A novel rationale for targeting FXI: Insights from the hemostatic miRNA targetome for emerging anticoagulant strategies

Authors:

Jamie Nourse[1,2] and Sven Danckwardt[1,2,3]

Affiliations:

[1] Center for Thrombosis and Hemostasis (CTH), University Medical Center of the Johannes Gutenberg University, Mainz, Germany

[2] Institute for Clinical Chemistry and Laboratory Medicine, University Medical Center of the Johannes Gutenberg University, Mainz, Germany

[3] German Center for Cardiovascular Research (DZHK), Rhine-Main

Corresponding author:

Sven Danckwardt, MD

University Medical Centre of the Johannes Gutenberg University

Langenbeckstrasse 1

55131 Mainz

Germany

Phone +49 6131 176041

Email sven.danckwardt@unimedizin-mainz.de

Abstract

The treatment and prevention of thrombosis is currently under a period of rapid change with the replacement of traditional anticoagulant vitamin K antagonists with direct oral anticoagulants (DOACs), which specifically target factors FXa or FIIa.

Nevertheless therapeutic targeting of blood coagulation is an inherently difficult task as it interferes with the delicate balance of pro- and anticoagulant activities. Although anticoagulants are employed in millions of thrombophilic patients worldwide each year, for a growing population with comorbidities who exhibit an increased risk of bleeding with DOAC treatment satisfying therapeutic options are still lacking and the quest for novel therapeutics continues.

Recently the targeting of factors FXI and FXII have emerged as new therapeutic strategies. As these factors play an important roles in thrombosis, however are more or less functionally dispensable for hemostasis, they may potentially overcoming the functional obstacle of treating or preventing thrombosis without affecting hemostasis.

A key question in following up this approach is which of these two factors is likely to be the more reliable target? Here we present and discuss a hitherto unrecognized rationale for the therapeutic targeting of FXI. This is based on mimicking endogenous FXI gene expression control by therapeutic delivery of miRNA mimics.

Studies are urgently needed to test this therapeutic principle in a clinical setting. This is particularly applicable in hemostaseology where monitoring of therapeutic effects remains a daily routine and thus assessment of the efficacy and safety of such therapeutic components could be simply implemented ushering in a novel therapeutic era with broad applicability.

Keywords

Dabigatranetexilat (Pradaxa), Apixaban (Eliquis), Rivaroxaban (Xarelto), Edoxaban (Lixiana)

Introduction

Therapeutic targeting of blood coagulation is an inherently difficult task as it interferes with the delicate balance of pro- and anticoagulant activities. Anticoagulants are employed in millions of thrombophilic patients worldwide each year to limit the otherwise essential function of blood clotting[1]. However this intervention inevitably leads to an elevated risk of undesired bleeding. While some bleeding events are clinically mild and less critical, others can be life threatening, such as intracranial or abdominal bleeding.

The dichotomy of therapies targeting the hemostatic system is also evidenced in hemophilic coagulopathies, where blood coagulation is insufficient. In these cases the treatment of acquired or inherited hemophilia requires substitution of the lacking blood coagulation factor(s), ideally without tilting the hemostatic system too far towards a procoagulant state with eventual predisposition to thrombosis[2].

The treatment and prevention of thrombosis is currently under a period of rapid change. Direct oral anticoagulants (DOACs), which specifically target blood coagulation factors FXa (Rivaroxaban, Apixaban, Edoxaban) or FIIa (Dabigatran), are replacing traditional anticoagulants, such as vitamin K antagonists which impair the biosynthesis of factors FII, FVII, FIX and FX (Figure 1A). Nevertheless, satisfying therapeutic options are still lacking for a growing population with comorbidities who exhibit an increased risk of bleeding with DOAC treatment. These include patients with moderate to severe renal failure, hepatic failure, mechanic heart valve or patients under therapy with CYP3A4 and P-Glycoprotein inhibitors[4-7]. This elevated risk primarily reflects interference with metabolic turnover or excretion mechanisms of the DOAC. Finally DOACs may be associated with an increased risk in patients with antiphospholipid syndrome (APS)[3] limiting their use in this critical population with a significant risk of thromboembolic events.

