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
identification of miRNAs involved in PR may also be relevant beyond thrombosis and bleeding, for example, in immune and oncologic disorders\textsuperscript{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.\textsuperscript{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.\textsuperscript{15,16} In humans, 2,300 mature miRNAs have been described including 1,917 with annotations in miBase V22.1.\textsuperscript{17} Over 500 miRNAs are found in platelets.\textsuperscript{18} Although platelets are anucleate cells, they have all the machinery for mRNA translation and protein production.\textsuperscript{19} In mammalian cells, including human platelets, miRNAs also affect epigenetic gene regulation, leading to modification of both platelet biogenesis and function.\textsuperscript{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,\textsuperscript{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.\textsuperscript{21} However, it seems that the contribution of exosomes is marginal with approximately 0.01 miRNA molecules per exosome,\textsuperscript{22} while circulating miRNA content is close to that measured in microvesicles making them the main miRNA carrier in circulating blood.\textsuperscript{23} Since 41 to 45% of the microvesicles are released from platelets,\textsuperscript{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.\textsuperscript{24} Of note, noncanonical miRNA biogenesis pathways have been described, such as production of pre-miRNA independently of Drosha or Dicer pathways.\textsuperscript{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 miRNA-targeting properties.\textsuperscript{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.\textsuperscript{14} Atypical sites with a seed mismatch or a compensatory site exist; however, their formation requires more energy.\textsuperscript{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.\textsuperscript{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 (\textit{Table 1}). As mentioned before, the platelet-derived miRNAs carried by microvesicles in the circulation reflect the platelet physiology and platelet content.\textsuperscript{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.\textsuperscript{32}

As illustrated in \textit{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,\textsuperscript{33} whereas no correlation was measured using qPCR in platelet samples and adenosine diphosphate-induced platelet aggregation in cardiovascular patients treated with aspirin and clopidogrel.\textsuperscript{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.\textsuperscript{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    | 2010 | Plasma      | Healthy volunteers                                                      | N/A                                                                       | Microarray            | LTA with epinephrine 1.5 µM                                            | miR-96                          |                                  |
| Zampetaki et al  | 2012 | Plasma      | Population-based survey                                                | No treatment, DAPT, or aspirin                                           | qPCR                  | Myocardial infarction                                                    | miR-126                         | miR-223                          | miR-197                          |
| Willeit et al    | 2013 | Platelet, MV, PRP, PPP, serum | Healthy volunteers, patients with diabetes or with symptomatic carotid atherosclerosis | None or various antiplatelet drugs regimen | qPCR                  | Modified LTA in a 96-well plate using various agonists and concentrations, serum TXB2 assay, and VerifyNow assay | miR-223                         | miR-191                          | miR-126                          | miR-150                          |
| Shi et al        | 2013 | Platelet    | Acute coronary syndrome                                                | Clopidogrel plus aspirin                                                 | qPCR                  | LTA with ADP 10 µM, VASP                                               | miR-223                         | miR-96                           |
| Zufferey et al   | 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  | 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  | 2016 | Plasma      | Diabetes mellitus                                                       | N/A                                                                       | qPCR                  | TF-mediated thrombogenicity                                              | miR-126                         |                                  |
| Peng et al       | 2017 | Platelet    | Acute coronary syndrome                                                | Clopidogrel plus aspirin                                                 | qPCR                  | LTA with ADP 20 µM                                                      | miR-223                         | miR-221                          | miR-21                           |
| Ding et al       | 2019 | Platelet    | Acute coronary syndrome                                                | Clopidogrel plus aspirin                                                 | qPCR                  | LTA with AA 500 µg/mL and ADP 5 µM                                     | miR-204                         |                                  |
| Tang et al       | 2019 | Plasma      | Stable coronary artery disease                                          | Clopidogrel plus aspirin                                                 | qPCR                  | Clinical outcomes                                                      | miR-142                         |                                  |
| Liu et al        | 2020 | Platelet    | Acute coronary syndrome                                                | Clopidogrel plus aspirin                                                 | qPCR                  | Thromboelastography                                                    | miR-126                         | miR-223                          | 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 debated; 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. When using plasma, heparin should be avoided since it interferes with nucleic acid amplification procedures. 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 artificial 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.

