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Review

Patrick Provost*

The clinical significance of platelet microparticle-associated microRNAs

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Abstract: Circulating blood platelets play a central role in the maintenance of hemostasis. They adhere to subendothelial extracellular matrix proteins that become exposed upon vessel wall damage, which is followed by platelet activation, further platelet recruitment, platelet aggregation and formation of an occlusive, or non-occlusive, platelet thrombus. Platelets host a surprisingly diverse transcriptome, which is comprised of ~9500 messenger RNAs (mRNAs) and different classes of non-coding RNAs, including microRNAs, as well as a significant repertoire of proteins that contribute to their primary (adhesion, aggregation, granule secretion) and alternative (RNA transfer, mRNA translation, immune regulation) functions. Platelets have the propensity to release microparticles (MPs; 0.1–1 μm in diameter) upon activation, which may mediate inflammatory responses and contribute to exacerbate inflammatory diseases and conditions. Carrying components of the platelets' cytoplasm, platelet MPs may exert their effects on recipient cells by transferring their content in platelet-derived bioactive lipid mediators, cytokines, mRNAs and microRNAs. Platelet MP-associated microRNAs may thus function also outside of platelets and play an important role in intercellular signaling and gene expression programming across the entire circulatory system. The role and importance of platelet MP-associated microRNAs in various aspects of biology and pathophysiology are increasingly recognized, and now provide the scientific basis and rationale to support further translational research and clinical studies. The clinical significance, pathophysiological role as well as the diagnostic and therapeutic potential of platelet MP-associated microRNAs in cardiovascular diseases, platelet transfusion and cancer will be discussed.

***Corresponding author: Patrick Provost,** CHU de Québec Research Center/CHUL Pavilion, 2705 Boulevard Laurier, Room T1-65, QC G1V 4G2 Quebec, Canada,
Phone: +1-418-525-4444 (Ext. 48842), Fax: +1-418-654-2765,
E-mail: patrick.provost@crchudequebec.ulaval.ca; and Faculty of Medicine, Université, Quebec, QC G1V 0A6, Canada

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Blood platelets

Circulating blood platelets play a central role in the maintenance of hemostasis, and participate to an increasing number of pathophysiological processes, ranging from atherosclerosis to cancer metastasis. Derived from bone marrow megakaryocytes, blood platelets have a circulating lifespan of 8–10 days [1, 2]. Patrolling the surface of the vessel wall, platelets bind to the subendothelial extracellular matrix that becomes exposed upon vessel damage [3]. More specifically, platelets come into contact with a number of adhesion molecules, including collagen, which interact with platelet surface receptors, leading to platelet arrest and activation [4]. Once activated, platelets release the content of their granules as well as adenosine diphosphate (ADP), serotonin and thromboxane A_2, leading to further platelet recruitment, aggregation and plug formation.

Platelet transcriptome

Platelets are enucleate in nature and, although they contain circular, ~16 kb mitochondrial DNA (there are 3–5 mitochondria per platelet) [5], they are devoid of genomic DNA. Incapable of de novo gene transcription, platelets thus rely on a rich repertoire of messenger RNAs (mRNAs), microRNAs, other non-coding RNAs and proteins to modulate their primary functions, such as adhesion, aggregation and secretion [6].

Human blood platelets are known to host an extremely diverse transcriptome, comprised of ~9500 different mRNAs [6–8] and different classes of non-coding RNAs, including antisense transcripts to protein-coding loci, long non-coding RNAs [9], microRNAs [10, 11] and, more recently, circular RNAs [12, 13].

Platelet microRNAs

microRNAs are endogenous, non-coding RNA species of 19–24 nucleotides (nt) in length that play a critical role in regulating mRNA translation into proteins in eukaryotic cells. Finely tuning expression of ~60% of our genes, microRNAs exert their regulatory effects mainly through recognition of specific binding sites located in the 3′ untranslated region of mRNAs [14–16].

