

Methods to Investigate miRNA Function: Focus on Platelet Reactivity

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Abstract

MicroRNAs (miRNAs) are small noncoding RNAs modulating protein production. They are key players in regulation of cell function and are considered as biomarkers in several diseases. The identification of the proteins they regulate, and their impact on cell physiology, may delineate their role as diagnostic or prognostic markers and identify new therapeutic strategies. During the last 3 decades, development of a large panel of techniques has given rise to multiple models dedicated to the study of miRNAs. Since plasma samples are easily accessible, circulating miRNAs can be studied in clinical trials. To quantify miRNAs in numerous plasma samples, the choice of extraction and purification techniques, as well as normalization procedures, are important for comparisons of miRNA levels in populations and over time. Recent advances in bioinformatics provide tools to identify putative miRNAs targets that can then be validated with dedicated assays. In vitro and in vivo approaches aim to functionally validate candidate miRNAs from correlations and to understand their impact on cellular processes. This review describes the advantages and pitfalls of the available techniques for translational research to study miRNAs with a focus on their role in regulating platelet reactivity.

Keywords

- ▶ microRNAs
- ▶ biomarkers
- ▶ experimental studies
- ▶ translational research
- ▶ platelet function

Introduction

Platelets are small megakaryocyte fragments mainly produced in bone marrow.¹ The primary role of platelets is to accumulate at sites of vessel injury to stop bleeding. In cardiovascular patients, platelets are pivotal in thrombus formation after atherosclerotic plaque rupture leading to acute ischemic events. Antiplatelet drugs, such as aspirin, decrease platelet reactivity (PR) and are a cornerstone in the

treatment of patients with cardiovascular risks. However, PR is highly variable among healthy subjects² and in patients taking antiplatelet drugs.^{3,4} This variability is associated with bleeding or thrombotic events.⁵ Family-based studies suggest a strong heritability of PR with⁶ or without antiplatelet drugs.² Several studies provided evidence of a correlation between microRNA (miRNA) levels and antiplatelet drugs, and pointed to miRNAs as putative biomarkers or therapeutic targets to regulate PR.⁷⁻¹¹ Moreover, the

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identification of miRNAs involved in PR may also be relevant beyond thrombosis and bleeding, for example, in immune and oncologic disorders^{12,13} where platelets play an important role. However, most studies have investigated the association between miRNA levels and PR or cardiovascular events. The causal relationship and the true impact of miRNA in platelet physiology have rarely been investigated to date.

miRNAs are small noncoding sequences, approximately 22 nucleotides (nt) in length, known to regulate messenger RNA (mRNA) translation and subsequently protein production. In humans, miRNAs are estimated to modulate at least 60% of the protein-coding transcriptome.¹⁴ miRNAs are responsible for the modulation of a wide variety of metabolic pathways and are involved in multiple biological processes such as inflammation and regulation of the immune system.^{15,16} In humans, 2,300 mature miRNAs have been described including 1,917 with annotations in miRBase V22.1.¹⁷ Over 500 miRNAs are found in platelets.¹⁸ Although platelets are anucleate cells, they have all the machinery for mRNA translation and protein production.¹⁹ In mammalian cells, including human platelets, miRNAs also affect epigenetic gene regulation, leading to modification of both platelet biogenesis and function.⁷

Circulating miRNAs have been studied in clinical trials. This pool of miRNAs consists of miRNAs bound to proteins, such as miRNAs stabilized by the Ago2 protein,²⁰ or those within extracellular vesicles. These vesicles include a heterogeneous population of various vesicular structures derived from different cell types and are represented mainly by microvesicles and exosomes. There are several uncertainties regarding these different extracellular vesicles in terms of biogenesis, heterogeneous RNA cargo, and, most importantly, the fate of their cargo.²¹ However, it seems that the contribution of exosomes is marginal with approximately 0.01 miRNA molecules per exosome,²² while circulating miRNA content is close to that measured in microvesicles making them the main miRNA carrier in circulating blood.²³ Since 41 to 45% of the microvesicles are released from platelets,²³ circulating miRNAs may provide information on platelet function and diseases where platelets play a major role.

This review describes the different strategies used to investigate miRNAs and their functional impact on platelets.

miRNA Biogenesis and Function

In mammalian cells, the gene that encodes for a miRNA is first transcribed by RNA polymerase II or III in the nucleus to produce a primary miRNA, the pri-miRNA. The microprocessor complex, Drosha-DGCR8, cleaves the single strand-double strand junction of the pri-miRNA hairpin to form a stem loop RNA double strand called the precursor-miRNA (pre-miRNA). Pre-miRNAs are exported into the cytosol by the exportin-5-Ran-GTP. In the cytoplasm, the RNase Dicer cleaves the pre-miRNA hairpin leading to the formation of a double-stranded miRNA duplex, with either strand potentially serving as a functional mature miRNA.²⁴ Of note, noncanonical miRNA biogenesis pathways have been described, such as production of pre-miRNA independently

of Drosha or Dicer pathways.²⁵ The 5' and 3' segments derived from the pre-miRNA within the mature double-stranded miRNA are called 5p and 3p, respectively. 5p and 3p are not perfectly complementary to each other, and can have different biological roles due to their dissimilar sequences and mRNA-targeting properties.²⁶

Mature miRNAs bind by imperfect complementarity to a seed region in the 3'UTR of a mRNA to form a duplex by the base-pairing of six to eight nucleotides at the 5' end of the miRNA.¹⁴ Atypical sites with a seed mismatch or a compensatory site exist; however, their formation requires more energy.^{27,28} The duplex together with argonaute 2 (Ago2) forms the RNA-induced silencing complex (RISC). RISC induces mRNA degradation, destabilization, or translational inhibition depending on the type of pairing, as described elsewhere.²⁶

One miRNA can target several mRNAs and therefore a single miRNA can regulate the expression of multiple proteins. Conversely, one mRNA sequence can have a seed region for multiple miRNAs, allowing a putative synergistic effect of several miRNAs on the production of a single protein.

