Large-scale identification of functional microRNA targeting reveals cooperative regulation of the hemostatic system

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To cite this article: Nourse J, Braun J, Lackner K, Hüttelmaier S, Danckwardt S. Large-scale identification of functional microRNA targeting reveals cooperative regulation of the hemostatic system. J Thromb Haemost 2018; 16: 2233–45.

Essentials
- MicroRNAs (miRNAs) regulate the molecular networks controlling biological functions such as hemostasis.
- We utilized novel methods to analyze miRNA-mediated regulation of the hemostatic system.
- 52 specific miRNA interactions with 11 key hemostatic associated genes were identified.
- Functionality and drugability of miRNA-19b-3p against antithrombin were demonstrated in vivo.

Summary. Background: microRNAs (miRNAs) confer robustness to complex molecular networks regulating biological functions. However, despite the involvement of miRNAs in almost all biological processes, and the importance of the hemostatic system for a multitude of actions in and beyond blood coagulation, the role of miRNAs in hemostasis is poorly defined. Objectives: Here we comprehensively illuminate miRNA-mediated regulation of the hemostatic system in an unbiased manner. Methods: In contrast to widely applied association studies, we used an integrative screening approach that combines functional aspects of miRNA silencing with biophysical miRNA interaction based on RNA pull-downs (miTRAP) coupled to next-generation sequencing. Results: Examination of a panel of 27 hemostasis-associated gene 3′UTRs revealed the majority to possess substantial Dicer-dependent silencing capability, suggesting functional miRNA targeting. miTRAP revealed 150 specific miRNA interactions with 14 3′UTRs, of which 52, involving 40 miRNAs, were functionally confirmed. This includes cooperative miRNA regulation of key hemostatic genes comprising procoagulant (F7, F8, F11, FGA, FGG and KLKB1) and anticoagulant (SERPINA10, PROZ, SERPIND1 and SERPINC1) as well as fibrinolytic (PLG) components. Bioinformatic analysis of miRNA functionality reveals established and potential novel links between the hemostatic system and other pathologies, such as cancer, bone metabolism and renal function. Conclusions: Our findings provide, along with an in-vivo proof of concept, deep insights into the network of miRNAs regulating the hemostatic system and present a foundation for biomarker discovery and novel targeted therapeutics for correction of de-regulated hemostasis and associated processes in the future. A repository of the miRNA targetome covering 14 hemostatic components is provided.

Keywords: anticoagulants; haemophilia; hemostasis; microRNAs; therapeutics; thrombosis.

Introduction

MicroRNAs (miRNAs) are short non-coding RNAs that post-transcriptionally regulate target genes, acting as fine-tuners of gene expression [1]. miRNAs are transcribed as primary transcripts, then processed by Drosha and Dicer to produce mature miRNAs [2]. These are incorporated into an RNA-induced silencing complex (RISC) consisting of Argonaute proteins [3], which targets mRNA, resulting in translational inhibition and/or transcript degradation [4]. In general, miRNAs tend to bind the 3′ untranslated regions (3′UTR) of messenger (m)RNAs, while to a lesser extent, miRNAs can also carry out their inhibitory function by binding to the coding region or the 5′UTR of target mRNAs [4]. miRNAs regulate the majority of human genes [5], with a single miRNA capable of targeting multiple genes, allowing broad regulation of molecular networks [6].

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Additionally, multiple miRNAs can cooperatively silence a single gene to gain regulatory specificity, with the targeting of particular network hub genes enabling the regulation of entire pathways [7]. Beyond their involvement in the control of various physiological mechanisms, miRNAs have been implicated in numerous pathophyslogies, including coagulation and hemostasis (Table S1). Further, they constitute targets for therapeutic intervention, with miRNA therapeutics currently the subject of several clinical trials [8], and offer potential as biomarkers for diagnosis and prognosis [9].

The hemostatic system controls the initiation and localization of blood clotting at sites of vascular injury and involves at least 50 components [10]. Thromboembolic conditions resulting from unresolved clotting, including myocardial infarction, ischemic stroke and venous thromboembolism (VTE), constitute a leading global cause of mortality, accounting for an estimated one in four deaths worldwide [11]. However, current therapies, including new oral anticoagulants (NOACs), used to prevent and treat thrombosis are associated with bleeding side-effects [12], and as such the quest for novel therapeutic strategies for prevention and treatment is ongoing [13], particularly for the growing population with comorbidities, including patients with moderate-severe renal failure, hepatic failure or mechanic heart valve, or patients under therapy with CYP3A4- and P-glycoprotein inhibitors [14–17]. Further, there is a lack of prospective markers for efficient prediction of recurrence of VTE, which could help stratify high-risk patients requiring more intensive treatment.

Although the implications of hemostatic factor plasma levels for disease have been widely described, expression regulation of hemostatic factors remains incompletely understood. In addition, despite the ubiquitous involvement of miRNAs in almost all biological processes, no extensive screening of coagulation factors has been carried out and their global role in blood coagulation has remained surprisingly poorly defined.

Here we advance the understanding of miRNA regulation in hemostasis in an extensive and unbiased manner. Previous studies have relied on in silico prediction tools to identify potential miRNA–target interactions; however, for large-scale studies these approaches suffer from impractical levels of false positive and negative predictions [18,19]. Further, other genetic events such as RNA editing after 24 h. For Dicer-rescue assays 100 ng reporter plasmid transfection reagent (Thermo Fisher Scientific) and assayed after 24 h. For Dicer-rescue assays 100 ng reporter plasmid and 100 ng pCAGGS-Flag-hsDicer [21] or empty vector were transfected into 2.5 × 10^5 MEF-Dicer-null-G5 cells in 75 μL in 96-well plates using 0.5 μL TurboFect transfection reagent (Thermo Fisher Scientific) and assayed after 24 h. For miRNA-mimic-rescue assays 100 ng reporter plasmid and 50 nmol L^-1 miRNA-mimics or miRNA-mimic Negative Control #1 (miRIDIAN; Dharmacon, Cambridge, UK) were transfected into 2.5 × 10^5 MEF-Dicer-null-G5 cells in 75 μL in 96-well plates using 0.4 μL DharmaFECT Duo transfection reagent (Dharmacon) and assayed after 24 h. For assays involving two miRNA-mimics, both were

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transfected at 50 nmol L⁻¹ and compared to controls transfected with 100 nmol L⁻¹ miRNA-Mimic Negative Control #1. Luciferase assays were performed using the Dual-Glo Luciferase Assay System (Promega). Luciferase activity was measured using a Fluoroskan luminometer (Thermo Fisher Scientific). Firefly luciferase activity was normalized to reporter internal renilla luciferase levels and silencing reported as fold of 3'UTR-containing reporter over empty pmirGLO reporter levels. As Dicer-rescue exhibited variability in silencing, values were normalized to a reference reporter for each replicate experiment (Figure S1). For Dicer and miRNA-mimic-rescue assays, impact of the rescue was determined as 1 + (Rescue – Null) (Fig. 1B). Significant silencing was defined as having a silencing of > 5% and a one-sample t-test against 1.0 P-value of < 0.1.

