Dicer Cleavage by Calpain Determines Platelet microRNA Levels and Function in Diabetes

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Rationale: MicroRNAs (miRNAs) are short noncoding RNA species generated by the processing of longer precursors by the ribonucleases Drosha and Dicer. Platelets contain large amounts of miRNA that are altered by disease, in particular diabetes mellitus.

Objective: This study determined why platelet miRNA levels are attenuated in diabetic individuals and how decreased levels of the platelet-enriched miRNA, miR-223, affect platelet function.

Methods and Results: Dicer levels were altered in platelets from diabetic mice and patients, a change that could be attributed to the cleavage of the enzyme by calpain, resulting in loss of function. Diabetes mellitus in human subjects as well as in mice resulted in decreased levels of platelet miR-142, miR-143, miR-155, and miR-223. Focusing on only 1 of these miRNAs, miR-223 deletion in mice resulted in modestly enhanced platelet aggregation, the formation of large thrombi and delayed clot retraction compared with wild-type littermates. A similar dysregulation was detected in platelets from diabetic patients. Proteomic analysis of platelets from miR-223 knockout mice revealed increased levels of several proteins, including kindlin-3 and coagulation factor XIII-A. Whereas, kindlin-3 was indirectly regulated by miR-223, factor XIII was a direct target and both proteins were also altered in diabetic platelets. Treating diabetic mice with a calpain inhibitor prevented loss of platelet dicer as well as the diabetes mellitus–induced decrease in platelet miRNA levels and the upregulation of miR-223 target proteins.

Conclusions: Thus, calpain inhibition may be one means of normalizing platelet miRNA processing as well as platelet function in diabetes mellitus. (Circ Res. 2015;117:157-165. DOI: 10.1161/CIRCRESAHA.117.305784.)

Key Words: diabetes mellitus ■ microRNA ■ platelets ■ signal transduction
circulating miRNA levels with decreased levels of several platelet-derived miRNAs. The molecular events leading to the decrease in platelet miRNAs are, however, unknown.

Platelet hyper-reactivity in patients with type 2 diabetes mellitus has previously been linked with the activation of the calpain family of Ca²⁺-activated cysteine proteases and the subsequent proteolytic cleavage of a series of substrate proteins that results in markedly altered protein function. Given the recent report that Dicer is a calpain substrate in cortical neurons, we hypothesized that the decrease in platelet miRNAs associated with diabetes mellitus is related to the calpain-dependent cleavage of Dicer leading to dysregulated miRNA generation. Moreover, targeting calpain pharmacologically may be a means of restoring the platelet miRNA profile.

**Methods**

**Animals**

C57BL/6 mice (6–8 weeks old) were purchased from Charles River (Sulzfeld, Germany). MiR-223-deficient mice were kindly provided by Fernando D. Camargo (Harvard University, Cambridge, MA). The miR-223 locus is located on the X chromosome and expressed in conditions that conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (National Institutes of Health publication no. 85-23). Both the University Animal Care Committees and the Federal Authorities for Animal Research at the Regierungspräsidium Darmstadt (Hessen, Germany; No. F28/44) approved the study protocols. Age-, sex-, and strain-matched animals (usually littermates) were used throughout.

**Statistics**

Data are expressed as the mean±SEM, and statistical evaluation was performed using Student t test for unpaired data, 1-way ANOVA followed by Bonferroni test, or ANOVA for repeated measures where appropriate. Values of P<0.05 were considered statistically significant.

Reagents and other detailed methods are described in the Online Data Supplement.

**Results**

**Cleavage of Dicer in Diabetic Platelets**

Platelets express part of the miRNA processing machinery and while argonaute 2 levels were comparable in healthy and diabetic platelets, Dicer levels were significantly decreased (79±3%) in platelets from diabetic patients (Figure 1A). However, in some samples the antibody used picked up a potential degradation product (arrow in Figure 1B) indicating that Dicer may have been targeted by proteases.

Given that Dicer is a calpain substrate in neurons, we determined whether Dicer can be cleaved by calpain in platelets. When platelets from healthy individuals were incubated with Ca²⁺ to activate the Ca²⁺-sensing receptor, the autolytic cleavage of calpain 1 (Capn1 or μ-calpain) occurred together with a slight

![Figure 1. Calpain-dependent cleavage of Dicer in platelets. A, Dicer and argonaute 2 (Ago2) levels in platelets from healthy (H) and diabetic (D) subjects. B, Dicer, calpain 1 (Capn1) and latent calpain 2 (L.Capn2) levels in platelets from healthy and diabetic subjects. C, Effect of calpain activation with Ca²⁺ (5 μmol/L) or Ca²⁺ and ionomycin (Iono; 1 μmol/L) on Dicer, Capn1 and Capn2 in platelets from healthy volunteers. Experiments were performed in the presence of solvent and calpeptin (10 μmol/L). D, Effect of acute calpain activation on Dicer levels in platelets from wild-type (WT) and Capn1−/− mice. Experiments were performed in the presence of solvent and calpeptin. The graphs summarize data from 5 to 8 individuals and 5 mice per group; **P<0.01, ***P<0.001 vs healthy or solvent-treated platelets; #P<0.01 vs the absence of Ca²⁺ and ionomycin.](http://circres.ahajournals.org/issue/96/2/158)
but distinct shift in the mobility of Dicer in SDS-PAGE (arrowhead on Figure 1C). No shift was observed in the presence of the calpain inhibitor, calpeptin. The combination of Ca2+ and ionomycin was, however, required to activate calpain 2 (Capn2 or m-calpain; decrease in latent levels), which in turn resulted in the further degradation of Dicer and the appearance of a degradation product similar to that detected in platelets from some diabetic individuals (arrow in Figure 1C). Again cleavage was not observed in the presence of calpeptin. A similar phenomenon could be demonstrated in murine platelets but the antibody used was unable to detect putative-cleaved products. Cleavage was not observed in the presence of calpeptin but was similar in platelets from wild-type and Capn1−/− mice (Figure 1D) indicating that Dicer is predominantly a Capn2 substrate.

