioate linkages sufficiently bind to plasma proteins and thus remain in circulation for longer periods [108].

Apart from ligand conjugation, chemically modified RNA nucleotides can be synthesized and locked to antagomirs to improve affinity, potency and specificity [109]. Among the most popular alterations in the design of synthetically modified oligonucleotides are so called locked nucleic acids (LNAs) in which the ribose moiety in the sugar-phosphate backbone is modified by the introduction of a bridge connecting the 2' oxygen and the 4' carbon residue. Such synthetically engineered LNAs are highly structured and stable [110, 111]. Moreover, LNA-antisense oligonucleotide targeting miRNA (LNA-AMO) complexes show a high binding affinity to complementary RNA molecules, low toxicity and appropriate bio-distribution *in vivo* [112, 113]. Nowadays, the LNA-AMO technology is frequently used in the design of miRNA-based therapeutics due to the excellent silencing efficiency observed *in vitro* and *in vivo* [106]. For instance, excellent therapeutic results were achieved in silencing miR-122. Using 2'-O-methoxyethyl phosphorothioate ASOs, a reduction of plasma cholesterol levels, increased hepatic fatty acid oxidation as well as a decreased synthesis

rate of hepatic fatty acids and cholesterol were observed in normal mice [114]. In a high fat fed obese mouse model a significant improvement in liver steatosis was achieved. In another example, an unconjugated high-affinity LNA-anti-miRNA system modified with phosphorothioate residues containing more than 50% LNA with a high melting temperature of 80°C was highly effective in silencing liver-expressed miR-122 after three repeated injections in non-human primates. The LNA-AMO complex was taken up into primate hepatocytes to form a stable duplex with miRNA-122. The treatment resulted in a long-lasting and reversible decrease in total plasma cholesterol [115]. The results were validated in a diet induced obesity mouse model after two weekly intraperitoneal injections showing a sustained decrease in total cholesterol without any evidence for liver toxicity or lipid accumulation in the liver. A related LNA-AMO complex with only 30% LNA and a melting temperature of 70°C was less effective in silencing miR-122 using the same dosing regime in chow diet fed mice [116]. Administering a cholesterol conjugated antagomir-122 complex with a partially or fully modified phosphorothioate backbone via intravenous injection to mice resulted in a specific dose dependent silencing of miR-122 in the liver [105]. However, the treatment doses required for efficient silencing were higher than for the unconjugated high-affinity LNA-AMO system described above. All these data support the notion that miR-122 is a highly promising therapeutic target for the treatment of metabolic diseases, but one has to keep in mind that a subtle balance and fine tuning in chemical modifications and sophisticated stabilization strategies are required to allow efficient carrier free delivery of miRNA mimics.

Despite considerable progress made through direct conjugation and chemical modifications of AMOs their efficiency in *in vivo* applications remains limited. When administered without a carrier system the AMOs are prone to degradation by nucleases and rapid excretion by the liver, kidney or spleen. Accordingly, carrier systems are designed to circumvent these problems by protecting the oligonucleotides from degradation; preventing non-specific interactions with proteins and cells; avoiding recognition by the immune system; enhancing the circulation time to improve tissue targeting; and facilitating cellular uptake. In the next chapter, major challenges in the engineering and application of miRNA-loaded delivery systems are discussed as well as recent approaches in the design of advanced miRNA-based entities to enable tissue specific and safe transport of active miRNA therapeutics. We will show key examples that highlight the therapeutic potential of miRNAs in the treatment of obesity related metabolic diseases, but we have to point out that most approaches so far are in the field of cancer research. In the following some of the most promising strategies for miRNA delivery are briefly discussed including lipid-, protein- and polymer-based nanoparticle systems.

4.2. miRNA replacement strategies using nanoparticles

As briefly mentioned above, the applications of nucleic acid-based therapeutics and their successful implementation in the clinic are hampered by several issues including limited stability of oligonucleotide molecules, short half-life in blood, poor cellular uptake and unwanted off target effects. To overcome these issues, miRNAs require proper transport systems to be delivered to the desired cells and tissues thereby enabling active uptake into the cells. Accordingly, the delivery system has to cross the cell membrane, to escape the endosome and release its cargo to the cytoplasm. Apart from viral delivery systems, a very promising strategy is to incorporate nucleic acids in delivery vehicles using non-viral vectors. In comparison to viral gene delivery, this approach has some limitations concerning loading efficiency, effective delivery, cytotoxicity or immunogenicity associated to the transport vehicle, but non-viral vectors have proven to be less toxic and immunogenic, and their production is much easier and less costly [117].

