Investigation of micro-RNAs as modulators of platelet reactivity: a translational approach

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INVESTIGATION OF MICRO-RNAS AS MODULATORS OF PLATELET REACTIVITY: A TRANSLATIONAL APPROACH

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Table of content

FIGURES INDEX

TABLES INDEX

ABBREVIATIONS

3'UTR	3'Untranslated region
ADAM9	Disintegrin and metalloproteinase domain 9
ADP	Adenosin diphosphate
Ago	Argonaute
AhR	Aryl hydrocarbon receptor
bp	Base pair
CCR4-NOT	Carbon catabolite repression-negative on TATA-less
CD	Cluster of Differentiation
CDC42	Cell division control protein 42
CMP	Common myeloid progenitor
C-mpl	Thrombopoietin receptor
CXCL1	C-X-C motif chemokine receptor 1
DAPT	Dual antiplatelet therapy
DGCR8	DiGeorge Syndrome Critical Region 8
DMS	Demarcation membrane system
ESCRT	Endosomal sorting complex required for transport
EV	Extra vesicle
FACS	Fluorescence-activated cell sorter scan
GAP	GTPase-activating protein
GEF	Guanine nucleotide exchange factor
GDP	Guanosine diphosphate
GPCR	G protein-coupled receptor
GTP	Guanosine triphosphate
hESC	Human embryonic stem cell
HSC	Hematopoietic stem cell
IL	Interleukin

iPSC	Induced pluripotent stem cell
JAK2	Janus kinase 2
LDL	Low density lipoprotein
LNA	Locked nucleic acid
LRO	Lysosome-related organelle
miRNA	microRNA
MPP	Multipotent progenitor
MSC	Mesenchymal stromal cell
MV	Microvesicle
MYH9	Myosin Heavy Chain 9
NBEAL2	Neurobeachin Like 2
PDI	Protein disulfide isomerase
PF4	Platelet factor 4
PLS	Platelet-like structure
PS	Phosphatidylserine
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
SCF	Stem cell factor
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
snoRNA	Small nucleolar RNA
SR1	StemRegenin 1
STAT	Signal transducer and activator of transcription
TEM	Transmission electronic microscopy
TGN	Trans-Golgi network
TLR9	Toll-like receptor 9
TNRC6	Trinucleotide repeat-containing gene 6A protein

TPO	Thrombopoietin
TRAP	Thrombin receptor agonist peptide
tRNA	Transfer RNA
TXA2	Thromboxane A2
VPS	Vacuolar protein sorting
VWF	Von Willebrand Factor
WASP	Wiskott-Aldrich syndrome protein

RÉSUMÉ

L'hémostase est un processus complexe qui se produit à la suite d'une lésion vasculaire pour prévenir l'hémorragie. Les plaquettes y jouent un rôle clé. En effet, l'exposition des composants sous-endothéliaux lorsque les vaisseaux sanguins sont endommagés provoque l'activation et l'agrégation des plaquettes afin de stopper le saignement. En outre, l'activation des plaquettes induit l'exposition de phosphatidylsérines à leur surface qui promeuvent la génération de thrombine et la formation de fibrine. La fonction (ou réactivité) plaquettaire est variable d'un individu à l'autre, avec une composante héréditaire importante qui peut être en relation avec la récidive d'évènements thrombotiques ou la survenue d'évènements hémorragiques. Les plaquettes sont la cible de médicaments inhibant leur fonction : les antiplaquettaires. Ces antithrombotiques sont prescrits principalement aux patients cardiovasculaires, avec une efficacité biologique variable d'un patient à l'autre. Les plaquettes ont également un rôle dans d'autres processus physiologiques comme la réponse immunitaire et la prolifération cellulaire. L'identification des déterminants de la réactivité plaquettaire peut donc avoir des répercussions dans de nombreux domaines en dehors de l'hémostase et pourrait permettre la mise au point de nouvelles stratégies thérapeutiques.

Au cours des dernières décennies, il a été démontré que de courtes séquences d'ARN non codant, appelées microARNs (miARNs), peuvent réguler un grand nombre de processus biologiques et la fonction de plusieurs types cellulaires. Les plaquettes contiennent un nombre élevé de miARNs qui sont libérés dans le plasma lors de l'activation des plaquettes. Les miARNs sont très stables dans la circulation, c'est pourquoi un nombre croissant d'études cliniques s'intéressent à la corrélation entre la réactivité plaquettaire et le niveau de certains miARNs circulants. Ces dernières ont identifié plusieurs miARNs qui pourraient être des biomarqueurs de la réactivité plaquettaire. A l'heure actuelle, l'impact des miARNs sur la fonction plaquettaire a très peu été étudié. Les modèles existants reposent sur l'utilisation de souris ou de lignées cellulaires immortalisées mais un modèle utilisant des cellules humaines fait défaut.

L'objectif de ce projet est de valider les miARNs candidats en tant que régulateurs de la fonction plaquettaire, et d'étudier les mécanismes sous-jacents avec une approche translationnelle comprenant notamment un modèle de cellule humaine.

La transfection ne permet pas de moduler le niveau de miARNs dans les plaquettes humaines sans altérer leur fonction. Nous avons donc utilisé des cellules souches hématopoïétiques pour produire *in vitro* des mégacaryocytes, qui peuvent être transfectés, et récolter ensuite les plaquettes qui en sont issues. La transfection de mégacaryocytes permet la modulation du niveau de miARNs dans les

plaquettes qui sont libérées en fin de différenciation. miR-126-3p, déjà connu pour augmenter l'agrégation plaquettaire dans un model murin, a été sélectionné pour étudier les performances de ce modèle. Ainsi, nous démontrons que la transfection dans des mégacaryocytes humains de séquences synthétiques mimant le miR-126-3p induit une augmentation de la réactivité plaquettaire. Dans cette étude, il a également été démontré que miR-126-3p régule l'expression de la plexine B2 (PLXNB2), un récepteur de la sémaphorine, et que la diminution de l'expression de le PLXNB2 avec un siRNA augmente la réactivité plaquettaire. L'une des voies permettant l'augmentation de la réactivité plaquettaire via miR-126-3p utiliserait donc un mécanisme impliquant PLXNB2. Cette étude confirme que ce modèle de cellules souches hématopoïétiques humaines permet d'analyser l'impact de miARNs sur la réactivité plaquettaire.

La deuxième partie du projet consiste à étudier miR-204-5p, précédemment décrit par notre groupe comme un possible régulateur de la fonction plaquettaire. L'étude morphologique et fonctionnelle de l'effet de miR-204-5p sur les mégacaryocytes et les plaquettes a montré que miR-204 modifie l'actine du cytosquelette par le biais des protéines Rho GTPase. Cela conduit à une perturbation qualitative de la biogenèse des plaquettes et à une augmentation de la réactivité plaquettaires via un accroissement de l'adhésion au fibrinogène.

Enfin, la dernière partie du projet est une étude clinique utilisant la cohorte ADRIE (Antiplatelet Drug Resistance and Ischemic Events). Le niveau d'une sélection de miARNs (miR-126-3p, miR-204-5p, miR-223-3p et miR-150-5p) a été mesuré dans le plasma de patients cardiovasculaires cliniquement stables sous aspirine. Les résultats ont montré une corrélation positive entre certains miARNs testés et l'agrégation plaquettaire avec différents agonistes ainsi qu'une corrélation positive avec les marqueurs de génération de thrombine *in vivo*. Une analyse *in silico* des cibles respectives des miARNs nous a conduit à proposer plusieurs gènes dont la régulation de l'expression par les miARNs pourrait expliquer l'augmentation de la réactivité plaquettaire.

Les analyses combinées de l'effet des miARNs sur la fonction plaquettaire et les résultats de l'étude clinique permettent de mieux comprendre l'action des miARNs sur la physiologie plaquettaire. De plus, le modèle de cellules souches hématopoïétiques humaines développé pourrait permettre d'identifier un grand nombre de miARNs comme régulateurs de la réactivité plaquettaire dans de futures études. Ces miARNs pourraient être utilisés comme biomarqueurs pour prédire la réactivité des plaquettes et le risque d'évènements cardiovasculaires ou comme de nouvelles cibles thérapeutiques.

ABSTRACT

Haemostasis is a complex process occurring to prevent bleeding following vessel injury. One of the key players of haemostasis are platelets, thanks to their ability to trigger the first step of the haemostatic process. Indeed, platelet activation and aggregation are promoted after close contact with sub-endothelial components exposed after vessel injury. In addition, platelet activation induces phosphatidylserine exposure at their surface, triggering thrombin generation and fibrin formation. Platelet function (or reactivity) varies from one individual to another, a phenotype that is largely inherited and may be related to the recurrence of thrombotic events or the occurrence of bleeding events. Platelets are targeted by drugs that inhibit their functions: antiplatelet drugs. These antithrombotics are prescribed mainly to cardiovascular patients, with a biological effectiveness that differs from one patient to another. Platelets also play a role in other physiological processes such as immune response and cell proliferation. The identification of determinants of platelet reactivity can therefore have implications in many areas beyond haemostasis and allows the development of new therapeutic strategies in several diseases.

Over the last decades, small non-coding RNAs, called microRNAs (miRNAs), have been shown to be regulators of several biological processes including biogenesis and function in several cell types. Platelets contain a high number of miRNAs that are released into the circulation during platelet activation. MiRNAs are highly stable in the plasma; this property suggests that miRNAs could be used as biomarkers of platelet reactivity. Numerous clinical trials showed a correlation between platelet reactivity and circulating miRNA levels and identified several miRNAs as putative biomarkers of platelet reactivity or clinical outcomes. The impact of miRNAs on platelet function has been scarcely investigated. The existing models use mice or immortalized cell lines; however, a human-derived cell model is lacking.

The aim of this project is to functionally validate candidate miRNAs as regulators of platelet function and to study the underlying mechanisms with a translational approach, including a human-derived cell model.

Human platelet transfection does not allow modulation of miRNAs content without impairing their function. Therefore, we developed a model of *in vitro* differentiation of hematopoietic stem cells to produce megakaryocytes and platelet-like structures. The transfection of megakaryocytes allows the modulation of miRNA levels in platelet-like structures subsequently released that can be used for functional evaluation. As a proof of concept, we used miR-126-3p, one of the validated regulators of platelet function in a mice model. We showed that synthetic miR-126-3p mimic sequences transfected

in human megakaryocytes increase the miR-126-3p level into platelet-like structures. Platelet function tests confirmed that miR-126-3p upregulation is associated with an increased platelet reactivity. The reporter gene assay showed that miR-126-3p targets the plexinB2 (PLXNB2) mRNA, a semaphorin receptor. In addition, the increased platelet reactivity observed after miR-126-3p overexpression was recapitulated by PLXNB2 silencing. This work validated our model of human hematopoietic stem cells which allows the functional investigation of miRNAs as modulators of platelet reactivity, and the study of underlying mechanisms.

The second part of the project investigates miR-204-5p, described as a putative regulator of platelet function in a previous study of our group. The morphological and functional investigations of the effect of miR-204-5p on megakaryocytes and platelet-like structures showed that miR-204-5p regulates actin cytoskeleton structures through modulation of Rho GTPase proteins. This led to a qualitative modification of platelet-like structures biogenesis and an increase of platelet reactivity by modulation of fibrinogen adhesion.

Finally, the last part of the project is a clinical study using the ADRIE cohort (Antiplatelet Drugs Resistance and Ischemic Events). The level of a set of miRNAs (miR-126-3p, miR-204-5p, miR-223-3p and miR-150-5p) was measured in plasma samples from stable cardiovascular patients with aspirin. The results showed an association between selected miRNAs level and platelet aggregation after stimulation with various agonists and *in vivo* thrombin generation markers. An *in silico* analysis of the respective miRNA-targets lead to the identification of candidate genes governing platelet reactivity via miRNAs regulation.

The combined analysis of the effect of miRNAs on platelet reactivity and the results of the clinical association studies allow to a better understanding of the interaction of miRNAs on platelet function. In addition, the model of human hematopoietic stem cells could allow to validate a large number of miRNAs as regulators of platelet reactivity in further studies. The validated miRNAs could then be used as biomarkers to predict platelet reactivity and cardiovascular events or as therapeutic targets.

1 INTRODUCTION

1.1 FOREWORD

Platelets (also called thrombocytes) are small megakaryocyte fragments mainly produced in the bone marrow.[1] Platelets play a crucial role in haemostasis and in multiple others processes including cell proliferation, innate immunity and inflammation.[2-5] Platelet reactivity with or without antiplatelet drugs is variable among individuals. In cardiovascular patients treated with these drugs, high or low on-treatment platelet reactivity has been associated with ischemic[6] or haemorrhagic events,[7] respectively. Therefore, the determinants of platelet reactivity have been largely investigated over the last decades. Family-based studies showed that platelet reactivity is an inherited phenotype,[8] even in patients taking antiplatelet drugs.[9] In that regard, a growing number of studies focused on the role of miRNAs in modulating biological processes related to haemostasis,[10] including platelet function, and as biomarkers to predict the course of diseases where platelet play a major role such as atherothrombotic diseases. During these last years, selected miRNAs have been identified as promising candidates to predict cardiovascular events and platelet reactivity.[11]

Clinical trials investigated the association between miRNA levels and platelet reactivity or recurrence of ischemic events in cardiovascular patients. However, these candidate miRNAs are identified through association studies and one of the major challenges is their functional validation to investigate a causative link between a given miRNA and platelet reactivity. Most of the recent studies aiming at validating candidate miRNA used mice or human immortalized cell lines but a model[12] using physiological human-derived cells is lacking. The modulation of miRNA content in platelets is not suitable since native platelets are refractory to the transfection procedure.[13] However, megakaryocytes derived from the differentiation of human haematopoietic stem cells can be transfected, thus offering a promising strategy for the production of platelet-like structures (PLS) with different miRNA levels.

Finally, the various mechanisms governing platelet reactivity through miRNA-dependent pathways are scarcely investigated. Studying the impact of miRNAs in a human cell-based model should ultimately help to translate findings on mechanistic issues in human diseases where platelets play a major role for the development a new biomarkers or therapeutic targets.

1.2 PLATELETS

1.2.1 BIOGENESIS

Hematopoietic stem cells (HSC) are multipotent blood cells precursors mainly present in the bone marrow. HSC are characterised by the expression of CD34+ Lin- CD38- CD90+CD45RA- receptors.[14] HSC have a self-renewal ability, meaning that they can proliferate without losing their differentiation potential. The self-renewed cells can be categorized in two populations: long-term and short-term HSC. The long-term HSC have a high self-renewal capability contributing to the repopulation of HSC while short-term HSC conserve this ability for only few weeks, and are dedicated to the differentiation process. Indeed, short-term HSC, also called precursor or progenitor cells, are more prone to differentiate into downstream cells. The established model of HSC differentiation includes a generation of multipotent progenitors (MPP) that differentiate in either myeloid or lymphoid lineage. The common myeloid progenitors (CMP) are parent cells of megakaryocytes, erythrocytes, mast cells, granulocytes and macrophages whereas common lymphoid progenitors (CLP) induce the production of natural killer cells, T lymphocytes and B lymphocytes.[15, 16]

Recent evidences suggest that megakaryocytes biogenesis may occur independently from other lineages and directly from HSC, bypassing the step-wise classical model. HSC, megakaryocytes and their progenitors express several similar receptors including CXCR4, CD150, CD41, CD9 and c-mpl receptor.[17] C-mpl is the receptor of the thrombopoietin (TPO) that plays a role in the maintenance of long-term HSC quiescence and induces the megakaryocytic lineage differentiation.[18] TPO is produced in the liver and is released into the circulation where it is sequestered by platelet through c-mpl dependent mechanism. The decrease of circulating platelets induces an increase in TPO plasma level, resulting in a TPO-mediated stimulation of megakaryocyte progenitors. TPO binding to c-mpl receptors of megakaryocyte progenitors induces JAK2 phosphorylation and STAT3/STAT5 activation. The downstream pathway leads to megakaryocyte-specific gene expression and differentiation of progenitor cells into megakaryocytes.[19] Of note, TPO deficiency results in a decrease of platelets production but low levels of platelet production remain, suggesting that another pathway exists.[19] Megakaryocytes are characterised by a polylobulated nucleus resulting to the accumulation of DNA during the maturation through the TPO-dependent endomitotic process. In the early stage of differentiation, the nucleus contents 2N and reaches 64N and even 128N in mature megakaryocytes. This allows to monitor the maturation process by measuring the megakaryocytes polyploidisation. During the megakaryocyte maturation process, platelet precursors production is initiated by invagination of megakaryocytes membrane and expansion through endoplasmic reticulum and Golgi

complex to form new platelet reservoir structures called demarcation membrane system (DMS).[20] DMS are polarized at the opposite site of polylobed nucleus via an actin-dependent mechanism linked to the WASP–WAVE pathway.[21] Actin remodelling also governs platelet formation through cytoskeletal structure modifications. Indeed, cytoskeleton-related proteins such as spectrin and MYH9, induce a stabilisation of the DMS into megakaryocytes and a premature initiation of proplatelets formation, respectively.[22] Along with the differentiation process, megakaryocytes migrate from osteoblastic niche to the vascular niche by chemotaxis. Cytoplasmic protrusions called proplatelets are extended from mature megakaryocytes in the sinusoidal vessels. A model called "stop and go signal" suggests that the balance between activation of the Rho GTPase proteins RhoA and CDC42 could govern proplatelets elongation. This cytoskeleton-dependent mechanism leads to megakaryocytes polarization and emission of proplatelets protrusion into sinusoidal bone marrow via a GPIb-dependent mechanism.[23] The proplatelets are then fragmented in the circulation according to partially understood and debated mechanisms. Indeed, some evidence suggests that platelet maturation occurs through blood shear forces[24] while other studies suggest that microtubule-based forces govern the final maturation step.[22]

As general note, recent evidence pointed lungs as another site of platelet biogenesis. Intra-vital microscopy in mice reveals that circulating megakaryocytes originated from bone marrow migrate to extravascular spaces of the lung and release about 50% of the total circulating platelets.[25]

1.2.2 PLATELET STRUCTURE

Platelets are anucleate discoid shape cells maintained by a cytoskeleton structure. Platelets measure 2 to 5 µm with a mean cell volume of 6 to 10 μm^3. Platelets have a life span of 5-7 days in the circulation. In healthy individual, the platelet count ranges from 150 to 350 × 10^9/L. The platelet membrane is rich in receptors that mediate internal and external signalling. Platelets content is compartmentalized with numerous organelles including mitochondria, Golgi apparatus, dense tubular system and membrane-delimited intracellular particles such as granules and lysosomes. The platelet structure is described in **Figure 1.**

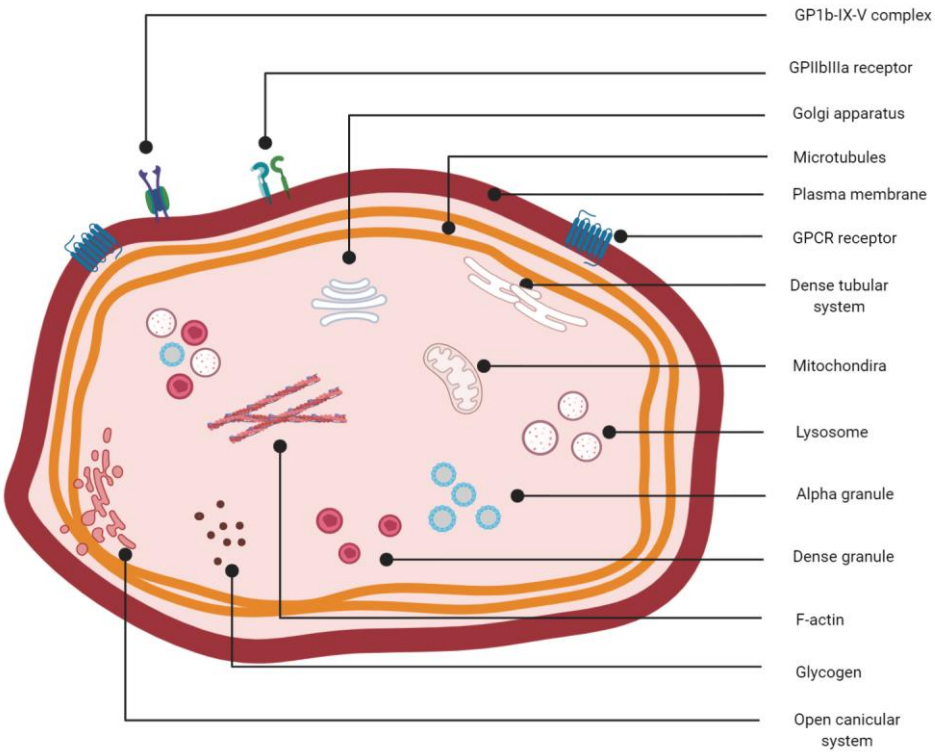

Figure 1: Platelet structure

Platelet cytoplasmic membrane supports several glycoproteins including GP1b-IX-V complex (VWF receptor), GPIIbIIIa (fibrinogen receptor), G-protein coupled receptor (GPCR, a family of transmembrane receptors) and surrounds internal membrane structures (dense tubular system and open canicular system), multiple of organelles (mitochondria and Golgi apparatus), storage organelles (lysosomes, alpha granules and dense granules) and glycogen. Platelet architecture is maintained by cytoskeleton (microtubules and F-actin).

1.2.2.1 Receptors

Platelets express at their surface integrins and transmembrane receptors called glycoproteins mediating platelet interaction with their environment. For example, glycoprotein GPIb/IX/V complex, GPVI as well as the integrins α5β1 and α6β1 recognize their specific ligand among the exposed subendothelial components and bind to VWF, collagen, fibronectin and laminin, respectively.[26] Platelet receptors are crucial for platelet physiology since they mediate the initial steps of haemostasis. Platelet receptors stimulation mediates platelet function by triggering bidirectional intraplatelet signals and release of vesicular cargo that in turn allows the initiation of platelet adhesion to exposed sub-endothelial structures at the site of vessel injury.

Platelet activation and adhesion trigger the release of paracrine and autocrine agonists that amplify the activation process and thrombus growth by recruiting the neighbouring platelets. For example, the dense granule secretion locally increases the concentration of platelet agonists such as ADP, thrombin

and platelet activation induces the production of TXA_2. These compounds bind to their specific platelet receptors to potentiate platelet activation.[27] ADP binds to two purinergic receptors, $P2Y_1$ and $P2Y_{12}$. TXA_2 binds thromboxane A_2/prostaglandin H2 (TXA_2/PGH2) receptor (TP) that induces an activation of phospholipase A2 and C[28]. Thrombin generation during the haemostasis process further increases the platelet activation process through binding the glycoprotein Ib (GPIb) and the protease-activated receptors 1 and 4 (PAR-1 and PAR-4).

The receptors of ADP, thrombin and TXA_2 are members of the seven transmembrane domains G-protein–linked protease-activated receptors family.[29] The G protein-coupled receptors (GPCRs) are composed by a single polypeptide chain with 7 transmembrane α-helices. The extracellular part of the receptors allows ligand binding while the intracellular part mediates downstream signalling cascade through a GDP/GTP dependent mechanism. The downstream signalling thus mediates platelet shape change through regulation of cytoskeleton structures, granules release and GPIIbIIIa activation.[30]

GPIIbIIIa also known as αIIbβ3 integrin is the receptor of fibrinogen. This receptor is divided in two parts GPIIb (CD41) and GPIIIa (CD61) and has the particularity to have a low affinity for its ligand while in a non-activated stage. Platelet adhesion to immobilized agonists such as collagen or VWF or platelet activation by soluble agonists such as ADP, thrombin or TXA2 that bind to their respective GPCR, trigger a downstream signalling that allows GPIIbIIIa activation through a mechanism called inside-out.[31] The inside-out signalling is associated with a conformational change of GPIIbIIIa receptor that enhances the affinity of this receptor for soluble and immobilized fibrinogen. Ligand binding to GPIIbIIIa triggers downstream a signalling cascade named outside-in. The outside-in signalling is triggered by the binding of the G protein subunit Gα13 to the cytoplasmic tail of the subunit β3. This promotes signal transduction regulated by the interaction of transmembrane proteins, intracellular adaptor molecules, kinases and phosphatases and Rho-family small GTPases.[31, 32]

1.2.2.2 Platelet cytoskeleton

Chemical (agonist) or physical (shear) stimulation of resting discoid platelets trigger platelet adhesion to the vessel sub-endothelial components and aggregation. Cytoskeleton rearrangement plays a major role in this process. Cytoskeleton network includes two types of polymers: the microtubules and the filamentous actin (F-actin) stabilised by binding proteins.

The microtubules are formed by protofilaments of α-tubulin and β-tubulin heterodimers. Microtubules play a major role in platelet biogenesis by governing the elongation of platelet protrusion from megakaryocytes. In platelets, the microtubules form a subcortical ring network called marginal ring.

This network links the cytoskeleton to the plasma membrane and maintains the specific platelets discoid shape. Upon stimulation, the marginal ring extends allowing the evolution of resting discoid platelets into spherical and slightly smaller activated platelets.[33]

F-actin is composed of two helical protofilaments of globular actin (G-actin) stabilised by actin binding proteins. F-actin results of the polymerization of successive G-actin recruited at its barbed-end extremity of the actin assembly. Upon activation, the F-actin/G-actin ratio increases from 30 to 70% in cells[34] and the cytoskeletal-associated rearrangement mediates the formation of various extensions of actin structures such as filopodia, lamellipodia, actin nodules and stress fibers **(Figure 2).** The main regulators of the actin assemblies are the proteins of the Rho GTPAse family including CDC42, RAC1 and RHOA that govern the formation of filopodia, lamellipodia and stress fibers, respectively.[35]

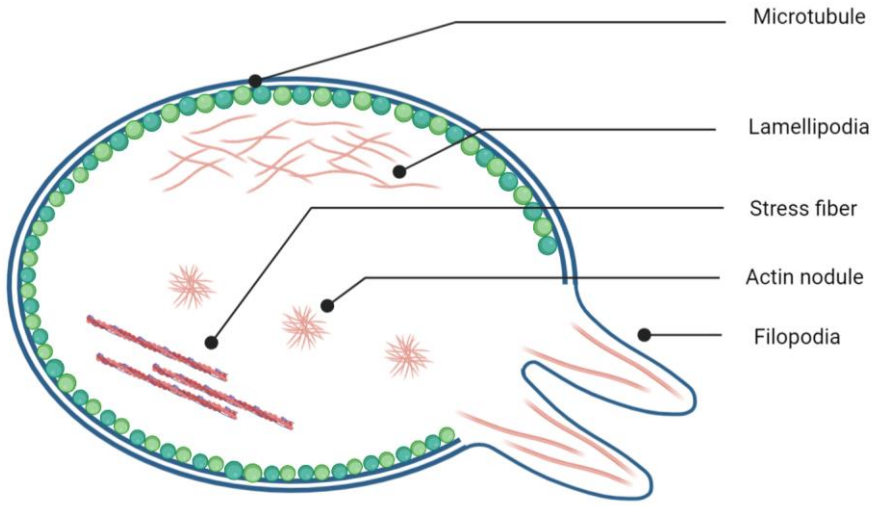

Figure 2. Platelet cytoskeleton network.

Stimulation of resting platelet triggers conformational changes of platelet into its activated form. This is mainly mediated by cytoskeleton network composed by microtubules, actin modules, and actin assemblies (lamellipodia, stress fibers and filopodia) that are governed by distinct mechanisms.

1.2.2.3 Granules

Secretion of platelet granule content is a crucial step for a sustained and irreversible platelet aggregation process. Platelets contain multiple distinct types of granules. α-granules and dense granules are the most abundant while there are only a few lysosomes and T granules. Granules are formed in megakaryocytes and are transferred to platelets during thrombopoiesis. The granules serve as cargo to drive their content to the extracellular environment by exocytose after platelet activation.[36]

α-granules are specific to platelets and approximately 50 to 80 α-granules are found in a single platelet. α-granules have a diameter of 200 to 500 nm and represent 10% of the platelet volume. They derive from both synthetic and endocytic pathways. They take their origin at the trans-Golgi network (TGN) or from plasma membrane endocytosis and are maturated in the cytoplasm via multi step-wise processes involving VPS33B, VPS16B, and NBEAL2 proteins. The morphologic granule characterisation is performed by transmission electron microscopy (TEM) where α-granules appear to be spherical organelles with an electron dense nucleoid delimited by a membrane. They contain up to 300 proteins involved in several platelet processes including haemostasis (e.g. VWF, PF4 and fibrinogen), inflammation (e.g. chemokines such as CXCL1 and interleukin-8) and platelet-derived receptors such as CD62P that are expressed on their plasma membrane.[37, 38] The composition of α-granules includes subpopulations with specific packages such as pro- or anti-angiogenic regulatory proteins. The existence of α-granules heterogeneity was further supported by the observation of VWF and fibrinogen in distinct platelet α-granules.[39]

Dense granules, also known as δ-granules, are platelet-specific lysosome-related organelles (LRO) and are the second most expressed granules with 3-8 dense granules per platelet. They have a diameter of 150 nm. Dense granules have a late endocytic origin and are mainly composed by bioactive amines, adenine nucleotides, polyphosphates and pyrophosphates as well as high concentrations of cations such as calcium. High cationic concentration induces electron-dense appearance in TEM, where their name takes its origin. Dense granules contain also ATP and ADP, which govern the dense granules exocytosis. The expression of LAMP2 and CD63 at their surface is used for dense granule sorting in flow cytometry.[40] Of note, CD63 is not a specific marker of dense granules as it is found at the surface of other intracellular vesicles.

Lysosomes are less represented in platelets, with 1 to 3 lysosomes per platelet. Their major cargo is the acid hydrolases and as the dense granules, they are characterised by CD63 and LAMP2 expression at their surface. In contrast to dense granules, their exocytosis can be evaluated by the measurement of lysosomal enzyme release. The specific role of lysosomes in platelet is only partially understood, however as in other cell types, platelet lysosomes play a role in the digestion of phagocytic and cytosolic components.[41]

The knowledge on T-granules emerged recently and their name was derived from their tubular morphology. In TEM, they appear as dense granules thanks to the intra granular presence of Toll-like receptor 9 (TLR9) and protein disulfide isomerase (PDI),[42] however, the existence of T-granules remains controversial since PDI is potentially packaged in non-granular compartment.[36, 43]

1.2.2.4 Platelet-derived structures

The role of extracellular vesicles (EV) has been unknown for a long time; they were considered first as "platelet dust" with no particular role since the techniques crucially limited their investigation. It seems now that EV are of utmost importance for several biological processes including haemostasis.[44] Platelet is a huge reservoir of signalling molecules that can be released in plasma in platelet-derived EV that trigger the delivering of their packaged molecules to neighbour cells.[45, 46] EV such as exosomes and microvesicles (MVs) have their own morphologic and content characteristics and mediate various biological functions.

Exosomes measure 40–100 nm in diameter and are derived from the exocytose of multivesicular bodies through endosomal sorting complex required for transport (ESCRT)-dependent mechanism. Exosomes represent approximately 25% of the total EV released by platelet in plasma.[47] Exosomes are characterised by the expression of the surface markers CD9, CD63, TSG101 and ALIX. In addition, platelet-derived exosomes are detected thanks to the expression of platelet specific proteins including CD31, CD41, CD42a, P-selectin, PF4 and GPIIb/IIIa.[45]

Exosomes packaging includes a large number of proteins, whose composition is influenced by the agonist selected for platelet stimulation,[48] as well as a low amount of miRNAs (approximately 0.1 per exosome).[49]

MVs are circulating small vesicles with a diameter of 100–1 000 nm derived from a wide variety of cells such as platelets, endothelial cells and leukocytes.[50] MVs are released by membrane blebbing upon platelet activation by soluble agonists, shear stress or GPIIb/IIIa inside-out signalling. Agonist stimulation and shear stress induce an elevated intracellular calcium level, which leads to loss of lipid asymmetry and cytoskeletal reorganisation via RhoA-cofilin pathway. The transmembrane receptors and lipids present in the MV membrane are similar to those of their parent cells. The platelet-derived MVs have at their surface several receptors including GPIIbIIIa, GP1b-IX-V, Annexin V and are enriched in factor X, prothrombin[45] and phosphatidylserine.[51] Platelet-derived MVs are packed with proteins (e.g., growth factors, cytokines/chemokines and Ago2), nucleic acid (RNA including mRNA and miRNA) and mitochondria.

Heterogeneity of the MV cargo is observed in mass spectrometry according to the stimulus driving MV generation. For example, ATP-related proteins are more present in platelet-derived MV after ADP stimulation.[52] In addition, MVs content including growth factors, chemokines, and plasma membrane receptors differ according to their size.[53]

The majority of circulating MVs (70 to 90%) originates from platelets[53] and shows a rapid clearance.[54] Therefore, characterisation of circulating MVs and their content may reflect platelet physiology, including platelet activation status in real time.

1.2.3 PLATELET FUNCTION

Beyond strictly cardiovascular disorders, platelets are involved in multiple biological processes.[5] Platelets contain immunomodulatory molecules including cytokines and chemokines that enhance or repress the immune response. In addition, platelets have the ability to adhere to a large variety of cells that promote innate and adaptive immune response. Therefore, platelet adhesion can induce bacteria phagocytosis, neutrophil tethering and dendritic cells activation.[3] In cancer, inhibition of platelet function using aspirin treatment induces a chemopreventive effect suggesting an oncologic role for platelets.[2] A role in metastases dissemination have also been reported and may be related to the adhesive function of platelets that bind cancer cells promoting extravasation process of tumor cells.[4] Therefore, the identification of modulators of platelet function may impact other pathophysiological processes and diseases.

As mentioned before, platelets are key players in the haemostasis process and thrombus formation. **Figure 3** summarises the stages of platelet function in haemostasis. Since platelets are small cell fragments, they circulate at the periphery of the blood vessel while larger cells (red and white blood cells) circulate in the centre. This phenomenon is called margination. Platelet margination allows them to circulate in the vicinity of the endothelium in order to be at the forefront of endothelium disruption in case of vessel injury. Throughout vessel injury, the sub-endothelial structures are exposed, triggering platelet adhesion to VWF, collagen, fibronectin and laminin via their receptors GPIb-V-IX, GPVI and the integrins α2β1, α5β1 and α6β1, respectively.[26] Platelet activation mediates intracellular modifications and leads to crucial modifications on platelet morphology. Discoid platelets become spherical and release autocrine and paracrine agonists including TXA_2, ADP, serotonin and thrombin in the circulation that triggers activation of adjacent platelets in order to potentiate platelets activation and form a platelet plug at the site of vessel injury. The platelet agonists can be divided in two categories: the strong and the weak agonists. The strong agonists include thrombin and collagen and induce an irreversible aggregation of platelet, TXA_2 synthesis and granules secretion, while platelet stimulation with a weak agonists such as ADP or epinephrine is associated with platelet aggregation but not secretion.[55]

Intracellular crosstalk initiated by platelet activation leads to inside-out signalling described in paragraph 1.2.2.1 resulting to an increase affinity of GPIIbIIIa receptor towards fibrinogen. Thereby platelets adhere to adsorbed fibrinogen at the site of the vessel injury and to plasma fibrinogen molecules allowing platelet-platelet binding. Finally, GPIIbIIIa-mediated outside-in signalling promotes platelets spreading, granule secretion and clot retraction.[31]

Once the thrombus is formed, activated platelets are in close contact and several processes, not yet all fully understood, take place to ensure thrombus stability. Outside-in signalling described previously can lead to protein interaction with their homologous *in trans* in order to form a "junction" between close platelets. Among those players, we find the cell adhesion molecule (CAM) family members including JAM-A, ESAM and PECAM-1 that mediate junction to adjacent platelets. The platelet-platelet interaction can be also mediated through receptor/ligand pairs such as Eph Kinase/ephrin and Plexin B1 and B2/sema4D. Moreover, the close contact between platelets provides a protected space allowing the diffusion and local accumulation of soluble platelet mediators including ADP, TXA_2 and α-granules content such as Gas-6, that enhances the platelet activation and thus the stability of the growing thrombus.[56] Finally, there is evidence of platelet-platelet communication through junctions involving connexin family members such as Cx37 and GJA4.[57] Those gap junction proteins assemble channels allowing the free transfer of small soluble molecules such as calcium between adjacent platelets that may in turn regulate thrombus growth.[58]

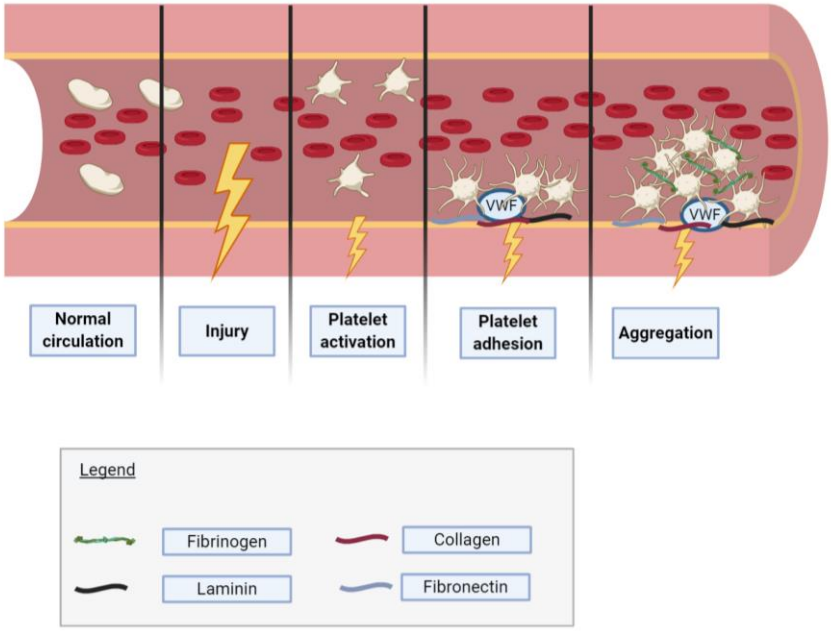

Figure 3. The role of platelet haemostasis

In asymptomatic vessels, platelets flow at the periphery of the vessels induced by the margination from red blood cells. The vessel injury exposed sub-endothelium components that trigger platelet activation, adhesion and aggregation to maintain haemostasis.

