MicroRNAs in platelet physiology and pathology

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Keywords
MicroRNAs

Summary
MicroRNAs (miRNAs), highly conserved, short (approx. 22 nucleotides) non-coding RNAs, exhibit a fine-tune control over gene expression by complementary sequence binding and translational repression of protein coding mRNA transcripts. Recently, the role of miRNAs has been increasingly investigated in various physiological or pathophysiological events. Circulating platelets are crucial for coagulation physiology to maintain haemostatic balance and are involved in various pathologies such as atherosclerosis and thrombosis. Anucleate platelets lack genomic DNA but inherit diverse array of functional coding or non-coding RNAs and translational machinery from their parent cells – megakaryocytes enabling activated platelets to synthesize proteins which suggests the possibility of post transcriptional gene regulation in platelets. Expression of functionally active miRNAs in platelets changes during platelet activation indicating a role in platelet biology. Here, we present a review on recently identified platelet miRNAs and their role in platelet physiology that is essential for maintaining haemostasis. MicroRNAs (miRNA) are small (21–23 nucleotides) non-coding RNAs regulating about two third of the mammalian protein coding genes at least in part by translational repression (1). MiRNAs are increasingly shown to play vital role in cellular development and various pathologies (2–7). Although most miRNAs expressed in multi-cellular organisms are well conserved throughout the animal kingdom (1) their expression varies in cellular content i.e. miRNA copy numbers (8) and tissue distribution patterns (5,9).

Platelets, the circulating anucleate blood cells, are crucial to maintain haemostasis and safeguard vascular integrity (10, 11). If disturbed, the platelet reactivity associates with various pathologies leading to morbidity and mortality (12–15) such as atherosclerosis, occlusive or thrombotic cardiovascular disorders, inflammation and cancer.

Platelets are released into circulation as cytoplasmic fragments, originated from bone-marrow megakaryocytes through commitment of the multipotent stem cells to the megakaryocyte lineage, proliferation of the progenitors and terminal differentiation of megakaryocytes in a precisely controlled microenvironment (16, 17). Despite of lacking nucleus, platelets are able to translate inherited miRNAs into proteins (18, 19) for example synthesis of integrin-alpha3 (glycoprotein-IIIa) protein, a fibrinogen receptor involved in platelet aggregation, is observed during their preservation (19, 20). Surprisingly, more than 32% of the human genes are represented as messenger RNA in circulating platelets. A strong correlation between platelet transcriptome and proteomic profile, in addition to inherited functional translation machinery e.g. rough endoplasmic reticulum,
ribo- somes and small amount of poly(A) positive RNAs from parent megakaryocytes supports de-novo translational capa- bilities of platelets and suggests the exciting possibility of post transcriptional regu- lation of gene expression within platelets (21).

The serial analysis of gene expression (SAGE) data has revealed the average length of 3'UTR (untranslated region) of platelet transcripts (1047 bases) are signifi- cantly longer than mRNAs of the nucleated cells (492 bases). Considering the depend- ency of miRNA mediated gene regulation on binding site availability and occupancy on the 3'UTR of target mRNAs, longer 3'UTR suggests more robust post tran- scriptional regulation in addition to mRNA stability via other mechanisms (22).

**MiRNA biogenesis**

MiRNAs are either co-expressed with the host gene transcript utilizing promoters of the host protein-coding genes or as stand- alone primary transcripts located in inter- genic regions of the genome using their own promoters (23). This early primary (pri)-miRNA transcript is processed se- quentially by the ribonuclease-III enzyme Drosha and the double stranded RNA- binding protein DGCR-8 into a short hairpin precursor (pre)-miRNA, and thereafter exported to cytosol by exportin-5 (1, 24, 25). Pre-miRNA is digested further into small RNA molecules by endonuclease Dicer followed by selective incorporation of one or both strands of duplex miRNA into Argonaute family protein leading its assembly into the RNA induced silencing complex (RISC). MiRISC protects and sta- bilizes mature miRNA strand against RNase and guides miRNA to its target mRNA (26).

MiRNAs are not subjected to translation but modulate the translation of other pro- tein coding genes by recognition of binding sites on their 3’ untranslated regions (UTR) harboring a sequence (2–8 nt) comple- mentary to miRNA seed sequence (27). Binding of miRNA-mRNA 3’UTR follows the Watson-Crick base pairing but does not mandates full complementarity (27). The complete homology usually initiates degradation of target mRNA and therefore results in on/off switching of functional proteins which is commonly seen in lower vertebrates than in mammals where the partial complementarity leads to the trans- lational repression exerting a fine-tune control over the functional protein ex- pression (28, 29).

This moderate control on gene expression by miRNA suggests a wide scope of miRNA-mRNA interactions following either single miRNA- many targets or many miRNAs- single target pathways of post- transcriptional regulation in living cells (29).

**Discovery of functionally active platelet miRNAs**

Expression of a huge number of platelet miRNAs (total 219) was first reported by Landry et al. in 2009 using locked nucleic acid (LNA) based microarray profiling (21). With advancement in miRNA detection techniques such as deep sequencing led to the discovery of 40 novel sequences, which has increased the total number of platelet expressed miRNAs to more than double of the initial findings, i.e. in total 490 as reported recently by Ple et al. (30). In addition to mature miRNAs, detection of pre-miRNA transcripts for some abundant platelet miRNAs (miR-223, let-7c and miR-19a) as well as presence of functional core components of miRNA effector complex i.e. Dicer and Ago2 suggested that partial biogenesis of mature miRNA from pre- miRNA templates could take place directly in platelets (21). Imprecise processing of platelet miRNAs by Drosha and/or Dicer may also occur in platelets generating nu- merous miRNA isomiRs for example expression of 5’ shifted isomiR transcripts with distinct miRNA targeting capabilities has been demonstrated by Ple et al.