In recent years the development of delivery platforms for nucleic acid therapeutics using non-viral vectors has tremendously advanced. Various formulations were designed using diverse nanoparticle delivery vehicles for nucleic acid molecules involving DNA, messenger RNA, small interfering RNA, microRNA or antisense oligonucleotides. Different classes of delivery vehicles including polymeric conjugates, polymer nanoparticles, proteins, dendrimers, lipid nanoparticles or liposomes are used as shown in Fig.6. To be applicable the nanoparticles have to meet a number of requirements. The manufacturing processes should be simple, preferentially by mixing of the single components for self-assembly and spontaneous formation of the nanoparticle-RNA complex, which is typically applied for lipid-based drug delivery systems. In case of polymers, emulsion technologies using either high-speed homogenization or ultrasonication are most frequently used [118]. To avoid clearance by the immune system or renal filtration the physicochemical characteristics, in particular the size of the nanoparticles has to be adjusted to approximately 10 to 200 nm in diameter. Other parameters being important for colloidal stability, bio-distribution and cellular uptake are the surface characteristics of the nanoparticle-RNA complexes including charge distribution, specific coatings or targeting moieties.

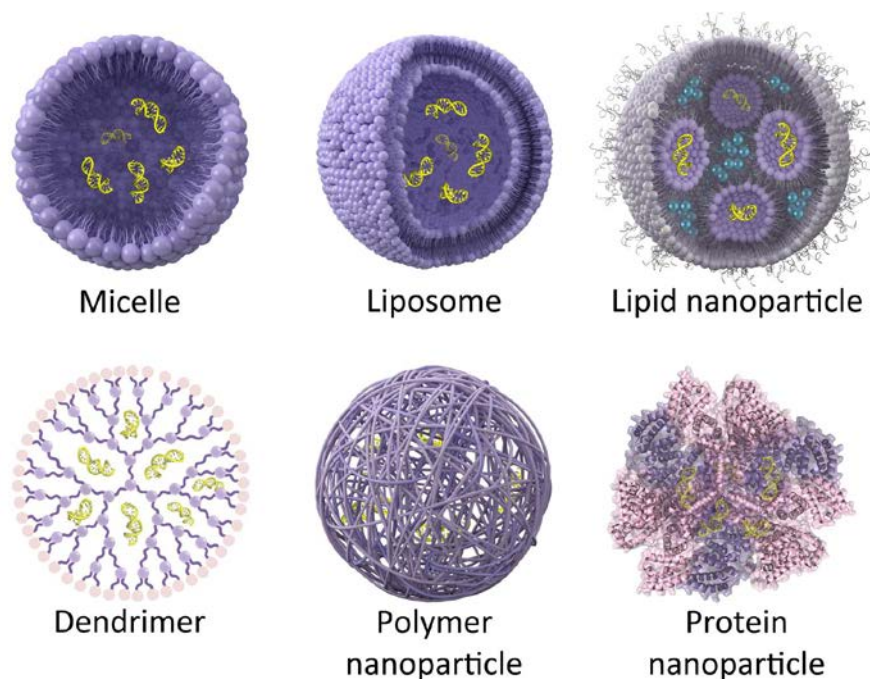


Fig.6 Nanoparticle platforms for miRNA delivery.

In the following section we will discuss some of the most promising delivery systems developed so far for miRNA delivery. By learning from current approaches in nanoparticle design, loading and targeting, the formulation protocols can be readily adopted for miRNA delivery to tissues other than cancer cells, and till now encouraging results have been achieved for different applications.

4.2.1 Lipid-based drug delivery systems for miRNA replacement

Among the most prominent representatives of drug delivery systems are liposomes, which play an increasingly important role as pharmaceutical carriers in both academic and industrial research. Liposomes are widely used, especially as homing devices for new molecules including biopharmaceuticals and nucleic acid-based drugs [119]. Currently, a great number of liposome-based formulations are in late-stage clinical tests, while some of them are already approved and marketed [120, 121]. However, only a very limited number of liposome based formulations for nucleic acid delivery are in clinical practice [122].

For an efficient transport of nucleic acids molecules, which have a negatively charged backbone, cationic lipids are typically used. The cationic lipids electrostatically interact with the oligonucleotides to form stable complexes called lipoplexes. The anionic nucleic acid molecules are thus neutralized by the cationic lipids and incorporated into the aqueous

