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Regulation of the Genetic Code in Megakaryocytes and Platelets

Matthew T. Rondina and **Andrew S. Weyrich**

The Molecular Medicine Program and Department of Internal Medicine, University of Utah, Salt Lake City, Utah, USA

Summary

Platelets are generated from nucleated precursors referred to as megakaryocytes. The formation of platelets is one of the most elegant and unique developmental processes in eukaryotes. Because they enter the circulation without nuclei, platelets are often considered simple, non-complex cells that have limited functions beyond halting blood flow. However, emerging evidence over the past decade demonstrates that platelets are more sophisticated than previously considered. Platelets carry a rich repertoire of messenger RNAs (mRNAs), microRNAs (miRNAs), and proteins that contribute to primary (adhesion, aggregation, secretion) and alternative (immune regulation, RNA transfer, translation) functions. It is also becoming increasing clear that the "genetic code" of platelets changes with race, genetic disorders, or disease. Changes in the "genetic code" can occur at multiple points including megakaryocyte development, platelet formation, or in circulating platelets. This review focuses on regulation of the "genetic code" in megakaryocytes and platelets and its potential contribution to health and disease.

Keywords

megakaryocytes; platelets; genes; human

Introduction

Despite lacking nuclei, there is growing evidence that platelets have complex molecular signatures that influence their function in health and disease. This includes a rich portfolio of mRNAs and miRNAs. We are just beginning to understand the importance of RNAs in platelets, and how the megakaryocyte contributes to mRNA and miRNA expression patterns in circulating platelets. This review focuses on regulation of the genetic code in megakaryocytes and platelets where the genetic code is defined as: transfer of genetic information in the form of mRNAs and miRNAs from megakaryocytes to platelets.

Address for correspondence: Andrew S. Weyrich, PhD, Professor of Internal Medicine, Eccles Institute of Human Genetics, Bldg 533, Rm 4220, University of Utah, Salt Lake City, Utah 84112, USA, andy.weyrich@u2m2.utah.edu.

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Megakaryopoiesis and Thrombopoiesis: Key Determinants of the Genetic Code

Megakaryocytes descend from pluripotent stem cells and undergo multiple DNA replications without cell divisions by a unique process referred to as endomitosis [1–2]. The process of megakaryocyte maturation and differentiation is called megakaryopoiesis [3–4]. Committed megakaryocytes eventually produce hundreds if not thousands of circulating platelets. Thrombopoiesis is the term ascribed to the generation of platelets (i.e., thrombocytes) [4–5]. Although the exact duration of the combined processes *in vivo* are not known, megakaryopoiesis and thrombopoiesis occurs over a two-week period in *ex vivo* human culture systems[6]. Although shorter in duration, the process of megakaryopoiesis and thrombopoiesis in murine fetal-liver cell-derived megakaryocytes is similar to humans [7].

Megakaryopoiesis and thrombopoiesis are controlled by multiple cytokines, although thrombopoietin (TPO) is the key regulator of megakaryocyte maturation and proliferation[8]. Different matrices and exposure to flow also regulate proplatelet formation [9–13]. Genetic studies indicate that transcriptional responses driven by GATA-1 and Nuclear Factor-Erythroid 2 (NF-E2) control megakaryocyte development and differentiation [1, 14–16]. Transcription of mRNA and subsequent translation of the message into protein is essential for producing requisite cytoskeletal, granule, intracellular signaling, adhesion, and other proteins that are packaged into platelets[1]. These proteins guide and control the primary functions of platelets, which involve thrombus formation and cessation of blood flow.

A primary thrust of investigations focused on megakaryopoiesis and thrombopoiesis has been to identify culture mediums, cytokine cocktails, and extracellular matrices that yield high numbers of platelets with morphologic characteristics and functions similar to platelets that are freshly-isolated from the bloodstream. Results from these studies indicate that slight alterations in the *in vitro* culture conditions and/or constituents can significantly influence the generation and phenotype of newly-formed platelets[17–23]. This strongly suggests that changes in the bone marrow milieu can alter transcriptional, translational, and post-translational processes throughout megakaryocyte development, differentiation, and proplatelet formation. It also infers that megakaryocytes transfer unique genetic codes to platelets that are fluid, especially in disease situations.