Results and Discussion

Recently a series of articles have documented the targeting of factors FXI and FXII as increasingly evolving new therapeutic strategies[4-8]. The rationale behind this approach is that both components, FXI and FXII, belong to the so-called “contact system”, which appears to play an important role in thrombosis, however they are more or less functionally dispensable for hemostasis[9-11] (Figure 1A). It is hoped that this approach may overcome functional obstacles in the quest for the ‘ideal anticoagulant’, that of treating or preventing thrombosis without affecting hemostasis. A key question in following up this approach is which of these two factors is likely to be the more reliable target in the challenging endeavor of inhibiting blood clotting while maintaining hemostasis?

Here we present an interdisciplinary contribution to this exciting discussion from the area of RNA biology providing a novel avenue for the therapeutic targeting of FXI. Based on the data generated in a recent large-scale determination of functional miRNAs targeting the hemostatic system in the search for novel and rationale therapeutic targets[12], we find FXI to be targeted by the largest number of miRNAs, while FXII does not appear to be regulated by miRNAs (Table 1). This observation is consistent with further miRNAs reported to target FXI[13-15], while no reports are found for factor FXII. Biologically, this finding is remarkable. It suggests that FXI expression is under tight control via 3'UTR mediated regulation to ensure modest levels of FXI protein while FXII is not. This is in line with tighter regulation reflected by the leptokurtic distribution (i.e. narrower range) and lower percent coefficient variation (%CV) of FXI plasma levels as compared to FXII in our cohort (Figure 1B), which is consistent with earlier observations[16]. The biological importance is corroborated by the functional consequences in patients where elevated FXI levels predispose to thrombosis[17-19], while patients with FXI deficiency are protected from venous thromboembolism (VTE) and ischemic stroke[20]. Further support for the regulatory importance of the FXI 3'UTR comes from a recent study in which 3'UTR single nucleotide polymorphisms were

found to be associated with plasma FXI activity and risk of venous thrombosis[13]. Remarkably while the FXII 3'UTR is relatively short (~150 nucleotides; nt) the FXI 3'UTR spans over ~1000 nt. In addition to the high number of miRNAs, using RNA affinity pull-downs coupled to mass spectrometry we identify here that the FXI 3'UTR also binds numerous RNA binding proteins (RBPs) in close proximity to, and sometimes even in the direct target region of miRNAs (Figure 1C-D).

With regards to the important question as to which of the two components of the upstream intrinsic pathway (FXI or FXII) is likely to be the better therapeutic target, our data suggests that the specific miRNA mediated control of FXI may be a hitherto unrecognized rationale for the therapeutic targeting of FXI. Additionally it may provide a novel avenue for this targeting.

While a number of FXI inhibitors, including polypeptides, active site peptidomimetic inhibitors, allosteric inhibitors, antibodies, aptamers and antisense oligonucleotides (ASOs), have been proposed[21], it is important to realize that by mimicking the endogenous function of miRNAs to limit excessive expression, therapeutic miRNA delivery may possibly reflect a more physiological state in limiting FXI levels to treat and prevent VTE while retaining sufficient hemostasis for the prevention of bleeding. Although miRNA targeting to silence FXI could potentially have off-target effects, the functional synergy of several targeting miRNAs in a therapeutic cocktail allows for an increase in effect size and a reduction in side effects[12]. Finally and most importantly, miRNA-mediated targeting may also preserve cell intrinsic regulatory mechanisms, such as modulated 'occlusion' of miRNA binding sites by RNA binding proteins (RBPs)[22-24], regulation through other noncoding RNAs or interaction with competing endogenous (ce)RNAs (i.e. miRNA sponges, circRNA) as well as other regulatory mechanisms[25], ensuring the normal physiological fine tuning of the availability of the factor is maintained[26] (Figure 1E). Thus even in the presence of the miRNA therapeutic, RBP-mediated regulation (and other posttranscriptional gene regulatory

mechanisms) may be in place to evade miRNA-mediated silencing for example in order to stock pile blood clotting factors in an acute event of blood loss.