We next determined the consequences of acute and prolonged calpain activation on Dicer activity. Let-7 was chosen for this assay as it is an abundant Dicer-regulated platelet miRNA. Acute treatment of washed platelets from healthy individuals with Ca2+ or Ca2+ and ionomycin increased the generation of mature let-7 in a calpeptin-dependent manner (Figure 2A), indicating that calpain-dependent cleavage acutely increases Dicer RNAse III activity. However, more maintained calpain activation in platelets from diabetic subjects could be separated into 2 groups, that is, those in which low levels of Dicer were still detectable; usually demonstrating better insulin sensitivity (homeostasis model assessment–insulin resistance, 4.6±0.54; n=8), and those with no detectable full-length or cleaved Dicer protein (homeostasis model assessment–insulin resistance, 7.6±1.47; n=8). Although the Dicer-containing diabetic human platelets were able to metabolize the pre-miR-substrate to the mature miRNA (cleavage was 25.7±0.2% versus 25.5±4.8% in platelets from healthy and diabetic individuals, respectively), they also gave products of an unexpected size (~35 nt; Figure 2B). Platelets from the Dicer-poor group, however, generated significantly less mature miRNA (Figure 2C). Thus, although the acute activation of calpain increased Dicer activity, the cleavage of the protein by Capn2 was linked to loss of expression, incomplete pre-miRNA processing and decreased activity.

Consequences of miR-223 Knockout on Platelet Function

Circulating levels of platelet-enriched miRNAs are decreased in diabetes mellitus.12 We confirmed that miR-142, miR-143, miR-155, and miR-223 were all expressed in platelets from nondiabetic individuals and that levels were reduced in age- and sex-matched diabetic subjects (Figure 3A). A similar phenomenon was observed in platelets from 5-month-old diabetic Ins2Akita mice versus their nondiabetic littermates (Figure 3B).

Given that platelet miR-223 was downregulated in diabetes mellitus, we studied the consequences of its deletion on platelet function, making use of the available male wild-type (miR-223+/+) and miR-223 knockout (miR-223−/−) mice.19 MiR-223-deletion was not associated with a clear difference in tail bleeding time (Figure 4A) but was linked with the enhanced in vivo formation of platelet–neutrophil aggregates (Figure 4B). Thrombus formation after FeCl3-induced carotid artery injury was also elevated in miR-223−/− mice (Figure 4C); clot formation was accelerated (time to maximum was 3.7±1.0 minutes in wild-type versus 2.5±1.0 minutes in miR-223−/− mice, n=6 per group; P<0.001) and the overall thrombus size was increased. In the microcirculation, however, embolism was increased in the miR-223−/− mice, with 3 of 11 wild-type vessels developing emboli compared with 13 of 15 vessels studied in miR-223−/− mice (Figure 4D).
The deletion of miR-223 enhanced aggregation in vitro induced by low concentrations of thrombin and collagen (Figure 5A), without affecting responses to higher concentrations of either agonist or aggregation induced by fibronectin or the thromboxane analog U46619 (Online Figure IA and IB). Platelets from miR-223y/− mice also demonstrated a delayed clot retraction to a low concentration of thrombin (0.1 U/mL; Figure 5B) but no differences when higher concentrations (0.75 U/mL) were used (data not shown). Although platelet adherence to collagen was not different between the 2 groups, spreading was increased in miR-223y/− platelets (Figure 5C). No significant differences in platelet adherence or spreading on fibronectin, laminin, or vitronectin were observed (Online Figure IC and IE). The most obvious phenotype of the miR-223y/− platelets was the spontaneous formation of large aggregates, a phenomenon that was independent of the matrix studied (Figure 5D; Online Figure II). These alterations were similar to those reported previously for streptozotocin-induced diabetes mellitus17 and comparable changes in platelet function were observed in animals made diabetic with a high-fat diet (Online Figure VIA). There was also no indication that α2 integrin could be directly regulated by miR-223. Given that kindlin-3 is an integrin-binding protein and that β1 integrin is targeted by miR-223 in endothelial cells20 we hypothesized that the changes in kindlin-3 may have been secondary to that of α2 integrin.

MiR-223 and the Platelet Proteome

Two-dimensional differential in-gel electrophoresis combined with mass spectrometry was used to identify miR-223 targets in murine platelets. A comparison of platelets from wild-type and miR-223y/− mice revealed marked differences in the expression of functionally relevant platelet proteins (Online Figure IV; Table I). Of the proteins identified, 2 were chosen for further functional studies, that is, fermitin family homolog 3 (kindlin-3) and coagulation factor XIII-A (FXIII-A).

The increase in kindlin-3 in miR-223y/− platelets was confirmed by Western blotting (Figure 6A) and was accompanied by an increase in the expression of β1 and α2 integrin, whereas the expression of β3 and α5 integrin were unaffected (Online Figure V). Comparable changes in kindlin-3, β1, and α2 integrin expression were also detected in platelets from patients with type 2 diabetes mellitus (Figure 6B) and in platelets from mice fed a high-fat diet (Online Figure VIA). Although kindlin-3 was picked up in the proteomic assay it could not be identified as a direct target of miR-223 (Targetscan, miRDB, and miRanda) and it was unaffected by miR-223 antagonism in wild-type mice (Online Figure VIB). There was also no indication that α2 integrin could be directly regulated by miR-223. Given that kindlin-3 is an integrin-binding protein and that β1 integrin is targeted by miR-223 in endothelial cells20 we hypothesized that the changes in kindlin-3 may have been secondary to that of α2 integrin.
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There was no effect of rhodocetin on integrin signaling, experiments were performed with the αw washed platelets from wild-type and miR-223y/− mice. The graphs show the number and size of large aggregates formed on collagen by type vs miR-223y/− mice, on collagen.

The second protein studied more closely was FXIII-A. The expression of the full-length protein was increased in platelets from miR-223y/− mice as was that of a band of lower molecular mass (∼55 kDa) recognized by the same antibody (Figure 7A). A significant, although less pronounced increase in FXIII-A and the 55 kDa band was also detected in platelets from diabetic versus healthy humans (Figure 7B). The altered protein levels were accompanied by an increase in FXIII activity (Figure 7C). A seeding sequence (6 base pairs of complementarity) for miR-223 was identified in 3′ UTR region of FXIII-A and cotransferring HEK-293 cells with increasing concentrations of pre-miR-223 and a luciferase construct containing the putative miR-223 binding sequence within the 3′ UTR of FXIII-A, resulted in a concentration-dependent decrease in luciferase activity (Online Figure IXA). No effect of pre-miR-223 was detected when the binding sequence was mutated, indicating that FXIII-A is directly targeted by miR-223. Moreover, miR-223 antagonism in mice over 12 days increased platelet FXIII-A levels (Online Figure VIB).

FXIII-A is involved in fibrin clot stabilization through fibrin–fibrin cross-linking,22 and as increased clot stability may delay clot retraction we reassessed clot formation and retraction in miR-223y/− platelets. Clot retraction was attenuated in platelet-rich plasma from miR-223y/− mice, and FXIII inhibition with putrescine,23 normalized responses. A similar effect was detected in samples from diabetic subjects (Figure 7D) and comparable results were obtained using tridegin,24 a second FXIII inhibitor (Online Figure IXB).