interior of the liposomes to be shielded from enzymatic degradation. Another advantage in the usage of cationic liposomes is that the transport across the negatively charged cellular membranes is facilitated. However, problems may arise from the fact the cationic lipoplexes injected systemically interact with negatively charged serum proteins to form a so-called protein corona surrounding the surface of the liposomes to form large liposome-protein aggregates that are subsequently eliminated by the liver and spleen [123, 124]. Thus, to reduce the positive net surface charge neutral lipids like phosphatidylcholines and/or cholesterol are incorporated into the lipid bilayer in combination with helper lipids like 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DOPE) [125]. DOPE is a fusogenic lipid that compensates for the lower cationic surface charge through destabilizing the lipid bilayer of the cell membrane to facilitate cellular entry via fusion [126]. Another problem associated to cationic lipids is that the lipids may be toxic to cells depending on their headgroup structure. To overcome this problem different new classes of cationic lipid-based compounds have been designed [127]. These new generations of lipids include ionizable lipids, which are neutral at physiological pH but become positively charged upon cellular uptake due to the acidic pH in the lysosomal compartment. Intracellularly, the ionizable aminolipid containing liposomes become destabilized and the nucleic acid is released in the cytoplasm. In addition, polyethylene glycol functionalized lipids (PEGylated lipids) can be introduced into the liposomes at low concentrations to confer steric stabilization. Such liposomal formulations are termed stable nucleic acid lipid particles (SNALPs) [128]. Due to the surface located PEG coat SNALPs show extended blood circulation times and low immunogenicity, but one has to keep in mind that the PEG moiety might mask the surface properties of the nanoparticles to modify cellular adhesion and uptake mechanisms [129].

Another class of molecules used for the design of lipid-based RNA carriers are lipoids, which are synthetically engineered lipid-like structures that contain multiple secondary and tertiary functionalized amino groups combined with two or more hydrophobic chains [130, 131]. Some representative structures of cationic and ionizable lipids currently used for nucleic acid delivery are shown in Fig. 7. Although most studies deal with the delivery of siRNA or ncRNA, it can be assumed that the lipid-based delivery systems are also appropriate for the delivery of miRNA molecules. As mentioned before, only a few studies of miRNA-loaded liposomes are described for the treatment of diseases except for cancer. Other fields of application in which miRNA-liposomes are successfully applied are tissue engineering and bone regeneration [132]. For the treatment of osteoporosis, anti-miR-148 was formulated into liposomes based on dioleoyl-3-trimethylammonium propane (DOTAP), DOPE and cholesterol targeted with an octa-aspartate sequence (D-8Asp) to specifically bind to hydroxyapatite to bone surface [133]. In another interesting study miRNA was delivered in gas bubble filled liposomes. The liposomes were loaded with miR-126 to

lower [135]. For instance, the injection of miR-124-DOPC complexes in the tail-vein in a mouse model of lung cancer showed that after 10 minutes the miRNA lipid complexes are found in blood, liver, kidney and lungs, while cellular uptake was only found in lung tissues [136]. In another example, Rupaimoole et al. [137] used anti-miR-630 DOPC particles to target miR-630 which is upregulated under hypoxic conditions. The authors found a rescue of Dicer expression and a decrease of tumor growth in orthotopic mouse model of ovarian cancer. However, to the best of our knowledge neutral liposomes and lipid emulsions have not been used to deliver miRNAs to non-carcinogenic tissues yet.

4.2.2. miRNA replacement by polymeric nanoparticles

Polymer-based nanoparticle systems are similar effective for the delivery of nucleic acids as lipid-based systems. Polymers are versatile structures to form nanoparticles of different size and surface characteristics in aqueous solutions. They are able to complex oligonucleotides within a well-defined polymer network. Common polymers used for the synthesis of nanoparticles for nucleic acid delivery are among others PLGA, PEG, polyethyleneimine (PEI) or chitosan. PLGA is a biocompatible and biodegradable polymer that is approved by the FDA for human clinical use as biodegradable sutures [138, 139]. PLGA-based delivery systems show a low toxicity *in vivo* but due to the negative surface charge of PLGA nanoparticles the complexation efficiency with miRNAs is limited. This shortcoming can be overcome by the use of copolymers like chitosan [140]. By this way the loading efficiency and the release rate can be triggered by varying the ratio of PLGA and copolymer. PLGA particles are predominantly taken up via endocytosis, escape the endolysosomes and enter the cytosol for sustained release of its cargo [141]. For example, miR-34 mimic was loaded into nanoparticles composed of a mixture of PLGA and chitosan in the presence of poloxamer as a surfactant to target myeloma cancer [142]. In another interesting recent study PLGA nanoparticles were used to deliver miR-145 to attenuate vascular smooth muscle cell (VSMC) proliferation, and to maintain a contractile state in order to prevent intimal hyperplasia [143]. The authors hypothesize that local administration of the PLGA-miR-145 nanoparticles could assist to inhibit vein graft disease and the development of vein atheroma in patients undergoing bypass surgery. The jugular vein graft containing the PLGA miRNA-145 nanoparticles were implanted in carotid arteries of a rabbit model as local slow and sustained release system. The changes in intimal hyperplasia and VSMC proliferation activity were monitored over a time period of two weeks showing promising results [143]. In a recent study, Malik et al. used synthetic cationic peptide modified nucleic acids incorporated into PLGA nanoparticles for selective inhibition of mi-155 [144]. The PLGA nanoparticles revealed a high loading capacity and excellent transfection efficiency *in*

vitro and *in vivo* in a xenograft mouse model. Although promising data on PLGA nanoparticles for miRNA delivery are available the adipose organ is not addressed yet.