We thus believe that despite the impressive 'juggernaut' of anticoagulants, there are untapped therapeutic options that can help meet unmet medical needs. miRNA based therapeutics, currently emerging as attractive therapies in many other disciplines[27], may represent an avenue for correction of de-regulated hemostasis and associated processes in the future. Given the plethora of miRNA based therapeutic possibilities, such as miRNA mimicking, silencing, target specific miRNA target site blockers, knowledge of the miRNA targetome in the hemostatic system is potentially a major trove for future targeted therapeutics, not only for the development of safe anticoagulants but also for developing treatments for coagulopathies. In aid of these endeavors the first comprehensive hemostatic miRNA targetome is provided as a repository accompanying our recent paper[12].

Future studies are urgently needed to test this therapeutic principle in a clinical setting. This is particularly applicable in hemostaseology where monitoring of therapeutic effects remains a daily routine and thus assessment of the efficacy and safety of such therapeutic components could be simply implemented ushering in a novel therapeutic era with broad applicability. Finally, although we have previously demonstrated the in-principal drugability of the hemostatic system in a miRNA-directed manner using intravenously administered LNA oligonucleotides[12], future therapies would ideally require a more convenient oral delivery route. This may be accomplished through novel delivery vehicles that allow oral miRNA uptake, for example milk exosomes[28].

Acknowledgments

This work was supported by grants from the BMBF, DFG, DGKL and the Hella Bühler Prize for cancer research.

Contribution

All authors contributed to the research and edited the manuscript; J.N. performed the data analysis; J.N. and S.D. wrote the manuscript.

Disclosure of Conflicts of Interest

The authors declare no competing financial interests.

Table 1. A. Functional miRNAs targeting the hemostatic system. The first two columns are based on the hemostatic miRNA targetome identified in Nourse et. al.[12] by miTRAP RNA pull-downs. The first column indicates interactions validated by functional miRNA-mimic-rescue luciferase assays, while the second column indicates interactions identified by miTRAP RNA pull-down alone[12]. The last column indicates miRNAs identified from the literature to directly target the gene 3'UTR of blood coagulation factors indicated. **B.** Identity of functionally validated FXI targeting miRNAs and their role in cardiovascular disorders (AMI, acute myocardial infarction; VTE, venous thromboembolism; PAD, peripheral artery disease; CAD, coronary artery disease; DIC, disseminated intravascular coagulation).

A.

		miRNAs identified in hemostatic miRNA targetome		Directly targeting miRNAs identified previously in literature
Gene	**Gene Description**	**Directly targeting**	**Potential targeting**	
F11	**Coagulation factor XI**	**10**	**126**	**4**
F8	Coagulation factor VIII	7	172	0
SERPINA10	Protein Z-Dependent Protease Inhibitor (ZPI)	7	122	0
SERPIND1	Heparin co-factor II	6	74	0
PROZ	Protein Z	6	57	0
PLG	Plasminogen	5	128	0
FGG	Fibrinogen gamma chain	4	127	0
FGA	Fibrinogen alpha chain	2	225	2
F7	Coagulation factor VII	2	103	0
SERPINC1	Antithrombin III	2	57	0
KLKB1	Kallikrein B1	1	48	0
FGB	Fibrinogen beta chain	0	145	1
PROC	Protein C	0	79	0
PLAT	Plasminogen activator tissue type (tPA)	0	76	2
GP1BA	Glycoprotein Ib platelet alpha subunit	0	0	2
ADAMTS13	ADAM metallopeptidase with thrombospondin type 1 motif 13	0	0	1
F12	**Coagulation factor XII**	**0**	**0**	**0**
F13B	Coagulation factor XIII B polypeptide	0	0	0
F2	Coagulation factor II (thrombin)	0	0	0
F5	Coagulation factor V	0	0	0
HABP2	Hyaluronan binding protein 2	0	0	0
HRG	Histidine-rich glycoprotein	0	0	0
KNG1	Kininogen 1	0	0	0
SERPINF2	Alpha-2-antiplasmin	0	0	0

B.

