HORMONE RESEARCH IN PÆDIATRICS

Functional Significance and Predictive Value of MicroRNAs in Pediatric Obesity: Tiny Molecules with Huge Impact?

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Abstract

Obesity is a major health concern. While some children develop comorbidities such as insulin resistance and low-grade systemic inflammation upon weight gain, others stay metabolically healthy. There is an urgent need for clinically relevant markers with prognostic value related to disease development and intervention success. MicroRNAs (miRNAs) are established biomarkers for several disease states. Herein, we give a brief overview of miRNA biogenesis and function and the potential role of circulating miRNA in the context of pediatric obesity.   © 2016 S. Karger AG, Basel

Introduction

A lot of research is performed to elucidate the pathophysiology of childhood obesity and the related metabolic disturbances. While some children show the phenotype of metabolically healthy obesity (MHO), others are affected by insulin resistance and low-grade inflammation [1, 2]. Depending on definitions and the classification system used, around 20–30% of the obese children aged between 8 and 17 years can be classified as MHO [1, 3, 4]. To date, it is still not clear why some children develop associated pathologies while others do not. Birth weight and postnatal weight gain seem to be important contributors. A low birth weight and intrauterine growth retardation is associated with insulin resistance, visceral obesity, metabolic syndrome, and cardiovascular disease in adulthood [1, 5–7].Vice versa, high birth weight together with early weight gain were identified as positive predictors for later insulin sensitivity [1, 8]. From this, one might conclude that insulin sensitivity might be programmed and contribute to a phenotype of MHO. Furthermore, waist circumference, dietary fat intake, and moderate-to-vigorous physical activity can serve as predictors for MHO [3]. In any case, different subtypes of obesity might develop through different pathophysiological processes. In this respect, there is a search for clinically relevant markers with prognostic value related to disease development and in-
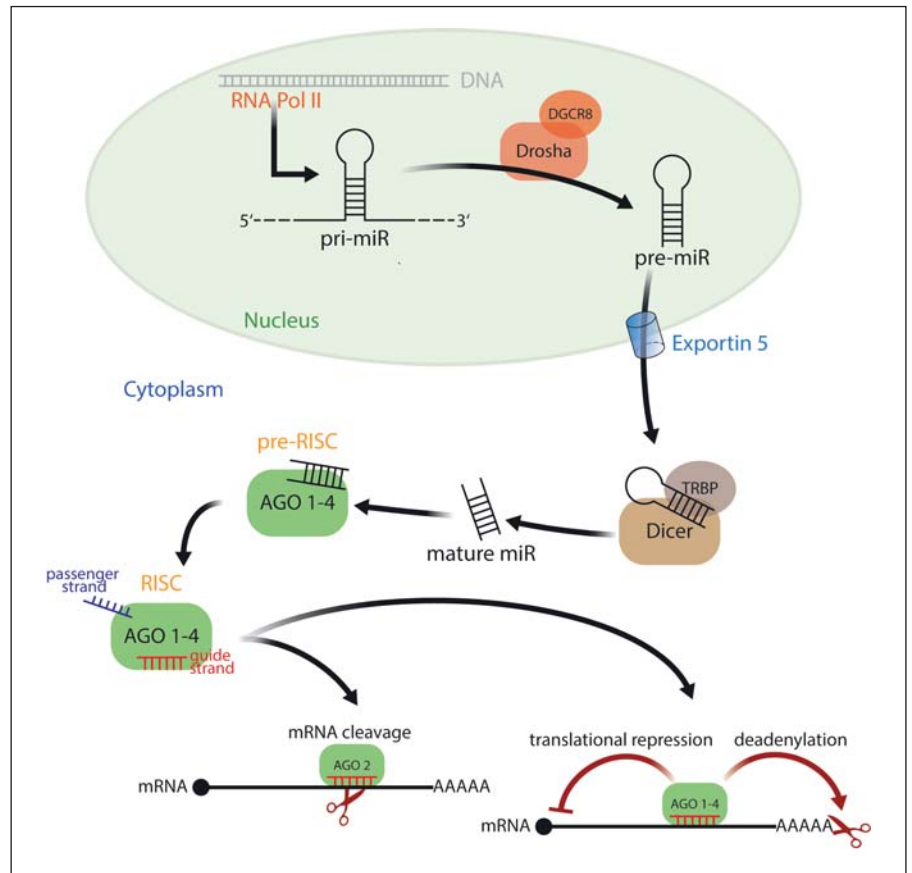
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Fig. 1. Overview of miRNA biogenesis and function.

tervention success. Recently, microRNAs (miRNA) have been established as biomarkers for several disease states [9] and have been repeatedly studied in the context of metabolic disease [10].