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. 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.

**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. The efficiency of miRNA extraction using a Trizol-based method remains unclear; therefore, a highly efficient and standardized technique dedicated to miRNAs, using purification columns, is preferred. 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) 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. 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) and Nanostring technologies 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. 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. 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.

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. 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 serum and could be undetectable. 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. 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-48446-55), serum (e.g., miR-23a, let7a, and miR-126046,56,57), and platelet samples (e.g., miR-28, miR-29c, and miR-15157,58). 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.

**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. The photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) method improves the previous technology by addition of photoactive 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. 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.63,66 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 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 miRNAs 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, and impair 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. Their differentiation leads to the production of functional platelet-like structures (PLS). 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) or human hematopoietic stem cells, and the platelets derived from them. CD34+ cells are typically purified from peripheral blood (buffy coat), apheresis, or from umbilical cord blood. PLS produced in vitro are functionally close to human platelets. 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. PLS adhesion can be assessed in static or dynamic conditions since these techniques require only low concentrations of platelets. 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. Megakaryocytes express the same platelet receptors at their surface as PLS and can conveniently be used in functional assays as a proxy to platelets for the measurement of P-selectin secretion and the activated form of GPIIb/IIIa after stimulation, as well as in flow assays.

**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. 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 myeloblasts). 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%. 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. 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. 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. Interestingly, similar results have been demonstrated in hPSCs. As a general note, transfection can lead to the delivery of nonphysiological amounts of miRNAs.

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. The concentration of synthetic miRNAs in transfection mixtures is adjusted to reflect the delivery efficiency into cells according to the technique used, 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, 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.\textsuperscript{94} Thanks to an additional lipid moiety at the nucleotide sequence extremity, LNA can freely cross the cytoplasmic membrane without the help of a transfection reagent, a mechanism known as gyromatic delivery.\textsuperscript{95} This method has been used to downregulate miRNA in megakaryocytes.\textsuperscript{85} however, it does not seem to be suitable for studying platelet function. Indeed, Flierl et al.\textsuperscript{96} 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.\textsuperscript{92}

Lentiviral transduction is one of the most effective systems to deliver a nucleotide sequence into hPSCs\textsuperscript{97} and to overexpress or repress miRNAs.\textsuperscript{73,98} The lentivector genome is integrated into the host genome, leading to stable miRNA expression in a transduced cell line.\textsuperscript{99} This is suitable for hematopoietic stem cell transduction.\textsuperscript{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.\textsuperscript{101}

Stable regulation of gene expression has been reported in different studies using CRISPR/Cas9 tools\textsuperscript{102} and Transcription Activator-Like Effector Nucleases (TALEN),\textsuperscript{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.\textsuperscript{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,\textsuperscript{105} and then in several species such as *Drosophila melanogaster* (fruit fly),\textsuperscript{106} *Danio rerio* (zebrafish),\textsuperscript{107} and *Mus musculus* (mouse).\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 GPIb and GPVI.\textsuperscript{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.\textsuperscript{111}

Zebrafish with green fluorescent protein expression under control of the cd41 promoter (*tiga2b:EGFP* or *cd41:EGFP*) enables visualization of thrombocytes by their fluorescence.\textsuperscript{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 miRNAs, which makes the zebrafish model particularly interesting to investigate the impact of candidate miRNA on thrombus formation in vivo.\textsuperscript{113,114}

Genetic modifications in zebrafish enable tissue-specific expression and constitutive or conditional mutations, depending on the regulatory elements used.\textsuperscript{109,114,115} The early zebrafish embryo is easy to microinjection, 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)\textsuperscript{116–118} and can be used to alter miRNA expression specifically in thrombocytes.\textsuperscript{114}

Forward genetic mutagenesis screens traditionally used chemical mutagens to induce random mutations followed by phenotype–gene correlations.\textsuperscript{119} Reverse genetics using gene targeting became commonplace with the use of nuclelease-based genome editing.\textsuperscript{120} Knockout zebrafish are produced by stable gene disruption obtained with TALEN\textsuperscript{121} or CRISPR-Cas9 systems.\textsuperscript{122} Tilling\textsuperscript{123} or zinc finger nucleases\textsuperscript{124} have also been used for reverse genetics, but these methods are less easily adopted than CRISPR-Cas9.\textsuperscript{123} The availability of high-quality, searchable reference genome sequences also greatly assists with genome targeting methods.\textsuperscript{126}