Clinical Association Studies: Sample Collection and miRNA Quantification

Clinical Studies

A growing number of studies have shown correlations between miRNA level and PR or ischemic events in healthy volunteers or in cardiovascular patients (►Table 1). As mentioned before, the platelet-derived miRNAs carried by microvesicles in the circulation reflect the platelet physiology and platelet content.^{29–31} This has led to the development of the hypothesis that miRNAs could be used as biomarkers of platelet function to predict recurrence of cardiovascular events or to tailor antiplatelet therapy.³²

As illustrated in ►Table 1, these clinical studies differ in many aspects including population selection (cardiovascular patients taking antiplatelet drugs or healthy volunteers), sample type (serum, plasma, or platelets), miRNA quantification assays (microarray or quantitative polymerase chain reaction [qPCR]), and platelet function assays. Therefore, it is not unexpected that results may diverge. An example is miR-96, which was quantified in plasma with microarray and found to be correlated with epinephrine-induced platelet aggregation in healthy volunteers,³³ whereas no correlation was measured using qPCR in platelet samples and adenosine diphosphate-induced platelet aggregation in samples from cardiovascular patients treated with aspirin and clopidogrel.³⁴ The selection of the platelet function assay is of utmost importance since it can evaluate a distinct facet of platelet physiology according to the parameter measured and the agonist used.³⁵ Altogether, the methodological heterogeneity of these clinical studies emphasizes the need for functional evaluation of candidate miRNAs to validate associations.

Sample Collection

There is no consensus on the optimal sample type or preparation procedure for isolating circulating miRNA. Clinical studies have used platelets, serum, or plasma with

Table 1 Selected association studies involving miRNAs in healthy volunteers and cardiovascular patients

References	Year	Samples	Setting	Treatment	miRNA quantification	Outcome	miRNA correlated with outcome	miRNA not correlated with outcome
Kondkar et al ³³	2010	Plasma	Healthy volunteers	N/A	Microarray	LTA with epinephrine 1.5 μM	miR-96	
Zampetaki et al ⁴⁵	2012	Plasma	Population-based survey	No treatment, DAPT, or aspirin	qPCR	Myocardial infarction	miR-126 miR-223 miR-197	
Willeit et al ⁸	2013	Platelet, MV, PRP, PPP, serum	Healthy volunteers, patients with diabetes or with symptomatic carotid atherosclerosis	None or various antiplatelet regimen	qPCR	Modified LTA in a 96-well plate using various agonists and concentrations, serum TXB ₂ assay, and VerifyNow assay	miR-223 miR-191 miR-126 miR-150	
Shi et al ³⁴	2013	Platelet	Acute coronary syndrome	Clopidogrel plus aspirin	qPCR	LTA with ADP 10 μM, VASP	miR-223	miR-96
Zufferey et al ¹¹	2016	Platelet	Stable cardiovascular patients	Aspirin	Microarray	LTA with epinephrine 0.4–10 μM, AA 1 mM, ADP 2 and 10 μM, and collagen 1 μg/mL	miR-135 miR-204	
Kaudewitz et al ⁹	2016	Plasma	Acute coronary syndrome	DAPT or aspirin	qPCR	LTA with ADP 20 μM, VerifyNow	miR-126 miR-223 miR-24 miR-191	
Witkowski et al ¹⁵⁴	2016	Plasma	Diabetes mellitus	N/A	qPCR	TF-mediated thrombogenicity	miR-126	
Peng et al ¹⁵⁵	2017	Platelet	Acute coronary syndrome	Clopidogrel plus aspirin	qPCR	LTA with ADP 20 μM	miR-223 miR-221 miR-21	
Ding et al ¹⁵⁶	2019	Platelet	Acute coronary syndrome	Clopidogrel plus aspirin	qPCR	LTA with AA 500 μg/mL and ADP 5 μM	miR-204	
Tang et al ¹⁵⁷	2019	Plasma	Stable coronary artery disease	Clopidogrel plus aspirin	High-throughput Illumina sequencing followed by validation with qPCR	Clinical outcomes	miR-142	
Liu et al ¹⁵⁸	2020	Platelet	Acute coronary syndrome	Clopidogrel plus aspirin	qPCR	Thromboelastography	miR-126 miR-223 miR-150 miR-130	miR-21 miR-96 miR-331 miR-326

Abbreviations: AA, arachidonic acid; ADP, adenosine diphosphate; DAPT, dual antiplatelet therapy; LTA, light transmission aggregometry; miRNA, microRNA; MV, microvesicles; PPP, platelet-poor plasma; PRP, platelet-rich plasma; qPCR, quantitative polymerase chain reaction; TF, tissue factor; TXB₂, thromboxane B₂; VASP, vasodilator-stimulated phosphoprotein phosphorylation assay.