**miTRAP in vitro RNA affinity assay**

miTRAP (miRNA trapping by RNA in vitro affinity purification) was performed as described by Braun et al. [22]. Briefly 3'UTRs were cloned into an MS2 fusion vector and bait RNA in vitro transcribed using the RiboMAX System (Promega). Bait RNA was immobilized to amyllose resin (New England Biolabs (NEB) GmbH, Frankfurt Main, Germany) via recombinant MBP-MS2BP protein and incubated with HuH-7 cell extracts. Following washing, bound complexes were eluted and miRNAs purified by phenol-chloroform extraction. miRNA was prepared for sequencing using the TruSeq Small RNA Kit (Illumina GmbH, Munich, Germany) and sequenced using the HighScan-SQ (Illumina). Pull-downs were performed in experimental duplicates. Reads were demultiplexed and trimmed and reads of 15–27 nucleotides aligned to human mirBase v21 using bowtie with an error rate of 1 nt per mature miRNA sequence being allowed. As the fold enrichment and significance parameters of functional miRNA/3'UTR interactions were not known it was decided to define cut-off values to result in a large, yet feasible number of candidate interactions that could then be further tested by the miRNA-mimic-rescue assay. This resulted in the selection of parameters of functional miRNA/3'UTR interactions.
of the criteria of at least 5-fold enrichment and t-test \(P\)-value of at least 0.165 of UTR over the empty MS2 control bait, which selected 149 interactions involving 96 miRNAs for further study.

**Western blotting**

For Dicer western blotting, Dicer-null MEFs were transfected with either pCIneo-hEST2 or the empty pCIneo vector. Twenty-four hours after transfection, cells were lysed and used for western blotting using an anti-Dicer rabbit polyclonal antibody (Bethyl A301-936A, 1:2000 dilution), which detects both mouse and human Dicer. Equal protein loading levels were determined by Ponceau S staining of the membrane after transfer. For protein recovery from miTRAP pull-down assay, 15 \(\mu\)L of maltose elute was used. Ago2 was detected using anti-Ago2 rabbit monoclonal antibody (ab156870, 1:500 dilution; Abcam, Berlin, Germany).

**Bioinformatic analysis**

miRNA target prediction algorithms were accessed via miRWalk (http://mirwalk.umm.uni-heidelberg.de) and limited to miRNAs expressed in our HuH-7 cells as determined by sequencing input lysate used in the miTRAP assays. Potential miRNA seed sites, as defined by Bartel [23], were identified within the 3' UTR sequences using the Motif feature of the Sequencher package (Gene Codes, Ann Arbor, MI, USA). For cluster analysis, miTRAP fold enrichment data were \(\log_2\) transformed and subjected to unsupervised hierarchical clustering based on Pearson uncentered distance and complete linkage agglomeration using the Genesis Suite [24]. For miRNA target enrichment analysis, miRNAs demonstrated to specifically target either procoagulant or anticoagulant gene 3'UTRs were then used for enrichment analysis against the disease terms of the Genetic Association Database [26] (GAD) using the DAVID bioinformatics resource [27].

**In vivo validation of miRNA functionality**

Seven-week-old C57BL/6J mice (Janvier Labs, Le Genest-Saint-Isle, France) were intravenously injected with fully phosphorothiolated LNA oligonucleotides (Exiqon, Vedbaek, Denmark) at 25 mg kg\(^{-1}\) bodyweight in phosphate buffered saline (PBS). After 6 days, citrated plasma was obtained, diluted 1/8 with calcium and magnesium-free PBS and antithrombin activity determined using the HemosIL Antithrombin assay (Instrumentation Laboratory). TOP 700 automatic hemostasis analyzer (Instrumentation Laboratory).

**Results**

miRNA-dependent 3'UTR-mediated silencing impacts expression of genes involved in the hemostatic system

The 3'UTR plays an important role in miRNA-mediated control of gene expression [4]. Accordingly, the impact of 3'UTR-mediated regulation on hemostasis was first assessed. An initial panel of 23 genes was selected based on involvement in clot formation or dissolution, pathological significance and expression in the liver (Table S2). Limitation of this panel to liver-derived factors was implemented, as this organ is the source of the majority of hemostatic factors, and to date hepatic delivery of therapeutic oligonucleotides has been the area with greatest progress [28]. From this panel, 27 unique 3'UTRs, including gene isoform variants (Table S3), were cloned into luciferase reporter constructs to enable assessment of 3'UTR activity, independent of the cellular expression level of the target gene. Here, silencing of expression resulting from factors interacting with the 3'UTR fused to the luciferase coding region can be measured by reduction in light output from luciferase enzymatic activity. Transfection of these constructs into the human hepatic cell line HuH-7 resulted in a wide range of activity (Fig. 1A), ranging from decreases in luciferase activity, suggestive of possible miRNA silencing interactions, to increases in activity, reflective of stabilization of the reporter mRNA and potentially resulting from interactions with stabilizing RNA binding proteins (RBPs, e.g. HuR). To exclude the possibility that scavenging of miRNAs by highly expressed endogenous transcripts (e.g. fibrinogens) may reduce levels available for binding to reporter transcripts, the relationship between 3'UTR silencing (Fig. 1A) and HuH-7 expression of the corresponding gene was examined (Figure S2). This revealed no significant correlation (Pearson \(r = 0.1635\), \(P = 0.4058\)), suggesting miRNAs are not exhausted by endogenous transcripts. This implies the reporter would reflect actual silencing, although these numbers may represent conservative estimates. Altogether these results indicate post-transcriptional modulation via the 3'UTR plays a significant role in the expression of many of these genes.

