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14. ABSTRACT

Human blood platelets play critical roles in normal hemostatic processes and pathologic conditions such as thrombosis (i.e. cardiovascular disease and stroke), vascular remodeling, inflammation, and wound repair. Despite the biological importance of platelets and their intact protein synthetic capabilities, remarkably little is known about platelet mRNAs. The pathogenesis of essential thrombocytosis (ET), a disease of platelet number and function, is poorly understood at the molecular level. The main goal of this project is to build on our preliminary data that suggests that patients with ET have distinct platelet transcript profiles that differ from those of normal platelets. The three main hypotheses to be tested are: (1) patients with ET have mRNA profiles that are distinct from those of normal controls; (2) these differences can be used to elucidate the molecular basis of ET; and (3) these differences can be used to differentiate ET from other causes of thrombocytosis (ET diagnostics). Completion of the specific aims as outlined below should (*i*) provide considerable insight into the molecular basis of ET, (*ii*) assist with molecular diagnostics, and (*iii*) help to devise rational approaches for pharmacological intervention.

15. SUBJECT TERMS
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I. INTRODUCTION. Human blood platelets play critical roles in normal hemostatic processes and pathologic conditions such as thrombosis (i.e. cardiovascular disease and stroke), vascular remodeling, inflammation, and wound repair. Despite the biological importance of platelets and their intact protein synthetic capabilities, remarkably little is known about platelet mRNAs. The pathogenesis of essential thrombocytosis (ET), a disease of platelet number and function, is poorly understood at the molecular level. The main goal of this project is to build on our preliminary data that suggests that patients with ET have distinct platelet transcript profiles that differ from those of normal platelets. The three main hypotheses to be tested are: (1) patients with ET have mRNA profiles that are distinct from those of normal controls; (2) these differences can be used to elucidate the molecular basis of ET; and (3) these differences can be used to differentiate ET from other causes of thrombocytosis (ET diagnostics). Completion of the specific aims as outlined below should (*i*) provide considerable insight into the molecular basis of ET, (*ii*) assist with molecular diagnostics, and (*iii*) help to devise rational approaches for pharmacological intervention. These three major hypotheses are the basis for the following specific aims:

Specific Aim I. Validate differences in microarray profiles between normal and ET patients

Specific Aim II. Establish the positive and negative predictive values of these profiles

Specific Aim III. Develop a pilot ET diagnostic microarray chip and evaluate a transcriptome-based approach to the diagnostics of ET.

II. BODY.

A. APPROACH (**Specific Aims 1 and 2**): Microarray analysis was used to study the molecular basis of essential thrombocythemia using highly-purified platelets isolated from peripheral blood (20 mL) or by apheresis. Differences were validated by quantitative PCR (Q-PCR) and/or protein analyses as necessary.

B. RESULTS: Initial one-way ANOVA identified 170 genes that were differentially expressed, the majority of which (141) were up-regulated in ET platelets; only 29 genes were down-regulated in ET compared to normal platelets. Analysis of the smaller subset of platelet-restricted genes demonstrated that only 13 genes were differentially-expressed (12 up-regulated, 1 down-regulated) in ET. A disproportionate number of upregulated genes encoded proteases or protease inhibitors (*HPSE*, *MMP1*, *SERPINI1*), a class of proteins known to be associated with tumor invasiveness and metastases. The single down-regulated gene (*HSD17B3*) was present in all normal samples and absent in all ET platelets. *HSD17B3* belongs to an extended family of 17BHSDs retaining oxido-reductase activity toward discrete substrates, and encodes an enzyme (type 3 17 -hydroxysteroid dehydrogenase) previously described as testis-specific. This enzyme is known to catalyze the penultimate step in testosterone biosynthesis. Using a functional assay of testosterone generation, we demonstrated that platelets retained 17βHSD3 activity, with nearly 10% of the capacity found in mouse testis, providing evidence for the first non-testicular source of this enzyme. Transcripts for two additional members of this family were found in human platelets, one of which (*HSD17B12*) was upregulated in the initial cohort of patients studied. Subsequent Q-PCR results were entirely concordant for all individuals studied, demonstrating that *17BHSD12:17BHSD3* transcript ratios reliably distinguished ET from normal patient platelets in all samples studied to date (N=20; 6 apheresis samples, 14 peripheral blood samples; $p < 0.0001$). Furthermore, these differential patterns of *HSD17B* expression appeared unrelated to the development of thrombocytosis *per se*, but rather, were restricted to the ET phenotype.

NB: This data is summarized in the manuscript D. Gnatenko, *et. al.* (Thromb. Haemost., 94:412-21, 2005).

Changes to Specific Aims 1 and 2: NONE

C. ONGOING STUDIES (**Specific Aim 3**): **A diagnostic platelet oligonucleotide microarray chip**

Human blood platelets play critical roles in normal hemostatic processes and pathologic conditions such as thrombosis (heart attacks and stroke), vascular remodeling, inflammation, and wound repair. We have identified a restricted list of platelet-expressed genes, and used these for fabrication of a focused, intermediate density oligonucleotide gene chip. For probe selection, we analyzed microarray data isolated from (*i*) normal patient peripheral blood leukocytes (WBC), (*ii*) patients with essential thrombocythemia ([ET]; i.e. those with high platelet counts), and (*iii*) normal platelets (NP). We hypothesized that such a strategy would maximize the identification of informative genes for chip fabrication. The final list was established by including the following cohorts: (*i*) a group of platelet-restricted genes with no expression in leukocytes (*N=126*); a preliminary group of discriminatory genes distinguishing between thrombohemorrhagic ET phenotypes (*N=71*); the list of genes with platelet expression > leukocyte expression by 10-fold (*N=285*); and the list of genes with leukocyte expression > platelet expression by 10-fold (*N=43*) [leukocyte contamination control]. After removal of duplicates, the final list contains 432 genes which clearly co-segregate by cell-type (**Fig. 1**).

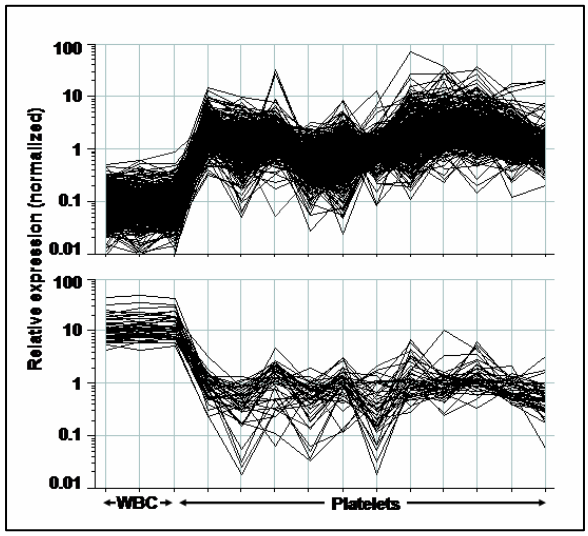
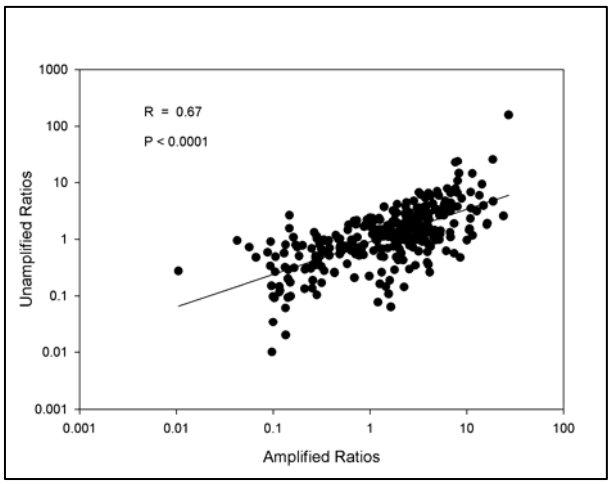


Fig. 1. *Gene rank-intensity plots.* Normalized data from individual samples (WBC=3, NP=5; ET=6) were analyzed by one-way ANOVA using parametric testing to identify a 432-member gene list. The *top panel* demonstrates the platelet-restricted genes (N=389) while the *lower panel* demonstrates the leukocyte-restricted genes (N=43). Note the clear difference in the expression patterns between the two groups, and the variability in expression among the various platelet populations (reflecting to some degree the different platelet populations (ET *versus* normal).

Since platelets have limited mRNA, we have also developed and refined methods for reproducible mRNA amplification, as an adjunct for chip development. Starting with **20 ng of RNA** (readily obtainable from 10 ml of whole blood), we have generated sufficient quantities of RNA that maintain excellent amplification fidelity for transcript profiling. After hybridization to our fabricated chip, we established correlation coefficients for the group of platelet genes (N=319) that were expressed in unamplified vs. amplified microarray experiments. These data were generated by calculating ratios against the co-hybridized reference RNA, and subsequently plotted (**Fig. 2**). For this subset of evaluable genes, the R-value was 0.67 with high statistical significance ($p<0.0001$).



We propose that this chip can be developed commercially for platelet diagnostics (anticipated 1 million tests annually in the US), and for establishment of microarray profiles for thrombosis-risk susceptibility in common diseases such as stroke and heart disease; additional applications include potential identification of novel genes as drug targets for modulating platelet function (gene and drug discovery).

Changes in Specific Aim 3: None. In the next funding period we will continue our validation studies of the chip in distinguishing ET from other causes of (reactive) thrombocytosis

III. KEY RESEARCH ACCOMPLISHMENTS.

- First demonstration that platelet microarray studies can be applied to a human platelet disorder
- First demonstration that ET platelets have a profile distinct from normal platelets
- First demonstration that a diagnostic assay may be available to distinguish ET from normal platelets

- Initial fabrication and characterization of a platelet-diagnostic oligonucleotide chip

IV. REPORTABLE OUTCOMES.

A. Manuscripts accepted and/or published

- Gnatenko, D., L. Cupit, E. Huang, A. Dhundale, P. Perrotta, and **W. Bahou**. Platelets express steroidogenic 17β-hydroxysteroid dehydrogenases: distinct profiles predict the essential thrombocythemic phenotype, Thromb. Haemost., 94:412-21, 2005.

