Platelet MicroRNAs: An Overview

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Abstract

MicroRNAs are short ~ 22 nucleotide non-coding RNA that have been found to influence the expression of many genes and cellular processes by either repressing translation or degrading mRNA transcripts. Platelet miRNA expression has been shown to be perturbed during ex vivo storage of platelets and in platelet associated disorders. While bioinformatics-based miRNA target predictions have been established, direct experimental validation of the role of miRNAs in platelet biology has been rather slow. Target prediction studies are nonetheless valuable in directing the design of appropriate experiments to test specific miRNA:mRNA interactions relevant to the underlying mechanisms of platelet function in general, and in disease as well as in ex vivo storage-associated ‘storage lesions’ (SL), a collective term used to include physiological, biochemical and morphological changes that occur in stored platelets. This brief review will focus on emerging human platelet miRNA studies to emphasize their potential role relevant to transfusion medicine field in terms of regulating platelet signaling pathways, markers of platelet associated disorders, and remote impactors of gene expression (intercellular bio-modulators) as well as potential platelet quality markers of storage and pathogen reduction treatments.

Keywords: MicroRNA, blood disorders, platelets, signaling pathways.

Introduction

MicroRNAs (miRNAs) are endogenously expressed small non-coding RNAs involved in post-transcriptional regulation of cellular gene (mRNA) expression [1-3]. It is estimated that over 60% of human protein-coding genes are regulated by miRNAs [2]. Being small in size, miRNAs are relatively stable molecules and easily quantifiable. Recent technical developments in the field have allowed researchers to measure the absolute and relative levels of many cellular miRNAs that further led to the recognition of their prognostic value as biomarkers [4-7]. Subsequently, this concept has further evolved and differential profiling of miRNAs as a tool to identify changes (i.e. biomarkers) in a normal 'biological entity' vs. its testing cohort, has emerged. The 'biological entity' could be a human, an animal, or a cell in culture etc. The testing cohort could be a genetic variant or a disease state, a drug or biologic treatment, , an infection state, radiation, or ex vivo stored human cells for therapeutics. Example of ex vivo stored cells includes concentrated human platelets, which are the most important transfused therapeutic products used in trauma, surgery and profusely bleeding patients [8]. For many years, investigators have studied the physiological and biochemical changes that occur in ex vivo stored platelets, which are collectively known as storage lesions (SLs). Since human mature platelet is enucleated, in order for it to be alive and functional, especially in ex vivo storage, and to regulate all important physiological processes, the platelet has to utilize its post-transcriptional regulatory machinery available in the cytoplasm. One such mechanism is miRNA based regulation of mRNA. Research on how miRNAs participate in the regulation of platelet mRNA expression is the key towards understanding the molecular mechanisms of stored platelets that could facilitate enhancements to the quality and shelf-life of ex vivo stored platelets.

This brief review will provide an overview on emerging trends in human platelet miRNA studies and the potential directions these studies will take in the near future to address a range of issues such as miRNA-based regulation of platelet signaling pathways, miRNA as markers of platelet associated disorders, and miRNA as remote impactors of gene expression (intercellular bio-modulators) as well as potential platelet markers of quality in storage and pathogen reduction treatments.

MicroRNAs as potential regulators of signaling pathways in platelets

Human platelets are generated from hematopoietic stem cells through a process known as hematopoiesis [9-11] and developmental differentiation of platelets from megakaryocytes is known as megakaryopoiesis. In these processes miRNAs have shown to play important roles as illustrated in Figure 1 [9-11, 15-18]. Different members of the cytoplasmic miRNA processing complex that includes proteins such as RNase III Dicer, Ago2 (Argonaute protein which is an essential component of the RNA-induced silencing complex aka RISC) and TRBP2 (TAR RNA binding protein 2) have been reported recently in platelets [19]. Although a functional cytoplasmic miRNA processing exists in platelets, the abundance of mature miRNAs over pre-miRNAs suggests that platelets inherit most of its mature miRNAs directly from the megakaryocytes. The observation of abundance of miRNAs in platelets relative to mRNAs suggests that the miRNA:mRNA ratio normally observed in nucleated cells of the hematopoietic system is significantly altered in platelets. In other words, a single mRNA can be targeted by multiple miRNAs. These observations support the idea that in a platelet, mRNAs are perhaps decorated at multiple sites by Ago2-miRNA complexes resulting in rendering a stronger mRNA repression than in other cells [19]. In platelets, more than 75% of the expressed miRNAs were

found to be predominantly from only five miRNA families which includes let-7, miR-199, miR-103, miR-25 and miR-140 [20]. And, among these 5 families, the members of let-7 and miR-103 are also in the list of top 30 highly expressed platelet miRNAs [21], which clearly suggests their key role in various biological processes of platelets. Another miRNA, miR-326 was markedly increased in platelet storage and was shown to directly target and inhibit anti-apoptotic Bcl-xL gene (i.e. its mRNA) [22, 23]. We have previously shown that two miRNAs, let-7b and miR-16, distinctly demonstrated an increasing trend, while miR-7 and miR-145 showed a decreasing profile during platelet storage [24]. Our previous observation of miR-16 increase during platelet storage and the increase of miR-326 in storage reported by another group [22], both of which target the genes involved in apoptosis, provides further circumstantial support that platelet apoptosis is regulated by miRNAs.