Transient overexpression can be made in zebrafish embryos by microinjection of miRNAs, miRNAs, or plasmids,\textsuperscript{127,128} whereas temporary gene or miRNA knockdowns can be achieved using antisense oligonucleotides, typically morpholinos\textsuperscript{129} 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. The use of antagonomirs stabilized with a phosphorothioate backbone is not encouraged since toxicity has been observed in zebrafish embryos.

Competitive miRNA inhibitors can be used in vivo, such as miRNA “sponges” or “decoys.” miRNA sponges are synthetic linear or circular nucleotide sequences presenting several consecutive binding sites for the miRNA of interest and lead to depletion of miRNA activity. Decoys are long noncoding RNAs forming a hairpin 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. The hairpin construct protects against RNases and miRISC-mediated degradation. Chimeric miRNA inhibition.

Mice

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

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 GPIb/IIa 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 evolution and Roux and coworkers 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. The first knockout resource for mice used recombination-mediated cassette exchange targeting vectors. This tool gave the possibility to alter miRNA expression and to create reporters or conditional mutants.

A genetically engineered mouse model (GEMM) generates mutations 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. This technology has been used to study the impact of miR-223 on platelet production and function in chimeric bone marrow. 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 mice with constitutive or conditional expression and tissue specificity. For example, mice carrying the platelet-factor 4-Cre transgene drive loxP recombination in megakaryocytes, platelets, and leukocytes, directing lineage-restricted regulation. Pf4-Cre knockout of Dicer in mice results in dysregulation of miRNA expression and platelet function, suggesting that miRNAs are key players in platelet mRNA regulation in mice, without identifying which miRNAs are important. 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 GPIba-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.

The CRISPR and TALEN technologies, as described above, are highly efficient tools used for genome editing. In mice, 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.

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. Although LNA sequences have been shown to impact PR in human platelets, Kaudewitz and coworkers 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 over-expression of a miRNA after injection in mice.