Beyond the aggregation process, platelets are essential in promoting thrombin generation that occurs at their surface. After activation, a subpopulation of platelets is particularly prone to support thrombin generation by exposition of procoagulant phospholipids. Over the last decades, this subpopulation has been referred to using several terms including COAT platelets (induced by the action of both thrombin and collagen) and blebbing or balloon(ing) platelets. [59-63]

The platelets stimulated via their GPVI and PAR1 and PAR4 receptors generate 20-40% of pro-coagulant platelets that are characterised by a rapid increase of cytosolic calcium concentration, an exposition of Annexin V and a destabilisation of the phospholipid membrane as well as an inactivation of GPIIbIIIa receptors due to calpain-2 proteolysis. The synergistic binding to GPVI, PAR1 and PAR4 results in an increase of calcium cytosolic level followed by an inhibition of the flippase activity and a "scrambling" of the phospholipids resulting to an exposition of phosphatidylserine (PS) at the platelets surface.[64] The PS exposure promotes the assembly of the tenase and prothrombinase complexes that contribute to the thrombin generation burst that is essential for haemostasis. Moreover, thrombin triggers the conversion of fibrinogen into fibrin. The fibrin monomers polymerize in a fibrin network that is stabilised by covalent crosslinking to factor XIIIa activated by thrombin through a calcium-dependant mechanism. The clot thus formed over the vessel injury prevents excessive bleeding.[65, 66]

Finally, upon vessel healing the fibrin clot is solubilised via a complex process called fibrinolysis. This mechanism is mediated via plasmin-dependent pathway, however the role of platelets in this mechanism remains unclear.[67, 68]

The importance of platelets in haemostasis is illustrated by several inherited defects of key genes in platelet physiology that influence platelet reactivity and are associated with various bleeding disorders. For example, Glanzmann's thrombasthenia is a rare autosomal recessive disorder associated with quantitative and/or qualitative defect of GPIIbIIIa receptor.[69] The Bernard-Soulier syndrome, another rare disorder, is due to mutations in GPIbα, GPIbβ and/or GPIX that decrease the complex GPIb-IX-V number at the platelet surface.[70] Platelet secretion defects may also cause haemorrhagic disorders, as illustrated by the Gray Platelet Syndrome (which is associated with a macrothrombocytopenia and defect of α granules).[71] Finally, the importance of procoagulant phospholipids exposure at the surface of platelets is illustrated by the Scott syndrome, a very rare disorder of the scramblase protein associated with a bleeding phenotype.[72]

To summarise, platelets are central in regulating haemostasis, from adhesion after vascular injury to the aggregation process and thrombin generation that promotes a stable thrombus formation to stop bleeding. Beyond platelet-related bleeding disorders due to major deficiencies of selected genes affecting platelet function, platelet reactivity exhibits a variable phenotype among individuals and is

supposed to be largely genetically determined. The Framingham Heart Study already demonstrated the inherited components of platelet reactivity[73] and several genetic polymorphisms of platelet receptors were found to be associated with platelet reactivity.[74-76] Platelet reactivity was shown to be associated with premature coronary artery disease in a family-based study[8] and predicts cardiovascular events.[77] The role of platelets in thrombotic events in patients with atherothrombotic disease prompted the development of several antiplatelet drugs that are the cornerstone of secondary cardiovascular prevention.

1.2.4 ATHEROTHROMBOSIS AND ANTIPLATELET DRUGS

Platelets are involved in the inflammation process at the early stage of atherosclerosis development as well as in the late stage, by contributing to the atherosclerosis plaque rupture. The role of platelets during inflammatory processes in chronic vascular pathologies is mediated through several mechanisms. Environmental and inherited conditions initiate damages to the vascular wall inducing an accumulation of cholesterol and lipids and vascular remodelling.[78] The subsequent complex immune reaction jeopardizes the vessel lumen by the formation of fatty streak lesions. The plaque vulnerability is characterised by multiple factors such as the size of the necrotic lipid core, the stability and the size of the thin fibrous cap, the presence of diffuse calcifications, a decrease of collagen and of vascular smooth muscle cells components at the site of lesions (from 95 to 50%) and finally the neovascularisation of the atheroma plaque.[67] The exposition of the vascular structure and the inflammatory microenvironment allow platelets activation and trigger the release of MV. In addition, exposition of surface platelet receptors leads to leukocytes recruitment and platelet adhesion/aggregation to the plaque. The evolution of the lesions in vulnerable plaques initiates the atherothrombotic process, hence, activated platelets form aggregates and trigger the coagulation cascade at the site of lesions which may then induce the formation of an occluding thrombus. This leads to acute vascular complications including myocardial infarction or stroke.

Beyond a healthy life style, the main strategy to reduce the risk of recurrence of acute ischemic events is to prevent platelet-mediated thrombus formation. Thereby, various drugs have been developed to specifically target key receptors such as $P2Y_{12}$ receptor inhibitors (e.g., thienopyridines and ticagrelor), GPIIbIIIa inhibitors and thrombin receptor inhibitors (vorapaxar), or to inhibit the production of platelet activators such as thromboxane A_2 (e.g., aspirin). Recent data suggest that, in addition to the antiplatelet therapy, a low dose anticoagulation may be beneficial to reduce recurrence of ischemic events, at least in high-risk patients without major bleeding risk.[79, 80] This further supports the

importance of the dual role of platelets (aggregation / thrombin generation) in thrombotic events of cardiovascular patients.

As mentioned above, platelet reactivity is a variable phenotype among individuals, but the clinical impact of this variability has been mostly studied in cardiovascular patients treated with antiplatelet drugs. Indeed, a large body of evidence indicates that platelet reactivity is still highly variable in cardiovascular patients treated with antiplatelet drugs (termed as on-treatment platelet reactivity),[81] even with more recent and more potent anti-$P2Y_{12}$ such as prasugrel and ticagrelor.[82] This variability has been associated with recurrence of ischemic events in selected situations,[83] but also to bleeding events,[84] particularly when using the more potent latest generation of anti-$P2Y_{12}$ drugs. These data have prompted several interventional trials aiming to tailor antiplatelet drugs according to platelet reactivity as assessed with various platelet function assays. These interventional trials gave rather mitigated results in low-risk patients to reduce the recurrence of ischemic events but were more promising in reducing bleeding events when prasugrel or ticagrelor were used as first-line therapy (de-escalation strategy).[85]

As in healthy individuals without antiplatelet drug, platelet reactivity is highly heritable in patients taking anti-$P2Y_{12}$ drugs.[86] Several genetic polymorphisms have been investigated, mostly in clopidogrel-treated patients. Overall, only one polymorphism of the CYP2C19 gene (rs4244285, implicated in the bioactivation of the drug) has been consistently associated with on-treatment platelet reactivity. However, this genetic variant only explains around 5% of the variability in cardiovascular patients[87] and high throughput techniques investigating DNA did not add much to our knowledge,[88] pointing to other possible mechanisms of gene regulation that may explain on-treatment platelet reactivity, such as miRNAs.

1.3 MIRNAS AS MODULATORS OF PLATELET REACTIVITY

miRNAs are small non-coding RNAs of approximately 22 nucleotides described for the first time in 1993 in C. elegans.[89] During the last decades, miRNAs have been investigated in a growing number of studies, as evaluated by the number of miRNA-related articles published in PubMed **(Figure 4).**

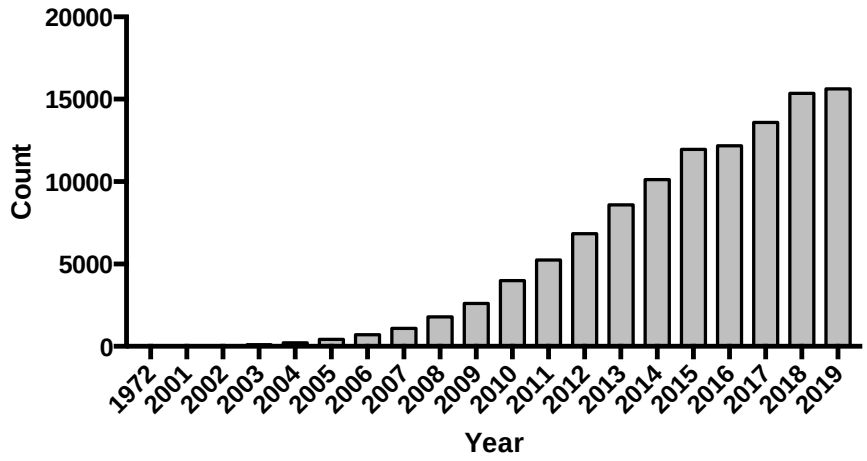

Figure 4. Number of published articles related to microRNAs (PubMed)

It is estimated that 2300 miRNAs are present in human and regulate at least 60% of the protein-coding transcriptome.[90] miRNAs have been described in most of eukaryotic cells and 1917 of them are annotated in miRBase V22.1.[91] miRNAs are present in the multiple subcellular compartments including nucleus, cytoplasm and vesicles. In mammalian cells, the miRNAs bind a specific sequence of their mRNA targets, allowing the cleavage of mRNAs or the inhibition of their translation. Although platelets are anucleate, they contain at least 500 different miRNAs[92] as well as mRNAs and all the machinery allowing miRNA-mRNA complex formation leading to post transcriptional regulation.[93] miRNAs are regulators of megakaryocyte maturation, platelet biogenesis and platelet function.[94] As described above, platelet activation leads to secretion of MV containing platelet-derived miRNAs and 41 to 45% of the MV in the circulation originate from activated platelets.[95] In addition, recent data suggest the presence of miRNAs outside of MV in the circulation, where they are probably transported and stabilised by proteins such as Ago2 that protect them from RNAse degradation. This observation suggests that platelets may be able to release miRNA-Ago2 complexes in the circulation.[96]

1.3.1 MIRNAS BIOGENESIS AND GENE REGULATION

miRNAs coding regions are present in introns and in exons as part of the genome.[97] In mammalian cells, the first step of the miRNA maturation process occurs in the nucleus. The primary miRNAs (pri-miRNAS) are transcribed by the RNA polymerase II (RNA pol II) and RNA polymerase III (RNA pol III) from intergenic region. Pri-miRNA is processed into precursor-miRNA (pre-miRNA) by a complex composed by the ribonuclease III enzyme Drosha and DiGeorge Syndrome Critical Region 8 (DGCR8)

protein. DGCR8 binds the N6-methyladenylated GGAC sequence at the 5' extremity of the pri-miRNA and Drosha cleaves the single strand-double strand junction of the pri-miRNA hairpin. The action of the microprocessor complex Drosha-DGCR8 results in the formation of stem loop structure of the RNA double strand. These structures, called pre-miRNA are then exported to the cytoplasm by the exportin-5-Ran-GTP for further processes. The double strand pre-miRNA is cleaved by the RNase III endonuclease Dicer at the hairpin site. This leads to the formation of 2 strands of mature miRNAs composed by primary stranded miRNA and its complementary strand.[98] The 3' part of the pre-miRNA results in miRNA-3p formation while the 5' extremity of the pre-miRNA allows the formation of the miRNA-5p (**Figure 5).** A pair 3p and 5p of one miRNA may exhibit an imperfect complementarity and the two strands of a single mature miRNA may have different biological functions.[99]

Mature miRNAs bind a specific region on the 3'UTR of the mRNA targeted. One miRNA can recognize several target sites of one or several mRNAs and conversely one mRNA can have seed sequences for multiple miRNAs. This characteristic confers putative synergistic effects of miRNAs for the expression regulation of one protein. The specificity of the interaction mRNA/miRNA called miRNA response elements (MREs) depends on multiple features including the size and the nucleotide composition of the pairing. Typically, 6 to 8 nucleotides located at the 5' extremity of the miRNA bind mRNA's 3'UTR.[90] However, atypical duplex with higher or fewer nucleotides pairing or including a mismatch can be formed, although their formation requires more energy.[100, 101] The duplex is loaded in Ago2 protein to form miRNA-induced silencing complex (RISC) **(Figure 5).** The binding of the downstream effector proteins, GW182/TNRC6 leads to the recruitment of the deadenylase complexes PAN2-PAN3 and CCR4-NOT. This is followed by mRNA decappping and a degradation, destabilisation or translational inhibition of mRNA through a complex mechanism dependent of the mRNA/mRNA pairing quality.[99, 102, 103]

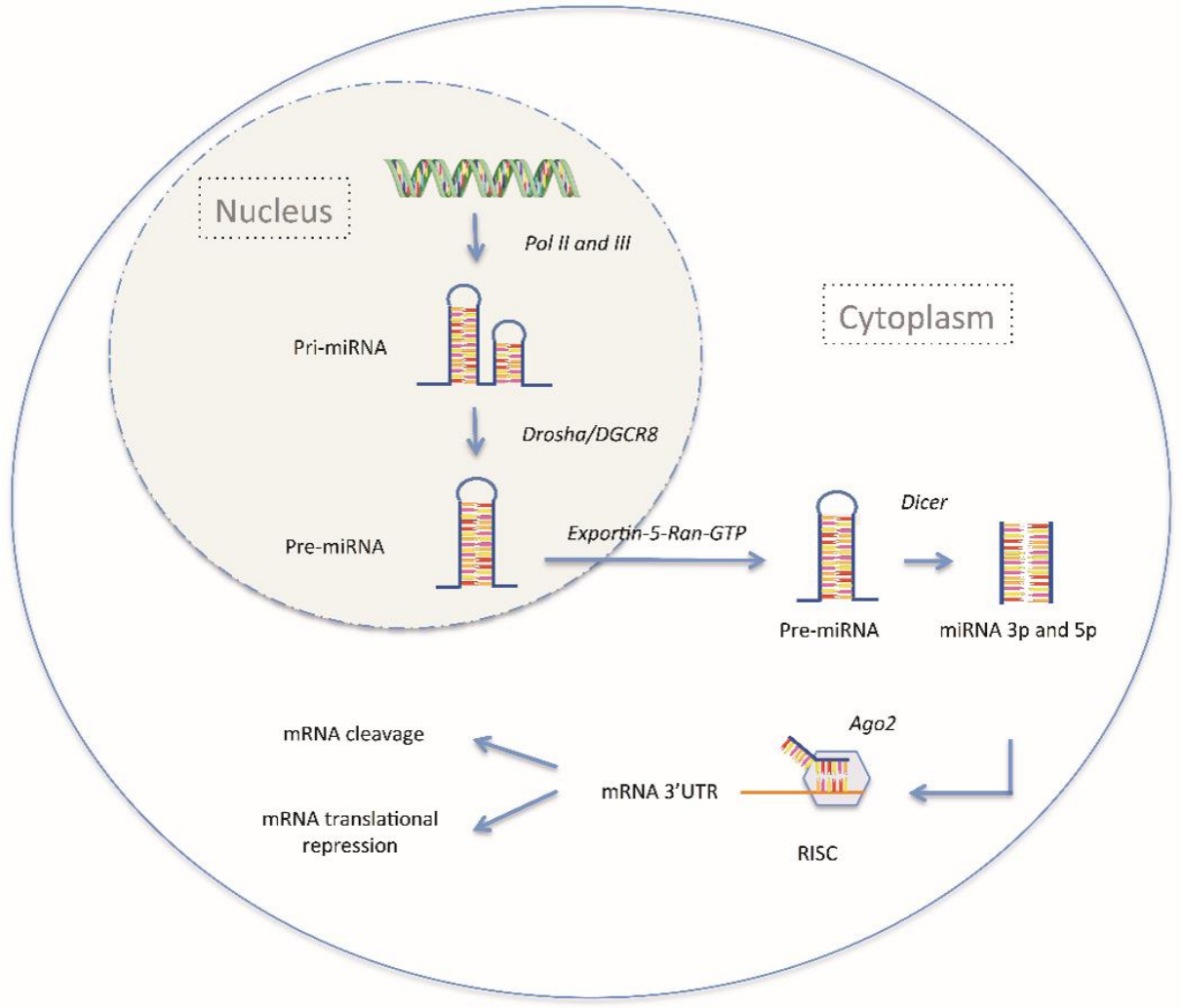

Figure 5. Canonical miRNAs biogenesis.

miRNAs are mainly transcribed from intronic region by the polymerase II/III. The pri-miRNA is processed into pre-miRNAs by the complex Drosha/DGCR8 and is then exported to the cytoplasm by the Exportin-5 for further maturation by Dicer protein. Mature miRNA binds by imperfect complementarity a seed sequence on the 3' untranslated region (UTR) of a mRNA. The duplex miRNA::mRNA is stabilised by Ago2 into a miRNA-induced silencing complex (RISC) triggering mRNA translational repression.

Multiple non-canonical pathways of miRNA maturation have been described; they allow the maturation of miRNAs by bypassing one of several steps of the canonical pathway described above. Indeed, the study of atypical pathways shows that miRNAs biogenesis could be formed through a Drosha/DGCR8 or Dicer-independent pathways[104] and could take their origin from introns, snoRNAs, endogenous short hairpin RNAs (shRNA) or transfer RNAs (tRNA).[105] The mirtrons are one example of the Drosha/DGCR8 independent pathways; in the nucleus, an intron is processed by spliceosomes and debranching enzymes to produce pre-miRNA hairpin. Pre-miRNA is then exported into the cytoplasm for canonical maturation and cleavage by Dicer, a RNase III endonuclease.[105] The Dicer-independent miRNA maturation occurs on short pre-miRNA sequence (approximately 18 nucleotides) not suitable

for Dicer cleavage. These short pre-miRNAs are bound by Ago1 protein or are cleaved and are subsequently loaded to Ago2 complex for processing the RISC and repressing mRNA translation.

Of note, reports indicated that miRNAs could bind other regions of the mRNA including 5'UTR, coding sequence and gene promoters that may result to gene expression repression or surprisingly, to gene activation. These atypical mechanisms are described elsewhere.[102]

1.3.2 PLATELET-ASSOCIATED MIRNAS

A high proportion of circulating miRNAs are of platelet origin. Indeed, as described below, platelet activation undergoes the release of MV where miRNAs are associated with Ago2 protein conferring a high stability to circulating miRNAs.[49] Circulating miRNAs can reflect platelet reactivity. Therefore, the circulating miRNAs profile has been investigated in cardiovascular patients and correlated to platelet function and recurrence of ischemic events. miR-223-3p and miR-126-3p are highly expressed in platelets.[106, 107] miR-223-3p is a very promising candidate as a major regulator of platelet function in cardiovascular patients since it regulates $P2Y_{12}$ expression, one of the two ADP receptors **(Table 1).** In addition, a clinical association study showed that miR-223-3p is negatively correlated to anti-$P2Y_{12}$ drugs effectiveness in cardiovascular patients upon DAPT and could identify clopidogrel poor responders.[108] Moreover, circulating miR-223-3p level was shown to be inversely associated with recurrence of ischemic events.[109] Functional validation studies using mice knocked-out for miR-223-3p showed an enhanced platelet function,[110] although this was not found by others.[111] Of note, mouse $P2Y_{12}$ mRNA does not seem to be a target for miR-223-3p[11] further supporting the need for functional validation models using human cells.[11]

The second most expressed miRNAs, miR-126, is of particular interest in view of the concordant data between clinical association studies and validation experiments. Indeed, miR-126 is positively correlated with platelet reactivity in several cardiovascular clinical trials as well as to cardiovascular events.[12, 109] Moreover, the systemic inhibition of miR-126 in mice results in a decrease of platelet aggregation.[12] The mechanisms governing miR-126-dependant modulation of platelet function was studied in an immortalized cell line showing that overexpression of miR-126 was associated with decreased ADAM9 mRNA level[12] one of the experimentally validated miR-126[112] targets coding for a protein implicated in platelet adhesion to collagen.[113] However, using this cellular model, miR-126 downregulation was associated with a decrease of TXA2-dependent platelet aggregation alteration, while there was no significant impact on collagen aggregation.[12] Beyond the limitations inherent of an

immortalized cell line, these data suggest that other mechanisms may mediate the effect of miR-126 on platelet reactivity.

Several platelet- and megakaryocyte-derived miRNAs have been investigated for their potential role on platelet biogenesis and/or platelet reactivity, (recently reviewed elsewhere[114]). The key miRNAs investigated in clinical association studies and the putative mechanisms of action are summarised in **Table 1 and 2**, respectively. Of note, the comparison of these results is challenging due to differences in patient populations and the lack of standardization with respect to the type of samples used, the miRNA extraction and purification methods and measurement techniques that may impact the detection of miRNA levels. The investigation of the effect of miRNAs and of the underlying mechanisms can be performed using numerous *in silico, in vitro* and *in vivo* strategies. The advantages and the limits of those techniques have been reviewed in **Appendix 1.**

References	Year	Significant association	No significant association	Samples	Studied group	Treatment	Test	Outcome
Kondkar *et al* [115]	2010	miR-96		Plasma	Healthy volunteers	/	Microarray	Platelet reactivity
Willeit *et al* [116]	2013	miR-223, miR-191, miR-126, miR-150		Platelet, MP, PRP, PPP, serum	Healthy volunteers	Prasugrel plus aspirin	qPCR	Platelet reactivity
					Symptomatic carotid atherosclerosis	Low-dose aspirin or dipyridamole or clopidogrel plus aspirin		
Shi *et al* [108]	2013	miR-223	miR-96	Platelet	Coronary heart disease patients	Clopidogrel plus aspirin	qPCR	Platelet reactivity
Zufferey *et al* [117]	2016	miR-135, miR-204		Platelet	Cardiovascular patients	Aspirin	microarray	Platelet reactivity
Kaudewitz *et al* [12]	2016	miR-126		Plasma	Acute coronary disease patients	Dual antiplatelet therapy	qPCR	Platelet reactivity
Witkowski *et al* [118]	2016	miR-126		Plasma	Diabetes mellitus patients	/	PCR	Blood thrombogenicity
Peng *et al* [119]	2017	miR-223, miR-221, miR-21		Platelet	Acute coronary syndrome patient	Clopidogrel plus aspirin	qPCR	Clopidogrel antiplatelet responsiveness
Ding *et al* [120]	2019	miR-204		Platelet	Acute coronary syndrome patients	Clopidogrel plus Aspirin	qPCR	Platelet reactivity
Tang *et al* [121]	2019	miR-142		Plasma	Coronary artery disease patients	Clopidogrel plus aspirin	High-throughput Illumina sequencing followed by qPCR validation	Clinical outcomes
Liu *et al* [122]	2020	miR-126, miR-223, miR-150, miR-130	miR-21, miR-96, miR-331, miR-326	Platelet	Acute coronary syndrome and Percutaneous coronary intervention	Clopidogrel plus aspirin	qPCR	Platelet reactivity
Zampetaki *et al* [109]	2012	miR-126, miR-223 and miR-197		Plasma	Healthy volunteers from their fifth to their eighth decades	/	qPCR	Myocardial infarction

Table 1. Clinical association studies between miRNA and platelet reactivity or cardiovascular outcome

References	Year	miRNAs	Model	miRNA modulation	Test	Direct target	Indirect target
Elgheznawy *et al* [110]	2015	miR-223	Mice	miR-223 deletion	Platelet aggregation and thrombus formation	Coagulation factor XIII-A	kindlin-3
Fejes *et al* [123]	2017	miR-26b and miR-140	MEG01 and K562 cells	mimics or anti-miRNAs transfection	qPCR	SELP mRNA levels	
Kaudewitz *et al* [12]	2016	miR-126	MEG01	mimics or LNA transfection	qPCR	ADAM9	$P2Y_{12}$
			Mice	LNA injection	Platelet aggregation		
Barwrari *et al* [124]	2018	miR-21	hPSC-derived	miR-21 mimic or LNA	qPCR	WASP	TGF-β1, VWF, fibronectin
			Mice	miR-21 deletion	Platelet TGF-β1 release, platelet and megakaryocyte count		
Basak *et al* [125]	2019	miR-15a	Cord Blood derived megakaryocytes	miRNA overexpression by transduction and downregulation using LNA	CRP- induced αIIbβ3 activation and α-granule release	FcRγ	GPVI

Table 2. Studies addressing the issue of the mechanisms related to miRNA-mediated modulation of platelet reactivity

1.4 *IN VITRO* PLATELETS PRODUCTION

Thrombocytopenia may be treated with infusion of platelet concentrates in selected situations to avoid haemorrhagic risks. Blood bank centres have recurrent concerns regarding platelet concentrates including the risk of shortage, of contamination and the immunologic risks.[126] Thereby, several groups aimed to elucidate the platelet production process and to develop techniques to produce *in vitro* a high amount of functional platelets.

The *in vitro* platelet production uses human pluripotent stem cells or hematopoietic progenitors. The induced pluripotent stem cells (iPSC) conserved the self-renewal activity of the embryonic stem cells (hESC) and have the advantage to allow cryopreservation. The engineering modifications of IPSC generate stable lineage allowing the production of element called “platelet-like structures” (PLS). However, the yield of PLS (10 PLS/megakaryocytes) remains low.[127]

An alternative is to use megakaryocyte progenitors (CD34+ cells) immune-purified from peripheral blood, cord blood, or bone marrow. Although the CD34+ expansion is limited, they have the advantage to produce 100-150 PLS/megakaryocytes. Recent studies improved the *in vitro* expansion and the differentiation process of CD34+ cells using several chemical components dedicated to mimic the hematopoietic microenvironment.[128] Proliferation of megakaryocytes is induced in presence of low density lipoprotein (LDL), recombinant human cytokines (SCF, IL-6, IL-9) and of StemRegenin 1 (SR1),

an antagonist of the aryl hydrocarbon receptor (AhR) that mimics the effect of mesenchymal stromal cells (MSC) on CD34+ environment in the bone marrow.[129] After the proliferation step, the differentiation is induced by addition of recombinant TPO into the medium that drives megakaryocytes maturation and differentiation as described previously in the "platelet biogenesis" section. The proliferation and the differentiation processes of the CD34+ have been developed in static conditions. However, recent biomechanical protocols proposed production of PLS in dynamic conditions mimicking the bone marrow environment. One of the first dynamic models describes a 3D structure composed by VWF coated porous hydrogel scaffolds that enhances the production of PLS compared to static model.[130] A promising alternative is the development of a 3D architecture dedicated to mimic bone marrow vascular niche using different types of scaffold.[131] Despite recent advances, the yield of platelets production is still not sufficient to cover the transfusion needs and a scale up in bioreactors is in development.[132]

The morphology of the PLS showed several similarities with human platelets regarding the distribution of the open canicular system and the α and δ granule content. The main differences between PLS and platelets relate to the lack of maturity of the former, including a larger size and an increased content of RNA. However, the recirculation of PLS produced *in vitro* in mice showed that PLS size is rapidly reduced probably due to a maturation step and a remodelling of PLS in the lung. PLS express a normal level of glycoproteins at their surface.[133] Upon activation PLS exhibit P-selectin secretion and express at their surface the active form of the GPIIbIIIa receptors. In addition, PLS form aggregates after activation with ADP or thrombin receptor agonist peptide (TRAP). *In vivo* investigation of PLS function showed that PLS have similar half-life than human platelets, they can be incorporated in thrombus and contribute to the limitation bleeding events in thrombocytopenic mice.[129, 134] These observations indicate that PLS constitute an adequate model to investigate the impact of a wide variety of components, such as miRNAs, on platelet reactivity.

1.5 AIM OF THE PROJECT

Platelets play a major role in several diseases, including atherothrombosis. Antiplatelet therapy is indeed a cornerstone in the secondary prevention of recurrent ischemic events in cardiovascular patients. The mechanisms governing platelet function regulation is poorly understood and a growing number of studies pointed miRNAs as major contributors of the variability of platelet reactivity in patients with or without antiplatelet drugs. The understanding of the mechanisms involved in the modulation of platelet reactivity may lead to a better tailoring of antithrombotic therapy and identify new targets for the secondary prevention in cardiovascular patients.

The goal of this project is to develop a model of human-derived cells dedicated to the study of miRNAs function in platelets. This model allows the investigation of the impact of miRNAs on platelet function and morphology, as well as the mechanisms involved. Finally, the available biobank of our group allows to challenge the association of these validated miRNAs with platelet function or other thrombotic markers in a cohort of cardiovascular patients.

Since platelets do not allow transfection procedure, the investigation of the impact of miRNAs is performed by modulation of miRNAs level in megakaryocytes derived from human progenitor cells and, in turn functional assessments of the generated PLS. This model is described in details in an original article (Thrombosis and Haemostasis, 2019) in **Section 2.1**. As proof of concept, miR-126-3p was selected for the development of functional tests to monitor PLS function after miRNA level modulation. In addition, this model allowed the evaluation of putative mRNA target of miR-126-3p on platelet function.

Section 2.2 describes the impact of another miRNA that was previously identified by our group – miR-204-5p – and the investigation of one of its putative targets, CDC42. In order to investigate the impact of the duplex miR-204-5p::CDC42, a morphologic and functional characterisation of the megakaryocytes and PLS using a large set of techniques was performed.

Finally, **Section 2.3** takes advantage of the ADRIE cohort to describe potential associations between platelet function tests and a set of miRNAs. The results of this clinical association study were combined to an *in silico* identification of the putative targets of these miRNAs to pave the way to the identification of the biological pathways behind the regulation of platelet function by miRNAs in cardiovascular patients.

2 RESULTS

2.1 MODEL VALIDATION USING MIR-126-3P

Functional Validation of microRNA-126-3p as a Platelet Reactivity Regulator Using Human Haematopoietic Stem Cells

Alix Garcia, Sylvie Dunoyer-Geindre, Veronika Zapilko, Séverine Nolli, Jean-Luc Reny and Pierre Fontana

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Personal contribution: I performed experiments, analysed the data (Fig.2, Fig.3 C and D, Fig. 4, Fig. 5) and wrote the first draft of the manuscript.

Overview

The study of the effect of miRNA on platelet function remains challenging. In this work, we developed a model of human hematopoietic stem cells differentiated into megakaryocytes and subsequently in PLS. The megakaryocytes are transfected to increase the miRNA content as well as in their derived PLS. To validate this new model, we used one of the most expressed miRNAs in platelets, miR-126-3p, that has been extensively studied and correlated with platelet function and recurrence of ischemic events in cardiovascular patients. miR-126-3p synthetic mimic sequences were transfected into mature megakaryocytes, the efficiency of transfection procedure as well as the impact of miR-126-3p on the expression of selected putative targets were quantified.

The *in vitro* generation of megakaryocytes yields a limited amount of PLS that does not allow the use of standard platelet function assays. Therefore, we developed original techniques to evaluate platelet function using a low amount of PLS such as FACS and cell perfusion into a flow chamber followed by automatic quantification of PLS adhesion. Lastly, a reporter gene assay allowed the validation of PLXNB2 as a target of miR-126-3p. PLXNB2 is a transmembrane receptor contributing to cell migration and the impact of down regulation of this protein on platelet function was validated using our model and functional assays.

Taken together, we show that our model allows the functional validation of candidate miRNAs in human-derived cells as well as the investigation of their underlying mechanisms. This work presents the first model of human derived-cells dedicated to the validation of miRNA in the field of platelet function.

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Functional Validation of microRNA-126-3p as a Platelet Reactivity Regulator Using Human Haematopoietic Stem Cells

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Thromb Haemost 2019;119:254–263.

Abstract

Background Platelets are an abundant source of micro-ribonucleic acids (miRNAs) that may play a role in the regulation of platelet function. Some miRNAs, such as miR-126-3p, have been noted as potential biomarkers of platelet reactivity and the recurrence of cardiovascular events. However, the biological relevance of these associations remains uncertain, and the functional validation of candidate miRNAs on human-derived cells is lacking.

Objective This article functionally validates miR-126-3p as a regulator of platelet reactivity in platelet-like structures (PLS) derived from human haematopoietic stem cells.

Materials and Methods $CD34^+$-derived megakaryocytes were transfected with miR-126-3p and differentiated in PLS. PLS reactivity was assessed using perfusion in a fibrinogen-coated flow chamber. miR-126-3p's selected gene targets were validated using quantitative polymerase chain reaction, protein quantification and a reporter gene assay.

Results $CD34^+$-derived megakaryocytes transfected with miR-126-3p generated PLS exhibiting 156% more reactivity than the control. These functional data were in line with those obtained analysing CD62P expression. Moreover, miR-126-3p transfection was associated with the down-regulation of a disintegrin and metalloproteinase-9 (ADAM9) messenger RNA (mRNA), a validated target of miR-126-3p, and of Plexin B2 (PLXNB2) mRNA and protein, an actin dynamics regulator. Silencing PLXNB2 led to similar functional results to miR-126-3p transfection. Finally, using a reporter gene assay, we validated PLXNB2 as a direct target of miR-126-3p.

Conclusion We functionally validated miR-126-3p as a regulator of platelet reactivity in PLS derived from human haematopoietic stem cells. Moreover, PLXNB2 was validated as a new gene target of miR-126-3p in human cells, suggesting that miR-126-3p mediates its effect on platelets, at least in part, through actin dynamics regulation.

Keywords
- microRNA
- platelet
- platelet function tests
- flow-based assay
- PLXNB2

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Introduction

Platelets play a crucial role, not only in haemostasis but also in inflammation, cell proliferation and immune system modulation.[1–3] Identifying the determinants of platelet reactivity may thus have clinical implications on the prognoses for several diseases and may help to tailor drug treatments. The discovery that platelets are an abundant source of microribonucleic acids (miRNAs)[4] and that miRNA expression profiles within platelets correlate with platelet reactivity[5,6] raised the exciting possibility of finding novel disease biomarkers and therapeutic targets.[7,8]

The vast majority of studies designed to address the role of miRNAs in platelet reactivity regulation are association-type studies investigating correlations between miRNA levels and platelet reactivity scores or the recurrence of ischaemic events. A functional approach is needed to understand the mechanisms involved and to validate the role of miRNAs in platelet function. Several studies have described methods using animal models[9,10] and immortalized cell lines,[11,12] but the functional validation of candidate miRNAs in human-derived cells is lacking. However, studying the function of miRNAs in anucleated platelets that are refractory to transfection is a major limitation.[13]

Platelet-like structures (PLS) derived from megakaryocytes can be used as a model to identify the functional impact of miRNAs on platelet reactivity. Over the last few years, several research groups have focused on producing PLS with the aim of creating in vitro transfusable platelets. PLS produced from haematopoietic stem cells are functionally close to human platelets.[14–18] Indeed, the secretion of PLS after agonist stimulation, as well as their function in relation to fibrinogen receptors, is comparable to the results observed with human platelets.[19,20]

Microfluidic flow chambers are currently used to study thrombotic processes by monitoring platelet incorporation into the clot, to study cell adhesion in dynamic conditions or to test the function of blood bank platelets in whole or reconstituted blood.[21,22] This process is recognized as a powerful, promising method to investigate platelet function.

