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Review

miR-223: An inflammatory oncomiR enters the cardiovascular field

Fatiha Taïbi [a], Valérie Metzinger-Le Meuth [a,b], Ziad A. Massy [a,c], Laurent Metzinger [a,d,*]

[a] INSERM U1088, University of Picardie Jules Verne, 1 Rue des Louvels, F-80037 Amiens, France
[b] Université Paris 13, UFR SMBH, 74 rue Marcel Cachin, F-93017 Bobigny, France
[c] Division of Nephrology, Ambroise Paré Hospital, Paris UVSQ University, 09 avenue Charles de Gaulle, 92100 Boulogne-Billancourt, France
[d] Centre De Biologie Humaine (CBH), Amiens University Hospital, Avenue René Laennec, F-80054 Amiens, France

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ABSTRACT

MicroRNAs (miRNAs) are small, noncoding RNAs of 18–22 nucleotides in length that regulate post-transcriptional expression by base-pairing with target mRNAs. It is now clearly established that miRNAs are involved in most of the cell's physiopathological processes (including carcinogenesis and metabolic disorders). This review focuses on miR-223, which was first described as a modulator of hematopoietic lineage differentiation. We outline the role of miR-223 deregulation in several types of cancers and highlight its inclusion in a newly identified and fast-growing family of miRNAs called oncomiRs. We then look at miR-223's emerging role in inflammatory and metabolic disorders, with a particular focus on muscle diseases, type II diabetes, atherosclerosis and vascular calcification. miR-223 is one of the growing number of RNA biomarkers of various human metabolic diseases and is thus of special interest to both researchers and clinicians in the cardiovascular field.

1. Introduction

MicroRNAs (miRNAs) are small, non-coding, regulatory RNAs that range from 18 to 22 nucleotides (nt) in length. They are mainly encoded by gene introns but are also sometimes encoded by dedicated genes. The miRNAs regulate the expression of specific target proteins by either inhibiting translation or degrading the corresponding mRNA. It has been estimated that miRNAs regulate between one and two thirds of the human genome [1] and are involved in most of the cell's main functions (including growth, proliferation, differentiation, signal transduction, apoptosis, metabolism and aging) [2]. At present, the majority of researchers consider that miRNAs act predominantly by inhibiting the translation of their target mRNAs, rather than by inducing degradation. However, Bartel's group has shown that mammalian miRNAs degrade their target mRNAs in most cases and thus decrease protein levels [8]. Regardless of the exact mechanism, miRNAs are posttranscriptional regulators that bind to their target mRNAs (mostly to the 3′ untranslated region (UTR) but sometimes within the coding region or the 5′ UTR) [1–3].

Many studies have demonstrated that miRNA expression is correlated with disease (first in cancer [4] and then in cardiovascular disease [5]). The miRNA miR-223 has a key role in the development and homeostasis of the immune system. To date, miR-223's involvement has been demonstrated for many types of cancers, inflammatory diseases, autoimmune diseases and other pathological processes. The present article reviews our current knowledge of the physiopathology of miR-223 microRNA (starting with the pioneering work in cancer and inflammation) and then focuses on this miRNA's emerging role in the cardiovascular field.

2. miR-223 biological functions

2.1. Location of the gene encoding miR-223 and regulation of its promoter

The fact that the sequence of miR-223 has been remarkably conserved during evolution suggests that this mRNA has an important

Abbreviations: AAA, Abdominal Aortic Aneurysm; AML, Acute Myeloid Leukemia; Apo-E KO, Apolipoprotein-E Knock-Out; C/EBP, CCAAT-Enhancer-Binding Proteins; CKD, Chronic Kidney Disease; D-HF, Diabetic Heart-Failure; DMD, Duchenne Muscular Dystrophy; E2F1, E2F transcription factor 1; EGF, Epidermal Growth Factor; EGFR2, Epidermal Growth Factor Receptor 2; EPB41L3, Erythrocyte Membrane Protein Band 4.1-like 3; FBW7, F-box and WD repeat domain-containing 7; GATA-1, GATA-binding factor 1; GLUT-4, Glucose Transporter Type 4; HCC, Hepatocellular Carcinoma; IGF-1R, Insulin-like Growth factor Receptor; IKK-α, IκB kinase subunit alpha; IL-4, Interleukin-4; KRAS, V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; LMO2, LIM domain only 2; MCSF, Macrophage Colony Stimulating Factor; Mef2c, Myocyte enhancer factor 2c; miRNA, MicroRNA; MMP9, Metallopeptidase 9; MYO, Myocardin; ND-HF, Non-Diabetic Heart-Failure; NFI-A, Nuclear Factor I-A; NF-κB, Nuclear Factor-kappa B; NLRP3, Nucleotide-binding oligomerization domain-like Receptor Protein 3; PI3K, Phosphoinositide 3-Kinase; PU.1, Purine-rich nucleic acid binding protein.1; RANKL, Receptor Activator of Nuclear factor kappa-B Ligand; SDF-1, Stromal Cell-Derived Factor-1; SR-BI, Scavenger Receptor class B type I; STMN1, Stathmin 1/oncoprotein 18; TNF-α, Tumor Necrosis Factor-α; VSMC, Vascular Smooth Muscle Cell; WT, Wild-Type

* Corresponding author at: INSERM U1088, Mécanismes physiopathologiques et conséquences des calcifications cardiovasculaires (MP3C), University of Picardie Jules Verne, 1 Rue des Louvels, F-80037 Amiens, France. Tel.: +33 22 82 77 91; fax: +33 22 82 54 25.
E-mail address: laurent.metzinger@u-picardie.fr (L. Metzinger).