Consequences of In Vivo Calpain Inhibition on Dicer and miR-223 Levels

To demonstrate a direct link between diabetes mellitus, calpain activation, and the proteolysis of Dicer, mice were made diabetic

Figure 6. Comparison of the consequences of miR-223 deletion in mice or diabetes mellitus in humans on platelet protein expression. A and B, Kindlin-3 (K3), β1 integrin, and α2 integrin in washed platelets from wild-type (y+/+) vs miR-223y/− (y−/−) mice (A), or platelets from healthy (H) vs diabetic (D) subjects (B). C, Effect of rhodocetin (Rdc; 5 μg/mL, 5 minutes) on the percentage of nonspread and spread platelets from wild-type or healthy, §P<0.01 vs wild-type or healthy, §§P<0.01 vs wild-type or healthy, §§§P<0.001 vs in the absence of putrescine.

β1 integrin. However, although the overexpression of precursor (pre) miR-223 efficiently decreased the expression of β1 integrin and kindlin-3 in human endothelial cells, the siRNA-mediated downregulation of β1 integrin did not significantly affect kindlin-3 expression (Online Figure VII).

To determine to what extent the functional defects in miR-223y/− platelet function could be attributed to alterations in β1 integrin signaling, experiments were performed with the α2β1 antagonist rhodocetin.21 There was no effect of rhodocetin on the adhesion of miR-223y/− platelets to collagen (not shown) but it normalized spreading (Figure 6C) and prevented aggregate formation (Figure 6D). The abnormal platelet spreading and aggregate formation by platelets from diabetic humans was also normalized in the presence of rhodocetin (Online Figure VIII).

Figure 7. Comparison of the consequences of miR-223 deletion in mice or diabetes mellitus in humans on platelet factor XIII-A (FXIII-A). FXIII-A (80 kDa) and the active Δ form (55 kDa) in washed platelets from (A) wild-type (y+/+) and miR-223y/− (y−/−) mice, or (B) from healthy (H) and diabetic (D) subjects. C, FXIII activity in platelet-rich plasma from wild-type and miR-223y/− mice (top) and healthy (H) vs diabetic (D) subjects (bottom). Experiments were performed in the absence and presence of putrescine (Put, 20 mmol/L). D and E, Effect of putrescine on thrombin-induced clot retraction in platelet-rich plasma from wild-type vs miR-223y/− mice (D) or healthy (H) vs diabetic (D) subjects (E). The graphs summarize data from 4 to 11 subjects or 4 to 13 mice per group; *P<0.05, **P<0.01 vs wild-type or healthy; §§P<0.01, §§§P<0.001 vs in the absence of putrescine.
with streptozotocin. Twelve weeks after the induction of diabetes mellitus, platelet Capn2 could not be detected in the latent form (Figure 8A) and Dicer levels were effectively decreased. Treating mice with the calpain inhibitor A-705253 for 12 days before euthanization significantly increased platelet Dicer levels in non-diabetic animals and prevented its loss in diabetic mice. In the same animals, the proteins previously found to be upregulated in miR-223y/− mice, that is, kindlin-3, β1 integrin, and FXIII-A were all increased by diabetes mellitus and largely reversed by calpain inhibition (Figure 8B). In the same animals, diabetes mellitus was associated with a decrease in mature miR-223 and an increase in pre-miR-223 levels (Figure 8C). A-705253 treatment, however, restored levels of both the species toward those detected in non-diabetic, vehicle-treated mice. Clearly, many miRNAs are affected by alterations in platelet Dicer levels, and the diabetes mellitus–associated decrease in mature miR-143 was also restored by calpain inhibition (Figure 8D) and a similar tendency was observed with miR-155 (Online Figure X). However, miR-451a, which is regulated by Dicer and the diabetes mellitus–associated decrease in miR-143 was also restored by calpain inhibition (Figure 8E) and a similar trend was observed with miR-155 (Online Figure X). However, miR-451a, which is regulated by Dicer-independent mechanisms,25,26 was not attenuated in platelets from streptozotocin-treated mice (Figure 8E), diabetic Ins2Akita littermates. G, MiR-451a in platelets from age-matched healthy and diabetic human subjects. The graphs summarize data from 5 to 18 mice or 9 to 10 human subjects per group; #P<0.05, **P<0.01, ***P<0.001 vs control, nondiabetic mice; #P<0.05; ##P<0.01 vs vehicle- and STZ-treated mice.

**Discussion**

The results of this investigation indicate that the decrease in platelet miRNA levels detected in diabetic subjects has consequences on platelet protein expression as well as function. Focusing on only 1 platelet-enriched miRNA, that is, miR-223, it was possible to relate changes in miRNA levels with a hyper-reactive and hyper-adhesive phenotype, as well as with altered levels of β1 integrin and FXIII-A. Moreover, altered platelet miRNA expression in diabetes mellitus could be linked to the activation of calpain and the proteolysis of the RNase III Dicer, eventually resulting in decreased Dicer activity.

That circulating levels of platelet miRNAs are altered in subjects with diabetes mellitus has been clearly demonstrated by others,12–14 and we were able to confirm those findings by analyzing a subset of miRNAs. To determine the molecular events responsible for the decrease in miRNA levels, we focused on the expression of Dicer and argonaute 2, both of which are donated to platelets by the parent megakaryocyte. Although Dicer was clearly expressed in platelets from healthy individuals, it was almost undetectable in platelets from severely diabetic patients (hemoglobin A1c, >7.4%). Levels of argonaute 2, however, were comparable in platelets from healthy and diabetic individuals. In some of the samples studied, the antibody used picked up possible Dicer degradation products indicating that the loss of expression may represent protein cleavage. In fact, proteases are assumed to be required for Dicer activation, as the full-length Dicer protein is part of a multimolecular complex and largely inactive. Proteases known to target Dicer include the cell death protease CED-3; that can generate 50 kDa C-terminal and 180 kDa N-terminal Dicer fragments that are catalytically inactive,24 and calpain.18 The acute activation of the latter protease has been linked to an increase rather than a decrease in Dicer activity. Certainly, the removal of the auto-inhibitory N-terminal domain increases Dicer activity and fragments of 75 and 50 kDa have been reported after the treatment of recombinant Dicer with Capn1, a step that also increases RNase III activity.16,20 In platelets from nondiabetic subjects, the acute activation of calpains resulted
in the accelerated processing of premature let-7a, indicating increased dicer activity. In platelets from diabetic subjects, however, the longer term activation of calpains resulted in the depletion of Dicer to such an extent that activity was either altered or markedly attenuated (depending on the degree of insulin insensitivity). Indeed, in a subgroup of diabetic individuals with detectable platelet Dicer levels, mature let-7a was generated together with apparently inappropriately processed miRNA products. Platelets from healthy subjects failed to generate these intermediates; producing only the mature miRNAs, whereas platelets from patients with little or no full-length Dicer failed to process the pre-let-7a used as substrate for the assay. Inappropriate or asymmetrical Dicer processing was previously reported to result in the generation of atypical miRNAs of ≈35 nt and may correspond to pre-miRNA substrates cleaved only once by Dicer, as proposed previously. Whether there are functional consequences of these intermediates on platelet protein expression or function is unknown.