The Genetic Code of Megakaryocytes

As described above, megakaryopoiesis and thrombopoiesis are complex processes where gene expression is under strict regulatory control[1]. As a result, there is ample opportunity for the genetic code to change as progenitor cells differentiate into mature megakaryocytes that produce platelets. Unfortunately, the exact genetic code of megakaryocytes is not known because megakaryocytes constitute less than 1% of all marrow cells[1]. Thus, it is extremely difficult to isolate sufficient numbers of purified bone marrow megakaryocytes, especially from human subjects. To overcome these challenges, investigators utilize immortalized or primary progenitor cell culture systems to study proplatelet-producing

megakaryocytes. These culture systems are designed to reflect the *in vivo* situation as much as possible, but results need to be viewed with caution since *in vitro* megakaryocyte cultures do not completely replicate the bone marrow milieu.

Studies in cultured systems do indicate that megakaryocytes transcribe thousands of mRNAs and express a diverse and rich repertoire of miRNAs, which regulate platelet biogenesis (reviewed by Edelstein and colleagues[24–25]). RNA expression studies in cultured megakaryocytes have several features including: proteins known to be present in platelets are generally detected at the mRNA level in megakaryocytes[26]; differentiated megakaryocytes have remarkably different mRNA expression patterns than non-megakaryocytic cells[27]; RNA expression patterns are fluid throughout megakaryocyte development and platelet biogenesis[28–29], and; by inference, RNAs present in megakaryocytes are thought to be transferred to circulating platelets[30].

mRNA and miRNA expression profiling in cultured megakaryocytes has primarily been used to identify key genes that regulate megakaryopoiesis and thrombopoiesis. Numerous studies have shown that miRNA regulation of mRNA stability and translation is critical for megakaryopoiesis and megakaryocyte differentiation [24–25]. However, very few studies in megakaryocytes have attempted to dissect how mRNAs and miRNAs are shuttled into platelets. Cecchetti et al.[26] has shown that proplatelet producing megakaryocytes differentially sort mRNAs for matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) into platelets. The mechanisms by which MMP and TIMP mRNAs are differentially transferred to platelets, however, remain unknown. There is also a paucity of studies examining whether changes in the megakaryocyte milieu alters the types and amounts of RNAs that are transferred to platelets.

In summary, the genetic code of megakaryocytes is incompletely understood and just recently being appreciated. However, there is strong circumstantial evidence that megakaryocytes transfer a rich and diverse repertoire of mRNAs and miRNAs to circulating platelets and that this transferable genetic code changes in disease (see below).

Megakaryocyte Investment of the Genetic Code into Platelets

It is becoming increasing more clear that variations in race, genetic mutations, and acute disease affect the types of transcripts transferred from megakaryocytes to platelets. Changes in the megakaryocyte genetic code are thought to be reflected in the platelet transcriptome (Figure 1). In regards to race, platelet miRNA levels, which are capable of destabilizing mRNA, have recently been shown to inversely correlate with the expression of target transcripts in black subjects [31–32]. In the Platelet RNA and eXpression-1 (PRAX1) study, Edelstein and coworkers[31] found that a number of platelet mRNAs and miRNAs were differentially expressed in black versus white subjects. One of these mRNAs encoded for phosphatidylcholine transfer protein (*PCTP*, a regulator of lipids). Compared to white subjects, black subjects had significantly higher expression of PCTP mRNA and protein, which correlated with increased PAR4-mediated platelet aggregation. These investigators also identified that platelets isolated from black subjects had a network of differentially expressed miRNA-mRNA pairs, compared to white subjects. Prediction analyses and