Another biocompatible polymer that is widely used for pharmaceutical applications is PEG [145]. As mentioned above, PEG is often used as polymer coat for liposomal formulations to achieve steric stabilization and longer circulation times after intravenous injection [135]. Alternatively, PEG molecules of different molecular weights, linear or branched can be directly coupled to oligonucleotides for gene transfection [146]. PEG molecules are also used to modify polycationic polymers like PEI to reduce their toxicity and to improve biodistribution [147]. PEI is one of the most commonly used polycations for electrostatic complexation and compression of negatively charged nucleic acids. The positive net charge of PEI strongly depends on the pH value and the protonation stage of the secondary amines [148]. As a proton acceptor PEI enables endosomal lysis and release of nucleic acids into the cytosol [149, 150]. Similar to PEG, PEI is biocompatible and commercially available in different molecular weights as linear or branched structures. In a recent study mesoporous silica nanoparticles were coated with PEI to modulate miR-24 levels in adipose-derived mesenchymal stem cells to elaborate the role of miR-24 in osteogenesis and adipogenesis [151].

Chitosan is another cationic polymer that is available as linear structure in different molecular weights. It has been extensively studied for drug delivery due to its biocompatibility and high safety profile. Accordingly, chitosan nucleic acid conjugates are promising entities for miRNA delivery. For instance, Nguyen et al. have used chitosan nanoparticles to functionally alter macrophage cholesterol efflux behavior by regulating ATP-binding cassette transporter A (ABCA1) expression [152]. MiR-33 was electrostatically loaded into chitosan-tripolyphosphate nanoparticles and delivered to macrophages to reduce the expression of ABCA1 resulting in a reduced cholesterol efflux to apolipoprotein AI. *In vivo* studies, confirmed a decreased reverse cholesterol transport to the plasma, liver, and feces. By delivery of miR-233 and miR-222, which are efflux promoting miRNAs, within the same chitosan nanoparticles cholesterol efflux and ABCA1 expression was improved. These findings show that chitosan entrapped miRNAs can be successfully delivered to macrophages to alter cholesterol homeostasis. The authors suggest that the same nanoparticles could be used to target miRNAs to atherosclerotic plaques to alter lesion progression, and are particularly suitable to treat cardiovascular and inflammatory diseases, where macrophages dominate and promote pathology, as it is the case in obesity and diabetes [152].

To achieve selective targeting of miRNAs, another promising approach is the conjugation of miRNA molecules to carbohydrates like N-acetyl-D-galactosamine (GalNAc).

GalNAc has a high affinity for asialoglycoprotein receptors and can be used to specifically target the liver where the particles are taken up into hepatocytes via clathrin-mediated endocytosis [109, 153]. GalNAc–miRNA conjugates are currently being evaluated in various preclinical and clinical settings and will be discussed later on in this review in greater detail.

To summarize, cationic polymers are among the most effective delivery vehicles for miRNAs due to the electrostatic interaction with anionic miRNA molecules and the enhanced cellular uptake due to the negatively charged cell membrane. However, similar to cationic lipoplexes systemic toxicity issues have to be considered and evaluated for each formulation individually.