Human platelets harbor a functional microRNA pathway

Human platelets harbor an extremely abundant and diverse array of microRNAs [10], which contrasts with their relatively low level of protein synthesis – unless it explains it? In other words, cells actively translating their pool of mRNAs require microRNAs for their regulation, and it may be that the abundance of microRNAs in platelets strongly inhibits platelet mRNA translation.

Human platelets contain all the cytoplasmic protein components of the microRNA pathway, such as Dicer [17], TAR RNA-binding protein 2 [18] and Argonaute 2 (Ago2) [10, 19]. Platelet Dicer can process microRNA precursors (pre-microRNAs) into mature microRNAs [10]. However, the relative abundance of the mature species over the precursors [10] is not consistent with Dicer actively processing pre-microRNAs in platelets; the study and comparison of subpopulations of platelets (e.g. old versus young platelets) may help clarify this question.

The complete microRNA repertoire of human platelets

Initial micro-array profiling of human platelet RNA identified 219 different microRNA sequences [10]. Subsequent RNA-Seq analyses revealed that the microRNA repertoire of human platelets is much more complex than previously reported micro-array results indicated, increasing their number to 532 [11]. Further bioinformatic analyses unveiled that platelet microRNAs bear important modifications at their 3′ end, mainly adenylation and uridylation. Whereas the former has been associated to microRNA stabilization [20], the latter may be involved in microRNA instability and degradation [21]. We also detected numerous variants of microRNA sequences of various length, which are collectively known as isomiRs, resulting from imprecise Drosha and/or Dicer processing. In some cases, these isomiRs were more abundant than the reference microRNA sequence, including isomiRs with microRNA seed region shifted towards the 3′ end and redirected mRNA targeting abilities [11].

A role for microRNAs in platelet function?

Increasing evidences suggest that platelet microRNAs are important for platelet biogenesis [22] and function [23–25]. Due to the inherent challenges of studying microRNA function in a cell type (anucleate platelets) that is refractory to transfection, initial reports of microRNA regulation of platelet mRNAs were based on data that were indirect and/or correlative. In 2010, Kondkar et al. [26] reported that increased levels of vesicle-associated membrane protein 8 (VAMP8)/endobrevin mRNA were associated with platelet hyperreactivity, and that miR-96 could regulate VAMP8/endobrevin mRNA levels in cultured cells. The authors concluded that these data support a role for VAMP8/endobrevin in the heterogeneity of platelet reactivity, and suggest a role for miR-96 in regulating expression of VAMP8, the primary platelet v-SNARE protein involved in the platelet secretion process [27].

A year later, the same group established a correlation between platelet microRNA-mRNA coexpression profiles with platelet reactivity [28]. Two years later, Edelstein et al. [29] narrowed the gap by showing that (i) miR-376c levels were inversely correlated with PCTP mRNA levels, PC-TP protein levels and PAR4 reactivity, and that (ii) miR-376c regulated expression of PC-TP in human megakaryocytes [29].

More recently, Zhou et al. [30] reported an interesting sequence of events, whereby antisense inhibition of miR-148a upregulated platelet T-cell ubiquitin ligand-2 mRNA expression, reduced platelet FcgammaRIIA signaling and decreased thrombosis in vivo in mice. These data suggest that modulating miR-148a expression is a potential therapeutic approach for thrombosis.

A year earlier, Londin et al. [31] had shown that, although the abundance of platelet mRNA transcripts was highly correlated across 10 healthy young males, there was only a weak connection between the platelet transcriptome and proteome, bringing some uncertainty as to whether microRNAs do regulate platelet mRNA translation.

A more recent study by Rowley et al. [32], however, showed that murine megakaryocyte-specific knockdown of Dicer1, the ribonuclease that cleaves miRNA precursors into mature miRNAs, reduced the level of the majority of

microRNAs in platelets, altered platelet mRNA expression profiles and induced mild thrombocytopenia. Dicer1 deletion resulted in increased surface expression of integrins alphaIIb and beta3, and enhanced platelet binding to fibrinogen in vivo and in vitro. These changes were the consequence of impaired microRNA regulation of alphaIIb and beta3 gene expression, supporting a regulatory role for microRNAs in platelet function.