different anticoagulants and different centrifugation protocols. The choice of using plasma or serum has been debated³²; plasma would reflect steady-state circulating miRNA levels while serum would reflect, in addition to circulating miRNAs, the miRNA content of platelets and other cells activated during the *in vitro* coagulation process.³⁶ When using plasma, heparin should be avoided since it interferes with nucleic acid amplification procedures.³⁷ Trisodium citrate is an option, although EDTA use is associated with a lower final calcium content and inhibits more profoundly platelet activation that may occur during the collection process. The preparation of platelet-poor plasma requires a double-centrifugation step to avoid any residual platelets, which would be lysed during sample freezing–thawing processes and lead to the artifactual release of miRNAs from the platelets into the plasma. Residual platelets and leukocytes in plasma samples should be assessed by quantification of specific markers (by qPCR or western blot), such as ITGA2B for platelets and CD45 for leukocytes.³⁸

To investigate miRNA content directly from platelets, they can be isolated and washed in HEPES buffer complemented by apyrase and PGI2 to prevent platelet activation during the procedure.³⁹ Depletion of leukocytes and erythrocytes increases the purity of platelet samples. For this purpose, anti-CD45- and anti-CD235a-labeled magnetic beads are added to platelet-rich plasma (PRP) before the washing procedure and transferred to a column that removes leukocytes and erythrocytes bound to the magnetic beads.^{38,40}

miRNA Dosage and Normalization

Although miRNAs are stable in biological samples over time, their low expression level is a major limitation for their quantification. Some technical variables such as the available sample amount, the collection procedure, storage conditions, and the miRNA isolation or reverse transcription efficiency can profoundly affect the amount of miRNA measured.^{41–43} The efficiency of miRNA extraction using a Trizol-based method⁴⁴ remains unclear; therefore, a highly efficient and standardized technique dedicated to miRNAs, using purification columns, is preferred.³⁴ Depending on the miRNA detection method chosen, the number of different miRNAs that can be evaluated simultaneously and the sensitivity vary considerably. Indeed, Taqman-based qPCR on complementary DNA (cDNA)^{34,45} has a high sensitivity and specificity and is therefore considered to be the gold standard to quantify miRNAs, although typically only few candidate miRNAs are tested in a single experiment. Given the low amount of miRNAs in samples, a preamplification step is often performed before the qPCR.^{44,45} This preamplification step increases the quantity of cDNA available for qPCR, decreasing Ct values and facilitating the analysis, but it does not impact sensitivity. Custom Exiqon locked nucleic acid (LNA)^{9,46} and Nanostring technologies^{11,47} allow measurement of the expression profile of approximately 100 miRNA candidates per run. Alternatively, small RNA sequencing (sRNAseq) has the advantage of investigating a large number of miRNAs in a single sample.⁹ However, technical bias can be introduced during preanalytical steps (e.g.,

adapter ligation, primer composition) for this latter assay, potentially introducing a distortion of miRNA levels compared with results from qPCR.⁴⁸ In addition, sRNAseq has a high cost per sample and can be time consuming if a pipeline of sequencing runs and bioinformatics support are not readily available.⁴⁹

To make sure that miRNA quantification is not affected by technical variability across a series of samples, a “spike-in” approach with a synthetic oligonucleotide used as an exogenous normalizing target (e.g., UniSP6⁵⁰ and cel-miR-39⁴⁴) can be added at a known concentration before the sample RNA extraction process. The variability of this reagent across samples should not exceed one qPCR cycle to confirm the efficiency and reproducibility of the extraction as well as preamplification and qPCR steps.³² However, the spike-in method does not account for other sources of variability such as sample quality or the total concentration of miRNAs. Therefore, an endogenous normalization is of utmost importance to identify true biological differences. The measurement of a stable endogenous miRNA used as a reference target is the optimal way to assess the relative amount of miRNAs of interest. Since there are no universally defined reference miRNAs, several normalization strategies have been proposed. RNU6, a small noncoding RNA, is frequently used, but it is not stable in serum⁵¹ and could be undetectable.³⁶ Moreover, RNU6 is not a miRNA. Therefore, the efficiency of its extraction, reverse transcription, and amplification can differ from miRNA, precluding the reliability of RNU6 as a normalizing RNA. A normalization with a reference belonging to the same RNA class is preferred. MiR-16 is often used, but was shown to be sensitive to hemolysis.⁵² Selection of endogenous miRNA depends on the sample. Stable endogenous miRNAs can be tissue- and disease-specific. For example, different normalizers are used for plasma (e.g., miR-638, miR-93, and miR-484^{53–55}), serum (e.g., miR-23a, let7a, and miR-1260^{46,56,57}), and platelet samples (e.g., miR-28, miR-29c, and miR-151^{57,58}). Algorithms such as geNorm or NormFinder may be used to identify the most stable endogenous miRNAs among potential reference miRNAs. Of note, the use of a panel of stable endogenous miRNAs increases the robustness of the normalization procedure compared with a single miRNA. The level of miRNAs of interest is then calculated relative to the panel of normalizers, according to the method described by Kok et al.⁵⁷

Identification and Validation of miRNA:mRNA Pairs

Clinical association studies pinpoint candidate miRNAs associated with biological or clinical outcome but do not provide information on underlying mechanisms. In that regard, the identification of the genes regulated by candidate miRNAs is of utmost importance to understand the biological pathways implicated in platelet function regulation and may lead to the identification of new targets for antithrombotic drugs. Several tools are available, both cell-based and *in silico*. These tools are complementary, but can give discordant results. Several parallel approaches should therefore be used to strengthen the mechanistic evidence of findings.

