Recent Advances in Platelet Transcriptomics

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Key Words

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Summary

Platelets are anucleated cells that contain small amounts of mRNA of megakaryocyte origin and possess the capacity for signal-dependent pre-mRNA splicing. They have an important role in diverse hemostatic and pathological disorders, including vascular remodeling, inflammation and wound repair. Recent progress in high-throughput mRNA profiling techniques permitted first insights into the platelet transcriptome – a catalogue of all mRNA transcripts expressed in platelets. It is evident that platelets have a unique and reproducible molecular signature. Detailed analysis of the platelet transcriptome has resulted in the identification of many genes and proteins, previously unknown to be expressed in platelets. Furthermore, microarray analysis was applied to platelet disorders: i) as potentially a powerful tool for differential diagnosis, ii) to study the molecular basis of the disease and iii) to identify potential targets for therapy. In this review, we focus on recent applications of transcript profiling to platelet studies. We briefly review current approaches to transcriptome analysis and describe practical application of these approaches to investigate molecular mechanisms of essential thrombocythemia, gray platelet syndrome and polycythemia vera.

Schlüsselwörter

Thrombozyten · mRNA-Profilierung · Transkriptom ·
Thrombozyten-mRNA · Thrombozytenerkrankungen

Zusammenfassung

Thrombozyten sind Zellfragmente ohne Zellkern, die eine geringe Menge mRNA megakaryozytären Ursprungs enthalten und über die Fähigkeit zu signalabhängigem pre-mRNA-Splicing verfügen. Sie spielen eine wichtige Rolle in verschiedenen hämostatischen und pathologischen Erkrankungen, einschließlich vaskulären Veränderungen, Entzündungen und Wundheilung. Jüngste methodische Fortschritte des umfassenden mRNA-Profilings ermöglichen ersten Einsichten in das Thrombozyten-Transkriptom – einer Auflistung aller in Thrombozyten vorkommender mRNA-Transkripte. Es wurde eindeutig gezeigt, dass Thrombozyten eine einzigartige und reproduzierbare molekulare Signatur aufweisen. Eine detaillierte Analyse des Thrombozyten-Transkriptoms führte zur Identifizierung vieler Gene und Proteine, von denen bisher nicht bekannt war, dass sie in Thrombozyten auftreten. Des Weiteren wurde die Mikroarray-Analyse auf Thrombozytenerkrankungen angewendet: 1) als mögliches wichtiges Instrument zur Differenzialdiagnose, 2) zur Untersuchung der molekularen Grundlage der Erkrankung und 3) zur Identifizierung möglicher Ziele im Rahmen einer Therapie. In diesem Artikel konzentrieren wir uns auf neueste Anwendungen des Transkript-Profilings zur Untersuchung von Thrombozyten. Wir gehen auf neue Ansätze in der Transkriptom-Analyse ein und beschreiben den praktischen Einsatz dieser Methoden bei der Untersuchung molekularer Mechanismen der essentiellen Thrombozythämie, des Gray-Platelet-Syndroms und der Polyzithämie vera.

Introduction

Platelets play critical roles in normal hemostatic processes and pathologic conditions, such as thrombosis, vascular remodeling, inflammation and wound repair. Generated as cytoplasmic buds from precursor bone marrow megakaryocytes, platelets are anucleate and lack nuclear DNA although retaining small amounts of megakaryocyte-derived mRNAs [1, 2]. Platelets on average contain as little as 2×10^{-3} fg mRNA/cell (approximately 3–4 logs less RNA than a typical nucleated cell [3]), although younger platelets contain relatively larger amounts of mRNA, as determined by use of fluorescent nucleic acid dyes such as thiazole orange [4]. Platelets contain rough endoplasmic reticulum and polyribosomes and retain the ability for protein biosynthesis from cytoplasmic mRNA [5, 6]. Quiescent platelets generally display minimal translational activity, although newly formed platelets synthesize various α -granule and membrane glycoproteins, including GPIb and GPIIb/IIIa ($\alpha_{IIb}\beta_3$). Furthermore, stimulation of quiescent platelets by agonists, such as α -thrombin, increases protein synthesis of various platelet proteins, including Bcl-3 [7].