subsequent validation identified one of these miRNAs as *miR-376c*. *miR-376c* targets the PCTP gene, was inversely correlated with PTCP mRNA and protein levels, and was inversely correlated with PAR4 reactivity in humans. Further work identified the locus mapping to chromosome 14q32.2, which contained a large cluster of miRNA genes (commonly called the *DLK1-DIO3* genomic region), the expression of which was significantly associated with race (black versus white race, $P = 1.09 \times 10^{-5}$), even after controlling for potential covariates. Follow-up studies by this same group of investigators also identified that platelets from blacks had 3 common single nucleotide polymorphisms in the PAR4 gene that were correlated with PAR4-induced platelet aggregation[32]. Together these data highlight that the genetic code of platelets – and presumably megakaryocytes, although this remains to be demonstrated – differs between black and white subjects and influences key platelet functional responses such as aggregation.

Inherited gene mutations in the megakaryocyte also contribute to an altered genetic code in released platelets. In this regard, mutations in just one gene may have profound effects on other mRNAs (and their protein products and associated functions) in megakaryocytes and platelets. One example in humans is gray platelet syndrome (GPS), for which the causative mutation was recently identified[33]. GPS, an autosomal recessive inherited platelet disorder, is characterized by thrombocytopenia and large platelets that lack α granules. Kahr et al.[33] used next-generation genome-wide RNA sequencing (RNA-seq) of platelets to identify that mutations in the *NBEAL2* are responsible for GPS. These RNA-seq studies also revealed that several transcripts (>100) were differentially expressed in platelets from GPS patients compared to platelets from unaffected individuals. The platelet transcriptome is also altered in patients with germline mutations in ETV6 (ets variant 6) that have thrombocytopenia and leukemia predisposition [34]. Together, these studies in humans suggest that targeted deletion of genes in mice is likely to have marked effects on the genetic code of megakaryocytes and platelets and their functional responses. They also raise the possibility that mouse models may be used to produce genetically-modified platelets through engineering of the genetic code.

Acute and chronic diseases also alter the platelet genetic code. Seminal work by Healy and colleagues[35] identified significant quantitative differences in the platelet transcriptome between patients with acute ST-segment-elevation myocardial infarction (STEMI) and patients with stable coronary disease. Specifically, these investigators found that by profiling platelet mRNA, they could identify that the expression of *CD69* (involved in platelet aggregation) and myeloid-related protein-8 (*MRP-8/14*, involved in calcium signaling, arachidonic acid metabolism, and cytoskeletal reorganization) was significantly increased in isolated platelets from STEMI patients. These findings suggested that the platelet transcriptome is dynamic and predicts functional alterations of clinical significance.

Another human disease state where the platelet transcriptome appears to be altered is in patients with systemic lupus erythematosis (SLE). SLE is an autoimmune disease known for injurious inflammatory responses in both solid organs and the circulatory system. Much of this inflammation is thought to be driven by an ongoing type I interferon response. SLE is also associated with an increased risk of cardiovascular disease and atherothrombosis. Recent work by Lood and colleagues[36] used real-time PCR to investigate whether

platelets isolated from patients with SLE displayed a type I interferon (IFN) gene signature. These investigators found that platelets from SLE patients, compared to healthy controls, had highly increased expression of many type I IFN-regulated genes. Among the genes validated in more detail, the mRNA expression of *CD69* in platelets from SLE patients was >6-fold higher relative to healthy controls. Expression of CD69 protein (and other type I IFN proteins) was also significantly increased in SLC patients compared to controls. Platelets from SLE patients also exhibited heightened platelet reactivity, as measured by binding of annexin V and the formation of platelet-monocyte aggregates, compared to platelets from control subjects [37–39]. In the immortalized megakaryocyte cell line MEG-01s, stimulation with IFNα led to significant increases in the expression of CD69 and other IFN-regulated proteins. Taken together, the authors concluded that platelets from SLE patients have a type I IFN transcriptional signature with increased levels of corresponding proteins, including CD69[36]. These findings also support the hypothesis that in SLE patients, circulating IFNα production by plasmacytoid dendritic cells may alter the genetic code of megakaryocytes within the bone marrow niche, leading to the production of platelets enriched for IFN-regulated proteins.