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

References
1 Thom JN, Italiano JE. Platelet formation. Semin Hematol 2010;47 (03):220–226
2 Bray PF, Mathias RA, Faraday N, et al. Heritability of platelet function in families with premature coronary artery disease. J Thromb Haemost 2007;5(08):1617–1623
3 Reny JL, De Moeroosse P, Dauzat M, Fontana P. Use of the PFA-100 closure time to predict cardiovascular events in aspirin-treated cardiovascular patients: a systematic review and meta-analysis. J Thromb Haemost 2008;6(03):444–450
4 Combescure C, Fontana P, Mallouk N, et al; CLOpidogrel and Vascular Ischemic Events Meta-analysis Study Group. Clinical implications of clopidogrel non-response in cardiovascular patients: a systematic review and meta-analysis. J Thromb Haemost 2010;8(05):923–933
5 Tantry US, Gurbel PA. Antiplatelet drug resistance and variability in response: the role of antiplatelet therapy monitoring. Curr Pharm Des 2013;19(21):3795–3815
6 Shuldiner AR, O’Connell JR, Blinden KP, et al. Association of cytochrome P450 2C19 genotype with the antiplatelet effect and clinical efficacy of clopidogrel therapy. JAMA 2009;302(08):849–857
7 Edelstein LC, McKenzie SE, Shaw C, Holinstat MA, Kunapuli SP, Bray PF. MicroRNAs in platelet production and activation. J Thromb Haemost 2013;11(Suppl 1):340–350
8 Willeit P, Zampetaki A, Dudek K, et al. Circulating microRNAs as novel biomarkers for platelet activation. Circ Res 2013;112(04):595–600
9 Kaudewitz D, Skroblin P, Bender LH, et al. Association of Micro-RNAs and YRNAs with platelet function. Circ Res 2016;118(03):420–432
10 Shi R, Zhou X, Ji WJ, et al. The emerging role of miR-223 in platelet reactivity: implications in antiplatelet therapy. BioMed Res Int 2015;2015:981841
11 Zufferey A, Liberson M, Reny JL, et al. New molecular insights into modulation of platelet reactivity in aspirin-treated patients using a network-based approach. Hum Genet 2016;135(04):403–414
12 Zufferey A, Kapur R, Semple JW. Pathogenesis and therapeutic mechanisms in immune thrombocytopenia (ITP). J Clin Med 2017;6(02):E16
13 Leblanc R, Peyruchaud O. Metastasis: new functional implications of platelets and megakaryocytes. Blood 2016;128(01):24–31
14 Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian miRNAs are conserved targets of microRNAs. Genome Res 2009;19(01):92–105
15 Semple JW, Italiano JE Jr, Freedman J. Platelets and the immune continuum. Nat Rev Immunol 2011;11(04):264–274
16 Leblanc R, Houssin A, Peyruchaud O. Platelets, autotaxin and lysophosphatidic acid signalling: win-win factors for cancer metastasis. Br J Pharmacol 2018;175(15):3100–3110
17 Alles J, Fehlmann T, Fischer U, et al. An estimate of the total number of true human miRNAs. Nucleic Acids Res 2019;47(07):3353–3364
18 Plö H, Landry P, Benham A, Coarfa C, Gunaratne PH, Provost P. The repertoire and features of human platelet microRNAs. PLoS One 2012;7(12):e50746
19 Landry P, Plante I, Ouellet DL, Perron MP, Rousseau G, Provost P. Existence of a microRNA pathway in anucleate platelets. Nat Struct Mol Biol 2009;16(09):961–966
20 Arroyo JD, Chevillet JR, Kroh EM, et al. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proc Natl Acad Sci U S A 2011;108 (12):5003–5008
21 O’Brien K, Breynke N, Ughetto S, Laurent LC, Breakfield XO. RNA delivery by extracellular vesicles in mammalian cells and its applications. Nat Rev Mol Cell Biol 2020
22 Provost P. The clinical significance of platelet microparticle-associated microRNAs. Clin Chem Lab Med 2017;55(05):657–666
23 Diehl P, Fricke A, Sander L, et al. Microparticles: major transport vehicles for distinct microRNAs in circulation. Cardiovasc Res 2012;93(04):633–644
24 Winter J, Jung S, Keller S, Gregory RL, Diederichs S. Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat Cell Biol 2009;11(03):228–234
25 Yang JS, Lai EC. Alternative miRNA biogenesis pathways and the interpretation of core miRNA pathway mutants. Mol Cell 2011;43(06):892–903
26 Desvignes T, Batzel P, Berezikov E, et al. miRNA nomenclature: a view incorporating genetic origins, biosynthetic pathways, and sequence variants. Trends Genet 2015;31(11):613–626
27 Crimmon A, Farh KK, Johnston WK, Garrett-Engele P, Lim LP, Bartel DP. MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Mol Cell 2007;27(01):91–105
28 Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009;136(02):215–233
29 Xia L, Zeng Z, Tang WH. The role of platelet microparticle-associated microRNAs in cellular crosstalk. Front Cardiovasc Med 2018;5:29
30 Camaioni C, Gustapeva M, Cialdella P, Della Bona R, Biasucci LM. Microparticles and microRNAs: new players in the complex field of coagulation. Intern Emerg Med 2013;8(04):291–296