High-Throughput Identification of miRNA::mRNA Pairs

High-throughput techniques are available to detect interactions between miRNAs and mRNAs in cells or tissues. These techniques take advantages of the RISC complex, which—as previously described—is formed by the binding of a miRNA on its mRNA target and is stabilized by the Ago2 protein. Immunoprecipitation of this complex followed by qPCR, microarray, or RNA-seq detects the nucleotide sequences in RISC, allowing duplex miRNA::mRNA identification. Given the limited strength of the complex, multiple techniques have been developed to improve the protein–RNA complex stability before purification. The cross-linking and immunoprecipitation (CLIP) technique covalently links miRNA, mRNA, and Ago2 via ultraviolet (UV) irradiation.⁵⁹ The photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) method improves the previous technology by addition of photoreactive ribonucleoside analogs (4-thiouridine [4-SU] and 6-thioguanosine [6-SG]) that increase the efficiency of RNA–protein UV cross-linking and convert thymidine into cytidine.⁶⁰ Other CLIP variants exist such as HITS-CLIP and iCLIP that increase the detection sensitivity of fixation sites of RNA-binding protein, or CLASH⁶¹ and iPAR-CLIP dedicated to enhance RNA–RNA interactions.⁶² High-throughput techniques use next-generation sequencing to generate a cDNA library based on the RNA immunoprecipitated during the CLIP, and computational approaches establish an annotated map of miRNA–target interactions often available online.^{59,60,63,64}

CLIP techniques enable the identification of duplex miRNA::mRNA in various cell types but, to the best of our knowledge, no such technique has been applied to platelets or megakaryocytes.

In Silico Identification of miRNA::mRNA Pairs

Bioinformatics approaches use knowledge of miRNA biology to develop algorithms that can detect putative miRNA binding sites on mRNA and predict gene expression modulation. Algorithms (e.g., TargetScan, PITA miRANDA, and DIANA-microT) predict the canonical and noncanonical miRNA binding sites on and outside the 3'UTR region of mRNAs.⁶⁴ The prediction strategy takes into account multiple pairing characterizations such as seed match, energy uptake, conservation across species, and multiple target sites.⁶⁵ The calculated scores rank the putative miRNA::mRNA duplexes and determine the best pairing according to their algorithm. Databases can exhibit inconsistent results across the bioinformatics tools, probably due to the different approaches used. Given the lack of accuracy and sensitivity of bioinformatics prediction tools alone, a large number of false-positive interaction predictions are generated. To avoid this, new bioinformatics tools predict miRNA targets by combining several prediction scores.^{65,66} In addition, some databases integrate data from in silico analyses and published biological observations from the PubMed literature.⁶⁷

Finally, miRNA targets may be analyzed as a target pool to identify biological pathways regulating cell physiology. Huntley et al provided comprehensive guidelines to identify the pathways impacted by miRNA regulation using gene

ontology (GO).⁶⁸ In addition, a recently published model (PmiRGO⁶⁹) built a network by associating multiple data sources to investigate the GO functions of miRNAs. GO has been used previously to identify biological pathways targeted by miRNAs in platelets.¹⁸

In Vitro Validation of miRNA Targets

The CLIP technique and in silico algorithms are prone to false-positive results and the biological relevance of identified miRNA::mRNA duplexes needs to be validated by in vitro approaches to directly evaluate the miRNA impact on gene expression.⁷⁰

One option is a pull-down assay. Briefly, synthetic biotinylated miRNAs are transfected into cells and miRNA::mRNA complexes are captured using streptavidin-coated beads. The mRNA level is measured by real-time PCR, microarray, or RNA-seq analysis. The pull-down assay monitors the direct association of one miRNA on several mRNA targets.⁷¹ This assay allows validation of interactions of one miRNA with its mRNA targets but does not provide information on miRNA interaction sequences. It is also based on results from an introduced miRNA. This demonstrates that it interacts with the mRNAs detected at the concentration used, but does not prove that the endogenous equivalent miRNA does so.

The reporter gene assay is widely used to identify the nucleotide sequence responsible for gene regulation. For miRNA, this method is typically based on the measurement of luciferase activity under the partial control of a predicted miRNA-targeted 3'UTR. To study gene regulation through miRNA modulation, the 3'UTR of interest is cloned in an expression vector downstream of the firefly luciferase gene sequence which itself is under the control of a promoter. A second reporter is expressed from a different expression cassette and used as a normalization control (e.g., renilla luciferase). The miRNA and the dual-reporter gene vector are cotransfected into cultured cells. Firefly and renilla luciferase activity are then evaluated after the addition of their respective substrates. Gene regulation is estimated by the ratio between firefly and renilla luciferase activities, measured by luminescence. The miRNA is expected to lower firefly luciferase activity if targeting the 3'UTR, compared with a control condition. To determine the specificity of the binding site of a miRNA, a mutated version of the cloned 3'UTR downstream of the firefly cassette is made, typically lacking the expected miRNA seed target sequence.^{58,72,73} If the miRNA fails to lower firefly luciferase activity in the mutated 3'UTR, it suggests a bona fide functional interaction in the native 3'UTR. This sensitive method allows the validation of the direct interaction between miRNA and its target through its binding site. Usually, this assay only investigates one miRNA::mRNA interaction duplex at a time, but it is considered a valuable validation of the interaction and can confirm the functional importance of the seed sequence.

In Vitro Study of Platelet Function

Although several miRNAs have been pinpointed as biomarkers of PR or recurrence of ischemic events, few of them have

been validated as true regulators of platelet function. The study of platelet function is challenging since platelets have a short life-span and their function remains intact for just a few hours after blood collection. In addition, the transfection of platelets has low efficiency,⁷⁴ and impairs any reliable functional evaluation. To address these issues, alternative methods have been developed.