This study describes the use of a flow-based assay, using PLS derived from human haematopoietic stem cells, to assess the functional impact of candidate miRNAs for regulating platelet reactivity. As a functional proof-of-concept experiment, we used miR-126-3p, one of the most promising platelet-derived miRNAs associated with platelet reactivity[11] and a potential biomarker of recurring cardiovascular events in humans.[23]

Materials and Methods

Cell Culture and Differentiation

Human $CD34^+$ cells were isolated from the buffy coats of healthy adult human donors provided by the Geneva University Hospitals' blood bank using a CD34 MicroBead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD34^+$ cell quantification was evaluated using an anti-CD34 PE antibody (Miltenyi Biotec) followed by flow cytometry analysis. More than 95% of the cells were positive for CD34. $CD34^+$ cells were cultured for 7 days in StemSpan Serum-Free Expansion Medium (Stemcell Technologies, Vancouver, Canada) supplemented with 20 ng/mL human low-density lipoprotein (LDL, Stemcell Technologies), StemSpan Megakaryocyte Expansion Supplement (CC220, Stemcell Technologies), penicillin-streptomycin-glutamine (PSG, Gibco, Thermofisher, Waltham, Massachusetts, United States) and 1 µM StemRegenin 1 (SR1, Cellagen Technology, San Diego, California, United States), as described elsewhere.[15] Next, the cells were washed, seeded and cultured in the presence of 0.5 µg/mL thrombopoietin (TPO, Stemcell Technologies), LDL, PSG and SR1 for an additional 8 days (►**Supplementary Fig. S1A**, available in the online version).

All the experiments described below were repeated with different, independent batches of cells from different blood bank donors.

Flow Cytometry

Megakaryocytes and PLS were analysed using flow cytometry with selected antibodies against specific markers of cell differentiation (CD34, CD41, CD42b and CD42d).

Anti-CD34 PE (Miltenyi Biotec), anti-CD41 fluorescein isothiocyanate (BioLegend, San Diego, California, United States) and anti-CD42b PE-vio770 (Miltenyi Biotec) antibodies were added directly to the cell suspension for 20 minutes, at room temperature, in the dark, and were then washed and re-suspended in phosphate-buffered saline.

Anti-CD42d mouse primary antibody (Santa Cruz, Dallas, Texas, United States) was added to the cells for 20 minutes. The cells were washed and then incubated with the secondary PE goat anti-mouse antibody (BioLegend) for 20 minutes.

Flow cytometry was performed using Attune (ThermoFisher, Ecublens, Switzerland) and Accuri C6 (BD Biosciences, Allschwil, Switzerland) cytometers. Positive cells were defined using appropriate isotypic control. The data acquired were analysed using the FlowJo software (TreeStar, Ashland, Oregon, United States).

Isolation and Quantification of Megakaryocytes and PLS

Megakaryocytes were isolated at day (D) 13 using a 10-minute centrifugation step at $400 \times g$. We used a Tali Image-Based Cytometer (ThermoFisher) to quantify megakaryocytes defined as elements > 6 µm.

At D15, PLS and megakaryocytes were quantified using the Tali Image-Based Cytometer; PLS were defined as elements ≥ 4 and ≤ 6 µm. The number of PLS was then normalized to the number of megakaryocytes to evaluate production.

Activation of PLS

Non-PLS cells were discarded at D15 using a 5-minute centrifugation step at $100 \times g$ after addition of 25 µM prostaglandin I_2 (Cayman Chemical Company, Ann Arbor, Michigan, United States) and 0.02 U/mL apyrase (Sigma, St. Louis, Missouri, United States). The supernatant was centrifuged at $1{,}000 \times g$ for 10 minutes and PLS were re-suspended in Tyrode's buffer (5.5 mM glucose, 137 mM NaCl, 2 mM KCl, 12 mM $NaHCO_3$, 0.3 NaH_2PO_4, 5 mM HEPES, 1 mM $MgCl_2$ and 2 mM $CaCl_2$; pH was adjusted to 7.3). After

1 hour at 37°C, PLS were stimulated with 1 U/mL thrombin (Sigma) and stirred for 10 minutes. PLS were labelled with CD62P PE antibody (BD Biosciences) and CD41 APC (BioLegend). PLS labelled with an isotypic antibody (PE, BD Biosciences, and APC, BioLegend) were used to define positive cells. Flow cytometry was performed using an Accuri C6 flow cytometer (BD Biosciences), and the data were analysed using the FlowJo software (TreeStar).

miR-126-3p Expression Profile during Differentiation and after Transfection

On selected culture days, cells were recovered in QIAzol lysis reagent (Qiagen, Hilden, Germany). Isolation of RNAs using the miRNeasy Mini Kit (Qiagen) was followed by a reverse transcription procedure using the TaqMan Advanced miRNA cDNA Synthesis Kit (Applied Biosystems, Foster City, California, United States). miRNAs were detected in triplicate on a 7900HT Sequence Detection System using TaqMan Advanced miRNA assays (Applied Biosystems) with pre-designed miRNAs probes (Applied Biosystems). miRNAs were quantified by normalizing miRNA reverse transcriptase-polymerase chain reaction (RT-PCR) data using a panel of stably expressed miRNAs. Using the geNorm algorithm, three of the six miRNAs constituting the platelet normalization panel given by Kok et al[24]—miR28, miR151 and miR29c—were identified as the best combination of normalizers in megakaryocytes and these were assessed in each sample.[25]

The transfection procedure's impact on miR-126-3p and miR-223-3p (a control platelet-derived miRNA) expression was quantified at D15 in PLS only.

Cell Transfection

Megakaryocytes isolated at D13 were seeded in an antibiotic-free medium at 1×10^6 cells per well for 2 hours in 6-well plates. They were then transfected at 200 nM using an hsa-miR-126-3p mimic, or scramble miRNA (ThermoFisher), which does not target any specific messenger RNA (mRNA), using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, California, United States). Plexin B2 (PLXNB2) small interfering RNA (siRNA) (SI00687603, Qiagen) or scramble siRNA were transfected at 50 nM using RNAiMAX Transfection Reagent (Invitrogen). Five hours after transfection, the medium was replaced and the cells were resuspended in StemSpan supplemented with 0.5 µg/mL TPO, 20 ng/mL LDL and 1 µM SR1. Results were compared to those of the mock condition (cells with transfection reagent only).

Transfection procedure efficiency was assessed at D13, 5 hours after megakaryocyte transfection, using siRNA AllStars negative Alexa Fluor 488 (Qiagen). The percentage of transfected cells was measured using Accuri C6 flow cytometer.

Flow Chamber Assay

Flow chambers (Vena8fluoro +, Cellix, Ireland) were coated overnight with 200 µg/mL fibrinogen (Sigma) at 4°C and washed with StemSpan Serum-Free Expansion Medium (Stemcell Technologies) for 1 minute at 80 µL/min. At D15, the cell suspension ($44 \pm 5.2\%$ PLS, the rest being $CD34^+$-derived megakaryocytes) was adjusted to 5×10^6 cells/mL. The cell suspension was perfused for 5 minutes at 2 µL/min (shear rate of 50 s^{-1}) and rinsed for 8 minutes at 3 µL/min using the StemSpan medium. Pictures of each channel were taken at five different points along them, 4 mm apart, with picture #1 closest to the injection site, using a Si-3000 camera and XLi Cap V18 driver software (Ceti, Medline Scientific, Chalgrove, England).

Flow chamber images were analysed using a custom-made software framework written in MATLAB (The MathWorks, Inc. Natick, Massachusetts, United States). Briefly, RGB images were first converted into greyscale. The flow area was then automatically detected within the field of view of the images; this enabled calibration of the image resolution in accordance with the chamber's 400 µm width. The content of the flow area was subsequently segmented using Otsu's method,[26] preceded by a background subtraction through a combination of low-pass filters. Separation of the multi-object content was done using watershed transformations[27] constrained by the regional extrema within the aggregate. This approach is also effective in cases involving single cells. Finally, each resulting individual object was classified by size as either a megakaryocyte cell (> 6 µm), a PLS (≥ 4 and ≤ 6 µm) or debris (< 4 µm). The number of PLS adhered to the immobilized fibrinogen was quantified and normalized to the mock condition.

Gene Expression Levels of Selected miR-126-3p Gene Targets

Forty-eight hours after transfection (D15), mRNA was extracted from the PLS using the trizol procedure. After RNA isolation, total RNA was reverse-transcribed using Improm-II reverse transcriptase (Promega, Madison, Wisconsin, United States) according to the manufacturer's instructions. A quantitative real-time RT-PCR (qPCR) was carried out using the $\Delta\Delta C_t$ method to evaluate the levels of a disintegrin and a metalloproteinase-9 (ADAM9) and PLXNB2 mRNAs. The level of HB2M was used as a housekeeping gene to normalize the qPCR results (►**Table 1**).

Western Blot

A Western blot analysis was performed 48 hours after transfection (D15) to quantify the PLXNB2 protein. Because the amount of protein in the PLS was too low, we performed

Table 1 Oligonucleotide sequences used in this study

Gene	Direction	Sequence
HB2M	Forward	TGCTCGCGCTACTCTCTCTTT
HB2M	Reverse	TCTGCTGGATGACGTGAGTAAAC
ADAM9	Forward	CCCCCAAATTGTGAGACTAAAG
ADAM9	Reverse	TCCGTCCCTCAATGCAGTAT
PLXNB2	Forward	GAAGACACCATCCACATC
PLXNB2	Reverse	ACTGAACCTGACCGTACAATGC ACGTCAAAGATGAAG
PmirGLO	Forward	GAGCTCTAGCGGCCGCCCTAC
PmirGLO	Reverse	CCTGCAGGCAGAGTGAAAAAGAC

this quantification in PLS and megakaryocytes. Proteins were labelled with an anti-PLXNB2 (AF5329, R&D, Minneapolis, Minnesota, United States) and an anti-β-actin antibody (Sigma). Horseradish peroxidase-conjugated secondary antibody (BioRad, Hercules, California, United States, and Abcam, Cambridge, England) was used for detecting chemiluminescence in a myECL imager (ThermoFisher). Band intensities were quantified using the ImageJ software (http//imagej.nih.gov/ij), and the PLXNB2 protein level was normalized to β-actin.

Reporter Gene Assay

The 3' untranslated region (UTR) of PLXNB2 mRNA, containing the miR-126-3p binding sequence wild-type (wt) or a mutant (mut) form (Invitrogen), was inserted into pmiRGLO Dual-Luciferase miRNA Target Expression Vector (Promega). The final sequences were validated using deoxyribonucleic acid sequencing (►**Table 1**). The empty pmirGLO plasmid containing no UTR sequence served as a negative control. HT1080 cells were plated in 96-well plates at 8,000 cells per well and co-transfected with pmirGLO-3'-UTR-wt, or pmirGLO-3'-UTR-mut and miRNA (50 nM scramble, 50 nM miR-126-3p mimic or 50 nM miR-126-3p inhibitor, which shares a complementary sequence with the miRNA mimic; ThermoFisher), using the Lipofectamine LTX and Plus^{M} reagent (Life Technologies). Each transfection was done in quadruplicate. After 48 hours, luciferase assays were carried out using the Dual-Luciferase Reporter Assay System (Promega). Individual luciferase activity was normalized to the corresponding Renilla luciferase activity constitutively expressed in the pmirGLO vector. Luminescence was measured using a Perkin Elmer Victor3 luminescence detection instrument (Perkin Elmer, Waltham, Massachusetts, United States).

Statistics

Data are expressed as means and standard error of the mean. The paired Student's *t*-test or the one-way analysis of variance followed by Dunnett's multiple comparison test was performed, when appropriate. Data were analysed using GraphPad Prism 7 software (GraphPad Software Inc., USA). *p*-Values of < 0.05 were considered statistically significant.

Results

Haematopoietic Stem Cell Differentiation

$CD34^+$ cells were cultured for 7 days before induction of differentiation via the addition of TPO. At D7, 69 ± 12.3% of the cells were positive for CD34, and 26 ± 4.1, 16 ± 2.8 and 17 ± 4.1% were positive for the CD41, CD42b and CD42d markers of differentiation, respectively. As expected, after induction of differentiation, the percentage of $CD34^+$ cells had decreased at D15 to 42 ± 6.4%, whereas cells positive for CD41, CD42b and CD42d had increased to 86 ± 0.7, 79 ± 2.0 and 74 ± 6.2%, respectively (►**Supplementary Fig. S1B**, available in the online version).

Finally, we investigated the time-course pattern of miR-126-3p levels during human haematopoietic stem cell differentiation. As shown in ►**Fig. 1**, the level of miR-126-3p decreased between D4 and D11, and then increased back to its initial level at D17.

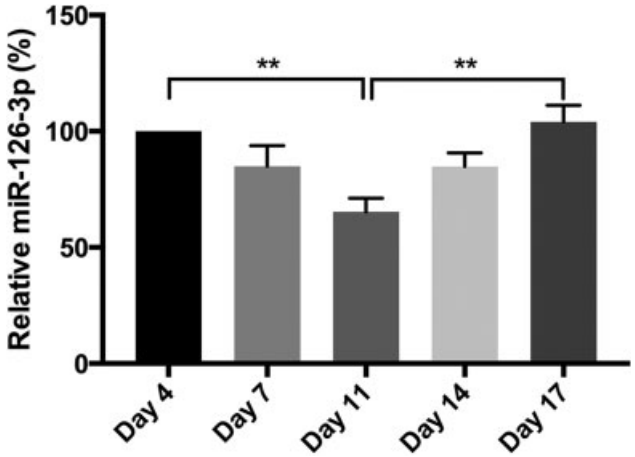

Fig. 1 Expression level of miR-126-3p during differentiation. The expression level of miR-126-3p is represented relative to the level in $CD34^+$ cells after 4 days of culture ($n = 4$). $^{**}p < 0.01$.

Transfection Procedure Efficiency

The transfection procedure was performed at D13. Tested 5 hours after the procedure, using siRNA labelled with Alexa Fluor 488, transfection efficiency was of 47 ± 3.2% ($n = 3$, ►**Supplementary Fig. S2A**, available in the online version). In the PLS, miR-126-3p expression had increased by a factor of 2.4 ± 0.28 more than the mock condition (►**Supplementary Fig. S2B**) 48 hours after the transfection procedure, without affecting either the differentiation process (►**Supplementary Fig. S2C**, available in the online version) or the production of PLS (►**Supplementary Fig. S2D**, available in the online version), which was of 4.1 ± 4.2 10^5 PLS/mL from 1 × 10^6 megakaryocytes/mL at D13 in the mock condition (►**Supplementary Fig. S2D**, available in the online version). Furthermore, transfection of miR-126-3p did not affect miR-223-3p level, one of the main platelet-associated miRNAs (►**Supplementary Fig. S2E**, available in the online version).

Functional Impact of Over-Expression of miR-126-3p on Platelet-Like Structures

At D15, corresponding to the end of megakaryocyte differentiation and maturation, the functional impact of miR-126-3p on the reactivity of PLS was assessed 48 hours after miR-126-3p transfection. A cell suspension containing mature megakaryocytes and PLS was perfused for 5 minutes at a shear rate of 50 s^{-1} in a microfluidic device coated with fibrinogen. The flow chamber assay showed that the number of PLS immobilized in the channel increased by 156 ± 14.9% after miR-126-3p transfection compared to the mock condition (►**Fig. 2A** and **B**). Moreover, in the PLS over-expressing miR-126-3p, we observed a 30% greater increase in CD62P expression after thrombin activation (►**Fig. 2C**) in comparison to the mean fluorescence after activation seen in the mock condition.

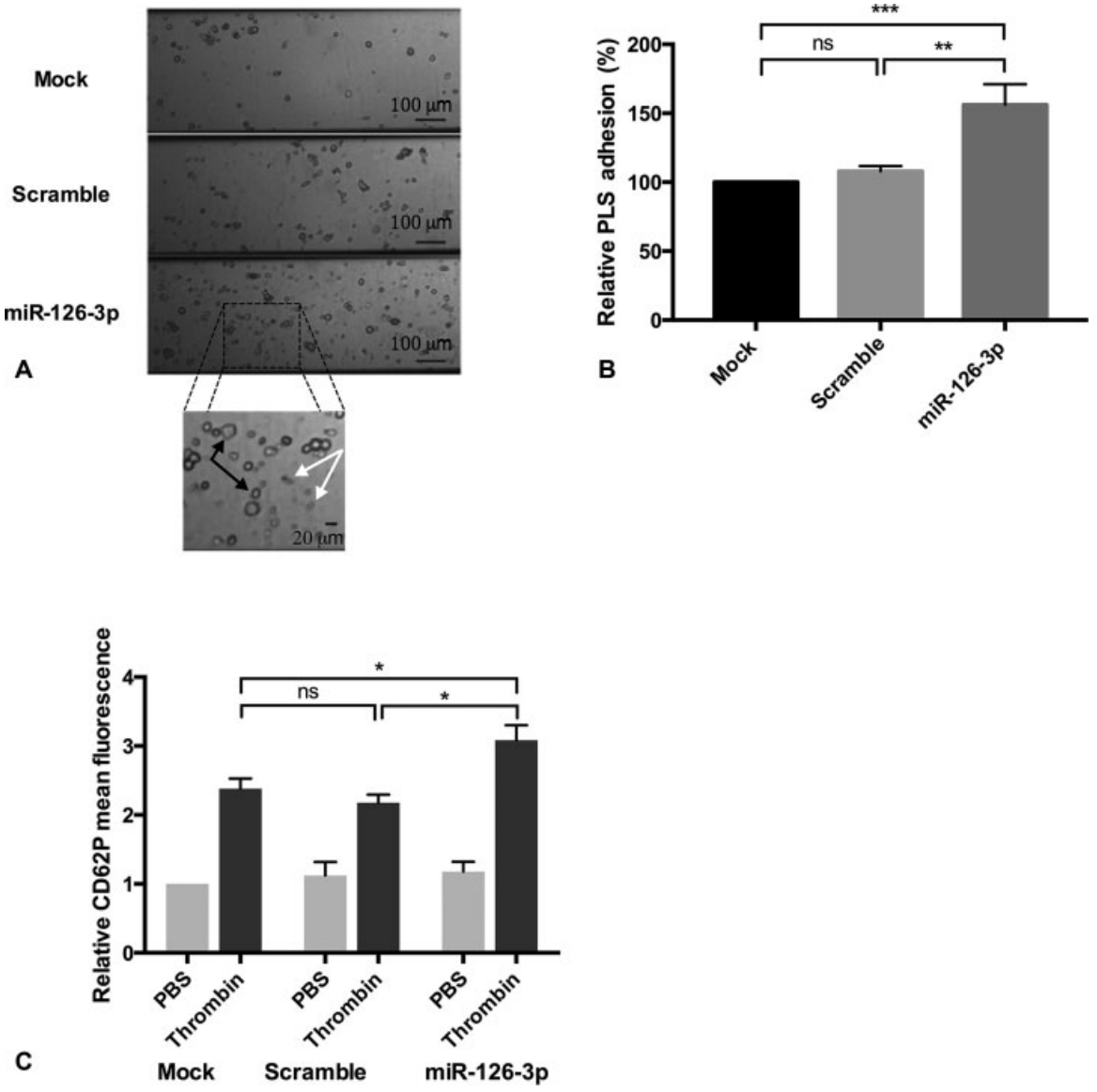

Fig. 2 Effect of miR-126-3p overexpression on megakaryocytes and platelet-like structure (PLS). (**A**) Inverted microscopy photographs of PLS and $CD34^+$-derived megakaryocytes in a fibrinogen-coated channel. Black arrows: megakaryocytes; white arrows: PLS. (**B**) miR-126-3p transfection increased the adhesion of PLS in a fibrinogen-coated channel ($n = 12$). Results are expressed relative to the mock condition. $^{***}p < 0.001$, $^{**}p < 0.01$. (**C**) CD62P relative mean of fluorescence of PLS before and after activation using thrombin 1 U/mL, compared to mock condition (n = 3). $^{*}p < 0.05$.

miR-126-3p Targets ADAM9 and PLXNB2 Genes in Platelet-Like Structures Derived from Human Haematopoietic Stem Cells

ADAM9 is a known miR-126-3p target, as previously shown in mouse and human immortalized cell lines.[11,28] In the PLS derived from human $CD34^+$ cells, we also observed that miR-126-3p transfection was associated with a 30 ± 3.7% down-regulation of ADAM9 mRNA levels compared to the mock condition (►**Fig. 3A**).

To screen for further putative miR-126-3p gene targets in human cells, we examined in silico databases and identified PLXNB2 as the best candidate, with its Context ++ score of –0.58 using TargetScan (http://www.targetscan.org) and an miRSVR score of –0.45 using miRANDA (http://www.microrna.org). In PLS, miR-126-3p over-expression was associated with a 29 ± 2.6% down-regulation of the PLXNB2 mRNA level (►**Fig. 3B**) and a 30 ± 6.1% down-regulation of the PLXNB2 protein level (►**Fig. 3C** and **D**).

Finally, silencing PLXNB2 in megakaryocytes induced a functional phenotype similar to that observed after transfection with miR-126-3p, and this was associated with a 148 ± 11.8% increase in the number of PLS immobilized in the flow chamber in comparison to the mock condition (►**Fig. 4**).

Validation of PLXNB2 as a Direct Target of miR-126-3p

To investigate the causal relationship between miR-126-3p and PLXNB2 down-regulation, a reporter gene assay was performed using the 3'UTR sequence of PLXNB2 wt or mut cloned into dualGLO luciferase reporter vector (►**Fig. 5A**).

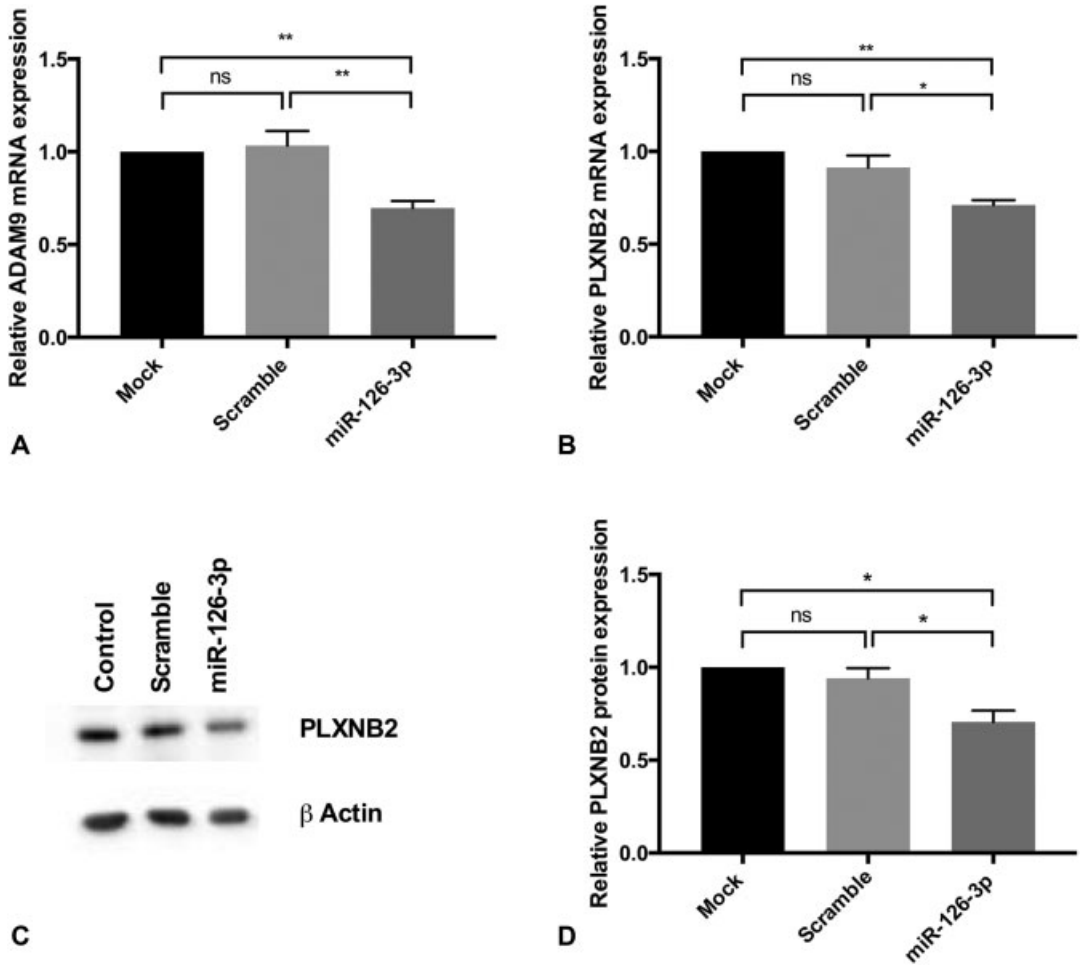

Fig. 3 Impact of miR-126-3p transfection on selected gene targets. Cell suspension was transfected at day (D) 13 using scramble micro-ribonucleic acid (miRNA) or the miR-126-3p mimic, and messenger RNA (mRNA) was extracted at D15. (**A**) Relative expression levels of a disintegrin and metalloproteinase-9 (ADAM9) mRNA in platelet-like structure (PLS) in different conditions ($n = 3$). $^{**}p < 0.01$. (**B**) Relative expression levels of Plexin B2 (PLXNB2) mRNA in PLS in different conditions ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$. (**C**) Western blot of PLXNB2 protein after scramble or miR-126-3p transfection in megakaryocytes and PLS. The picture is representative of three independent experiments. (**D**) Quantification of PLXNB2 protein relative to the mock condition ($n = 3$). $^{*}p < 0.05$.

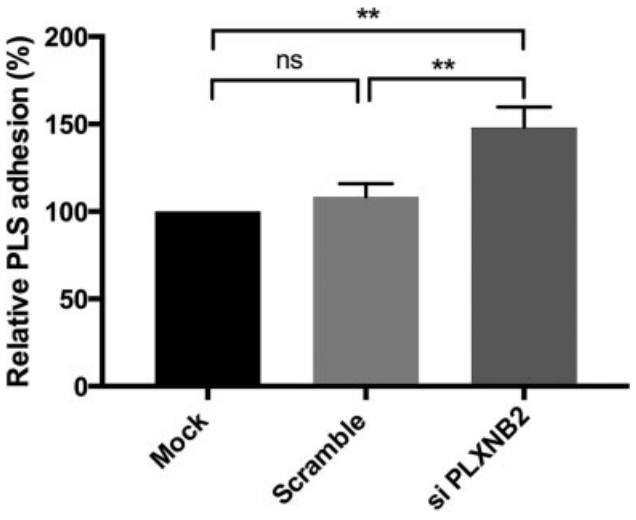

Fig. 4 Plexin B2 (PLXNB2) silencing modulates platelet-like structure (PLS) reactivity. Silencing PLXNB2 increased the adhesion of PLS on the fibrinogen-coated channel. Results are expressed relative to the mock condition ($n = 6$). $^{**}p < 0.01$.

Co-transfection of HT1080 cells with pmirGLO-3'UTR PLXNB2 wt and miR-126-3p resulted in a 42 ± 2.4% reduction in luciferase activity compared with cells transfected in the absence of miR-126-3p. Moreover, the addition of miR-126-3p inhibitor reversed the effect of the miR-126-3p mimic (►**Fig. 5B**). No significant difference in luciferase activity was observed upon transfection with scramble miRNA. Co-transfection of miR-126-3p and the pmirGLO mut did not lead to a significant change in luciferase activity (►**Fig. 5C**).

Discussion

In this study, we demonstrated the feasibility of functionally validating a candidate miRNA of the regulation of platelet reactivity by using human-derived PLS and a flow chamber assay. Moreover, using this model, we validated PLXNB2 as a target gene of miR-126-3p and as a potential regulator of platelet reactivity.

PLS were derived from human haematopoietic stem cells following a protocol described elsewhere that allows the

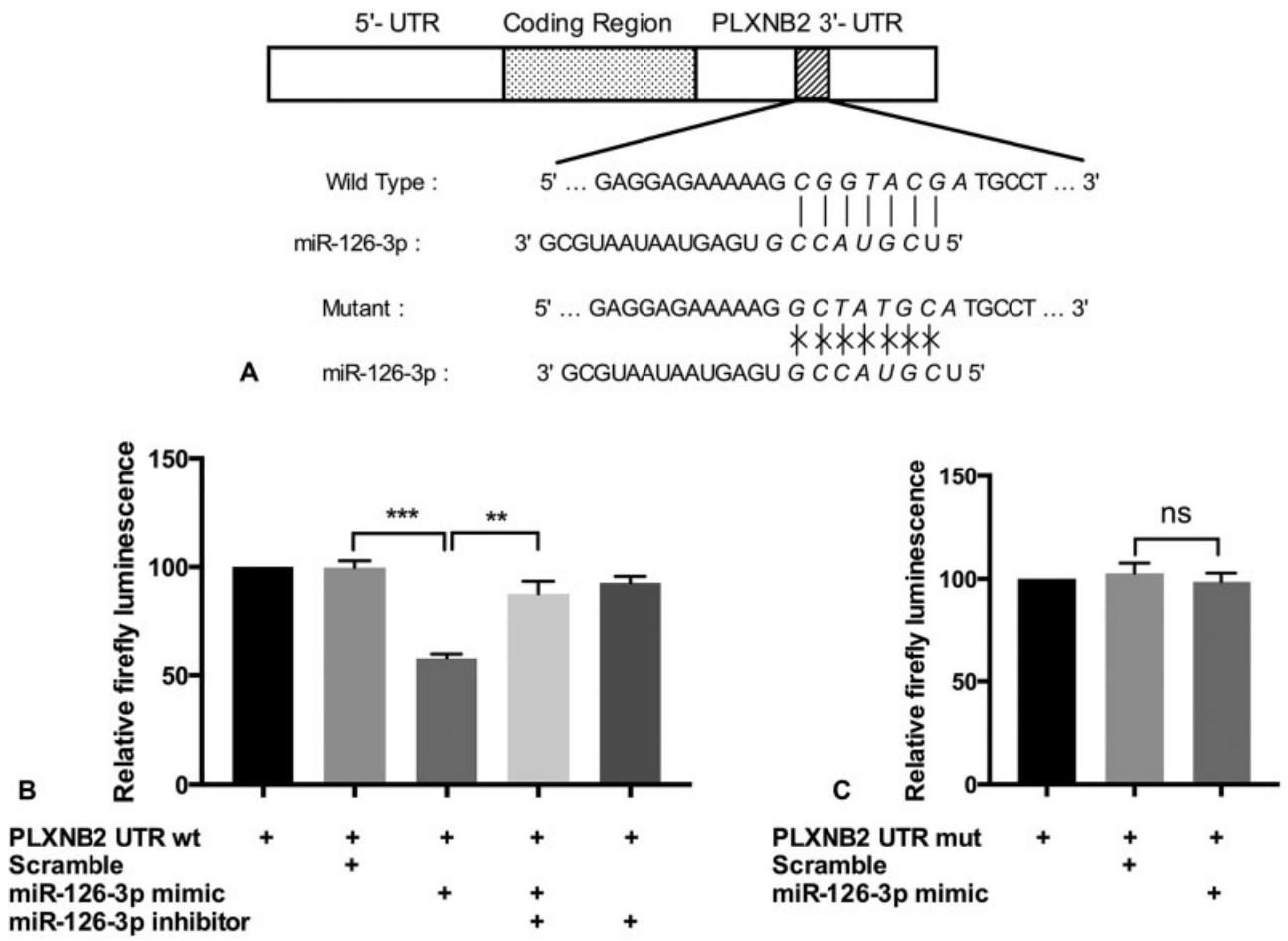

Fig. 5 3' Untranslated region (UTR) sequence of Plexin B2 (PLXNB2) is directly targeted by miR-126-3p. (**A**) The 3'UTR sequence of PLXNB2 wild-type (wt) and mutant (mut) were designed and cloned into PmirGLO vector. (**B**) The PmirGLO-3'UTR PLXNB2 wild-type was co-transfected with the miR-126-3p mimic and/or inhibitor in HT1080 cells. Luciferase activity was normalized on Renilla luminescence ($n = 4$). $^{**}p < 0.01$, $^{***}p < 0.001$. (**C**) The PmirGLO-3'UTR PLXNB2 mutant was co-transfected with the miR-126-3p mimic and/or inhibitor in HT1080 cells ($n = 4$).

production of elements ultra-structurally and functionally similar to circulating platelets.[15] At D15, 86% of cells were positive for CD41, and the proportions of CD42b and Cd42d positive cells were in line with those expected during the differentiation process.[15] Moreover, the functional characterization of the PLS generated in this study using flow cytometry showed a 2.4-fold increase in CD62P expression after stimulation, in line with the twofold increase in glycoprotein (GP) IIb/IIIa activation[15] and the 1.8-fold increase in CD62P expression[29] described in other studies. Finally, recent data have demonstrated significant correlations between the miRNA profile of primary human bone marrow megakaryocytes and cultured megakaryocytes derived from umbilical cord blood progenitors at D13.[30] This further supports our approach based on in vitro haematopoietic stem cell culture and differentiation.

We quantified the miR-126-3p level during the differentiation procedure and observed an initial decrease from D4 to D11 followed by an increase up to D17. This U-shaped curve is in line with independent data on in vitro-differentiated megakaryocytes derived from $CD34^+$ haematopoietic progenitors. This suggests that miR-126-3p may play a role in the regulation of the megakaryocyte differentiation process by unblocking miR-126-3p's target genes during the proliferation stage (D7) and the early stages of differentiation (D11).[31] The initial decrease in miR-126-3p was also evidenced during the first steps of mouse megakaryocyte differentiation.[32] We thus speculate that the relatively low level of miR-126-3p could be associated with an up-regulation of the proteins involved in megakaryocyte differentiation.

It is of note that the transfection procedure with miR-126-3p at D13 did not seem to alter the last stage of differentiation (D13–D15) since it had no impact on PLS production and the expression of differentiation markers.

Unstimulated PLS were perfused in a flow chamber assay, at a relatively low shear rate, to evaluate PLS adhesion on immobilized fibrinogen. Indeed, at a low shear rate, platelet immobilization is primarily mediated by fibrinogen through the GPIIb/IIIa receptor, the main receptor involved in the platelet aggregation process,[33,34] whereas immobilization at higher shear rates relies more on a von Willebrand factor-dependent adhesion process.[34] Using a higher shear rate (up to 1,000 s^{-1}) in our assay was indeed associated with no PLS adhesion on the fibrinogen-coated flow chamber. It is of note that, instead of PLS only, we perfused a cell suspension that included both megakaryocytes and PLS through the flow

chamber assay: this was to favour the margination process of PLS that is of the utmost importance in flow assays.[35] In addition to the flow chamber assay results, miR-126-3p transfection was associated with a 30% increase in CD62P expression in PLS after thrombin stimulation, compared to the mock condition. These data further support the existence of miR-126-3p-mediated platelet reactivity modulation and are in line with data showing a correlation between miR-126-3p levels and soluble P-selectin in plasma.[11]

As expected, we observed that miR-126-3p transfection was associated with a 30% down-regulation in the level of ADAM9 mRNA compared to the mock control. This result was in line with data using locked nucleic acid and a miRNA mimic in a human megakaryoblastic cell line.[11] Since immortalized cell lines can have different regulation mechanisms than cells derived from human progenitors, our result provides a valuable confirmation that ADAM9 is indeed a target gene for miR-126-3p in PLS derived from human haematopoietic stem cells.