The ability of human platelet miRNAs to mediate gene specific silencing was examined by incubating 32P-labeled miR-223 sensor transcript with platelets or megakaryocyte extracts in separate RISC activity assays. Ago2 association with endogenous mature miRNA- miR-223 was confirmed in northern blotting of platelets, Ago2 immunoprecipitation and finally the regulatory function of the Ago2-miR-223 complex was validated by investigating the gene expression of P2Y12 in platelets. MiR-223 pairing with purinergic receptor P2Y12 3’UTR leads to repression of gene expression in reporter gene assay perform- ed in miR-223 null HEK-293 cell lines, in which mutation of miR-223 binding site disrupted miR-223:P2Y12 pairing (21). Platelet surface expression of P2Y12 recep- tor is highly increased upon their activa- tion influencing the platelet functions and also serves as a target receptor for new anti-platelet drugs (31–33). These studies confirm functionally efficient platelet miRNA system to regulate platelet proteins important for their activation.

**Platelet derived miRNAs affect platelet function**

The role and importance of miRNA in pla- telet function is gradually emerging with progress in miRNA research techniques. Platelet activation, adhesion and aggre- gation are essential for coagulation physi- ology and tissue repair. Hyper-reactivity of platelets symbolizes a risk of thrombolic conditions such as myocardial infarction, peripheral artery disease and stroke, whereas platelet hypo-reactivity indicates risk of haemorrhagic disorders (12). In ab- sence of transcriptional control, differences in platelet miRNA profiles during platelet activation and various pathological condi- tions may arise due to their

- partial biogenesis from pre-miRNAs,
- loss in microparticles secretion,
- change in stability upon activation or posttranscriptional regulation of miRNA transcripts such as terminal adenylation or uridylation (30,34).

A role of miRNAs in platelet reactivity was first addressed among healthy individuals demonstrating hyper- or hypo-reactivity of platelets (35, 36). Elevated RNA and pro- tein levels of VAMP8, a miR-96 predicted target and critical component of granule exocytosis were observed in hyper-reactive platelets (35). Dose dependent decrease in VAMP8 mRNA and protein upon miR-96 overexpression in VAMP8 expressing cell-
lines further confirmed miR-96:VAMP8 relation.

Likewise, miRNA-mRNA co-expression profiles of leukocyte depleted platelets and platelet reactivity was reported by Nagalla et al., where 74 miRNAs were differentially expressed in subjects grouped according to epinephrine induced experimental platelet reactivity response (36). Negative correlation among miR-200b:PRKAR2B, miR-495:KLHL5 and miR-107:CLOCK was validated by in-vitro miRNAs mediated gene knockdown approach. Moreover, differences in miRNA signature in resting and thrombin stimulated human platelets has also been investigated using qPCR coupled with an annotation network for predicted target genes. MiR-15a, miR-339–3p, miR-365, miR-495, miR-98 and miR-361–3p were differentially regulated in thrombin activated platelets (37).

Post-transcriptional modification of miRNA transcripts by terminal uridylation and adenylation may affect the stability or target recognition of miRNAs (38). In vitro uridylation and adenylation assays indicate capability of platelets to uridylate single stranded miRNAs, which correlated with Uridyltransferase enzyme TUT-4 expression in the platelets. MiRNA terminal adenylation activity was not significant. However, other endogenous small RNAs could be adenylated in platelets (30). These studies unveiled a potential link between miRNA system and platelet functionality to provide a platform for yet undiscovered facts of platelet physiology.

**Extra-platelet effects of platelet derived miRNAs**

Platelet microparticles released upon platelet activation are rich in variety of effector proteins and contain RNAs that may exert extraplatelet effects (34). As RNase, abundant in circulating blood, rapidly degrades RNA in plasma, packaging in microparticles renders protection against degradation and therefore stabilizes miRNAs for their safe transport to the site of action. Conventional plasma preparations contain substantial numbers of microparticles of platelet, leukocytes and endothelial origin containing large numbers of specific miRNAs, which may be associated with cardiovascular disorders. MiRNA profiles of microparticles differ from their source cells, indicating a selective packaging of miRNAs from cells to microparticles (34).

Therefore, platelet-secreted miRNAs contributing to the plasma miRNA pool may serve as a potential source of novel biomarkers associated with various pathological conditions (39).

A recent report on miRNA recovery from platelet derived microparticles in plasma suggests platelets as a delivery vehicle at the site of action in cardiovascular system (40). However, the contribution of platelet-secreted miRNAs in microparticles remains to be clearly defined, the application of network analysis may prove advantageous to design and collect further experimental evidences (39).

**Conclusion**

Platelets being anucleate cytoplasm bodies were underestimated in genomic research and thought to function by inherited ready made proteins. Differential platelet miRNA expression patterns have been highlighted in platelet functions and associated disorders since the discovery of miRNAs in platelets (41, 42) such as *overexpression of miR-28 in platelets derived from myeloproliferative neoplasm patients (42) and* increased levels of miR-340* and miR-624* in platelets of premature coronary artery disease patients (41).

Platelet miRNA research represents its own unique challenges such as e.g. tedious preparation of pure platelet concentrates from platelet rich plasma, insufficient clinical blood sample size for RNA recovery, lack of molecular mechanistic tools for gene expression modification in platelets (43). Nevertheless, with current progress in miRNA detection techniques and computational target-predicting algorithms, the platelet miRNA research represents an enormous scope for novel findings to unravel yet undiscovered molecular mechanisms regulating platelet physiology which may identify new therapeutic platelet or extraplatelet targets in thrombotic or occlusive cardiovascular disorders.

**Conflict of interest**

The authors declare, that they have no conflict of interest.

**References**

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