- Watson, S., **W. Bahou**, D. Fitzgerald, W. Ouwehand, A. K. Rao, and A. Leavitt. Mapping the platelet proteome: a report of the ISTH platelet physiology subcommittee. J. Thr. Haemost. 3:2098-2101, 2005.

- Gnatenko, D. and **W. Bahou**. Advances in platelet transcriptomics. Trans. Med. Hemotherapy, *in press.*

- **Bahou, W.** Platelet genetic disorders, in Genomics and Clinical Medicine, D. Kumar, editor, Oxford University Press, 2006.

B. Invited Reviews (*Pending publication*)

Gnatenko, D., P. Perrotta, and **W. Bahou**, Advances in platelet proteomics: one-half the story, Blood, *submitted*

C. Oral presentations

- *"Global platelet profiling*", Platelets Symposium (Jerusalem, Israel; May, 2006)

- "*Transcript profiling of essential thrombocythemic platelets"*, Dept. Defense Peer Reviewed Medical Research Program (San Juan, Puerto Rico; May, 2006)

- *"Progress and limitations of platelet transcript profiling*", International Society of Thrombosis and Hemostasis (Sydney, Australia; 2005)

V. CONCLUSION: We have made considerable progress in all aims of the research, specifically in (*i*) providing initial proof-of-principal that ET has unique mRNA transcript profiles, (*ii*) that these profiles can be used to develop potential diagnostic tests for the disease, and (*iii*) in identifying a novel steroidogenic pathway in normal and platelets. During the subsequent funding cycle, we will continue validation of these initial observations by recruiting a broader cohort of subjects, and initiating preliminary studies to dissect the role of steroidogenic pathways in megakaryocyte proliferation and/or proplatelet formation (thrombopoiesis).

VI. REFERENCES. SEE SECTION IV. Reportable Outcomes (*above*)

VI. APPENDICES.

- Gnatenko, D., L. Cupit, E. Huang, A. Dhundale, P. Perrotta, and **W. Bahou**. Platelets express steroidogenic 17β-hydroxysteroid dehydrogenases: distinct profiles predict the essential thrombocythemic phenotype, Thromb. Haemost., 94:412-21, 2005.

- Watson, S., **W. Bahou**, D. Fitzgerald, W. Ouwehand, A. K. Rao, and A. Leavitt. Mapping the platelet proteome: a report of the ISTH platelet physiology subcommittee. J. Thr. Haemost. 3:2098-2101, 2005.

VII. SUPPORTING DATA. As outlined in Figures 1 and 2 (*above*) and in two appended articles.

Platelets express steroidogenic 17β–hydroxysteroid dehydrogenases

Distinct profiles predict the essential thrombocythemic phenotype

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Summary

Human blood platelets have important, regulatory functions in diverse hemostatic and pathological disorders, including vascular remodeling, inflammation, and wound repair. Microarray analysis was used to study the molecular basis of essential thrombocythemia, a myeloproliferative disorder with quantitative and qualitative platelet defects associated with cardiovascular and thrombohemorrhagic symptoms, not infrequently neurological. A platelet-expressed gene (*HSD17B3*) encoding type 3 17β-hydroxysteroid dehydrogenase (previously characterized as a testis-specific enzyme catalyzing the final step in gonadal synthesis of testosterone) was selectively down-regulated in ET platelets, with reciprocal induction of the type 12 enzyme (*HSD17B12*). Functional 17β-HSD3 activity corresponding to ~10% of that found in murine testis was demonstrated in normal platelets. The induction of *HSD17B12* in ET platelets was unassociated with a concomitant increase in androgen biosynthesis, suggesting distinct functions and/or substrate specificities of the types 3 and 12 enzymes. Application of a molecular assay distinguished ET from normal platelets in 20 consecutive patients ($p < 0.0001$). These data provide the first evidence that distinct subtypes of steroidogenic 17β–HSDs are functionally present in human blood platelets, and that the expression patterns of *HSD17B3* and *HSD17B12* are associated with an uncommon platelet disorder manifest by quantitative and qualitative platelet defects.

Keywords

Platelets, hydroxysteroid dehydrogenases, microarray, essential thrombocythemia

Thromb Haemost 2005; 94: 412-21

Introduction

Circulating blood platelets are anucleate although they retain small amounts of megakaryocyte-derived mRNAs and a fully functional protein biosynthetic capacity (1). Essential thrombocythemia (ET) represents a myeloproliferative disorder subtype, characterized by increased proliferation of megakaryocytes, elevated numbers of circulating platelets, and considerable thrombohemorrhagic events, not infrequently neurological (2). ET is seen with equal frequency in males and females, although an additional female incidence peak at age 30 may explain the apparent higher disease prevalence in females. The molecular basis of ET remains unestablished although historically it has been considered a "clonal" disorder (3). Causative mutations have been identified in the thrombopoietin gene, but these appear to be uncommon, and restricted to rare individuals with familial thrombocythemia (4). Other than the exaggerated platelet volume and inconsistent platelet aggregation abnormalities evident in subsets of ET platelets, no functional or diagnostic test is currently available for ET and it remains a diagnosis of exclusion (5).

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 remains unestablished. Previous work from this laboratory demonstrated the feasibility of

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platelet profiling using apheresis techniques (1, 6), prompting us to adapt this method for the molecular study of ET. We now demonstrate that human blood platelets express a previously described (testis-specific) enzyme (type 3 17β-hydroxysteroid dehydrogenase) known to catalyze the penultimate step in testosterone interconversion; furthermore, distinct expression patterns of genes encoding this enzyme are associated with the ET phenotype. These data identify 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.

Methods

Patient selection and characterization

Patients were enrolled from the larger pool of patients referred to the Division of Hematology for evaluation of thrombocytosis. All patients provided informed consent for an IRB (Institutional Review Board)-approved protocol completed in conjunction with the Stony Brook University Hospital General Clinical Research Center. Standard hematological criteria were followed for the diagnosis of essential thrombocythemia, reactive thrombocytosis, or other myeloproliferative disorders (7, 8). Both sex- and age-distribution paralleled prevalence figures for ET, with a M:F ratio of 1:2.3, and age at diagnosis ranging 23–78 years. Platelet counts at the time of blood isolation ranged from normal (reflecting treatment) to 1,724,000/μl; patient utilization of platelet-lowering drugs (i.e. hydroxyurea, analgrelide, or untreated) was recorded at the time of platelet isolation and purification (refer to Table 1 for detailed patient characteristics).

Platelet molecular studies

Platelets were obtained by apheresis or from peripheral blood (10 ml), and were isolated essentially as previously described, utilizing gel-filtration and CD45-coupled magnetic micro-beads for leukocyte immunodepletion (1). The final platelet-enriched product contained no more than 3–5 leukocytes per 1 X 10^5 platelets; peripheral blood leukocytes from three healthy donors were isolated as previously described (1). Pure cellular pellets were resuspended in 10 ml of Trizol reagent (Invitrogen, Carlsbad, CA), transferred into DEPC (diethylpyrocarbonate)-treated Corex tubes, and serially purified and precipitated using isopropanol (9). Platelet total mRNA quantification and integrity were established using an Agilent 2100 Bioanalyzer, and mean platelet RNA concentrations between the two groups were nearly identical: ET platelets contained ~0.8 ± 0.2 fg/platelet while normal platelet RNA concentrations were ~0.6 ± 0.3 fg/platelet. Quantitative reverse transcription (RT)-PCR was performed using fluorescence-based real-time PCR technology (TaqMan Real-Time PCR, Applied Biosystems, Foster City, CA). Oligonucleotide primer pairs were generated using Primer3 software (www-genome.wi.mit.edu), designed to generate ~200-bp PCR products. *HSD17B3*-specific primers were: forward (5'-3' AAATGTGATAACCAAGACTGC [bp 755–775]; reverse (5'-3' CTTGGTGTTGAGCTTCAGGTA [bp 956–936]; *HSD17B12*-specific primers were: forward (5'-3' TGAATACTTTTGGATGTTCCTGA [bp 496–519]); reverse (5'-3' AGTCTTGGTTGCAGAATAGATGGT, [bp 634–611]; *HSD17B11*-specific primers were: forward (5'-3' TGGATATAAAATGAAAGCG CAATA [bp 1067–1090]; reverse (5'-3': ATCAGCTTTTG GCTAAAGAACAAG [bp 1265–1242]; *F7*-specific primers were: forward (5'-3': TCCTGTTGTTGGTGAATGG [bp 734–753]; reverse (5'-3': GTACGTGCTGGGGATGATG [bp933–915]; β-actin-specific primers were as previously described (1). Purified platelet mRNA (4 μg) was used for first strand cDNA synthesis using random hexamers and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). For RT-PCR analysis, the RT reaction was equally divided among primer pairs and used in a 40-cycle PCR reaction for each target gene using three-step cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 min; mRNA levels were quantified by monitoring real-time fluorimetric intensity of SYBR green I. Relative mRNA abundance was determined from triplicate assays performed in parallel for each primer pair, calculated and standardized to β-actin as previously described (6, 10). For some patients, high molecular-weight genomic DNA was isolated from peripheral blood leukocytes for PCR-based amplification and sequencing of exons and exon-intron boundaries (11).

Gene expression profiles were completed using the HU133A GeneChip containing a 22, 283 probe set (Affymetrix, Santa Clara, CA). Total cellular RNA (5.8 μg) was used for cDNA syn-

Table 1: Patient characteristics.