Recently we have profiled both miRNAs and mRNAs in ex vivo stored platelets at different days of storage to examine storage-associated changes in mRNA levels and correlated them with differentially expressed miRNAs. The data analysis was performed using Ingenuity Pathway Analysis (IPA) and identified top canonical pathways in which potential genes (mRNAs) that are targeted by miRNAs were uncovered (Table 1). Some of these pathways such as those involved in ‘Actin Cytoskeleton Signaling’ and ‘Integrin Signaling’ pathways that we identified in our analysis here were also previously identified by others [25] as important players of platelet biology which lends support to our observation. Furthermore, it is known that Ras-related protein 1 (Rap 1) signaling pathway increases during platelet storage and inhibition of Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) was shown to reduce Rap 1 activity and concomitant improvement in platelet integrity and quality [26]. Through the network analysis of potential miRNA targets, we identified a set of 28 mRNAs that were implicated in PI3K

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4 signaling pathway (Figure 2), which again argues in favor of the idea that miRNAs do play
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6 important roles in platelet biology.
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10 11 **MicroRNAs as potential markers of platelet associated disorders** 12 13

14 The first human platelet miRNA profiling was reported as part of a study testing for
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16 differentially expressed miRNA in polycythemia vera patients and found that miR-26b was
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18 significantly higher in these patient platelets compared to healthy controls, which has opened the
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20 field towards identifying miRNAs as potential biomarkers of platelet disorders [27]. Platelet
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22 reactivity is another disorder in which miR-96 was shown to downregulate vesicle-associated
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24 membrane protein 8 (VAMP8) mRNA expression and the protein levels [28]. In atrial fibrillation
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26 (AF) associated with poor prognosis in heart failure (HF) patients , platelets contribute to the
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28 prothrombotic state and in a recent study, miR-150 expression levels in platelets evaluated from
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30 systolic HF patients with AF were found to be significantly reduced and correlated to the cell-
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32 free circulating levels of this miRNA [29]. This suggests that perhaps for this disease, circulating
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34 miR-150 levels could serve as a surrogate marker. In sickle cell disease (SSD), increased platelet
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36 activation contributes to a state of hypercoagulability and thereby increasing the risk of
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38 thrombotic complications where 40 miRNAs were implicated in the pathogenesis of SSD in
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40 which three distinct miRNA families (hsa-miR-154, hsa-miR-329 and hsa-miR-376) were down
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42 regulated when compared to healthy controls [30]. This is another example where miRNAs
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44 appears to be useful as markers of platelet-associated disease. In megakaryocytes and platelets,
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46 miR-223 is the most abundant miRNA and one of the genes (mRNA) regulated by this miRNA is
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48 the ADP receptor P2Y₁₂, a key target for current antiplatelet therapy. Human P2Y₁₂ mRNA
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50 3'UTR has one miR-223 target site, a requirement for miRNA based gene regulation. Many
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4 studies have demonstrated favorable trend towards a regulatory function of miR-223 in several
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6 clinical disorders, some of which include platelet associated disorders such as high on-treatment
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8 platelet activity (HTPR). HTPR is a strong predictor of major cardiovascular events (MACEs) in
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10 coronary heart disease (CHD) patients receiving antiplatelet treatment, suggesting the value of
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12 presumably platelet derived miR-223 as a diagnostic tool for HTPR in clinical settings [31].
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19 **Platelet microparticle (MP)-derived miRNAs as *in vivo* bio-modulators**

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21 Earlier studies on circulating MPs suggest that most of these MPs are platelet derived
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23 microparticles (PMPs) [32] and PMPs contain various cytoplasmic components including
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25 miRNAs [33]. PMPs act as inter-cellular carriers to deliver and/or transfer genetic information
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27 from one cell to the other across the circulatory system and modulate cellular gene expression,
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29 vascular homeostasis and inflammation [32-39].
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33 One of the miRNAs identified in PMPs, the miR-126, is known to mediate vascular
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35 repair and progenitor cell recruitment to maintain vascular homeostasis [35, 39]. Another
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37 miRNA, miR-223, while abundant in platelets and in PMPs, it is scarce in endothelial cells.
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39 When human umbilical vein endothelial cells (HUVEC) were incubated with PMPs containing
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41 Ago2-miR-223 complex, it was observed that in 6 hours, the expression of two endothelial genes
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43 (mRNAs), the FBXW7 and EFNA1 that contain miR-223 binding sites in their 3' UTR was
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45 significantly down-regulated and so was their protein expression in 18 hours and 96 hours,
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47 respectively [40, 41]. These observations further generate a testable hypothesis that when stored
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49 platelets containing substantial amount of PMPs are transfused, a variety of miRNAs trapped in
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51 PMPs are also transferred to the recipient and modulate the gene (mRNA) expression in the
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53 recipients. Testing this hypothesis first in preclinical animal studies and subsequently through
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prospective clinical trials will unravel the long-term consequences if any, of the stored platelet derived PMP-miRNAs in transfusion recipients.