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role in physiological processes (Fig. 1A). The gene encoding miR-223 is located within the q12 locus of the X chromosome (Fig. 1B) [6]. It was discovered *in silico* [7] and then detected in a hematopoietic system [8]. Expression of the miR-223 is regulated by several transcription factors, including transcription factor PU.1, CCAAT-enhancer-binding proteins (C/EBP)-α and -ß and nuclear factor I-A (NFI-A) (Fig. 1C). More precisely, PU.1 is involved in osteoclast differentiation and binds to two separate sites within the miR-223 promoter region. Expression of PU.1 is induced by macrophage colony stimulating factor (MCSF) and the receptor activator of nuclear factor kappa-B ligand (RANKL). It has been shown that PU.1 binds to the miR-223 promoter to activate expression of the miRNA, which in turn induces osteoclastogenesis by targeting osteoclast-specific target mRNAs [8,9]. Similarly, C/EBP-α binds to the miR-223 promoter, which increases miR-223 expression and, as a result, promotes granulocyte differentiation [10]. In contrast, NFI-A binds to the promoter of the *miR-223* gene, inhibits its expression and thus decreases granulocyte and osteoclast differentiation [8–10]. It is noteworthy that miR-223 also targets NFI-A — thus producing a negative feedback loop between the target gene and its corresponding miRNA [10]. During this regulation, miR-223 is translocated into the nucleus, where it binds to and silences the NFI-A promoter. As with miR-223, the (complimentary) sequence of the NFI-A promoter has been highly conserved during evolution (Fig. 2) [11]. The same researchers demonstrated that miR-223 is also able to suppress expression of the NFI-A gene *via* heterochromatin repression. The biogenesis of miR-223, with its regulation by NFI-A, is comprehensibly described in Fig. 2.

A pioneering study demonstrated the existence of semi-microRNAs; these are 12 nt in length and correspond to the 5′ or 3′ half of the mature miRNA. The semi-miRNAs are produced during miRNA maturation [12] and are devoid of any direct regulation effects on the corresponding miRNA's target mRNA. The semi-miRNA derived from miR-223 (smiR-223) nevertheless modulates miR-223's ability to repress translation or degradation of target transcripts and thus controls indirectly gene expression [12].

2.2. Physiological function

The first important role of miR-223 was discovered in the field of hematology, since it was shown to modulate the differentiation of hematopoietic lineages [7]. This function takes place in the hematopoietic bone marrow and affects hematopoietic stem cells and myeloid, erythroid and lymphoid cells at various stages of their development [7]. In the bone marrow, miR-223 expression is mainly confined to myeloid cells and is induced during the lineage differentiation of myeloid progenitor cells. These myeloid cells will differentiate in monocyte/macrophage and granulocyte cells. Expression of miR-223 is highly lineage-specific, since it is repressed when granulocyte–monocyte progenitors start to differentiate into monocytes. Conversely, miR-223 is highly expressed when granulocyte–monocyte progenitors enter the granulocyte differentiation phase [7]. In contrast, the same researchers developed miR-223 −/− mice and showed that miR-223 is not strictly essential for granulocyte differentiation but was required for normal maturation of granulocytes and regulation of the granulocyte compartment size. The researchers observed marked neutrophilia and hyperplasia of the bone marrow granulocyte compartment in these miR-223 −/− mice. Another study demonstrated that miR-223 −/− mice suffer from lung inflammatory damage and display tissue destruction after endotoxin treatment [13].

miR-223 has also a role in monocyte/macrophage differentiation, since it targets and represses IκB kinase subunit alpha (IKK-α), a component of nuclear factor-kappa B (NF-κB) pathway. During macrophage differentiation, a fall in miR-223 expression induces an increase of IKK-α expression which induces the expression of p52 followed by the repression of NF-κB pathways [13,14] (Table 1). miR-223-rich microvesicles induce the differentiation of recipient monocytes, activate hematopoietic cell production in the bone marrow and then induce the release of more microvesicles [15]. Aucher et al. [16] have demonstrated that miR-223 transfer from human macrophages to hepato-carcinoma cells and inhibit their proliferation. In this elegant paper, they demonstrated that miR-223 expression cells increased in HuH7 transformed hepatic cells after co-incubation with macrophages. However, this increase was prevented by chemical fixation of macrophages. In this experience, surface proteins still interacted with other cells but dynamic processes, such as secretion, were inhibited. Using several inhibitors of gap junction activity, they further demonstrated that endogenous macrophages transfer miRNA to HuH7 cells in a process dependent on cell–cell contacts and gap junction. Yuan et al. [17] studied the erythroid–megakaryocyte cell line K562 and found that miR-223 was downregulated during erythroid