In order to further investigate the mechanism associated with the increased reactivity of PLS mediated by miR-126-3p over-expression, we selected the most promising in silico-predicted gene target of miR-126-3p, which was PLXNB2. Interestingly, this gene has also been identified as differentially expressed in cardiovascular patients with an extreme platelet reactivity phenotype.[6] We thus observed that miR-126-3p transfection induced a 29% down-regulation in PLXNB2 mRNA in PLS, and a 30% down-regulation at the protein level in megakaryocytes and PLS. Moreover, the reporter gene assay validated that miR-126-3p directly regulates PLXNB2 gene expression, in line with recent data on ovarian cancer.[36] Finally, transfection with miR-126-3p or the silencing of PLXNB2 using siRNA resulted in a similar functional phenotype. This suggests that miR-126-3p mediates its effect, at least in part, through the down-regulation of PLXNB2. The relative contributions of ADAM9, PLXNB2 or other gene targets to this hyper-reactivity phenotype are unknown.

Plexins—a family of transmembrane proteins—act as receptors for semaphorins, which control RhoGTPase signalling. There are several clues as to the possible roles of PLXNB2 and semaphorins in the regulation of actin dynamics. Semaphorin 4D, a PLXNB2 ligand, was recently shown to support communication between platelets in the early stages of thrombus formation in mice.[37–39] In human glioblastoma cells, semaphorin 4C was shown to stimulate PLXNB2-inducing cellular actin dynamics regulation.[40] In mouse macrophages, PLXNB2 negatively regulates the Rac1 and CDC42 Rho family proteins.[38,41] Finally, by modulating Rho/Rac activity, PLXNB2 enables the modification of actin dynamics and, due to their effect on Ras/PI3K, they can modulate cell adhesion and migration.[38] These observations support the hypothesis that the PLXNB2 gene might regulate platelet actin dynamics, and therefore platelet function, through the regulation of Rho family protein.

This work represents a significant step forward since, until now, the functional validation of miRNA in platelet physiology has been scarce. A first attempt was made in an animal model via the systemic administration of an antagomiR sequence against miR-126-3p in mice, followed by ex vivo platelet aggregation.[11] However, predictive mRNA/miRNA pairs are species-dependent, and mouse-model prediction does not always reflect human gene expression and regulation.[42]

It is worth noting that this study's main limitation is that its flow assay may not be suitable for the functional validation of all the candidate miRNAs regulating platelet reactivity: it will be dependent on the mechanism involved in the modulation of platelet reactivity.

In conclusion, we have described a method for the functional validation, using human-derived cells, of a candidate miRNA in the regulation of platelet reactivity. This model also enables the investigation of potential gene targets to improve our understanding of the regulation of platelet reactivity in humans. Using this method—and for the first time—we validated the impact of miR-126-3p and one of its targets, the PLXNB2 gene, as regulators of platelet function in human-derived cells.

What is known about this topic?

- MicroRNAs have been noted as potential biomarkers of the recurrence of cardiovascular events.
- miR-126-3p is a putative regulator of platelet reactivity.
- Functional validation of microRNAs in human-derived cells is lacking.

What does this paper add?

- We used a flow-based assay with platelet-like structures derived from human haematopoietic stem cells.
- We functionally validated miR-126-3p as a regulator of platelet reactivity using human cells.
- We validated PLXNB2 as a target gene of miR-126-3p, regulating the reactivity of platelet-like structures derived from human haematopoietic stem cells.

Authors' Contributions

A. Garcia, S. Dunoyer-Geindre, J.-L. Reny and P. Fontana designed the study and analysed the data. A. Garcia, S. Dunoyer-Geindre, S. Nolli and V. Zapilko performed the experiments and analysed the data. A. Garcia, S. Dunoyer-Geindre and P. Fontana wrote the first draft of the manuscript, and all authors revised the intellectual content and approved the final version.

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Conflict of Interest

P. Fontana reports non-financial support from Novo Nordisk and Bayer, outside the scope of the submitted work. J.-L. Reny reports non-financial support from Bayer and

grants from Daichii-Sankyo, outside the scope of the submitted work. The other authors have no conflicts of interest to report.

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2.2 IMPACT OF MIR-204-5P ON PLATELET MORPHOLOGY AND FUNCTION

miR-204-5p and platelet function regulation: insight into a mechanism mediated by CDC42 and GPIIbIIIa.

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Submitted to Thrombosis and Haemostasis

Personal contribution: I developed and performed morphological and functional investigations of megakaryocytes and PLS (western Blot, FACS, microscopy and flow chamber assay), analysed the data and wrote the first draft of the manuscript.

Overview

Over the last decades, clinical association studies pointed several candidate miRNAs as regulators of platelet function, however the underlying mechanisms are still unclear. The development of human-derived cell model, described in the previous chapter, allows to study the impact of selected miRNAs on platelet function. In a previous clinical study from our group, the association between miRNAs level in platelets and platelet reactivity pointed miR-204-5p as a modulator of platelet function. In addition, the combined 'omics analysis predicted the involvement of seven putative targets, including CDC42 already validated as miR-204-5p target and described as putative regulator of platelet function. Therefore, the impact of miR-204-5p and its target CDC42 were investigated using our model.

CDC42 is a Rho GTPase protein mediating actin polymerization and formation of cytoskeleton structures. Recent observations reveal that CDC42 may also be involved in the regulation of platelet biogenesis and function through mechanisms that are still not fully understood. Therefore, we studied the impact of miR-204-5p on megakaryocytes differentiation, platelet biogenesis and platelet reactivity. The results showed that miR-204-5p increases actin polymerization in megakaryocytes. In addition, miR-204-5p overexpression enhanced the expression of GPIIbIIIa, the fibrinogen receptor, and was associated with the increase of PLS interaction to soluble and immobilized fibrinogen. Moreover, we validated that miR-204-5p decreases CDC42 level of both mRNA and protein expression in megakaryocytes and PLS. Finally, the phenotype observed after miR-204-5p overexpression was recapitulated by CDC42 silencing. Those data further support that miR-204-5p increases platelet reactivity at least in part through a CDC42-dependent mechanism.

miR-204-5p and platelet function regulation: insight into a mechanism mediated by CDC42 and GPIIbIIIa

Running heads: miR-204 regulates platelet function via GPIIbIIIa

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Abstract

Background: Several platelet-derived miRNAs are associated with platelet reactivity (PR) and clinical outcome in cardiovascular patients. We previously showed an association between miR-204-5p and PR in stable cardiovascular patients, but data on functional mechanisms are lacking.

Aims: To validate miR-204-5p as a regulator of PR in platelet-like structures (PLS) derived from human megakaryocytes and to address mechanistic issues.

Methods: Human hematopoietic stem cells were differentiated into megakaryocytes, enabling the transfection of miR-204-5p and the recovery of subsequent PLS. The morphology of transfected megakaryocytes and PLS was characterized using flow cytometry and microscopy. The functional impact of miR-204-5p was assessed using a flow assay, the quantification of the activated form of the GPIIbIIIa receptor and a fibrinogen-binding assay. qPCR and western blot were used to evaluate the impact of miR-204-5p on a validated target, CDC42. The impact of CDC42 modulation was investigated using a silencing strategy.

Results: miR-204-5p transfection induced cytoskeletal changes in megakaryocytes associated with the retracted protrusion of proPLS, but it had no impact on the number of PLS released. Functional assays showed that the PLS produced by megakaryocytes transfected with miR-204-5p were more reactive than controls. This phenotype is mediated by the regulation of GPIIbIIIa expression, a key contributor in platelet–fibrinogen interaction. Similar results were obtained after CDC42 silencing, suggesting that miR-204-5p regulates PR, at least in part, via CDC42 downregulation.

Conclusions: We functionally validated miR-204-5p as a regulator of the PR that occurs through CDC42 downregulation and regulation of fibrinogen receptor expression.

Keywords: miRNA, CDC42, GPIIbIIIa, platelet reactivity, biomarker

Summary table:

What is known on this topic?

- miR-204-5p is a candidate biomarker of platelet reactivity
- CDC42 is a direct target of miR-204-5p

What does this paper add?

- miR-204-5p modulates megakaryocyte structure and platelet biogenesis via CDC42
- miR-204-5p transfection enhances platelet reactivity via the regulation of GPIIbIIIa expression
- CDC42 silencing induces a similar phenotype than miR-204-5p transfection suggesting than miR-204-5p increases platelet reactivity at least in part through regulation of CDC42

Introduction

Platelets are involved in several human processes, including the regulation of inflammation, the immune system, and cancer.[1, 2] They play a key role in regulating hemostasis and are involved in cardiovascular ischemic events. Platelet reactivity (PR) is a variable phenotype, either with[3] or without[4] antiplatelet drugs, and monitoring platelet function is now being investigated as a mean to tailor antiplatelet drug strategies.[5] Understanding the mechanisms driving PR could lead to the development of new biomarkers or new therapeutic targets in diseases where platelets play a major role.

Human platelets express more than 450 megakaryocyte-derived microRNAs (miRNA) molecules.[6] Over the past years, platelet-derived miRNAs have been identified as biologically and clinically relevant.[7] miRNAs are a class of short, non-coding RNAs of approximately 17 to 25 nucleotides. miRNAs bind by imperfect complementarity to a sequence of the three-prime untranslated region (3'UTR) of mRNAs, repressing the translation and/or degradation of endogenous RNA and thus modulating protein expression. Several miRNAs expressed in platelets are supposed to regulate platelet function, such as miR-223,[8] miR-200b,[9] miR-107,[9] miR-96,[10] or miR-126-3p,[11, 12] and they are associated with PR and/or cardiovascular outcome.[7, 9] However, their exact role in the modulation of human platelet function remains poorly understood.

In a previous study, we pointed out miR-204-5p as a putative biomarker of platelet function.[13] Recently, Ding *et al*.[14] showed a positive correlation between miR-204-5p and patients with high PR. Databases such as Targetscan or miRanda indicate that miR-204-5p has between 700 to 3000 potential target genes (TargetScan release 7.2 and miRanda august 2010 release, respectively). However, the combined analysis of proteomic and transcriptomic data from patients with high or low PR identified differentially expressed genes, targets of miR-204-5p, including *THBS1*, *CDC42*, *CORO1C*, *SPTBN1*, *TPM3*, *GTPBP2* and *MAPRE2*.[13] Among these putative targets, *CDC42*[15] has been validated as direct targets of miR-204-5p using reporter gene assay.

CDC42 belongs to the RHO family of GTPases that contributes to cytoskeletal changes resulting in the regulation of cell morphogenesis and cell adhesion. Furthermore, CDC42 regulates actin polymerization, demarcation membrane system (DMS) formation, proplatelet biogenesis,[16, 17] and platelet function.[18] We thus addressed the issue of the impact of miR-204-5p and CDC42 on platelet biogenesis and function using an *in vitro* model allowing to generate megakaryocytes and platelet-like structures (PLS) from human hematopoietic stem cells.[12, 19]

Material and methods

Human CD34+ hematopoietic progenitor cells

CD34+ purification, differentiation, transfection, and measurement of transfection efficiency were performed as previously described.[19, 20] Overexpression of miR-204-5p and the knock-down of CDC42 were performed using an hsa-miR-204-5p mimic (ThermoFisher, Waltham, USA) and small interfering RNA (siRNA) (Qiagen, Hilden, Germany), respectively. Morphological and functional characterizations were performed on day (D) 15, 48 hours after transfection unless otherwise specified.

Megakaryocytes were recovered and centrifuged at 100g for 10 minutes at 37°C in the presence of 25 μM prostaglandin I2 (PGI2 Cayman Chemical Company, Ann Arbor, USA) and 0.02 U/ml apyrase (Sigma, St Louis, USA) to avoid activation. The supernatant containing the PLS was centrifuged at 1000g for 10 minutes and resuspended in dedicated buffer. The concentration was monitored using Tali™ Image-Based Cytometer (ThermoFisher), megakaryocytes were defined as elements > 6 μm and PLS were defined as elements <6 μm.

miRNA quantification

miRNA content in PLS was quantified using Taqman qPCR and normalized using a panel of stably expressed miRNAs (miR-28, miR-29c, and miR-151) previously identified using the geNorm algorithm.[21, 22]

RNA extraction and mRNA analysis

mRNA analysis was performed as previously described.[12] The oligonucleotide sequences used for qPCR were:

HB2M-forward: 5'-TGCTCGCGCTACTCTCTCTTT-3'

reverse: 5'-TCTGCTGGATGACGTGAGTAAAC-3'

CDC42-forward: 5'-ACATCTGTTTGTGGATAACTCA-3'

reverse: 5'-GGGAGCCATATACTCTTGGA-3'

Western blot analysis

Megakaryocytes and PLS were activated by stirring for 6 minutes with TRAP 20 μM at 37°C and were then lysed in a dedicated buffer. The protein extracts were separated onto 12% polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane (Amersham Biosciences, NY, USA). Proteins of interest were labeled with primary anti-GAPDH and anti-CDC42 (Cell Signaling Technology, Danvers, USA). Band intensities were quantified using ImageJ software, and the CDC42 protein level was normalized to GAPDH.

Megakaryocyte differentiation

In order to assess the possible role of miR-204-5p transfection on megakaryocyte differentiation, megakaryocyte pellet was resuspended in 200 µl phosphate-buffered saline (PBS) and incubated for 1 hour at 4°C in 70% ethanol. The cells were centrifuged at 1000g for 10 minutes, resuspended in 100 µl propidium iodide (PI) (BD Biosciences, Allschwill, Switzerland) at 50 µg/ml in PBS, and then incubated for 30 minutes at 37°C. PI labeling was measured using fluorescence-activated cell sorting (FACS) to evaluate the megakaryocytes' ploidy. Analyses were performed using an Accuri C6 flow cytometer (BD Biosciences).

Megakaryocyte differentiation was also analyzed using FACS with selected antibodies against specific markers of differentiation (CD41, CD42b, and CD42d) as previously described.[20]

Megakaryocyte polarization

Megakaryocytes were fixed and permeabilized using 4% paraformaldehyde (PAF) and 0.05% triton in PBS. They were subsequently centrifuged at 250g for 10 minutes onto coverslips previously coated with 100 µg.ml^{-1} poly-L-Lysine (Sigma) for 5 minutes and then dried at room temperature. The DMS were visualized using co-localization of the filamentous (F)-actin and GP1bβ[23, 24] in differentiated megakaryocytes, defined as megakaryocytes exhibiting at least two nuclear lobes. GP1bβ was labeled using a mouse antibody anti-GP1bβ (CD42c, Santa Cruz Biotechnology, Dallas, USA) and then stained with AF-488 anti-mouse antibody (Life Technologies, Carlsbad, USA). F-actin was visualized using TRITC-phalloidin (Sigma). Quantification was performed blindly on at least 30 mature megakaryocytes per condition.

The coverslips were mounted using the Vectashield® Hardset™ mounting medium with DAPI (Reactolab SA, Servion, Switzerland). The cells were visualized using an LSM 700 microscope (Zeiss, Oberkochen, Germany), and the images were analyzed blindly using ImageJ software (http://rsb.info.nih.gov/ij/). The 3D reconstitution of z-stacks was performed using a background subtraction and the application of a Gaussian filter using Imaris 9.5.1 Software (Bitplane AG, Zurich, Switzerland).

ProPLS formation

On D13, 5 hours after transfection, megakaryocytes were seeded at 5000 cells/well in a 96-well plate. On D15, pictures were taken at magnification x 100 and analyzed using ImageJ software. Megakaryocytes bearing proPLS were defined as megakaryocytes with at least one protrusion (500

megakaryocytes per condition were analyzed). The surface areas of the protrusions were also evaluated for 50 megakaryocytes bearing proPLS.

PLS production

The PLS suspension (300 µL) released from 500,000 megakaryocytes was labeled with Hoechst (ThermoFischer) and anti-CD41-APC antibody (BioLegend, San Diego, USA). PLS were washed and resuspended in 300 µL PBS and 50 µL Precision Count Beads (BioLegend). Samples were analyzed on an Attune NxT flow cytometer (ThermoFischer), and data processing was performed using FlowJo software. PLS were defined as Hoechst-/CD41+ events. The concentration of PLS was evaluated using the following equation:

C PLS = (PLS count x V Beads x C Beads) / Bead count x V PLS, where PLS count = number of PLS quantified, V Beads = Volume of Precision Count Beads, C Beads = Concentration of Precision Count Beads, Bead count = number of beads quantified, and V PLS = Volume of the PLS suspension.

PLS size

To study the PLS size at rest, PLS were recovered and incubated for 1 hour at 37°C on poly-L-Lysine-coated coverslips. Non-adherent PLS were removed by washing with PBS-0.1% BSA. Adherent PLS were fixed using 4% PAF and then permeabilized using 0.5% Triton X-100 in Hank's Balanced Salt Solution.

F-actin was stained with TRITC-phalloidin, and coverslips were mounted with Vectashield® Hardset™ mounting medium with DAPI. Images were acquired using an LSM700 and analyzed blindly to the experimental condition using ImageJ software. The PLS area was measured in arbitrary units relative to the mock condition. At least 100 PLS were analyzed per condition.

PLS receptor expression

PLS were adjusted at 1.3 x 10^6 PLS/mL in PBS. PLS were labeled with anti-CD42b-BV650 (BioLegend), anti-CD41-V450 (BD Biosciences), anti-CD61-APC (Miltenyi Biotec), and anti-CD49b-FITC (BD Biosciences) antibodies or with their corresponding isotypic antibodies. PLS were washed in PBS and fixed in 2% PAF. Flow cytometry was performed using an Attune NxT flow cytometer. The data were analyzed using FlowJo software.

PLS activation

To investigate the potential effect of miR-204-5p on GPIIbIIIa activation, PLS were adjusted at 1.10^6 PLS.mL^{-1} in Tyrode's albumin (TA) buffer25 and then activated with 1 U/mL thrombin (Sigma) for 6 min at 37°C without stirring. PLS were labeled with anti-CD31-APC (BioLegend), anti-CD62P-PE (BD

Pharmingen, Allschwil, Switzerland) and PAC1-FITC (BD Biosciences), and antibodies or with their corresponding isotypic antibodies. PLS were then fixed with 2% PAF. Flow cytometry was performed using an Accuri C6 flow cytometer, and the data were analyzed using BD Accuri™ C6 Plus software (BD Biosciences).

PLS adhesion to fibrinogen

A spreading assay was performed by adhesion of PLS onto fibrinogen-coated coverslips (100 µg/mL) for 1 hour at 37°C. PLS were fixed, permeabilized, and labeled with TRITC and DAPI, as described above. Pictures were taken using an LSM700, and the PLS spreading areas were quantified using ImageJ software.

To investigate the binding of fibrinogen on activated PLS surfaces, PLS were adjusted to 1.3 x 10^6 cells/mL in TA buffer. PLS were then activated with 0.05 U/mL of human thrombin (Calbiochem, Los Angeles, USA) in the presence of 50 µg/mL of AF-488-labeled fibrinogen (ThermoFisher) for 10 minutes at 37°C without stirring. PLS were labeled with anti-CD31-APC antibody and fixed with 2% PAF. Flow cytometry was performed using an Accuri™ C6 flow cytometer with an FL-1 90% filter, and the data were analyzed using BD Accuri™ C6 Plus software. Double-labeled anti-CD31-APC and AF-488 events were considered to be PLS binding to fibrinogen.

Megakaryocyte cytoskeleton

To study the impact of miR-204-5p on megakaryocyte cytoskeleton, these were put onto fibrinogen-coated coverslips and examined using confocal microscopy. Briefly, coverslips were coated with 100 µg/mL of fibrinogen (Sigma) at 4°C overnight. Coverslips were washed with PBS-0.1% BSA and blocked with 1% BSA. Megakaryocyte adhesion occurred at 37°C for 1 hour. Megakaryocytes were then permeabilized and labeled with TRITC and DAPI, as described above. Pictures were taken using an LSM700, and at least 20 mature megakaryocytes per condition were analyzed, blindly to the experimental condition, using ImageJ software.

F/G actin ratio measurement

Megakaryocytes were activated by stirring for 6 minutes at 37°C with 20 µM Thrombin Receptor Activator Peptide-6 (TRAP, Bachem, Dubendorf, Switzerland). F and G (globular) actin fractions were recovered using the G-actin/F-actin *in vivo* assay kit (Cytoskeleton, Denver, USA) according to the manufacturer's instructions. Briefly, the megakaryocytes were harvested by centrifugation at 1000g for 10 minutes, resuspended in a lysis buffer, and homogenized by passing them through a 25G needle. After 10 minutes of incubation at 37°C, the lysates were ultra-centrifuged at 100,000g for 2 hours at 37°C. The supernatants containing G-actin were retrieved, and the pellets containing F-

actin were resuspended for 1 hour on ice in a volume of depolymerization buffer equal to the corresponding supernatant. Equal volumes of G- and F-fraction were loaded onto a 12% polyacrylamide gel and subjected to western blot analysis using rabbit anti-actin antibody (Cytoskeleton). G- and F-actin band intensities were quantified separately using ImageJ software.

Flow chamber assay

A flow chamber assay was performed as previously described.[12] Briefly, megakaryocytes and PLS were perfused at 50 s^{-1} for 5 minutes onto fibrinogen-coated slides (Vena8 Fluoro+™, Cellix, Dublin, Ireland). Pictures were taken at five different points along the channel (Si-3000 camera, Ceti, Medline Scientific, Chalgrove, England), and image analysis was performed using a custom-made framework written in MATLAB (The MathWorks, Inc. Natick, USA) and dedicated to quantifying PLS individually and megakaryocytes according to their size.

Statistical analysis

To avoid batch effect from different donors of buffy coat, all the data are expressed relatively to mock control. All the data are presented as the mean ± SEM of at least three independent experiments. Statistical significance was evaluated using one-way ANOVA followed by a *post hoc* Tukey test or a student *t*-test, when appropriate. Data were analyzed using GraphPad Prism 7 software (GraphPad Software Inc., San Diego, USA). P-values<0.05 were considered statistically significant.

Results

PLS function

PLS activation with thrombin induced an increase in P-selectin expression (31.8 ± 5%), activation of GPIIbIIIa (47.8 ± 13%) and binding of AF488 soluble fibrinogen (42.3 ± 10%) **(Fig. S1).** These data are in line with the characteristics of PLS produced elsewhere with the same protocol.[12, 19]

The impact of miR-204-5p transfection on CDC42 downregulation

As expected, miR-204-5p transfection was associated with a 21.2 ± 4% downregulation of the mRNA level **(Fig. 1A)** and a 31 ± 6% downregulation of the protein level **(Fig. 1B**) of CDC42, which is a validated target of miR-204-5p.

miR-204-5p transfection did not affect megakaryocyte differentiation but modulated megakaryocyte polarization

FACS analysis showed that miR-204-5p transfection at D13 had no effect on the ploidy of megakaryocytes at D15 **(Fig. 2A)**. In addition, the percentage of megakaryocytes expressing the differentiation markers CD41, CD42b and CD42d was unchanged 48h after transfection showing that miR-204-5p transfection does not impact the differentiation process **(Fig. 2B).** Labeling of megakaryocytes with DAPI and GPIbβ/F-actin enables the study of the localization of both the nucleus and DMS, respectively. In both mock and scramble conditions, the nuclei were located at the periphery of the megakaryocytes, whereas their DMS were present at the opposite edge. Conversely, in megakaryocytes overexpressing miR-204-5p, nuclei were in the center of the cells and were surrounded by DMS, indicating an absence of polarization in these megakaryocytes. **(Fig. 2C).** In mock condition, 85 ± 2.3% of megakaryocytes were polarized, whereas after transfection with miR-204-5p, polarization was observed in only 49 ± 6.4% of megakaryocyte cells **(Fig. 2D).** This indicates that transfection of miR-204-5p 48 hours before the end of the differentiation had no effect on the differentiation markers nor on the ploidy, but it did modulate megakaryocyte polarization.

miR-204-5p overexpression modulated the production of PLS without affecting their size

miR-204-5p transfection had no effect on the proportion of megakaryocytes releasing proPLS (data not shown). However, the megakaryocytes transfected with miR-204-5p produced abnormal protrusions with retracted networks of proPLS tips. Protrusions were more compact after miR-204-5p transfection, showing a 50% decrease in proPLS protrusion areas **(Fig. 3A and 3B).**

In addition, miR-204-5p overexpression increased the production of PLS slightly but significantly as compared to the mock condition **(Fig. 3C)** without affecting their size **(Fig. 3D)**. This was confirmed by an analysis of the median forward scatter (FSC) of the PLS in flow cytometry **(Fig. S2).**

miRNA-204-5p overexpression increased GPIIb and GPIIIa expression in PLS

To further characterize the PLS produced by miR-204-5p-transfected megakaryocytes, we quantified the expression of selected platelet receptors. **Fig. 3E** shows that miR-204-5p transfection had no effect on CD42b (GPIb) expression, a late marker of PLS differentiation, nor on CD49b (GPIa) expression, a subunit of a collagen receptor. In contrast, miR-204-5p transfection enhanced the expression level of two subunits of the fibrinogen receptor: CD41 (GPIIb) and CD61 (GPIIIa). Their median fluorescence increased by 26 ± 9.6% and 18 ± 1.5%, respectively.

miR-204-5p overexpression increased PLS reactivity and adhesion

Since we evidenced an increase in GPIIbIIIa expression in PLS derived from miR-204-5p-transfected megakaryocytes, we focused functional assays using fibrinogen as a matrix. Thrombin stimulation did not induce a significant modulation in P-selectin (CD62P) expression after miR-204-5p transfection **(Fig. 4A).** Among activated PLS (defined as CD31+/CD62P+ PLS), the median fluorescence of PLS positive for the activated form of GPIIbIIIa was 30 ± 9% higher after miR-204-5p transfection than under the mock or scramble conditions **(Fig. 4B)**. In static conditions, PLS adhesion on immobilized fibrinogen was associated with a 45 ± 13% greater PLS spreading area after miR-204-5p transfection than in the mock or scramble conditions **(Fig. 4C** and **4D)**. Finally, stimulation of PLS in the presence of soluble AF488-labeled fibrinogen showed a 51 ± 15% greater capacity of miR-204-5p-transfected PLS to bind soluble fibrinogen than in the mock condition **(Fig. 4E)**.

miR-204-5p modulated megakaryocyte adhesion in dynamic conditions and induced cytoskeletal reorganization

In flow conditions, we found 50 ± 16% more megakaryocytes adhering to the fibrinogen-coated channel after miR-204-5p transfection than in the mock condition **(Fig. 5A)**. However, PLS adhesion did not differ significantly with this assay (data not shown). Since cytoskeletal rearrangement plays a critical role in cell adhesion and is regulated by CDC42, the actin cytoskeleton of megakaryocytes was analyzed using confocal microscopy. This revealed a markedly disturbed cytoskeletal organization. In control cells, actin filaments were located at cell periphery, whereas transfection with miR-204-5p increased the content of stress fibers **(Fig. 5B)**. Analysis of actin dynamics showed that upon activation with TRAP, the F/G actin protein ratio increased more in megakaryocytes transfected with miR-204-5p than in mock or scrambled conditions **(Fig. 5C).** This indicates that miR-204-5p overexpression promotes the polymerization of G-actin into F-actin.

CDC42 mediated the effect of miR-204-5p on PLS

SiCDC42 transfection represses CDC42 protein expression about 42 ±7% **(Fig. S3)**. Downregulation of CDC42 produced very similar data to that obtained from the miR-204-5p transfection of megakaryocytes. Using our flow chamber assay, CDC42 silencing induced a 46 ± 14% increase in megakaryocyte adhesion **(Fig. 6A)** but had no effect on PLS adhesion (data not shown)**.** Silencing of CDC42 and the overexpression of miR-204-5p showed similar F-actin organization, with greater stress fiber formation in megakaryocytes **(Fig. 6B)**. The area of the proPLS network decreased by 38 ± 7% after CDC42 silencing **(Fig. 6C)**. The pattern was similar to that observed after the overexpression of miR-204-5p, with a less expansive proPLS network and shorter protrusions than in control conditions **(Figs 6D and 3B)**. Finally, CDC42 silencing had no effect on P-selectin secretion **(Fig. 6E)** but increased

the activated form of GPIIbIIIa by 22 ± 3% in PLS **(Fig. 6F)** and the PLS spreading area by 34 ± 6% **(Fig 6G** and **6H).**

Discussion

This study investigated the functional impact of miR-204-5p in regulating PR using human cells. This step is of utmost importance to validate platelet-derived miRNA identified through association studies and proposed as biomarker of PR and/or recurrence of cardiovascular events.[25] PLS were derived from human hematopoietic stem cells according to a protocol which enables the production of elements ultra-structurally and functionally similar to circulating platelets.[19, 26] PLS show a distribution of the open canicular system and a content α and δ granule similar to human platelets but are slightly larger than circulating platelet. PLS are functional, they can be incorporated into a thrombus formed *in vitro* or *in vivo* and their activation leads to the exposure of the active form of GPIIbIIIa and secretion of P-selectin.[19]

We first studied the effect of miR-204-5p on megakaryocyte differentiation and PLS formation. Our results showed that miR-204-5p had no effect on the polyploidization of megakaryocytes, nor on the proportion of megakaryocytes expressing the differentiation markers. However, transfection of megakaryocytes with miR-204-5p resulted in a modification of the localization of the DMS within megakaryocytes and of the nucleus polarization, associated with the abnormal generation of a PLS network and retraction of the proPLS buds. The DMS is a membrane reservoir for proplatelet formation and extension,[24, 27] and abnormal development of the DMS has been implicated in several hereditary diseases associated with macrothrombocytopenia.[28, 29] Moreover, Mountford *et al*[30] showed that alteration of DMS structures did not affect platelet production but dysregulated platelet adhesion, resulting in the formation of large, unstable thrombi. Taken together, those studies clearly indicated that DMS reorganization in megakaryocytes is critical for platelet production and function; therefore modifications in the localization of the DMS, induced by miR-204-5p overexpression, may lead to the production of functionally altered PLS.

Confocal microscopy analysis of megakaryocytes showed that miR-204-5p transfection was associated with a marked modification of cytoskeletal organization and an increase in stress-fiber content. Stress fibers are mainly composed by polymerized actin. G-actin can polymerize into the F form, and F-actin can depolymerize into G-actin, according to cell activity. In resting cells, about 40% of actin is in F form and this proportion rises to 80% after activation.[31] Dysregulation of the actin cytoskeleton was also shown to impair the generation of mature platelets,[32, 33] indicating an effect of

F-actin on both megakaryocyte architecture and platelet formation. The stimulated megakaryocytes in our study exhibited a greater F/G actin ratio in megakaryocytes transfected with miR-204-5p than in non-transfected cells or cells transfected with scrambled miRNA, indicating that miR-204-5p enhances F-actin polymerization. Actin polymerization is regulated by the Rho GTPases family, including RHOA, RAC1, and CDC42, which control the formation of stress fibers, lamellipodia, and filopodia, respectively.[34] Since CDC42 is a direct target of miR-204-5p,[15] we investigated the role of CDC42 in the phenotype observed after miR-204-5p transfection. In our study, downregulation of CDC42 using a siRNA and miR-204-5p transfection both resulted in an increase in stress-fiber content in megakaryocytes and an abnormal network of proPLS formation. This suggests that the changes in megakaryocyte morphology associated with miR-204-5p transfection were mediated, at least in part, via CDC42 downregulation. Palazzo *et al*[35] showed that CDC42 is involved in cytoskeletal changes during differentiation via its effector N-WASP. The knock-down of N-WASP enhanced stress-fiber formation, further supporting the hypothesis that CDC42 regulates stress-fiber formation. Moreover, Antkowiak *et al*[17] showed that a deficiency in CDC42 dramatically impairs DMS biogenesis in megakaryocytes. They observed a similar pattern with the formation of abortive protrusions, bearing enlarged tips, which retracted into the cell after drug-induced inhibition of CDC42 in megakaryocytes. Lastly, they showed that formation of mature DMS is intimately linked to F-actin turnover through a microtubules-independent mechanism. Taken together, these results suggest that miR-204-5p modulates DMS formation through downregulation of CDC42 and an effect on F-actin polymerization into stress fibers.

The mechanism by which CDC42 increases stress fiber formation remains unclear. Due to several experimental limitations – notably the low number of megakaryocytes and PLS obtained *in vitro* and subsequently the small amount of protein recovered – it was not possible to use dedicated techniques allowing the study of protein phosphorylation, or the degree of activation of the Rho GTPases, the detection limits being reached. However, at least two hypotheses can be put forward. Stress fibers formation pass through a RHOA-dependent process, and Rho GTPases interact with each other.[36] For example, in the initial steps of cell adhesion and spreading, RHOA-GTP level is inhibited whereas RAC1 and CDC42 activation increases, resulting in suppressed actomyosin contractility and enhanced actin-mediated protrusion. At a later phase, RAC1 and CDC42 decrease and the activity of RHOA increases gradually inducing the formation of stress fibers and the maturation of focal adhesion.[37] In addition, RAC GTPase negatively regulates the RHOA-induced stress fiber formation and focal adhesion assembly through its effector p21-activated kinases (PAK) at the leading edge.[38] Those studies indicate that Rho GTPases ensure a coordinated control of cytoskeleton modification. We can then speculate that the downregulation of CDC42 mediated via miR-204-5p induces an

enhanced activity of other Rho GTPase family members, in particular RHOA leading to the increase formation of stress fibers.

An alternative hypothesis is based on the actin depolymerization factor (ADF/cofilin) function. This family of actin-binding proteins acts as a negative regulator of actin stress fibers by depolymerizing and severing the existing actin filaments. Phosphorylation of cofilin by LIM kinases inhibits cofilin-induced actin depolymerization resulting in the formation of stress fibers.[39] LIM kinases are common downstream effectors of Rho GTPases and are activated by p65PAK and p160 ROCK downstream of CDC42, RAC1 and RHOA. We can thus speculate that in miR-204-5p transfected cells, downregulation of CDC42 induces an increased LIM kinases activity, leading to an enhanced amount of F-actin. Further studies are needed to understand the molecular pathways underlying the overexpression of stress fibers in miR-204-5p-transfected cells.

Conversely to our *in vitro* observations, proplatelet formation in *Cdc42*$^{-/-}$ mice was moderately reduced.[40, 41] In addition, the deletion of either *Rac1* or *Cdc42* was associated with thrombocytopenia in mice.[41] In our study, human megakaryocyte transfection with miR-204-5p had no impact on the number of megakaryocytes releasing a proPLS network, but it did induce a slight but significant increase in the number of PLS produced. Those conflicting results might be explained by the fact that, in our study, PLS were released in static conditions and were collected mechanically via flushing, whereas in the *in vivo* model, the elongation process enables proplatelet release into the bloodstream. It is of note that the miR-204-5p transfection procedure induced only a partial downregulation of CDC42, whereas the knockout of *Cdc42* in mice resulted in a constitutive absence of the protein which may also have contributed to these different results. Taken together, these findings indicated that miR-204-5p plays a role in the conformational changes in megakaryocyte cytoskeleton and in proplatelet formation via the modulation of CDC42.

The functional impact of miR-204-5p was firstly investigated using a model of cell adhesion in dynamic conditions already described in our previous study.[12] Flow experiments showed an increase in megakaryocyte adhesion on fibrinogen after miR-204-5p transfection that was recapitulated after silencing. This indicates that miR-204-5p plays a role in megakaryocyte adhesion in flow conditions, at least partly due to its effect on CDC42. Surprisingly, PLS adhesion in dynamic conditions, after either miR-204-5p transfection or silencing CDC42, remained unaffected. These results may be due to the absence of red blood cells that contribute to PLS margination and enhance contact between the platelets and the channel,[42] while this margination effect is less important for larger cells such as megakaryocytes. It is of note that megakaryocyte function is often used as a proxy for platelet function,[43] suggesting that the functional data obtained with megakaryocytes might be translatable to PLS.

miR-204-5p transfection had no effect on the size of the PLS produced but induced an upregulation of CD41 and CD61, the two subunits of the fibrinogen receptor. Although the magnitude of this modulation may be considered as modest, it is noteworthy that moderate variation of platelet receptors of similar magnitude may have functional consequences.[44] The magnitude of this upregulation is in line with the 30%-increase of the GPIIbIIIa active form after PLS activation. In addition, we demonstrated that miR-204-5p transfection of megakaryocytes produced PLS with an increase of fibrinogen binding and spreading area by approximately 50%. This suggests that miR-204-5p mediates its effect on PLS binding to fibrinogen at least in part via GPIIbIIIa upregulation, and via an increase of its affinity for fibrinogen. Taken together, these data demonstrated that miR-204-5p increases PLS binding to fibrinogen, and this may contribute to the higher PR phenotype.