31 Anene C, Graham AM, Boyne J, Roberts W. Platelet micro-particle delivered microRNA-Let-7a promotes the angiogenic switch. Biochim Biophys Acta Mol Basis Dis 2018;1846(08):2633–2643
32 Sunderland N, Skroblin P, Barwari T, et al. MicroRNA biomarkers and platelet reactivity: the clot thickens. Circ Res 2017;120(02):418–435
33 Kondkar AA, Bray MS, Leal SM, et al. VAMP8/endobrevin is over-expressed in hyperreactive human platelets: suggested role for platelet microRNA. J Thromb Haemost 2010;8(02):369–378
34 Shi R, Ge L, Zhou X, et al. Decreased platelet miR-223 expression is associated with high on-clopidogrel platelet reactivity. Thromb Res 2013;131(06):508–513
35 Fontana P, Roffi M, Reny JL. Platelet function test use for patients with coronary artery disease in the early 2020s. J Clin Med 2020;9(01):E194
36 Wang K, Yuan Y, Cho JH, McClarty S, Baxter D, Galas DJ. Comparing the MicroRNA spectrum between serum and plasma. PLoS One 2012;7(07):e41561
37 Kaudewitz D, Lee R, Willeit P, et al. Impact of intravenous heparin on quantification of circulating microRNAs in patients with coronary artery disease. Thromb Haemost 2013;110(03):609–615
38 Fejes Z, Pölüska S, Czimrer Z, et al. Hyperglycaemia suppresses microRNA expression in platelets to increase P2RY12 and SELP levels in type 2 diabetes mellitus. Thromb Haemost 2017;117 (03):529–542
39 Cazenave JP, Ohlmann P, Cassel D, Eckly A, Hechler B, Gachet C. Preparation of washed platelet suspensions from human and rodent blood. Methods Mol Biol 2004;272:13–28
40 Tran JQD, Pedersen OH, Larsen ML, et al. Platelet microRNA expression and association with platelet maturity and function in patients with essential thrombocytopenia. Platelets 2020;31(03):365–372
41 Binderup HG, Madsen JS, Heegaard NH, Houlin K, Andersen RF, Brasen CL. Quantification of microRNA levels in plasma -
impact of preanalytical and analytical conditions. PLoS One 2018;13(07):e0201069
42 Cheng HH, Yi HS, Kim Y, et al. Plasma processing conditions substantially influence circulating microRNA biomarker levels. PLoS One 2013;8(06):e64795
43 Tiberio P, Callari M, Angeloni V, Daidone MG, Appierto V. Challenges in using circulating miRNAs as cancer biomarkers. BioMed Res Int 2015;2015:731479
44 Jansen F, Schäfer L, Wang H, et al. Kinetics of circulating MicroRNAs in response to cardiac stress in patients with coronary artery disease. J Am Heart Assoc 2017;6(08):e005270
45 Zampetaki A, Williet P, Tilling I, et al. Prospective study on circulating MicroRNAs and risk of myocardial infarction. J Am Coll Cardiol 2012;60(04):290–299
46 Blondal T, Jønksen Nielsen S, Baker A, et al. Assessing sample and miRNA profile quality in serum and plasma or other biofluids. Methods 2013;59(01):51–56
47 Simon LM, Edelstein LC, Nagalla S, et al. Human platelet microRNA-miRNA networks associated with age and gender revealed by integrated plateletomics. Blood 2014;123(16):e37–e45
48 Coenen-Stass AM, Magen I, Brooks T, et al. Evaluation of methodologies for microRNA biomarker detection by next generation sequencing. RNA Biol 2018;15(08):1133–1145
49 Pordzik J, Jakubik D, Jarosz-Popek J, et al. Significance of circulating microRNAs in diabetes mellitus type 2 and platelet reactivity: bioinformatic analysis and review. Cardiovasc Diabetol 2019;18(01):113
50 Jakob P, Kacprowski T, Briand-Schumacher S, et al. Profiling and validation of circulating microRNAs for cardiovascular events in patients presenting with ST-segment elevation myocardial infarction. Eur Heart J 2017;38(07):511–515
51 Benz F, Roderburg C, Vargas Cardenas D, et al. U6 is unsuitable for normalization of serum microRNA levels in patients with sepsis or liver fibrosis. Exp Mol Med 2013;45:4e2
52 McDonald JS, Milosevic D, Reddi HV, Grebe SK, Algeciras-Schimnich A. Analysis of circulating microRNA: preanalytical and analytical challenges. Clin Chem 2011;57(06):833–840
53 Tanaka M, Oikawa K, Takanashi M, et al. Down-regulation of miR-92 in human plasma is a novel marker for acute leukemia patients. PLoS One 2009;4(05):e5532
54 Zalewski K, Misiek M, Kowalik A, et al. Normalizers for microRNA quantification in plasma of patients with vulvar intraepithelial neoplasia lesions and vulvar carcinoma. Tumour Biol 2017;39(11):1010428317717140
55 Mompeón A, Ortega-Paz L, Vidal-Gómez X, et al. Disparate miRNA expression in serum and plasma of patients with acute myocardial infarction: a systematic and paired comparative analysis. Sci Rep 2020;10(01):5373