In Vitro Models

The use of immortalized human megakaryocyte cell lines offers a strategy to elucidate the impact of miRNAs on megakaryocyte morphology and function. A variety of immortalized megakaryocyte cell lines are commercially available, including MEG01 (human megakaryoblast line from chronic myelogenous leukemia), DAMI (human megakaryocytic cell line from a patient with megakaryoblastic leukemia), and K562 (human immortalized myelogenous leukemia cell line). Immortalized megakaryocytes easily proliferate, providing sufficient RNA and protein to study the biological impact of miRNAs.^{9,75} Their differentiation leads to the production of functional platelet-like structures (PLS).⁷⁶ While immortalized cells have numerous advantages, their major limitation is the occurrence of mutations that could alter cell morphology and function.

An alternative is the use of human-derived cells such as megakaryocytes differentiated from human pluripotent stem cells (hPSCs)⁷⁷ or human hematopoietic stem cells,⁷⁸ and the platelets derived from them.⁷⁹ CD34+ cells are typically purified from peripheral blood (buffy coat),⁵⁸ apheresis,⁷⁶ or from umbilical cord blood.^{73,80} PLS produced *in vitro* are functionally close to human platelets.⁸¹ Given the low number of PLS generated from megakaryocyte progenitors, specific methods dedicated to test PLS at low concentrations have been developed. Similarly to human platelets, activation of PLS is associated with specific surface markers which can be assessed by fluorescence-activated cell sorting, such as P-selectin (CD62P) and the activated form of GPIIb/IIIa.^{76,82} PLS adhesion can be assessed in static or dynamic conditions since these techniques require only low concentrations of platelets.^{58,83} It is noteworthy that PLS may differ substantially from their native counterparts and that, depending on the culture conditions, the reported findings might not replicate the *in vivo* situation. miRNA-related *in vitro* findings might also be affected by potential contamination of cell-culture reagents with miRNAs.⁸⁴ Megakaryocytes express the same platelet receptors at their surface as PLS and can conveniently be used in functional assays as a proxy to platelets⁸⁵ for the measurement of P-selectin secretion and the activated form of GPIIb/IIIa after stimulation, as well as in flow assays.⁵⁸

Modulation of miRNA Content

The modulation of miRNA content in megakaryocytes is fundamental to evaluate the functional impact of a candidate miRNA. The effects of changing the amount of a miRNA present within a system can be rapidly quantified by measuring target mRNA expression or protein production from primary or immortalized transfected cells, with the results

reflecting the direct or indirect effect of a given miRNA.^{33,58,73} miRNA content can be modulated using numerous strategies. Lipofection uses a transfection reagent's ability to encapsulate nucleotide sequences into liposomes that deliver a given cargo into the cell cytoplasm following fusion with the cytoplasmic membrane. Transfection reagents show toxicity, in particular in nonadherent cells such as megakaryocytes and CD34+ cells. Transfection efficiency largely depends on the cell type and the reagent used (from less than 10% in HL60 to over 95% in primary myoblasts).⁸⁶ Balancing between transfection efficiency and cell viability is challenging since transfection efficiency is generally positively correlated with the toxicity. The transfection of CD34+ and megakaryocyte progenitors by lipofection showed an efficiency of 50 to 75%.^{58,87,88} The impact of the transfection reagent on morphology and function of the cells can be evaluated using the transfection reagent alone (mock condition).

Nucleofection allows the delivery of nucleotide sequences by electroporation in the presence of a commercial nucleofection reagent.^{73,89} Successive electrical impulses induce the formation of transient pores on the cytoplasmic membrane allowing the delivery of the nucleotide sequence into cells. The efficiency is cell type-specific and the optimum voltage range must be investigated for each cell type used. This method is efficient and is less toxic than lipofection for most nonadherent and adherent cells tested.⁹⁰ The efficiency of the transfection by nucleofection ranges from 40 to 90% depending of the cell type and, similarly to lipofection, the efficiency is approximately 55% in hematopoietic stem/progenitor cells.⁹⁰ Interestingly, similar results have been demonstrated in hEPSC.⁹¹ As a general note, transfection can lead to the delivery of nonphysiological amounts of miRNAs.⁹²

The majority of nucleotide sequences delivered into the cells by lipofection or nucleofection to assess the functional impact of candidate miRNAs are synthetic mimic or inhibitor sequences, allowing the overexpression or the repression of miRNA content. Synthetic mimic nucleotide sequences used to overexpress the miRNA content have the same nucleotide sequence as an endogenous one. Conversely, synthetic miRNA inhibitors, such as antagomir sequences, reduce miRNA content by binding to endogenous miRNAs. There is no standardization for the concentration of synthetic transfected miRNA mimics or inhibitors, and the concentration used in different studies is highly variable.^{58,75,80,89} The concentration of synthetic miRNAs in transfection mixtures is adjusted to reflect the delivery efficiency into cells according to the technique used,⁹² with the aim of achieving a concentration close to that observed in clinical studies. Furthermore, to control for a possible impact of the transfection procedure on cell function, transfection using a scrambled sequence, a short nucleotide sequence unable to target any mRNA sequence and without known biological impact, is required. Alternatively, the modulation of miRNA content can also be performed by transfection of a recombinant plasmid with a pri-miR sequence,⁹³ leading to overexpression or downregulation of a miRNA level. Recombinant plasmids increase the number of miRNA copies or decrease

the level of available miRNAs by binding endogenous miRNAs using antisense sequences.

LNAs are antisense miRNA sequences. Their phosphorothioate backbone enhances their affinity for complementary miRNA. This confers a higher repression efficiency than other synthetic inhibitors.⁹⁴ Thanks to an additional lipidic moiety at the nucleotide sequence extremity, LNA can freely cross the cytoplasmic membrane without the help of a transfection reagent, a mechanism known as gymnotic delivery.⁹⁵ This method has been used to downregulate miRNA in megakaryocytes;⁸⁵ however, it does not seem to be suitable for studying platelet function. Indeed, Flierl et al⁹⁶ have shown that the LNA phosphorothioate backbone could activate human platelets.