There is currently great interest in the role played by platelet-derived miRNAs for use as biomarkers of disease, but little is known about the consequences of decreased miRNA levels on platelet function. One miRNA, miR-223, was selected for closer study as although its expression increases during megakaryocyte differentiation and it is highly expressed in healthy platelets, and low levels have been reported to predict platelet reactivity as well as responsiveness to clopidogrel. Moreover, miR-223 was previously shown to affect platelet function and was 1 of only 2 circulating miRNAs inversely associated with the risk of myocardial infarction. We found that in miR-223-deficient mice, thrombus size and embolism were increased in vivo and ex vivo the aggregation elicited by low concentrations of thrombin and collagen was increased. There was also a significant delay in clot retraction and a marked increase in platelet spreading and the formation of platelet aggregates by samples from miR-223-deficient mice. Such changes were highly reminiscent of the phenotype of platelets from diabetic subjects. However, although our results are in agreement with those of others who observed that alterations in miR-223 levels affect platelet function, at least partly by affecting P2Y₁₂ purinergic receptor expression and activity, other researchers recently failed to see any consequence of the loss of miR-223 on platelet activation. The reason for these conflicting results are unclear but may be related to the concentration of the platelet agonists studied, as the clearest effects in this study were observed using lower agonist concentrations.

Two-dimensional in-gel electrophoresis was used to identify platelet proteins altered in miR-223-deficient mice and of the proteins identified, we concentrated on 2 candidates known to regulate platelet function, that is, kindlin-3 and FXIII-A. These proteins were selected for further study because of their role in the regulation of platelet function and because of the fact that they are not known calpain targets. Both kindlin-3 and FXIII-A were upregulated in platelets lacking miR-223 as well as in platelets from diabetic mice and human individuals. Kindlin-3 is an integrin-binding protein, and its altered expression was paralleled by increased expression of β1 and β2 integrins in platelets from miR-223 mice and diabetic subjects. Although it was regulated in platelets from miR-223 mice, kindlin-3 does not seem to be a direct target of this miRNA because no apparent seeding sequence was detectable and kindlin-3 was unaffected by miR-223 antagonism in wild-type mice. Given that β1 integrin is directly targeted by miR-223, we postulated that an increase in β1 expression may affect the expression or stability of its binding partner. However, the siRNA-mediated downregulation of β1 integrin had no effect on kindlin-3 levels (at least in cultured endothelial cells) suggesting that different, more indirect, mechanisms underlie the effects observed. The increases in α2β1 integrin were, however, functionally relevant as while the adherence of miR-223-deficient mouse platelets and diabetic human platelets to different matrices was not altered, spreading was increased in miR-223-deficient platelets. Also, an α2β1 antagonist was able to prevent the increase in spreading as well as the spontaneous formation of large platelet aggregates in samples from miR-223-deficient mice and diabetic human platelets, indicating that elevated α2β1 integrin signaling contributes to the phenotype observed.

The second protein investigated in more detail was FXIII-A, which makes up half of an inactive transglutaminase tetrameric complex. The FXIII-A subunit contains the active site of the enzyme and is present in large amounts as a homodimer in platelets. Once activated, it cross-links platelet fibrin, making clots mechanically stronger and protecting them against fibrinolysis. Given its role in determining the mechanical stability of the clot, increased FXIII-A levels could account for the larger and apparently stable clots generated after FeCl₃ injury in the carotid artery. We also found that the defective clot retraction observed using platelets from miR-223 mice or diabetic humans was normalized by 2 different FXIII inhibitors. Our observation that FXIII-A levels were increased in platelets from diabetic wild-type mice and human subjects correlates well with reports of increased plasma FXIII levels in subjects with type 2 diabetes mellitus. Until now, there has been no explanation for the increase in FXIII activity in diabetes mellitus but the observation that platelet FXIII-A levels and FXIII activity are directly regulated by miR-223, which declines in diabetic platelets, goes a long way to explaining this phenomenon.

Could the activation of calpain in diabetic megakaryocytes or platelets be responsible for the limited proteolysis and altered activity of Dicer that ultimately leads to altered platelet miRNA levels? To make a link between calpain activity, miRNA levels and the miR-223 targets identified, animals made diabetic with streptozocin were treated with the calpain inhibitor A-705253 for 12 days. Although streptozocin is a model for type 1 rather than type 2 diabetes mellitus, we have previously shown that this model elicits a similar platelet calpain activation as seen in human type 2 diabetes mellitus. Calpain inhibition in vivo restored levels of full-length Dicer to control (and above) at the same time as increasing mature miR-223 and decreasing β1 integrin, kindlin-3 and FXIII-A levels. Although this study focused on miR-223 for functional analyses, it is clear that alterations in Dicer activity would be expected to affect a spectrum of miRNAs and it was possible to demonstrate diabetes induced decreases in miR-143 and miR-155 levels. However, platelet levels of miR-451a, which is known to be regulated by Dicer-independent mechanisms, tended to increase in streptozotocin-treated mice; a finding that fits well with a previous report. However, in a genetic model of murine diabetes mellitus or in human platelets, miR-451a was unaffected. Thus, Dicer is a novel substrate of calpain in platelets and the reported
improvement in platelet function after calpain inhibition, previously assumed to be the consequence of the direct cleavage of specific platelet proteins,\textsuperscript{37} may also be related to the increased expression of platelet proteins whose expression is determined by Dicer-regulated miRNAs.\textsuperscript{40} By targeting calpain, particularly when the altered miRNA profile is linked to changes in calpastatin levels or Dicer activity, it may be possible to prevent or reverse changes in several miRNAs or even restore the healthy miRNA profile.

The fact that the acute activation of calpain was required for Dicer activation may imply that long-term calpain inactivation may be detrimental to global miRNA production. This seems unlikely to be the case as although studies assessing changes in miRNA levels as a consequence of calpain inhibition in healthy animals have not been performed, the long-term treatment with A-705253 in vivo is well tolerated and can protect against platelet hyper-reactivity,\textsuperscript{37} retinal nerve\textsuperscript{41} as well as penile nitrergic nerve dysfunction in diabetic mice.\textsuperscript{42} In this study, calpain activation in platelets was clearly demonstrable while there was no evidence of protease activation in hearts or kidneys from the same animals (I. Fleming, unpublished data, 2014). One reason for the apparent lower threshold for calpain activation in platelets than other cells and tissues may be related to the comparatively low platelet expression of the endogenous calpain inhibitor; calpastatin.\textsuperscript{43} Whether calpain activation; such as that linked with cardiac ischemia reperfusion injury\textsuperscript{44} or myocardial infarction\textsuperscript{45} are also linked to an altered tissue miRNA profile subsequent to the cleavage of Dicer remains to be determined.