Some diseases originating from either inherited (both quantitative and qualitative) or acquired platelet disorders are characterized by the deficiency of key platelet-specific proteins [8, 9]. Better understanding of the molecular mechanisms of platelet pathology requires detailed studies of the entire pool of platelet mRNA transcripts and proteins. Until recently, low levels of mRNA in platelets made mRNA profiling analysis challenging. But advances in gene profiling technologies, coupled with the completion of the Human Genome Project, have permitted first insights into the platelet transcriptome – the catalogue of the entire population of mRNAs transcripts present in platelets. Historically, the platelet mRNA content was considered invariant, gradually declining with cell senescence, presumably at various rates for distinct transcripts. The traditional paradigm was re-configured with the discovery of signal-dependent pre-mRNA splicing in platelets [10]. This observation established that platelets possess essential spliceosome components, such as U1 70K protein and all U snRNAs, as well as select unspliced pre-mRNAs from megakaryocytes. Interestingly, these spliceosomes retain the unique ability of pre-mRNA splicing in the cytoplasm (as opposed to the typical nuclear location) – a functional capacity undescribed in any other type of mammalian cells. Specifically, platelets precisely excise introns from interleukin-1 β pre-mRNA in response to surface receptor activation. Signal-dependent splicing allows platelets to alter the pool of translatable messages from the same invariable pool of transcripts in response to cellular activation/stimulation. This discovery emphasizes the importance of accurate platelet transcript profiling – along with studies focusing on the correlation between mRNA abundance and protein expression – in order to optimally dissect complex processes of platelet function.

Fig. 1. Schema of microarray mRNA analyses. Two-color microarray analysis provides information on the differential expression of genes between two tissue samples. One-color microarray analysis measures gene expression profile from a single tissue and have enough built-in controls and standards to normalize data so that different arrays can be analyzed simultaneously. Both analyses include transcript labeling (either cDNA or cRNA), hybridization to the chip, washing, scanning and data processing.

Two complimentary techniques are predominantly used for mRNA transcript profiling – microarray and serial analysis of gene expression (SAGE). Microarray technology represents a ‘closed’ profiling strategy limited by the target genes imprinted onto gene chips. In contrast, SAGE is an ‘open’ architectural system that can be used to identify novel genes and to quantify differentially expressed mRNAs [11, 12]. Taken together, these techniques provide a panoramic definition of the entire pool of mRNA transcripts, constituting the transcriptome. In this review, we focus on the application of recent platelet transcriptome findings to the general understanding of molecular mechanisms of platelet function and provide perspectives of molecular diagnostics of platelet-associated diseases.

Approaches to Transcriptome Analysis (mRNA Profiling)

Microarray Analysis

Microarray analysis adapts artificially constructed grids of known DNA samples such that each element of the grid probes for a specific RNA sequence. These are then used to capture and quantify RNA transcripts [13]. Many different

protocols and types of microarray have been developed to date – oligonucleotide and cDNA arrays, commercially available whole-genome arrays and custom-made tissue-specific arrays. All of them require i) isolation of RNA from biological sample, ii) copying of RNA into either cRNA or cDNA, simultaneously incorporating either fluorescent nucleotides or a tag that is later used for fluorescent labeling, iii) hybridization to a microchip containing a grid of DNA probes, iv) washing, v) labeling (if necessary, depending on the protocol), vi) scanning under laser light, and vii) image processing, data extraction and analysis (fig. 1).

Both cDNA and oligonucleotide arrays are used in microarray experiments, either purchased commercially or generated for tissue-specific analyses. Irrespective of the approach, bioinformatic clustering tools are required for delineation of closely related expression patterns that might be controlled by common regulatory mechanisms. Oligonucleotide microarrays provide for an absolute signal intensity for a single sample at a specific point in time, whereas spotted cDNA microarrays provide comparative signal intensities between two simultaneously processed tissue sources. The sources for cDNA arrays are generally PCR products from expressed sequence tags (ESTs) or from cell (tissue)-specific sources, followed by robotic fabrication onto glass slides ('spotted cDNA microarray'). In contrast, the technology for oligonucleotide arrays has been pioneered commercially using a combination of photolithography and solid-phase DNA synthesis. The latter technology generates relatively short (25-mer) oligonucleotides specific for transcripts of interest, with sequence-specific mismatches (1-base pair/oligonucleotide) that serve as important internal controls to assess hybridization specificity and 'system noise'. In addition, concomitant use of multiple oligonucleotide primers encompassing different regions of the same transcript allow for more accurate and reliable profiling. Software algorithms compare the relative intensities of each transcript-specific signal, quantifying integrated signal specificities (perfect match) adjusted for non-specific hybridization (mismatch) to the transcript control. These average difference values, typically reported in logarithmic (base 2) transformations, generally reflect relative transcript abundance. Oligonucleotide arrays have been used to distinguish acute myelogenous leukemia from acute lymphoblastic leukemia, suggesting that transcript signatures may provide for novel, discriminatory methods for molecular classification [14].