miRNA	Thrombosis/Hemostasis associations	References (PMID)
miR-103a-3p	**Targets FXI**	30207063
miR-103a-3p	Involved in atherosclerosis and vascular inflammation by suppression of KLF4	26837267
miR-103a-3p	Biomarker for VTE	26235746
miR-1255a	**Targets FXI**	30207063
miR-1255a	Biomarker for stroke	30135469
miR-145-5p	**Targets FXI**	28053049, 28053049
miR-145-5p	Biomarker for CAD, AMI, stroke, long-term outcome	28051023, 25465803, 20886220, 26238333, 25008143, 25938589, 28205634, 26096228
miR-145-5p	Impedes thrombus formation in atherosclerosis by targeting tissue factor and influencing platelet reactivity	29217135, 29311920
miR-148b-3p	**Targets FXI**	30207063
miR-151a-3p	**Targets FXI**	30207063
miR-15b-5p	**Targets FXI**	30207063
miR-15b-5p	Involved in development of hemophilic arthropathy	27070581
miR-15b-5p	Biomarker for PAD	24129592
miR-15b-5p	Influences platelet reactivity and clopidogrel response	29311920
miR-181a-5p	**Targets FXI**	28053049, 25379760
miR-181a-5p	Biomarker for AMI and PAD	27997916, 24129592
miR-181b-5p	**Targets FXI**	30207063
miR-181b-5p	Serum levels altered in patients with atherosclerosis	26420120
miR-191a-5p	**Targets FXI**	25379760
miR-24-3p	**Targets FXI**	30207063
miR-24-3p	Biomarker for acute cerebral infarction, arteriosclerosis obliterans, atherosclerosis and severe trauma	24841240, 26159387, 29250154, 28694557
miR-30a-3p	**Targets FXI**	30207063
miR-30a-3p	Biomarker for AMI and ischemic stroke	23236408, 24237608
miR-30d-3p	**Targets FXI**	30207063
miR-544a	**Targets FXI**	28444748
miR-96-5p	**Targets FXI**	30207063
miR-96-5p	Biomarker for DVT and DIC	27588107, 24806323