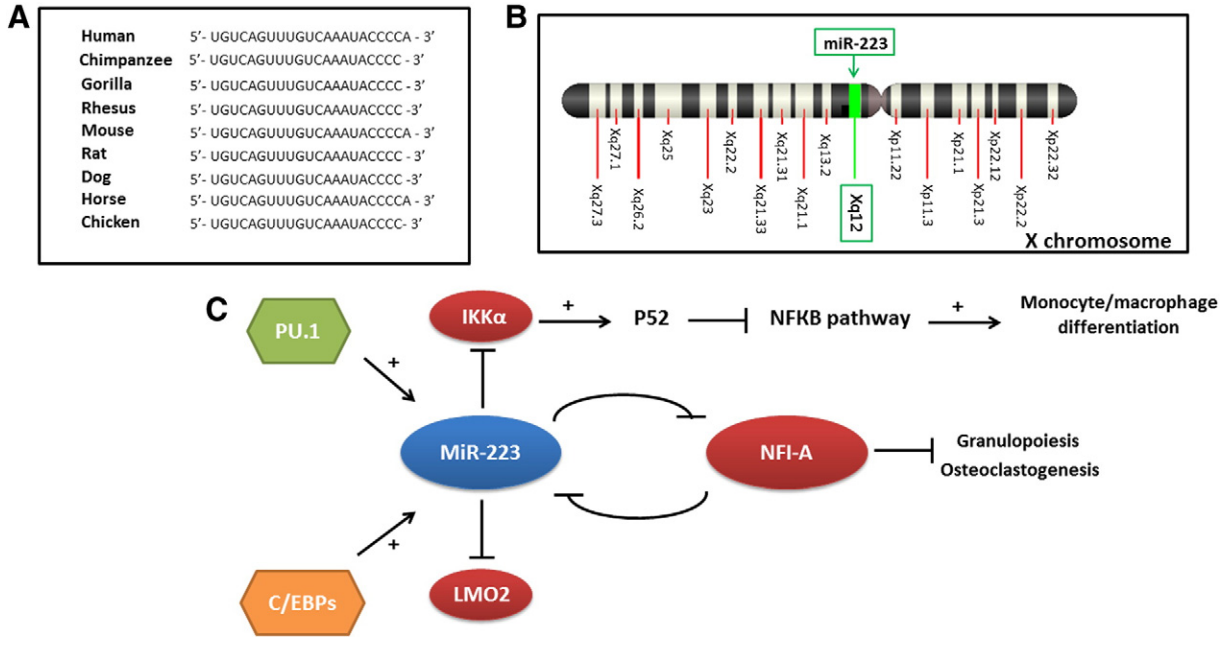

Fig. 1. A. Sequence alignment of the mature miR-223, showing its remarkable conservation during the evolution. B. The genomic location of miR-223 on the X chromosome. C. A pictorial representation of the transcription factor network that regulates the expression of miR-223. Relevance in granulopoiesis, monocyte/macrophage differentiation and osteoclastogenesis.

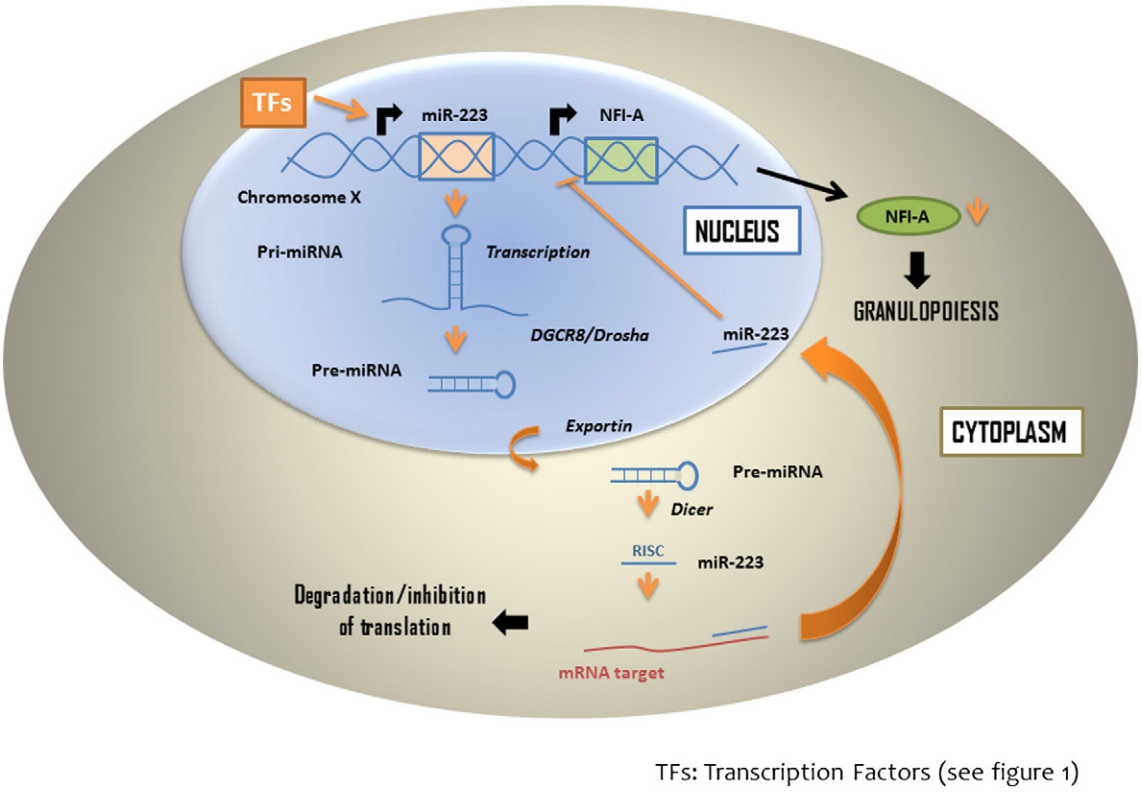

Fig. 2. Schematic representation of miR-223 biogenesis and regulation. Various transcription factors (such as PU.1 and NFIA) bind the *miR-223* promoter and activate transcription of the gene encoding miR-223. MiR-223 is transcribed as a single pri-miRNA. The Drosha and DiGeorge Syndrome Critical Region 8 (DGCR8) complex processes the pri-miRNA into a hairpin-structured pre-miRNA. The pre-miRNA corresponding to miR-223 is exported by a nucleocytoplasmic shuttle protein (exportin) from the nucleus to the cytoplasm and is then cleaved by the Dicer complex into a miRNA duplex. Lastly, the miRNA duplex is unwound to obtain the mature miR-223, which is incorporated into the RNA-induced silencing complex (RISC) and binds various mRNA targets in their 3′ UTR. miR-223 targets the NFIA transcript in the cytoplasm but also acts in the nucleus by inhibiting the production of NFIA, which in turn decreases the production of miR-223 in a feedback loop.

differentiation and upregulated during megakaryocyte differentiation. In fact, miR-223 targets and regulates the expression of the LIM domain only 2 (rhombotin-like 1) (LMO2) transcription factor, a bridging molecule that serves to assemble an erythroid DNA-binding complex including GATA-binding factor 1 (GATA-1) [17] (Table 1). During erythroid differentiation, LMO2 levels increase as miR-223 levels decrease, which promotes the differentiation of K562 cells into erythrocytes.

miR-223 also has an important role in osteoclast formation and the regulation of bone remodeling. Indeed, the miRNA is expressed in osteoclast precursors (RAW 264.7 cells); both under- and over-expression of miR-223 expression diminish the osteoclast-like cell formation induced by RANKL [9]. This indicates that the expression of miR-223 must be fine-tuned for normal osteoclastogenesis. NFI-A is involved in this process too, since it suppresses osteoclastogenesis. In fact, NFI-A inhibits the expression of the MCSF receptor, which in turn is essential for osteoclast differentiation, function and survival [10,18,9] (Table 1).

miR-223 was recently demonstrated to regulate human embryonic stem cell (hESC) differentiation by targeting the IGF-1R/Akt signaling pathway [19]. The inhibition of miR-223 expression maintained hESCs in the undifferentiated state, while addition of exogenous miR-223 induced their differentiation. These effects were dependent of the IGF-1R/Akt pathway, since IGF-1R mRNA was demonstrated to be a target of miR-223. The level of phosphorylated Akt, a downstream kinase of the IGF-1R signaling pathway was higher in cells where miR-223 was depleted than in those expressing exogenous levels of miR-223 (Table 1).