Control of gene expression via the 3'UTR is mediated by miRNAs as well as other UTR-interacting components, such as RBPs [29]. To determine the miRNA-dependent impact on the 3'UTR panel, the role of Dicer in the biogenesis of mature miRNAs [30] was exploited. To this end, a Dicer-rescue assay was developed employing Dicer-null MEFs, previously demonstrated to lack almost all miRNAs [31] (Fig. 1B). Here, co-expression of Dicer with the luciferase reporter construct restores miRNA levels, allowing assessment of the impact of all cellular miRNAs on the 3'UTR. The performance of this
miRNA regulation of the hemostatic system

Dicer-rescue assay was confirmed using luciferase reporter constructs containing reverse complement miRNA sequences to functionally demonstrate miRNA restoration with Dicer-rescue (Fig. 1C). Application of this assay to the 3′UTR reporter panel described above revealed the majority of 3′UTRs to possess substantial Dicer-dependent silencing capability (Fig. 1D).

Consistent with observations that miRNA sites preferentially cluster at the boundaries of 3′UTR ends (i.e. near the stop codon and polyadenylation site), whereas RNA binding protein sites are organized in clusters throughout the 3′UTR [32,33], Dicer-independent but not Dicer-dependent silencing was found to strongly correlate with UTR length (Figure S3).

Implementation of a novel integrative screening approach identifies a large number of novel hemostatic miRNA/3′UTR interactions

To identify specific miRNA/3′UTR interactions, an unbiased screen using the miTRAP [22] in vitro RNA affinity assay (Fig. 2A) was performed on a panel of 14 3′UTRs. In this assay miRNAs are identified by their binding to in vitro transcribed and immobilized 3′UTR RNA probes, followed by next-generation sequencing of bound miRNAs. Selection for inclusion in the miTRAP assay was based on the observation of strong silencing in the Dicer-rescue assay (Fig. 1D), suggestive of significant miRNA interaction with the UTR. In addition, UTR selection was further limited based on the UTR being a major expressed isoform, and thus highly silenced minor isoforms (such as FGA-alphaE and PLGv2) were not selected. Capture of functional miRNA by this assay was confirmed by western blotting for the Ago2 miRNA silencing complex [3] (Fig. 2B). Here, substantial correlation of total counts of identified miRNA with silencing complexes bound to bait 3′UTR RNA (as determined by Ago binding) was observed (Pearson $r = 0.71$). Using a criterion of at least 10 counts to one sample, 4466 potential miRNA/3′UTR interactions involving 319 miRNAs were identified, and from these 149 interactions involving 96 miRNAs with silencing complexes bound to bait 3′UTR RNA [32,33], Dicer-independent but not Dicer-dependent silencing was found to strongly correlate with UTR length (Figure S3).

Validation of the identified hemostatic miRNA/3′UTR interactions reveals a high rate of functional miRNAs, reflecting the strength of the miTRAP screening approach

To determine the functionality of candidate miRNA/UTR interactions identified by the miTRAP assay, miRNA-rescue luciferase assays using specific miRNA-mimics transfected along with reporter constructs into Dicer-null MEFs were employed. This alleviates bias arising from endogenous miRNA cellular-expression levels and as both UTR and miRNA are exogenously supplied, these experiments are independent of the endogenous expression levels of both the target gene and miRNA in MEF cells. A clear miRNA-mimic dose–response curve demonstrates the reliability of this assay (Figure S4).

Application of the miRNA-mimic-rescue assay revealed 52 of the selected 149 miTRAP candidate interactions (35%) to exhibit significant silencing activity, indicative of functional targeting of the 3′UTR by miRNA (Fig. 3A). Interestingly, two interactions (miR-193b-3p/PROC and miR-192-5p/F8) resulted in significant enhancement of luciferase activity, suggestive of indirect or competitive interactions with the 3′UTR. The proportion of candidate interactions functionally validated varied greatly between 3′UTRs, with levels ranging from 75% for PROZ to none for FGB and PROC (Fig. 3B). This suggests that specific, as yet unidentified, factors may be independently affecting the functionality of miRNA interactions for each 3′UTR.

In total, despite limited functional validation, we identify 11 hemostatic components to be significantly regulated by multiple miRNAs (Fig. 3C). Although the top quartile of interactions identified by miTRAP enrichment exhibited a significantly higher level of functional validation (58%) than the bottom three quartiles (27%) (Figure S5), this lower level suggests that substantial numbers of potentially strong silencing interactions can be found below our cut-off of 5-fold enrichment (see miRNA targetome, Table S4).

The strength of this experimental set-up is reflected by the abundance of novel interactions identified, with miTRAP identifying six functional interactions not found by the union of predictions from three top bioinformatic algorithms (Fig. 4A, Table S5). Importantly, miTRAP produces a substantially higher rate of true positive interactions in relation to the number of predicted candidate interactions (Fig. 4B).

miRNA targeting via a short perfect match to nucleotides 2–8 in the 5′ end of a miRNA, termed the ‘seed’ region, is regarded to be the most important feature for target recognition [23]. As such, in silico miRNA target site prediction algorithms concentrate on target sites equipped with perfect seed matches. Examining the 3′UTR sequences for potential miRNA seed sites (Fig. 4C, Figure S6) revealed that many miRNAs functionally target hemostatic 3′UTRs that do not possess canonical nor marginal seed sites and are therefore not detected by target prediction algorithms. Conversely, many 3′UTRs possessing marginal and even canonical seeds are not functionally targeted by the corresponding miRNA, thus further demonstrating the limitation of bioinformatic assays and the advantage of the assay system employed here.