ID	Age[1]	Sex	Diagnosis	Sample Source[2]	Platelet count[3] (X 10^9/L)	Treatment[4]	Genetic analysis[5]
ET1	31	F	ET	B,P	1,308	H	M,P
ET3	49	M	ET	B,P	565	H	M,P
ET4	33	M	ET	B,P	1,515	N	M,P
ET5	23	F	ET	B,P	1,566	N	M,P
ET6	37	F	ET	B,P	539	A	M,P
ET7	65	F	ET	B	991	A	P
ET9	64	F	ET	B	588	A	P
ET10	57	M	PV/ET6	B	945	H	P
ET11	71	F	ET	B	477	H	P
ET12	40	F	ET	B	456	N	P
ET14	65	F	ET	B	853	A	P
ET15	64	F	ET	B	511	H	P
ET18	78	M	ET	B	329 (940)	H	P
ET19	77	F	ET	B	488	H	P
ET20	69	M	ET	B	345 (1,063)	A	P
ET27	50	F	PV/ET6	B,P	1,724	H	M,P
ET28	29	F	RT7	B	402	N	P
ET29	66	F	ET	B	495	N	P
ET31	70	M	ET	B	347 (880)	A	P
ET32	50	F	ET	B	513	N	P
ET33	75	F	ET	B	641	H	P

[1]Age at diagnosis
[2]Sample source: B – peripheral blood; P – plateletpheresis
[3]Platelet count at time of sample collection (normal range 150 – 350); note that for patients with normal platelet counts at time of blood isolation, the platelet counts in parentheses are highest pre-treatment determinations
[4]Refers to treatment at the time of platelet isolation; A – Anagrelide; H- Hydroxyurea; N – Not treated 5M – Microarray; P – Quantitative RT-PCR
[6]Originally given the diagnosis of polycythemia rubra vera (PV)
[7]Secondary (post-splenectomy) thrombocytosis

thesis using SuperScript Choice system (Life Technologies, Rockville, MD) and an oligo (dT) primer containing the T7 polymerase recognition sequence, followed by cDNA purification using phenol/chloroform extraction and ethanol precipitation (1). *In vitro* transcription was completed in the presence of biotinylated ribonucleotides using a BioArray HighYield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY), and after metal-induced fragmentation, 10 μg of the biotinylated cRNA was hybridized to the GeneChip array for 16 hours at 45°C. After washing, the cRNA was detected with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR) and analysis completed using a Hewlett-Packard Gene Array Scanner. The fluorescence intensity of each probe was quantified using Affymetrix GeneChip software (MAS version 5.0), calculated as an average difference for each gene set obtained from 16 to 20 paired (perfectly matched and single nucleotide-mismatched) 25-bp oligonucleotides. The software is designed to exclude "positive calls" in the presence of high average differences with associated high mismatch intensities.

Bioinformatic and statistical analyses

Microarray data were analyzed and visualized using GeneSpring (version 7.0) software (Silicon Genetics, a subsidiary of Agilent Technologies, Palo Alto, CA). Megakaryocyte expression data were obtained from the National Center for Biotechnology (NCBI) Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/projects/geo/; platform GPL96), using profiles obtained from thrombopoietin-stimulated normal and ET bone marrow $CD34^+$ cells differentiated in liquid culture for 14 to 16 days (12). $CD34^+$-derived megakaryocytes (Mks) were purified using a megakaryocyte-specific anti-CD41a monoclonal antibody, followed by microarray analysis using the identical HU133A GeneChip. Data were normalized by dividing each measurement by the 50th percentile of all measurements in that sample, and each gene was divided by the median of its measurements in all samples. Normalized median ratios of individual genes were filtered for presence across arrays, and selected for expression levels as detailed. Prior to unsupervised hierarchical clustering of the uncentered Pearson correlation similarity matrix, the platelet microarray data were filtered for gene expression across phenotypic cohorts, defined as those genes present or marginal in a minimum of 80% of platelet samples (yielding 2,906 transcripts). A subset of genes was culled from the 2,906-gene list to specifically delineate those transcripts uniquely expressed in platelets; this platelet-restricted subset (N=126) was delineated by removing genes expressed in 3/3 leukocyte microarrays. A non-parametric analysis of variance test (ANOVA) was performed to identify differentially expressed genes using the Benjamini and Hochberg method to lower the false discovery rate ($p < 0.01$). All statistical analyses were completed using SPSS (Statistical Package for Social Sciences, version 11.5) software.

Functional 17β–HSD studies

Functional studies for platelet 17β–HSD3 activity were completed using gel-filtered platelets (GFP). Briefly, 1.5 X 10^8 platelets were solubilized and freeze-thawed in HSD buffer containing {20 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 50 mM NaCl, 5% Glycerol, 10 mM DTT, 1.5 mM NAD (D-5755, Sigma Co., St. Louis, MO)}, and the reaction started by addition of 1 μl (12.7 pmol) of [1,2,6,7-^{3}H(N)]-testosterone (specific activity 78.5 Ci/mM) (Dupont/NEN) to equivalent protein aliquots. A 30– or 90-minute reaction was allowed to proceed at 25^0 C, and quenched at –20°C in the presence of cold testosterone (1 mg/ml) and androstenedione (1 mg/ml). Steroid extraction was performed twice using 150 μl of ethyl acetate, both fractions were combined and air-dried under a vacuum. Androstenedione and testosterone were separated by thin layer chromatography (Uniplate Silica Gel GF plates (250 μm), Analtech Inc, Newark, DE) using 4:1 (v/v) chloroform:ethyl acetate. Discrete steroid fractions were visualized and extracted from silica gels using ethanol, and quantified by liquid scintillography. Purified hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* (Sigma Co., St. Louis, MO) diluted to 10 mg/ml in HSD buffer served as control for some experiments. Mouse testis extract prepared from a C57/Bl6 mouse served as standard for platelet 17β-HSD3 quantification, and was prepared by homogenization in 3 ml of HSD buffer. After centrifugation at 5000 g for 5 minutes, supernatants were gel-filtered in HSD-equilibrated Centrisep spin columns prior to use; protein quantification of all samples was determined by optical density as previously described (13).

Results

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 (Fig. 1). 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 (1). 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 for normal controls (compared with ~10,500 transcripts identified in all three leukocyte microarrays). More stringent analyses (i.e. marginal or present in all of the arrays within a single group) extended these differences, with 1,840 transcripts expressed in ET platelets *versus* 1,086 transcripts expressed in platelets from healthy controls ($p < 0.03$). Thus, while bone marrow megakaryocyte expansion is known to accompany the thrombocythemic phenotype (2), this cellular proliferation is also associated with a nearly 45% increase in overall gene transcription.

An unsupervised, hierarchical clustering algorithm was used to group normal and ET platelet genes on the basis of similarities of gene expression (Fig. 1a,b). This direct comparison against a genetically normal platelet pool highlighted genes that consistently distinguish normal from diseased platelets (14). Normal or ET platelets have genetic profiles distinct from leukocytes, and notable differences between normal and ET platelets are clearly evident. 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 – several methods of computational analyses were applied. Initial one-way ANOVA identified 170 genes that were differentially expressed, the majority of which (141) were up-regulated in ET platelets; only 29 genes were down-regulated

in ET compared to normal platelets (*see below*). Functional cluster analysis of this limited set of differentially-expressed genes (Fig. 2) demonstrated that genes involved in adhesion and catalytic activity represented the largest subgroups, although a sizable number of genes (35%) remained unclassified.

Because of inherent difficulties in analysis of microarray data sets (15), we re-analyzed the data, now computing *t*-statistics of ET *versus* normal platelets for each gene; the *t*-values were ranked by absolute magnitude (thereby incorporating inter-sample variability in expression ratios); they were then ranked by