56 Li Y, Xiang GM, Liu LL, et al. Assessment of endogenous reference gene suitability for serum exosomal microRNA expression analysis in liver carcinoma resection studies. Mol Med Rep 2015;12(03):4683–4691
57 Kok MG, Halliani A, Moerland PD, Meijers JC, Creemers EE, Pinto-Sietsma SJ. Normalization panels for the reliable quantification of circulating microRNAs by RT-qPCR. FASEB J 2015;29(09):3853–3862
58 Garcia A, Dunoyer-Geindre S, Zappillo V, Nolli S, Reny JL, Fontana P. Functional validation of microRNA-126-3p as a platelet reactivity regulator using human haematopoietic stem cells. Thromb Haemost 2019;119(02):254–263
59 Chou CH, Lin FM, Chou MT, et al. A computational approach for identifying microRNA-target interactions using high-throughput CLIP and PAR-CLIP sequencing. BMC Genomics 2013;14(Suppl 1):S2
60 Hafner M, Landthaler M, Burger L, et al. PAR-CLIP—a method to identify transcriptome-wide the binding sites of RNA binding proteins. J Vis Exp 2010;(41):2034
61 Helwak A, Kudla G, Dudnakaova T, Tollervey D. Mapping the human miRNA interactome by CLASH reveals frequent non-canonical binding. Cell 2013;153(03):654–665
62 Steinkraus BR, Toegel M, Fulga TA. Tiny giants of gene regulation: experimental strategies for microRNA functional studies. Wiley Interdiscip Rev Dev Biol 2016;5(03):311–362
63 Hafner M, Landthaler M, Burger L, et al. Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP. Cell 2010;141(01):129–141
64 Betel D, Koppal A, Agius P, Sander C, Leslie C. Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites. Genome Biol 2010;11(08):R90
65 Quillet A, Saad C, Ferry G, et al. Improving bioinformatics prediction of microRNA targets by ranks aggregation. Front Genet 2020;10:1330
66 Chu YW, Chang KP, Chen CW, Liang YT, Soh ZT, Hsieh LC. miRgo: integrating various off-the-shelf tools for identification of microRNA-target interactions by heterogeneous features and a novel evaluation indicator. Sci Rep 2020;10(01):1466
67 Moore AC, Winkjer JS, Tseng TT. Bioinformatics resources for MicroRNA discovery. Biomark Insights 2016;10(Suppl 4):53–58
68 Huntley RP, Sitnikov D, Orlic-Milacic M, et al. Guidelines for the functional annotation of microRNAs using the Gene Ontology. RNA 2016;22(05):667–676
69 Deng L, Wang J, Zhang J. Predicting gene ontology function of human MicroRNAs by integrating multiple networks. Front Genet 2019;10:3
70 Cloonan N. Re-thinking miRNA-mRNA interactions: intertwining issues confound target discovery. BioEssays 2015;37(04):379–388
71 Tan SM, Lieberman J. Capture and identification of microRNA targets by biotin pulldown and RNA-seq. Methods Mol Biol 2016;1358:211–228
72 Jin Y, Chen Z, Liu X, Zhou X. Evaluating the microRNA targeting sites by luciferase reporter gene assay. Methods Mol Biol 2013;936:117–127
73 Bianchi E, Bulgarelli J, Ruberti S, et al. MYB controls erythropoiesis versus megakaryocyte lineage fate decision through the miR-486-3p-mediated downregulation of MAF. Cell Death Differ 2015;22(12):1906–1921
74 Hong W, Kondkar AA, Nagalla S, et al. Transfection of human platelets with short interfering RNA. Clin Transl Sci 2011;4(03):180–185
75 Edelstein LC, Simon LM, Montoya RT, et al. Racial differences in human platelet PAR4 reactivity reflect expression of PCTP and miR-376c. Nat Med 2013;19(12):1609–1616
76 Dhenge A, Kuhikar R, Kale V, Limaye L. Regulation of differentiation of MEG01 to megakaryocytes and platelet-like particles by valproic acid through Notch3 mediated actin polymerization. Platelets 2019;30(06):780–795
77 Barwari T, Eismana S, Mayr U, et al. Inhibition of probiotic microRNA-21 affects platelets and their releasate. JCI Insight 2018;3(21):123335
78 Woolthuis CM, Park CY. Hematopoietic stem/progenitor cell commitment to the megakaryocyte lineage. Blood 2016;127(18):1242–1248
79 Sim X, Poncz M, Gadue P, French DL. Understanding platelet generation from megakaryocytes: implications for in vitro-devised platelets. Blood 2016;127(10):1227–1233
80 Romania P, Lulli V, Pelosi E, Biffoni M, Peschle C, Marziali G. MicroRNA 155 modulates megakaryopoiesis at progenitor and precursor level by targeting Ets-1 and Meis1 transcription factors. Br J Haematol 2008;143(04):570–580
81 Choi ES, Nichol JL, Hokom MM, Hornkohl AC, Hunt P. Platelets generated in vitro from proplatelet-displaying human megakaryocytes are functional. Blood 1995;86(02):402–413
82 Sangkhu K, Shuldiner AR, Klein TE, Altman RB. Platelet aggregation pathway. Pharmacogenet Genomics 2011;21(08):516–521
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83 Kamat V, Muthard RW, Li R, Diamond SL. Microfluidic assessment of functional culture-derived platelets in human thrombocytopoiesis. J Thromb Haemost 2015;13(10):2023-2032