In contrast, spotted cDNA arrays typically can be adapted for delineation of relative transcript abundance between two cellular sources (i.e. control and transformed cells). In this case, one mRNA population is labeled with one fluorophore (i.e. Cy3) and the other with a wavelength-restricted alternative fluorophore (i.e. Cy5), and both pools are competitively co-hybridized to the same slide for quantification of relative expression of individual transcripts. The utility of such an approach has demonstrated distinct mRNA profiles in patients with diffuse large B-cell lymphoma. Hierarchical clustering

Fig. 2. Schema of SAGE as an approach to mRNA transcript profiling. AE = anchoring enzyme, TE = tagging enzyme.

tools were used to sub-classify these lymphomas and to correlate gene expression patterns with disease outcome [15]. Similarly, gene expression profiling appeared to predict the outcome of young patients with breast cancer [16], suggesting that molecular classification may become a more broadly utilized technology for rational therapy of certain human diseases. Several different microarray systems are commercially available, including whole-genome microarrays from Affymetrix (Santa Clara, CA, USA), Applied Biosystems (Foster City, CA, USA), MWG-Biotech AG (Ebersberg, Germany) and others.

Serial Analysis of Gene Expression

Classical SAGE [11] relies on the observation that short (<10 bp) sequences within 3'-mRNAs can stringently discriminate among the approximately 30,000 genes comprising the human genome, assuming a random nucleotide distribution along a 9-bp stretch (4^9 bp = 262,244 random nucleotide combinations). SAGE is completed by i) isolation of RNA from biological sample, ii) preparation of a 3' end-specific cDNA library, iii) generation and concatenation of tags from the cDNA library using an anchoring enzyme, such as NlaIII, and a tagging enzyme, such as BsmFI, and iv) cloning of tag concatemers into a sequencing vector and performing automated sequencing (fig. 2). The sequence of each tag along with its positional location, uniquely identifies the gene from which it is derived, and differentially expressed genes can be identified in a quantitative manner since the frequency of tag detection

Table 1. Megakaryocyte and platelet microarray studies

	Gnatenko et al., 2003 [22]	Gnatenko et al., 2005 [45]	Bugert et al., 2003 [21]	McRedmond et al., 2004 [23]	Shim et al., 2004 [24]	Tenedini et al., 2004 [25]
Genes studied, n ^a	12,599	22,200	9,850	12,599	12,599	22,200
P/M ² , n	1,500–2,147	NR	NR	NR	NR	appr. 10,000
P, n	NR	appr. 1,668	appr. 1,526	2,928	NR	4,637 – 4,655
Arrays, n	3	5	6	1 in duplicate from 23 pooled donors	3	4 pooled (6 samples/array)
Cells studied	platelets	platelets	platelets	platelets	megakaryocytes	megakaryocytes
Cellular source	apheresis	apheresis	concentrates	blood (50 ml)	CD34+ ex vivo expanded	CD34+ ex vivo expanded

M = Marginal, NR = not reported, P = present, appr. = approximately.

^aNumber of probes (genes) represented on individual microarray slides.

reflects the steady-state mRNA level of the cellular transcriptome [11, 12] (www.sagenet.org). With relatively deep sampling (> 50,000 tags), genes expressed at low levels (< 0.01% of total mRNA) can be readily identified. Two limitations of the current SAGE technology are the relatively short (14 bp) and variable tag lengths caused by the tagging enzyme BsmFI not always cleaving a fixed distance 3' into the covalently attached cDNA, and the large amount of redundant DNA sequencing needed for a comprehensive analysis of low-abundant mRNA transcripts [11]. Modifications to the original protocols have been devised for i) generation of longer tags as a means of providing more definitive tag-to-gene identification, ii) efficient identification of low-abundant transcripts using subtractive SAGE techniques, and iii) amplification techniques to circumvent small mRNA starting material [17–19].

Quantitative Reverse Transcription PCR

Although quantitative reverse transcription PCR (Q-PCR) can be used to monitor expression levels of only a limited subset of transcripts and therefore cannot be applied for whole-genome mRNA profiling, it represents an invaluable tool for the validation of mRNA profiling findings. Q-PCR techniques have also been successfully used to characterize inherited platelet disorders (such as Glanzmann's thrombasthenia and Bernard-Soulier syndrome) and to identify platelet-specific alloantigens [9, 20].