Platelet microRNAs may be of clinical significance

Whether microRNAs regulate platelet mRNA translation or not does not prevent their use as disease biomarkers. A disease in which circulating platelets are exposed to extreme conditions, and thus expected to be the most affected, is chronic kidney disease (CKD). Indeed, platelet exposure to uremic toxins and contact with artificial surfaces during dialysis had been reported to induce platelet abnormalities and alter the platelet proteome. Comparing the circulating platelets of CKD patients with age- and sex-matched healthy subjects, we observed an alteration of platelet mRNA and microRNA levels in CKD patients, which appeared to be corrected by dialysis [33]. Reduced in platelets of uremic patients, WD repeat-containing protein 1 was found to be regulated by miR-19b, a microRNA increased in platelets of uremic patients and involved in platelet reactivity [33]. These results support a mRNA regulatory role of platelet microRNAs, and suggest that an alteration of microRNA-based mRNA regulatory mechanisms may underlie the platelet response to uremia and entail the development of platelet-related complications in CKD. These results also open the possibility of using platelet microRNAs as biomarkers. However, their monitoring would imply isolating platelets prior to qPCR detection, for example, which may not be easily adapted to high-throughput screening in a clinical setting. A biomarker assay based on microRNA measurements in plasma, which is a clinically available biofluid, would, by far, be more straightforward.

Extracellular microRNAs in the circulation

While the majority of microRNAs are found and mediate their regulatory effects on gene expression intracellularly, a number of microRNAs are also present in the extracellular milieu and in various biological fluids of the human body, such as maternal milk [34–38], saliva [39, 40], urine [41], nasal secretions [42, 43], sperm [44] and plasma [45–48], and may represent interesting biomarkers of diseases [49, 50].

Circulating microRNAs are associated with Ago2 proteins [46, 51], which may explain their striking stability in plasma [51], an extracellular milieu rich in nucleases [51]. Extracellular microRNAs may also be packaged with high-density lipoprotein [45] and nucleophosmin 1 [52], and function as secreted signaling molecules that influence the function and phenotype of recipient cells. Significant amounts of extracellular microRNAs are also found in small membranous vesicles, such as exosomes [53], shedding vesicles [54], apoptotic bodies [55] and microparticles (MPs) [56].

Platelets release extracellular vesicles upon activation

Extracellular vesicles (EVs) may form a barrier against the degradative components (e.g. nucleases)/conditions of the extracellular milieu and contribute to the stability of microRNAs outside of cells. Platelets have been shown to release EVs upon activation by physiological agonists/mediators, such as (i) collagen, which mimics the matrix exposed upon damage of the vessel wall, (ii) ADP, which is released from activated platelets, and (iii) thrombin, which is generated during coagulation and is simulated by thrombin-receptor activating peptide (TRAP) [57]. Platelets are also activated by the increased shear stress caused by a reduced lumen at sites of atherosclerotic plaques. The platelet EV nature and microRNA content, like that of proteins [58], is expected to vary depending on the agonist(s) or stimulus(i) to which platelets are exposed, but will invariably miror that of the platelets from which they derive.

Platelets have been shown to release two types of EVs upon stimulation with thrombin receptor agonist peptide SFLLRN (TRAP) or alpha-thrombin: exosomes, derived from exocytosis of multivesicular bodies and alpha-granules, and MPs, produced by surface shedding [59].

Platelet exosomes

In 1999, Heijnen et al. [59] reported that activated platelets release exosomes measuring 40–100 nm in diameter, analogous to the vesicles termed exosomes secreted by other cell types. Exosomes have been proposed as vehicles

for microRNA-based intercellular communication and a source of microRNA biomarkers in bodily fluids [60]. However, quantification of both the number of exosomes and the number of microRNA molecules isolated from five diverse sources (i.e. plasma, seminal fluid, dendritic cells, mast cells, and ovarian cancer cells) unveiled ~ 0.01 microRNA molecules per exosome [60]. This microRNA:exosome stoichiometry is hardly compatible with a role for exosomal microRNAs in intercellular communications, unless exosomes compensate by their relative abundance and/or microRNA delivery efficiency. Whether platelet-derived exosomes exhibit a similar empoverishment in microRNAs remains unknown.