Table 1
Targets of miR-223 known to be involved in physiopathological processes.

miR-223			
Function	Target(s)	Clinical relevance	References
Osteoclastogenesis	NF-IA	Vascular calcification?	[8–10]
Granulopoiesis	IKKα	?	[13,14]
	NF-IA	?	[8–10]
	E2F1	Leukemia	[21]
Erythropoiesis	LMO2		[17]
Cell invasiveness	MEF2C	Breast cancer	[22]
Tumor suppressor	EPB4IL3	Gastric cancer	[24]
Tumorigenesis	STMN1, FBW7	Hepatocellular carcinoma, gastric cancer	[25,26]
	KRAS,EGF,EGFR2, MMP9, SEPTIN6	Ovarian cancer	[28]
Inflammation	NLRP3	IL-1ß production?	[29]
	Pknox1	Anti-inflammatory response	[30]
Glucose uptake	GLUT-4	Diabetes, heart failure	[37]
VSMC proliferation	IGF-1R	Vascular stretch stress (essential hypertension?)	[41]
VSMC contractile phenotype	Rho B, MEF2C	Vascular calcification	[43]

To briefly recap, miR-223 is located within the X chromosome and regulated during hematopoiesis by a number of transcription factors including PU.1, C/EBP-α and -ß, and NFI-A. miR-223 modulates the differentiation of the hematopoietic lineage and is confined in myeloid cells. It is repressed when granulocyte–monocyte progenitors start to differentiate into monocytes and is highly expressed when granulocyte–monocyte progenitors enter the granulocyte differentiation phase. Its expression is also required for megakaryocyte differentiation. In addition to the hematopoietic differentiation, miR-223 also regulates osteoclastogenesis, and the differentiation of human embryonic stem cells.

3. miR-223 is involved in the carcinogenesis process

miR-223 expression is deregulated in many types of cancer; miR-223 is thus a member of an emerging family of miRNAs called oncomiRs.

3.1. Leukemia

miR-223 is poorly expressed in acute myeloid leukemia [20] (AML) and several other types of leukemia. Eyholzer et al. found a low level of miR-223 in AML and demonstrated that this could not be attributed to mutation and/or hypermethylation of miR-223 gene regulatory elements (*i.e.* the NFI-A, CEBP and PU.1 binding sites; Table 1). The researchers concluded that miR-223 suppression in AML patients was probably due to the deregulation of transcription factors and/or miRNAs upstream of miR-223. Another study has demonstrated that miR-223 targets E2F1, a regulator of the cell cycle during granulopoiesis [21]. However, miR-223 is downregulated in several subtypes of leukemia, which in turn induces an increase in E2F1 and thus the risk of carcinogenesis. Furthermore, the same researchers demonstrated that E2F1 binds to the miR-223 promoter in AML cells and thus worsens the phenomenon by inhibiting transcription of miR-223.

3.2. Breast cancer

miR-223 was found to promote breast cancer invasiveness [22]. Indeed, tumor-associated macrophages induced by interleukin-4 (IL-4) promote breast cancer invasion and metastasis through macrophage-secreted exosomes that deliver miR-223 to breast tumor cells. The researchers also showed that the invading power of the co-cultivated breast cancer cells faded when miR-223 expression in IL-4-activated macrophages was decreased by treatment with miR-223 antisense oligonucleotide. Myocyte enhancer factor 2c (Mef2c, a target of miR-223) is instrumental in promoting breast cancer invasiveness through the Mef2c–ß-catenin pathway (Table 1). Furthermore, Mef2c targeting by miR-223 has been shown to inhibit proliferation and granulocyte function in myeloid progenitor cells [7].

3.3. Gastric cancer

Li et al. [23] examined miRNAs in several human gastric cell lines and showed that miR-223 is specifically overexpressed in metastatic gastric cells, stimulating migration and invasion. Indeed, Li et al. demonstrated that expression of miR-223 (induced here by the transcription factor Twist) downregulates that of erythrocyte membrane protein band 4.1-like 3 (EPB41L3) [24] by directly targeting its 3′-UTR. Interestingly, EPB41L3 is believed to link membrane receptors to the cytoskeleton and display tumor-suppressive capabilities and its expression is progressively lost in gastric cancers. Another study [25] demonstrated that the oncogenic cytosolic microtubule-destabilizing protein stathmin1/oncoprotein 18 (STMN1) is targeted by miR-223 in gastric cancer. In both gastric cancer cell lines and primary gastric adenocarcinoma cells, upregulation of STMN1 promotes cell proliferation, invasion and migration (Table 1). The researchers also showed that miR-223 was downregulated in several cancer gastric cell lines, so miR-223 specifically targets STMN1 expression. miR-223 expression is also upregulated in gastric cancer tissue samples [26]. This study clearly showed that levels of miR-223 and a combination of six other miRNAs formed a powerful biosignature for the prediction of overall survival and disease-free survival in gastric cancer patients. Furthermore, transfection of miR-223 into the SGC7901 gastric cancer cell line induced a reduction in apoptosis and a concomitant increase in *in vitro* proliferation and invasion. Similar results were found in tumorigenesis assays performed in nude mice. A molecular explanation was found when miR-223 was shown to target FBW7, which has a role in the ubiquitin-dependent proteolysis of several oncoproteins [26].