miRNAs target hemostatic factors in a cooperative manner

Several genes have been previously described to be targeted by multiple miRNAs [37], with cooperative effects having considerable biological significance [7]. As multiple miRNAs were found to target individual 3′UTRs in this study (Fig. 3C), a proof of concept examination was made combining the two strongest silencing miRNAs,
Fig. 2. Biophysical miRNA interaction screening based on RNA pull-downs (miTRAP) coupled to next-generation sequencing to identify functional 3’UTR/miRNA interactions in the hemostatic system. (A) Schematic of the miTRAP in vitro miRNA pull-down assay. Full description of the assay is presented in Methods. (B) Total counts of miRNA bound to 3’UTR RNA baits as identified by next-generation sequencing as well as miRNA counts of the input lysate used for pull-downs. Ago2 western blotting of protein binding to RNA baits and input lysate as a measure of silencing complexes formed on RNA baits. MS2 refers to pull down using the empty MS2 fusion construct. Input refers to HuH-7 lysate used for pull-downs. (C) Volcano plot of fold enrichment and significance of identified miTRAP miRNA/UTR interactions indicating selection of candidate miRNA/3’UTR interactions for further validation. A complete repository of the miRNA targetome covering 14 hemostatic components is provided in Table S4. [Color figure can be viewed at wileyonlinelibrary.com]
miR-128-3p and miR-375, against the PROZ 3'UTR. Transfection of these miRNAs in combination resulted in significantly increased silencing as compared with the individual miRNAs (Fig. 5A). This confirms that the multiple miRNAs found to target individual genes in this study may act cooperatively to gain regulatory specificity, which could provide a rationale for future targeted therapeutics.

Bioinformatic analysis identifies target-specific miRNAs and regulatory hubs linking hemostatic miRNAs to many novel pathophysiological processes

To dissect the target specificity of the identified miRNAs, cluster analysis was used to group miRNAs by their binding to the 3'UTRs (Fig. 5B). This reveals a high proportion of miRNAs (18/52) to uniquely target individual 3'UTRs, with the remaining 34 miRNAs targeting multiple 3'UTRs, including groups of miRNAs specifically targeting procoagulant genes. This may reflect a functionally coupled, (patho)physiologically relevant regulatory network.

To gain further insight into the association of the identified hemostasis-interacting miRNAs with other disorders, disease-term enrichment analysis was performed using gene sets consisting of known targets of the hemostasis-interacting miRNAs, and over- or under-representation of a set of genes with a disease term statistically determined. Strikingly, this revealed miRNAs specifically targeting procoagulant factors to be enriched in pathophysiological processes related to tumorigenesis.
Fig. 4. miTRAP with miRNA-mimic-rescue validation exhibits superior and distinctive performance compared with bioinformatic target prediction. A comparison of interactions identified from the miTRAP assay with a panel of prediction algorithms was performed. (A) miTRAP identifies interactions not found by the union of the three top-performing algorithms. TargetScan searches for seven to eight nucleotide canonical seeds [34], whereas PITA and RNAhybrid also allow six nucleotide marginal sites [35,36]. (B) Comparison of rate of functional interactions identified by the miTRAP assay (red) with that of the full panel of prediction algorithms (green) and combinations of algorithms (blue). (C) Analysis of the presence of potential seed sequences in the identified miRNA/3'UTR interactions reveals many functional miRNAs (red) do not have canonical or marginal seed sequences and many miRNAs with canonical seeds are non-functional (black). Analysis of the silencing conferred by each interaction revealed interactions possessing a canonical site exhibited significantly more silencing as compared with interactions possessing a marginal or no site. Bars indicate significance of the differences in mean silencing for interactions possessing seed sequences (as categorized by Bartel [23]) determined by Student’s t-tests. A more detailed examination is presented in Figure S4, including comparisons using miTRAP enrichment. [Color figure can be viewed at wileyonlinelibrary.com]
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Fig. 5. Cooperative and systems-specific miRNA-mediated silencing in the hemostatic system. (A) Functional cooperation of miRNAs in the silencing mediated by the PROZ 3′UTR. Bars indicate fold change in impact of double miRNA as compared with single miRNA-mimic-rescue assays. Values are presented as mean ± SEM of four independent experiments. Stars indicate statistical significance as measured by Student’s t-test, *p < 0.05, **p < 0.01. (B) Heatmap of miRNA/3′UTR interactions. Results of miTRAP assays were divided into three functional categories of procoagulant, anticoagulant or fibrinolytic, and for miRNAs targeting multiple 3′UTRs each category subjected to unsupervised hierarchical clustering as indicated by tree on left. Interactions functionally validated in Fig. 3(C) are boxed. [Color figure can be viewed at wileyonlinelibrary.com]

...and other functional categories, whereas those targeting anticoagulant factors are not (Table 1). Thus, apart from deeper insights into the regulatory capacity of miRNAs in the hemostatic system, the data obtained here provide instructive information linking the regulated (patho)physiology of the hemostatic system with other known, and as yet not connected, (patho)physiological processes, such as cancer, bone metabolism and renal function, on the level of miRNA-mediated control of gene expression.

In vivo validation demonstrates miR-19b-3p functionality against SERPINC1 (antithrombin)

To finally evaluate the biological functionality of the identified miRNA/3′UTR interactions, an in-vivo proof of concept study was undertaken. In light of a recent successfully applied antisense strategy [38] and the critical role of antithrombin deficiency in a variety of clinical conditions [39], the miR-19b-3p/SERPINC1 (antithrombin) interaction was assessed initially by miRNA-mimic-rescue assay using the mouse SERPINC1 3′UTR (Fig. 6A). This was followed-up in vivo using a specific phosphorothioate backbone modified locked nucleotide acid (LNA) mir-19b-3p inhibitor oligonucleotide, which blocks miRNA-mediated silencing, ultimately resulting in an upregulation of expression. As phosphorothioate backbone modified RNAs can have effects on platelet activation and coagulation [40], both control and targeting miRNA LNA oligonucleotides were phosphorothioatylated to control for non-specific effects.

Here, a small, albeit functionally consistent and statistically significant, increase of antithrombin activity was observed (Fig. 6B). Although these changes resulting from the inhibition of a single miRNA were modest, it is worth noting that even very subtle differences in expression of the hemostatic components can have substantial clinical consequences [41–43]. Additionally, the cooperativity of miRNAs (Fig. 5A) suggests synergy of multiple miRNAs may be more drastic, which offers appealing therapeutic avenues with reduced off-target effects. Together these results document the functional validity of the miRNAs identified here. They also illustrate the in-principal drugability of the hemostatic system in a miRNA-directed manner.