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Disclosures

None.

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Novelty and Significance

**What Is Known?**

- Platelet-derived microRNAs (miRNAs) contribute to the circulating miRNA pool.
- Diabetes mellitus is associated with a marked change in the circulating miRNA profile.
- Diabetes mellitus–induced platelet hyper-reactivity is linked to calpain activation.

**What New Information Does This Article Contribute?**

- miRNA maturation is attenuated in platelets from diabetic humans and mice because of the lack of functional Dicer.
- Dicer is a calpain 2 substrate in human and murine platelets and Dicer cleavage by calpain inhibits Dicer activity.
- Calpain inhibition in vivo prevents the diabetes mellitus–induced decrease in platelet miRNA levels and the increase in target protein levels.

Platelets contain high levels of miRNAs but as they are anucleate they are unable to generate novel primary miRNA transcripts, rather platelet Dicer and Argonaute 2 convert precursor miRNAs donated from the parent megakaryocyte into mature miRNA. Diabetes mellitus is associated with a marked change in the profile of platelet miRNAs but the reasons underlying this effect are unknown. We report that the activation of calpain 2 in diabetic platelets cleaves Dicer and prevents the normal maturation of platelet precursor miRNAs. Taking the decrease in miR-223 as an example, it was possible to show that the increased expression of the miR-223 targets; β1 integrin and factor XIII-A could be linked to changes in platelet function similar to those observed in diabetes mellitus. In vivo calpain inhibition was able to prevent the decrease of a spectrum of Dicer-regulated platelet miRNAs (miR-223, miR-143, and miR-155) without affecting levels of miR-451a, which is regulated by dicer-independent mechanisms. Thus, by targeting calpain in diseases such as diabetes mellitus, it may be possible to prevent or reverse changes in several miRNAs or even restore the healthy miRNA profile.
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Supplemental Material

Expanded Methods and Results

Reagents

Type I collagen and fibronectin were from BD transduction laboratories (Heidelberg, Germany). Vimentin was from Millipore, calpeptin was from Calbiochem (Darmstadt, Germany) and thrombin from Hemochrom Diagnostica (Essen, Germany). U46619 was from Enzo Life Science (Lörrach, Germany) and the α2β1 blocker; rhodocetin, was kindly provided by Johannes Eble (Frankfurt, Germany). The calpain inhibitor N-(1-benzyl-2-carbamoyl-2-oxoethyl)-2-[E-2-(4-diethyl-aminomethylphenyl)ethen-1-yl]benzamide (A-705253) was provided by AbbVie Deutschland GmbH & Co. KG (Ludwigshafen, Germany). Laminin, putrescine, 3,3′-dihexyloxacarbo-cyanine iodide (DiOC6) and all other compounds were from Sigma-Aldrich (Steinheim, Germany). Tridegin was from Zedira (Darmstadt, Germany).

The anti-β1 integrin, CD61 FITC, and CD45 PE were obtained from BD Biosciences (Heidelberg, Germany). The antibodies against α2 integrin and α5 integrin were from Millipore (Schwalbach, Germany), anti-FXIIIA was from Acris (Montluçon, France), anti-Dicer from Abcam (Cambridge, UK) and anti-Ago2 was from cell signaling (Frankfurt, Germany). The anti-β3 integrin antibody was from Epitomics (Berlin, Germany) and anti-kindlin-3 was from Abnova (Heidelberg, Germany). The antibodies recognizing β-actin, µ-calpain, and m-calpain were from Sigma-Aldrich (Munich, Germany).

Animals

C57BL/6 mice (6-8 weeks old) were purchased from Charles River (Sulzfeld, Germany). miR-223-deficient mice were kindly provided by Fernando D. Camargo (Harvard University, Cambridge, MA 02138m USA) and heterozygous C57BL/6-Ins2 Akita/J mice were purchased from The Jackson Laboratory (Farmington, CT, USA). Calpain 1 (Capn1)−/− mice, generated by crossing “floxed” Capn1 (Capn1tm1.1Arte) mice with animals expressing the Cre-recombinase under the control of the CMV promoter, were bred by the animal facility at the University of Frankfurt. Mice were housed in conditions that conform to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication no. 85-23). Both the University Animal Care Committees and the Federal Authorities for Animal Research at the Regierungspräsidium Darmstadt (Hessen, Germany; # F28/21, F28/44 and FU1012) approved the study protocols. Age-, gender-, and strain-matched animals (usually littermates) were used throughout.

Some of the C57BL/6 mice were also treated with either control antagonim (GFP specific) or antagonim directed against miR-223 (both 8 mg/kg, 100 µl; VBC Biotech, Vienna, Austria) three times over 12 days. Diabetes was induced with a single intraperitoneal injection of streptozotocin (STZ; 150 mg/kg) or by feeding animals a high fat diet (58% fat; ssniff, Soest, Germany) for 20 weeks. Animals were considered diabetic when fasting plasma glucose was over 250 mg/dL. In some experiments STZ-treated animals were treated by oral gavage with the calpain inhibitor A-705253 (30 mg/kg/day) for 12 days after 12 weeks untreated diabetes.

Human material

A total of 22 patients (12 women, 10 men; mean age, 45.78±3.12 years; age range, 30 to 60 years) with type 2 diabetes mellitus attending the clinic for routine control visits were included in the study; hemoglobin (Hb)A1c over 7.4% (9.01±0.37%) and fasting plasma glucose, 8.35±0.82 mmol/L. All patients were treated with insulin alone or in combination with metformin. Nondiabetic, age-matched subjects (12 women, 10 men; mean age, 41.6±7.5 years; age range, 25 to 60 years; HbA1c, 4.98 ±0.58%; fasting plasma glucose, 5±0.19 mmol/L) who had not taken
any medication known to interfere with platelet aggregation for at least 10 days before the experiment served as the control group. The study protocol was approved by the ethics committee of the Goethe University Hospital (No. E 61/09 geschäfts Nr 86/09) and all of the participants gave written informed consent.