MicroRNAs as potential platelet markers of storage and pathogen reduction treatments

While ex vivo stored platelets at 22°C-24°C (room temp) with continuous gentle agitation do well in transfusion recipients, both the platelet storage solutions and the storage temperature are conducive to bacterial growth. As a result, often undetectable bacterial contamination (i.e. residual) that occurs during collection from the donor and downstream processing has the potential to grow rapidly. This is an added risk for sepsis in patients, especially those with impaired immune systems. Several different techniques are available to reduce the bacterial burden in stored platelets. The two most common methods used for pathogen reduction (PR) are Mirasol Pathogen Reduction Technology (PRT) (riboflavin + ultraviolet-B [UVB] light) [42] and Intercept Blood System (amotosalen + ultraviolet-A [UVA] light) [43]. We have previously reported that among 52 selected apoptosis associated miRNAs, a few are differentially expressed during various time points of platelet storage which suggested that miRNA profiles do respond to platelet treatments (stored vs. fresh) and therefore, selected differentially expressed platelet miRNAs have the potential to serve as storage biomarkers of quality [24]. In line with this concept, recently Osman et al have reported changes in platelet miRNAs associated with different PR treatments relative to respective controls [44], which further suggests that miRNAs of platelets have the potential to be the markers for a given context.

Conclusions and future prospects

Within the last five years, identification and evaluation of platelet-associated miRNAs in different areas related to platelet function has expanded our understanding of molecular mechanisms underlying some of the platelet functions. The examples we provided in this brief review clearly suggests that the platelet miRNA as a field has crossed the threshold of mere predictions through bioinformatics about what these tiny RNAs may or may not do functionally, to a stage where we can rely on miRNA with respect to unravelling many poorly understood events of platelets in storage, such as platelet storage lesions. Such studies should help facilitate improvements to platelet quality in storage for better patient outcomes following transfusion as well as enhancements to their storage shelf-life beyond current 5-day expiration.

Disclaimers

None.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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Table 1. Top canonical pathways identified by Ingenuity pathway analysis.

Top Canonical Pathways	p-value	Ratio	Genes
Day 5			
Amyloid Processing	9.21E-04	2/61 (0.033)	PRKACB, CSNK2A1
Putrescine Biosynthesis III	1.77E-03	1/4 (0.25)	ODC1
IGF-1 Signaling	3.28E-03	2/107 (0.019)	PRKACB, CSNK2A1
NF-κB Signaling	1.01E-02	2/181 (0.011)	PRKACB, CSNK2A1
RAR Activation	1.05E-02	2/195 (0.01)	PRKACB, CSNK2A1
Day 9			
Actin Cytoskeleton Signaling	1.02E-12	32/242 (0.132)	RAF1, MPRIP, PDGFA, ARPC5, EGF, SSH1, SLC9A1, PDGFC, ROCK2, PTK2, SHC1, DIAPH1, FGF18, MAP2K2, FLNA, EZR, MAP2K1, ARHGEF12, GRB2, CSK, CRKL, GNA12, ITGA2, RAC1, GSN, ROCK1, PIP5K1C, CYFIP1, MYH9, WASF2, ACTN4, PIP4K2A
Integrin Signaling	9.2E-11	28/208 (0.135)	RAF1, FYN, RAP2A, MPRIP, ARPC5, ILK, ITGB3, PTK2, NCK2, SHC1, AKT1, MAP2K2, MAP2K1, VASP, SRC, GRB2, ASAP1, CRKL, RALB, ITGA2, RAC1, TNK2, ROCK1, ARF1, ARF3, LIMS1, ACTN4, FNBP1
Ephrin Receptor Signaling	1.6E-10	26/210 (0.124)	RAF1, FYN, AXIN1, PDGFA, ARPC5, GNB5, EGF, PDGFC, GNB1,

			ROCK2, PTK2, NCK2, SHC1, AKT1, MAP2K2, ATF4, MAP2K1, SRC, GRB2, CRKL, GNA12, ITGA2, RAC1, GNAZ, ROCK1, ACP1
Molecular Mechanisms of Cancer	1.15E-09	36/388 (0.093)	RAF1, FYN, RAP2A, AXIN1, CTNNA1, PTK2, TGFBR2, CASP6, SHC1, AKT1, MAP2K2, ARHGEF3, MAP2K1, RALGDS, PRKCA, SRC, ARHGEF12, PRKCQ, CASP3, GRB2, GNA12, ADCY3, RALB, RAC1, GNAZ, RALBP1, FADD, BCL2L1, CBL, FOXO1, IRS1, CDKN1A, ARHGEF18, MAP2K3, DIABLO, FNBP1
VEGF Signaling	1.18E-09	18/109 (0.165)	EIF1AY, SRC, RAF1, PTPN6, GRB2, PDGFC, ROCK2, PTK2, ROCK1, SHC1, BCL2L1, AKT1, FOXO1, MAP2K2, FOXO3, ACTN4, MAP2K1, PRKCA

Figure 1

Figure 2