Obesity and insulin resistance are complex conditions. Thus, it is advantageous and reasonable to examine potential causal and/or prognostic new factors early in life when environmental influences are less pronounced and significant associations might represent causal relationships. There is even the possibility to longitudinally examine such associations over several years when young individuals are recruited in order to strengthen these associations and to establish a prognostic value. Therefore, studies on the functional significance and predictive value of miRNAs in pediatric obesity currently increase in numbers. Herein, we give a brief overview of miRNA biogenesis and function and the potential role of circulating miRNA in the context of pediatric obesity on the basis of recently published research data.

miRNA Biogenesis and Function

miRNA are short, approximately 19–24 nucleotide-long, non-coding ribonucleic acids. They were first discovered in 1993 by Ambros and co-workers [11]. Studying *Caenorhabditis elegans* development, they described a gene *(lin-4)* encoding for small RNAs, which are not translated into a protein but regulate the expression of another protein-encoding gene via an antisense RNA-RNA interaction. It took another 7 years until the second miRNA – let-7 – was also found in *C. elegans* [12]. Soon after these two seminal reports, the existence of similar small RNA was reported in various species, and they were collectively termed 'microRNA' [13–15].

In humans, most miRNAs are encoded by intronic regions [16]. miRNA loci may localize in close proximity to each other and form polycistronic transcription units [16]. miRNAs are mainly transcribed by RNA polymerase II [17]. The first transcript comprises the primary miRNA (pri-miRNA), which is typically over 1 kb long and contains a local stem-loop structure (Fig. 1). The canonical

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miRNA maturation process is realized in a protein complex called Microprocessor, which consists of the nuclear RNase III Drosha and its cofactor DGCR8. In this complex, Drosha cuts of the stem-loop releasing an approximately 65 nucleotide-long, small hairpin-shaped RNA, the pre-miRNA [18]. In a noncanonical pathway, pri-miRNAs are processed in a spliceosome-dependent mechanism [16, 19]. Pre-miRNAs are then transported from the nucleus to the cytoplasm via a protein called exportin-5 [16, 20]. In the cytosol, the pre-miRNA forms a complex with Dicer and transactivation-response RNA-binding protein. Within this complex, the terminal loop is cropped yielding the mature double-stranded, approximately 22 nucleotide-long miRNA [16, 21]. The duplex is then loaded onto particular types of AGO proteins (AGO 1–4) to form an effector complex called RNA-induced silencing complex (RISC) in a two-step process. The pre-RISC is formed by loading of the miRNA to AGO proteins, which then by removal of the passenger RNA strand generates the mature RISC that harbors the guide strand (for review see [16]). This assembly is then guided to specific target sequences in mRNAs and induces translational repression, mRNA deadenylation, and mRNA cleavage. The initial recognition between miRNA and mRNA is mediated by Watson-Crick base-pairing by the nucleotides 2–8 in the mature miRNA (seed sequence) with mRNA target sequences, which are mainly located in the 3′ untranslated region. Computational and experimental approaches indicate that a single miRNA may have several, even hundreds of possible mRNA targets [22]; affinity and targeting efficiency is regulated by additional base pairing, e.g., by nucleotide 8 or nucleotides 13–16 of the miRNA [16]. In addition, more than 60% of the human protein-coding genes are predicted to contain miRNA binding sites within their 3′ untranslated region [23]. These features, coupled with their conservation in eukaryotic organisms, suggest that miRNA possess a vital and evolutionarily ancient role in gene regulation [24].

Circulating miRNA

The majority of miRNA are found inside the cells [9]. A significant number though has also been detected without a cellular context, for example in body fluids such as plasma, serum, urine, saliva, or breast milk [9]. Surprisingly, miRNA seem to be quite stable, suggesting that they are somehow packaged and therefore protected from ribonucleases. RNA-binding proteins have been proposed to shield the small RNA molecules from degradation [9]. Specifically, miRNAs are often found outside the cell in complexes with AGO proteins [25, 26]. Another concept is that miRNAs are encapsulated into membrane vesicles. Indeed, they have been identified in both microvesicles (approx. 100–1,000 nm) and exosomes (approx. 30–100 nm) but also in apoptotic bodies [26]. Some studies have detected miRNA complexes with circulating high-density lipoprotein and to a lesser extent also with low-density lipoprotein [26–28].