3.4. Hepatocellular carcinoma

Expression of miR-223 is repressed in hepatocellular carcinoma (HCC) in chronic carriers of hepatitis B virus and hepatitis C virus and in nonviral-associated patients [27]. Conversely, the transfection of either precursor or mature miR-223 into the corresponding HCC cell lines reduced the cancer cells' viability. Similarly, STMN1 was found to be over-expressed in HCC. A strong inverse correlation between STMN1 mRNA expression and miR-223 levels was demonstrated. STMN1 has a functional role in HCC cell line Hep3B, in which STMN1 knockdown by siRNA resulted in a 30% decrease in cell viability (Table 1). The researchers concluded that miR-223 has a role in HCC through STMN1.

3.5. Ovarian cancer

miR-223 is strongly expressed in ovarian cancer and is the most upregulated miRNA in recurrent tumors (relative to primary tumors) [28] (Table 1). Depending on the context, miR-223 acts as either an oncogene or a tumor suppressor gene; it targets V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS), Epidermal Growth Factor (EGF) and its receptor Epidermal Growth Factor Receptor 2 (EGFR2) (which have well characterized roles in ovarian cancer) and also Septin 6 and matrix metallopeptidase 9 (MMP9) (which were found to be deregulated in a transcriptome study of primary and recurrent ovarian cancer samples from several different patients).

miR-223 has thus an instrumental role during carcinogenesis in numerous tissues. It can act as either a tumor suppressor (in leukemia, hepatocellular carcinoma) or an oncogene function (breast cancer), or both depending on the clinical context (gastric and ovarian cancers).

4. miR-223 is an inflammatory miRNA

4.1. Involvement in inflammatory mechanisms

miR-223 regulates the production of nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) and IL-1ß [29]. The NLRs form the inflammosome complex induced by a variety of pathogens or cell stresses. NLRP3 is probably the best characterized NLR and is activated by the presence of toxins, uric acid crystals, amyloid and other related factors. In turn, this induces IL-1ß processing *via* caspase-1 activation. Following stimulation by a Toll-like receptor ligand, many cell types need the NLRP3 inflammosome to initiate an inflammatory response and induce IL-1 ß production. Haneklaus et al. found that (i) miR-223 targets the NLRP3 3′ UTR, (ii) miR-223 expression decreases as monocytes differentiate into macrophages and (iii) NLRP3 protein levels increase. Overexpression of miR-223 prevented the accumulation of NLRP3 protein and inhibited IL-1 ß production by the inflammosome.

Zhuang et al. [30] demonstrated that miR-223 is involved in the adipocyte inflammation associated with morbid obesity. Indeed, miR-223 −/− mice on a high-fat diet (HFD) displayed more severe systemic insulin resistance, as demonstrated by a decrease in insulin-induced adipose tissue Akt phosphorylation, an increase in resistin mRNA levels and an increase in the insulin response to glucose (despite similar

fasting insulin and glucose levels). A marked increase in adipose tissue inflammation was also observed in these mice, including increased phosphorylation of nuclear factor-κB p65 and enhanced secretion of inflammatory mediators (tumor necrosis factor (TNF)-α, IL-1b and IL-6). When fed an HFD, miR-223 −/− mice displayed a high proportion of pro-inflammatory macrophages in adipose cells and stromal cells (when compared to WT mice) [31]. The specific role of miR-223 in regulatory effects in myeloid cell-mediated regulation of adipose tissue inflammation and insulin resistance was confirmed by transplantation of miR-223 −/− myeloid cells into WT recipient mice. The same study [30] highlighted a role for miR-223 as a novel regulator of macrophage polarization because it stimulated the alternative anti-inflammatory pathway and suppressed the classic pro-inflammatory (IL-1ß/IL-6/TNF-α) pathways. In this context, miR-223 acted by targeting PBX/knotted 1 homeobox 1 (Pknox1). Indeed, Pknox1 protein levels in adipose tissue collected from HFD-fed mice were inversely correlated with miR-223 expression levels. Furthermore, knockdown of Pknox1 in miR-223 −/− bone-marrow-derived macrophages (BMDMs) decreased pro-inflammatory cytokine production. Moreover, Meng et al. [32] suggested that miR-223 secreted by BMDMs may directly target adipocytes and modulate their insulin sensitivity. They noted that plasma miR-223 levels are low in patients with type 2 diabetes [33] and that insulin-stimulated Akt phosphorylation in adipocytes is attenuated by co-culture with miR-223-null BMDMs. One can thus hypothesize that *in vivo*, the miR-223 secreted by macrophages in microvesicles regulates metabolic signaling in other tissues.

4.2. Involvement in inflammatory diseases

4.2.1. Rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disease characterized by inflammation of joint synovial tissue and progressive damage to cartilage and bone tissue (leading to permanent disability in late-stage disease). Fulci's group [34] demonstrated that miR-223 is upregulated in T-lymphocytes from RA patients (when compared to those from healthy donors). The researchers found that miR-223 is predominantly expressed in CD4+ T helper lymphocytes, which are crucial players in RA. However, miR-223 levels did not differ significantly when comparing patients treated with low-dose corticosteroids and untreated patients. Furthermore, miR-223 levels were not correlated with RA severity, age and/or disease duration. As Th17 T helper lymphocytes were recently shown to have a key role in RA [35], Fulci's group measured miR-223 expression levels in these cells and found that they were lower than control naïve CD4+ cells from the peripheral blood of patients with RA. The predominant expression of miR-223 in CD4+ naïve cells seems to be involved in the etiology of RA disease. However, little is known about miR-223's exact role(s) in T-lymphocytes.

Moreover, Li et al. [36] demonstrated that the symptoms of mice with collagen-induced arthritis (CIA) were alleviated by lentivirus-mediated miR-223 silencing (LVmiR-223 T). miR-223 is significantly overexpressed in the synovium of RA patients (compared to osteoarthritis patients) and the ankle joints of mice with CIA (compared to normal mice). Treatment with LVmiR-223T reduced osteoclastogenesis and concomitantly increased levels of NFI-A and macrophage colony-stimulating factor receptor. The latter is crucial for osteoclastogenesis and also reduces bone erosion in mice with CIA. Taken as a whole, these data strongly suggest that miR-223 is involved in the disease mechanism of RA.