Uncovering the Platelet Transcriptome

Microarray

The limited yield of RNA means that potential interference by contaminating leukocyte-derived RNA may bias transcript profiling approaches. Despite this technical concern, a limited

number of published microarray studies using megakaryocyte- or platelet-derived mRNAs have been described, with generally concordant agreements on transcript quantitation and gene expression patterns (table 1). Not surprisingly, platelets have less transcripts than those found in nucleated cells, ranging from 1,600 to 3,000 mRNAs [21–23]. In particular, platelets express much less transcripts than leukocytes [24, 25]. The small number of platelet-expressed transcripts represents the lack of ongoing transcription in the anucleate platelet. The quantitative differences between studies presumably reflect, in part, the methods of isolation.

Several well-characterized platelet transcripts are amongst the most abundant genes identified in the platelet transcriptome (i.e. platelet factor (PF) 4, glycoprotein 1b β), while numerous transcripts encoding proteins involved in cytoskeletal organization (actin, thymosin β 4, cofilin) are also readily identified [26] (table 2). A large percentage of genes with miscellaneous (25%) or unknown (32%) function as annotated by Affymetrix and RefSeq databases are also evident [27]. Thus, nearly one half of platelet genes and gene products presumably have important but poorly characterized functions in platelet and/or megakaryocyte biology. The mRNAs encoding the actin-related machinery are overrepresented in these analyses [22] – not unexpected, given prior estimates that 20–30% of the total platelet proteome is comprised of actin or actin-related proteins, such as myosin and talin [28]. In unstimulated platelets, 30–40% of actin is polymerized as F-actin, while the balance of G-actin monomers are polymerization-inhibited by sequestering proteins, such as profilin and thymosin β 4 [29]. The high thymosin β 4 transcript expression not only correlates with its known abundance in platelets, but supports the importance of actin inhibitory proteins in maintaining the non-stimulated state of circulating platelets. Genes involved in the immune response (chemokines, growth factors and human leukocyte antigen (HLA) proteins) are also evident, consistent with the role of platelets in inflammation and immunity [21]. Finally, unexpected transcripts encoding ery-

Table 2. Most highly expressed platelet transcripts (n = 50)

Platelet transcript ^a	Gene symbol
Platelet factor 4 (chemokine (C-X-C motif) ligand 4	PF4
H3 histone, family 3A	H3F3A
Rho guanine nucleotide exchange factor (GEF) 10	ARHGEF10
β-Actin mRNA	ACTB
H3 histone, family 3B	H3FB
β2-microglobulin	B2M
Neurogranin (protein kinase C substrate)	NRGN
Pro-platelet basic protein (chemokine (C-X-C motif) ligand 7	PPBP
Guanine nucleotide-binding protein G(s), γ-subunit	GNAS
Thymosin β4	TMSB4X
Ferritin, light polypeptide	FTL
Ferritin, heavy polypeptide 1	FTH1
Glutathione peroxidase 1	GPX1
Vinculin	VCL
Major histocompatibility complex, class I, B	HLA-B
Glycoprotein Ibβ	GP1BB
Major histocompatibility complex, class IC	HLA-C
Nuclear receptor coactivator 4	NCOA4
Ornithine decarboxylase antizyme 1	OAZ1
Protein tyrosine phosphatase, receptor-type O	PTPRO
Cofilin 1 (non-muscle)	CFL1
Chemokine (C-C motif) ligand 5	CCL5
Transgelin 2	TAGLN2
Coagulation factor XIII, A1 polypeptide	F13A1
Ribosomal protein L41	RPL41
SH3 domain binding glutamic acid-rich protein like 3	SH3BGR13
Clusterin (complement lysis inhibitor)	CLU
Nerve growth factor receptor-associated protein 1	NGFRAP1
Major histocompatibility complex, class I, E	HLA-E
Major histocompatibility complex, class I, A	HLA-A
Tumor protein, translationally-controlled 1	TPT1
Regulator of G-protein signaling 10	RGS10
SPARC/osteonectin mRNA	SPARC
Homo sapiens nuclear protein SDK3 mRNA	PNN
Monocyte to macrophage differentiation-associated	MMD
Hemoglobin β-gene	HBB
MutL homolog 3 (<i>Escherichia coli</i>)	MLH3
Ubiquitin C	UBC
Chromosome 21 open reading frame 7	C21orf7
Ubiquitin B	UBB
CD99 antigen	CD99
Spermidine/spermine N1-acetyltransferase	SAT
Glyceraldehyde-3-phosphate dehydrogenase	GAPD
Progesterone receptor membrane component 1	PGRMC1
Guanine nucleotide binding protein (G protein), gamma 11	GNG11
Myosin, light polypeptide 6, smooth muscle and non-muscle	MYL6
Nucleosome assembly protein 1-like	NAP1L1
Integral membrane protein 2B	ITM2B
Hemoglobin, α ₁ -gene	HBA1
Actin related protein 2/3 complex, subunit 1B	ARPC1B