The function of extracellular miRNAs is still a matter of debate. The fact that exosomes or apoptotic bodies were found to be involved in transferring genetic information from one cell to another [29] together with the identification of miRNAs in those particles led to the idea that miRNAs might be involved in cell-cell or perhaps even inter-organ communication [26]. A role of miRNA in paracrine or endocrine communication processes would first of all require a regulated process of secretion. The mechanisms of miRNA export are only poorly understood. The finding that miRNAs and AGO proteins co-localize in cellular compartments that are linked to endosomes and microvesicles suggests that they are subject to a sorting procedure [26]. A ceramide-dependent secretory pathway seems to be involved in the release of exosomal miRNAs (for review see [26]). In support of this theory, the modulation of the activity of the enzyme controlling the ceramide biosynthesis, the neutral sphingomyelinase, led to altered levels of extracellular miRNAs [30]. Other reports, however, rather indicate that miRNAs are nonspecific remnants from cells occurring after cell death or upon cellular damage [26, 31].

Some studies have demonstrated that exosomal miRNAs are taken up by recipient cells and are able to exert specific functions (reviewed in [26]). A short-distance, paracrine mode of cell-cell communication appears conceivable because local concentrations of miRNAs should be sufficiently high to ensure delivery from a donor cell to an acceptor cell. However, long-distance, endocrine-like functions of miRNAs seem not feasible because the levels of circulating miRNAs are very low [32]. Steroid hormones such as estrogen or testosterone are present in the nanomolar range, and even hormones with very low concentrations such as adrenocorticotropic hormone or parathyroid hormone show circulating concentrations in the picomolar range. Far from that, deep sequencing analyses showed that the concentration of total miRNAs in human plasma lies within the 100 femtomolar range, the single miRNA being present at a fraction [32]. miRNAs exert their function on mRNA targets on a 1:1 basis, and approximately 1,000 copies of a miRNA are required to exert a measurable activity [32]. Hormonal signals, however, are amplified upon receptor-binding and

signal transduction processes. Therefore, a hormone-like action appears not plausible unless a so far undescribed, highly sensitive miRNA receptor exists [26, 32].

miRNAs as Biomarkers

Although the in vivo function of circulating miRNAs is still a matter of debate, they are currently studied as biomarkers in the context of many diseases.

A good biomarker fulfills several important criteria (summarized in [9]). The marker should be specific for the affected organ or tissue and suitable to differentiate between pathologies. The marker should be sensitive, i.e., rapidly released and significantly altered upon the development of pathology, and it should proportionally reflect the severity of the pathology. Ideally, it has a long half-life in the clinical sample, can be detected with a rapid, simple and inexpensive method, and is not confounded by the environment or other unrelated conditions. Furthermore, it should be translatable to help building the bridge between preclinical and clinical results. Finally, and most importantly, it should be easily accessible with a noninvasive method. miRNAs fulfill many of those criteria [9]. They are stably present in body fluids and can easily be measured by polymerase chain reaction. The sequence of most miRNAs is conserved across species, and some miRNAs are expressed in a tissue-specific manner. As such, it is not surprising that they are increasingly studied as biomarkers in the context of many diseases, for example cancer [33], Alzheimer's disease [34], and cardiovascular diseases [35], just to name a few of them, and recently also in the context of obesity.

miRNAs in Obesity

One hallmark of obesity is the excessive accumulation of white adipose tissue (WAT). For decades considered a passive storage organ only, WAT is nowadays well recognized as an important endocrine organ [36–38]. It secretes several hundreds of different factors collectively called adipokines, including classical hormones such as leptin, growth factors such as insulin-like growth factor-1 or platelet-derived growth factor, inflammatory mediators such as interleukins (e.g. IL-6 or IL-8) or tumor necrosis factor-alpha, but also metabolites such as fatty acids [36–38]. By these collectively called adipokines, WAT is in permanent crosstalk with other organ systems in the body and signals the filling state and storing capacity of the energy pool. Upon obesity, WAT undergoes pathological alterations. Both hyperplastic (increase in number) and hypertrophic (increase in volume) growth of adipocytes can be observed with diameters exceeding the

maximal diffusion rate of oxygen [39]. It is supposed that local hypoxia, cell death, and infiltration of macrophages occur as a consequence and lead to an altered adipokine secretion profile with an upregulation of inflammatory factors, which contribute to the chronic low-grade inflammation observed in obesity [39].