Platelet MPs

In contrast to exosomes (< 0.1 μm) that are released from intracellular compartments, platelet MPs are essentially vesicular fragments of platelet cytoplasm that are shed from the cytoplasmic membrane and express antigens from their parent cells at their surface, such as CD41 and CD62p (P-selectin); they can be distinguished from apoptotic bodies (> 1 μm) based on size [61]. Similar to exosomes and apoptotic bodies, MPs can exhibit phosphatidylserine, in consequence to the loss of membrane asymmetry that occurs during their release [62–65]. All of these markers may be detected by flow cytometry and used to distinguish platelet MPs from other circulating MPs derived, for instance, from endothelial cells and red blood cells.

Boilard et al. [56] have reported that platelets may generate MPs (0.1–1 μm in diameter) through stimulation of the collagen receptor glycoprotein VI, at least in arthritis pathophysiology. The authors detected proinflammatory platelet MPs in joint fluid from patients with rheumatoid arthritis and other forms of inflammatory arthritis, and observed that platelet depletion attenuated murine inflammatory arthritis. Containing several potent bioactive mediators, platelet MPs may amplify inflammation and arthritis in mice [56, 62].

Platelets transfer microRNAs through MPs

Platelet MPs may mediate pathophysiological effects and participate in hemostasis and thrombosis, inflammation, malignancy infection transfer, angiogenesis, and immunity [66] by acting as intercellular carriers that deliver, not only bioactive proteins (e.g. cytokines) and mRNAs [67], but also microRNAs [68, 69] to recipient cells.

Platelet MPs mediate microRNA transfer to endothelial cells

Indeed, we found that MPs isolated from thrombin-activated platelets contained microRNA miR-223, in complex with the microRNA effector protein Ago2 [69]. We showed that these MPs can be internalized by human umbilical vein endothelial cells, leading to the accumulation of platelet-derived miR-223 and the regulation of two endogenous endothelial genes (FBXW7 and EFNA1), both at the mRNA and protein levels [69]. Platelet MPs may thus act as intercellular carriers of functional Ago2•microRNA complexes that may exert heterotypic regulation of gene expression in endothelial cells (Figure 1, lower left).

Platelet MPs reprogram macrophage gene expression and function

We transposed our findings to another recipient cell type of the circulatory system, and observed that platelet MPs can be internalized by primary human macrophages and deliver functional miR-126-3p [70]. Platelet MPs dose-dependently downregulated expression of four predicted mRNA targets of miR-126-3p. These effects were abrogated by expression of a neutralizing miR-126-3p sponge, implying the involvement of miR-126-3p. The changes induced by platelet MPs included downregulation of cytokines/chemokines chemokine (C-C motif) ligand 4 (CCL4), colony-stimulating factor 1 (CSF1) and tumor necrosis factor (TNF) mRNA levels and reduced CCL4, CSF1 and TNF cytokine/chemokine release, accompanied by a marked increase in their phagocytic capacity [70]. These findings demonstrate that platelet MPs can be taken up by macrophages to modify their transcriptome and reprogram their function towards a phagocytic phenotype (Figure 1, lower right).

Platelet MP-derived microRNAs may thus function outside of platelets and play an important role in microRNA transfer, intercellular signaling and gene expression programming across the entire circulatory system [71].