^aTranscripts are rank-ordered using log₂ intensities of average-difference values from 5 normal donors normalized across individual arrays; data and methods updated from [22, 45].

throid genes (i.e. globin, ferritin) have been consistently identified in reported platelet microarray studies. While this may suggest reticulocyte contamination during platelet isolation, residual expression from precursor cells cannot be excluded. Similarly, histone transcripts (notably histone H2A, but also H3.3, H2B.1 and H2A.2) have been observed in transcriptome analysis of highly purified platelets, the significance of which remains unestablished [22].

As a means of better dissecting the molecular anatomy of the platelet, expressed genes grouped on the basis of assigned gene annotations have been used to provide a panoramic definition of the platelet transcriptome. Of the genes that could be catalogued within assigned clusters, those involved in metabolism (11%) and receptor/signaling (11%) represented the largest groups. Also evident in these analyses is the relatively large percentage of genes involved in functions unrelated to these key groups (i.e. miscellaneous, 25%), and the overrepresentation of genes with unknown function (32%) as annotated by Affymetrix and RefSeq databases [30]. These results identify a vast array (nearly one half) of platelet genes and gene products that presumably have important but poorly characterized functions in platelet and/or megakaryocyte biology.

Platelet SAGE

In addition to microarray analysis, limited platelet SAGE has been completed, initial studies cataloguing a total of 2,033 tags [22]. Interestingly, 1,800 tags (89%) corresponded to mitochondrial-derived genes. The unusually high preponderance of mitochondrial-derived genes is consistent with the known enrichment of these genomes in human platelets [31], and presumably reflects persistent transcription from the mitochondrial genome in the absence of nuclear-derived transcripts. In addition, preliminary observations indicate that nuclear-encoded mitochondrial transcripts are also highly expressed in platelets, suggesting that preservation of mitochondrial functions is crucial for platelet function [32]. The mitochondrial genome is a compact, approximately 16.6 kb sequence encoding 13 genes and 2 ribosomal RNAs. The overall distribution of platelet-derived mitochondrial SAGE tags is quite similar to that found in muscle [33]. Like muscle, platelets are metabolically adapted to rapidly expend large amounts of energy required for aggregation, granule release and clot retraction. Platelet mitochondria represent the primary source of adenosine triphosphate, and mitochondria are also responsible for most of the toxic reactive oxygen species generated as by-products of oxidative phosphorylation. The mitochondrial DNA (mtDNA) encodes polypeptides found within 4 of the 5 multifunctional complexes that regulate oxidative phosphorylation within the platelet mitochondria [34]. Whether the continued generation of these polypeptides has a role in platelet energy metabolism and/or the apoptotic mechanisms regulating platelet survival, remains speculative, although not incon-

Table 3. Platelet and leukocyte RNA yields from variable blood volumes

Blood, ml	Platelets		WBC	
	number	RNA, µg	number	RNA, µg
1	3×10^8	0.05–0.01	5×10^6	10–25
10	3×10^9	0.5–1	5×10^7	100–250
100	3×10^{10}	5–10	5×10^8	1,000–2,500
1,000	3×10^{11}	50–100	5×10^9	10,000–25,000

sistent with prior evidence that platelets retain a fully functional translational apparatus [7].

Given the small number of non-mitochondrial SAGE tags available for analysis ($n = 233$), limited conclusions can be drawn using traditional (non-subtraction) platelet SAGE libraries as presented here. Overall, a total of 126 unique tags have been identified, the majority of which (94) were represented only once. Of the genes with unique tags identified more than once, there was excellent concordance with microarray expression analysis, with nearly all of the SAGE tags corresponding to platelet top 75 microarray transcripts. The PF 4 variant represented a single aberration since this was rank-ordered approximately 350 by microarray, although its SAGE tag frequency was identical to that of the predominant PF4 transcript. Furthermore, a preponderance of the total unique tags was not represented on the microarray chip, thus highlighting the utility of two complimentary systems – open and closed gene profiling approaches.