In conclusion, miR-223 acts as an inflammatory miRNA, by regulating the production of the inflammosome complex. This miRNA is also involved in the adipocytic inflammation where it is associated with morbid obesity. Finally, miR-223 is implicated in inflammatory diseases such as RA, by modulating the functions of T helper lymphocytes in patients.

5. MiR-223 in muscles

5.1. miR-223 in cardiac tissues

Lu et al. [37] analyzed 155 miRNAs in left ventricle biopsies from patients suffering ventricular dysfunction in the presence or absence of type 2 diabetes. miR-223 was found to be upregulated in these diabetic patients. The researchers also observed an increase in glucose uptake by rat cardiomyocytes transfected with miR-223. In transfection experiments, over-expression of miR-223 in cardiomyocytes induced an increase in GLUT-4 expression, which was necessary and sufficient for increasing glucose uptake by these cells. This relationship was confirmed by transfection of murine left ventricle cardiomyocytes with anti-mmu-miR-223, which induced a decrease in GLUT-4 expression (relative to matched controls). Greco et al. [38] showed that miR-223 is differently expressed in diabetic patients with heart failure (D-HF) or matched non-diabetic patients with heart failure (ND-HF). miR-223 levels were abnormally low in both groups, although the decrease was less marked in D-HF patients than in ND-HF patients. Moreover, this decrease was attenuated in the distant zone in D-HF patients and the border zone in ND-HF patients. The researchers hypothesized that the observed, diabetes-associated induction of miR-223 [37] might counteract the decrease in miR-223 levels provoked by other stimuli. In support of this hypothesis, it had been previously reported [39] that miR-223 levels are elevated in end-stage ischemic cardiomyopathy. One could thus hypothesize that the decrease in miR-223 is an adaptive mechanism that is active in early-stage patients but lost in end-stage patients.

5.2. An emerging role for miR-223 in vascular damage, including atherosclerosis

De Rosa et al. [40] measured concentration gradients of several miRNAs throughout the coronary circulation, with a focus on miR-223 (which is known to be an enriched miRNA in platelets). Plasma was simultaneously obtained from the aorta and coronary venous sinus of patients with stable coronary artery disease (CAD) or troponin-positive acute coronary syndrome (ACS). Aortic plasma levels of miR-223 were higher (although not significantly) in patients with ACS than in patients with CAD, whereas there was no difference between the two groups for samples from the coronary sinus. To distinguish between heart-specific and systemic alterations in circulating miRNA levels, they calculated the transcoronary concentration gradients by subtracting plasma miRNA levels in the aorta from the levels in the coronary sinus. There was no significant intergroup difference in miR-223 concentrations, although a trend towards a decrease in levels during transcoronary passage was identified.

Song et al. [41] used a microarray analysis of RNA isolated from vascular smooth muscle cells (VSMCs) cultured under dynamic or static conditions to demonstrate that miR-223 is downregulated under stretch stress. A qPCR experiment showed that miR-223 levels were four-fold lower under stretch stress conditions than under static conditions. Overexpression of miR-223 (*via* retroviral infection of VSMCs) decreased protein levels of IGF-1R (which are elevated after 24 h of exposure to stretch stress). Inhibition of IGF-1R production (using siRNA) and PI3K respectively prevented and significantly decreased the stretch stress-enhanced proliferation of VSMCs. Hence, stretch stress was found to induce VSMC proliferation *via* an increase in IGF-1R functionality and downstream PI3K–Akt signaling. Lastly, overexpression of miR-223 in VSMCs inhibited stretch-stress-enhanced proliferation and prevented the activation of PI3K–Akt signaling. In conclusion, the downregulation of miR-223 induced by stretch stress contributes to VSMC proliferation by enhancing the activity of IGF-1R and its downstream PI3K–Akt signaling.

Kin et al. [42] studied tissue- and plasma-specific microRNA signatures for atherosclerotic abdominal aortic aneurysm (AAA). Tissue wall samples from patients undergoing AAA repair and patients

undergoing aortic valve replacement surgery were analyzed with microRNA arrays and RT-PCR assays. miR-223 was found to be significantly upregulated in AAA tissue, and there was a significant negative correlation between monocyte chemoattractant protein-1 and TNF-α expression levels on one hand and miR-223 expression levels on the other. Interestingly, plasma miR-223 levels were low in patients with AAA.

In our laboratory, Rangrez et al. [43] demonstrated that miR-223 is expressed in VSMCs and is significantly upregulated *in vitro* in the presence of inorganic phosphate (Pi, a well-known calcifying uremic toxin). Over-expression of miR-223 in VSMCs in our static conditions increased cell proliferation and migration. In the same conditions we observed elevated levels of miR-223 and concomitantly low expression of two miR-223 targets (Mef2c and RhoB). Interestingly, we also found that