Since silencing CDC42 recapitulates the phenotype associated with miR-204-5p transfection, this validated target of miR-204-5p plays a major role in the regulation of PR. The role of CDC42 on PR has been studied in mice, using different knockout approaches and leading to conflicting results. Using an Mx-cre;*Cdc42*$^{lox/lox}$ mouse model, Akbar *et al*.[45] showed that *Cdc42* knockout was associated with an inhibition of filopodia formation, a decrease in P-selectin release, and an inhibition of agonist-induced platelet aggregation. Conversely, Pleines *et al*.,[40] using Pf4-cre *Cdc42*$^{-/-}$ knockout mice, found normal filopodia formation, increased aggregation upon low agonist stimulation, accelerated thrombus formation, a shorter time to occlusion of arterioles injured by ferric chloride, an enhanced aggregate formation on collagen under flow despite a slight decrease in GPIIbIIIa activation. Overall, this latter study supports the idea that *Cdc42* knockout is associated with an upregulation of PR. Different methodological approaches may explain these discrepancies between both studies.

The exact mechanism allowing an increase in GPIIbIIIa expression after miR-204-5p transfection remains unclear. Since the Rho family GTPase proteins have been described as modulators of GPIIbIIIa activity,[16, 46] we speculate that miR-204-5p may modulate GPIIbIIIa expression and activity, either directly or indirectly via the modulation of other members of the Rho family of GTPases. The small Rho GTPase RAP1b is known to play a key role in regulating outside-in signaling and could be a link between GPIIbIIIa activation and the cytoskeleton.[36, 47, 48] Indeed, inhibition of actin polymerization by cytochalasin D or latrunculin A impaired RAP1b-dependent fibrinogen binding in stimulated megakaryocytes[47] and RAP1b can enhance agonist-induced ligand binding to GPIIbIIIa possibly by modulating the interactions of GPIIbIIIa with the actin cytoskeleton.[47] We can thus speculate that in our study the increased actin polymerization induced by miR-204-5p transfection could result in an enhanced integrin affinity mediated at least in part by RAP1.

In conclusion, our findings demonstrated that the overexpression of miR-204-5p had an impact on the actin cytoskeleton and the localization of DMS in megakaryocytes which produce PLS. In addition, miR-204-5p overexpression upregulate PR through, at least in part, an increased expression of the GPIIbIIIa receptor. These structural and functional effects are mediated via the downregulation of CDC42. To the best of our knowledge, this study is the first dedicated to the functional validation of miR-204-5p as a regulator of PR in human-derived cells, and it supports the idea that miR-204-5p has a potential role as a biomarker for PR, a phenotype of importance in a variety of clinical conditions.

Conflict of interest

The authors have no conflicts of interest to report.

Author's contribution

A. Garcia, S. Dunoyer-Geindre, C. Strassel, J.-L. Reny, and P. Fontana designed the study and analyzed the data. A. Garcia, S. Dunoyer-Geindre, and S. Nolli performed the experiments and analyzed the data. A. Garcia, S. Dunoyer-Geindre, and P. Fontana wrote the first draft of the manuscript, and all authors revised the intellectual content and approved the final version.

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Figure legends

Figure 1. miR-204-5p transfection efficiency

miR-204-5p transfection induced a decrease of the relative expression of both (A) CDC42 mRNA and (B) protein level in megakaryocytes and PLS. n = 4 to 5 independent experiments; * $p < 0.05$, ** $p < 0.01$.

Figure 2. Effect of miR-204-5p on megakaryocyte differentiation

Fluorescence-activated cell sorting analysis at D15 showed that miR-204-5p (A) had no effect on megakaryocyte ploidy, and (B) no effect on the expression of the specific differentiation markers CD41, CD42b, and CD42d. miR-204-5p decreased megakaryocyte polarization observed as the co-localization of F-actin and GP1bβ (C and D, LSM700 microscope, background subtraction and application of a Gaussian filter using Imaris 9.5.1 Software for 3D rendered, scale bars equal 10 µm). N = 3 to 5 independent experiments; * $p < 0.05$, *** $p < 0.001$.

Figure 3. Characterization of PLS overexpressing miR-204-5p

miR-204-5p overexpression (A) significantly decreased the relative proPLS network area, and (B) affected the pattern of proPLS elongation (Si-3000 camera, scale bars equal 100 µm, arrows show proPLS network). (C) The number of PLS produced was slightly but significantly higher, whereas (D) no differences in PLS size were observed after adhesion on poly-L-lysine. (E) miR-204-5p increased expression of CD61 and CD41 receptors without affecting CD42b nor CD49b expression. The results are expressed relative to the mock condition. n = 3 to 6 independent experiments; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 4. miR-204-5p overexpression affected PLS reactivity

miR-204-5p overexpression (A) did not impact CD62P expression after thrombin stimulation but (B) induced a higher PAC-1 relative median of fluorescence. miR-204-5p transfection (C and D) was associated with an increase in PLS spreading area on fibrinogen-coated coverslips (LSM700 microscope, scale bars equal 20 µm) and (E) enhanced the binding of soluble fibrinogen to PLS. Results are expressed relative to mock. n = 3 to 4 independent experiments; * $p < 0.05$.

Figure 5. miR-204-5p modulated megakaryocyte adhesion and induced cytoskeletal reorganization.

(A) Flow chamber experiments showed that miR-204-5p transfection increased the adhesion of megakaryocytes onto a fibrinogen-coated channel. (B) Confocal analysis of the megakaryocytes adhering to fibrinogen-coated coverslips in static conditions showed that miR-204-5p induced

modification in actin cytoskeleton organization, with an increase in stress-fiber content. Polylobed nuclei were stained with DAPI (blue), and F-actin was stained with TRITC Phalloidin (red, LSM700 microscope, Scale bars equal 20 µm) (C) miR-204-5p transfection enhances the F/G actin ratio in megakaryocytes. n = 3 to 6 independent experiments; * $p < 0.05$.

Figure 6: CDC42 mediated the effect of miR-204-5p on PLS

(A) CDC42 knock-down increased the adhesion of megakaryocytes in a fibrinogen-coated channel in dynamic conditions and (B) led to an actin cytoskeleton organization similar to that observed after miR-204-5p transfection, that is an increase in the stress-fiber content. Images were taken using confocal microscopy of megakaryocytes, with polylobed nucleus labeled with DAPI (blue) and F-actin labeled with TRITC Phalloidin (red) (scale bars equal 20 µm). (C) Knock-down of CDC42 decreased the relative proPLS network area and (D) displayed a similar pattern to that observed after miR-204-5p transfection (Si-3000 camera, scale bars equal 100 µm, arrows show proPLS network). (E) CDC42 silencing did not impact CD62P expression and (F) increased relative GPIIbIIIa expression in activated PLS and (G and H) enhanced the PLS spreading area on fibrinogen-coated coverslips (LSM700 microscope, scale bars equal 20 µm). Results are expressed relative to the mock condition. n = 3 to 5 independent experiments; * $p < 0.05$, ** $p < 0.01$.

Figure S1: **PLS derived from CD34+-cells are functional**

Thrombin stimulation of PLS increased P-selectin secretion (CD62P), activation GPIIbIIIa receptors (PAC-1) and induced the adhesion of soluble fibrinogen by FACS compared to non-activated PLS. n = 4 independent experiments; * $p < 0.05$, ** $p < 0.01$. T test student

Figure S2: miR-204-5p does not impact PLS morphology

FACS analysis showed no difference in the normalized forward side scatter (FSC) of PLS after miR-204-5p transfection compared to mock condition. The figure is representative of 4 independent experiments.

Figure S3: CDC42 silencing

Western blot analysis showed that transfection of si CDC42 decreased CDC42 protein expression. n = 5 independent experiments; * $p < 0.05$, *** $p < 0.001$.

Figure 1

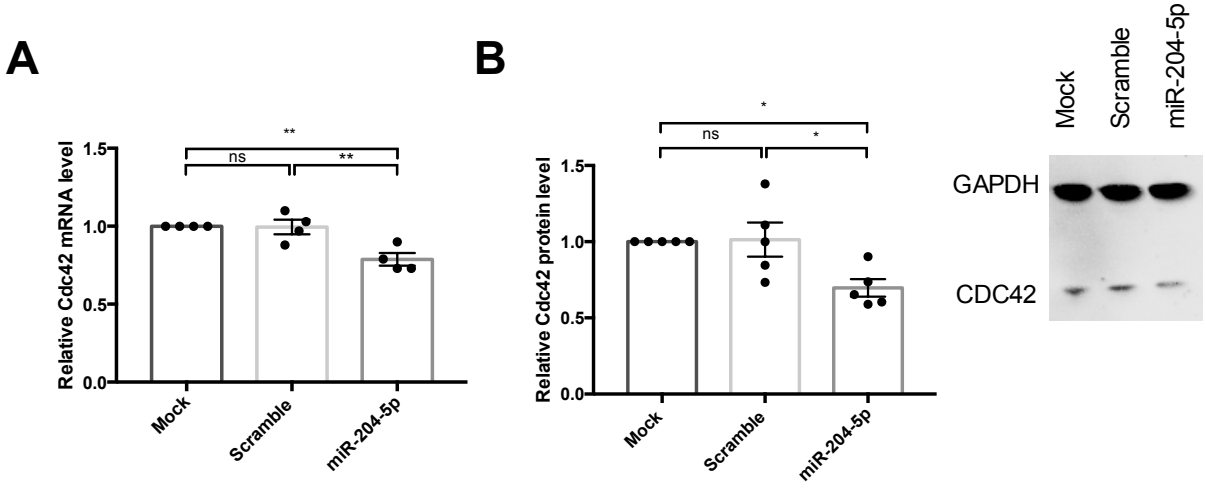

A
B
Relative Cdc42 mRNA level
1.5
1.0
0.5
0.0
ns
**
**
Mock
Scramble
miR-204-5p
Relative Cdc42 protein level
1.5
1.0
0.5
0.0
ns
*
*
Mock
Scramble
miR-204-5p
Mock
Scramble
miR-204-5p
GAPDH
CDC42

Figure 2

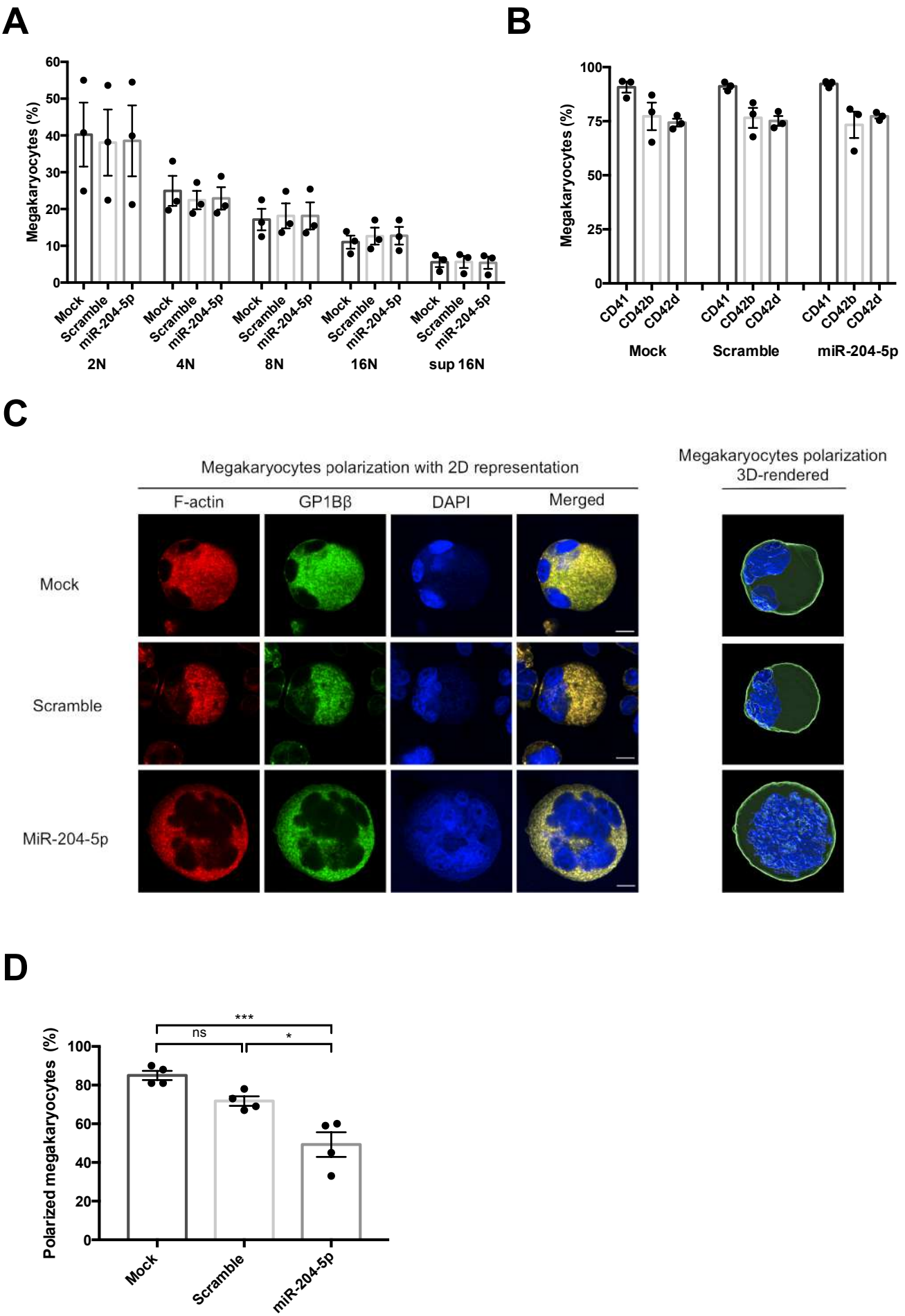

A
Megakaryocytes (%)
60
50
40
30
20
10
0
Mock
Scramble
miR-204-5p
2N
4N
8N
16N
sup 16N
B
Megakaryocytes (%)
100
75
50
25
0
CD41
CD42b
CD42d
Mock
Scramble
miR-204-5p
C
Megakaryocytes polarization with 2D representation
F-actin
GP1Bβ
DAPI
Merged
Megakaryocytes polarization 3D-rendered
Mock
Scramble
MiR-204-5p
D
Polarized megakaryocytes (%)
100
80
60
40
20
0
***
ns
*
Mock
Scramble
miR-204-5p

Figure 3

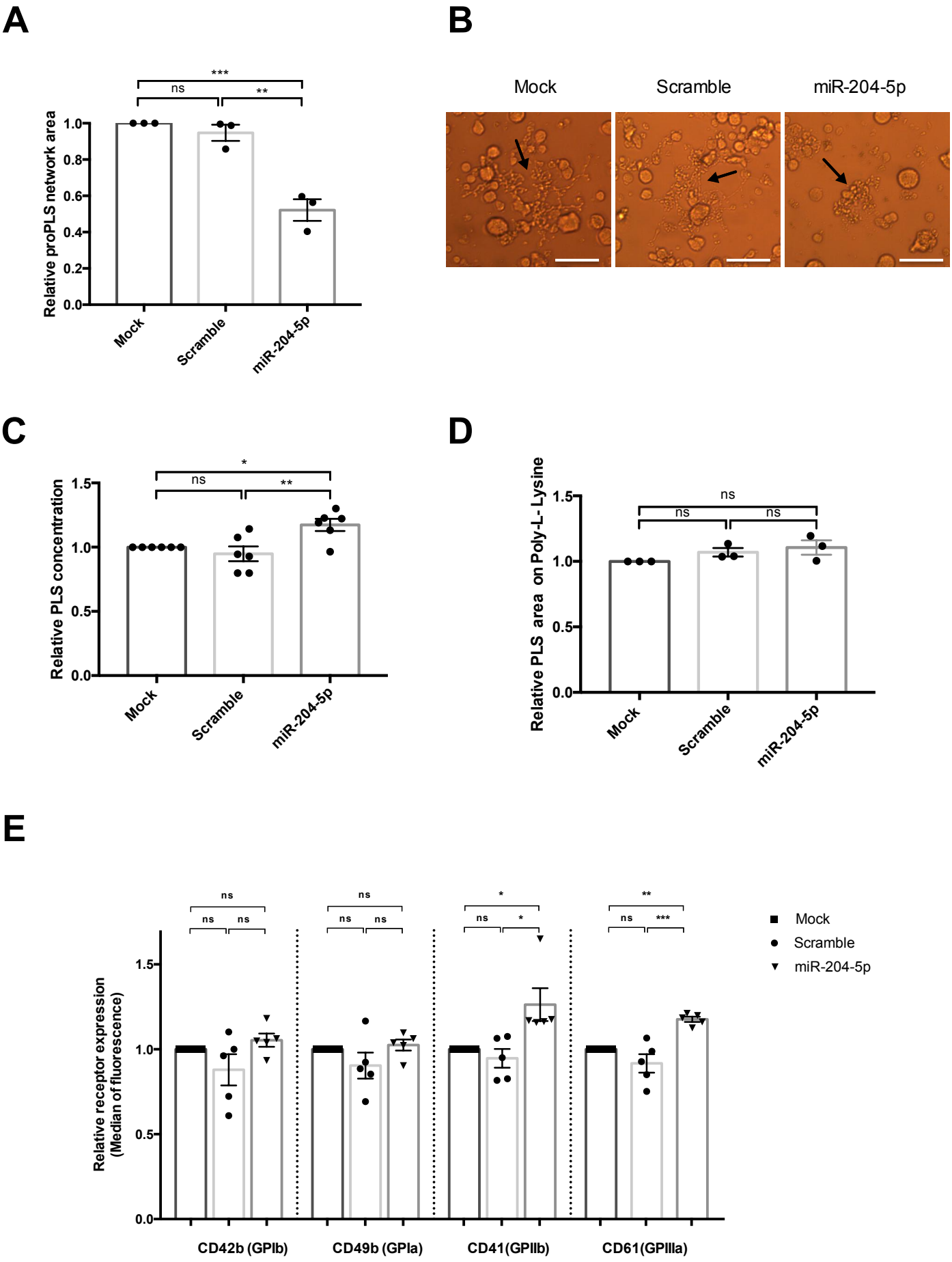

A
***
ns
**
Relative proPLS network area
1.0
0.8
0.6
0.4
0.2
0.0
Mock
Scramble
miR-204-5p
B
Mock
Scramble
miR-204-5p
C
*
ns
**
Relative PLS concentration
1.5
1.0
0.5
0.0
Mock
Scramble
miR-204-5p
D
ns
ns
ns
Relative PLS area on Poly-L- Lysine
1.5
1.0
0.5
0.0
Mock
Scramble
miR-204-5p
E
ns
ns
ns
ns
ns
ns
*
ns
*
**
ns
***
Relative receptor expression
(Median of fluorescence)
1.5
1.0
0.5
0.0
CD42b (GPIb)
CD49b (GPIa)
CD41(GPIIb)
CD61(GPIIIa)
Mock
Scramble
miR-204-5p

Figure 4

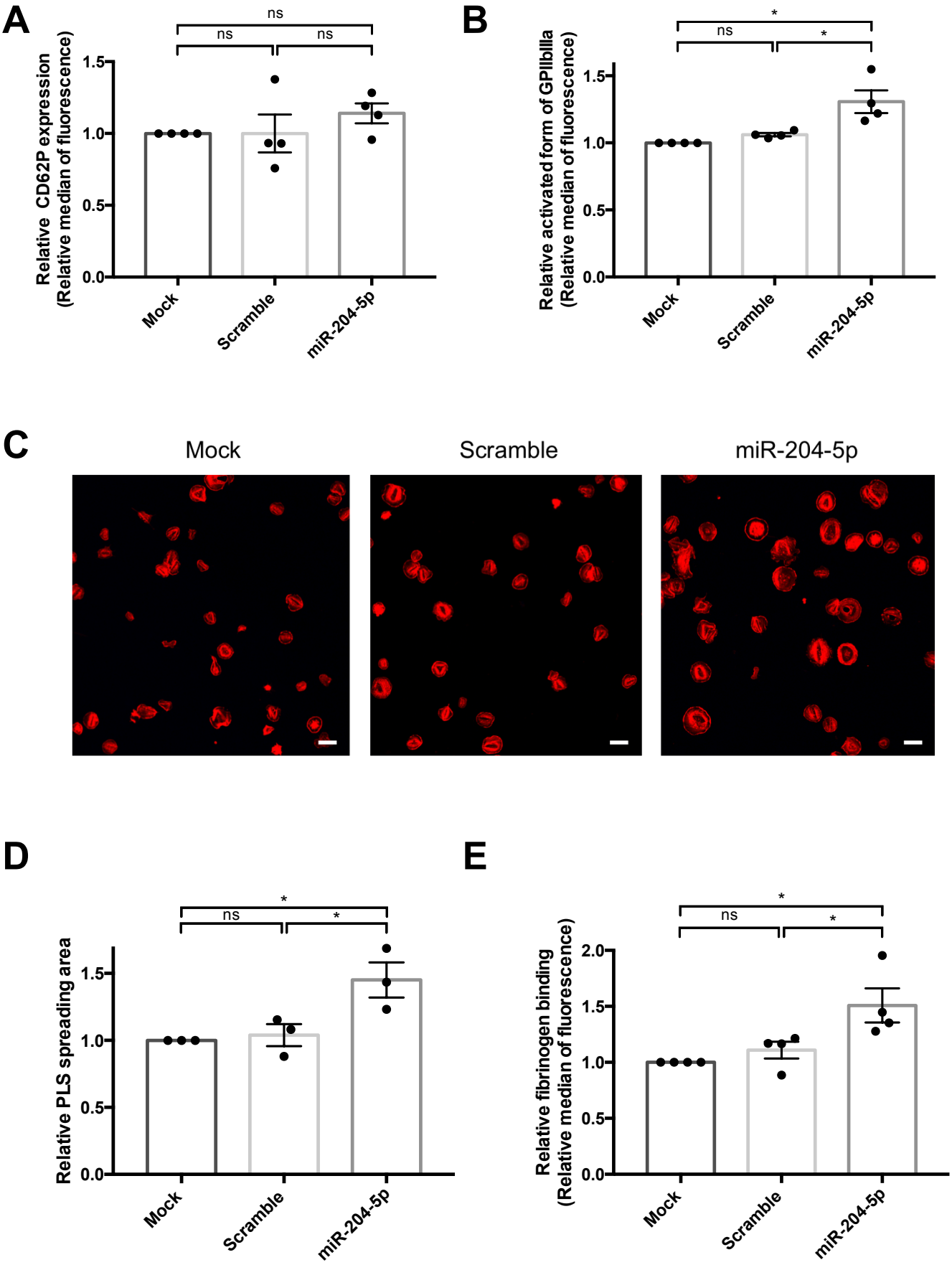
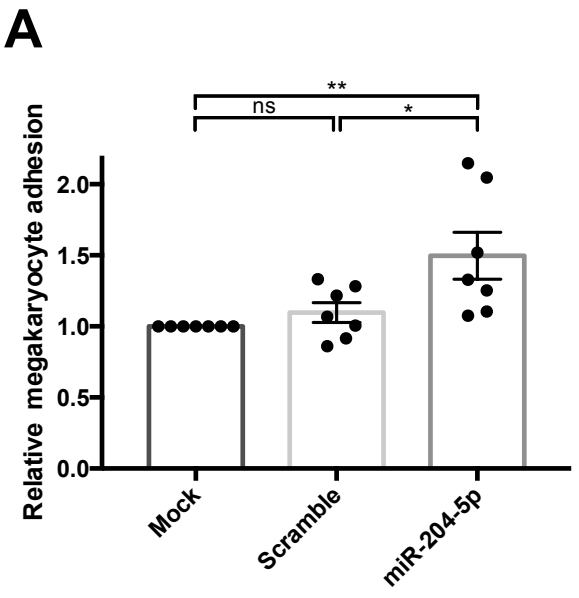
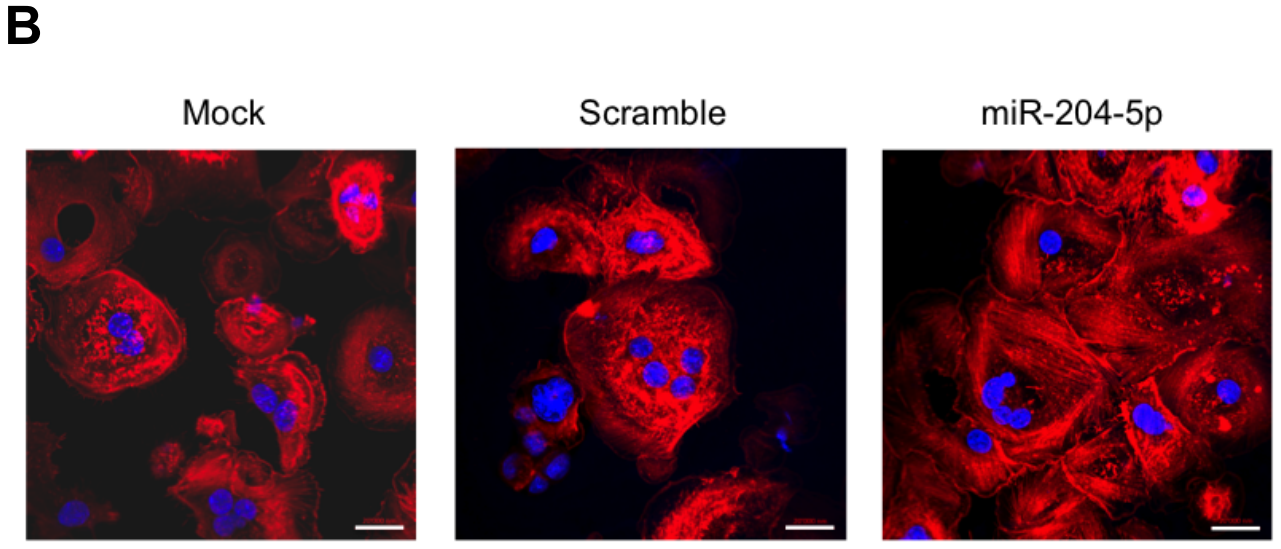

A
ns
ns
ns
Relative CD62P expression
(Relative median of fluorescence)
1.5
1.0
0.5
0.0
Mock
Scramble
miR-204-5p
B
*
ns
*
Relative activated form of GPIIbIIIa
(Relative median of fluorescence)
1.5
1.0
0.5
0.0
Mock
Scramble
miR-204-5p
C
Mock
Scramble
miR-204-5p
D
*
ns
*
Relative PLS spreading area
1.5
1.0
0.5
0.0
Mock
Scramble
miR-204-5p
E
*
ns
*
Relative fibrinogen binding
(Relative median of fluorescence)
2.0
1.5
1.0
0.5
0.0
Mock
Scramble
miR-204-5p

Figure 5


A
**
ns
*
Relative megakaryocyte adhesion
2.0
1.5
1.0
0.5
0.0
Mock
Scramble
miR-204-5p


B
Mock
Scramble
miR-204-5p

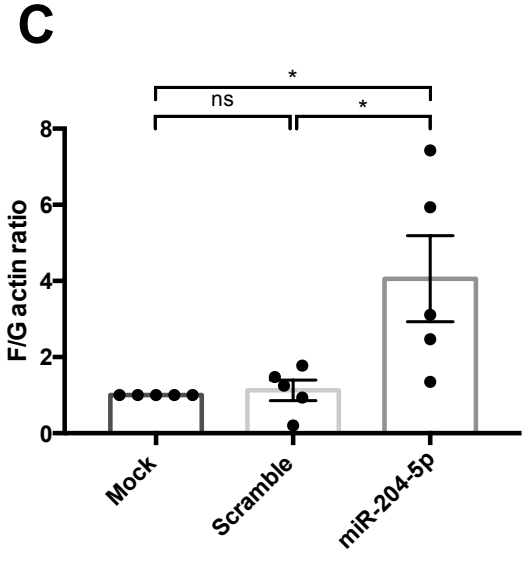
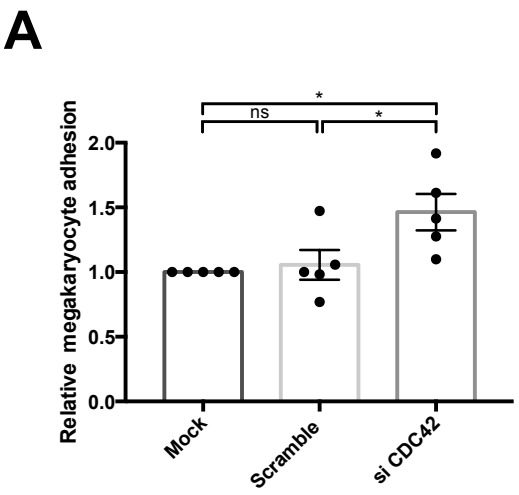
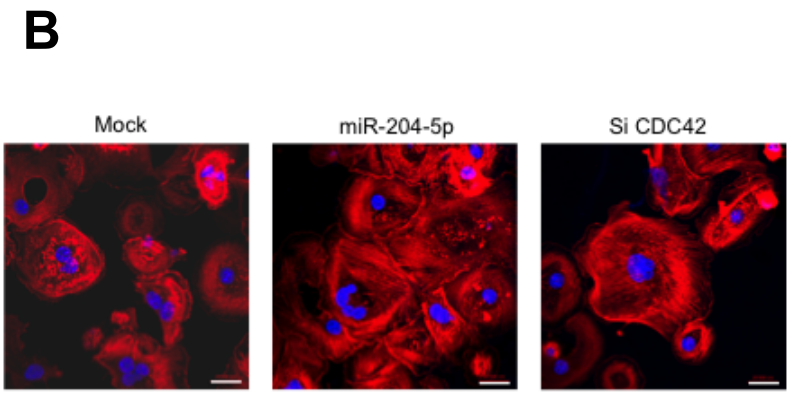
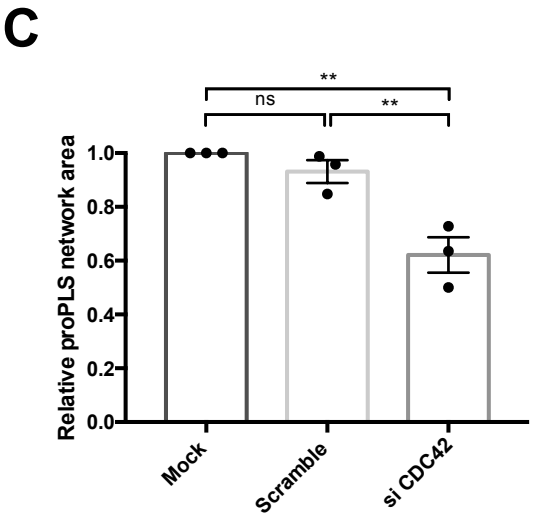
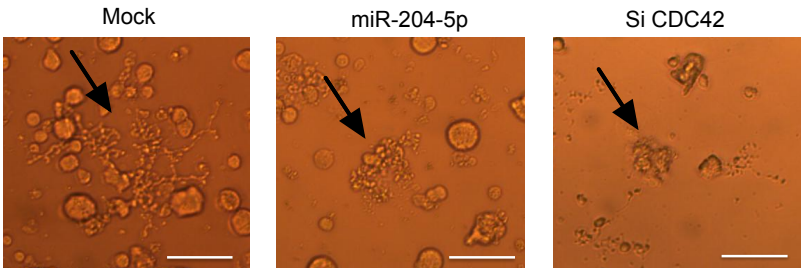

C
*
ns
*
F/G actin ratio
8
6
4
2
0
Mock
Scramble
miR-204-5p

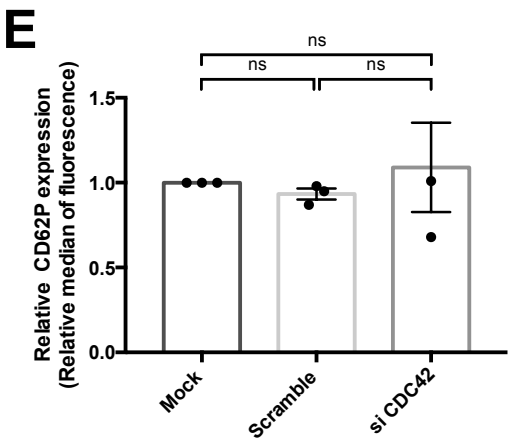
Figure 6

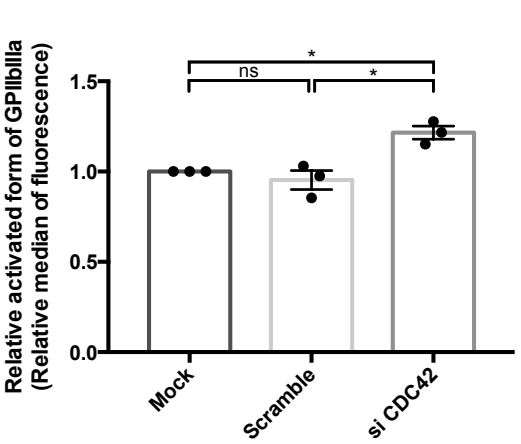

A
Relative megakaryocyte adhesion
ns
*
*
2.0
1.5
1.0
0.5
0.0
Mock
Scramble
si CDC42

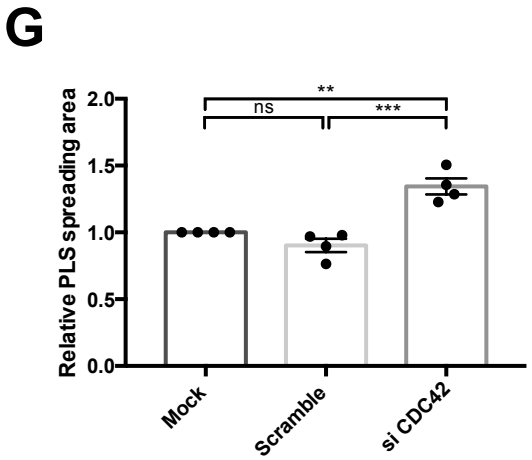

B
Mock
miR-204-5p
Si CDC42


C
Relative proPLS network area
ns
**
**
1.0
0.8
0.6
0.4
0.2
0.0
Mock
Scramble
si CDC42


D
Mock
miR-204-5p
Si CDC42


E
Relative CD62P expression
(Relative median of fluorescence)
ns
ns
ns
1.5
1.0
0.5
0.0
Mock
Scramble
si CDC42


F
Relative activated form of GPIIbIIIa
(Relative median of fluorescence)
ns
*
*
1.5
1.0
0.5
0.0
Mock
Scramble
si CDC42


G
Relative PLS spreading area
ns
**
***
2.0
1.5
1.0
0.5
0.0
Mock
Scramble
si CDC42

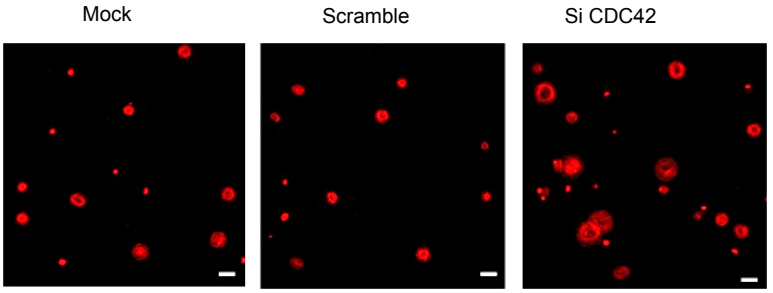

H
Mock
Scramble
Si CDC42

Figure S1

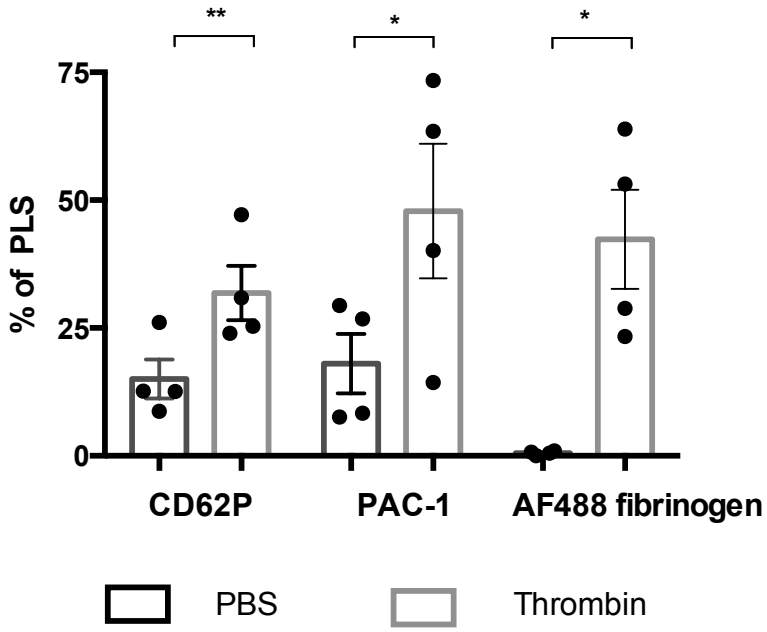


Figure S2

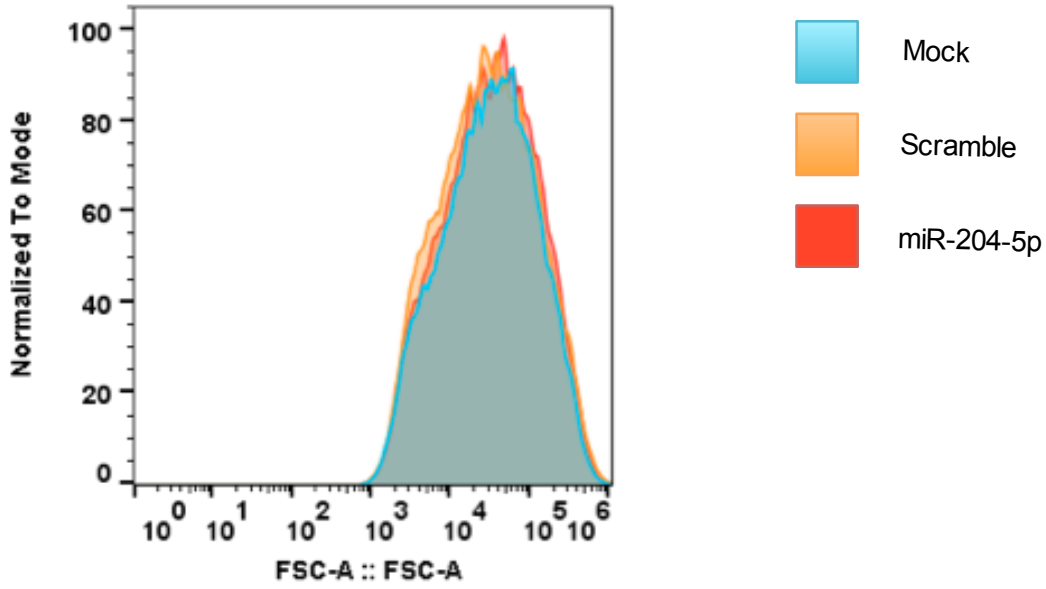
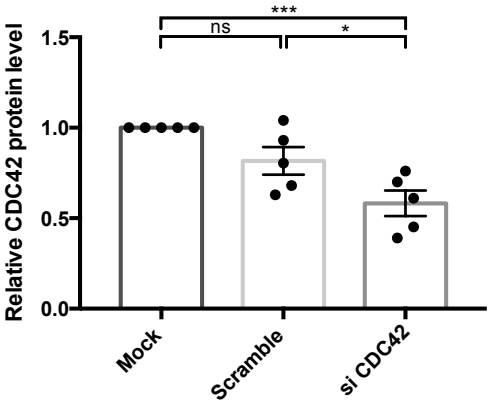
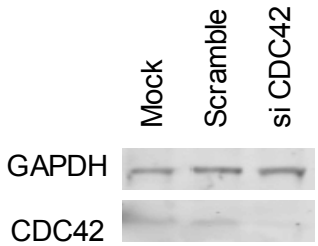

Normalized To Mode
100
80
60
40
20
0
10^0 10^1 10^2 10^3 10^4 10^5 10^6
FSC-A :: FSC-A
Mock
Scramble
miR-204-5p

Figure S3


***
ns
*
Relative CDC42 protein level
1.5
1.0
0.5
0.0
Mock
Scramble
si CDC42


Mock
Scramble
si CDC42
GAPDH
CDC42

2.3 CLINICAL STUDY

Insights into the association of miRNAs level and platelet reactivity in stable cardiovascular patients: an *ex vivo* and *in silico* study

Alix Garcia, Sylvie Dunoyer-Geindre, Séverine Nolli, Jean-Luc Reny, Pierre Fontana

Manuscript under preparation

Personal contribution: I participated to the miRNAs extraction from plasma samples and to the measurement of miRNAs level at the genomic platform. I performed statistical and *in silico* analyses and wrote the first draft of the manuscript.