84 Auber M, Fröhlich D, Drechsel O, Karaulanov E, Krämer-Albers EM. Serum-free media supplements carry miRNAs that co-purify with extracellular vesicles. J Extracell Vesicles 2019;8(1):1656042

85 Basak I, Bhatelekar S, Manne BK, et al. miR-15a-5p regulates expression of multiple proteins in the megakaryocyte GPVI signaling pathway. J Thromb Haemost 2019;17(03):511–524

86 Wang T, Larcher LM, Ma L, Veedu RN. Systematic screening of commercially available common transfection reagents towards efficient transfection of single-stranded oligonucleotides. Molecules 2018;23(10):2526

87 Spinello I, Quaranta MT, Pasquinii L, et al. PLZF-mediated control on c-kit expression in CD34+ cells and early erythropoiesis. Oncogene 2009;28(23):2276–2288

88 Diener Y, Bosio A, Bissels U. Delivery of RNA-based molecules to human hematopoietic stem and progenitor cells for modulation of gene expression. Exp Hematol 2016;44(11):991–1001

89 Zhou Y, Abraham S, Andre P, et al. Anti-miR-148a regulates platelet FcγRIIA signaling and decreases thrombosis in vivo. Blood 2015;126(26):2871–2881

90 Maurisse R, De Semir D, Emamekhoo H, et al. Comparative transfection of DNA into primary and transformed mammalian cells from different lineages. BMC Biotechnol 2010;10:9

91 Starili Tabar M, Hesarak M, Esfandari F, Sahneshin Samani F, Vakilian H, Baharvand H. Evaluating electroporation and liposome transfection of DNA into primary and transformed mammalian cells. J Cell Mol Med 2009;13(11):3333–3346

92 Kurreck J. Antisense technologies. Improvement through novel mechanisms of clustered microRNA regulation and function of miR-219 promotes differentiation of human induced pluripotent stem cells. J Cell Mol Med 2009;13(11):3018–3030

93 Fish RJ, Di Sanza C, Neerman-Arbez M. Targeted mutation of the zebrafish genome sequence and its relationship to the human genome. Genome Res 2003;13(12):2707–2716

94 Nazari B, Soleimani M, Ebrahimi-Barough S, et al. Overexpression of miR-219 promotes differentiation of human induced pluripotent stem cells into pre-oligoendrocyte. J Chem Neuroanat 2018;73:16–26

95 van Til NP, Wagemaker G. Lentiviral gene transduction of mouse and human hematopoietic stem cells. Methods Mol Biol 2014;1185:311–319

96 Sebrow J, Goff SP, Griffin DO. Successfully transfected primary peripherally mobilized human CD34+ hematopoietic stem and progenitor cells (HSPCs) demonstrate increased susceptibility to retroviral infection. Virol J 2020;17(01):22