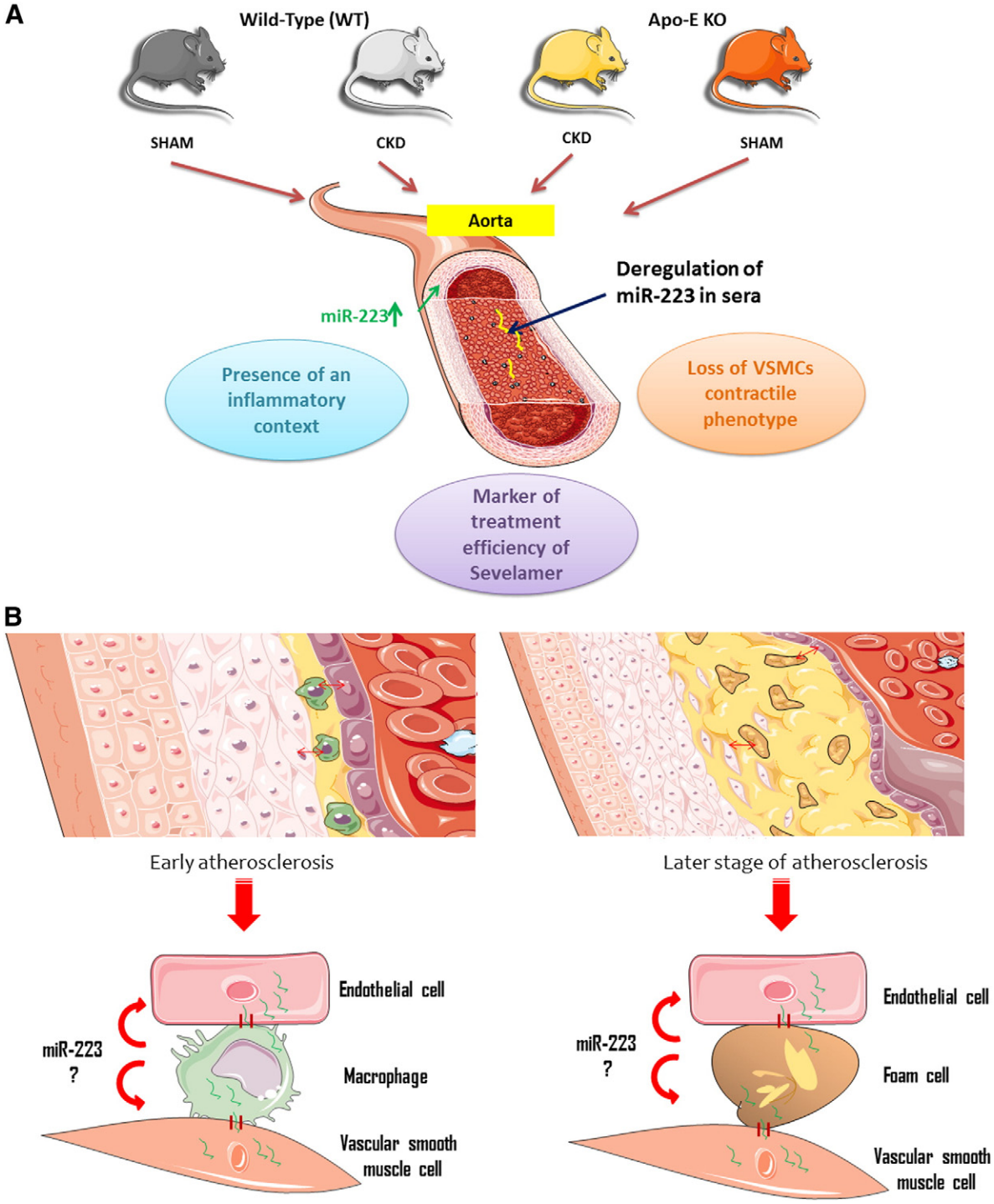

Fig. 3. A. mir-223 is a biomarker of Chronic Kidney Disease (CKD), atherosclerosis and vascular calcification. Recapitulative representation of miR-223 relevance in murine models of Chronic Kidney Disease (CKD), atherosclerosis and vascular calcification. In the presence of an inflammatory context in CKD, atherosclerosis and vascular calcification, contractile vascular smooth muscle cells (VSMCs) transdifferentiate into a synthetic phenotype, reflective of a pathogenic context, and endothelial cells become dysfunctional. In this inflammatory context, miR-223 is increased in the aorta and conversely, the expression of this miRNA decreases in the serum. Sevelamer treatment alleviates symptoms of CKD and reestablishes at least partly miR-223 levels. B. At the early stage of atherosclerosis, macrophages are recruited in the atheromatous plaque by diapedesis and are converted into foam cells at the later stages of atherosclerosis. In the atherome plaque we propose that they could interact with endothelial cells and vascular smooth muscle cells using gap junctions to transfer miR-223 into recipient cells. miR-223 could as a consequence modulate in these cells a phenotypic switch towards dedifferentiated/proliferative states.

miR-223 is upregulated in aorta samples collected from ApoE knock-out mice, which display vascular calcification — a major risk factor in kidney disease and subsequent vascular complications. Indeed, patients in the later stages of chronic renal disease (CKD) develop vascular calcification, which is associated with higher cardiovascular morbimortality [44]. The scarce literature data suggest that miRNAs are involved in the physiopathology of CKD, since they are important regulators of VSMC and endothelial cell plasticity. In studies of murine models of CKD and atherosclerosis, we recently reported that miR-223 is deregulated in atherosclerotic aortas, which develop vascular calcifications [45]. Our data in mice demonstrate that miR-223 can be detected in normal, uremic and atherosclerotic aortas. Importantly, levels of miR-223 and its targets GLUT-4 and NFIA are altered during the crucial stages of CKD and atherosclerosis (Table 1 and Fig. 3A). miR-223 levels were found to be correlated with classical biomarkers of CKD and atherosclerosis, such as cholesterol, urea and calcium levels. Furthermore, the observation that the calcium-free phosphate-binding drug sevelamer-carbonate alleviates miRNA deregulation suggests a direct link between miRNA alterations and CKD/atherosclerosis. Until very recently, it was considered that miR-223 was not present in endothelial cells [46]. However, Fleming's group [47] has shown that miR-223 is strongly expressed in endothelial cells but can only be detected in freshly isolated cells. The authors claim that miR-223 mainly acts by targeting integrin-beta 1 (a transmembrane receptor involved in cell–cell and cell–matrix interactions and communication) and by preventing growth factor signaling. Interestingly, Fleming's group also observed increases in arteriogenesis in ischemic muscles and endothelial sprouting in aortic rings in miR-223 −/− mice. These results strongly argue in favor of an endothelial role for miR-223 (along with its role in VSMCs [43]) in vasculogenesis.