2D-DIGE analysis

Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE)\(^3,4\) was performed using the CyDye DIGE Fluors minimal labeling kit (GE Healthcare \#28-9345-30, Freiburg, Germany) according to the manufacturer’s instructions, with minor modifications. Briefly, four samples (each representing platelets pooled from 6 mice), from either wild-type or miR-223 \(^{-/-}\) mice were solubilized in DIGE labeling buffer (7 mol/L urea, 2 mol/L thiourea, 4 % Chaps and 30 mmol/L Tris/HCl pH 8.6), sonicated for a few seconds on ice and centrifuged (10,000 g, 10 min, 4°C). The protein content of the supernatant was determined using Bradford-UltraTM reagent (Expedeon, Harston, UK). Two of each control and miR223 \(^{-/-}\) samples were labeled either with Cy3 or Cy5 minimal dye by setting a dye/protein ratio of 200 pmol/25 µg. A mixture of all samples was labeled with Cy2 minimal dye to generate an internal standard. The labeling reaction was quenched by 1 mmol/L L-lysine (final concentration). Samples were mixed in 7 mol/L urea, 2 mol/L thiourea, 2% (w/v) Chaps, 0.002 % bromphenol blue, 2 % (v/v) pharmalytes\(^\text{TM}\) carrier ampholytes pH 3-10, 13 mmol/L DTT and each set was supplemented with equal amounts of internal standard. Samples were loaded onto Immobiline\(^\text{TM}\) DryStrip gels (pH 3-10, 24 cm) rehydrated in 7 mol/L urea, 2 mol/L thiourea, 2% (w/v) Chaps, 0.002 % bromphenol blue, 0.5 % pharmalytes\(^\text{TM}\) carrier ampholytes pH 3-10, 13 mmol/L DTT. Proteins were separated by isoelectric focusing (IEF). Before 2D separation on a SDS gel, strips were equilibrated for 15 minutes in buffer (6 mol/L urea, 2% (w/v) SDS, 50 mmol/L Tris/HCl pH 8.8, 0.02 % (w/v) bromphenol blue and 30 % (v/v) glycerol) containing 65 mmol/L DTT, and then replaced the same buffer but containing 135 mmol/L iodoacetamide for another 15 minutes. The second dimension SDS-PAGE was carried out using 12.5 % acrylamide gels in an Ettan Dalt-twelve system (Amersham Biosciences). Labeled proteins were detected by a Typhoon\(^\text{TM}\) fluorescence scanner and gels were analyzed by DeCyder 2-D 7.0 software (GE Healthcare).

For mass spectrometric protein identification a preparative gel generated using ~200 µg of a mixture of all samples was prepared, fixed in 50% methanol, 10% acetic acid, stained in SYPRO Ruby (Sigma S4942), then destained in 10% methanol, 7% acetic acid and washed in water. The preparative gel was placed on a frame fixed cellophane sheet (GE Healthcare), scanned and matched to the analytical gels in the BVA module of DeCyder 2-D 7.0 software (GE Healthcare). Spots of interest (Student’s t-test, p-value < 0.05, average ratio ± 1.15) were picked by Ettan spot picker (GE Healthcare) into perforated 96 well plates and proteins were identified by mass spectrometry.

In-gel tryptic digests for mass spectrometry

The in-gel digestes were performed essentially as described.\(^5\) Briefly, gel pieces in perforated 96-well plates were washed twice in 50% methanol, 50 mmol/L ammonium hydrogen carbonate and then incubated for 30 minutes at 4°C in 5µL 5 ng/µL trypsin in 50 mmol/L ammonium hydrogen carbonate, 10% acetonitrile and 1 mmol/L calcium chloride. Each gel slice was covered by 20 µL of 50 mmol/L ammonium hydrogen carbonate and trypptic digestion was carried out overnight at 37°C. The peptide containing supernatants were collected by centrifugation into a new 96 well plate. The gel pieces were incubated with 50% acetonitrile, 1% formic acid for another 45 minutes to extract remaining peptides. Supernatants were combined and dried in a SpeedVac.
Mass spectrometry

Dried tryptic peptides were dissolved in 15 µL 5% acetonitrile, 0.5% formic acid and analyzed by liquid chromatography/mass spectrometry (LC-MS). Briefly, a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Dreieich, Germany) was coupled to an Agilent 1200 series nano-HPLC system and tryptic peptides from each 2-D gel spot separated on a column consisting of 10 cm x 75 µm ID fused silica emitter tip filled with 3 µm C18 reversed phase silica (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) using 40 minute gradients of 5% to 50% acetonitrile with 0.1% formic acid, followed by a column wash and re-equilibration for 10 minutes each. Eluted peptides were electrosprayed in positive ion mode and analyzed by a MS method programmed to fragment top ten most abundant precursor ions using dynamic exclusion for 3 minutes with a resolution of 30,000 at 400 Th. Single-charged precursor ions were rejected, doubly and higher charged ions were fragmented in the linear ion trap by CID at 35% normalized collision energy. Database searches were performed using Mascot server 2.2 as database search engine. Following parameters were set: 5 ppm deviation on the precursor and 0.8 Da on fragment masses, fixed carbamidomethylation of cysteine, variable oxidation of methionine and trypsin as protease (full tryptic with one missed cleavage allowed). Spectra were matched against *Mus musculus* reviewed protein database containing 16541 sequences obtained from www.uniprot.org. Database search results were filtered by individual peptide ion score cut-off value calculated by Mascot to indicate identity or extensive homology (P<0.05).

Plasmid construction and reporter gene assays

The 3' UTR of FXIIIA as a reporter gene construct was purchased from Origene (Frankfurt am Main, Germany) and the mutation of the FXIIIA miR-223 seeding sequence was generated using the QuickChange kit (Stratagene, Waldbronn, Germany) with miR-223 mutation primers. The primer sequences used for cloning and mutation were:

<table>
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<th>Cloning primer</th>
<th>Sequence</th>
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For the 3' UTR reporter gene assay, the appropriate plasmids were transfected into HEK293 cells in the presence and absence of pre-miR-223 and pcDNA3.1CMV-β-gal (Invitrogen, Darmstadt, Germany) for 48 hours.

Reverse transcription and real time PCR (RT-qPCR)

Total RNA from platelets was extracted using a miRNA easy kit (Qiagen, Hilden, Germany) and equal amounts (50 ng) of total RNA was reverse transcribed (Superscript III, Invitrogen, Heidelberg, Germany). mRNA levels were detected using SYBR Green (Absolute QPCR SYBR Green Mix; Thermo Scientific, Hamburg, Germany). To quantify miRNAs, specific stem-loop primers were used for reverse transcription then specific primers, conserved reverse primer and FAM-labeled oligonucleotides (universal probe library probe #13, Roche Diagnostics, Mannheim, Germany) were used for the subsequent real-time qPCR. The relative expression level of miRNA and genes were calculated using the $2^{-\Delta\Delta C_T}$ method relative to 18S RNA.