4.2.3. miRNA replacement by dendrimers

Dendrimers are a special class of hyper-branched polymers consisting of a varying number of repeating monomer units, which are branched in all directions starting from the central core [154]. Dendrimers can be engineered in different sizes, surface charge and terminal functional groups. The most prominent dendrimer structures for gene delivery are polyamidoamine (PAMAM) dendrimers. MiRNA molecules can be embedded in the internal cavities of the dendrimer structure being complexed by the cationic structures. Disadvantages of dendrimers are their low transfection efficiency and potential cytotoxicity [155]. Similar as for liposomes and polymer nanoparticles, most of the miRNA-dendrimer complexes are designed for cancer treatment [156]. They are characterized by different compositions and conjugated surface groups including folic acids [157], thiol containing groups [158], chondroitin sulfate [159] or targeting/cell penetrating peptides [160]. In the latter study the authors have synthesized a Janus- faced dendrimer particle in which the one side of the cationic PAMAM dendrimer was complexed with miR-126 and the other side of the focal point with a polyarginine or RGD peptide to facilitate cellular uptake. These dendrimeric bowties were highly effective in promoting angiogenesis by enhancing cell proliferation and tube formation in endothelial cells. The delivery system was suggested for miRNA-based therapies in case of myocardial infarction or ischemic diseases [160]. In another example, systemic delivery of a chemically stabilized anti-miR-122 complexed with a so called interfering nanoparticle (iNOP) lead to effective silencing of liver-expressed miR-122 in mice, which was accompanied by regulating gene expression in liver and lowering of plasma cholesterol levels [161]. Such iNOPs are nanoparticles synthesized from lipid-functionalized poly-L-lysine dendrimers [162]. Notably, numerous dendrimer-based nanoparticles developed to date are in preclinical and clinical trials while first products are already available on the market but so far only as *in vitro* diagnostics or transfection reagents [163].

5. DELIVERY ROUTES, TARGETING STRATEGIES and CLINICAL TRANSLATION

One of the greatest challenges in drug delivery is to reach the specific target tissue. After systemic administration the drug carriers should remain in the circulation till they reach their desired target. On site, the delivery system has to cross physical and biological barriers depending on the target tissue. In case of miRNAs, the delivery vehicle has to protect its cargo from rapid degradation by nucleases that are abundantly present in the blood stream. Once in contact with biological fluids plasma proteins bind to the surface of the carrier system to form the previously mentioned protein corona. The attachment of the protein corona is associated with some drawbacks for *in vivo* application. Importantly, the protein coating might readily change the physicochemical properties of the delivery system, mask its surface characteristics, cause colloidal instabilities, modify bio-distribution or clearance behavior by the mononuclear phagocyte system (MPS) [164]. Another fact that has to be considered is host triggered immune response, meaning that the innate immune system can be activated by the miRNA-therapeutics causing severe toxic reactions and unexpected side effects. Thus, local delivery of miRNA therapeutics is expected to show less toxic and immunogenic effects when compared to systemic administration. Local deposition could be approached by injection of the delivery system directly into the target tissue using a slow-release depot formulation of the miRNAs. This could be achieved by embedding the miRNA delivery system within the matrix of a biopolymer such as a hydrogel, which serves as a local release reservoir. Local and sustained miRNA delivery from a hyaluronic acid hydrogel injected into the heart of mice resulted in cardiomyocyte proliferation and functional cardiac regeneration [165]. In other studies, miRNA containing hydrogels are applied for tissue repair [166], or as adhesive hydrogel system to promote chronic wound healing by acting immunomodulatory upon macrophage polarization [167]. All these results indicate that hydrogels are an appropriate matrix for local miRNA treatment. Although no studies are available for direct administration of miRNA loaded nanoparticles in adipose organ, local treatment by subcutaneous application appears to be a viable option.

Concerning liposome based delivery systems for miRNAs one of the major challenges is a non-specific bio-distribution of the nanoparticles in the body following systemic administration [168]. To enhance the specificity of the delivery system to selected tissues and cells different strategies have been employed using targeting ligands covalently coupled to the surface of the liposomes. These targeting moieties include proteins, which preferentially bind to cellular receptors. Among the most widely used ligands for cancer cell targeting are transferrin and folic acid [169]. Antibodies to matrix metalloproteases, integrins, adiponectins, interleukin-10, vascular cell adhesion molecules or vascular endothelial growth