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The therapeutic maintenance of hemostasis involves treating coagulopathy without inducing thrombosis, in addition to treating thrombosis without inducing bleeding. This requires molecular targets that specify differences between hemostasis and thrombosis. As reliable therapeutic targeting requires comprehensive and bona fide target identification, we utilized an innovative, large-scale and unbiased method to screen for novel miRNAs interacting with components of the hemostatic system. Expression of miRNAs is known to vary greatly between different cell types; in addition, other variables can affect miRNA binding in a cell type-dependent manner [44]. Thus bona fide identification of miRNAs may critically depend on the experimental system used. This work utilized an in vitro binding miTRAP assay using liver-derived HuH-7 lysates for the identification of candidate miRNA/3′UTR interactions, as compared with previous studies where in silico prediction was primarily employed (Table S1). This approach resulted in the order of 150 potential candidate miRNA/mRNA interactions, of which up to 75% were functionally validated for several genes using stringent criteria (Fig. 3B).

### Table 1 Procoagulant-targeting, but not anticoagulant-targeting, miRNA/3′UTR interactions are associated with pathophysiological processes.

| GAD disease term       | Enrichment – procoagulant | Enrichment – anticoagulant |
|------------------------|----------------------------|----------------------------|
|                        | Genes involved in term     | % of term genes            | Benjamini-corrected P-value | Genes involved in term | % of term genes | Benjamini-corrected P-value |
| Mouth neoplasms        | 5                          | 6.1                        | 3.69E-04                   | 0                      | 0.0           | N/A                         |
| Ovarian cancer         | 11                         | 13.4                       | 6.41E-04                   | 5                      | 4.2           | 9.25E-01                   |
| Head and neck neoplasms| 7                          | 8.5                        | 3.71E-03                   | 5                      | 4.2           | 9.50E-01                   |
| B-cell lymphoma        | 4                          | 4.9                        | 7.90E-03                   | 0                      | 0.0           | N/A                         |
| Breast cancer          | 11                         | 13.4                       | 1.11E-02                   | 10                     | 8.4           | 8.91E-01                   |
| Squamous cell carcinoma| 3                          | 3.7                        | 1.26E-02                   | 0                      | 0.0           | N/A                         |
| Non-Hodgkin’s lymphoma | 3                          | 3.7                        | 1.26E-02                   | 2                      | 1.7           | 8.69E-01                   |
| Bone mineral density   | 9                          | 11.0                       | 1.69E-02                   | 0                      | 0.0           | N/A                         |
| Head and neck cancer   | 5                          | 6.1                        | 2.81E-02                   | 0                      | 0.0           | N/A                         |
| Lung cancer            | 10                         | 12.2                       | 3.40E-02                   | 0                      | 0.0           | N/A                         |
| Chronic renal failure  | 12                         | 14.6                       | 4.12E-02                   | 0                      | 0.0           | N/A                         |
| Pancreatic neoplasms   | 6                          | 7.3                        | 4.27E-02                   | 0                      | 0.0           | N/A                         |
| Leukemia               | 5                          | 6.1                        | 4.41E-02                   | 4                      | 3.4           | 8.41E-01                   |

Validated targets of miRNAs specific to either procoagulant or anticoagulant gene 3′UTRs were used for enrichment analysis of terms of the Genetic Association Database (GAD). Data for terms exhibiting significant (< 0.05) Benjamini-corrected Fischer exact test P-value as shown, with significant P-values indicated in bold.

**Fig. 6.** In vivo validation of miR-19b-3p functionality against SERPINC1 (antithrombin). (A) Validation of the miR-19b-3p/SERPINC1 interaction using the mouse 3′UTR in miRNA-mimic-rescue reporter assays. Values are presented as mean ± SEM of four independent experiments. (B) In vivo validation of miR-19b-3p functionality against SERPINC1 (antithrombin). Mice were injected with either a miR-19b-3p LNA inhibitor (antagomir) or LNA-negative control and plasma antithrombin activity assayed after 6 days. Values are presented as mean ± SEM of 22 mice for pretreatment, 10 mice for LNA-antagomir and 12 mice for LNA-control. Bars indicate significance as determined by Student’s t-test. [Color figure can be viewed at wileyonlinelibrary.com]
Of note, only nine unique interactions have been previously identified for the genes examined in this study (Table S1). Of these, six were not expected to be found as the miRNAs are not expressed in HuH-7 cells, as determined by miRNA sequencing (F11/miR-145, F11/miR-544, FGA/miR-759, FGB/miR-409-3p, PLAT/miR-133a and PLAT/miR-144). In contrast, the F11/miR-181a interaction [45,46] was identified in the miTRAP assay in addition to F11/miR-181b, a member of the same seed-family; however, only F11/miR-181b functionality was confirmed by miRNA-mimic-rescue. PLAT/miR-340 [47,48] showed a significant enrichment in the miTRAP assay of 4.7-fold. However, as this was below the cut-off of 5-fold enrichment, this interaction was not selected for validation with miRNA-mimic-rescue. Finally, only PLAT/miR-21 [49] was not identified in the miTRAP assay, even though this miRNA is highly expressed in HuH-7 cells.

In light of the overall very sparse number of papers reporting functional miRNAs in the hemostatic system, this limited overlap is not entirely surprising, and even expected. More importantly, none of the 52 interactions identified in this study have been previously identified, suggesting the novel experimental approach used in this study produces results distinct from the previously used methodologies. Interestingly, 23% of the functional interactions had no canonical or marginal site in the 3’UTR sequence, and are therefore missed by bioinformatic algorithms. Additionally, 59% of the non-functional interactions did possess a canonical or marginal site (with 21% having a canonical site), reinforcing the need for biological validation of bioinformatically predicted miRNA-target interactions.

The strongest silencing observed was for two miRNAs targeting PROZ, which also demonstrated cooperative activity (Fig. 5A). PROZ deficiency is linked with a procoagulant state in several thrombotic disorders and pregnancy complications [50]. Interestingly, in a mouse model of the acute-phase inflammatory response, plasma PROZ levels significantly increased, whereas levels of mRNA in the liver remained unchanged [51], consistent with release of miRNA-mediated silencing.