Overview:

miR-126-3p and miR-204-5p have been previously validated as modulators of platelet reactivity in our model of human derived-cells. The results make these miRNAs interesting candidates as biomarkers of platelet reactivity. Therefore we investigated the expression of circulating miR-126-3p, miR-204-5p as well as two other miRNAs described in the literature as promising biomarkers; miR-150-5p and miR-223-3p. The vast majority of clinical association studies are performed in cohort of cardiovascular patients under dual anti-platelet therapy and considering only one platelet function test. This strategy may hide information regarding the impact of anti-P2Y12 variability response and on the regulation of other facets of platelet reactivity.

In this work, we quantify the circulating miRNA levels from cardiovascular patients taking aspirin only. In addition, a particular interest has been given to the normalisation procedure performed using a set of stable endogenous miRNAs previously validated using geNorm. The originality of this study is based on the correlation of miRNA level with multiple facets of the platelet reactivity including thrombin generation markers and the aggregation induced by several agonists (ADP, arachidonic acid and collagen).

The causative link between circulating miRNA level and platelet function was then investigated using a computational approach. The strategy was to compare the predicted miRNA's targets to the genes involved in different facets of platelet function tested. Finally, the analysis of the literature enables to highlight the putative mechanisms that may explain the correlation between miRNA level and platelet function.

Insights into the association of miRNAs level and platelet reactivity in stable cardiovascular patients: an *ex vivo* and *in silico* study

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[2]Division of General Internal Medicine, Geneva University Hospitals, Geneva, Switzerland
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Abstract

Background: Platelet reactivity is a variable phenotype, associated with recurrence of thrombotic or occurrence of bleeding events in cardiovascular patients treated with antiplatelet drugs. Among determinants of platelet reactivity, platelet-derived microRNAs (miRNAs) focused attention during these last years and circulating miRNAs have been suggested as biomarkers to predict platelet reactivity and clinical outcome in these patients. However, the impact of miRNA on platelet function and the mechanisms behind are largely unknown.

Material and methods: Selected miRNAs were extracted and quantified in 191 plasma samples of stable cardiovascular patients. miR-126-3p, miR-150-5p, miR-204-5p and miR-223-3p levels were normalized using geNorm algorithm. Each miRNA level was correlated with platelet reactivity as assessed with light transmission aggregometry with various agonists, and with *in vivo* thrombin generation markers. Finally, a miRNA's targets network was built based on the gene ontology of pathways mediating platelet reactivity.

Results: Each miRNA was associated with a different pattern of platelet reactivity according to the agonists used. In addition, miR-126-3p and miR-223-3p were positively correlated with *in vivo* thrombin generation markers. *In silico* analysis pointed putative genes regulated by those miRNAs implicated in platelet function regulation.

Conclusion: The platelet-derived circulating miRNA profile is associated with different aspects of platelet function including platelet aggregation and platelet-supported thrombin generation, through regulation of different genes. This paves the way to a personalized antithrombotic treatment according to miRNA profile in cardiovascular patients.

Keywords: miRNA, biomarker, cardiovascular disorders, platelet aggregation, thrombin generation

Introduction

Platelets are involved in several processes such as haemostasis, inflammation, cell proliferation and immune system modulation.[1] The exposition of the sub-endothelium following vessel injury induces platelet activation and triggers thrombus formation. A similar process is observed at the site of atherosclerosis where platelets play a pivotal role in plaque disruption that trigger acute thrombotic events.[2, 3] The role of platelets in thrombus formation is mediated via several processes, including platelet aggregation and thrombin generation occurring at the surface of activated platelets following exposition of prothrombotic phospholipids.[4, 5] Platelet reactivity is highly variable and is governed by heritable determinants.[6]

miRNAs are small, noncoding RNAs that regulate gene expression at the post-transcriptional level and may modulate cells biogenesis and function. The vast majority of circulating miRNAs originates from activated platelets[7] and are mainly transported in the circulation by microvesicles where they are stabilized by argonaute.[8] Therefore circulating miRNA profile mirrors platelet miRNA profile, and selected circulating miRNAs were shown to be correlated with recurrence of ischemic events in cardiovascular patients,[9] suggesting that platelet-derived miRNAs could be used as biomarkers for the prediction, diagnosis and prevention of cardiovascular diseases.[10] Circulating miRNAs can thus shed light on the genomic content of platelets, on platelet function, on response to antiplatelet therapy and on cardiovascular risk.[11]

However, to the best of our knowledge, only few studies investigated the association of miRNAs with different assays assessing platelet reactivity.[12] Therefore, this work aims to correlate a set of platelets derived miRNAs with platelet reactivity using light transmission aggregometry (LTA) as well as *ex vivo* thrombin generation markers. The causative link between miRNAs level and platelet function patterns observed was then investigated using *in silico* strategy.

Material and methods

Study Population

Patients were selected out of the multi-centric ADRIE (Antiplatelet Drug Resistance and Ischemic Events; clinicalTrials.gov identifier NCT00501423) population described in detail elsewhere[13, 14] that included 771 cardiovascular patients treated with aspirin (n=223), clopidogrel (n=111), or both (n=437). In this work, we focused on the 191 patients treated with aspirin only. Patients with symptomatic documented ischemic atherothrombotic disease were included between June 2006 and December 2008. This study was approved by the Central Ethics Committee of University Hospital of

Geneva (Switzerland) and by the Ethic Committee of Bezier (France). Written informed consent was obtained from all individual patients included in this study.

Blood collection

Samples were collected by venipuncture and blood was collected in EDTA- and citrated tubes. Plasma was obtained by centrifugation of whole blood at 2300g during 15min at room temperature and was stored at -80°C until analysis.

Platelet function evaluation

Platelet function was assessed by LTA on an 8-channel aggregometer (TA-8V, SD Medical, Heillecourt, France) using adenosine diphosphate (ADP) 5 µmol/L and 20 µmol/L (Sigma, St Louis, USA), Horm collagen 1µg/mL (Nycomed, Linz, Austria) or arachidonic acid (AA) 1mmol/L (BioData Corps, Horsham, PA).[13, 14]

Thrombin-antithrombin complexes (TAT) and prothrombin fragments F1+2 (F1+2), were quantified in citrated plasma samples using Enzygnost TAT micro and Enzygnostat F1+2 kits (Siemens Healthcare Diagnostics Products, Marburg, Germany), respectively, according to the manufacturer instructions.[15]

Selection of candidate miRNAs

The selection criteria for the miRNA quantified in this study was based on their expression profile in platelets; miR-126-3p, miR-150-5p and miR-223-3p belong to the most expressed miRNAs[16] (http://www.plateletomics.com/) and they were consistently associated with platelet reactivity in clinical studies.[17] We also investigated miR-204-5p that was firstly described by our group as associated with platelet reactivity.[18] In addition, a positive correlation between miR-204-5p level and platelet reactivity was reported thereafter in an independent cohort,[19] further supporting the role of miR-204-5p in platelet function regulation. These four miRNAs are referred below as candidate miRNAs.

Plasma miRNA extraction and analysis

1mL Qiazol and 5 fmole of *Caenorhabditis elegans* miR-39 were added to 2x100µL each EDTA plasma sample. MiR-39 was used as a spike-in control in order to assess the efficiency of the purification and reverse transcription procedures. miRNA isolation was performed using the miRNeasy mini kit according to the manufacturer's instructions (Qiagen, Hilden, Germany).

The reverse transcription procedure was done using a fixed volume of 2µl of the elute RNA and the reverse transcription products were preamplified with the TaqMan Advanced miRNA cDNA Synthesis Kit (Applied Biosystem, Foster City, CA, USA). Real time qPCR analysis was performed using Taqman

advanced miRNA assays and Taqman Fast advanced Master mix (Applied Biosystem). PCR reactions were run on 7900HT SDS system (Applied Biosystems). Results of miRNA quantitation with Ct above 35 were excluded from the analysis.

Normalization procedure

The review of the literature pointed out miR-16, miR-93 miR-106-5p and miR-484 and as putative normalizers in plasma samples.[20-23] These four normalizers were tested in plasma samples of the present study and the best panel was determined using geNorm algorithm. The relative level of each miRNA investigated was then calculated according to the technique described by Kok *et al*.[24]

Plasma sample quality control

To assess a possible contamination of plasma samples with residual platelets, red blood cells and white blood cells a quality control was performed in 20% of randomly selected plasma samples.

A western blot analysis was performed using an anti-ITGA2b (CD41) and anti-PTPRC (CD45) antibody, a specific marker of platelets and white blood cells, respectively. Briefly, 2 µL of plasma was diluted in 18µL of PBS and boiled 15 min at 95°C in reducing buffer. Samples were loaded on NuPAGE 4-12% Bis-Tris Gel 10 wells (Invitrogen, Carlsbad, CA, USA) then transferred to nitrocellulose membrane. The membrane was blocked with non-fat dry milk 5% and labeled with primary antibody anti-ITGA2b (Sigma) and anti-PTPRC (Abcam, Cambridge, England). An IRDye 680RD secondary antibodies antibody (LI-COR Biosciences, Lincoln, Nebraska USA) was used for detection on Odyssey Imaging Systems (LI-COR Biosciences).

The ratio of miR-451 (an haemolysis indicator) to miR-23a-3p (an haemolysis-insensitive miRNA) evaluated by qPCR reflects haemolysis and is currently used as a quality control.[8, 25] A ratio below 7 indicates the absence of significant haemolysis in the plasma sample.[8]

miR-39 was measured by qPCR. Standard deviation of Ct value was determined in order to establish the quality control of extraction and RT procedure.

Gene ontology and pathway analysis

Three databases (miRANDA, TargetScan and PicTar) were used to determine the putative gene targets of selected miRNAs. Genes included in at least two independent databases were included for further analysis.

Gene ontology (GO, https://geneontology.org) is a tool allowing the characterization of the association between genes. Pathways were selected according to the results of the correlation with platelet aggregation assays and *in vivo* thrombin generation markers: “aggregation”, “P2Y12”, “actin cytoskeleton”, “thrombin” and “calcium homeostasis”. The GO terms associated to these selected

pathways were defined using Amigo 2 (http://amigo.geneontology.org/amigo) **(Table 1)**. The genes belonging to the selected GO-terms that were also predicted to be the target of candidate miRNA were selected for further analysis. These targets were then represented in a network built using Cytoscape (https://cytoscape.org/). Finally, a literature review allowed to link candidate miRNA targets present in GO-terms and platelet function.

Statistics

Correlation analyses were performed using a Spearman test. Data were analysed using GraphPad Prism 7 software (GraphPad Software Inc., San Diego, USA). P-values < 0.05 were considered statistically significant.

Results

Plasma sample quality controls

Western blot analysis in plasma samples reveals the absence of glycoprotein ITGA2b and PTPRC, indicating that no residual platelets and leukocytes are present in the samples **(Figure 1).** The ratio of miR-451 to miR-23a-3p evaluated by qPCR was below 7 in all the samples tested, indicating the absence of haemolysis.

In addition, qPCR analysis of cel-miR-39 added as a spike-in control before the extraction procedure showed a mean cycle threshold (Ct) value of 23.09, the coefficient of variation (CV) of the Ct values across the samples was 6.84% indicating consistent extraction efficiency between the samples tested.

miRNA reference panel for the normalization procedure

We determined the most stable endogenous miRNAs that can be considered as reference for the normalization procedure in our study. The geNorm cut-off value of 1.5[26] was used, below which the endogenous miRNA was considered as stable across samples and suitable to use as a reference. miR-16, miR-93 and miR-484 were thus identified as the best combination of normalizers in plasma samples and they were quantified in each sample **(Table 2)**.[27] The expression of the candidate miRNAs was then normalized against the set of stable miRNAs as described elsewhere.[24] The relative expression of the miRNAs of interest is given in arbitrary unit (AU).

Correlation between miRNA level and platelet aggregation

Among the 191 plasma samples, hsa-miR-126-3p was successfully quantified in 183 samples, hsa-miR-150-5p and hsa-miR-223-3p were quantified in 189 samples and hsa-miR-204-5p in 142 samples.

Relative plasma hsa-miR-126-3p level ranged from 0.5 to 9.2 arbitrary units (AU),[15] with a median value of 2.1 AU (IQR: 1.3-2.9). Plasma hsa-miR-150-5p level ranged from 0.06 to 6.2 AU, with a median value of 0.7 AU (IQR: 0.4-1.2).[15] Plasma hsa-miR-223-3p level ranged from 0.04 to 3.04 AU with a median value of 0.14 (IQR:0.10-0.22) and plasma hsa-miR-204-5p level ranged from 0.03 to 31.8 AU with a median value of 0.65 (IQR:0.25-1.36).

The circulating miRNA levels correlated with platelet aggregation results but with a different pattern for each miRNA **(Table 3).** While the magnitude of the correlation with hsa-miR-126-3p level was the strongest using AA as agonist, the aggregation response to ADP and collagen correlated mostly with the other 3 miRNAs **(Table 3).**

Correlation between miRNA level and thrombin-generation markers

A positive correlation between miR-126-3p or miR-223-3p level and TAT or F1+2 was observed in plasma samples from cardiovascular patients taking aspirin while no correlation was observed between miR-204-5p or miR-150-5p and these thrombin generation markers **(Table 3).**

Gene ontology analysis

The miRNAs prediction databases indicated 146, 4156, 5820 and 3584 predicted targets for miR-126-3p, miR-150-5p, miR-204-5p and miR-223-3p, respectively, in at least one of the databases interrogated. When considering the predicted targets present in at least 2 databases, 16, 232, 600 and 100 targets were identified for miR-126-3p, miR-150-5p, miR-204-5p and miR-223-3p, respectively. The putative targets for miR-150-5p, miR-204-5p and miR-223-3p present in at least two databases were used for further analysis. Given the small number of common putative targets identified for miR-126-3p in 2 distinct databases, all the 146 putative targets predicted by at least one database were used for further analyses.

The predicted targets of each miRNA were matched against selected gene ontology pathways including *aggregation*, *P2Y12*, *thrombin generation*, *actin cytoskeleton* and *calcium homeostasis*. **Figure 2** depicts a network based on gene-gene interactions of the predicted genes of each miRNA. miR-150-5p and miR-223-3p are predicted to target 21 and 29 genes, respectively including 10 commonly targets belonging to the P2Y12 pathway. The other genes potentially regulated by miR-150-5p and miR-223-3p are mainly a part of calcium homeostasis and actin cytoskeleton pathways, respectively. miR-126-3p is predicted to target 18 genes including RORB, a nuclear hormone receptor which is also a putative target for both miR-150-5p and miR-223-3p. Finally, miR-204-5p is predicted to modulate the expression of 49 genes including a majority of genes involved in actin cytoskeleton and P2Y12 pathway modulation. As miR-150-5p, miR-204-5p is predicted to target METAP1 (methionine aminopeptidase1 involved in signalling by G protein-coupled receptors (GPCR)).

Moreover, Ral GEF with PH Domain and SH3 Binding Motif 2 (RALGPS2) that may modulate cytoskeletal organization and ATPase Plasma Membrane Ca2+ Transporting 1 (ATP2B1) are putatively targeted by both miR-204-5p and miR-223-3p.

Discussion

Circulating miRNAs are predominantly derived from platelets and are often associated with platelet reactivity in a wide variety of clinical studies. However, the vast majority of these studies investigated healthy subjects or cardiovascular patients on dual antiplatelet therapy (aspirin and anti-$P2Y_{12}$ – mostly thienopyridines) and used mostly platelet aggregation assays. The main original features of our approach is to select patients treated with aspirin only in order to overcome the variability of clopidogrel response mainly due to a variability in liver-dependant activation of the pro-drug, rather than to the variability of platelet metabolism.[28] Moreover, the available correlation studies between circulating miRNA levels and platelet reactivity usually used platelet aggregation assays. In our work, we also addressed the issue of platelet-supported thrombin generation, which is a crucial step in thrombus formation.
Pre-analytical issues are of utmost importance in order to avoid contamination with other source of miRNAs **(Appendix 1).** We paid particular attention to rule out any significant contamination of the plasma samples with platelets, red and white blood cells.

We measured the correlation between candidate miRNA levels and maximal platelet aggregation in response to 3 agonists including collagen, AA and ADP. Collagen is considered as a strong agonist that binds to the platelet GPIaIIa and GPVI receptors and is dependant of amplification pathways tested with AA and ADP. AA induces the formation of the TxA2 under the successive action of enzymes including COX-1, target of aspirin. TxA2 binds to its specific G protein-coupled receptor (GPCR, TP) and triggers platelet activation. ADP is a soluble agonist released by platelet dense granules and mediates its effect via two GPCR, P2Y1 and P2Y12. Our results show that our candidate miRNAs correlate with platelet aggregation response of at least two different agonists.

miR-223-3p is positively, although weakly, associated with aggregation with ADP and collagen. The association of miR-223-3p and platelet reactivity or patient outcome has been largely investigated but divergences have been highlighted.[29-34] For example, a low level of miR-223-3p was associated to a poor response to anti-P2Y12 drugs treatments [29] while miR-223-3p was positively correlated to platelet inhibition in another study.[35] These differences may be explained by the setting of the studies that differ in patient selection, treatment, samples, outcomes and experimental strategy for

miRNA quantification. **(Appendix 1)** Although the results are conflicting, the authors agree that miR-223-3p may regulate platelet function through a P2Y12-dependent mechanism.[36, 37] Consistently, network in **Figure 2** showed that miR-223-3p is predicted to mainly target proteins from P2Y12 pathway including the Glucagon-like peptide-1 receptor (GLP1R), that is known to decrease platelet activation, aggregation and thrombosis.[38] The downregulation of GLP1R expression by miR-223-3p may thus induces an increase of platelet reactivity.

GLP1R is also a predicted target of miR-150-5p. Moreover, as GLP1R, 9 other genes belonging to the P2Y12 pathway are predicted targets of both miR-150-5p and miR-223-3p. This suggests a possible synergistic effect of these two miRNAs on the P2Y12 pathway. The correlation of circulating miR-150-5p with ADP and collagen-aggregation as well as the high number of putative targets among P2Y12 pathway showed that miR-150-5p modulation is closely linked to P2Y12 pathway that was already described in patient treated with anti-$P2Y_{12}$ drugs.[35, 36]

In the ADRIE cohort, miR-126-3p is weakly associated to AA and ADP-induced platelet aggregation. These results are in line with the results of Kaudewitz and co-workers[39] who showed that the inhibition of miR-126-3p in mice reduced platelet aggregation to AA and PAR4-AP. They also showed a positive association between miR-126-3p level and P-selectin secretion in a clinical study.[39] These results are in agreement with our study carried out on platelet-like structures derived from megakaryocytes where miR-126-3p transfection increased P-selectin secretion **(section 2.1**).[40] Taken together, these data further support the hypothesis of a causative link between miR-126-3p expression and platelet aggregation. Our *in silico* investigations showed that A-Kinase Anchoring Protein (AKAP13), a member of actin cytoskeleton and P2Y12 pathways, may be down regulated by miR-126-3p. AKAP13 is known to be a regulator of cAMP that blocks calcium release, G protein activation, adhesion, granule release as well as aggregation.[41-43]

A positive association between miR-204-5p level in plasma and aggregation after stimulation with AA, ADP and collagen response was also observed. The results of the present study showed that miR-204-5p may regulate platelet aggregation to different agonists. This is consistent with the results of Ding and coworkers[19] who observed that miR-204-5p level in platelets is upregulated in acute coronary syndrome patients with high platelet reactivity. Among platelet activation and aggregation pathways, 5 putative targets of miR-204-5p have been found while 20 and 21 targets were related to P2Y12 and actin cytoskeleton pathways, respectively. Among the targets related to actin cytoskeleton, CDC42, a validated target of miR-204-5p,[44] is of major interest since as shown in our previous study **(section 2.2),** miR-204-5p regulates the expression of CDC42 that induces an increase of platelet reactivity via the regulation of its adhesion to fibrinogen. Fibrinogen adhesion is one of

the key players of the formation of platelet-aggregates that could explain the positive correlation between miR-204-5p expression and the platelets aggregation using all three agonists tested.

Platelets promote thrombus formation via an aggregation process and the generation of thrombin triggered by the exposition of procoagulant phospholipids at their surface. We previously showed with human-derived cells that overexpression of miR-126-3p in megakaryocytes was associated with an increased generation of procoagulant platelet-like structures and induced an increased thrombin generation profile in human plasma.[45] In the present work, we extend the results that we found with miR-126-3p and miR-150-5p, and identified another miRNA, miR-223-3p that is also associated with *in vivo* thrombin generation markers in cardiovascular patients. This suggests that both miRNAs could have a synergetic effect, and argues to a miRNA profile as predictor of the ability of platelets to generate thrombin at their surface.

The present *in silico* study does not predict any direct miR-126-3p target belonging to the thrombin generation pathway. Therefore, we speculate that the potential effect of miR-126-3p on thrombin generation could be mediated through an indirect mechanism. This indirect mechanism could be mediated by AKAP13 targeting resulting to an increase of calcium release required for pro-coagulant activity.[5] Conversely, the *in silico* analyses predict Stathmin 1 (STMN1), a validated target of miR-223-3p[46] that may govern thrombin generation pathway. In a human breast cancer cell line, it was shown that transfection of miR-101 induced a decrease STMN1 expression associated with an increase Annexin V exposure.[47] In platelets, the exposition of annexin V is related to their procoagulant activity required for thrombin generation.[5] These findings suggest that miR-223-3p may increase the thrombin generation via a down regulation of STMN1. This may be validated using a translational strategy as already performed for the miR-126-3p in a previous study of our group[15] where an *ex vivo, in vitro* and *in vivo* investigation were done.

Taken together, the combination of the different analyse suggests that miRNAs regulate platelet reactivity through different mechanisms, related to platelet aggregation or platelet-supported thrombin generation. The **Figure 3** provides a model of the putative association between miRNAs and platelet reactivity profile. miR-150-5p and miR-223-3p are both predicted to target GLP1R mRNA that mediates ADP-induced aggregation through a P2Y12-dependent mechanism. miR-204-5p regulates platelet function at least in part by CDC42 regulation and an increase of fibrinogen binding **(section 2.2)**. Finally, miR-126-3p regulates platelet aggregation through actin cytoskeleton and P2Y12-related pathways mediated by AKAP13, while the causative link of miR-126-3p on thrombin generation is not elucidate. This effect is probably indirectly mediated by modulation of calcium release through AKAP13. In addition, miR-223-3p may promote thrombin generation possibly via a

STMN1 regulation. Finally only a part of the miRNA targets described in this work is validated using luciferase reporter gene assay. Therefore, further studies should be carried out to validate these targets and the underlying mechanisms.

We identified the relationship between four miRNAs and various facets of platelet function in stable cardiovascular patients taking aspirin. Since circulating miRNA level reflects platelet activation status in real time, the miRNA profile could be used to predict platelet reactivity and tailor antithrombotic therapy, favouring low dose anticoagulant in patients with a miRNA profile associated with an increased platelet-mediated thrombin generation.

Author's contribution

A. Garcia, S. Dunoyer-Geindre, J.-L. Reny, and P. Fontana designed the study and analysed the data. A. Garcia, S. Dunoyer-Geindre, and S. Nolli performed the experiments. A. Garcia, S. Dunoyer-Geindre, and P. Fontana wrote the first draft of the manuscript, and all authors revised the intellectual content and approved the final version.

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Conflict of interest

The authors have no conflict of interest to report.

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Legend of figures and tables

Figure 1. Representative western blot of ITGA2b (CD41) and PTPRC (CD45) proteins observed in control sample (+) and in 8 plasma samples randomly chosen.

Figure 2. Network of the miRNAs predicted targets and their involvement in platelet function-related pathways including aggregation, P2Y12, actin cytoskeleton thrombin and calcium homeostasis in blue, yellow, red, pink and green, respectively.

Figure 3. Putative associations between miRNAs expression and platelet function. The biologically validated miRNAs targets are shown in pink boxes and the in silico-predicted putative targets are in orange boxes.

Table 1. GO terms related to pathways mediated the several facets of platelet reactivity monitored in this work.

Table 2. Reference panel determination.

Table 3. Correlation between miRNAs level and aggregation and thrombin generation markers in stable cardiovascular patients.

Figure 1.

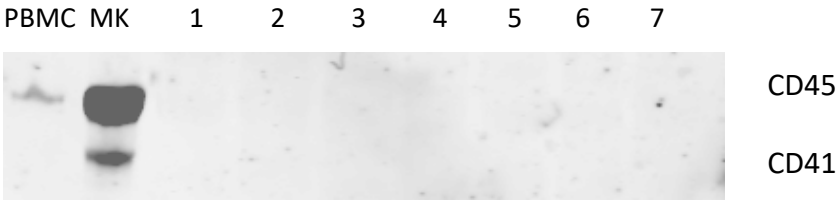

Figure 2.

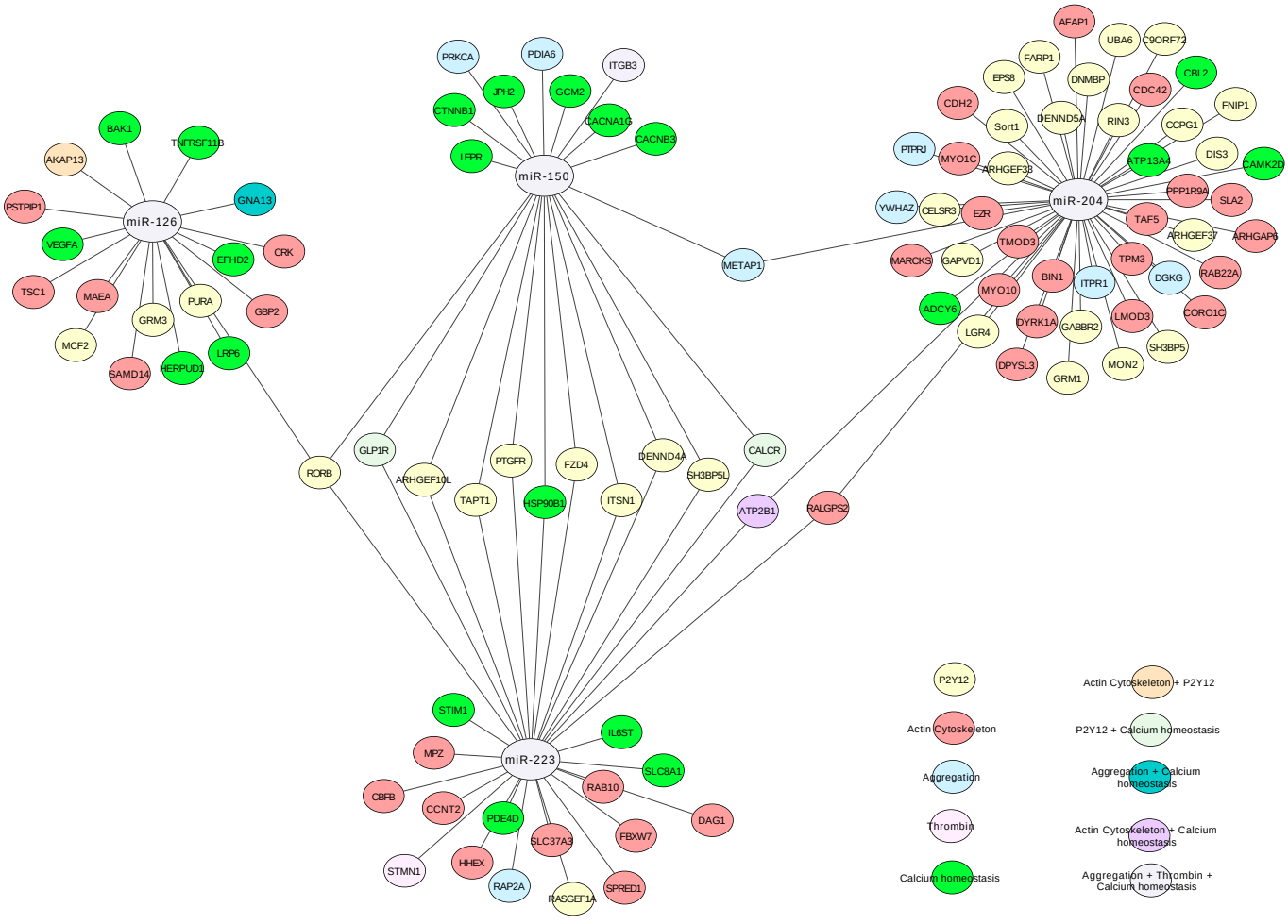

Figure 3.

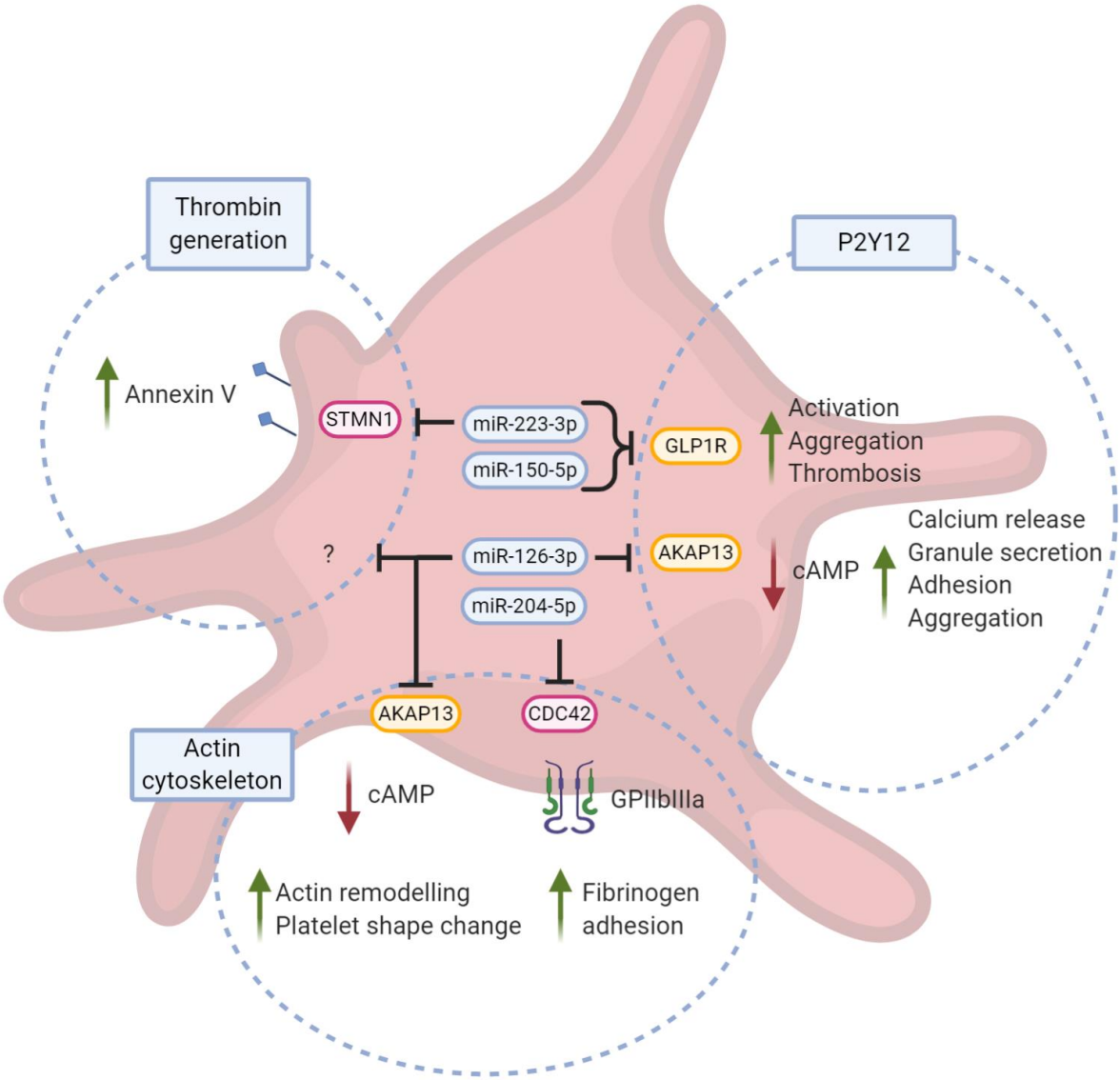
Thrombin generation
P2Y12
Annexin V
STMN1
miR-223-3p
miR-150-5p
GLP1R
Activation
Aggregation
Thrombosis
?
miR-126-3p
AKAP13
cAMP
Calcium release
Granule secretion
Adhesion
Aggregation
miR-204-5p
AKAP13
CDC42
Actin cytoskeleton
cAMP
GPIIbIIIa
Actin remodelling
Platelet shape change
Fibrinogen adhesion

Table 1.