factors are the most promising ligands to address inflammation in metabolic disorders. In this respect, a peptide targeted cationic liposome formulation containing anti-miR-712 was developed to target the vascular cell adhesion molecule 1 (VCAM1) for treatment of atherosclerosis [170]. Optical imaging validated disease-specific accumulation of anti-miR-712 in inflamed mouse aortic endothelial cells *in vitro* and *in vivo* without observable changes in the expression profile of miR-712 in non-targeted organs. Only recently, peptide sequences were identified to preferentially target the vasculature of adipose tissue to deliver peptide-conjugate vascular endothelial growth factor B (VEGF-B) with the aim to improve HFD induced obesity and fatty liver disease [171]. The same peptide sequence (KGGRAKD) was coupled to the surface of liposome via a PEG spacer. These circular peptide functionalized liposomes were highly successful in targeting adipose tissues after intraperitoneal injection into mice [172, 173]. Various cell penetrating peptides (CPP) are frequently conjugated as targeting sequences to liposomes to enhance cellular uptake as well as intracellular trafficking [174]. For instance, a cyclic RGD peptide was coupled to hyaluronic acid containing liposomes to deliver anti-miR-296 to inhibit endothelial cell migration by specifically targeting integrin receptors. These anti-miR-296 particles efficiently inhibited cell proliferation and angiogenesis after subcutaneous implantation in mice using a microgel plug assay [175]. Another approach to achieve miRNA delivery is by direct association of CPPs to miRNA molecules. CPPs are cationic peptides that electrostatically bind to the phosphate backbone of the nucleic acids to form stable complexes [176]. More generally, noncovalent package of oligonucleotides with cationic peptides is considered to be a promising strategy to shield oligonucleotides from degradation and to enhance cellular uptake [177]. One interesting example using a commercially available cationic peptide sequence, termed N-TER peptide, shows successful transfection and anti-adipogenic effects of miRNA-27a in 3T3-L1 mouse adipocytes [178]. MiRNA-27a is a recognized negative regulator in fat metabolism and the transfection efficiency was validated in a reduction of lipid droplet formation in mature adipocytes [178, 179]. Whether such loose assemblies of peptides and miRNAs are sufficiently stable *in vivo* to protect miRNAs from degradation after systemic administration needs to be further validated. An alternative strategy could be to use protamine, a cationic peptide naturally derived from salmon sperm that self-assembles in aqueous solution to form nanoparticles [180]. Due to its positive charge protamine forms stable complexes with negatively charged oligonucleotides. To enhance tissue specificity, the protamine nanoparticles could further be functionalized with specific targeting sequences. For instance, nucleic acid loaded protamine nanoparticles were conjugated to interleukin 10 or adiponectin to enable detection and *ex vivo* imaging of atherosclerotic plaques in a HFD fed Apo E deficient mice model of atherosclerosis [181, 182]. In another study, miR-29b was delivered with low molecular weight protamine to hMSC. The protamine-miR-29b complex

was efficiently taken up by hMSCs to promote the expression of osteoblast differentiation markers in a dose dependent manner [183].

More recent strategies involve the coupling of aptamers that bind to the selected cell surface receptors [184]. Aptamers have been identified using cell-SELEX technology to specifically recognize mature adipocytes. The aptamers show high affinity for mature 3T3-L1 mouse adipocytes [185, 186] to serve as selective markers for detecting and targeting adipocytes, however, the knowledge of cell surface biomarkers specific for adipocytes is still limited [186]. Only recently, the potential of aptamer adipo-8 to recognize and interact with the adipocyte plasma membrane associated protein (APMAP) was highlighted to reduce lipid accumulation and fat deposition [187]. Intraperitoneal injection of adipo-8 in high fat diet fed mice for 21 days resulted in body weight loss and a decrease of triglyceride levels in blood without causing liver or renal dysfunction. These data suggest that the aptamer adipo-8 could act both as potential anti-obesity drug and as targeting sequence to specifically address adipocytes. In support of this assumption the authors have constructed a PEGylated PLGA nanoparticle conjugated to adipo-8 encapsulating the model drug emodin. The author's results not yet published revealed a better lipid-lowering efficiency compared to the administration of adipo-8 alone [187].

Today, peptide sequences [188], proteins, antibodies and aptamers [185, 187] have been identified as most promising targeting sequences to become attached to the surface of nanoparticles to improve adipose tissue targeting. An overview of common targeting strategies for nanoparticles is depicted in Fig. 8 using liposomes as an exemplary nanoparticle. To date a comprehensive screening of suitable targeting moieties for adipocytes is largely missing.