LNA as well as synthetic miRNA mimics or inhibitors allows rapid study of the impact of candidate miRNAs, avoiding time-consuming preparations. Moreover, transfection and nucleofection enable the testing of the effects of multiple combinations of candidate miRNAs in a transient manner, facilitating the exploration of potential synergistic effects, an option that is not possible with transduction.⁹²

Lentiviral transduction is one of the most effective systems to deliver a nucleotide sequence into hPSC⁹⁷ and to overexpress or repress miRNAs.^{73,98} The lentiviral vector genome is integrated into the host genome, leading to stable miRNA expression in a transduced cell line.⁹⁹ This is suitable for hematopoietic stem cell transduction.¹⁰⁰ Of note, a recent study showed that prior transfection of CD34+ cells with plasmid DNA increases the efficiency of the following transduction procedures by threefold.¹⁰¹

Stable regulation of gene expression has been reported in different studies using CRISPR/Cas9 tools¹⁰² and Transcription Activator-Like Effector Nucleases (TALEN),¹⁰³ which are robust gene-editing systems to modify miRNA levels. In a recent study, over 80% of hPSCs were shown to undergo targeted genome editing using CRISPR-Cas9 reagents and nucleofection.¹⁰⁴ Promising advances could emerge from these studies. The combination of these methods could lead to the generation of heritable overexpression or deletion of miRNAs in platelets differentiated from transduced hematopoietic stem cells.

In Vivo Models

miRNAs were first described in *Caenorhabditis elegans* in 1993,¹⁰⁵ and then in several species such as *Drosophila melanogaster* (fruit fly),¹⁰⁶ *Danio rerio* (zebrafish),¹⁰⁷ and *Mus musculus* (mouse).¹⁰⁸ The miRNA database, miRBASE V22.1, describes 355, 1,234, and 1,917 miRNA entries for *D. melanogaster*, *D. rerio*, and *M. musculus*, respectively. miRNAs are conserved among vertebrates. Therefore, vertebrate animal models are of particular interest to study the functional impact of miRNA in or ex vivo.¹⁰⁹ However, a single miRNA is predicted to modulate several hundred mRNAs expressed in different tissues. Therefore, experimental studies of the effects induced by miRNA should ideally be tissue-specific.

Zebrafish

The zebrafish model is used for the study of hemostasis. Despite the fact that the cellular equivalents of platelets in zebrafish are nucleated thrombocytes, these cells are functionally close to human platelets. Thrombocytes are activated at sites of vessel injury and interact with vascular components to form a thrombus.¹¹⁰ However, some hemostasis-related gene orthologs are absent from zebrafish; for instance, zebrafish lack the coagulation factors XI and XII and the platelet receptors GP1b and GPVI.¹¹⁰

An advantage of the zebrafish model is the transparency of the zebrafish embryos and larvae between 24 hours and 5 days postfertilization, allowing the observation of vessels in simple microscopy set-ups. This characteristic is used in thrombotic assays to determine the time to occlusion, a general measure of hemostasis, by visualizing in real time the formation of an occlusive thrombus at the site of vessel injury, typically induced by a laser.¹¹¹

Zebrafish with green fluorescent protein expression under control of the cd41 promoter (*itga2b:EGFP* or *cd41:EGFP*) enables visualization of thrombocytes by their fluorescence.¹¹² In *cd41:EGFP* transgenic larvae, the number of thrombocytes accumulated at the site of vessel injury can be quantified and the size of the thrombus can be assessed. Of note, several human miRNAs, such as miR-223, have orthologs in zebrafish and bind the same region on target mRNAs, which makes the zebrafish model particularly interesting to investigate the impact of candidate miRNA on thrombus formation in vivo.^{113,114}

Genetic modifications in zebrafish enable tissue-specific expression and constitutive or conditional mutations, depending on the regulatory elements used.^{109,114,115} The early zebrafish embryo is easy to microinject, which makes the introduction of exogenous nucleic acids or proteins into the rapidly developing embryo straightforward.

Transgenesis allows stable genomic insertions and is particularly useful for introducing a reporter gene using transgenic constructs to produce a zebrafish line (e.g., with I-SceI or, Tol2, BACs, or by gene trapping)¹¹⁶⁻¹¹⁸ and can be used to alter miRNA expression specifically in thrombocytes.¹¹⁴

Forward genetic mutagenesis screens traditionally used chemical mutagens to induce random mutations followed by phenotype-gene correlations.¹¹⁹ Reverse genetics using gene targeting became commonplace with the use of nuclease-based genome editing.¹²⁰ Knockout zebrafish are produced by stable gene disruption obtained with TALEN¹²¹ or CRISPR-Cas9 systems.¹²² Tilling¹²³ or zinc finger nucleases¹²⁴ have also been used for reverse genetics, but these methods are less easily adopted than CRISPR-Cas9.¹²⁵ The availability of high-quality, searchable reference genome sequences also greatly assists with genome targeting methods.¹²⁶

Transient overexpression can be made in zebrafish embryos by microinjection of mRNAs, miRNAs, or plasmids,^{127,128} whereas temporary gene or miRNA knockdowns can be achieved using antisense oligonucleotides, typically morpholinos¹²⁹ with partial or complete complementarity to the miRNA studied. Morpholinos block

translation, pre-mRNA splicing, or miRNA activity by forming a base-paired duplex with the target RNA sequence. As an example of miRNA targeting, morpholinos have been used for inhibition of miR-126, demonstrating their role in vascular integrity.¹³⁰ The use of antagomirs stabilized with a phosphorothioate backbone is not encouraged since toxicity has been observed in zebrafish embryos.¹³¹