Body mass index and circulating levels of C-reactive protein (CRP) and interleukin-6 are also associated with changes in platelet gene expression [40–41], and there are many other published lines of evidence consistent with the supposition that human diseases are associated with changes in the genetic code of platelets[30, 42–45]. While an in-depth discussion of these established and emerging findings are beyond the scope of the current brief review, the reader is encouraged to keep an eye on this rapidly evolving area of investigation. It is also important to note that the aforementioned studies infer that differential transfer of mRNA occurs during proplatelet formation. While this may be true, there is evidence that tumor cells transfer RNA into platelets [46]. The study by Nilsson and colleagues [46] suggests that RNA profiling in blood platelets may serve as a diagnostic platform for cancer, but it is unknown if this type of RNA transfer has functional consequences in platelets.

Regulation of the Genetic Code in Circulating Platelets

When mRNAs reach platelets their structural features mimic their expression in megakaryocytes. In other words, they are of predicted length (i.e., not degraded) and each mRNA is capped and polyadenylated on the 5′- and 3′-untranslated region, respectively [47–50]. Platelet miRNAs also resemble miRNAs found in megakaryocytes and nucleated cells [51–52]. Surprisingly, platelets have functional machinery that allows them to splice pre-mRNAs into mature mRNAs and process pre-miRNAs into miRNAs [51, 53–54]. These unexpected splicing/processing functions provide anucleate platelets with alternative paths of gene regulation that can affect their genetic code.

RNAs are presumably fairly stable in platelets, but *in vivo* studies of RNA stability have not been performed. *In situ* hybridization studies from freshly-isolated human platelets suggest that all subpopulations of platelets express mRNA [53]. However, these studies are limited to select candidates and their qualitative nature does not distinguish whether mRNA

expression levels vary among platelet subpopulations (i.e., older versus younger platelets). These caveats need to be considered as one evaluates the genetic code of platelets.

Studies over the last 50 years have demonstrated that platelets are capable of translating a subset mRNAs into proteins and this list continues to grow. Translation of platelet mRNAs is reviewed in detail elsewhere [50, 55]. Recent studies not included in these reviews, however, demonstrate that dengue infected platelets synthesize interleukin-1β that is packaged into MPs and induces endothelial permeability [56]. Corduan and colleagues[57] also recently demonstrated rapid dissociation of Ago2 protein complexes from serpine1 mRNA, which codes for plasminogen activator inhibitor-1 (PAI-1) that is known to be translated by activated platelets [58–59]. This disassembly process is another exquisite example of platelets possessing unexpected, complex gene expression pathways.

Transfer of the Platelet Genetic Code to Target Cells

Although RNAs are typically considered intrinsic regulators of cellular function, emerging evidence suggests that mRNAs and miRNAs can be transferred from one cell to another. In this regard, several studies have demonstrated that platelets are capable of transferring RNAs to recipient cells (reviewed by Clancy and Freedman[60]). Risitano and colleagues [61] found that labeled RNA from platelet-like particles is transferred to leukocytes and endothelial cells, and this transfer alters gene expression patterns in the target cells. Using a green fluorescent-based system, they also showed that RNA from the platelet-like particles is translated by the recipient cells. This same group also demonstrated that platelet RNA transfer to leukocytes occurs *in vivo*.

In addition to mRNA transfer, platelets transfer miRNAs to endothelial cells. Laffont and coworkers[62] found that platelets transfer miR-223 to human umbilical vein endothelial cells (HUVECs). miR-233 was transferred in platelet microparticles (MPs), which contained a functional Argonaute-2 (Ago2)-miR233 complex that regulated the expression of a reporter construct in recipient HUVEC's. Similarly, Gidlöf et al.[63] found that platelets isolated from patients with myocardial infarction release specific miRNAs that can regulate endothelial cell gene expression. Gidlöf and colleagues also found that activated platelets can transfer fluorescently-labeled miRNA and endogenously released miR-39 to endothelial cells.