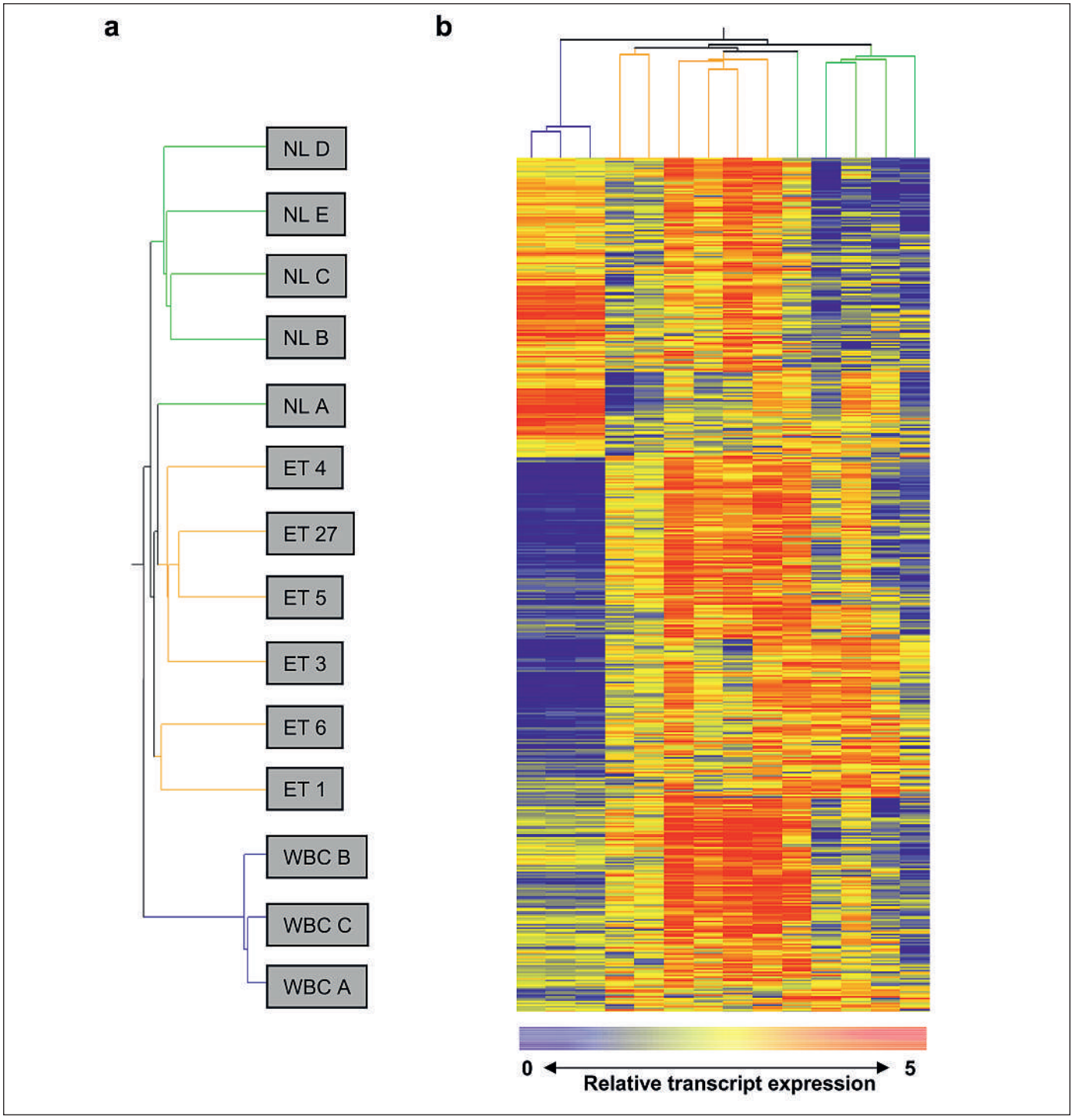


Figure 1: Molecular signature of normal and essential thrombocythemic platelets. Gene expression profiles from 14 experimental samples (eleven apheresis donors [5 normal, NL A-E; 6 patients with essential thrombocythemia (ET)], or 3 normal leukocyte [WBC A-C] donors) are displayed. a. Relationships between the experimental samples are displayed as dendrograms, in which the pattern and length of the branches depict sample cohort relatedness among the experimental groups. b. Unsupervised hierarchical clustering using the 2,906-gene set demonstrates the distinct variation in gene expression pattern among defined cohorts; each row represents a single gene.

the magnitude of the test statistic numerator, a measure of the biological difference in expression ratios. By applying a rigid 5-fold difference in pair-wise expression as the cut-off, we identified 163 genes that were up-regulated in ET, but only a small number (5) of down-regulated genes (Fig. 3a). To further pare this list, the analyses were repeated using the database of genes whose expression was restricted to platelets. This subset of platelet-restricted genes was delineated by excluding genes from the 2,906-gene list that were expressed in all 3 leukocyte arrays, leaving only 13 genes that were differentially-expressed (12 up-regulated, 1 down-regulated in ET)(Fig. 3b). Of the small subset of platelet-restricted, differentially expressed genes identified by this analysis, 7 were also in the top 40 list identified by the one-way ANOVA, establishing an independent layer of validation to these findings (Fig. 3c); while only 17% of the 170 differentially-expressed genes were under-expressed in ET platelets by one-way ANOVA, down-regulated genes were over-represented in the platelet-restricted gene list, accounting for 36% (9/25) of the total. Of the top 20 over-expressed genes, 3 encoded proteases or protease inhibitors (*HPSE*, *MMP1*, *SERPINI1*), a class of proteins well-associated in tumor invasiveness and cancer metastases (16). Interestingly, matrix metalloproteinases have recently been shown to mediate megakaryocyte transendothelial migration and proplatelet formation, although this effect appeared restricted to MMP-9 to the exclusion of MMP-2 (17); while the latter report did not specifically study MMP-1, our data would implicate MMP-1 as having a specific or ancillary role in the basement degradative process known to accompany proplatelet formation.

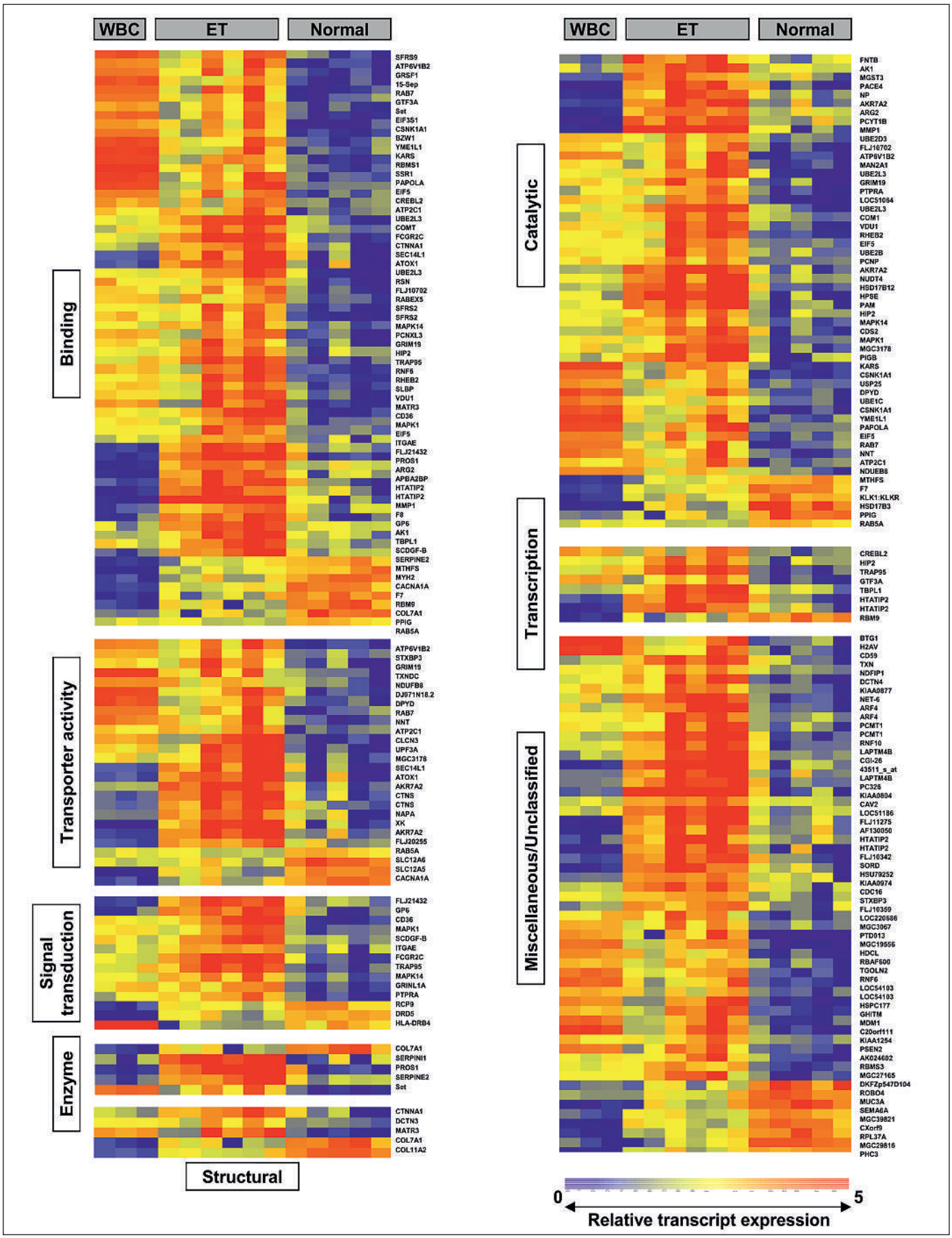
A single platelet-restricted gene *HSD17B3* (encoding the type 3 17β-hyrdroxysteroid dehydrogenase [17β-HSD3]) was expressed in all normal platelet arrays, and uniquely under-expressed in ET compared to normal platelets (Fig. 3b). The large family of steroid dehydrogenases retain oxidoreductase activity in a wide range of biological processes, although the 17β-HSDs uniquely function in the formation and inactivation of all active androgens and estrogens, with substrate-product interconversion regulated by the oxidative state of the NADP/NAD(P)H cofactors (18). The HSD type 3 enzyme is generally regarded as testes-specific, although rare SAGE (19) tags have been identified in CGAP tissues including brain (7×10^{-6}), skeletal muscle (9.3×10^{-6}), and prostate (1.6×10^{-5}) (20). The 17β-HSD3 enzyme specifically mediates the catalytic interconversion involving 4-androstenedione and testosterone, and molecular defects of the *HSD17B3* gene are causally implicated in male pseudohermaphroditism (21). While steroidogenic pathways are incompletely characterized in platelets, previous data have demonstrated that megakaryocytes (Mk) express the glucocorticoid receptor, and that both Mk and platelets selectively express estrogen receptor β (ERβ) and androgen receptor mRNA and protein, to the exclusion of ER α or progesterone receptor (22). Furthermore, Mk express functional 3β-HSD that is known to catalyze the essential step in the transformation of 5-pregnen-3β-ol and 5-androsten-3β-ol steroids into the corresponding Δ^4–3-keto-steroids, i.e. progesterone as well as the precursors of all androgens, estrogens, glucocorticoids and mineralocorticoids. Indeed, Mk-derived estradiol triggers megakaryocyte proplatelet formation *in vitro*, a process that is blocked through inhibition of 3β-HSD activity (23).

Figure 2: Functional clusters of differentially expressed genes from 14 experimental samples (see legend to Fig. 1). Gene Ontology classifications are delineated for the differentially-expressed gene set (N=170) as identified by one-way ANOVA ($p<0.01$).

To date, genes encoding twelve types of 17β-HSD enzymes have been described, although the type 6 and 9 genes have been only characterized in rodents (24). Oligonucleotide probes specific for all ten *HSD17B* genes are represented on the Affymetrix HU133a gene chip; probes for type 6 and 9 are not represented. Examination of the microarray data demonstrated that platelet *HSD17B* transcript expression was limited to three isoforms: *HSD17B3*, *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 (Fig. 4 a-c). 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. To compare these platelet profiles with those from ET and normal megakaryocytes, we downloaded Mk profiles from the NCBI GEO database for bioinformatic analyses using these published data sets (12). ET Mks demonstrated the identical patterns as those found in ET platelets, i.e. absence of *HSD17B3* expression with enhanced expression of the *HSD17B12* gene. Somewhat unexpectedly, these same patterns were also evident in normal Mks; while these comparisons are of potential relevance, they are nonetheless limited by the exogenous cytokine supplementation required for *ex vivo* Mk differentiation.