Competitive miRNA inhibitors can be used *in vivo*, such as miRNA “sponges” or “decoys.”¹³² miRNA sponges are synthetic linear or circular nucleotide sequences¹³³ presenting several consecutive binding sites for the miRNA of interest and lead to depletion of miRNA activity.¹²⁷ Decoys are long noncoding RNAs forming a hairpin¹³⁴ containing multiple high affinity binding sites for miRNAs. These sequences aim to de-repress miRNA targets by preventing the binding of miRNAs on their predicted seed sequence.^{132,135} The hairpin construct protects against RNAses and miRISC-mediated degradation. Chimeric sequences created by the addition of a decoy structure to sponge sequences increases competition efficiency for miRNA inhibition.^{133,136}

Mice

The mouse model is widely used for hemostasis-related research thanks to a hemostatic system functionally close to that in humans.¹³⁷ However, mice platelets are smaller and more numerous than human platelets and exhibit a greater granule heterogeneity. Moreover, some platelet receptors are differentially expressed. Mice platelets express PAR1 and PAR3 as their thrombin receptors, whereas human platelets are stimulated by thrombin through the PAR1 and PAR4 receptors. In addition, mice platelets do not express FcγRIIA which participates in platelet activation induced by von Willebrand factor.¹³⁸

Blood sampling in mice allows collection of several hundred microliters of blood, which is sufficient to test platelet function by light transmission aggregometry. In addition, dedicated antibodies such as anti-CD62P and Jon Ab (equivalent of PAC1 in human) allow monitoring of platelet secretion and GPIIb/IIIa activation respectively, by flow cytometry. Moreover, the mouse model also allows assessments such as tail bleeding time and saphenous vein bleeding. These tests cover global aspects of hemostasis in contrast to tests performed with PRP or washed platelets.

miRNA target sites were conserved during mammalian evolution¹⁴ and Roux and coworkers¹³⁹ underlined the existence of a tissue-specific evolutionary pattern of miRNA::mRNA pairs in humans and mice. However, the difference between the number of miRNAs in mice and humans suggests that not all the miRNA::mRNA pairs are shared and therefore the mouse model cannot be used for the study of all human miRNAs.

Most of the knowledge about miRNA function in vertebrates emerged from loss-of-function studies. The generation of miRNA-specific knockouts in mice are dedicated to study the impact of one miRNA of interest on physiological functions.¹⁴⁰ The first knockout resource for mice used recombination-mediated cassette exchange targeting vec-

tors. This tool gave the possibility to alter miRNA expression and to create reporters or conditional mutants.¹⁴¹

A genetically engineered mouse model (GEMM) generates mutants via transgenesis. A transgene is injected into the male pronucleus in fertilized eggs, which are transplanted into a female. This allows a random insertion of the transgene. GEMM can use homologous recombination; the vector containing a transgene flanked by a homologous DNA sequence is transfected into embryonic stem cells that are then implanted into a surrogate female which generates chimeric mice.⁶² This technology has been used to study the impact of miR-223 on platelet production and function in chimeric bone marrow.¹⁴² Homologous recombination using the Cre/loxP system is a powerful tool giving multiple possibilities of gene regulation. The Cre/loxP system enables the generation of knockout or knockin mice¹⁴³ with constitutive or conditional¹⁴⁴ expression and tissue specificity. For example, mice carrying the platelet-factor 4-Cre transgene drive loxP recombination in megakaryocytes, platelets, and leukocytes,¹⁴⁵ directing lineage-restricted regulation. Pf4-Cre knockout of Dicer in mice results in dysregulation of mRNA expression and platelet function, suggesting that miRNAs are key players in platelet mRNA regulation in mice, without identifying which miRNAs are important.^{146,147} To the best of our knowledge, no study has used a conditional knockout of a miRNA in platelets, although this strategy could lead to a better understanding of the involvement of a given miRNA in platelet function *in vivo*. The recent generation of GPIIb-Cre transgenic mice offers a more specific model with recombination only in megakaryocytes and platelets. This model should therefore detect effects mediated in these cells, independently from leukocytes.¹⁴⁸

The CRISPR and TALEN technologies, as described above, are highly efficient tools used for genome editing. In mice, TALEN¹⁴⁹ and CRISPR¹⁵⁰ systems can successfully induce miRNA deletions. In addition, comparative studies have shown that CRISPR is more efficient for single-step biallelic mutations in mice than the other technologies.¹⁵¹

LNAs, also described above, can be used as chemical inhibitors to transiently decrease miRNA levels after injection in mice and lead to modulation of PR.⁸⁹ Although LNA sequences have been shown to impact PR in human platelets,⁹⁶ Kaudewitz and coworkers⁹ measured the absence of platelet aggregation after incubation of mouse PRP with LNA.

Alternatively, miRNA transduction can be performed by use of lentiviral vectors designed to lead to stable overexpression of a miRNA after injection in mice.¹⁵²

Finally, intramuscular injection of miRNA using poly lactic-co-glycolic acid nanoparticles has the advantage of directly regulating mRNA levels in a specific tissue.¹⁵³

Altogether, a large panel of methods is available to modulate miRNA levels in animal models, allowing transient or permanent overexpression, downregulation, or gene mutation for selected miRNAs, systemically or with tissue specificity. These tools offer numerous possibilities for *in vivo* or *ex vivo* functional evaluation of miRNAs on PR and thrombus formation.