miRNAs are involved in many different aspects of adipose tissue biology. A study comparing the miRNA expression pattern in subcutaneous and visceral WAT identified a total of 106 miRNA species [40]. None of those was exclusively expressed in either fat depot, but sixteen displayed a significant depot-specific expression pattern. miR-17–5p, miR-132, miR-99a, miR-134, miR-181a, miR-145, and miR-197 showed a significant correlation with adipose tissue morphology and metabolic parameters. Among them were fasting plasma glucose, HbA(1c), and circulating adiponectin levels. A study performed by Ortega et al. [41] identified 50 miRNAs differentially regulated in adipocytes obtained from subcutaneous WAT of lean versus obese subjects. Seventy miRNAs were differentially regulated between preadipocytes and mature adipocytes, suggesting that they play a role in adipogenesis. In the meantime indeed, several miRNA species were identified as regulators of adipogenic differentiation. For example, miR-130 inhibits adipogenic differentiation by suppressing the expression of PPARγ, the master regulator of adipogenesis [42]. Likewise, miR-27b also targets PPARγ and impairs adipogenesis [43]. An overview of miRNAs involved in adipogenic differentiation was provided by Peng et al. [44].

miRNAs are also important for brown adipose tissue biology and the development of brown or beige adipocytes [45]. Finally, miRNAs have been identified as mediators of the inflammatory process in WAT. Incubation with macrophage-conditioned media leads to an altered miRNA expression profile in human adipocytes [46]. Interestingly, miRNA species, which are generally involved in inflammatory processes, showed up here and they were not only detected within cells but also in media supernatants [46], strongly supporting a paracrine cross-talk of immune cells and adipocytes in vivo.

Circulating miRNA in Obesity

Circulating miRNAs in obesity is still a young research area. While a PubMed search performed on the terms 'circulating microRNA AND cancer' retrieved >780 results, a search on 'circulating microRNA AND obesity' gave only 27 hits. Table 1 summarizes the current knowl-

Table 1. Summary of the current knowledge on circulating miRNA in the context of human obesity

Year	Studied miRNA	Results	Study population	First author [ref.]
2015	miR-335, miR-143, miR-758, miR-27, miR-370, miR-378	low in obese patients high in obese patients	45 obese vs. 41 lean children	Can [50]
2015	miR-130b miR-221	low in obese patients with HF high in obese patients with HF miR-221/-130b ratio increased in obese HF and associated with body fat	40 patients with HF (20 obese, 20 lean) vs. 17 healthy, lean subjects	Thomé [58]
2015	miR-223	miR-223 lower in overweight and obese patients miR-223 increased upon lifestyle intervention	41 normal weight 40 overweight 40 obese subjects	Wen [59]
2015	miR-122	miR-122 was associated with obesity and insulin resistance	112 obese and control subjects	Wang [60]
2013	miR-138 miR-15b miR-376a	miR-138, miR-15b, and miR-376a have potential as predictive biomarkers in obesity	13 patients with T2DM 20 obese subjects 16 obese patients with T2DM 20 healthy controls	Pescador [61]
2013	miR-21, miR-27, miR-103	low in obesity miR-21/27b/103/155 reduced in obesity in males and females but tend to increase in PCOS	12 female controls 12 male controls 12 patients with PCOS (each group 50% lean and 50% obese)	Murri [62]
2013	miR-221, miR-28-3p miR-586-3p/5p, miR-142-3p, miR-130, miR-423-5p	low in obesity high in obesity associations with BMI, fat distribution/HOMA-IR	5 lean/5 obese boys 85 lean vs. 40 obese children	Prats-Puig [48]
2013	miR-130b	miR-130b reflects degree of obesity	normal, overweight and obese groups (only men)	Wang [49]
2013	miR-140-5p, miR-142-3p, miR-222 miR-532-5p, miR-125b, miR-130b, miR-221, miR-15a, miR-423-5p	high in obesity low in obesity	6 male patients after gastric surgery 80 male subjects cross-sectional, 22 patients longitudinally	Ortega [46]

HF = Heart failure; PCOS = polycystic ovary syndrome; HOMA-IR = homeostatic model assessment of insulin resistance; T2DM = type 2 diabetes mellitus.

edge on circulating miRNA in the context of human obesity.