<table>
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<th>Primer</th>
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<td>miR-142 RT primer</td>
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Platelet isolation

**Human platelets:** Platelets were obtained by centrifugation (900g, 7 minutes) of platelet-rich plasma, as described.6 The resulting pellet was washed in Ca²⁺-free HEPES buffer (mmol/L: NaCl, 136; KCl, 2.6; MgCl₂, 0.93; NaH₂PO₄, 3.26; glucose, 5.5; HEPES, 3.7; pH 7.4 at 37°C). Platelet pellets were either resuspended in Ca²⁺-free HEPES buffer to a density of 4x10⁸ platelets/mL for Western blotting or to a density of 1x10⁷ platelets/mL for the adhesion assay. In some cases, platelet pellets were snap frozen with liquid nitrogen and stored at -80°C until use.

**Murine platelets:** Mice were anesthetized with isoflurane and blood was collected from the retro-orbital plexus into a tube containing 10% acidic citrate dextrose (120 mmol/L sodium citrate, 110
mmol/L glucose, 80 mmol/L citric acid) as anticoagulant. Platelets were prepared from whole blood by differential centrifugation and resuspended in Ca$^{2+}$-free HEPES buffer to a density of 4x10$^8$ platelets/mL for Western blotting, 1x10$^8$ platelets/mL for aggregation studies and 1x10$^7$ platelets/mL for adhesion assays.

For miRNA analyses, high purity platelets were obtained using a hematopoietic progenitor cell depletion kit (STEMCELL, EasySep negative selection, Grenoble, France) according to the manufacturer's protocol.

**Platelet aggregation**

Aggregation of washed murine platelets (1x10$^8$ platelets/mL in 250 µL) was measured using an 8-channel aggregometer (PAP8, Mölab, Germany) as described.7

**Clot retraction**

Platelet-rich plasma (adjusted to 300x10$^5$ platelets/mL for human platelets and 5x10$^5$ platelets/mL for murine platelets, 300 µL) obtained by centrifugation of whole blood at 250g for 10 minutes, was stimulated with thrombin (0.1 U/mL) in the presence of CaCl$_2$ (20 mmol/L) and 10 µL erythrocytes to enhance the contrast of the clot. The clots were allowed to retract for up to 1 hour at 37°C and were photographed at different time points. The extent of retraction was quantified using imageJ software (ImageJ 1.44p, USA).

**Bleeding time**

Mice were restrained in appropriate restrainer and placed on a heated mat. A 1 mm section of the tail tip was cut, and the tail tip was immediately immersed in sterile saline at 37°C. The bleeding time (i.e, the time between initial flow of blood and its cessation) was recorded. When no blood was observed on the saline after 60-s intervals, bleeding was considered to have ceased. The experiment was stopped after 20 minutes. The mice were monitored for an additional 10 minutes.

**Intravital assessment of arteriolar thrombus formation**

To assess thrombus formation in vivo, mice were anesthetized by intraperitoneal injection of ketamine and xylazine and placed on a heated mat. The fluorescent dye 3,3’ dihexyloxacarbocyanine iodide (DIOC6; Invitrogen, Darmstadt, Germany) was injected into the jugular vein (5 µL of a 100 µmol/L solution/g body weight) to allow visualization of the thrombus. Thereafter, a segment of the right carotid artery was exposed and injury was induced by the topical application of FeCl$_3$ for 2 minutes (Whatmann paper 1 mm$^2$ soaked with 0.2 µL of 10% FeCl$_3$) as described.7 The artery was then rinsed with saline and thrombus formation was monitored for 30 minutes by placing the carotid artery under a fluorescence microscope equipped with a camera (AxioScope, Carl Zeiss, Jena, Germany). Fluorescent images were acquired sequentially (1 image/second) and thrombus size was quantified using AxioVision 4.8.2 imaging software (Carl Zeiss).

To assess thrombus formation in a resistance bed thrombus formation in vivo was assessed in the dorsal skinfold chamber in mice using a light dye injury model. Briefly after surgical preparation of the dorsal skinfold chamber as described previously,6 the animals fulfilling the criteria for an intact microcirculation (accurate arteriolar blood flow, absence of surgery-related artefacts) underwent carotid artery catheterization for application of the dye. After injection of 4 µL/g of 5% FITC-dextran (150,000 MW) photoactivation was initiated by exposing the vessel to a 100 W mercury lamp (Fluoarc HBO100, Carl Zeiss) using the respective fluorescence filter. Intravital fluorescence microscopy was performed using a Zeiss AxioTech Vario microscope (Zeiss, Göttingen, Germany) and thrombus formation was recorded with a digital camera (AxioCam HSm, Carl Zeiss) in 2-4 arterioles per mouse with at least 40 µm in diameter. Onset
of thrombus formation, stability of thrombi (emboli with a diameter larger than half of the vessel diameter) and time to complete thrombotic vessel occlusion resulting in flow cessation were analyzed.

**Platelet adhesion and spreading assays**

Static adhesion assays were performed as described. Washed human or murine platelets were suspended into platelet buffer containing physiological concentration of 1.8 mmol/L CaCl₂ in the absence or in the presence of the selected inhibitor. Platelet suspensions (1x10⁴ platelets/µL in 300 µL) were seeded on plastic slides (µ-Slide 8 well, ibidi, Martinsried, Germany), coated with fibronectin (100 µg/mL), vitronectin (1 µg/mL) collagen (50 µg/mL) or laminin (10 µg/mL) and incubated at 37°C for 30 minutes. Non adherent platelets were washed off and adherent and spread platelets as well as the large platelet aggregates were fixed. Images were captured by an AxioCam MRm on a Cell Observer microscope (Zeiss, Jena, Germany) and analyzed using the imaging software AxioVision 4.8 (Zeiss, Jena, Germany).

**Flow cytometry analysis of platelet-leukocyte aggregates**

Anticoagulated whole murine blood (100 µL) was incubated for 15 minutes at room temperature in the dark with a phycoerythrin-conjugated anti-mouse CD45 antibody and a FITC-conjugated anti-mouse CD61 antibody or matched mouse IgG isotype controls. Then 1 mL erythrocyte lysis buffer was added to lyse erythrocytes (30 minutes, room temperature). After centrifugation and washing pellets were fixed with formalin (2% vol/vol in PBS) then washed and resuspended in PBS. Samples were then analyzed by flow cytometry (FACS Calibur flow cytometer; BD Biosciences).

**FXIII activity assay**

Human or murine platelet-rich plasma was incubated with or without the FXIII inhibitor (putrescine, 20 mmol/L or tridegin, 50 µg/mL) for 30 minutes at 37°C. FXIII activity was measured with a microtiter assay using fibrinogen and 5-(biotinamido) pentyamine as substrates, as described.