Overcoming Technical Limitations of Platelet Gene Profiling

To date, microarray analyses are not standardized, with considerable inter-laboratory variability related to fundamental issues pertaining to platform type (i.e. spotted cDNA vs. oligonucleotide array), RNA extraction and labeling techniques, hybridization conditions and software algorithms for analysis of large datasets. Such issues hinder the exchange of microarray data among various investigators and create considerable challenges to the scientific community in terms of test sensitivity, specificity, reproducibility, reliability and accuracy. The issue of platform reproducibility and quality control may be addressed by the expanded use of commercially available slides (with built-in controls), although the high cost of individual slides creates a barrier for the extensive profiling required to define 'normal'. Thus, even for a microarray with 99% accuracy ($p < 0.01$), readouts of 10,000 data points would result in 100 false-positive signals by chance alone. Guidelines for experimental design, data reporting and analysis of microarray experiments have been set forth by the scientific community (www.mged.org), while regulatory issues pertaining to diagnostic testing using microarrays have been outlined [35]. In addition, reliable and reproducible transcript profiling imposes high standard on the purity of platelets and requires

excessive purification and leukodepletion. Coupled with exceptionally small RNA content in platelets, this limits wide-scale studies of platelet transcriptome in patients. Table 3 outlines the relative yield of platelet- and leukocyte-derived RNAs from various starting blood volumes, based on prior determinations of cellular RNA content [36]. Microarray experiments typically require an absolute minimum of 5 µg of total cellular RNA, although with appropriate controls and reproducibility issues, 20–100 µg requirements are more typical. As evident, nearly 1 l of blood would be required to ensure the availability of sufficient RNA for reliable studies, an amount that would be overwhelmed by leukocyte-derived RNA. Nonetheless, current methodologies entailing approximately 3-log leukodepletion result in sufficiently pure platelet mRNA for reliable interpretation of these experiments [22]. Given the importance of leukocyte depletion in transfusion medicine, it is likely that more efficient and stringent approaches will be developed in the foreseeable future. Finally, while sophisticated technologies, such as laser microdissection, have been described for removal of leukocytes, the general applicability of such methodology remains to be determined [3]. Several groups have devised modified protocols which require significantly less starting material, typically less than 100 ng of mRNA. Most of these procedures incorporate the known ability of Moloney murine leukemia virus reverse transcriptase to efficiently add several unencoded dC residues when it reaches the 5' end of the RNA. When cDNA synthesis is primed by oligo(dT) in the presence of a second oligonucleotide with a short stretch of dG residues at its 3' end, this second oligo anneals to the polyC extensions and provides a template for further cDNA extension by the reverse transcriptase [37]. The enzyme is therefore able to switch templates and incorporates the complement to the template switching (TS) oligonucleotide. In principle, every first-strand cDNA will now have a common sequence at its 3' end which is complementary to the TS sequence. Thus, a TS oligonucleotide can be used in combination with a biotinylated oligo(dT) primer to PCR-amplify the cDNA, allowing much smaller amounts of starting mRNA to be utilized. Comparable adaptations have been used to generate high-quality cDNA for microarray analysis starting from as little as 1 pg of total starting RNA isolated from 9 human oocytes [38]. When combined with methods for high-fidelity in vitro transcript amplification using T7 RNA polymerase, it is likely that platelet analysis from small blood volumes will be feasible. Recently, a thorough study of the feasibility of the analysis of platelet transcriptome from a single donor using RNA amplification technique has been reported [39]. The aim of this study was to establish a protocol that makes analysis of platelet transcriptome applicable to a single patient with suspected platelet disorder. Platelets were depleted from leukocytes by centrifugation and filtration, and total RNA was amplified using a commercially available PCR-based RNA amplification kit (switching mechanism at the 5' end of RNA templates, SMART, BD Biosciences Clontech, Mountain

Fig. 3. Pathways of androgen formation and differential expression of 17 β -hydroxysteroid dehydrogenases type 3 (HSD17B3) and type 12 (HSD17B12) in normal and ET platelets. Changes in gene expression were calculated from microarray data. Function of HSD17B12 in the steroid metabolism pathway remains unknown.

View, CA, USA). Reliability and precision of RNA amplification was evaluated by Q-PCR using selected platelet-expressed transcripts. Microarray analysis was performed using a glass chip containing probes to 9,850 genes (MWG-Biotech AG). Microgram quantities of cDNAs were produced from as little as 50 ng of total platelet RNA or 40 ml of a whole blood. Amplification of all monitored transcripts was exponential to the cycle number 16. Gene profiling gave similar results for 9,815 of the 9,850 genes, with 1,576 positive and 8,239 negative. Interestingly, only 8 transcripts failed to be amplified, and amplification efficiency was slightly higher for low-abundant transcripts. In addition, day-to-day variability in the expression of selected transcripts in 3 healthy individuals has been addressed and found to be minor. The authors concluded that the SMART amplification protocol does not cause significant distortion of the gene expression profile and can potentially be applied for routine studies of the platelet transcriptome for individual patients.