Pathways	Go terms
Aggregation	Platelet aggregation Platelet activation Regulation of platelet aggregation
P2Y12	G protein-coupled adenosine receptor activity, ADP receptor activity G protein-coupled receptor activity Guanyl-nucleotide exchange factor activity, G protein-coupled purinergic nucleotide receptor activity)
Thrombin generation	Thrombin-activated receptor activity, Thrombin-activated receptor signalling pathway, Fibrinogen binding Blood coagulation common pathway
Actin cytoskeleton	Actin cytoskeleton
Calcium homeostasis	Vacuolar calcium ion homeostasis Golgi calcium ion homeostasis, Cellular calcium ion homeostasis Mitochondrial calcium ion homeostasis Mitochondrial, calcium ion homeostasis, Endoplasmic reticulum calcium ion homeostasis Smooth endoplasmic reticulum calcium ion homeostasis Regulation of cytosolic calcium ion concentration, Circadian regulation of calcium ion oscillation Bone remodeling

Table 2.

miRNA	geNorm M Value
miR-106-5p	1.018
miR-93	0.926
miR-484	0.874
miR-16	0.760

Table 3.

	miR-126-3p		miR-204-5p		miR-150-5p		miR-223-3p	
	Rho	**P-value**	**Rho**	**P-value**	**Rho**	**P-value**	**Rho**	**P-value**
AA	0.210	0.004	0.183	0.029	0.079	0.282	0.058	0.431
ADP5	0.074	0.321	0.184	0.028	0.132	0.071	0.015	0.837
ADP20	0.148	0.046	0.158	0.06	0.257	<0.001	0.166	0.023
Collagen	0.144	0.053	0.279	0.001	0.260	<0.001	0.178	0.014
F1+2	0.329	<0.001	0.173	0.040	0.124	0.092	0.224	0.002
TAT	0.182	0.014	0.115	0.174	0.046	0.533	0.281	<0.001

3 DISCUSSION

Platelets play a pivotal role in the regulation of haemostasis and a large body of evidence suggest that platelet reactivity is associated with thrombotic or bleeding events in cardiovascular patients.[135-137] The role of platelets in the haemostatic process is characterized by a large variety of mechanisms promoting thrombus formation via aggregation and thrombin generation but also limiting the thrombus growth by different biological pathways.[57, 138] The identification of the determinants of platelet reactivity could thus lead to a better management of cardiovascular patients, especially when considering the growing arsenal of antithrombotic drugs and strategies that are available nowadays. Numerous association studies described a correlation between miRNA expression and platelet reactivity **(Appendix 1)**.[11] However, the underlying mechanisms remain unclear. miRNAs repress the translation of several mRNAs and may govern the regulation of a wide variety of biological pathways. Therefore, miRNAs could regulate one or several facets of platelet function and circulating miRNAs could thus be used as biomarkers for predicting platelet reactivity, subsequent clinical outcome and as a determinant for antithrombotic therapy.

In this work, a human-derived cell model was developed to study the mechanisms governing the regulation of platelet function by miRNAs. This model was used to investigate the role of miR-126-3p and miR-204-5p on the regulation of platelet biogenesis and function. Moreover, the correlation between a set of selected miRNAs and multiple platelet function assays was then evaluated in a cohort of cardiovascular patients. Finally, a network-based approach was used to delineate the putative genes regulated by these selected miRNAs.

3.1 THE HUMAN HEMATOPOIETIC STEM CELLS MODEL

Transfection of human platelets using current technology is limited by the transfection efficiency and is inappropriate to study the impact of miRNA on platelet function.[13] In addition, the conventional protocol describes a transfection duration of several hours, which is not compatible with the preservation of native human platelets. Indeed, platelets retain their function for a few hours after blood collection, and according to the recommendations of the Standardization Subcommittee of the ISTH, platelet function tests such as light transmission aggregometry must be carried out within 4 hours after blood sampling.[139] Therefore, an alternative method was needed to study the role of miRNAs in human-derived cells. The use of platelets derived from megakaryocyte-differentiated hematopoietic

stem cells has been developed over the past decades to meet the demand for platelet transfusion. This promising *in vitro* platelet production strategy has been transferred for the investigation of the miRNA on platelet function. We have developed, in collaboration with the INSERM1255, the production of PLS *in vitro* from human hematopoietic stem cells. CD34+ cells were purified from healthy human buffy coat, expended and differentiated using TPO. The proportion of megakaryocytes expressing differentiation markers, including CD41, CD42b, and CD42d, increased significantly indicating that a high proportion of cells were successfully differentiated into platelet progenitors.[140] The efficiency of differentiation was comparable to other studies using a similar model.[129, 133, 141] It is interesting to note that the supplementation of the medium with Stem Regenin 1 (SR1), an aryl hydrocarbon receptor (AhR) antagonist dedicated to increase CD34+ cell expansion and PLS production maintains CD34+ expression[129, 142] while CD34 expression is switched off during the differentiation of human native platelets.

The megakaryocytes thus produced were then transfected by lipofection with synthetic miRNA to modulate the level of miRNA in the megakaryocytes and in the subsequently released PLS. The efficiency of the transfection reached 50%, which in line with the level described in the literature.[143, 144] Only a portion of the megakaryocytes are transfected and therefore only a part of the derived PLS overexpress the transfected miRNA, suggesting that the quantification of the morphological and functional effect of miRNA transfection is underestimated. Other mechanisms for delivering nucleotide sequences into cells exist, including nucleofection. Their advantages and limitations are described in the review in **Appendix 1.**

Our delivery method increases the miRNA content within a physiological range. Indeed, in our study described in **Section 2.1,**[140] the transfection procedure increases the endogenous miRNA level by 2.4 ± 0.28 times, which seems to be close to the variability observed in a population of cardiovascular patients.[145] The use of a lipofection reagent only (mock condition) or transfection of non-effective miRNA sequences (scramble condition) does not impact PLS function.[140]

The PLS activation remains lower than that of native platelets in same condition. In line with our results, PLS stimulation by thrombin was shown to double the expression of the active form of GPIIbIIIa[129] and soluble P-selectin.[146] Interestingly, the expression level of CD62P and the active form of GPIIbIIIa before stimulation suggest that the produced PLS are pre-activated **(Section 2.2)** as already described in other studies.[147, 148]

Of note, results obtained with *in vitro* models such as with immortalized cell line[12] or CD34+derived megakaryocytes[125] should be taken with caution since the miRNA profile of these cells and their PLS counterparts may differ from that of native platelets. The platelets inherit the majority of their miRNA

content from megakaryocytes[94] for which the *in vitro* culture does not mirror the hematopoietic niche. In addition, platelets are able to uptake the miRNA derived from other cell types, that cannot be mimicked *in vitro* where only one cell type is present.[149]

3.2 TRANSLATIONAL INVESTIGATIONS OF THE MECHANISMS GOVERNING PLATELET FUNCTION THROUGH REGULATION OF MIRNAS

The transfer of miRNA from stimulated platelets to the plasma[150] suggests that circulating platelet-derived miRNAs may reflect platelet activity in real time. Our translational approach combining human-derived cells model and the observation of the association between circulating miRNA level and several facets of platelet reactivity, including aggregation in response to a set of agonists and *in vivo* thrombin generation markers, unveils that one miRNA may govern one or several aspects of platelet reactivity.

In this work, the mechanism of action of two distinct miRNAs was investigated (miR-126-3p and miR-204-5p). miR-126-3p is one of the most widely expressed miRNA in platelets.[106] miR-204-5p was shown for the first time, in a previous study of our group, as associated with platelet reactivity in stable cardiovascular patients,[117] and latter an independent study showed that miR-204-5p was positively associated to platelet aggregation.[120]

3.2.1 MIR-126-3P

A large number of studies have shown that circulating miR-126-3p correlates with platelet reactivity and predict cardiovascular complications.[109, 116, 149] The first investigation dedicated to unveil the underlying mechanism was performed in mice.[12] In this model, the systemic inhibition of miR-126-3p induced a significant decrease in platelet aggregation after AA and ADP stimulation but did not affect significantly collagen-induced platelet aggregation. This indicates that miR-126-3p may regulate several platelet activation pathways. However, the possible non-conservation of seed sequences during the evolution suggests that results from animal model may differ from those obtained in human cells, further supporting our approach to use human-derived cells.

Due to the low concentration of PLS harvested after differentiation of human hematopoietic stem cells, assessment of PLS function cannot be performed using conventional methods such as light transmission aggregometry (LTA). Therefore, we have developed original assays to monitor the effect of miR-126-3p on PLS function. In **Section 2.1**[140] a flow chamber assay was developed where both PLS and megakaryocytes were perfused onto fibrinogen-coated slides. It should be noted that the flow chamber assay only partially mimics the vessels since the matrix does not mirror the sub-endothelial components and the engineering of the channel does not mimic turbulence at the site of atherosclerosis plaque. In addition, no red blood cells were added to the cell suspension to promote margination of PLS that allows the circulation of platelets at the periphery of the blood vessel and enhances the contact between the platelets and the vessel wall. However, the perfusion of megakaryocytes may overcome, at least in part, this issue.

Our model allowed us to go further in the understanding of the mechanisms governing miR-126-3p-incuded modification of platelet reactivity since silencing of one its validated target recapitulated the phenotype. This suggests that the regulation of platelet reactivity by miR-126-3p is, at least in part, mediated by direct regulation of PLXNB2. miR-126-3p is predicted to regulate a high number of mRNAs. Among them, ADAM9, a protein involved in collagen adhesion[113] has already been validated by a reporter gene assay.[112]

In the present study, miR-126-3p overexpression was associated with P-selectin secretion, which was also observed in an independent cohort.[12] The secretion of P-selectin is observed in both activated[151] and procoagulant platelet[152] that is consistent with the results of our clinical study **(Section 2.3 and Appendix 2)** where miR-126-3p was positively associated to both platelet aggregation and *in vivo* thrombin generation markers. Although several other studies showed an association between miR-126-3p and platelet aggregation,[12] the impact of miR-126-3p on platelet-supported thrombin generation is new. Indeed, we showed in a zebrafish model, that miR-126-3p overexpression in thrombocytes induces a thrombus sensitive to an anti-thrombin treatment, but not aspirin, and that miR-126-3p overexpression in PLS induces a procoagulant phenotype.[145] Of note, the gene network described in **section 2.3** pointed miR-126-3p as putative regulator of AKAP13, a protein known to modulate calcium release[153-155] which is closely linked to the platelet procoagulant activity.[63] Although this predicted interaction may explained the impact of miR-126-3p on thrombin generation, AKAP13 remains unvalidated as a target of miR-126-3p yet. In addition, miR-126-3p is predicted to target hundreds of mRNA targets, and we cannot exclude that other mechanisms are involved.

3.2.2 MIR-204-5P

Previous omic's and *in silico* investigations predicted that miR-204-5p was associated with platelet reactivity in stable cardiovascular patients by regulation of a set of actin binding proteins.[117] Among them, CDC42, a Rho GTPase protein has already been biologically validated as a target of miR-204-5p.[156] In addition, several studies described the impact of CDC42 on the modulation of platelet biogenesis and platelet function, further supporting that the pair miR-204-5p/CDC42 is of interest to be functionally validated using our model.[23, 157, 158]

We showed that miR-204-5p regulates CDC42 that in turn induces megakaryocyte cytoskeleton defects and leads to the alteration of PLS biogenesis. Moreover, miR-204-5p overexpression and CDC42 silencing increase both the activation of the GPIIbIIIa receptor after PLS stimulation and the PLS adhesion to immobilized and soluble fibrinogen. In addition, in a cohort of cardiovascular patients under aspirin treatment, miR-204-5p is positively correlated with the aggregation to AA, ADP and collagen. Taken together, these results suggest that miR-204-5p may regulate platelet aggregation via the regulation of fibrinogen binding through a CDC42-dependent mechanism. Although the role of Rho GTPase protein in the regulation of actin cytoskeleton has been largely investigated, its impact on platelet function is poorly understood. The Rho GTPase protein function is mediated by a rapid activation or inactivation characterized by GTP and GDP-binding, respectively.[159] The Rho GTPase activation is closely linked to guanine nucleotide exchange factors (GEFs) while the GTPase-activating proteins (GAPs) mediate the Rho GTPase inactivation. There are more than 80 GEF and 70 GAP, suggesting that the regulation of Rho GTPase family activity is highly complex.[160] In addition, the rapid switch between activated and non-activated form as well as some technical issues makes difficult the investigation of the protein activity status. This is even more true in the case of platelets for which Rho GTPase proteins activation is closely linked to the platelet activation status.[161] The Rho GTPase proteins mediate cytoskeleton remodelling via various and distinct downstream effectors. CDC42, RHOA and RAC1 are known to regulate the formation of filopodia, lamellipodia and stress fibers, respectively.[35] In the **Section 2.2**, we observed a modulation of stress fibers formation in megakaryocytes transfected with miR-204-5p as well as after silencing of CDC42 suggesting a regulation of RHOA activity via down regulation of CDC42. The mechanism has not been entirely elucidated. However, Dutting and colleagues[23] showed a functional link between CDC42 and RHOA during platelet biogenesis. They proposed a model of "*Stop and Go"* signal showing that a balance between CDC42 and RHOA activation may exist in downstream of GPIbα. A decrease of CDC42 may be associated to an increase of RHOA mediating the "*Stop*" signal characterized by an alteration of DMS polarization and platelet formation. In contrast, the "*Go*" signal is associated with an increase of CDC42 and a reduced level of RHOA that

mediates megakaryocytes transmigration into sinusoidal vessels.[162] Although the "*Stop and Go"* signal has been investigated in platelet biogenesis, a similar mechanism could be involved in platelet activation and could explain the impact of miR-204-5p via CDC42 regulation on platelet reactivity. In addition, actin remodelling by RhoGTPase proteins may govern the GPIIbIIIa inside-out and outside-in signalling that contribute to enable GPIIbIIIa in its active form that dramatically increases its affinity for fibrinogen and activation of GPIIbIIIa's downstream effectors triggering platelet spreading, aggregation and clot retraction.[31] Moreover, miR-204-5p overexpression and silencing of CDC42 increase PLS adhesion to fibrinogen that suggests the existence of a miR-204-5p/CDC42/GPIIbIIIa axis. Taken together, these data suggest that miR-204-5p may govern outside-in and/or inside-out signalling mediating platelet aggregation.

3.3 MIRNAS AS BIOMARKERS

In this work, we validated the functional role of two miRNAs in human-derived cells; miR-126-3p increases platelet aggregation and platelet-supported thrombin generation via regulation of ADAM9, PLXNB2 and possibly AKAP13, while miR-204-5p increases platelet reactivity at least in part via regulation of CDC42 and GPIIbIIIa.

The identification of these two miRNAs as regulators of platelet aggregation and platelet-supported thrombin generation opens the way of a circulating miRNA profile that would allow tailoring of antithrombotics in cardiovascular patients. In addition to these 2 miRNAs, we quantified two additional miRNAs largely described in the literature as associated with platelet reactivity,[11, 163] miR-223-3p and miR-150-5p in a sub-population of the ADRIE study. Our clinical association study followed by *in silico* study showed that miR-223-3p, as miR126-3p, is positively correlated to both platelet aggregation and *in vivo* thrombin generation markers that could be mediated via regulation of GLP1R and STMN1, respectively**. (Section 2.3)** Finally, miR-150-5p, mainly known to regulate platelet biogenesis by targeting c-Myb, the TPO receptor,[164] has been investigated. We showed that miR-150-5p was positively associated with platelet aggregation but not with *in vivo* thrombin generation markers. The underlying mechanisms might involve GLP1R regulation. Of note, miR-223-3p and miR-150-5p are positively associated with each other and target both GLP1R, suggesting a putative synergistic effect.

Platelets may transfer their miRNA content to other cells, and thus modify protein expression in other tissues implicated in diseases where platelets play a role. Indeed, the extracellular miRNAs enclosed in

microvesicles or associated to lipoproteins mediate extracellular communication and influence recipient cell function[165] including macrophage and hepatocytes.[166] For example, in mice, miR-126-3p uptake has an anti-inflammatory role and limits atherosclerosis progression[167] while miR-126-5p, but not miR-126-3p, has a protective role by targeting Dlk1 in mice.[168] Finally, miR-126 was described as a biomarker of atherosclerosis and its overexpression in mice may prevent the progression and development of the disease.[169] These results are not what one would expect given that in human, miR-126-3p is positively associated with platelet reactivity[12] **(Section 2.1[140] and Appendix 2[145])** and predicts myocardial infarction;[109] these conflicting results pinpoint differences in the model used (murine vs human) or other yet-to-be defined compensatory mechanisms in atherosclerotic lesions. Finally, platelet-derived miRNAs may regulate a large variety of cellular processes outside the scope of cardiovascular disorders, such as cancer progression where miR-223-3p released from activated platelets can be delivered into lung cancer cells and promotes cell invasion.[170]

4 CONCLUSIONS AND PERSPECTIVES

A growing number of studies dedicated to understand the role of miRNAs in platelet function emerged over the last decades. Since the miRNA profile in plasma mirrors the one in platelets, quantification of circulating miRNA could be used as biomarkers of platelet reactivity, to predict ischemic or bleeding events and allow tailoring the antithrombotic treatment. However, clinical association studies are very heterogeneous in terms of methodological issues, which may contribute to some inconsistent results across different studies **(Appendix 1).** A standardization initiative could certainly help in this regard. This lack of standardization and some inconsistent results emphases the need for tools aiming at functionally validate candidate miRNAs within a true translational approach.

Behind strictly prediction of platelet reactivity or cardiovascular events, circulating miRNAs may help to identify patients who would particularly benefit a low-dose of anticoagulant in addition to antiplatelet therapy.[79, 80] Indeed, the duality of platelet function (aggregation and support for thrombin generation) together with the growing arsenal of antithrombotic therapies render the selection of a therapeutic strategy complex and the circulating miRNA signature could allow the optimisation of the benefit/risk ratio of the treatment.

It is reported that platelet-derived miR-223-3p is negatively associated with high on-clopidogrel platelet reactivity showing that miR-223-3p may predict anti-P2Y12 therapy responsiveness.[171]

Conversely, some data in the literature indicate that antiplatelet drugs may interfere with the level of circulating miRNA[149] however, the causes are scarcely investigated. This could be due to a decrease of miRNA released from inhibited platelets or to a modulation of miRNA production. The miRNA production depends of several factors including the representation of the miRNA loci in the genome. In addition, it has been described that miRNA translation is affected by epigenetic modifications including DNA methylation and histone modifications.[172, 173] Conversely, miRNAs can regulate epigenetic modifications. Indeed, it was shown that SIRT1, a histone deacetylase known to prevent pulmonary thrombus formation[174] is targeted by miR-204-5p.[175] This underlines the existence of a crosstalk between miRNA expression and epigenetic regulators. The interactions between microRNAs and epigenetic regulations as well as the consequences of this crosstalk on platelet function have been scarcely studied. A better knowledge regarding these interactions could shed light on poorly understood mechanisms.

The miRNAs-based therapy provides also multiple opportunities of treatment in disorders where a regulation of miRNA induces pathological changes.[176] A synthetic miRNA mimic may reconstitute the expression level of a specific miRNA, while an elevated pathological level miRNA level can be repressed using an anti-miR sequence (antagomiR). The miRNA-based therapy emerged very recently and a number of issues need to be resolved in order to reliably consider its use in clinical practice. The first clinical trials testing the use of miRNAs-based therapy pointed some dosage toxicity and side off-target effects. The development of a cell or tissues specific delivery system must be considered before reaching the pharmaceutical breakthrough. Finally, effort must be done regarding the galenic to get an *in vivo* delivery tool preventing miRNAs degradation by RNAses.[177, 178]

To conclude, since 1993 and the discovery of an antisense RNA able to bind 3'UTR of mRNAs that marks the start point of the investigations of this small RNA (so-called miRNA), the number of miRNA-related studies increased exponentially. Over the last decades, clinical and fundamental studies have been conducted in parallel in order to understand the role of miRNAs in multiple disorders. Translational studies are of utmost importance and open the perspectives of the use of miRNA as biomarkers for diagnostic and miRNA-based therapy for a preventive medicine. We are now at a pivotal step where the new advances will promote the integration of the basic research with clinical observations taking research from the "bench to bedside".

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APPENDICES

APPENDIX 1: METHODS TO INVESTIGATE MIRNA FUNCTION: FOCUS ON PLATELET REACTIVITY

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Methods to Investigate miRNA Function: Focus on Platelet Reactivity

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Thromb Haemost

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 subjects[2] 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 with[6] 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 drugs 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 1mM, 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; TXB_2, thromboxane B_2; VASP, vasodilator-stimulated phosphoprotein phosphorylation assay.

different anticoagulants and different centrifugation protocols. The choice of using plasma or serum has been debated[32]; 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 method[44] 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 approximately100 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., UniSP6[50] and cel-miR-39[44]) 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 serum[51] 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 crosslinking 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 CLASH[61] 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 (PmiRGO[69]) 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 GPIIbIIIa.[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 platelets[85] for the measurement of P-selectin secretion and the activated form of GPIIbIIIa 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 al[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.[92]

Lentiviral transduction is one of the most effective systems to deliver a nucleotide sequence into hEPSC[97] 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 tools[102] 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 TALEN[121] or CRISPR-Cas9 systems.[122] Tilling[123] or zinc finger nucleases[124] 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 morpholinos[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.[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 sequences[133] 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 hairpin[134] 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 FcgRIIA 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 PACI in human) allow monitoring of platelet secretion and GPIIbIIIa 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[14] and Roux and coworkers[139] 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 vectors. 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 mice[143] with constitutive or conditional[144] 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 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.[148]

The CRISPR and TALEN technologies, as described above, are highly efficient tools used for genome editing. In mice, TALEN[149] and CRISPR[150] 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 coworkers[9] 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.

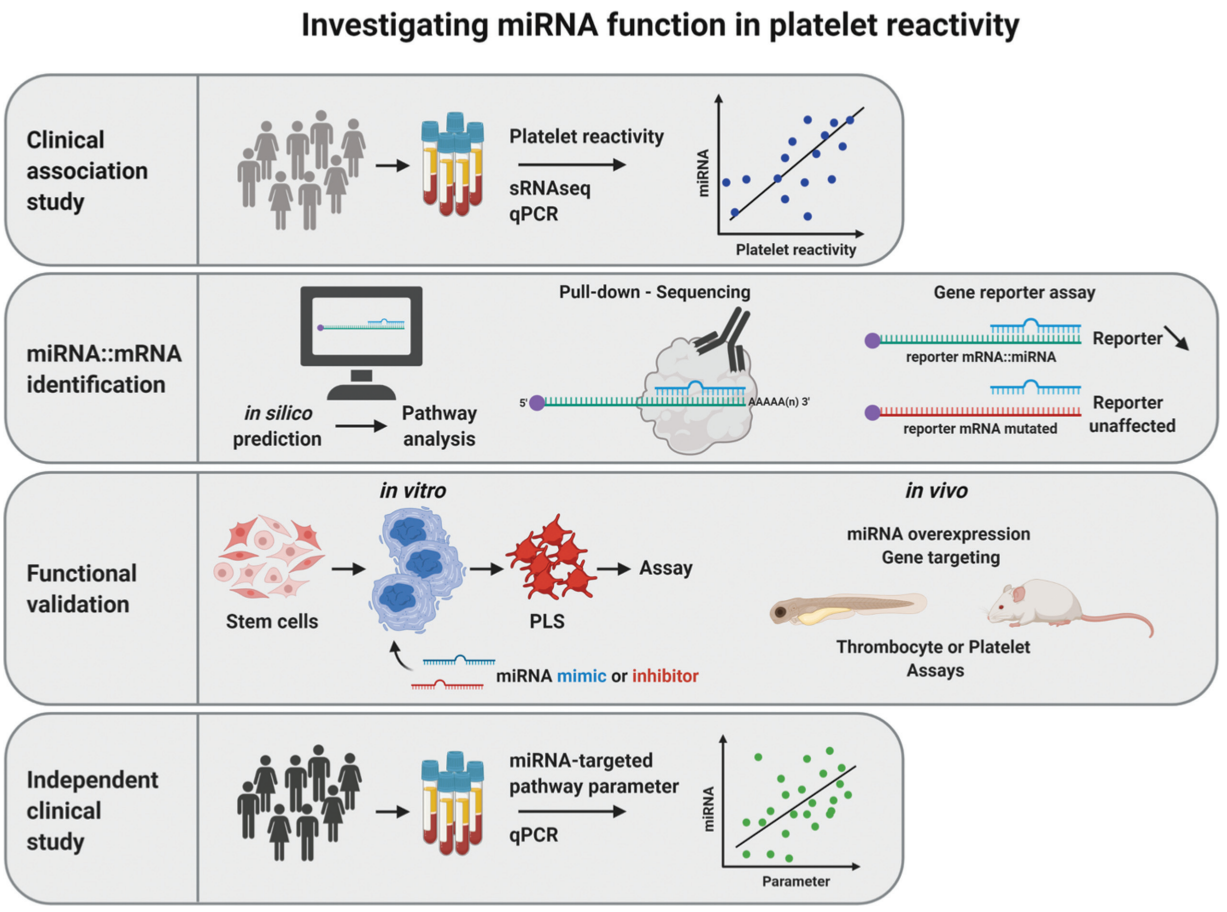

Fig. 1 Suggested general workflow guidelines for the identification and functional validation of miRNA involved in platelet reactivity.

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

Alix Garcia had the initial idea and conceptualized this work, Alix Garcia, Sylvie Dunoyer-Geindre, and Pierre Fontana wrote the first draft of the manuscript. Jean-Luc Reny, Marguerite Neerman-Arbez, and Richard J. Fish critically revised the manuscript. All authors approved the manuscript.

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Conflict of Interest
None declared.

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MICRORNA-126 IS A REGULATOR OF PLATELET-SUPPORTED THROMBIN GENERATION

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MicroRNA-126 is a regulator of platelet-supported thrombin generation

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Abstract

Circulating microRNA (miRNA) expression profiles correlate with platelet reactivity. MiR-126 is a promising candidates in this regard. We generated a transgenic zebrafish line with thrombocyte-specific overexpression of miR-126. Laser injury of the posterior cardinal vein of 5 day-old larvae was performed with or without antithrombotic pre-treatment. Platelet-like structures (PLS) derived from human megakaryocytes transfected with miR-126 were also evaluated for procoagulant activity. Finally, we studied the correlation between miR-126 level and thrombin generation markers in a cohort of stable cardiovascular patients. Control zebrafish developed small thrombocyte-rich thrombi at the site of vessel injury, without vessel occlusion. The miR-126 transgenic line developed an occluding thrombus in 75% (95% CI: 51–91%) of larvae. Pre-treatment with the direct thrombin inhibitor argatroban, but not aspirin, prevented vessel occlusion in the transgenic line (0% occlusion, 95%CI: 0–18%). Upon activation, human PLS showed an increased procoagulant profile after miR-126 transfection compared to control. Finally, the plasma levels of miR-126, but not a control platelet-derived miRNA, correlated with markers of *in vivo* thrombin generation in a cohort of 185 cardiovascular patients. Our results from three complementary approaches support a key role for miR-126 in platelet-supported thrombin generation and open new avenues in the tailoring of antithrombotic treatment.

Keywords

Platelet, platelet reactivity, thrombin, thrombosis, zebrafish

History

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Introduction

After vascular injury, platelets adhere to sub-endothelial components that promote platelet activation, secretion of their granule contents, aggregation and exposure of the necessary phospholipids to support thrombin generation and fibrin formation [1]. Modulators of platelet reactivity may therefore have an impact on the hemostasis balance, from excessive thrombus growth to bleeding.

Platelets are known to host a diverse transcriptome, including more than 700 microRNAs (miRNAs) [2]. MiRNAs are small non-coding RNAs, 19–24 nucleotides in length, that regulate mRNA translation and provide a fine-tuning of protein expression, even in platelets [3]. Indeed, platelets contain all the necessary components for the maturation of miRNAs [4], and increasing evidence suggests that platelet miRNAs are important for the regulation of platelet function. Several studies in cardiovascular patients pointed out selected platelet-derived miRNAs as potential biomarkers for cardiovascular outcome [5,6]. MiRNAs found in plasma or serum could thus be of help as a diagnostic tool [2], reflect platelet reactivity [7] and allow prediction of recurrence of cardiovascular events [5,6], However, the impact of these candidate miRNAs on thrombus formation is largely unknown. To address this issue, the zebrafish model is of particular interest. The zebrafish bears a conserved coagulation system [8], centered upon the controlled conversion of fibrinogen to fibrin and the activity of hemostatic thrombocytes, which while nucleated, unlike platelets, are the functional equivalents of human platelets [9]. MiRNAs are also largely conserved across vertebrates [10], supporting functional studies on miRNAs identified in human systems in the zebrafish. Of key importance, several research groups have used the *Tg(cd41:EGFP)* transgenic zebrafish line to monitor thrombocyte aggregation and thrombus formation in response to a vascular injury [11,12]. This transgenic line has green fluorescent thrombocytes that can be observed in real-time in larval zebrafish, without invasive procedures.

In this translational study, we used a zebrafish model, human-derived cells and samples from cardiovascular patients to investigate the functional impact of miR-126, a platelet-derived miRNAs associated with platelet reactivity [13] and proposed as a potential biomarker for the recurrence of cardiovascular events in humans [6].

Methods

DNA Constructs

The zebrafish has two miR-126-3p orthologues; dre-miR-126a-3p (which has an identical sequence to the human hsa-miR-126-3p) and dre-miR-126b-3p that differs by one nucleotide in the 3′ part downstream of their common seed sequences (supplemental Figure 1a) [14]. These miRNAs are involved in lymphatic and vascular development as well as in the regulation of the cell fate decision between

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erythroid and megakaryocytic lineages during definitive hematopoiesis [15,16]. Thus, to avoid possible indirect effects on hemostasis in the course of functional studies, we established transgenic zebrafish lines expressing dre-miR-126a-3p under the control of the cd41 promoter (*itga2b* promoter) specifically in the thrombocyte lineage using the Tol2-transposon system. In order to identify cells expressing these transgenic miRNAs, the gene encoding the red fluorescent protein mCherry was integrated upstream of the dre-miR-precursor-sequence in a Tol2 transposon system vector (supplemental Figure 1b). The plasmid pDestTol2 CG2-cd41:mcherry-pri-miR-126a was made with plasmids from the Tol2kit and from Ablain *et al*. using Gateway™ cloning technology [17,18]. pDestTol2 CG2-U6:gRNA (ID: 63156, Addgene, Cambridge, USA, a gift from L. Zon) was used as the final destination template plasmid. The zebrafish cd41 promoter was sub-cloned from a pCRIITOPO4 vector clone, a gift from Professor Julien Bertrand (University of Geneva) and mCherry was PCR-amplified from pRSET-B-mCherry (a gift from the laboratory of Roger Tsien, University of California). The Dre-miR-126a precursor was amplified by PCR and assembled as 3 tandem copies, using restriction sites downstream of the mCherry sequence. Verification of the plasmids was performed with Sanger sequencing analysis and restriction digests.

Generation of miR-126a Transgenic Zebrafish

Tg(cd41:EGFP) zebrafish, referred to below as the control zebrafish line, were mated and embryos at the single-cell stage were collected [12]. Forty picograms (pg) of Tol2 transposon transgenesis vector containing the *myl7*:EGFP (*CG2*) transgenesis marker and 20 pg of Tol2 mRNA were injected into the cytosols of approximately 200 embryos. Three days after injection, embryos with modest mosaic and strong EGFP fluorescence signal in the developing heart, and with green fluorescent thrombocytes, were selected under a fluorescence stereomicroscope, raised to adulthood, and outcrossed to the TU strain. Founder fish were identified by the production of F1 offspring with green fluorescent hearts. F1 fish with green fluorescent thrombocytes and green fluorescent hearts were then again outcrossed to the wild-type TU strain to generate F2 families.

Zebrafish larvae experimentation was made before 6 days post fertilization. Until this developmental age, in accordance with Swiss guidelines and in line with Directive 2010/63/EU of the European Parliament, the larvae are not independently feeding and therefore not under experimental restriction. The generation of transgenic animals was made in accordance with the local Swiss regulations. When necessary during stock breeding cycles, adult fish were euthanized with an analgesic overdose of 300 µg/ml Tricaine methanesulphonate with 300 µg/ml sodium bicarbonate, buffered at pH 7.0.

Thrombocyte Isolation Procedure

Hearts were manually removed from 5 day-old Tg(*cd41*:EGFP) zebrafish larvae. Subsequently, the remaining tissue was dissociated with Liberase (5 mg/ml in 0,9x PBS, Roche, Basel, Switzerland) at 33°C for 2–2.5 h in a 1.5 ml Eppendorf tube while shaking. The reaction was stopped with 1% FCS/0,9x PBS and thrombocytes were sorted based on the intensity of the GFP fluorescence signal with a Biorad S3 cell sorter, as previously described [12].

Quantification of thrombocytes relative to the total number of cells was performed by dissociating 5 zebrafish larvae. Thrombocytes were then sorted as described above and counted on a Biorad S3 cell sorter. This latter experiment was performed 5 times and the results were averaged and expressed as number of thrombocytes for 10′000 cells.

Quantification of microRNA in Zebrafish Larvae

MiRNAs were isolated from thrombocytes and other cells using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) and this was followed by a reverse transcription procedure using the TaqMan™ Advanced miRNA cDNA Synthesis Kit (Applied Biosystems, Foster City, USA). MiRNAs were detected in triplicate on a 7900HT SDS system using TaqMan™ Advanced miRNA assays (Applied Biosystems) with predesigned probes for miR126-3p, miR223-3p and 18 S rRNA (used as a control miRNA and gene, respectively).

Laser-induced Thrombus Formation and Analysis

Five day-old zebrafish larvae with the Tg(*cd41*:EGFP) transgene, were anesthetized with tricaine (MS-222, Sigma-Aldrich, Saint Louis, USA) at a concentration of 164 mg/L and mounted with low gelling agarose (Sigma-Aldrich) on glass slides. Zebrafish were positioned on their right sides, with anterior to the left. Laser injury was performed blinded from the genotype. Following injury of the posterior cardinal vein of these specimens at the level of the third somite posterior to the cloaca, using a laser (Cryslas Laser, power: 15) coupled to an inverted microscope (Leica DM 6500, objective HCX PL FLUOTAR L 20x/0.40 corr.), thrombi developed. Videos of the green fluorescent and accumulating thrombocytes were taken (Leica DFC 360 FX camera, LAS-AF software, 5 frames/sec). To analyze the thrombus formation and the quantification of thrombocyte accumulation at the site of vessel injury, the fluorescent area in each captured picture (μm^2) of the recordings was calculated with the imaging analysis software MetaMorph (v.7.8.12) and plotted against time. Thrombocyte accumulation at the site of vessel injury was quantified at 130 sec and defined below as "thrombocyte-content" (μm^2). Clots obstructing the blood flow within 2 min after laser injury were counted as occlusive thrombi. For this, occlusion was defined at 2 min, as no flow at the site of laser injury. After imaging, larvae were not revived and euthanized in a bleach solution as described in the National Institutes of Health (2009) Final Report to OLAW on Euthanasia of Zebrafish.