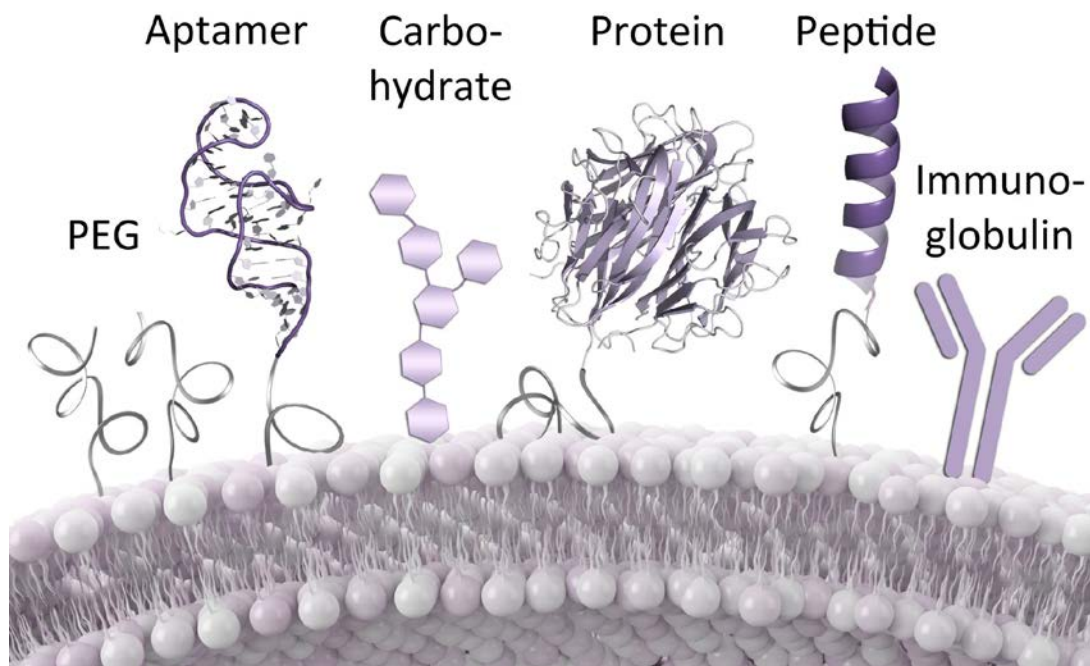


Fig.8 Common targeting strategies for nanoparticles.

5.1. miRNAs therapeutics in clinical trials

In total more than 2000 true mature miRNAs are estimated to exist in humans [189], and it is well established that miRNAs have manifold biological functions and regulatory roles. Consequently, miRNAs are deeply involved in the development and progression of diseases and, thus modulation and regulation of miRNA expression has evolved as emerging therapeutic strategy over the past few years. Despite many efforts, only a few miRNA-based therapeutics are undergoing clinical trials so far and none of them has reached phase III, while some of the studies had to be discontinued due to adverse side effects in patients. In comparison, about sixty siRNA drugs have completed clinical trials and two siRNA-based products have been approved recently by the FDA for clinical use in humans. The siRNA-products are ONPATTRO® (patisiran) and GIVLAARI™ (givosiran), both being achieved by Alnylam Pharmaceuticals. For further details on clinical trials of siRNA/miRNA therapeutics the interested reader is referred to recent comprehensive reviews on this matter [190-192]. The miRNA-based therapeutics are briefly discussed in the following. MRX34 was the first miRNA therapeutics approaching clinical trials. MRX34 is a liposome-based formulation of miR-34a mimics developed by Mirna Therapeutics for the treatment of solid tumors [193]. The miRNA mimic is intended to restore the lost suppressor function of miR-34 in p53 and wnt/ β -catenin cellular pathways [194]. The efficacy of miR-34a loaded liposomes to target the liver and to promote tumor regression was verified in a clinically relevant mouse model of hepatocellular carcinoma [195]. Then, a phase 1 clinical study was launched in patients with

advanced solid tumors critically evaluating dosage issues, safety aspects, pharmacokinetics and off-target effects [196]. In 2016, the clinical study was terminated due to multiple immune-related severe adverse events in five patients dosed with MRX34. The reported cytokine release was first attributed to the liposome delivery technology, termed NOV340 technology. Interestingly, using the same lipid formulation in combination with a single-stranded DNA oligonucleotide did not show any severe side effects in a clinical study with lymphoma patients. Thus, the reason for the failure of MRX34 in clinical use was suggested to be due to the miRNA itself that is targeting multiple genes and pathways simultaneously causing undesirable side effects [197]. To tackle this problem, it would be important to address the molecular mechanisms involving single miRNAs *in vivo* more specifically. Moreover, local administration of the miRNA therapeutics at the diseased site and tissue targeted delivery could be promising possibilities to impede adverse effects in the future.

Santaris Pharma developed a 15-mer LNA-ribonucleotide complementary to miR-122 (Miravirsen; SPC3649), which was modified with phosphorothioate linkages [198]. The modified miRNA showed remarkable *in vitro* antiviral activity and generated viral suppression in all hepatitis C virus (HCV) genotypes without producing viral resistance *in vivo* [199]. These promising pharmaceutical effects allowed to launch Miravirsen into the clinic [200], which proved to be safe and effective when injected intravenously or subcutaneously in primates and humans with HCV [201] and is currently undergoing multiple phase II clinical trials [191]. MRG-106 is an anti-miRNA-155 LNA-modified antisense inhibitor that is employed for patients with cutaneous T cell lymphoma and mycosis fungoides in a Phase II clinical trial. MRG-201 (Remlarsen) is a miR-29 mimic with a cholesterol-conjugated miRNA duplex that is used for patients with scleroderma, and the initiation of a Phase II clinical trial was announced. RG-012, named Lademirsen, by Sanofi Genzyme is a formulation of anti-miR-21 that is currently recruiting patients of a clinical phase II study for the treatment of Alport syndrome by reducing the decline in renal function. MRG-201 by MiRagen Therapeutics is an LNA- miR-29b mimic that is injected intradermal for the treatment of cutaneous fibrosis among other fibrotic events and is currently in clinical phase II [202]. The most interesting clinical trials with regard to obesity is RG-125/AZD4076 (Clinical Trials.gov Identifier: NCT02612662 and NCT02826525) by Regulus Therapeutics/ Astra Zeneca using a GalNAc- anti-miR-103/107 conjugate for the treatment of non-alcoholic steatohepatitis (NASH) in patients with type 2 diabetes. Although the study is in a Phase I/II with successful results, some concerns were raised related to cardiac implications associated with the systemic use of anti-miR-103/107. Anti-miR-103/107 was shown to impair the cardiac mitochondrial function in healthy mice. Thus, the authors of this study recommend a careful cardiac phenotyping of patients within the ongoing clinical trial [203].