Application of Platelet Transcriptomics to Hematological Diseases

Essential Thrombocythemia as a Paradigm

The utility of gene expression profiling for the molecular classification of human cancer is well-documented, although its applicability to poorly-understood myeloproliferative disorders, such as essential thrombocythemia (ET) remains unestablished. ET represents a myeloproliferative disorder subtype, characterized by increased proliferation of megakaryocytes, elevated numbers of circulating platelets and considerable thrombohemorrhagic events, not infrequently neurological [40]. The molecular basis of ET remains unestablished, and his-

torically it has been considered a clonal disorder [41], although recent discovery of an acquired mutation in the Janus kinase 2 (JAK2) gene may shed a light on the molecular mechanism of this disease [42–44]. Recently, a microarray approach was used to gain further insight into the specific changes associated with the ET phenotype and to identify genes that could discriminate between morphologically indistinguishable ET and normal platelets [45]. Initial analysis of the genetic profiles of highly purified apheresis platelets isolated from 6 ET patients (4 females, 2 males) and 5 normal, healthy controls demonstrated distinctly different molecular signatures. ET platelets collectively demonstrated higher numbers of expressed transcripts compared to normal controls, but considerably less than the transcript numbers generally found in nucleated cells [22]. Of the genes classified as marginal or present in a minimum of 4 microarrays, ET patient samples expressed an average of 3,562 transcripts compared to 1,668 in normal controls. Normal or ET platelets have genetic profiles distinct from leukocytes, and notable differences between normal and ET platelets were clearly evident. Initial one-way analysis of variance identified 170 differentially expressed genes, the majority of which (141) were up-regulated in ET platelets. Only 29 genes were down-regulated compared to normal platelets. Functional cluster analysis of this limited set of differentially expressed genes demonstrated that genes involved in adhesion and catalytic activity represented the largest subgroups (38 and 32%, respectively), although a sizable number of genes (35%) remained unclassified.

It was found that normal human blood platelets express HSD17B3 enzyme (type 3 17 β -hydroxysteroid dehydrogenase), previously described as testis-specific. This enzyme belongs to a large family of 17BHSDs and is known to catalyze the penultimate step in testosterone biosynthesis (fig. 3). Molecular defects of the *HSD17B3* gene are causally implicated in

male pseudohermaphroditism [46]. In addition to *HSD17B3*, transcripts for two more members of this family were found in human platelets – *HSD17B11* and *HSD17B12*. While the *HSD17B11* mean, normalized signals between the normal and diseased cohorts were low-level and not statistically different, there was a striking change in the pattern of *HSD17B3* and *HSD17B12* expression between ET and normal platelets. Absence of *HSD17B3* transcript expression was evident in all 6 ET patients, changes that occurred concomitantly with elevated transcript levels of *HSD17B12* in the same patient subgroup. In contrast, expression of *HSD17B3* in normal platelets was accompanied by negligible to low-level *HSD17B12* expression. Several independent methods were used to validate and extend studies of 17 β -hydroxysteroid dehydrogenases in normal and ET platelets. Initial findings were confirmed both at the mRNA level by Q-PCR and at the protein level by the analyses of functional activity of *HSD17B3*. Recent studies demonstrated that another sex hormone, estradiol, is produced in megakaryocytes [47]. Moreover, proplatelet formation of megakaryocytes was triggered by autocrine-synthesized estradiol. It was clearly demonstrated that the inhibition of another key enzyme for steroid hormone biosynthesis, 3 β -hydroxysteroid dehydrogenase (3 β -OH-steroid dehydrogenase, fig. 3), blocks proplatelet formation. In addition, estrogen receptor antagonists inhibit platelet production in vivo. Taken together, these data suggest that sex hormones and enzymes involved in steroid metabolism may play an important role in megakaryocyte maturation and proplatelet formation. In summary, transcript profiling demonstrated noticeable difference between the spectrum of mRNAs expressed in normal and ET platelets, thus contributing to the generation of the molecular signature of ET. Moreover, these data establish a novel link between platelet function and hormone steroidogenic pathways that may be causally implicated in platelet-mediated thrombotic events and/or platelet production.