In 2013, the first study investigating circulating miRNAs in the context of obesity was published by Ortega et al. [47]. They performed miRNA profiling using a Taqman miRNA array in plasma samples from a cohort of 32 male subjects with a BMI ranging from 20 to 60 kg/m^2. All in all, they detected 108 miRNA species in the circulation. Including an extended cohort of 80 subjects, they identified 18 miRNAs that were different between obese, morbidly obese, and control subjects. They report increased levels of miR-142–3p and miR-140–5p in obese and morbidly obese subjects compared to lean and overweight subjects. miR-222 was significantly higher in morbidly obese compared to lean, overweight, and obese subjects. Furthermore, they detected decreased circulating concentrations of miR-221, miR-15a, miR-520c-3p, miR-423–5p, and miR-130b in morbidly obese subjects. Most interestingly, however, the study revealed that miRNAs are differentially regulated by weight loss induced by gastric surgery. miR-140–5p, miR-142–3p, miR-16–1, and miR-122 were decreased, whereas miR-221 and miR-130b were upregulated by weight loss. However, those changes were not observed in diet-induced weight loss, a finding possibly explained by the fact that weight loss was less than in the surgery group. This seminal study first demonstrated that morbid obesity may be associated with a distinct miRNA pattern in the circulation.

Soon thereafter, the first study on circulating miRNAs in the context of pediatric obesity was published by the same group [48]. Also performing Taqman array analysis, Prats-Puig et al. [48] profiled the circulating miRNAs in prepubertal children. Corroborating the findings from the adult study, miR-221 was decreased upon obesity and miR-142–3p was increased. Surprisingly, miR-130b was higher in obese compared to lean children, which is in contrast to earlier findings of the group in adults [47]. An independent study found miR-130b increased in the circulation of obese Chinese subjects and also in mouse models of obesity [49]. Of note, miR-130b was among those miRNAs, which were regulated in a longitudinal expression analysis. In children whose BMI remained stable or decreased over 3 years, decreased levels of miR-130b were identified [48]. This clearly demonstrates that age, growth and presumably also pubertal status should be taken into account when analyzing circulating miRNA levels in children.

In a recent study, Can et al. [50] studied circulating miRNAs in lean and obese children using qPCR for quantification. They found that miR-335, miR-143, and miR-758 were lower, and miR-27, miR-378, and miR-370 were higher in obese children compared to lean controls. These alterations were associated with elevated triglycerides and low-density lipoprotein levels and the low level of high-density lipoprotein in obese subjects [50].

Notably, the so far performed studies on circulating miRNAs in the context of obesity revealed statistically significant differences between lean and obese patients, but the biological significance of those findings is not clear yet. Specific miRNA signatures are proposed; however, there is no overlap in either single miRNA or patterns of miRNAs in studies published so far. This lack of reproducibility may be explained by the use of different methods, different platforms and products used from different vendors [51, 52]. In any case, further studies are required to understand the pathophysiological relevance of circulating miRNAs in obesity.

Future Perspectives

Although there are still many issues and drawbacks related to miRNA measurement [9], miRNAs are on everybody's lips as promising biomarkers. They have indeed proven very valuable not only in terms of diagnosis or prognosis, but also as therapeutic tools.

A prominent example is miR-34, which is among the first miRNAs that entered phase I clinical studies [53]. miR-34 family members, mainly miR-34a, act as tumor suppressors in several cancers [54, 55] and are direct targets of p53 [56]. In mice, systemic delivery of miR-34 mimic led to reduced tumor burden and prolonged survival [57]. Since April 2013, MRX34, a double-stranded miRNA mimic of miR-34, has been investigated in an open-label, multicenter, dose-escalation study to examine pharmacokinetics, pharmacodynamics, and safety in patients with unresectable primary liver cancer or advanced or metastatic cancer with or without liver involvement or hematologic malignancies (https://clinicaltrials.gov/ct2/show/NCT01829971). MRX34 is encapsulated in liposomal nanoparticles and is administered daily by intravenous injection for 5 consecutive days with 2 weeks off.

The example of miR-34 demonstrates how fast research results can be translated from bench to bedside and how it can encourage further miRNA studies in the field of obesity and metabolic disease.

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