**Dicer activity assay**

Washed platelets from healthy or diabetic individuals were isolated as described and incubated with or without Ca²⁺ and ionomycin for 30 minutes at 37°C. Thereafter platelets were washed and resuspended in Dicer assay buffer and Dicer activity was assessed using ³²P randomly labeled human pre-let-3, as described.

**Immunoblotting**

Washed human or murine platelets (4x10⁸ platelets/mL) were solubilized in SDS sample buffer, separated by SDS-PAGE and subjected to Western blotting. Proteins of interest were visualized by enhanced chemiluminescence using a commercially available kit (Amersham, Freiburg, Germany), as described.

**Statistics**

Data are expressed as the mean ± SEM, and statistical evaluation was performed using Student’s t test for unpaired data, 1-way analysis of variance (ANOVA) followed by Bonferroni t test, or ANOVA for repeated measures where appropriate. Values of P<0.05 were considered statistically significant.
References


### Supplementary Table I: Proteins differentially expressed in platelets from miR-223<sup>−/−</sup> mice. Spots from 2D DIGE analysis with an average ratio ± 1.15 and p<0.05 were considered to be proteins of interest.

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Figure I. Consequences of miR-223 deletion on platelet aggregation, adhesion, and spreading. A&B Washed platelets were isolated from wild-type (y+/+) and miR-223<sup>y/-</sup> (y/-) mice and aggregation assessed in response to fibronectin (A) and the thromboxane analogue U46619 (B). C-E Washed platelets (3x10<sup>4</sup> platelets/µL) were and incubated for 30 minutes on fibronectin (C), laminin (D) or vitronectin (E) and the number of adherent platelets as well as the percentage of non spread and spread platelets were counted. The graphs summarise data from 4-10 mice per group.
Figure II. Consequences of miR-223 deletion on the formation of spontaneous platelet aggregates and expression of platelet surface receptors. Washed platelets were isolated from wild-type (y/+ ) and miR-223y/- (y/-) mice and incubated for 30 minutes on (A) fibronectin, (B) vitronectin, or (D) laminin. Thereafter, the number and size of the aggregates formed were counted. The graphs summarize data from 4-10 mice per group; *P<0.05 and **P<0.01 versus +/+.
Figure III. Consequences of a high fat diet and miR-223 antagonism on platelet function. (A-C) Male wild-type mice were treated with normal chow (NC) or a high fat diet (HFD) for 20 weeks. (A) Aggregation of washed platelets in response to thrombin. (B) Platelet adherence and spreading on collagen. (C) Number and size of aggregates on collagen (no flow conditions, 30 minutes). (D-G) Male wild-type mice were treated with control oligonucleotides (CTL) or antagonirs directed against miR-223 (AmiR) 3 times over 12 days. Aggregation of washed platelets in response to thrombin (D) and collagen (E). (F) Platelet adherence and spreading on collagen. (G) Number and size of aggregates on collagen. The graphs summarize data from 5 mice per group; *P<0.05; **P<0.01; ***P<0.001 versus NC or CTL.
Figure IV. 2-D DIGE analysis of platelets from wild-type (y/+) and miR223\textsuperscript{y/-} (y/-) mice. Pseudo colors visualize proteins from miR223\textsuperscript{y/-} mice (green) versus wild-type mice (red). Yellow spots reflect proteins that did not change between the experimental groups. Numbers indicate proteins of interest (average ratio $\pm$ 1.15 and $P<0.05$) identified by mass spectrometry and listed in Supplemental Table 1.
Figure V. Consequences of miR-223 deletion or downregulation on the expression of β3 and α5 integrins in platelets. (A) Representative blots showing the expression of β3 and α5 integrin in washed platelets from wild-type (y/+) and miR-223 y/- (y/-) mice. (B) Levels of β3 and α2 integrin in washed platelets from healthy (H) and diabetic subjects (D). Similar results were obtained with a total of 4-10 mice/donors per group.
Figure VI. Consequences of a high fat diet and miR-223 antagonism on platelet protein expression. Male wild-type mice were treated with (A) normal chow (NC) or a high fat diet (HFD) for 20 weeks, or (B) treated with control oligonucleotides (CTL) or antagomirs directed against miR-223 (AmiR) 3 times over 12 days. Western blots show the expression of β1-integrin (β1), Dicer, FXIII-A and the active Δ form (55 kDa) in washed platelets. The graphs summarize data from 5 mice per group; *P<0.05; **P<0.01 versus NC or CTL.
Figure VII. Consequences of pre-miR-223 overexpression and β1 integrin downregulation on kindlin-3 expression in human endothelial cells. Representative blots showing the levels of kindlin-3 (K3) in cells transfected with a control pre-miRNA, Pre-miR-223, a control siRNA (si-CTL) or siRNA directed against β1 integrin (si-β1) for 48 hours. Similar results were obtained in 3 additional experiments.
Figure VIII. Phenotype of diabetic human platelets and sensitivity to rhodocetin. (A) Clot size 60 minutes after the addition of thrombin (0.1 U/ml) to platelet rich plasma from healthy (H) and diabetic (D) individuals. (B) Effect of rhodocetin (Rdc; 5 µg/mL, 5 minutes) on the percentage of non spread and spread platelets on collagen from healthy and diabetic individuals. (C) Representative images showing aggregate formation on collagen by platelets from healthy and diabetic patients under no flow conditions for 30 minutes. Experiments were performed in the absence and presence of rhodocetin; bar=10 µm. The graphs summarize data from 3-5 subjects per group; **P<0.01, ***P<0.01 versus healthy/solvent; #P<0.05, ##P<0.01 versus diabetic/solvent.
Figure IX. FXIII-A is targeted by miR-223 and affects platelet function. (A) Effect of a control (CTL) miR and pre-miR-223 on the activity of the wild-type (WT) or mutated (Mut) FXIII-A 3'UTR in HEK-293 cells. The graph summarizes data from 4 independent experiments; *P<0.05; **P<0.01. (B) Effect of tridegin (50 µg/ml) on FXIII activity (upper panel) and thrombin-induced clot retraction (lower panel) in platelet rich plasma from wild-type versus miR-223 y/- mice. The graph summarizes data from 9-13 mice per group; *P<0.05; **P<0.01 versus wild-type, §P<0.05; §§P<0.01 versus in the absence of tridegin.
Figure X. Consequence of diabetes and *in vivo* calpain inhibition on miR-155. Mature miR-155 levels in platelets from control (CTL) and diabetic (STZ) mice treated with vehicle or A-705253. The graph summarize data from 5-13 mice per group.