Indirect Studies of Platelet Disorders: Gray Platelet Syndrome and Polycythemia vera

Molecular mechanisms of other platelet disorders, such as gray platelet syndrome (GPS), were also studied by transcript profiling, although these studies were not focused on platelets as a source of RNA. GPS is a rare congenital bleeding disorder of unknown origin, with phenotypic and genetic heterogeneity, characterized by thrombocytopenia, increased platelet size and decreased alpha-granule content [48–50]. In order to investigate mechanisms involved in GPS, the microarray approach was applied [51]. In pursuing the etiology of GPS, fibroblasts were employed as a model system, partly because the myelofibrosis in this subtype of GPS was likely to involve these cells. Normal and GPS fibroblasts were cultured from skin biopsy, and the differential expression of 4,900 unique genes was evaluated by microarray techniques. Differ-

entially expressed genes were clustered according to the change in their expression, and genes within each cluster were tested for functional similarities. Significant up-regulation of expression of the cytoskeleton proteins fibronectin 1, thrombospondins 1 and 2 and collagen VI α was demonstrated. Overexpression of fibronectin and thrombospondin 1 was independently confirmed at the RNA level by Northern blotting. In addition, up-regulation of expression of fibronectin was confirmed at the protein level by immunostaining. These results clearly demonstrate the feasibility of a transcriptome approach to identify gene candidates involved in GPS and suggest that cytoskeleton proteins may play an important role in the molecular mechanisms of this rare disorder.

Polycythemia vera (PV) is a myeloproliferative disorder of unknown molecular pathogenesis. It is characterized by increased proliferation of all 3 myeloid lineages with subsequent overproduction of mature red cells, granulocytes and platelets [52]. Initially, microarray analysis was employed to identify genes which are differentially expressed in normal and PV granulocytes [53]. Evaluation of expression levels of 6,000 genes revealed that 147 genes were up-regulated in the majority of PV patients compared to normal. 11 of these genes were elevated in all 11 patients studied. In addition, elevated expression of several protease inhibitors with affinity for proteases involved in apoptosis and a number of antiapoptotic factors has been documented.

Another study focused on the differential diagnosis between PV and secondary erythrocytosis (SE) [54]. Granulocytes were purified from peripheral blood of 40 PV patients, 12 SE patients and 50 healthy volunteers. RNA was isolated and hybridized to the customized cDNA microarray chip, containing probes for 7,497 genes. Two independent sets of data were obtained. First, a set of 64 genes was determined, which correctly distinguish RV from SE, thus representing a valuable diagnostic tool. Second, 644 genes were identified as differentially expressed in PV patients compared to normal (253 up-regulated, and 341 down-regulated in PV, $p < 0.01$). Many genes which are up-regulated in PV are controlled by Spl-like transcription factors. One of them, transcription factor NF-E2 was overexpressed 2–40-fold in PV patients compared to normal. In addition, overexpression of this transcription factor was documented in erythroid and myeloid precursors and in megakaryocytes of PV patients. These data establish the feasibility of the differential diagnosis of myeloproliferative disorders based on the molecular signature of the diseases and suggest that transcription factor NF-E2 may be involved in the molecular etiology of PV.

Conclusion

Taken together, platelet mRNA profiling studies have defined the basic platelet transcriptome. It is clear that platelets have a distinct and reproducible transcript profile. Moreover, tran-

script profiles of normal and diseased platelets are significantly different, as was demonstrated for ET and PV. Transcript profiling not only defines the molecular signature of platelets in normal and diseased states, but also can be used for identification of novel proteins previously unknown to be expressed in platelets. To date, genetic profiling techniques have focused on well-defined (but rare) congenital platelet disorders, although their applicability to more common disorders affecting platelets (i.e. cardiovascular and cerebrovascular diseases) remains unestablished.

Recent advances in platelet proteomic studies give further insight into mechanisms of platelet function and permit identification of novel proteins at an unprecedented scale [55–60]. The discovery of pre-mRNA splicing in platelets [10] opens new perspectives in studying the complexity of transcriptome-proteome correlations. Platelet-restricted signal-dependent splicing contributes to previously unrecognized complexity

regulating the platelet proteome, although its physiological consequences remain unestablished. Whether alternative splicing occurs in platelets in response to various exogenous factors or diseases, is also unknown, although this issue can be partially addressed by the next generation of microarray chips which should allow the detection of alternatively spliced transcripts. Integrated platelet transcriptomics and proteomics will represent a powerful tool to study complex processes underlying platelet regulatory functions and identify potential new therapeutic targets in platelets.

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