Notably, among the genes studied here the largest number of miRNAs targeted F11. Recent studies suggest that while the intrinsic pathway, of which F11 is a key factor, plays a relatively minor role in normal hemostasis, it plays a significant role in thrombosis [52,53]. Inhibition of the intrinsic pathway via F11 leaves the extrinsic and common pathways of coagulation intact, making F11 an attractive antithrombotic target [13]. Accordingly, targeting F11 provides antithrombotic benefits with reduced bleeding [54]. It is tempting to speculate that in vivo administration of miRNA-mimics of bona fide functional miRNAs targeting F11 may thus have analogous benefits.

The in-principal druggability of the hemostatic system in a miRNA-directed manner is demonstrated in vivo by the inhibition of miR-19b-3p against antithrombin (SERPINC1, Fig. 6). As a major inhibitor of active clotting factors, particularly thrombin and factor Xa, antithrombin is a critical regulator of the hemostatic system. Deficiency of antithrombin is associated with an increased risk of venous thrombosis and pulmonary embolism and insensitivity against antithrombin-dependent anticoagulants, and occurs in a range of conditions, including nephrotic syndrome, extracorporeal membrane oxygenation, hemodialysis, disseminated intravascular oxygenation or asparaginase therapy [55]. In these conditions, restoration of antithrombin levels is clinically relevant and reversal of miR-19b-3p (and/or other miRNA) mediated inhibition of antithrombin expression may represent an alternative to antithrombin replacement therapies.

Given the broad role of miRNAs targeting almost all human genes [5], either individually or in combination [6], such a therapeutic principle may become relevant for a variety of hemostatic disturbances, including common and rare hemophilic conditions. Additionally, the use of miRNA target-site blockers may allow new tailored therapies with reduced off-target effects [56]. Finally, such miRNA-based therapeutics may be less antigenic, which still places a burden on many of the currently existing replacement therapies.

Analysis of miRNAs specifically targeting procoagulant genes reveals these miRNAs to also target genes associated with other pathophysiological processes, whereas miRNAs targeting anticoagulant genes do not (Table 1). In particular, cancer stood out among these processes. Of note, VTE is a common, and clinically highly relevant, complication in tumor patients [57]. Dysregulation of miRNA networks is well characterized in many cancers [58], and such dysregulation may also result in perturbations of the coagulation system. Indeed, examples of miRNA dysregulation of the coagulation system in human cancers exist [59].

Together these findings present a foundation for understanding the role of miRNAs in hemostatic control and, in the context of recent endeavors and success in developing more efficient and sustainable targeted therapies for thrombosis and hemostasis [38], provide avenues for the development of novel therapeutic approaches for the correction of deregulated hemostasis in the future. Given the broad role of the hemostatic system in various other pathogenic processes, including atherosclerosis [60,61], these findings may have wide therapeutic implications beyond blood coagulation. Ultimately, this repository of hemostatic miRNA/3’UTR interactions (Table S4) may also constitute a valuable resource for rationalized biomarker discovery.

Addendum

J. Nourse and S. Danckwardt conceived and designed the research. J. Nourse and J. Braun performed experiments and bioinformatic and statistical analyses. J. Nourse and S. Danckwardt interpreted data and wrote the paper. K. Lackner and S. Hüttelmaier provided research tools. All
authors critically reviewed and approved the final version of the manuscript.

Acknowledgements

The authors thank K. Krohn of the Core Unit DNA-Technology, Leipzig University, for miRNA sequencing and miRNA mapping, in addition to K. Friedemann and J. Satrapa for assistance with the mouse studies. The authors also acknowledge the gifts of the Dicer-Null MEF cell line from G. Meister, the pCIneo-hEST2 plasmid from B. Weinberg (Addgene plasmid #1781) and the pCAGGS-Flag-hsDicer plasmid from P. Sharp (Addgene plasmid #41584). Work in the laboratory of S. Danckwardt is supported by grants from the BMBF, DFG, DGKL and the Hella Bühler Prize for cancer research.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article:

Fig. S1. Normalization of Dicer-rescue luciferase reporter assays.
Fig. S2. High levels of endogenous transcripts do not compete in 3′UTR reporter assays.
Fig. S3. Association of 3′UTR length and silencing.
Fig. S4. The miRNA-mimic rescue-assay reliably quantifies silencing.
Fig. S5. miTRAP repository (Table S4) includes many additional and potentially functional interactions.
Fig. S6. Presence of established miRNA seed sites is not entirely reflective of functionality.

Table S1. Published literature on miRNAs determined to target genes associated with hemostasis.
Table S2. Hemostatic-associated genes.
Table S3. Primers used in production of luciferase reporter and miTRAP bait RNA expression constructs.
Table S4. Repository of miRNA/3′UTR interactions identified by miTRAP assay.
Table S5. miRNA target predictions.