References

1. Wendelboe Aaron, M. & Raskob Gary, E. Global Burden of Thrombosis. *Circ Res* **118**, 1340-1347 (2016).
2. Martin, K. & Key, N.S. How I treat patients with inherited bleeding disorders who need anticoagulant therapy. *Blood* **128**, 178 (2016).
3. Pengo, V.*, et al.* Rivaroxaban vs warfarin in high-risk patients with antiphospholipid syndrome. *Blood* **132**, 1365 (2018).
4. Stoll, G., Kleinschnitz, C. & Nieswandt, B. Molecular mechanisms of thrombus formation in ischemic stroke: novel insights and targets for treatment. *Blood* **112**, 3555 (2008).
5. Löwenberg, E.C., Meijers, J.C.M., Monia, B.P. & Levi, M. Coagulation factor XI as a novel target for antithrombotic treatment. *J Thromb Haemost* **8**, 2349-2357 (2010).
6. He, R., Chen, D. & He, S. Factor XI: Hemostasis, Thrombosis, and Antithrombosis. *Thromb Res* **129**, 541-550 (2012).
7. Gailani, D. & RennÉ, T. The intrinsic pathway of coagulation: a target for treating thromboembolic disease? *J Thromb Haemost* **5**, 1106-1112 (2007).
8. Fredenburgh, J.C., Gross, P.L. & Weitz, J.I. Emerging anticoagulant strategies. *Blood* **129**, 147 (2017).
9. van Montfoort, M.L. & Meijers, J.C.M. Recent insights into the role of the contact pathway in thrombo-inflammatory disorders. *ASH Education Program Book* **2014**, 60-65 (2014).
10. Younis, H.S.*, et al.* Antisense inhibition of coagulation factor XI prolongs APTT without increased bleeding risk in cynomolgus monkeys. *Blood* **119**, 2401 (2012).
11. Furie, B. & Furie, B.C. In vivo thrombus formation. *J Thromb Haemost* **5**, 12-17 (2007).
12. 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* **16**, 2233-2245 (2018).
13. Vossen, C.Y.*, et al.* Identification of coagulation gene 3′UTR variants that are potentially regulated by microRNAs. *Brit J Haematol* **177**, 782-790 (2017).
14. Sennblad, B.*, et al.* Genome-wide association study with additional genetic and post-transcriptional analyses reveals novel regulators of plasma factor XI levels. *Hum Mol Genet* **26**, 637-649 (2017).
15. Salloum-Asfar, S.*, et al.* Regulation of coagulation factor XI expression by microRNAs in the human liver. *PloS one* **9**, e111713-e111713 (2014).
16. Chen, Q.*, et al.* Biological and Analytical Variations of 16 Parameters Related to Coagulation Screening Tests and the Activity of Coagulation Factors. *Sem Thromb Hemost* **41**, 336-341 (2015).
17. Yang, D.T., Flanders, M.M., Kim, H. & Rodgers, G.M. Elevated Factor XI Activity Levels Are Associated With an Increased Odds Ratio for Cerebrovascular Events. *Am J Clin Pathol* **126**, 411-415 (2006).
18. Siegerink, B., Rosendaal, F.R. & Algra, A. Antigen levels of coagulation factor XII, coagulation factor XI and prekallikrein, and the risk of myocardial infarction and ischemic stroke in young women. *J Thromb Haemost* **12**, 606-613 (2014).
19. Folsom, A.R.*, et al.* Prospective study of circulating factor XI and incident venous thromboembolism: The Longitudinal Investigation of Thromboembolism Etiology (LITE). *Am J Hematol* **90**, 1047-1051 (2015).
20. Salomon, O., Steinberg, D.M., Koren-Morag, N., Tanne, D. & Seligsohn, U. Reduced incidence of ischemic stroke in patients with severe factor XI deficiency. *Blood* **111**, 4113 (2008).
21. Al-Horani, R.A. & Afosah, D.K. Recent advances in the discovery and development of factor XI/XIa inhibitors. *Med Res Rev* **38**, 1974-2023 (2018).
22. Danckwardt, S., Hentze, M.W. & Kulozik, A.E. 3' end mRNA processing: molecular mechanisms and implications for health and disease. *EMBO J* **27**, 482-498 (2008).
23. Lu, Y.-C.*, et al.* ELAVL1 modulates transcriptome-wide miRNA binding in murine macrophages. *Cell Rep* **9**, 2330-2343 (2014).
24. Jens, M. & Rajewsky, N. Competition between target sites of regulators shapes post-transcriptional gene regulation. *Nat Rev Genet* **16**, 113 (2014).
25. Tay, Y., Rinn, J. & Pandolfi, P.P. The multilayered complexity of ceRNA crosstalk and competition. *Nature* **505**, 344 (2014).
26. Danckwardt, S. & Kulozik, A. A p38 MAP kinase controlled novel gene regulatory switch links blood coagulation with tumor progression and outcome. *Hämostaseologie* **1**, A75 (2011).
27. Rupaimoole, R. & Slack, F.J. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. *Nature Reviews Drug Discovery* **16**, 203 (2017).
28. Manca, S.*, et al.* Milk exosomes are bioavailable and distinct microRNA cargos have unique tissue distribution patterns. *Sci Rep* **8**, 11321 (2018).
29. Danckwardt, S.*, et al.* p38 MAPK controls prothrombin expression by regulated RNA 3' end processing. *Mol Cell* **41**, 298-310 (2011).
30. Bartel, D.P. MicroRNAs: target recognition and regulatory functions. *Cell* **136**, 215-233 (2009).
31. Selbach, M.*, et al.* Widespread changes in protein synthesis induced by microRNAs. *Nature* **455**, 58-63 (2008).