To validate and extend these findings, we developed a quantitative RT-PCR (qRT-PCR) assay, and applied this assay to the original ET cohort and an expanded cohort of normal controls, specifically collected to exclude potential gender-bias in *HSD17B* gene expression. These results confirmed and paralleled those found by microarray, demonstrating ~4.5-fold greater *HSD17B3* transcript levels in normal platelets (compared to ET, $p \leq 0.001$) and concomitant ~27-fold greater *HSD17B12* transcript expression in ET platelets (compared to normal, $p \leq 0.03$); these reciprocal changes amount to an aggregate ~2-log change in intracellular *HSD17B3*:*HSD17B12* transcript levels between normal and ET platelets (Fig. 4d). Since the qRT-PCR data established an absolute decrease in *HSD17B3* transcript level in ET platelets, preliminary genomic analyses of *HSD17B3* were completed in 4 of the 6 ET patients. The 11 exons and intron-exon boundaries were amplified and sequenced, resulting in identification of a single heterozygous A insertion [not involving the splice junction site (25)] in the first intron of one patient (ET1). Thus, there was no evidence that a small deletion or missense mutation affecting *HSD17B3* transcript stability was causally implicated in reduced *HSD17B3* transcript expression (data not shown).

The distinct patterns of *HSD17B* expression identified by microarray and confirmed by qRT-PCR were then extended to a


WBC
ET
Normal
Binding
SFRS9
ATP6V1B2
GRSF1
15-Sep
RAB7
GTF3A
Set
EIF3S1
CSNK1A1
BZW1
YME1L1
KARS
RBMS1
SSR1
PAPOLA
EIF5
CREBL2
ATP2C1
UBE2L3
COMT
FCGR2C
CTNNA1
SEC14L1
ATOX1
UBE2L3
RSN
FLJ10702
RABEX5
SFRS2
SFRS2
MAPK14
PCNXL3
GRIM19
HIP2
TRAP95
RNF6
RHEB2
SLBP
VDU1
MATR3
CD36
MAPK1
EIF5
ITGAE
FLJ21432
PROS1
ARG2
APBA2BP
HTATIP2
MMP1
F8
GP6
AK1
TBPL1
SCDGF-B
SERPINE2
MTHFS
MYH2
CACNA1A
F7
RBM9
COL7A1
PPIG
RAB5A
Transporter activity
ATP6V1B2
STXBP3
GRIM19
TXNDC
NDUFB8
DJ971N18.2
DPYD
RAB7
NNT
ATP2C1
CLCN3
UPF3A
MGC3178
SEC14L1
ATOX1
AKR7A2
CTNS
CTNS
NAPA
XK
AKR7A2
FLJ20255
RAB5A
SLC12A6
SLC12A5
CACNA1A
Signal transduction
FLJ21432
GP6
CD36
MAPK1
SCDGF-B
ITGAE
FCGR2C
TRAP95
MAPK14
GRINL1A
PTPRA
RCP9
DRD5
HLA-DRB4
Enzyme
COL7A1
SERPINI1
PROS1
SERPINE2
Set
CTNNA1
DCTN3
MATR3
COL7A1
COL11A2
Structural
WBC
ET
Normal
Catalytic
FNTB
AK1
MGST3
PACE4
NP
AKR7A2
ARG2
PCYT1B
MMP1
UBE2D3
FLJ10702
ATP6V1B2
MAN2A1
UBE2L3
GRIM19
PTPRA
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UBE2L3
COM1
VDU1
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EIF5
UBE2B
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MAPK14
CDS2
MAPK1
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PIGB
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USP25
DPYD
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YME1L1
PAPOLA
EIF5
RAB7
NNT
ATP2C1
NDUEB8
MTHFS
F7
KLK1:KLKR
HSD17B3
PPIG
RAB5A
Transcription
CREBL2
HIP2
TRAP95
GTF3A
TBPL1
HTATIP2
HTATIP2
RBM9
Miscellaneous/Unclassified
BTG1
H2AV
CD59
TXN
NDFIP1
DCTN4
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NET-6
ARF4
ARF4
PCMT1
PCMT1
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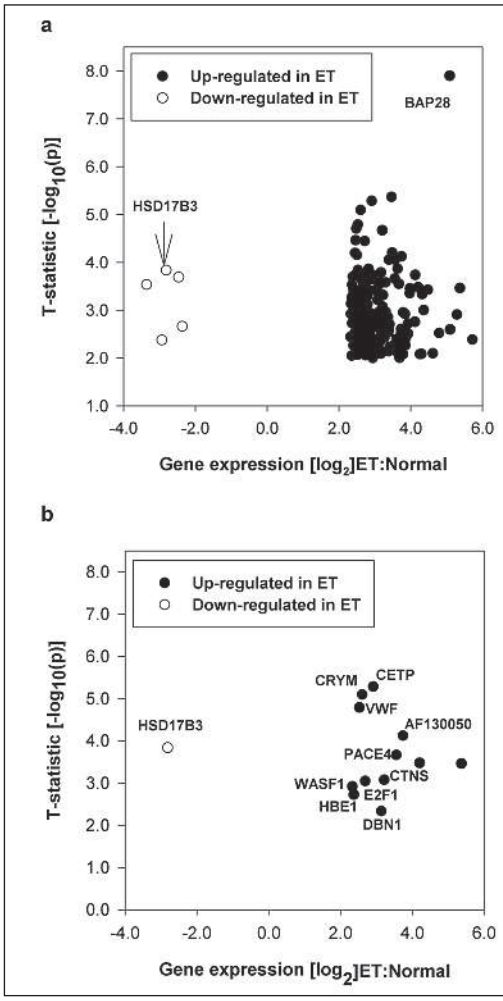
GHITM
MDM1
C20orf111
KIAA1254
PSEN2
AK024602
RBMS3
MGC27165
DKFZp547D104
ROBO4
MUC3A
SEMA6A
MGC39821
CXorf9
RPL37A
MGC29816
PHC3
0
5
Relative transcript expression

larger cohort of 20 ET patients (6 original ET patients and 14 newly-studied individuals), now uniformly analyzed using peripheral blood as the starting source for platelet analysis (bypassing the need for cumbersome apheresis technique used in the original cohorts). The qRT-PCR results were entirely concordant for all individuals studied, demonstrating that *17BHSD12:17BHSD3* transcript ratios reliably predicted the ET phenotype in all patients studied to date ($p < 0.0001$) (Fig. 5). Furthermore, these differential patterns of *HSD17B* expression appeared unrelated to the development of thrombocytosis *per se*, but rather, were restricted to the ET phenotype. This observation is based on the results of four individuals: ET28 who has secondary thrombocytosis and ratios predictive of the normal phenotype; and ET18, ET20, and ET31 with aggressively-treated ET and normal platelet counts who maintain *17BHSD12:17BHSD3* transcript ratios predictive of ET. The causes of thrombocytosis are varied, and larger cohorts of patients with etiologically diverse causes for thrombocytosis will need to be studied for confirmation, and for potential discriminatory value. Nonetheless, these initial data suggest that the intracellular signals regulating MK/platelet *17BHSD* expression may be associated with the pathophysiological mechanism(s) of the ET phenotype.

To confirm that platelets retained functional 17β–HSD3 activity (and to compare this activity between normal and ET platelets), we quantified the oxidative conversion of testosterone to 4-androstenedione. Entirely consistent with the gene expression data, normal platelets retained 17β–HSD3 activity, providing (to our knowledge) the first evidence that non-testicular sources retain functional capacity in the penultimate step of androgen biosynthesis; furthermore, the platelet-derived 17β-HSD3 activity was not inconsequential, providing nearly 10% of the capacity found in testis (Fig. 6). Finally, in the initial cohort of patients studied, ET platelets demonstrated total 17β–HSD3 activity that was not statistically different from that found in normal platelets.



c

Gene Symbol	Gene Description	(ET:Normal)
HPSE	Heparanase	52.657
LAPTM4B	**Lysosomal associated protein transmembrane 4 beta**	41.344
FLJ10359	Protein BAP28	33.924
PROS1	Protein S (alpha)	22.212
SEC14L1	SEC14-like 1 (*S. cerevisiae*)	20.559
SERPINI1	Protease inhibitor 12 (neuroserpin)	19.707
FCGR2C	Fc fragment of IgG, low affinity IIb, receptor for (CD32)	19.104
NDFIP1	Nedd4 family interacting protein 1	19.007
CLCN3	Chloride channel 3	18.609
ATOX1	**ATX1 antioxidant protein 1 homolog (*yeast*)**	18.456
EIF3S1	Eukaryotic translation initiation factor 3, subunit 1 alpha	17.322
UPF3A	UPF3 regulator of transcripts homolog A (*S. cerevisiae*)	17.213
KIAA1254	Phosphatidylinositol glycan, class B	16.168
XK	Kell blood group precursor (McLeod phenotype)	15.777
MMP1	**Matrix metalloproteinase-1**	15.393
KIAA0877	KIAA0877 protein	15.184
HSD17B12	Hydroxysteroid (17-beta) dehydrogenase 12	14.522
PCMT1	Protein-L-isoaspartate (D-aspartate) O-methyltransferase	14.449
UBE2L3	Ubiquitin-conjugating enzyme E2L 3	14.039
TRAP95	Thyroid hormone receptor-associated protein, 95-kD subunit	13.416
HSD17B3	**Hydroxysteroid (17-beta) dehydrogenase 3**	0.142
MUC3A	Mucin 3A, intestinal	0.225
PSEN2	Presenilin 2 (Alzheimer disease 4)	0.229
RBMS3	**RNA binding motif, single stranded interacting protein**	0.235
PPIG	Peptidyl-prolyl isomerase G (cyclophilin G)	0.268
COL7A1	Collagen, type VII, alpha 1	0.297
RPL37A	Ribosomal protein L37a	0.301
SLC12A6	Solute carrier family 12 (K/Cl transporters), member 6	0.341
CXorf9	Human DNA sequence on Xq25-26.1	0.353
CACNA1A	Alpha1A-voltage-dependent calcium channel	0.363
RBM9	**RNA binding motif protein 9**	0.374
COL11A2	Collagen, type XI, alpha 2	0.376
(AK024602)	Transcribed locus, similar to gasdermin domain	0.380
SLC12A5	**Solute carrier family 12, (K-Cl transporter) member 5**	0.381
MGC29816	Hypothetical protein MGC29816	0.409
ROBO4	Roundabout homolog 4, magic roundabout (*Drosophila*)	0.414
DKFZp547D104	Acyl-CoA synthetase long-chain family member 6	0.415
HLA-DRB4	Major histocompatibility complex, class II, DR beta 4	0.418
F7	Coagulation factor VII	0.472
PHC3	Polyhomeotic like 3 (*Drosophila*)	0.501

Figure 3: Identification of most significant differentially-expressed ET genes. Gene selection was calculated for each gene by applying a 5-fold cut-off and a computed t-statistic, using the aggregate (N=2906) gene list (a), or the list of platelet-restricted (N=126) genes (b). c. The list of 40 genes (20 over-expressed, 20 under-expressed) demonstrating greatest differential expression by one-way ANOVA is presented, ranked by the ratio of the mean group normalized signals (ET:Normal); shaded column delineates genes with highest expression in ET whereas unshaded column refers to genes with lowest expression. Genes are delineated by gene symbol, or if unassigned, are specified by GenBank ID in parentheses. Genes in bold are platelet-restricted; 2/12 genes in Panel b (*ATOX1*, *LAPTM4B*) are not depicted because they are present in Panel c.