Clinical significance of platelet MPs and microRNAs

Interestingly, platelet MPs are the most abundant cell-derived MP subtype in the circulation [72], where relatively

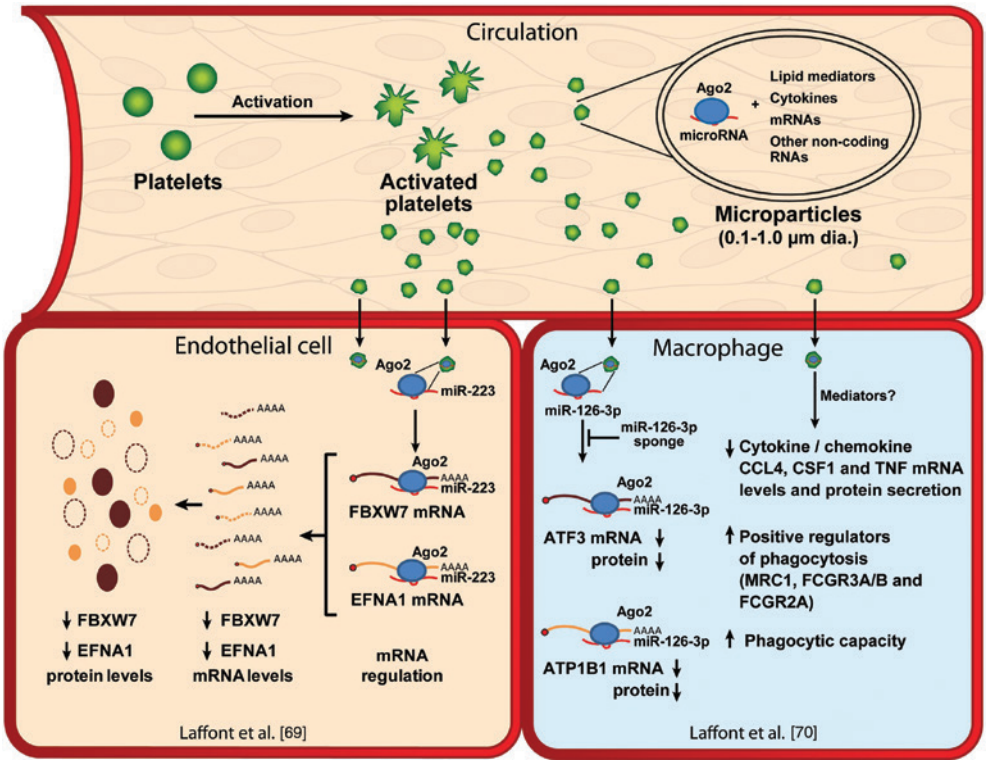

Figure 1: Platelet MPs mediate microRNA transfer to other cells of the circulatory system, and regulate recipient cell gene expression and function.

Activated platelets release MPs that contain microRNAs as well as bioactive lipid mediators, cytokines, mRNAs and, as suggested by a genomic analysis of human platelets [9], a wide variety of additional non-coding RNAs (upper panel). The results of our studies suggest that human platelets can efficiently transfer their microRNA content, as Ago2 · microRNA complexes, to human (umbilical vein) endothelial cells [69] (bottom left panel) and primary human macrophages [70] (bottom right panel) through the release of MPs. These findings support a scenario by which platelet MPs may protect microRNAs from extracellular nucleases and act as intercellular carriers that deliver functional Ago2 · microRNA complexes [69], through which they may exert heterotypic regulation of endogenous gene expression and modulate the function of recipient cells in the circulatory system, such as endothelial cells [69] and macrophages [70]. Ago2, Argonaute 2; ATF3, activating transcription factor 3; ATP1B1, sodium/potassium-transporting ATPase subunit beta-1; CSF1, colony-stimulating factor 1; CCL4, chemokine (C-C motif) ligand 4; EFNA1, Ephrin A1; FBXW7, F-box and WD-40 domain protein 7; FCGR2A, Fc gamma receptor IIA; FCGR3A/B, Fc gamma receptor IIIA/B; MPs, microparticles; MRC1, mannose receptor, C type 1; mRNA, messenger RNA; TNF, tumor necrosis factor. Adapted from Laffont et al. [69].

high levels of microRNAs originating from platelets are also found [73].

Considering the pro-inflammatory properties of platelet MPs, it is very likely that platelet MPs and their microRNA cargo may be involved both in acute and chronic blood vessel inflammatory conditions, such as those associated with cardiovascular diseases related to atherosclerosis, platelet transfusion and cancer.