Figure 1

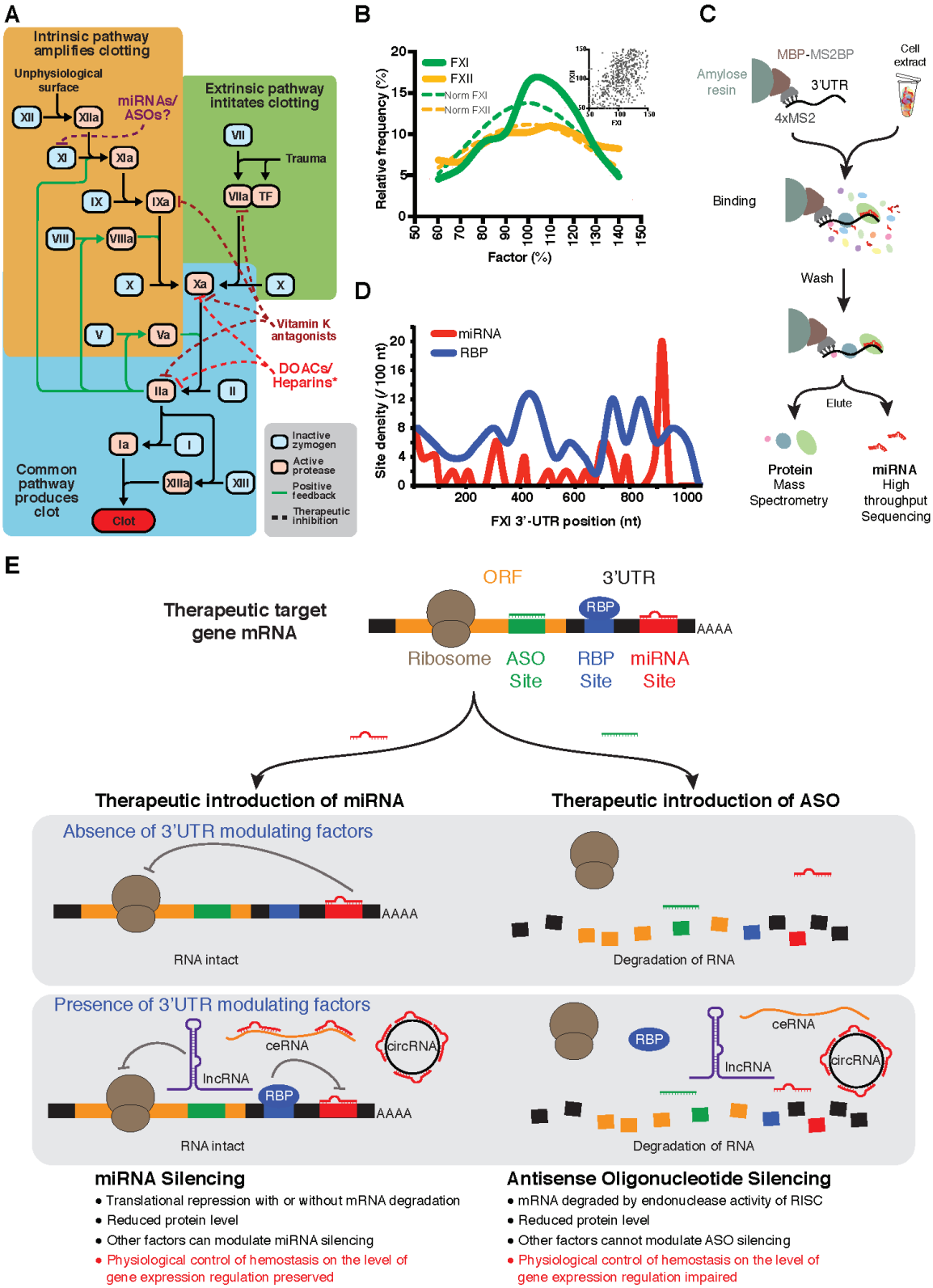
A
Intrinsic pathway amplifies clotting
Unphysiological surface
miRNAs/ ASOs?
Extrinsic pathway intitates clotting
Trauma
Vitamin K antagonists
DOACs/ Heparins*
Common pathway produces clot
Clot
Inactive zymogen
Active protease
Positive feedback
Therapeutic inhibition
B
FXI
FXII
Norm FXI
Norm FXII
Relative frequency (%)
Factor (%)
C
MBP-MS2BP
Cell extract
Amylose resin
3'UTR
4xMS2
Binding
Wash
Elute
Protein Mass Spectrometry
miRNA High throughput Sequencing
D
miRNA
RBP
Site density (/ 100 nt)
FXI 3'-UTR position (nt)
E
Therapeutic target gene mRNA
ORF
3'UTR
RBP
AAAA
Ribosome
ASO Site
RBP Site
miRNA Site
Therapeutic introduction of miRNA
Therapeutic introduction of ASO
Absence of 3'UTR modulating factors
RNA intact
Degradation of RNA
Presence of 3'UTR modulating factors
ceRNA
circRNA
lncRNA
RBP
AAAA
RNA intact
Degradation of RNA
miRNA Silencing
• Translational repression with or without mRNA degradation
• Reduced protein level
• Other factors can modulate miRNA silencing
• Physiological control of hemostasis on the level of gene expression regulation preserved
Antisense Oligonucleotide Silencing
• mRNA degraded by endonuclease activity of RISC
• Reduced protein level
• Other factors cannot modulate ASO silencing
• Physiological control of hemostasis on the level of gene expression regulation impaired