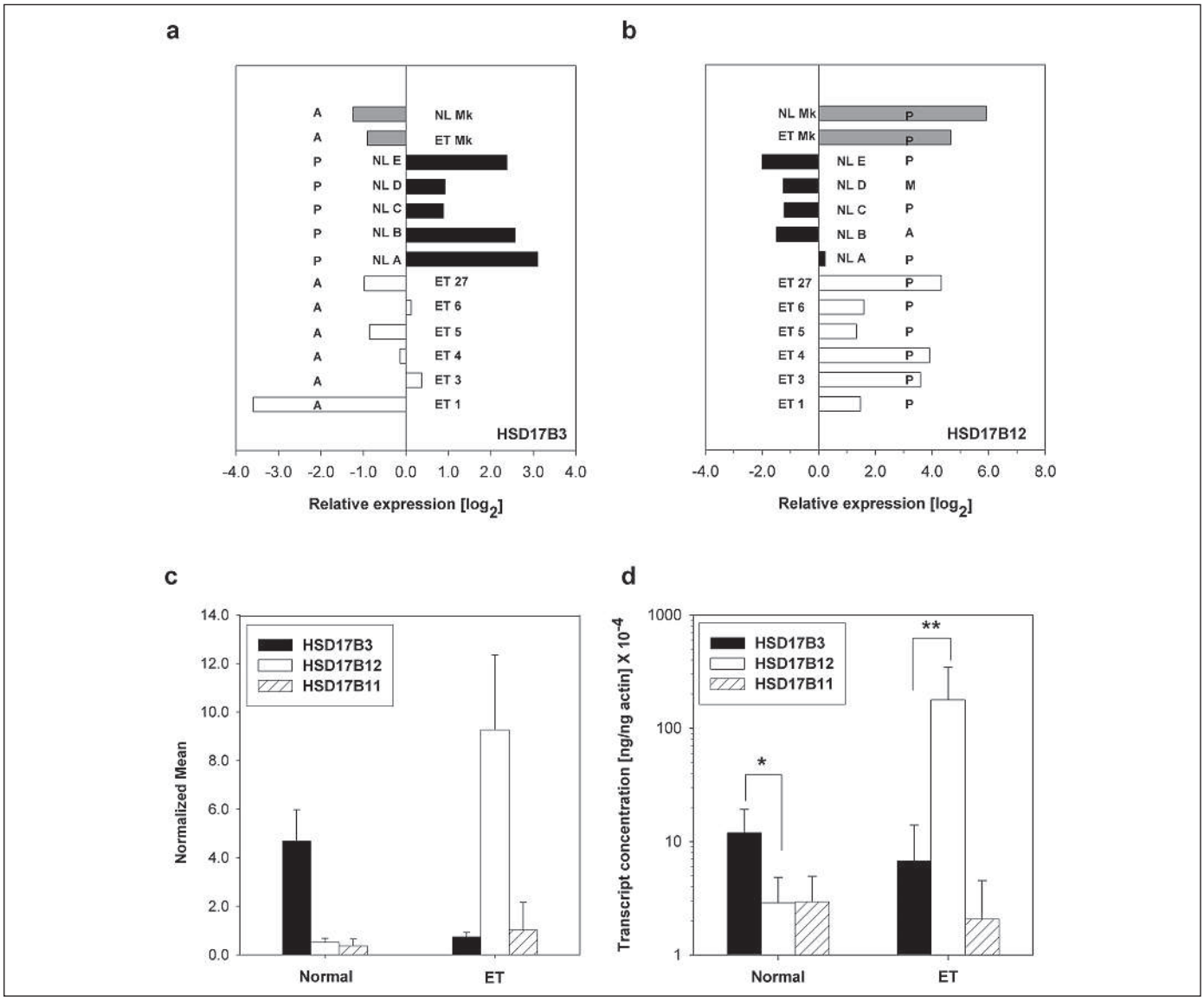


Figure 4: Transcript analysis of platelet-expressed *HSD17Bs*. Normalized microarray values for individual patients (Panels a, b) or the normalized aggregate means by group (c) are shown. In Panels a (*HSD17B3*) and b (*HSD17B12*), expression levels are $\log_2$-transformed, such that negative numbers reflect decreased expression compared to the normalized mean of 14 chips (6 ET, 5 normals, 3 WBC); for individual patients, delineation of transcript expression using Affymetrix MAS 5.0 software is specified (P – *present*; M – *marginal*; A- *absent*). The megakaryocyte (Mk) data in (a) and (b) were downloaded from the GEO platform GPL96 database, and analyzed using GeneSpring software. d. Quantitative RT-PCR was completed on the original cohort of 6 ET patients and a new cohort of 10 normal controls (5 males, 5 females) using *HSD17B3*-, *HSD17B11*-, *HSD17B12*-, or *F7*-specific oligonucleotide primers (platelet coagulation factor VII (*F7*) is primarily endocytosed from plasma with negligible to no platelet mRNA expression (34), thereby establishing the lower limit of assay sensitivity [$1.1 \times 10^{-5} \pm 2.6 \times 10^{-7}$ ng/ng actin, *not shown*]). *p ≤0.03; **p ≤0.001.

Thus, the high level expression of *HSD17B12* transcript in ET platelets is unassociated with overall enzymatic capabilities in androgen biosynthesis. To date, the potential role of 17β–HSD12 in human steroidogenesis remains unknown, although our data would suggest that its substrate specificity is distinct from that of 17β–HSD3. Phylogenetic analysis of the recently identified *HSD17B3* and *HSD17B12* orthologs from the zebrafish *Danio rerio* (26) suggest an evolutionarily conserved and potentially important physiological role for *HSD17B12*, although its precise function remains speculative and offers little insight to the function of its human ortholog.

Discussion

Our data provide the first evidence that distinct subtypes of the steroidogenic 17β–HSDs are functionally expressed in human blood platelets, and that the reciprocal expression patterns of *HSD17B3* and *HSD17B12* are distinctly associated with a rare platelet disorder manifest by quantitative and qualitative platelet defects. In all ET patients studied to date, a simple and reliable assay predicts the ET phenotype, providing a potentially diagnostic molecular marker for this disease; to date, we have insufficient evidence that this assay can distinguish ET from reactive

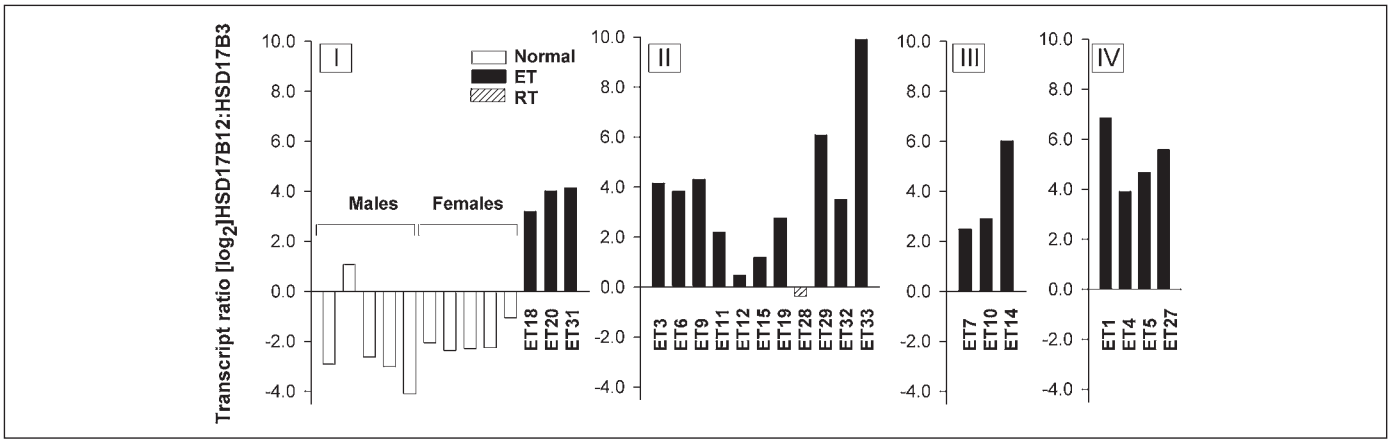


Figure 5: Genetic assays of normal and ET platelets. a. qRT-PCR was completed on platelets isolated by routine phlebotomy (10 ml) from ET or normal controls, using *HSD17B*-specific primers as outlined in Fig. 4d. The (log_2) ratio of *HSD17B12:HSD17B3* transcript expression is depicted for individual patients, categorized by platelet count (10^9/L) at time of platelet isolation; Category I: 0 – 350; Category II: 351–675; Category III: 676–999; Category IV: >1,000 (refer to Table 1 for detailed patient characteristics). Note that three Category I ET patient had normalized platelet counts with treatment at the time of analysis, and retained an *HSD17B12:HSD17B3* ratio predictive of the ET phenotype; the single patient with secondary thrombocytosis (RT) studied to date had a normal ratio.

thrombosis. Given the broad etiologies for thrombocytosis, a more comprehensive study is currently being designed for large cohort analysis and applicability.