Investigating miRNA function in platelet reactivity

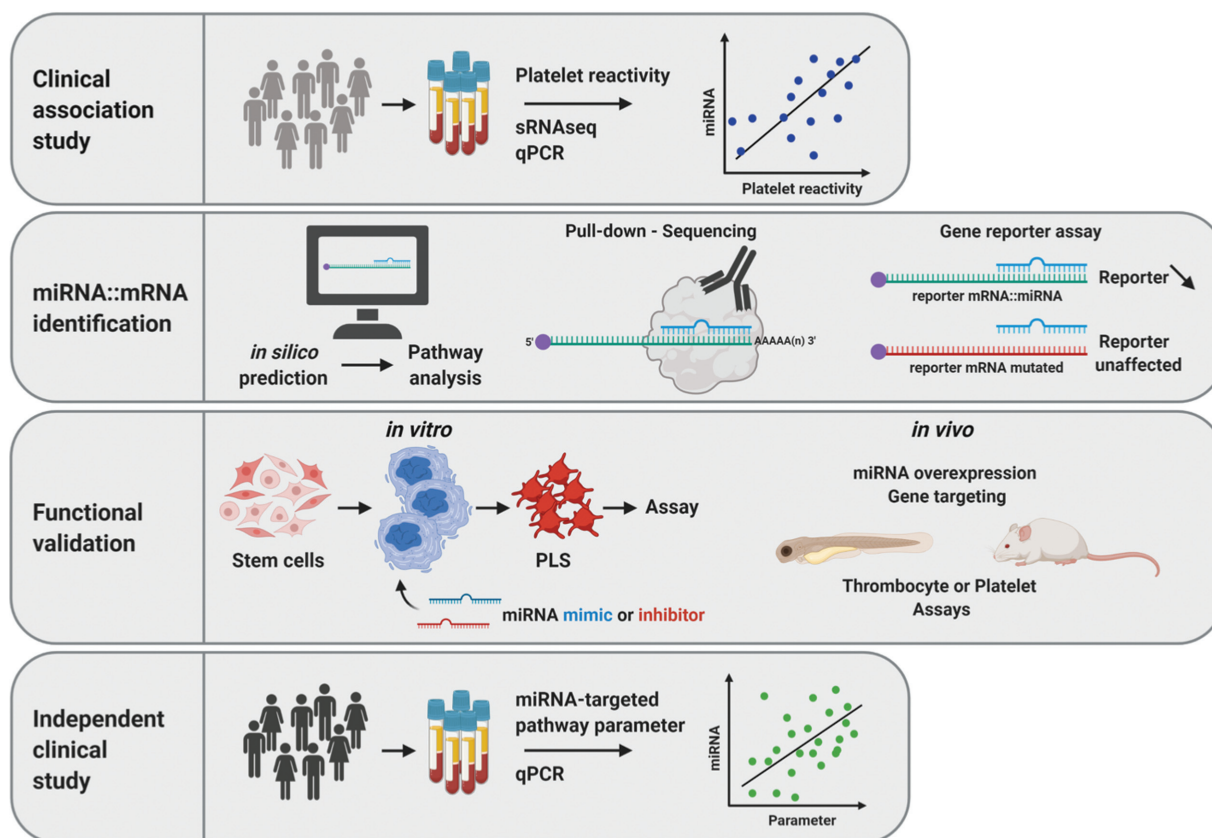


Fig. 1 Suggested general workflow guidelines for the identification and functional validation of miRNA involved in platelet reactivity.

Conclusion and Perspective

In recent decades, multiple lines of evidence point toward miRNAs as regulators of platelet function. The high stability of miRNAs in biological samples enables them to be used as potential biomarkers for diseases where platelets play a major role. Moreover, the identification of their key gene targets and the biological pathways they regulate may help in understanding the mechanisms of several diseases and identify new therapeutic options. Although a high number of clinical studies associated miRNA levels to PR or cardiovascular outcome, only a few have investigated the mechanisms involved.

► **Fig. 1** summarizes a proposed workflow to studying miRNA in the context of PR, with some of the techniques available at each step, from the identification of candidate miRNAs to their functional validation and the identification of their target genes and the biological pathways they regulate.

Clinical association studies using high-throughput techniques are usually the first step, with the identification of candidate platelet-derived miRNAs associated with the outcome, mostly PR or cardiovascular events. Identification of target genes using *in silico* and *in vitro* validation then allows the identification of the putative biological pathways involved. The impact of candidate miRNAs on platelet

morphology and function is then assessed in cellular models with various approaches used to modulate miRNA content and methods to measure platelet function. Promising candidate miRNAs may then be evaluated *in vivo* using animal models to investigate their roles directly in platelets on hemostatic properties and thrombus formation. Finally, selected miRNAs could be used to predict a biological outcome related to their functional impact on hemostasis in an independent clinical study,¹¹⁴ further supporting their role in modulating PR.

The identification of miRNA modulating PR and the mechanisms behind their activity is a growing research field that needs a true translational approach. In this review, we describe at least some of the pitfalls that should be carefully addressed and highlight the technical differences between some of the available clinical studies as well as the advantages and limitations of *in vitro* and *in vivo* strategies. A standardization initiative would probably allow a better comparison and replication of data among research groups and favor translation toward clinical application.

Authors' Contributions

A.G. had the initial idea and conceptualized this work, A.G., S.D.-G., and P.F. wrote the first draft of the manuscript. J.-L.R., M.N.-A., and R.J.F. critically revised the manuscript. All authors approved the manuscript.

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Conflict of Interest

None declared.

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