We also quantified serum miR-223 levels in our murine models after two and ten weeks of exposure to CKD (see [45] and Fig. 3A). Two weeks after the induction of CKD, serum miR-223 levels were significantly lower in Apo-E KO mice than in WT counterparts. This clearly suggests that miR-223 is a potential early biomarker for atherosclerotic damage. Serum miR-223 levels were also low after ten weeks of CKD. Lastly, at that stage of the disease, levels of miR-223 were lower in serum from CKD mice than from sham-operated mice, while the opposite was true for aortic samples. As mentioned above, a serum *vs.* tissue difference in miR-223 expression levels was also found in patients undergoing AAA repair [42]. Serum levels of miR-223 were also found to be inversely associated with disease severity in patients with type 2 diabetes [33], who often suffer from vascular calcification and atherosclerosis [48]. Taken as a whole, these recent data suggest that miR-223 may be a valuable, independent, noninvasive biomarker in patients with CKD, atherosclerotic and/or type 2 diabetes patients. In addition, we propose that miR-223 deregulation could contribute to the development of metabolic and vascular disorders in CKD patients by modulating GLUT-4 expression, and thus glucose uptake which will in turn induce the insulin resistance common in these disorders [49]. In this respect, it is interesting that miR-223 was also found to be upregulated in diabetic patients [32], the number one cause of CKD. Also, since inflammation is prevalent in the pathophysiology of CKD [44], the increase of the inflammation-regulated miR-223 [29] we found in CKD could be a consequence of this. In this regard, Bao et al., when studying renal injuries in IgA nephropathy published that miR-223 was able to inhibit cell proliferation and alleviate the inflammatory status in endothelial cells [50]. Since it was already demonstrated that miR-223 can transfer from human macrophages to hepato-carcinoma cells and inhibit their proliferation [16], one can hypothesize that, in CKD and atherosclerosis, miR-223 transfers from monocytes and spumous macrophages present in atheromatous and vascular calcified lesions to vascular smooth muscle cells from the media and/or endothelial cells from the intima. Mir-223 could thus play a pivotal role in the modulation of the phenotype of these cells, and consequently of the vascular calcification processes (Fig. 3B).

Furthermore, miR-223 was recently described as a regulator of cholesterol uptake [51]. In fact, miR-223 targets the scavenger receptor class B, type I (SR-BI), the levels of which are low in diabetic mice. Hence, miR-223 may be a potential biomarker for the intracellular trafficking of cholesterol and may thus constitute a novel drug target for normalizing this trafficking (notably in atherosclerotic disease).

5.3. miR-223 in skeletal muscular dystrophy

Greco et al. [52] studied the miRNAs involved in skeletal muscle damage and regeneration during the course of Duchenne muscular dystrophy (DMD). They found that 11 miRNAs (including miR-223) were deregulated in both *mdx* mice (a murine genetic model of DMD) and DMD patients and referred to this set as the DMD signature. The expression of miR-223 in areas of damaged muscle is correlated with the presence of infiltrating inflammatory cells and myofibril necrosis/regeneration — all of which are hallmarks of DMD patients and *mdx* mice. Using a murine model of acute ischemia (the 129 SvEv strain) in which femoral artery was removed to induce a rapid increase in apoptosis and necrosis throughout the muscle followed by a regeneration phase, Greco et al. observed that expression of miR-223 was strongly induced in the two days following ischemia and then declined over time. The areas of damaged muscle contained not only necrotic and apoptotic myofibrils but also infiltrating inflammatory cells. miR-223 was strongly induced in the damaged myofibrils. As expected, miR-223 was not modulated in the skeletal muscle of newborn mice because of the absence of inflammation during normal growth. Moreover, Chen et al. [53] also showed that miR-223 levels increased immediately after cardiotoxin injury of the murine tibialis anterior muscle.

Several studies show thus a clear trend towards an upregulation of miR-223 in various clinical disorders associated with cardiac, smooth and skeletal muscle cells. This is particularly obvious in the cardiac tissue from diabetic patients, in the smooth muscle cells from atherosclerotic tissue, and in the skeletal muscle from DMD. Further work will clearly be needed to determine if the molecular mechanisms implicated in the physiopathology of these various muscle cell types are identical or not.

6. miR-223 is a promising, non-invasive biomarker in cancer and cardiovascular disease

Due to the complexity of disease processes, novel predictive factors are becoming increasingly useful for diagnosis. Over the last decade, miRNAs have emerged as potential biomarkers of many diseases (including cardiovascular disorders). Mitchell et al. [54] first demonstrated the presence of circulating miRNAs in human plasma. These miRNAs are transported by microvesicles (which protect their cargo against RNase activity [55]) or as a complex with the chaperon argonaute 2 and/or lipoproteins. Marsh's group found that (i) macrophage-derived microvesicles contain miRNAs (including miR-223), (ii) these vesicles were transported to target cells (including endothelial cells, monocytes and fibroblasts) and (iii) miR-223 was functionally active in the target cells [15]. In contrast, Arroyo's group found that non-vesicular miR-223 was associated with an argonaute2-containing ribonucleoprotein complex [56]. Resolution of this discrepancy will require further research. Several studies have demonstrated a correlation between plasma miRNA levels and diseases such as cancer [57] — thus highlighting the miRNAs' potential roles as non-invasive biomarkers [58]. Stephanie Dimmeler's group was the first to show that serum miRNA levels are altered in the serum of patients with CAD (relative to healthy counterparts) [59].

In studies of cardiovascular disease, endogenous, circulating miRNAs (*e.g.* miR-454, U6 and miR-17-5p) have been used to normalize circulating serum miRNA levels. However, the use of spiked-in, exogenous non-human miRNA (*e.g.* synthetic *Caenorhabditis elegans* miR-39) is increasingly common, as no additional experimental bias is added

[54]. Lastly, other body fluids may be suitable for diagnosis, since it was recently shown that miR-223 and other miRNAs can be detected in human saliva [60].

7. Conclusions/perspectives

Given the abundant, recent literature, it is now increasingly clear that miR-223 is involved in the regulation of a broad range of important cellular processes (including cell cycle regulation) and the invasiveness of many different cell types, hematopoietic differentiation and immune cell function. The role of miR-223 in the cardiovascular system is becoming increasingly clear and we have described its involvement in metabolism-related disorders such as diabetes, obesity-induced inflammation and cholesterol transport. Serum and tissue levels of miR-223 are becoming useful clinical parameters. The next challenge will now be to therapeutically modulate levels of this miRNA and relieve symptoms in animal models and then patients.

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