What is the biological relevance of 17β–HSD expression in platelets, and is this causally associated with the molecular defect regulating megakaryocytopoiesis and/or proplatelet formation? Platelet production is complex, but is clearly affected by endogenous or exogenous sex hormones. Androgens improve platelet counts in patients with distinct types of thrombocytopenia (27), and diminished thrombopoiesis is evident in castrated mice, with correction upon testosterone supplementation (28). Furthermore, murine Mk retain the machinery for estradiol synthesis *in vitro*, and the secreted hormone's ability to bind estrogen receptor β triggers proplatelet formation in an autocrine manner (23). Although we did not characterize *HSD17B* expression in Mk, diminished platelet *HSD17B3* transcripts (with exaggerated *HSD17B12* expression) presumably reflects comparable changes in Mk. While *HSD17B3* transcript is down-

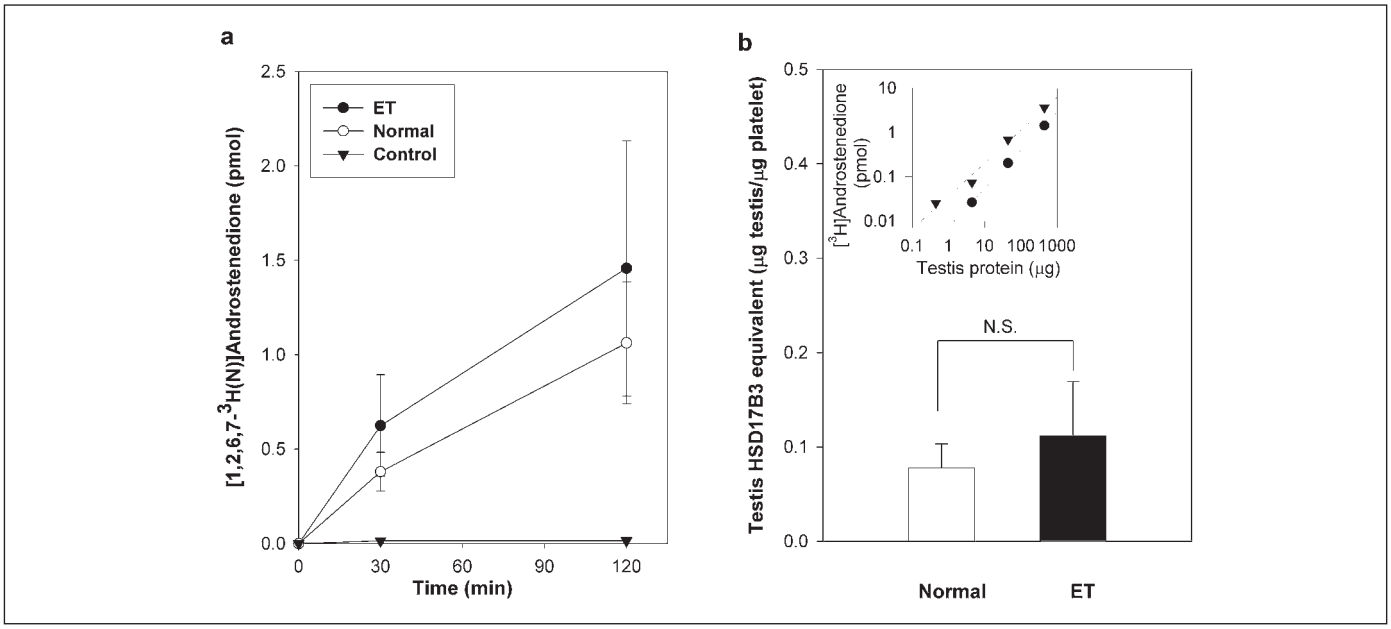


Figure 6: Functional 17β-HSD assays in platelets. Lysates of platelets isolated from 3 normal or 3 ET patients were used for determination of functional 17β-HSD3 activity at distinct time points, and expressed as standardized mean ± SEM (control is HSD buffer alone). In Panel b, curves generated for 30-minute (●) or 120-minute (▲) 17-βHSD3 assays (inset) were used as standards for comparative determination of platelet 17β-HSD3 assays performed in parallel. Data are the mean ± SEM from 6 determinations; note that 0.1 testis equivalent units correspond to 10% of the activity found in protein equivalent of mouse testes.

regulated in ET platelets, the changes are not as pronounced as those of *HSD17B12* where transcript expression is ~25-fold higher than in normal platelets. Despite this exaggerated increase, our data demonstrate that *HSD17B12* induction is unassociated with enzymatic changes in total platelet 17β–HSD3 activity. Thus, the function and substrate specificity of human 17β-HSD12 appears distinct from that of 17β–HSD3, and elucidation of its substrate(s) may provide further insights into the roles of 17β–HSDs in platelet and/or Mk functions.

Human platelets express both androgen and estrogen β receptors, and platelet function is known to be modulated by gender differences (29), the menstrual cycle (30), and exogenous testosterone (31). Similarly, hormonal replacement in postmenopausal females, or oral contraceptive use in menstruating females are known to predispose to thrombotic diseases (32, 33), although the mechanism(s) of this effect remain unclear. These effects could be mediated at either genomic (Mk transcriptional activity regulated by hormonal/receptor binding) or nongenomic (non-transcriptional) levels, although an interplay at both levels is equally plausible. The evidence that human blood platelets retain 17β-HSD3 activity, express distinct subtypes of 17β-HSD enzymes, and demonstrate altered *HSD17B* expression patterns in a disorder known to be association with thrombohemorrhagic risk, provides novel insights into the interplay between sex hormones, platelet function, and vascular diseases, both cerebro- and cardiovascular.

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Accession numbers

All microarray data were submitted to the GEO database in MIAME-compliant form, reported under the accession number GPL1716.

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Mapping the platelet proteome: a report of the ISTH Platelet Physiology Subcommittee

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Summary. Proteomic technology has the potential to transform the way we analyze platelet biology, through the determination of platelet protein composition and its modification upon stimulation and with disease. We are a considerable way from achieving these goals, however, because of significant limitations in current methodology. It is therefore important to consider the extent to which these aims can be met and the way that proteomic data should be presented and used. These issues are discussed in the present paper by the Platelet Physiology Subcommittee of the ISTH Scientific Standardisation Committee (SSC). It is recommended that proteomic information be combined with data from other experimental approaches to establish a database on protein expression and function in platelets.

Introduction

The proteomic revolution has been driven by the ability of mass spectrometry techniques to identify and map partial protein amino acid sequences and thereby enable predictions of protein identification. Potentially, this approach could lead to mapping of the full protein composition of a cell, including levels of protein expression and sites of post-translation modification, e.g. phosphorylation. Importantly, proteomics can further be used to investigate how these events are altered upon stimulation and with disease. This is vital information on which to build our understanding of platelet biology, with the ultimate goal of defining the function of every platelet protein.

There are however considerable hurdles to be overcome in realizing these aims and a significant potential for errors to accrue with regard to protein identification and modification. The present article discusses the reasons underlying these issues, and considers the way in which proteomic information should be presented and used. The need for caution and independent evidence supporting expression and function is emphasized.

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To what extent can we use proteomics to map the platelet proteome?

Proteomic technology involves either fractionation of proteins, digestion into peptides and sequencing by mass spectrometry or immediate protein digestion, fractionation of peptides and then sequencing [Multidimensional Protein Identification Technology (MuDPit)]. Purification is an essential step in mapping the full proteome because of the wide range of protein levels within a cell, which vary by up to 8 orders of magnitude. As a consequence, competition for sequencing between peptides will favor those derived from highly expressed proteins over other, often very interesting but lower expressed proteins, such as kinases and receptors. Significant sequence coverage is also only achieved for proteins that are present in high amounts, and this coverage is seldom more than 50%. Depending on the criteria and methodology, the largest group of proteins is often those identified on the basis of a single peptide [1]. For these and other proteins with low coverage, there is a need for independent confirmation of expression. Thus, at present, we are a long way from achieving the goal of mapping all proteins within a cell.

Protein purification plays an important role in facilitating protein identification. The more concentrated the protein in the mixture, the more likely that sequence information will be obtained. The partial or full purification of a protein sample, for example through isolation of a cellular organelle or by affinity chromatography, can have a dramatic effect on protein

identification. The most frequently used high throughput platform for protein identification is two-dimensional (2D) gel electrophoresis, based on sequential isoelectric focussing (IEF) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) steps. This technique not only favors identification of highly expressed proteins, but is also capable of detecting proteins over several orders of magnitude using high-resolution 2D gels and very sensitive protein dyes. There are however special problems associated with 2D gel electrophoresis in identifying hydrophobic, basic and high-molecular-weight proteins. Both 1D gel and non-gel-based methods can be used to identify these groups of proteins, but with the caveats of increasing the complexity of the peptide mixture and favoring identification of the most highly expressed proteins. In addition, this approach loses valuable information on p*I* values which facilitates protein identification and provides information on possible post-translational modifications. Alternative methodologies that identify subgroups of peptides, such as those that contain cysteine residues, have the advantage of providing a less-complex peptide mixture for analysis, although information is limited by the number of peptides recovered. Given the above considerations, several complementary proteomic approaches will usually be required to map the composition of a complex sample.

There is a considerable potential for error and misinformation in proteomics that can severely hamper research. This includes errors relating to sample preparation and contamination, peptide sequencing and database analysis as illustrated below. It is therefore important to consider the methodological aspects relating to platelet preparation, and the way that we should present and interpret proteomic information. The latter is of particular relevance in view of the recent plethora of proteomic publications on platelets that has been driven by the relative ease of access to material and the important role of platelets in atherothrombotic and inflammatory disease. It is however beyond the scope of this article to provide full experimental details on the way to prepare and analyze platelets for analysis, and there is also unlikely to be widespread agreement on this in view of the differing ways that platelets are prepared in experimental laboratories throughout the world. A number of the factors that must be taken into consideration in the proteomic analyses are highlighted below.