References

1 Mukherji S, Ebert MS, Zheng GXY, Tsang JS, Sharp PA, van Oudenaarden A. MicroRNAs can generate thresholds in target gene expression. Nat Genet 2011; 43: 854–9.
2 Krol J, Loedige I, Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. Nat Rev Genet 2010; 11: 597–610.
3 Meister G. Argonaute proteins: functional insights and emerging roles. Nat Rev Genet 2013; 14: 447–59.
4 Fabian MR, Sonenberg N, Filipowicz W. Regulation of mRNA translation and stability by microRNAs. Ann Rev Biochem 2010; 79: 351–79.
5 Friedman RC, Friedman KK-H, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 2009; 19: 92–105.
6 Megiorni F, Cialfi S, Dominici C, Quattrucci S, Pizzuti A. Synergistic post-transcriptional regulation of the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) by miR-101 and miR-494 specific binding. PLoS One 2011; 6: e26601.
7 Li X, Jiang W, Li W, Lian B, Wang S, Liao M, Chen X, Wang Y, Lv Y, Wang S, Yang L. Dissection of human MiRNA regulatory influence to subpathway. Brief Bioinform 2012; 13: 175–86.
8 Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. Nat Rev Drug Discov 2017; 16: 203–22.
9 Arroyo AB, de Los Reyes-García AM, Teruel-Montoya R, Vicente V, González-Conjero R, Martínez C. microRNAs in the haemostatic system: more than witnesses of thromboembolic diseases? Thromb Res 2018; 166: 1–9.
10 Basmadjian D, Setfon MV, Baldwin SA. Coagulation on biomaterials in flowing blood: some theoretical considerations. Biomaterials 1997; 18: 1511–22.
11 Wendelboe AM, Raskob GE. Global burden of thrombosis: epidemiologic aspects. Circ Res 2016; 118: 1340–7.
12 Geddings JE, Mackman N. New players in haemostasis and thrombosis. Thromb Haemost 2014; 111: 570–4.
13 Fredenburgh JC, Gross PL, Weitz JI. Emerging anticoagulant strategies. Blood 2017; 129: 147–54.
14 Chan KE, Giugliano RP, Patel MR, Abramson S, Jardine M, Zhao S, Perkovic V, Maddux FW, Piccini JP. Nonvitamin K anticoagulants in patients with advanced chronic kidney disease or on dialysis with AF. J Am Coll Cardiol 2016; 67: 2888–99.
15 Dobrovolski C, Clark EG, Sood MM. Venous thromboembolism in chronic kidney disease: epidemiology, the role of proteinuria, CKD severity and therapies. J Thromb Thrombolysis 2017; 43: 241–7.
16 Burnett AE, Mahan CE, Vazquez SR, Oertel LB, Garcia DA, Ansell J. Guidance for the practical management of the direct oral anticoagulants (DOACs) in VTE treatment. J Thromb Thrombolysis 2016; 41: 206–32.
17 Di Minno A, Frigerio B, Spadarella G, Ravan A, Sansaro D, Amato M, Kitzmiller JP, Pepi M, Tremoli E, Baldassarre D. Old and new oral anticoagulants: food, herbal medicines and drug interactions. Blood Rev 2017; 31: 193–203.
18 Witkos TM, Koscianska E, Krzyzosiak WJ. Practical aspects of microRNA target prediction. Curr Mol Med 2011; 11: 93–109.
19 Lee T, Wang N, Houel S, Couts K, Old W, Ahn N. Dosage and temporal thresholds in microRNA proteomics. Mol Cell Proteomics 2015; 14: 289–302.
20 Borchert GM, Gilmore BL, Spengler RM, Xing Y, Lanier W, Bhattarcharya D, Davidson BL. Adenosine deamination in human transcripts generates novel microRNA binding sites. Hum Mol Genet 2009; 18: 4801–7.
21 Gurtan AM, Lu V, Bhutkar A, Sharp PA. In vivo structure-function analysis of human Dicer reveals directional processing of precursor miRNAs. RNA 2012; 18: 1116–22.
22 Braun J, Misiai D, Busch B, Krohn K, Hüttelmaier S. Rapid identification of regulatory microRNAs by miTRAP (miRNA trapping by RNA in vitro affinity purification). Nucleic Acids Res 2014; 42: e66.
23 Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009; 136: 251–33.
24 Sturm A, Quackenbush J, Trajanoski Z. Genesis: cluster analysis of microarray data. Bioinformatics 2002; 18: 207–8.
25 Grimson A, Farh KK-H, Johnston WK, Garrett-Engele P, Lim LP, Castoldi M, Vujic Spasic M, Altamura S, Elm Danckwardt S, Gehring NH, Neu-Yilik G, Hundsdoerfer P, Kertesz M, Iovino N, Unnerstall U, Gaul U, Segal E. The role of Dweep H, Gretz N. miRWalk2.0: a comprehensive atlas of microRNA-target interactions. Nat Methods 2015; 12: 697.

26 Becker KG, Barnes KC, Bright TJ, Wang SA. The genetic association database. Nat Genet 2004; 36: 431–2.

27 Huang D-W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 2009; 4: 44–57.

28 Sehgal A, Vaishnaw A, Fitzgerald K. Liver as a target for oligonucleotide therapeutics. J Hepatol 2013; 59: 1354–9.

29 Danckwardt S, Ganzert A-S, Macher-Goeppinger S, Probst HC, Gentz M, Wilm M, Grüne H-J, Schirmacher P, Hentze MW, Kulozik AE. p38 MAPK controls prothrombin expression by regulated RNA 3′end processing. Mol Cell 2011; 41: 298–310.

30 Castoldi M, Vujic Spasic M, Altamura S, Elm Jén M, Lindow M, Kiss J, Stolte J, Sparla R, D’Alessandro LA, Klingmüller U, Fleming RE, Longerich T, Grüne HJ, Benes V, Kauppinen S, Hentze MW, Muckenthaler MU. The liver-specific microRNA miR-122 controls systemic iron homeostasis in mice. J Clin Invest 2011; 121: 1366–96.

31 Kim B-M, Thier M-C, Oh S, Sherwood R, Kanellopoulou C, Edenhofer F, Choi MY. MicroRNAAs are indispensable for reprogramming mouse embryonic fibroblasts into induced stem cell-like cells. PLoS One 2012; 7: e92339.

32 Gaidatzis D, Van Nimwegen E, Haasser J, Zavolan M. Inference of miRNA targets using evolutionary conservation and pathway analysis. BMC Bioinformatics 2007; 8: 69.

33 Dassi E, Zucotti P, Leo S, Provenzani A, Assfalg M, D’Onofrio M, Riva P, Quattrone A. Hyper conserved elements in vertebrate mRNA 3′-UTRs reveal a translational network of RNA-binding proteins controlled by HuR. Nucleic Acids Res 2013; 41: 3201–16.

34 Grimson A, Farh KK-H, Johnston WK, Garrett-Engele P, Lim LP, Bartel DP. MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Mol Cell 2007; 27: 91–105.

35 Kertesz M, Ivovino N, Unnerstall U, Gual U, Segal E. The role of site accessibility in microRNA target recognition. Nat Genet 2007; 39: 1278–84.

36 Krüger J, Rehmsmeier M. RNAhybrid: microRNA target prediction easy, fast and flexible. Nucleic Acids Res 2006; 34: W451–4.

37 Krek A, Grün D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, MacMenamin P, da Piedade I, Gunsalus KC, Stoffel M, Rajewsky N. Combinatorial microRNA target predictions. Nat Genet 2005; 37: 495–500.