Cardiovascular diseases

Accumulating data suggest mechanistic roles for platelet-derived microRNAs in haemostasis, thrombosis, and unstable coronary syndromes [74]. Using RNA-Seq, Gidlöf et al. [75] found nine differentially expressed microRNAs in patients with myocardial infarction compared with healthy controls, of which 8 were decreased in patients. Of these, miR-22, -185, -320b, and -423-5p increased in the supernatant of platelets after aggregation and were depleted in thrombi aspirated from MI patients, indicating the release of certain microRNAs from activated platelets [75]. The authors confirmed that endothelial cells could take up the released platelet microRNAs and see their intercellular adhesion molecule-1 (ICAM-1) expression regulated by platelet-derived miR-320b. Therefore, platelets activated during myocardial infarction may release functional microRNAs, which can be taken up by endothelial cells, regulate ICAM-1 expression and possibly modulate the adhesiveness of the blood vessel wall lining. Platelet microRNAs may even modulate endothelial cell viability in diabetes, as platelet-secreted miR-223 was

found to promote endothelial cell apoptosis induced by advanced glycation end products by targeting the insulin-like growth factor 1 receptor [76].

Circulating microRNAs have been proposed by as novel biomarkers for platelet activation [73] and coronary artery disease [77]. The group of Manuel Mayr demonstrated a substantial platelet contribution to the pool of circulating microRNAs, noting, in doing so, that the microRNA content of platelet-poor plasma differs from that of serum [73]. However, the authors cautioned that (i) antiplatelet medication (e.g. aspirin) attenuates the release of platelet-derived microRNAs [73], and (ii) heparin inhibits the polymerase chain reactions, in particular the amplification of the exogenous Caenorhabditis elegans spike-in control, thereby resulting in an artefactual rise of endogenous microRNAs [78]. These findings prompted the authors to caution that antiplatelet therapy and preparation of blood samples could be confounding factors in case-control studies relating plasma microRNAs to cardiovascular disease. This has to be taken into account when designing studies to investigate the relation of circulating microRNAs to acute cardiovascular events or coronary intervention [78].

A meta-analysis of studies that tested the plasma concentration of platelet MPs in patients with acute coronary syndrome (ACS) showed (i) a significant difference in plasma platelet MP levels between the patients with ACS and healthy controls, and (ii) that patients with ACS tend to have higher plasma platelet MPs concentration than patients with stable angina [79]. Interestingly, ACS patients undergoing percutaneous coronary intervention (PCI) saw their plasma concentration of platelet MPs decrease, leading the authors to conclude that platelet MPs may be both a promising biomarker for the development of ACS and a prognostic factor of PCI. A role for microRNAs in restenosis and thrombosis after vascular injury has also been proposed [80].

A study published 2 years earlier had shown that EVs containing miR-126 and miR-199a, but not freely circulating microRNA expression, could predict the occurrence of cardiovascular events in patients with stable coronary artery disease; increased expression of miR-126 and miR-199a in circulating EVs was significantly associated with a lower major adverse cardiovascular event rate [81].

In a more recent study, Hartopo et al. [82] reported that the number of platelet MPs was increased in acute myocardial infarction as compared to unstable angina, and were associated with the extent of myocardial damage.

It is tempting to speculate that platelet MPs may normally have a physiological function in health, but convey an "alert" signal that would condition the entire circulatory system in response to the emergence of a (new) pathophysiological state, and also possibly contribute to its exacerbation.

Platelet transfusion

Several studies have unveiled a potential role for human platelet microRNAs in the field of transfusion medicine, in terms of regulating platelet signaling pathways, markers of platelet associated disorders, and remote impactors of gene expression (intercellular biomodulators) as well as potential platelet quality markers of storage and pathogen reduction treatments [83], some of which have been reviewed recently [84].

Platelet MPs and associated microRNAs may also be involved in the pathophysiological response to platelet transfusion. Platelet concentrates prepared from blood donations are stored under blood bank conditions (gentle rocking at room temperature), during which platelets gradually deteriorates and become activated.