Treatment of Zebrafish with Antithrombotic Drugs and Subsequent Analysis of Thrombus Formation

Aspirin (1 mM) was delivered into 5 day-old zebrafish larvae by direct soaking for 1 h at 27°C in the dark. Argatroban (Argatra®, Mitsubishi Tanabe Pharma, Düsseldorf, Germany), dissolved in ethanol (1 mg/ml), was administered by pericardial injection using a glass needle. The injection was performed under anesthesia with tricaine (164 mg/L), 20 min before the laser injury. As control, fish were injected with ethanol alone. To identify a possible effect of both drugs on thrombus formation, we analyzed laser-induced thrombocyte aggregation and evaluated occlusive thrombus formation as described above.

Generation of Platelet-like-structures from Hematopoietic Stem Cells

Platelet-like structures (PLS) produced from hematopoietic stem cells are functionally close to human platelets [19–21]. Indeed, the secretion of PLS after agonist stimulation, as well as their function in relation to fibrinogen receptors, is comparable to the results observed with human platelets [22,23]. PLS were derived from megakaryocytes produced from human hematopoietic stem cells as described elsewhere [24]. Briefly, human CD34+ cells were isolated from the buffy coats of healthy adult human donors provided by the Geneva University Hospitals' blood bank using a CD34 microbead kit (Miltenyi Biotec, Bergisch Gladbach,

Germany). CD34+ cells were cultured for 7 days before addition of 0.5 μg/mL thrombopoietin (TPO, Stemcell Technologies, Vancouver, Canada) for an additional 8 days [24]. Megakaryocytes isolated at D13 were then transfected with a hsa-miR-126-3p mimic, or scrambled miRNAs (200 nM, ThermoFisher, Waltham, USA) as described elsewhere [24]. Using this method, we increased hsa-miR-126-3p level by a factor of 2.4 ± 0.28 compared to the mock condition (cells with transfection reagent only referred to as control) [24]. PLS and megakaryocytes were quantified using Tali Image-based cytometer (Thermofisher). The megakaryocytes were defined as elements > 6 μm and the PLS as elements ≥ 4 and ≤ 6 μm [24].

Generation and Quantification of Procoagulant Platelet-like Structures

Megakaryocytes were discarded at D15 using a 5-min centrifugation step at 100 g as described [24]. The supernatant was centrifuged at 1000 g for 10 min and PLS were resuspended in Tyrode's buffer (138 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 1 mM $CaCl_2$, 5 mM Hepes, 3.5 mg/ml human serum albumin, 5.5 mM glucose, pH 7.3).

Procoagulant platelets possess an elevated cytosolic Ca^{2+}-concentration and expose phosphatidylserine [25]. Procoagulant activity was induced by the addition of the agonists convulxin (10 nM) and thrombin (1 U/ml) in the presence of Annexin V Alexa Fluor™ 647 conjugate (1:1000, Molecular probes, Eugene, USA), an indicator of phosphatidylserine exposure at the outer leaflet of the platelet plasma membrane, fluo 3-AM (final concentration 2 μM, Molecular probes), a calcium indicator, and a mouse anti-human CD41 antibody (1:40) conjugated with PE (Biolegend, San Diego, USA) for PLS identification, at 37°C in the dark. Appropriate isotypic controls were used to setup cutoff values. The formation of PLS with a procoagulant phenotype (++ Annexin V/++fluo-3 AM) within 10 min post-activation, was detected using a BD Accuri C6 flow cytometer (BD Biosciences Allschwill, Switzerland) and the percentage of procoagulant PLS was determined with FlowJo software (TreeStar, Ashland, Oregan, United States).

Thrombin Generation Assay

A thrombin generation assay using the Calibrated Automated Thrombogram® (Stago, Asnières sur Seine, France) was performed to evaluate the procoagulant potential of megakaryocytes and PLS collected at D15. The cell suspension was centrifuged at 1000 g for 10 min at 37°C, with 25 μM PGI_2 (Cayman Chemical Company, Ann Arbor, USA) 0.02 U/mL apyrase (Sigma-Aldrich), and resuspended in human plasma (Cryocheck pooled normal plasma, PrecisionBiologic, Dartmouth, Canada) at 3.125×10^6 cells/mL. This cell suspension contained megakaryocytes and 33 ± 1.8%, 33 ± 1.4%, 32 ± 1.7% of PLS in mock, scramble and hsa-miR-126-3p-transfected samples, respectively. The thrombin generation assay was performed with 250′000 cells/well in triplicate using the PRP reagent (Stago) according to the manufacturer's instructions, with or without previous activation with a mixture of 15 μM TRAP (Bachem Holding, Bubendorf, Switzerland) and 20 μg/mL of Horm collagen type I (Takeda Pharmaceutical, Tokyo, Japan) for 10 min at 37°C in order to induce procoagulant cells [26]. The thrombin burst was evaluated with the velocity parameter (a parameter that recapitulates the lag-time, the thrombin peak and the time to peak parameters) and the endogenous thrombin potential (ETP).

Cardiovascular Patients

Patients were selected out of the ADRIE (Antiplatelet Drug Resistance and Ischemic Events; clinicalTrials.gov identifier NCT00501423) population described in detail elsewhere [27]. The study protocol was approved by the Central Ethics Committee of the University Hospitals of Geneva (Geneva center) and the Ethics Committee of Montpellier Saint-Eloi (Béziers and Montpellier centers). Briefly, the ADRIE study is a prospective study focusing on the clinical relevance of the platelet response to aspirin and/or clopidogrel in stable cardiovascular outpatients. EDTA and citrate-anticoagulated plasma samples were collected at inclusion and stored at −80°C. For the present study, we used samples of cardiovascular patients treated with aspirin as their sole antiplatelet drug.

Quantification of microRNAs in Cardiovascular Patients

For each sample 2 × 100 μl of plasma were mixed with 1 ml QIAzol® lysis reagent (Qiagen). RNA was isolated using the miRNeasy mini kit (Qiagen). The TaqMan Advanced miRNA cDNA Synthesis Kit (Applied Biosystems) was used for reverse transcription, using 2 μl of the RNA eluate. Reverse transcription products were preamplified and expression of miRNAs were assessed in triplicate on a 7900 HT SDS system using TaqMan™ Advanced miRNA assays (Applied Biosystems) with predesigned probes for hsa-miR126-3p and hsa-miR150-5p, another platelet-derived miRNA positively correlated with platelet reactivity in clinical association studies, used here as a negative control [28].

Using the geNorm algorithm, we previously determined a set of three stably expressed miRNAs (hsa-miR-106-5p, hsa-miR-16-5p, and hsa-miR-484) as the best combination for normalization in plasma among panels given by several groups [29,30] and was assessed for each sample [31]. MiRNA levels are expressed as a ratio of miRNA to these normalizers and are referred to below as arbitrary units.

Quantification of in Vivo Thrombin Generation Markers in Cardiovascular Patients

Thrombin-antithrombin complexes (TAT) and prothrombin fragment F1 + 2 (F1 + 2), were quantified in citrated plasma samples using Enzygnost® TAT micro and Enzygnostat F1 + 2 kits (Siemens Healthcare Diagnostics Products, Marburg, Germany), respectively, according to the manufacturer instructions.

Statistical Analysis

Data are presented as mean and standard error of the mean (SEM). Proportions were compared with the Fisher's exact test. The unpaired student's t-test or the one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was performed, when appropriate. A Spearman rho test was used to evaluate the correlation between plasma miRNAs levels and thrombin generation markers. A p value < .05 was considered as significant. Data were analyzed using Prism7 software (GraphPad software Inc., San Diego, USA).

Results

Generation of Zebrafish with Thrombocyte-specific Overexpression of dre-miR-126a-3p

The DNA construct for the generation of the zebrafish line overexpressing miR-126a is represented in Supplemental Figure 1b. Three founder animals that express dre-miR-126a-3p in their

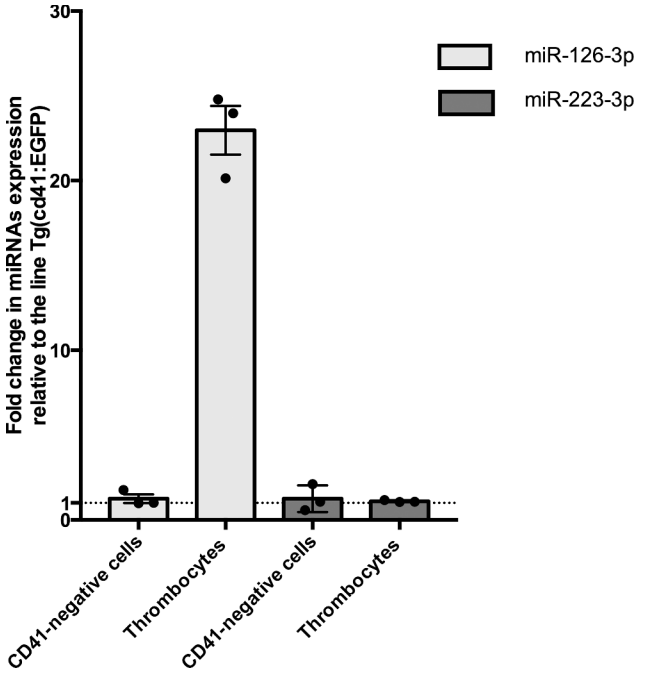

Figure 1. Expression of dre-miR-126a-3p and dre-miR-223-3p in thrombocytes and in a random population of EGFP-negative cells of double-transgenic zebrafish lines compared to control. Results are expressed as fold change relative to control (n = 3, $p < .01$ using t-test).

thrombocytes were obtained. We observed specific upregulation of the mature forms of dre-miR-126a-3p in EGFP-positive cells of the double-transgenic larvae (dre-miR-126a and EGFP) at 5 days post-fertilization, while dre-miR-223, another abundant miRNA in thrombocytes was not affected (Figure 1). Despite a clear upregulation of dre-miR-126a-3p in EGFP-positive cells, the mCherry fluorescence was hardly detectable in thrombocytes of double transgenic animals. This might be due to competition between miRNA production and mCherry translation. Indeed, we used a design described elsewhere [32] and the inclusion of several miRNAs into the same backbone may dramatically decrease fluorescent protein expression [32].

Overexpression of dre-miR-126a-3p in Thrombocytes Leads to Laser-induced Occluding Thrombi

We measured thrombus formation at the site of a laser-induced injury of the venous vessel wall in zebrafish larvae containing EGFP-tagged thrombocytes (Figure 2A,B). With this approach, in the control zebrafish line (n = 16), laser injury was followed by the formation of a thrombocyte-rich thrombus (mean thrombocyte-content 521 ± 65 μm^2, Figure 2C and video 1 in the supplemental material), that never occluded the vessel. In the double-transgenic line *Tg((cd41:mCherry-pre-miR-126a); cd41:EGFP))* referred below as (miR-126a/EGFP), 5-day-old larvae developed large thrombi with few thrombocytes at the site of vessel injury, with a mean thrombocyte-content of 265 ± 37 μm^2, Figure 2C, and video 2 in the supplemental material, $p < .01$ compared to control zebrafish line. Of note, while none of the control zebrafish developed an occluding thrombus ((0%, 95% CI: 0–20%), 75% (95% CI: 51–91%) of the miR-126a/EGFP line (n = 20) did. Thrombus formation in bright field mode in control and miR-126a/EGFP lines are illustrated in video 3 and 4 in the supplemental material, respectively.

Since we overexpressed dre-miR-126a specifically in thrombocytes, we addressed the issue of platelet count in the transgenic line compared to control. The proportion of thrombocytes compared to other cells in miR126a/EGFP larvae was 2.8 ± 0.3 per 10′000 cells (n = 5 independent experiments), compared to 5.4 ± 0.6 thrombocytes per 10′000 cells in controls (n = 5, $p < .01$).

Following this observation, we hypothesized that overexpression of dre-miR-126a-3p in zebrafish thrombocytes is, at least in part, associated with an increased thrombin generation leading to vessel occlusion at the site of laser injury. We thus challenged this hypothesis with prior administration of two antithrombotics; an anticoagulant and an antiplatelet drug.

Argatroban, but Not Aspirin, Prevents Vessel Occlusion in miR-126a/EGFP Larvae

As expected, incubation of control larvae with aspirin at the concentration of 1 mM for 1 h before laser-injury was associated with a decrease in the amount of immobilized thrombocytes at the site of vessel injury (181 ± 56 μm^2 in the condition with aspirin, n = 7, compared to 472 ± 79 μm^2 in controls, n = 5, $p < .05$). When assessing the incidence of vessel occlusion after laser injury in the dre-miR-126a/EGFP zebrafish line, aspirin had no effect on this phenotype, while injection of argatroban completely prevented vessel occlusion (Table I).

Overexpression of hsa-miR-126-3p in PLS derived from human megakaryocytes is associated with a procoagulant phenotype after stimulation

PLS activation with convulxin and thrombin generated 19.1 ± 5.1% of PLS positive for both calcium and annexin V in the mock condition (Figure 3A). While transfection of megakaryocytes with a scrambled miRNA did not significantly affect the proportion of procoagulant PLS ($p = .57$), transfection with hsa-miR-126-3p mimic increased it significantly (n = 4, $p = .012$, Figure 3B).

Overexpression of hsa-miR-126-3p in human-derived cells is associated with an increased thrombin generation profile

Figure 4A is representative of a typical thrombin generation profile, with and without previous activation and in different conditions. Compared to the mock condition, transfection of megakaryocytes at D13 with scramble miRNA did not affect the velocity parameter, while there was an increase of 2.6 ± 0.5 fold when megakaryocytes were transfected with hsa-miR-126-3p (Figure 4B, $p = .016$ compared to mock and $p = .028$ compared to scramble, n = 5 independent experiments). The ETP did not differ between groups (data not shown).

Circulating hsa-miR-126-3p levels correlate with in vivo thrombin generation markers in cardiovascular patients

The characteristics of the ADRIE study population according to antiplatelet drug regimen is described elsewhere [33]. Out of the 223 patients treated with aspirin as their sole antiplatelet drug, seven were deemed non-compliant to treatment [33] and were excluded from the present analysis. Plasma samples were available in 191 patients out of the remaining 216 and hsa-miR-126-3p was successfully quantified in 185 while hsa-miR-150-5p was successfully quantified in all 191 samples.

Plasma hsa-miR-126-3p levels ranged from 0.5 to 16.2 arbitrary units (AU), with a median value of 2.1 AU (IQR: 1.3–2.9). Plasma hsa-miR-150-5p levels ranged from 0.06 to 6.2 AU, with a median value of 0.7 AU (IQR: 0.4–1.3).

TAT and F1 + 2 were successfully quantified in 189 samples. Median TAT and F1 + 2 values were 4.8 μg/l (IQR: 3.7–6.7) and 252.2 pmol/l (IQR: 186.1–366.3), respectively. There was a positive correlation between thrombin generation biomarkers

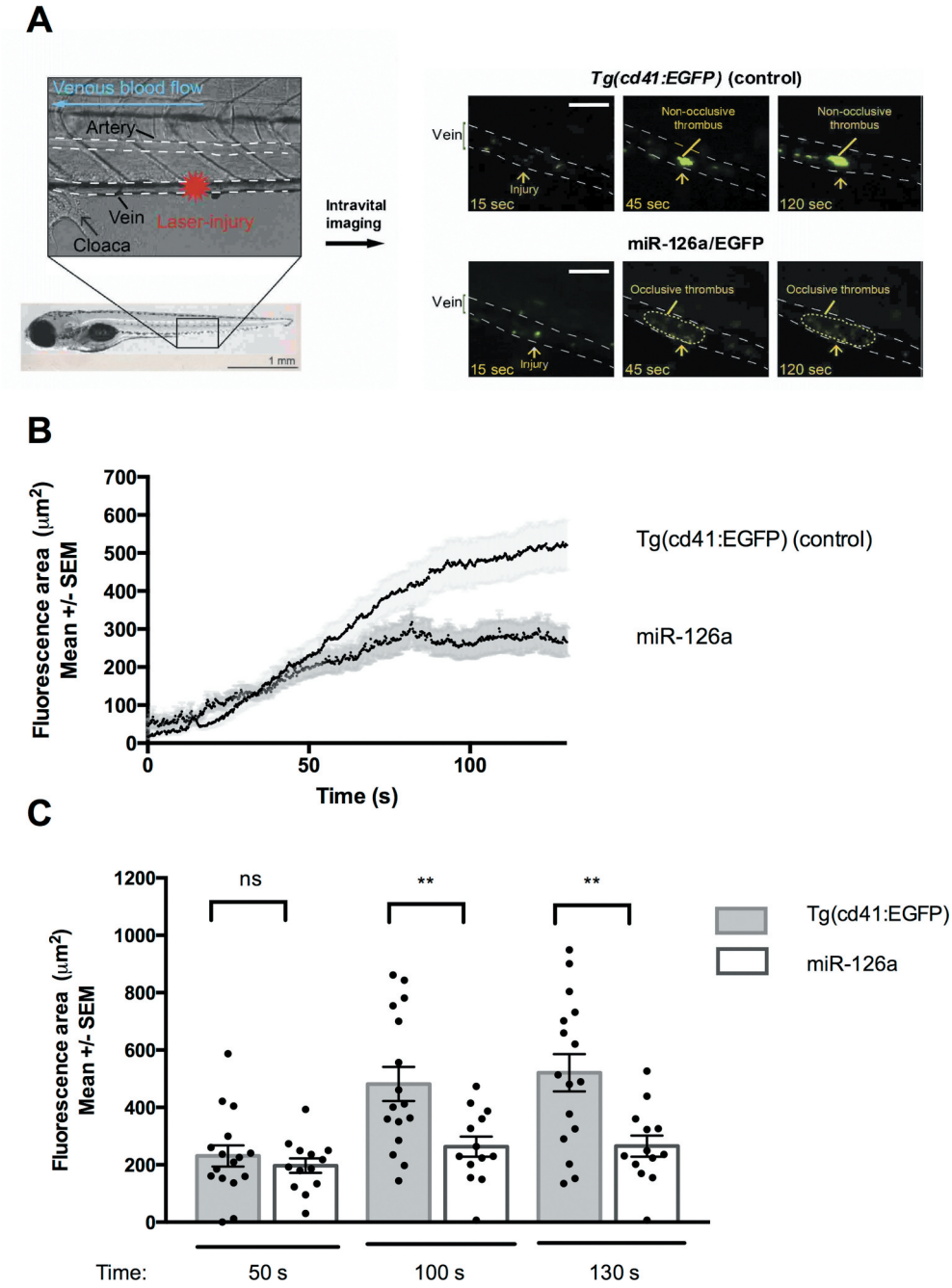

Figure 2. Thrombus formation after laser injury of the posterior cardinal vein of different zebrafish lines. A. representative pictures 15 s, 45 s and 2 min after laser injury. White scale bar: 30 µM. B and C. Quantification of thrombocytes at the site of laser injury. Control (n = 16) vs miR-126a/EGFP (n = 13, $p < .01$ using t-test).

Table I. Effect of selected antithrombotic drugs on vessel occlusion after laser injury in miR-126a/EGFP line. P calculated with Fisher's exact test.

	Control	Aspirin	p
% occlusion (95% CI)	75 (51–91) (n = 20)	79 (49–95) (n = 14)	1.0
% occlusion (95% CI)	60 (32–84) (n = 15)	Argatroban 0 (0–18) (n = 18)	<0.01

and hsa-miR-126-3p level, while there was none with hsa-miR -150-5p, a known platelet-derived miRNA (Table II).

Discussion

In this study, we provide evidence for a role for miR-126, one of the most abundant miRNAs in platelets, in the regulation of platelet-supported thrombin generation.

The zebrafish is a validated *in vivo* model for studying hemostasis and has been employed, for example, for loss-of-function studies demonstrating bleeding and thrombotic phenotypes in fibrinogen [34], factor X [35] and antithrombin [36] deficiencies. We used the *Tg(cd41*:EGFP) transgenic zebrafish line, with fluorescent thrombocytes [12], to monitor thrombocyte aggregation and thrombus formation in response to a laser-induced vascular injury that can be observed in real-time.

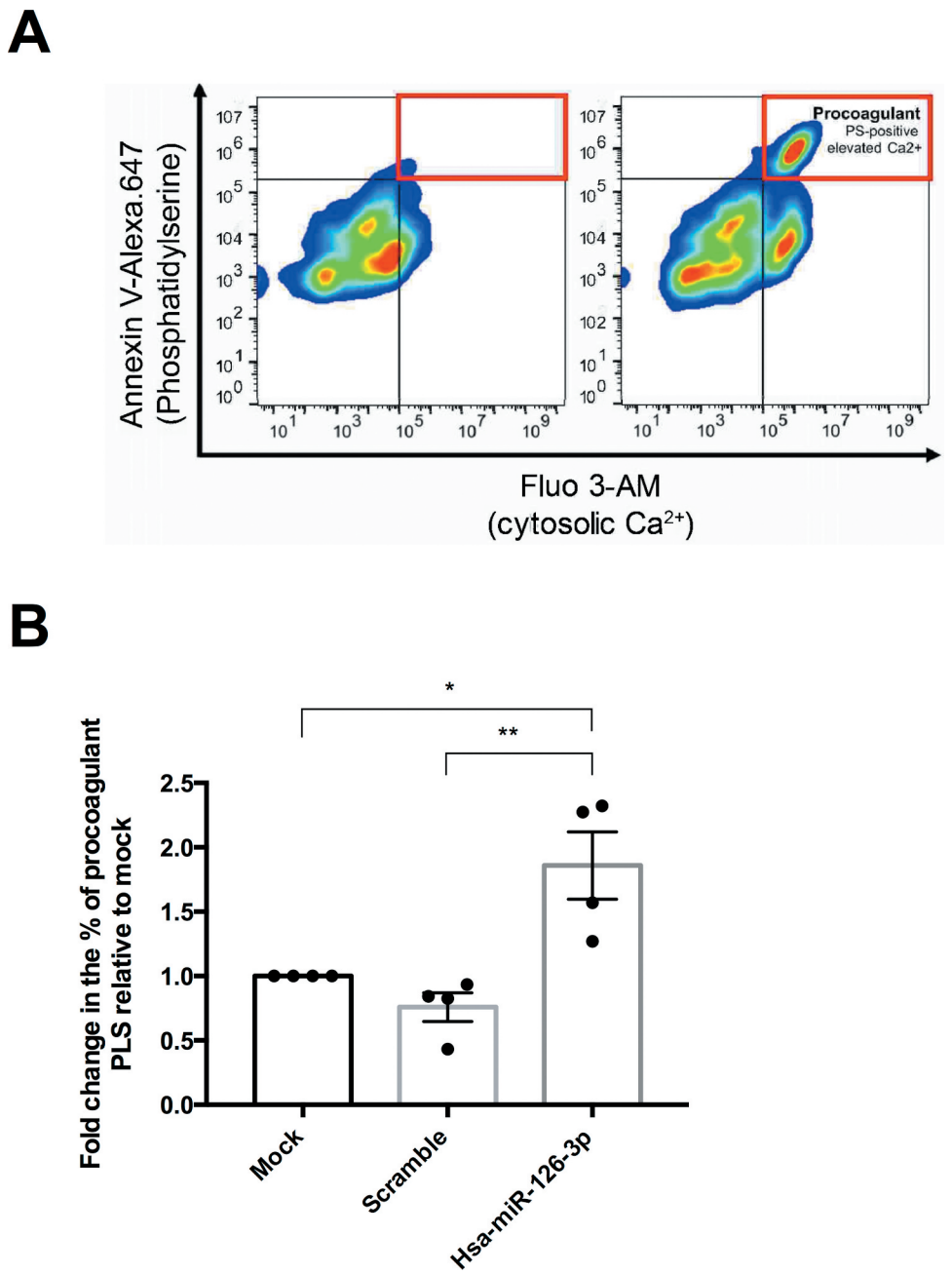

Figure 3. A. Representative procoagulant PLS by flow cytometry before (left panel) and after (right panel) activation. B. Quantification of procoagulant PLS after activation with thrombin and convulxin. Data are expressed as fold change relative to mock condition (n = 4 independent experiments, $p = .012$ relative to mock condition and $p = .003$ relative to scramble using ANOVA followed post-hoc Tukey test).

Our double transgenic lines expressed a 23-fold increase of dre-miR-126a with the sequence of dre-miR-126a repeated 3 times. We acknowledge that this might have influenced the phenotype and we cannot rule out that different number of repeats would have been associated with a different phenotype. Of note, the clot is three dimensional, and there is variability in assessing thrombus growth with 2D-imaging in our assay. Nevertheless, overexpression of dre-miR-126a – a miRNA with an identical sequence to the human hsa-miR-126 – in zebrafish thrombocytes lead to a striking difference in laser-induced thrombus formation with vessel occlusion that was prevented by argatroban, but not by aspirin. The quantification of fluorescent thrombocytes at the site of vessel injury showed fewer cells in the miR-126a/EGFP line compared to control. This may be in relation with the decreased thrombocyte count in this line, highlighting the potential role of dre-miR-126a in thrombocyte production in zebrafish. Another explanation is that rapid formation of a large fibrin-rich thrombus induced by a small number of thrombocytes, with 23-fold more dre-miR-126a than controls, decreased the blood flow and the ability of circulating thrombocytes to be exposed to the site of vessel injury. Cessation of blood flow would also prevent accumulation of thrombocytes behind the formed thrombus.

The zebrafish is a recognized model to test thrombin inhibitors, and several compounds have already been tested using the laser injury in the posterior cardinal vein [37]. Some of them such as bivalirudin are used in humans and were effective to prevent vessel occlusion in zebrafish [37]. Our data showing that argatroban, but not aspirin, prevents vessel occlusion is consistent with the hypothesis of an increased thrombin generation leading to the formation of an occluding thrombus. To support this hypothesis, we investigated the generation of procoagulant PLS derived from human megakaryocytes transfected with hsa-miR

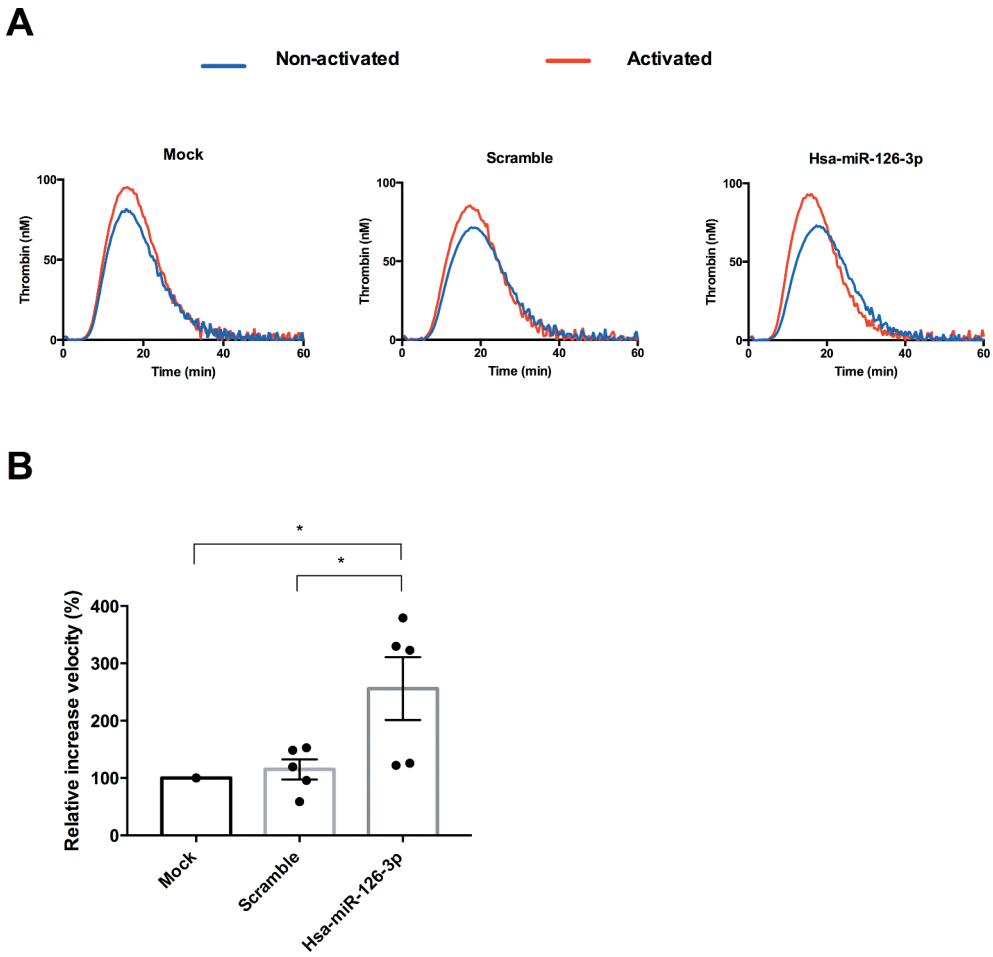

Figure 4. Impact of hsa-miR-126-3p on thrombin generation in human plasma. A. representative thrombin generation profile before (blue) and after (red) cell activation. B. Quantification of the velocity parameter. Data are expressed relative to mock condition (n = 5 independent experiments, $p = .016$ compared to mock and $p = .028$ compared to scramble using ANOVA followed post-hoc Tukey test).

Table II. Correlation of thrombin generation markers and selected miRNA levels in stable cardiovascular patients. F1 + 2: prothrombin fragment 1 + 2. TAT: thrombin-antithrombin complex (Spearman correlation test).

	hsa-miR-126-3p		hsa-miR-150-5p	
	rho	P	rho	P
TAT	0.19	0.01	0.06	0.43
F1 + 2	0.34	<0.01	0.14	0.06

-126-3p mimic [24]. Although the increase in hsa-miR-126-3p level is modest in PLS, compared to our zebrafish model (less than 3-fold increase in PLS derived from transfected megakaryocytes compared to control [24]), we demonstrated that upon activation, those PLS are more prone to generate a procoagulant phenotype than PLS derived from control megakaryocytes. Procoagulant human platelets, referred to as collagen (or convulxin) and thrombin (COAT)-platelets, have been described as a subpopulation of activated platelets [38] expressing procoagulant phosphatidylserine and harboring procoagulant proteins on their surface and on the surface of the microvesicles generated [39] through a rise of intracellular free calcium [25], thus supporting thrombin generation. This subpopulation of procoagulant platelets represents around 17% of platelets in healthy volunteers and rises to about 25% in patients with cardiovascular disease [40]. Interestingly, aspirin has no effect on the generation of procoagulant platelets in cardiovascular patients [40], in line with our observations in the zebrafish model with no effect of aspirin on the incidence of vessel occlusion. Using human cells, 19% of control PLS met the definition of COAT-platelets after activation, with a significant 2-fold increase in those derived from hsa-miR-126-3p-transfected megakaryocytes. Of note, results with PLS derived from megakaryocytes transfected with the scrambled miRNA did not differ from control, suggesting that this increase in procoagulant PLS is indeed specific to hsa-miR -126-3p overexpression. These results together with the 2-fold increase in the proportion of procoagulant PLS are consistent with the 2.6-fold increase in thrombin burst associated with hsa-miR-126-3p transfection when cells are suspended in human plasma. The increase of the velocity parameter, with no difference in ETP, is indeed typical of accelerated thrombin formation via platelet content release and platelet-derived extracellular vesicle formation [41]. Finally, it should be emphasized that the impact of hsa-miR-126-3p transfection in human cells is probably underestimated since only half of the megakaryocytes are indeed transfected at D13 after the procedure [24].

We [24] and others [42] identified several gene targets of miR-126 that may explain the phenotype observed in this study. In human cells, miR-126 downregulates plexin B2, a Semaphorin 4D ligand [24,43]. Since Semaphorin 4D promotes platelet-platelet interactions, this may contribute to the lower amount of thrombocytes found at the site of laser injury in the miR-126a/EGFP transgenic line. Recent data also showed that after stimulation of rat mast cells, miR-126 enhances a PI3 K/Akt signaling

pathway by promoting calcium influx [42]. Since calcium is of utmost importance for platelet activation and generation of COAT-platelets [25], one can speculate that the same mechanism holds true for platelets. Altogether, the changes in gene expression associated with miR-126 overexpression lead to reduced platelet-platelet interactions and increased platelet-mediated thrombin generation, a well-described phenotype of COAT-platelets [44].

Circulating miRNAs are proposed to be mostly of platelet origin [45], which supports our approach to test the hypothesis of a correlation between *in vivo* thrombin generation markers and plasma hsa-miR-126-3p level in cardiovascular patients. TAT and F1 + 2 are indeed validated thrombin generation markers that have been shown to correlate with thrombotic events in different settings [46,47]. We found a correlation between these thrombin generation markers and hsa-miR-126-3p level, but not hsa-miR -150-5p, a known platelet-derived miRNA which is, as hsa-miR -126-3p, positively correlated with platelet reactivity [7] and cardiovascular events [28]. Of note, the distribution of hsa-miR -126-3p plasma levels in our population showed a 2.2-fold difference between the 25th and 75th percentile, a similar magnitude measured in our PLS with a 2.4-fold increase after transfection. This further supports our model of transfected human-derived cells with differences of miR-126 levels in line with the distribution observed in cardiovascular patients. The ADRIE study included stable cardiovascular outpatients. This is noteworthy since there was no ongoing thrombus formation at inclusion and we can speculate that the correlation between hsa-miR-126-3p and thrombin generation markers might be even stronger in acute coronary syndrome situations, when platelets are activated during thrombus formation. Of note, we selected samples of patients treated with aspirin only. Indeed, there is a large variability in the biological response to clopidogrel, largely mediated by the variability in the active metabolite generated by liver cytochromes. Since antiplatelet drugs may affect the plasma levels of platelet-derived miRNAs [7], we speculated that this liver cytochrome-mediated variability of clopidogrel response might have biased any correlation between platelet-derived hsa-miR -126-3p and thrombin generation markers.

The main limitation of this study is that we did not investigate the functional impact of miR-126 inhibition. In our hands, and with our *in vitro* model, the available antagomirs/miRNA inhibitors did not induce any significant up-regulation of validated target mRNAs. Systemic injection of an antagomir in our zebrafish model was not considered, mostly because the putative functional effect of systemic injection would not be thrombocyte-specific or expected to have long-lasting effects. The laser injury in zebrafish larvae was not performed on an artery, but on the posterior cardinal vein, a vessel very close to the tail surface allowing a technically more robust and reproducible injury than in the dorsal aorta. Nevertheless, we clearly demonstrate that in the wild-type fish cardinal vein, we generate a thrombocyte-dependant thrombus sensitive to aspirin, as one would expect in an artery. Noteworthy, the zebrafish model was a starting point, which raised an original hypothesis, validated afterward with human cells and in cardiovascular patients. Another limitation is that the thrombin generation assay was performed on both megakaryocytes and PLS, and not on PLS alone. The production of PLS was not sufficient to provide the phospholipid concentration necessary to induce detectable thrombin generation. Of note, functional assays on megakaryocytes have already been used as a surrogate marker of platelet function, supporting our approach [48]. These limitations are balanced by the 3 different approaches that we used, including *in vivo* data of cardiovascular patients, all pointing to the same conclusion.

The present work echoes clinical trials assessing the safety and efficacy of low dose anticoagulant in the prevention of recurrence of ischemic events in cardiovascular patients, such as the COMPASS trial [49]. This trial randomized more than 27′000 stable atherosclerotic patients to rivaroxaban 2.5 mg twice daily with aspirin, rivaroxaban 5 mg twice daily, or aspirin 100 mg once a day [49]. This study showed that those patients assigned to rivaroxaban 2.5 mg twice daily plus aspirin had better cardiovascular outcomes but more major bleeding events than those assigned to aspirin alone. In view of our results, one could speculate that the miRNA profile may help in tailoring the antithrombotic medication to optimize the benefit/risk ratio, by prescribing anticoagulant to those patients with high hsa-miR-126-3p.

In summary, our results provide mechanistic insights on the association between miR-126 and cardiovascular events through modulation of platelet-mediated thrombin generation potential. These data pave the way for the use of miRNA profiling to tailor antithrombotic therapy in cardiovascular patients.

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Authorship Contributions

V.Z., R.J.F, J.-L.R and M.N-A. designed the study and analysed the data. V.Z, A.G., S.D-G performed the experiments and analysed the data. All authors revised the intellectual content of the manuscript and approved the final version.

Declaration of interest statement

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Supplementary material

Supplemental data for this article can be accessed on the publisher's website.

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