1 *Platelet preparation*. It is essential to minimize the degree of platelet activation during blood collection and cell isolation to limit activation-dependent changes in post-translation modification, e.g. protein phosphorylation. Special consideration should be given to the use of prostacyclin and other cAMP-elevating agents during the isolation procedure as their use will generate new protein forms through phosphorylation.
2 *Protein extraction*. Experimental reactions should be stopped as quickly as possible to minimize post-activation changes in proteins, notably degradation. Reactions should be terminated rapidly, e.g. by rapid freezing, and a cocktail of protease inhibitors should be used, although this complicates the use of MuDPit through the introduction of new peptide species.
3 *Platelet purity*. The degree of contamination of the platelet sample with other cell types and plasma should be kept to a minimum. This is of particular significance in mapping proteins that are expressed at low levels, as contamination could lead to identification of more highly expressed proteins in other blood cells or plasma. Sample purity applies also to use of polymerase chain reaction (PCR)-based techniques in analysis of gene identification in platelets. It may be prudent to use the same method of platelet preparation in proteomic and real time (RT)-PCR-based analyses, especially as the latter can be used to look for tissue-specific genes (and therefore give an indication of purity).
4 *Endogenous and exogenous proteins*. Platelets have the ability to take up proteins from the surrounding medium via surface receptors and through endocytosis, and also bind plasma proteins on their surface. The protein composition of the platelet and profile of expressed genes are therefore distinct. Factors that influence uptake of proteins and protein binding (e.g. buffer) will alter the composition of the platelet proteome and must be appreciated by the investigator.
5 *Peptide sequencing*. Errors can accrue in peptide sequencing as a result of ambiguous patterns of fragmentation, necessitating use of stringent criteria on which to base acceptance of expression. However, because of variations between the resolution power of mass spectrometers and differences in methodology, it is difficult to arrive at absolute guidelines on this subject. It should therefore be recognized that there is a potential for error in peptide sequencing, albeit one that is low ($< 5\%$) [1], and that this is governed by the criteria used for acceptance of the predicted sequence. The potential for error can be lessened by manual inspection of records and through use of other supporting information, if available, such as molecular weight and p*I* values. There is also the issue of splice variants, which cannot be predicted from genomic databases.
6 *Protein databases*. There is a potential for error in searching protein databases such as NCBI, SWISS-PROT and TrEMBL. For example, not all proteins are present and the databases themselves almost certainly contain errors, although considerable efforts are being made to correct these and other groups such as HuPO and IPI are attempting to produce better databases. Nevertheless, the predictive value of sequenced peptides does not take into account factors such as alternative splicing, polymorphisms and post-translational modifications.

How should we use proteomic information?

It is important to consider the extent to which we can rely on proteomic information to establish expression of a protein in a cell. For highly expressed proteins, this is not a major issue, as such proteins are routinely picked up in a variety of proteomic-based methods and through repeat studies, with a large degree of supporting peptide sequence information being obtained. It is generally accepted that a sequence of eight amino acids or more is sufficient to serve as a unique

identifier of a protein, although not all peptides of this length will map to a single protein. Bearing in mind the potential for a sequence error, however, an increased degree of confidence in protein identification is gained through identification of two or more unique peptides of this length. The probability of obtaining two different sequences for the same protein from a sample via sequencing errors is extremely low, although this is dependent on the stringency of the acceptance criteria for a peptide.

Special consideration needs to be given to proteins that have been identified on the basis of a single peptide, the so-called 'one-hit wonders' [1]. This group of proteins often makes up the largest group of identified proteins in a study. While it is important to report this information available, it is even more critical that such data be published with the clear statement that supporting information is required to confirm expression. The field will move backward, not forward, if it has to spend an inordinate amount of time correcting mistakes.

Current status of platelet proteomic studies

Several reports on the mapping of the platelet proteome have now been published, most notably over the last twelve months. These reports have used a variety of approaches, including classical 2D gel electrophoresis [2,3], combined fractional chromatography (COFRADIC), gel chromatography based on labelling of specific amino acids or regions in the protein, e.g. N-terminal labelling [4,5], or through use of MuDPit, which avoids the need for gel chromatography [6,7]. The largest number of proteins identified in a single approach is just over 400, via 2D gel chromatography using narrow-range IEF gels [2,3]. The other approaches have identified 80–250 proteins. However, while the quantity is impressive, the relative quality of the data from these different approaches will only be understood over time.

There is a considerable degree of overlap in the types of proteins that have been found in these studies. This includes groups of proteins that are expressed at high level, such as those involved in the regulation of the cytoskeleton and in protein synthesis. Proteins are also carried over from the megakaryocyte and this may be of particular relevance for the latter group, since platelets have a minimal capacity for supporting protein synthesis. These studies have also identified many unique proteins, some of which have only previously been recognized as hypothetical proteins in the database, and so provided first reported expression in any cell. However, many of these identifications have been based on a single peptide sequence necessitating extreme caution as discussed above.

An impressive feature of these studies is the number of proteins that have not been previously reported in platelets, which is estimated to be in the order of 50% of those identified. On the contrary, it is of concern that several major regulatory groups of proteins are absent, such as G protein-coupled receptors and key signaling molecules, e.g. phospholipases and tyrosine kinases. This is likely to reflect their low level of expression as well as other factors that interfere with some methodologies, e.g. the hydrophobicity of membrane proteins is a recognized problem in 2D gel electrophoresis.

Recommendation for mapping of the platelet proteome

It is not possible, at present, to establish a specific set of criteria on which to base acceptance of protein expression through proteomics because of the widespread variation in factors such as the sensitivity of mass spectrometers, mode of sample preparation and so forth. This has been discussed in a recent editorial in *Molecular and Cellular Proteomics*, which also provides important guidelines on the way that proteomic information should be presented [8]. We are in full support of the adoption of these guidelines in the reporting of platelet proteomic information. However, until strict criteria are established on which to base acceptance of expression, the Platelet Physiology Subcommittee strongly recommends that information derived from other techniques be used to support proteomic data. The information on protein expression should be compiled into a database as follows:

1 *Proteins that are known to have a functional role in platelet regulation*. There are many proteins that are known to play an important role in platelet biology, whether or not they have been identified through proteomics. Such information has been obtained through studies on mutant mice, use of pharmacological-specific agents etc. Examples of proteins in this class include G protein-coupled receptors and signalling proteins.
2 *Proteins of unknown function, but for which expression is beyond doubt*. Expression of many proteins in platelets has been established through protein chemistry and related techniques, such as flow cytometry and radioligand binding, even though their function remains unclear. This group almost certainly includes proteins that have been 'carried over' from the megakaryocytes, which may or may not have a functional role in platelets, e.g. transcription factors.
3 *Proteins identified only by proteomic and/or PCR-based techniques*. The largest group of proteins have been identified solely on the basis of proteomic and genomic technologies. In such cases, the need for independent supporting information on expression such as Western blotting of demonstration of function is necessary.

The database will serve to guide development of platelet-specific antibody and gene microarrays that could be used to study individuals with platelet- and megakaryocyte-related disorders. These platelet-specific arrays have several advantages over the use of proteomics as a high throughput platform for patient-based studies, including a focus on proteins that are known to play important roles in regulating platelet reactivity.

Future developments

It is interesting to speculate on the number of proteins that are expressed in platelets, and the proportion of these that are

likely to be of functional importance. The number of proteins corresponding to separate gene products that have been identified in platelets using proteomics is approaching 1,000, which is a considerable achievement. However, this is a fraction of the number of platelet-based genes predicted using microarrays, which is in the order of 4–6000 [9–11], although not all of these mRNAs may have been translated. Proteomics has also failed to identify many important platelet proteins, including surface receptors, protein kinases and signalling proteins, and so this technology presently falls far short of serving as a high-throughput approach for identifying platelet proteins.

The analysis of post-translational modifications of proteins in basal and activated platelets brings in a further level of complexity in the analysis, but has the important potential to provide novel information on factors such as degradation and protein phosphorylation [12]. Analysis of non-activated platelet by 2D gel electrophoresis has indicated that expressed genes are, on average, represented by 2.5 features (i.e. distinct spots that are detected with the dye), although this ratio is likely to be much higher in a stimulated cell (and will also be dependent on the nature of the stimulus). Even under basal conditions, a small number of proteins could be resolved into as many as ten separate features or more suggesting extensive protein modifications [3]. It will take a considerable time and effort to map the nature of these changes, and we are far from being able to achieve this at present.

A further issue is that of quantitative proteomics. This is likely to be the major way of applying the technology to patient samples, for example in analyzing specific diseases and providing a peptide map that could be used in phenotyping patients. There are also a number of technologies, including isotope-coded affinity tag (ICAT), mass-coded abundance tagging (MCAT) and ^{18}O-labeling, that could be used for investigating the effect of drug treatment by comparing samples. However, the ultimate goal is to compare samples from individual patients which would require absolute quantitation. We are a considerable way from being able to achieve this at present, and it is highly likely that other techniques such as antibody arrays will also have to be used to achieve this.

An urgent immediate goal is to establish a database that will describe proteins expressed in platelets and the way they change upon activation and in disease. The Platelet Physiology Subcommittee recommends the setting up of a database overseen by a subgroup to evaluate evidence for protein expression and modification, with all related information maintained in the public domain. It will be important for the database to be maintained in collaboration with the widely available and established databases such as SwissProt, HuPO and the International Protein Index of EMBL-EBI. The need for caution is stressed, but the database will serve as a powerful resource to further our understanding of platelet biology.

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Addendum of the Editor-in-Chief

This paper has been submitted as a regular manuscript and was accepted after peer-review, but since it is an SSC Communication we chose to put it in the corresponding section in spite of its length (exceeding 1000 words).

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