Figure 1. New rational therapeutic targets in the hemostatic system. (A) The hemostatic system is tightly balanced to reduce blood loss by formation of blood clots (hemostasis), while at the same time preventing pathological clot formation (thrombosis). Deficiencies of the intrinsic pathway factors XII and XI are usually not associated with abnormal hemostasis but appear to prevent thrombus formation, qualifying them as attractive therapeutic targets. As FXI is exclusively targeted by miRNAs it provides an attractive avenue for therapeutic targeting. (B) FXI plasma levels exhibit a leptokurtic distribution as compared to platokurtic distribution type of FXII (levels of FXI and FXII from patients admitted to the University Medical Center Mainz from 2012 to 2018; values were limited to patients for which both FXI and FXII assays were performed and extremes (levels ≥150% and ≤50%) have been excluded, normal distributions with standard deviations corresponding to those of FXI and FXII are shown dashed). (C) Schematic of the in vitro pull down assay used to identify 3'UTR interacting miRNAs and RBP. Briefly baits consisting of in vitro transcribed 3'UTRs fused to copies of the bacteriophage MS2 stem-loop were immobilized by binding to amylose resin via recombinant MS2 coat protein-maltose binding (MBP-MS2BP) fusion protein. Immobilized baits were incubated with cell extracts to allow binding of 3'UTR interacting factors. Following washing protein–RNA complexes were eluted with maltose and either RNA or proteins were purified and identified using RNA-sequencing and mass spectrometry. (D) Density of potential sites for miRNA and RNA-binding proteins (RBPs) across FXI 3'UTR. 125 FXI-3'UTR/miRNA interactions were identified by miTRAP/RNA-seq[12] and of these 41 are mapped to the FXI 3'UTR using miRWalk target site prediction. 392 FXI-3'UTR/RBP interactions identified by miTRAP/MS (unpublished) and of these 66 could be mapped to the FXI 3'UTR using RBPDB target site prediction. Site density calculated by number of sites present in 50 nt windows over length of the FXI-3'UTR. (E) Function and consequences of therapeutic miRNA targeting versus antisense oligonucleotide (ASO) silencing. Protein expression depends not only on the rate of transcription, but on other regulatory mechanisms, including mRNA processing, stability, transport and translational regulation. These post-transcriptional mechanisms are mainly mediated by the binding of RNA-binding proteins (RBPs), lncRNAs and miRNAs to regulatory elements in 3'UTRs of mRNAs. The emerging picture is that of complex and multifaceted layers of 3'UTR-mediated regulation, including competition and cooperativity of these regulatory components, to adjust protein synthesis in a spatiotemporal manner[24,25]. Of note such mechanisms also tune the hemostatic system, for example in an acute event of blood loss to stock pile blood coagulation factors[29]. As opposed to ASO silencing, and in many cases siRNAs, (right), which inevitably results in degradation of the target mRNA, the partial base-pairing of miRNAs (left) prevents the cleavage activity of RISC with silencing predominately resulting from translational repression, and in some cases deadenylation, decapping and finally mRNA degradation[30]. This appears to be particularly relevant for secretory proteins (such as components of the hemostatic system) produced at the endoplasmic-reticulum[31]. Thus, miRNA-mediated therapeutic targeting without degradation of such target mRNAs preserves cell intrinsic regulatory mechanisms executed by 3'UTRs and their 'interacting partners' such as RBPs, miRNAs and/or other layers of control, such as ceRNA, circRNA miRNA sponges. Finally miRNA sites in proximity to RBP sites are often subject to mechanisms of physiological control[22-25], which may play important regulatory roles to control the delicate balance of hemostatic system in response to environmental cues[29].