An important consideration is that intercellular transfer of platelet RNAs and proteins to recipient cells can occur via MPs. Given that the vast majority of circulating MPs are derived from platelets, and the numbers of MPs increases significantly in disease situations due to their increased uptake and/or decreased consumption, it is plausible that platelet MPs transfer significant amounts of genetic information to recipient cells [64–66]. This transfer has widespread implications for several fields, especially if the genetic code bestowed into MPs changes in disease situations.

Conclusion

There is no doubt that the basic functions of platelets are similar between individuals. However, it is becoming increasingly clear that platelets from different individuals and races

have distinct genetic codes that personalize and predict how they react and respond. Furthermore, variations in the platelet genetic code become more pronounced in acute and chronic disease situations. Identifying what these differences are, how they occur, and the means by which the platelet genetic code affects and/or causes human disease will be an exciting element of future studies. In addition, disease-specific interrogation of the megakaryocyte and platelet genetic code will undoubtedly reveal surprises that broaden our understanding of how circulating platelets perform in sickness and in health.

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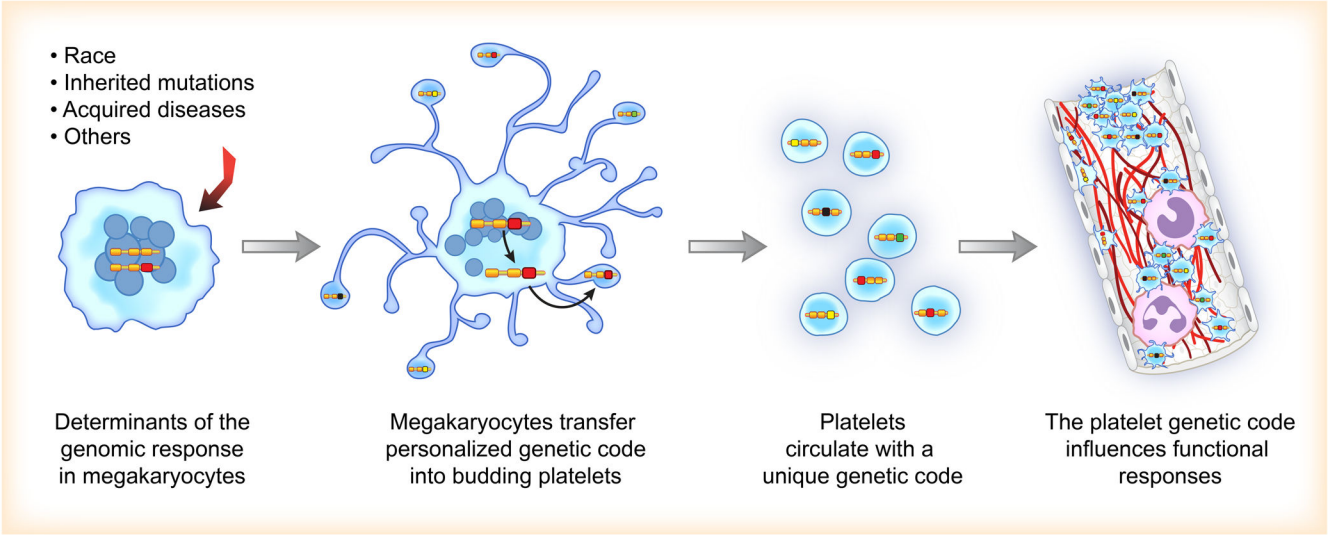

Figure 1. Megakaryocyte Investment of the Genetic Code into Platelets

Variations in race, genetic mutations, or acute disease are associated with changes in the genetic code (i.e., mRNA and miRNA expression patterns) in megakaryocytes. Changes in the megakaryocyte genetic code are then transferred into developing platelets that are released into the circulation. Alterations in the platelet transcriptome and corresponding proteins influence functional responses in both health and disease.