97 Lataniotis I, Albrecht A, Kok PO, et al. CRISPR/Cas9 editing reveals novel mechanisms of clustered microRNA regulation and function. Sci Rep 2017;7(01):8358

98 Mir P, Ritter M, Welte K, Skolikova J, Klimiankou M. Gene knockout in hematopoietic stem and progenitor cells followed by granulocytic differentiation. Methods Mol Biol 2020;2115:455–469

99 Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 1993;75(05):843–854

100 Carthew RW, Agbu P, Giri R. MicroRNA function in Drosophila melanogaster. Semin Cell Dev Biol 2017;65:29–37

101 Schier AF, Giraldez AJ. MicroRNA function and mechanism: insights from zebra fish. Cold Spring Harb Symp Quant Biol 2006;71:195–203

102 Lewis MA, Steel KP. MicroRNAs in mouse development and disease. Semin Cell Dev Biol 2010;21(07):774–780

103 Pal AS, Kasinski AL. Animal models to study MicroRNA function. Adv Cancer Res 2017:135:53–118

104 Lang MR, Gih G, Gawaz MP, Müller B. Hemostasis in Danio rerio: is the zebrafish a useful model for platelet research? J Thromb Haemost 2010;8(06):1159–1169

105 Gregory M, Hanumanthaihara R, Jagadeesanwar P. Genetic analysis of hemostasis and thrombosis using vascular occlusion. Blood Cells Mol Dis 2002;29(03):286–295

106 Lin HF, Traver D, Zhu H, et al. Analysis of thrombocyte development in CD41-GFP transgenic zebrafish. Blood 2005;106(12):3803–3810

107 Roberto VP, Tiago DM, Gauvitsk K, Cancela ML. Evidence for the conservation of miR-223 in zebrafish (Danio rerio): Implications for function. Gene 2015;566(01):54–62

108 Apelikov B, Fish RJ, Garcia A, et al. MicroRNA-126 is a regulator of platelet-supported thrombin generation. Platelets 2020;31(06):746–755

109 Dong M, Fu YF, Du TT, et al. Heritable and lineage-specific gene knockdown in zebrafish embryo. PLoS One 2009;4(07):e6125

110 Thermes V, Grabber C, Ristoratore F, et al. I-SceI meganuclease mediates highly efficient transgenesis in fish. Mech Dev 2002;118(1–2):91–98

111 Abe G, Suster ML, Kawakami K. Tol2-mediated transgenesis, gene trapping, enhancer trapping, and the Gal4-UAS system. Methods Cell Biol 2011;104:23–49

112 Suster ML, Abe G, Schouw A, Kawakami K. Transposon-mediated BAC transgenesis in zebrafish. Nat Protoc 2011;6(12):1998–2011

113 Haffter P, Granato M, Brand M, et al. The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio. Development 1996;123:1–36

114 Housden BE, Muhar G, Gemberling M, et al. Loss-of-function genetic tools for animal models: cross-species and cross-platform differences. Nat Rev Genet 2017;18(01):24–40

115 Bassett AR, Azaam G, Wheatley L, et al. Understanding functional miRNA-target interactions in vivo by site-specific genome engineering. Nat Commun 2014;5:4640

116 Hwang WY, Fu Y, Reyon D, et al. Efficient genome editing in zebrafish using a CRISPR-Cas system. Nat Biotechnol 2013;31(03):227–229

117 Wienholds E, van Eeden F, Kosters M, Mudde J, Plasterk RH, Cuppen E. Efficient target-selected mutagenesis in zebrafish. Genome Res 2003;13(12):2700–2707

118 Fish RJ, Di Sanza C, Neerman-Arbez M. Targeted mutation of zebrafish fga models human congenital afibrinogenemia. Blood 2014;123(14):2278–2281

119 Gut P, Reischauer S, Stainer Dyr, Arnaout R. Little fish, big data: zebrafish as a model for cardiovascular and metabolic disease. Physiol Rev 2017;97(03):889–938

120 Howe K, Clark MD, Torroja CF, et al. The zebrafish reference genome sequence and its relationship to the human genome. Nature 2013;496(7446):498–503

121 Yin VP, Lepilina A, Smith A, Poss KD. Regulation of zebrafish heart regeneration by miR-133. Dev Biol 2012;365(02):319–327