38 Pasi KJ, Ranagaran J, Georgiev P, Mant T, Creagh MD, Lisichkov T, Bevan D, Austin S, Hay CR, Hegemann I, Kazmi R, Chowdry P, Gercheva-Kychukhova L, Mamonov V, Timofeeva M, Soh C-H, Garg P, Vaishnaw A, Akinc A, Sørensen B, et al. Targeting of antithrombin in Hemophilia A or B with RNAi therapy. N Engl J Med 2017; 377: 819–28.

39 Maclean PS, Tait RC. Hereditary and acquired antithrombin deficiency: epidemiology, pathogenesis and treatment options. Drugs 2007; 67: 1429–40.

40 Flierl U, Nero TL, Lim B, Arthur JF, Yao Y, Jung SM, Gitz E, Polit AY, Zaldavia MTK, Jandrot-Perrus M, Schäfer A, Niesenwander B, Andrews RK, Parker MW, Gardiner EE, Peter K. Phosphorothioate backbone modifications of nucleotide-based drugs are potent platelet activators. J Exp Med 2015; 212: 129–37.

41 Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3′-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. Blood 1996; 88: 3698–703.

42 Danckwardt S, Gehring NH, Neu-Yilik G, Hundsdoerfer P, Pförsch M, Frede U, Hentze MW, Kulozik AE. The prothrombin 3′end formation signal reveals a unique architecture that is sensitive to thrombophilic gain-of-function mutations. Blood 2004; 104: 428–35.

43 Danckwardt S, Hartmann K, Katz B, Hentze MW, Levy Y, Eichele R, Deutsch V, Kulozik AE, Ben-Tal O. The prothrombin 2020 C→T mutation in Jewish-Moroccan Caucasians: molecular analysis of gain-of-function of 3′ end processing. J Thromb Haemost 2006; 4: 1078–85.

44 Kulkarni V, Naqvi AR, Uttamani JR, Nares S. MiRNA-target interaction reveals cell-specific post-transcriptional regulation in mammalian cell lines. Int J Mol Sci 2016; 17: 72.

45 Sennblad B, Basu S, Mazur J, Suchon P, Martinez-Perez A, vanHylckama Vlieg A, Truong V, Li Y, Gädin JR, Tang W, Grossman v, deHaan HG, Handin N, Silveira A, Souto JC, Franco-Cereceda A, Morange P-E, Gagnon F, Soria JM, Eriksson P, et al. Genome-wide association study with additional genetic and post-transcriptional analyses reveals novel regulators of plasma factor XI levels. Hum Mol Genet 2017; 26: 637–49.

46 Salloum-Asfar S, Teruel-Montoya R, Arroyo AB, Garcia-Barberá N, Chaudhry A, Schuetz E, Luengo-Gil G, Vicente V, González-Conejero R, Martínez-C. Regulation of coagulation Factor XI expression by microRNAs in the human Liver. PLoS One 2014; 9: e111713.

47 Li S, Zhang R, Yuan Y, Yi S, Chen Q, Gong L, Liu J, Ding F, Cao Z, Gu X. MiR-340 regulates fibrinolysis and axon regrowth following sciatric nerve injury. Mol Neurobiol 2016; 54: 4379–89.

48 Yamashita D, Kondo T, Ohue S, Takahashi H, Ishikawa M, Matoba R, Suehiro S, Kohno S, Harada H, Tanaka J, Ohnishi T. miR340 suppresses the stem-like cell function of glioma-initiating cells by targeting tissue plasminogen activator. Cancer Res 2015; 75: 1123–33.

49 Terao M, Fratelli M, Kuroski M, Zanetti A, Guarnacci V, Paroni G, Tsykin A, Lupi M, Gianni M, Goodall GJ, Garattini E. Induction of miR-21 by retinoic acid in estrogen receptor-positive breast carcinoma cells: biological correlates and molecular targets. J Biol Chem 2011; 286; 4027–42.

50 Sofi F, Cesari F, Abbate R, Gensini GF, Broze G, Fedi S. A meta-analysis of potential risks of low levels of protein Z for diseases related to vascular thrombosis. Thromb Haemost 2010; 103: 749–56.

51 Girard TJ, Lasky NM, Tuley EA, Broze GJ, Protein Z, protein Z-dependent protease inhibitor (serpinA10), and the acute-phase response. J Thromb Haemost 2013; 11: 375–8.

52 Seligsohn U. Factor XI deficiency in humans. J Thromb Haemost 2009; 7(Suppl 1): 64–7.

53 Furie B, Furie BC. In vivo thrombus formation. J Thromb Haemost 2007; 5(Suppl 1): 12–7.

54 Chen Z, Seiffert D, Hawes B. Inhibition of Factor XI activity as a promising antithrombotic strategy. Drug Discov Today 2014; 19: 1435–9.

55 Muszbek L, Bereczky Z, Kovács B, Komáromi I. Antithrombin deficiency and its laboratory diagnosis. Clin Chem Lab Med 2010; 48(Suppl 1): S67–78.

56 Wei Y, Corbalán-Campos J, Gurung R, Natarella L, Zhu M, Exner N, Erhard F, Greulich F, Geißler C, Uhlenhaut NH, Zimmer M, Schober A. Dicer in macrophages prevents atherosclerosis by promoting mitochondrial oxidative metabolism. Circulation 2018; [Epub ahead of print].

57 Timp JF, Brekkan SK, Versteeg HH, Cannegieer SC. Epidemiology of cancer-associated venous thrombosis. Blood 2013; 122: 1712–23.

58 Tuna M, Machado AS, Calin GA. Genetic and epigenetic alterations of microRNAs and implications for human cancers and other diseases. Genes Chromosom Cancer 2016; 55: 193–214.

59 Eischen AC, Leppert U. The impact of microRNAs on the regulation of tissue factor biology. Trends Cardiovasc Med 2014; 24: 128–32.

60 Borissoff JJ, Sprok HMH, Cate ten H. The hemostatic system as a modulator of atherosclerosis. Schwartz RS, editor. N Engl J Med 2011; 364: 1746–60.

61 Danckwardt S, Hentze MW, Kulozik AE. Pathologies at the nexus of blood coagulation and inflammation: thrombin in hemostasis, cancer, and beyond. J Mol Med 2013; 91: 1257–71.