Taken together, it is evident that further intense research is required in the field of miRNA therapeutics, whereas the promising results achieved so far in clinical trials give confidence that miRNA-based therapeutics will find their way to approved and marketed products within the next few years. Concerning the delivery systems, liposomes and lipid nanoparticles seem to be highly promising, since the first COVID-19 vaccines that received approval for human use are lipid-based mRNA vaccine BNT162b by BioNTech/Pfizer and mRNA-1273 by Moderna, and according to the manufacturer a few more lipid-based mRNA vaccines are in the pipeline [204]. In particular, the manufacturing technology for pharmaceutical production and proper upscaling procedures are readily in place for the lipid-based delivery platform, thus reducing the hurdles and costs in pharmaceutical technological development of special drug delivery systems.

6. DISCUSSION

MiRNAs are deeply involved in many cellular processes, and their dysregulation actively causes various pathological diseases. Therefore, they are highly interesting candidates as therapeutics or as therapeutic targets.

However, miRNA mimics and inhibitors in therapeutic applications typically require a delivery system to improve their stability and efficiency *in vivo* and to enhance their therapeutic index. This additional necessity of a delivery system was supposed to be a disadvantage in the past but now turns out to be an advantage for future therapeutic applications. One reason for that is that miRNAs have a very narrow spectrum of physicochemical properties such as length and charge, irrespective of the miRNA mimic or inhibitor sequence. That means, once developed and established, a miRNA delivery system for a specific target cell or organ opens the avenue to deliver any kind of miRNA mimic or inhibitor, also accelerating and optimizing miRNA drug development in the near future. Consequently, the vision for the future is a modular system for one to combine therapeutic miRNA mimics or inhibitors of interest with an optimized targeted delivery system suitable for target organs.

To enhance the efficiency and stability of miRNAs *in vivo* numerous non-viral delivery systems have been designed and optimized over the last decades resulting in improved targeting specificity, tolerable toxicity and reduced off-target effects. To date, also the delivery of siRNA is strongly on the rise. Despite the similarity of the oligonucleotide structures, their biochemical characteristics have to be carefully assessed for *in vivo* applications as biological and metabolic activities are distinct from miRNAs.

For the development of therapeutics most preclinical studies are performed in mice but one has to keep in mind that miRNA profiles and activities differ between mouse and human. In case of brown adipose tissue only a small sub-set of 145 miRNAs was identified to be common in both mouse and human BAT [205]. Out of the 145 miRNAs, 25 miRNAs have been identified to potentially target genes involved in cellular growth, proliferation and development. While most endogenous miRNAs which are currently known to be involved in BAT biology are characterized in mouse models, only three miRNAs, i.e. miR-26, let-7i, and miR-125b, have also been functionally characterized in humans [18]. The underlying biological processes are highly complex making the identification of putative mRNA-miRNA pairs associated with a specific disease challenging and urgently asking for an integrative bioinformatics approach of global transcriptome data. To this end, extended cross-species studies with miRNA and mRNA profiling are highly in demand to identify molecular interactions and targets in a comparative manner.

To conclude, considering the broad spectrum of aforementioned nanoparticle platforms already available for the delivery of miRNAs we are very optimistic that new miRNA-therapeutics will enter clinical trials in the near future. It also might be possible to propose the inclusion of two or more miRNA and/or miRNA inhibitors in the same nanoparticles in order to enhance or probably synergize the physiological response. Although today, the applicability and potential of nanoparticles as delivery systems are mainly explored in the field of cancer research considerable efforts have been made to exploit the same delivery platforms for other areas of disease. Encouraging results achieved so far give a great outlook for the establishment of miRNA-based therapies in various fields of applications including metabolic diseases such as diabetes and obesity.

Declaration of Competing Interest

The authors declare no conflict of interest in the publication of this work.

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