In addition, we have shown that pathogen reduction technologies, which aim to reduce the risk of transfusion-transmitted infections and are being implemented worldwide, induce platelet activation and a decrease in platelet mRNA [85] and microRNA [57] levels. These changes in RNA levels were concomitant with a reduction in platelet mean volume and an increase in MP concentration in platelet concentrates. Therefore, it appears likely that pathogen reduction technologies may enhance the formation of platelet MPs in platelet concentrates. Although this hypothesis remains to be validated, preliminary data support it [57].

Platelet MPs and associated microRNAs may thus be transfused with platelets to the patients. Considering the pro-inflammatory properties of platelet MPs, it is reasonable to hypothesize that platelet MPs may exacerbate both acute and chronic blood vessel inflammatory conditions, such as those associated with platelet transfusion and atherosclerosis, respectively.

Koberle et al. [86] reported that the release of vesicle-associated microRNAs from blood cells can occur in blood samples within the time elapsing in normal clinical practice until their processing, without significant hemolysis, which should be taken into consideration when studying EVs and associated microRNAs in blood samples.

Cancer

Tumor growth/cancer development is a major pathophysiological condition that may be influenced by platelet MPs

and associated microRNAs. Indeed, miR-223 delivered by platelet-derived EVs was found to promote lung cancer cell invasion via targeting of tumor suppressor EPB41L3 [87]. The authors noted that platelets and platelet-secreted EVs from non-small cell lung cancer patients contain higher level of miR-223 than that from healthy subjects. Incubation of human lung cancer A549 cells with platelet-secreted EVs resulted in rapid delivery of miR-223 into A549 cells, in which platelet miR-223 targeted EPB41L3 to promote A549 cell invasion [87].

Although platelet MPs may have beneficial effects by supporting tissue repair and regeneration, as well as hemostasis, they may, on the other hand, be a pro-coagulant promoter leading to the thrombotic events seen in the context of cancer [88]. Platelet MPs may also act as a direct tumor growth enhancer, through the release of potent growth factors in the tumor microenvironment, and favor tumor dissemination through their propensity to trigger thrombosis and support tumors.

Perspectives

The role and importance of platelet MP-associated microRNAs in various aspects of biology and pathophysiology are increasingly recognized, and now provide the scientific basis and rationale to support further translational research and clinical studies.

Use of platelet MPs and microRNAs as biomarkers of disease

microRNAs are present in all the biological fluids of the human body, such as saliva [39, 40], urine [41], nasal secretions [42, 43], sperm [44] and plasma [45–48], which are relatively easy to collect from patients. Can monitoring of platelet-specific MPs and microRNAs in biological samples collected from patients help establish correlations between a given microRNA and specific diseases or conditions, and identify a more sensitive biomarker [89]?

The therapeutic potential of platelets, MPs and microRNAs

Alternatively, applied basic research may help develop the therapeutic potential of platelets, MPs and associated microRNAs. For instance, one of the miR-223-regulated genes is ADP P2Y12 receptor, a key target for current antiplatelet drug therapy. Recent studies showed that a blunted response to P2Y12 antagonist, that is, high on-treatment platelet reactivity (HTPR), is a strong predictor of major cardiovascular events (MACEs) in coronary heart disease (CHD) patients receiving antiplatelet treatment. Recent clinical cohort study showed that the level of circulating miR-223 is inversely associated with MACE in CHD patients. In addition, the level of both intraplatelet and circulating miR-223 is an independent predictor for HTPR, thus providing a link between miR-223 and MACE. These evidences indicate that miR-223 may serve as a potential regulatory target for HTPR, as well as a diagnostic tool for identification of HTPR in clinical settings [90].

Can we envision inhibiting platelet MP internalization by recipient cells? Inhibiting (or enhancing) the function of MP-associated microRNAs? Using platelet MPs to deliver a therapeutic RNA, aimed to restore normal gene expression and cell function?

Such work is to be pursued if we wish to take advantage of, and develop, the full diagnostic and therapeutic potential of platelet MP-associated microRNAs.

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