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Graphical abstract

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Platelet-derived miR-223 promotes a phenotypic switch in arterial injury repair.

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Abstract

Upon arterial injury, endothelial denudation leads to platelet activation, and delivery of multiple agents (e.g. TXA2, PDGF) promoting VSMC dedifferentiation, and proliferation, in injury repair (intimal hyperplasia). Resolution of vessel injury repair, and prevention of excessive repair (switching VSMC back to a differentiated quiescent state) is a poorly understood process. We now report that internalization of activated platelets by VSMCs promotes resolution of arterial injury by switching on VSMC quiescence. Ex vivo and in vivo studies using lineage tracing reporter mice (PF4-Cre x mTmG) demonstrated uptake of green platelets by red vascular smooth muscle cells upon arterial wire injury. Genome-wide miRNA sequencing of VSMCs co-cultured with activated platelets identified significant increases in platelet-derived miR-223. miR-223 appears to directly target PDGFRβ (in VSMCs) reversing the injury-induced dedifferentiation. Upon arterial injury platelet miR-223 knockout mice exhibit increased intimal hyperplasia, whereas miR-223 mimics reduced intimal hyperplasia. Diabetic mice with reduced expression of miR-223, exhibited enhanced VSMC dedifferentiation, proliferation and increased intimal hyperplasia. Horizontal transfer of platelet-derived miRNAs into VSMCs provide a novel mechanism for regulating VSMC phenotypic switching. Platelets thus play a dual role in vascular injury repair, initiating an immediate repair process, and concurrently, a delayed process to prevent excessive repair.

Key words: Vascular smooth muscle cells, arterial injury, platelets, diabetes mellitus, microRNA
Introduction

At sites of vascular injury where endothelium is damaged or removed, vascular smooth muscle cells (VSMC) are directly exposed to activated platelets (1, 2). Platelets provide a first and crucial line of defense against vascular injury to maintain normal hemostasis (3, 4). Upon activation, platelets also release bioactive mediators such as PDGF and thromboxane promoting VSMC dedifferentiation from a quiescent contractile phenotype to a highly synthetic and proliferating cell type, promoting injury repair (5). Excessive repair, such as observed with intimal hyperplasia in diabetes mellitus, can result from enhanced VSMC dedifferentiation and proliferation (6). The mechanism for dysregulation of VSMC phenotypic switching in vascular injury repair in diabetic patients is unclear. This is clinically important as diabetic patients are more likely to suffer restenosis (intimal hyperplasia) after a vascular procedure (7, 8).

Platelets are anucleate cells without genomic DNA. However, they contain an abundant and diverse array of mRNAs and miRNAs, which appears disproportionately high relative to their low level of protein synthesis (9). Recent advances have demonstrated that activated platelets (APs) contain microRNAs (miRNAs) in complex with the miRNA effector protein Ago2, which can also be released into the circulation and engaged in horizontal miRNAs transfer into other vascular cells, modulating gene expression and function in the recipient cells (10-13). Platelet derived miRNAs can be internalized by recipient cells including endothelial cells, macrophages, hepatocytes and tumor cells, and play a role in regulating their functions (14-16). Platelet miRNAs are thus recognized to play important roles in interacting with and modulating many cells, however, whether platelet miRNA can modulate VSMCs in vivo and the pathophysiological consequences of such interactions is unknown. Such interactions are plausible with arterial injury, denudation of endothelium and subsequent platelet activation and thrombosis on the exposed vessel wall. The study of platelet VSMC interactions may provide important new insights into arterial injury repair and intimal hyperplasia.

We now demonstrate that internalized activated platelets release key functional miRNAs (miR-143, miR-145 and miR-223), which inhibit the expression of their targets, namely Kruppel-like factor 4 (KLF4), Kruppel-like factor 5 (KLF5) and PDGFRβ, thereby suppressing VSMC dedifferentiation (promoting resolution of repair). DM platelets exhibit significantly reduced expression of miR-223. Deficiency of miR-223 in platelets under DM conditions and in miR223 knockout mice, contribute to enhanced VSMC dedifferentiation, leading to exacerbated intimal
hyperplasia. Targeting the mechanisms by which platelet miRNAs regulate the gene expression of VSMCs represents a potential therapeutic strategy for treatment of smooth muscle related vascular diseases such as excessive repair as observed with intimal hyperplasia.

Results

Platelets are internalized by VSMCs and induce VSMC differentiation. At sites of arterial injury where endothelium is damaged, vascular smooth muscle cells are directly exposed to activated platelets (1, 2). We initially co-cultured activated platelets (APs) (Cell Tracker green CMFDA-labeled) with human aortic vascular smooth muscle cells (VSMC). We were anticipating that activated platelets would induce VSMC dedifferentiation as part of the recognized wound repair response. Unexpectantly, the protein expression of VSMC differentiation markers ACTA2, TAGLN and CNN1 were significantly higher in VSMCs co-cultured with APs than those co-cultured with resting platelets (RPs) or without platelets (Ctrl) (Figure 1A). Concurrent significant decreases in VSMC dedifferentiation markers including KLF4, KLF5, and OPN in VSMCs further supported the induction of a differentiated state by activated platelets (Figure 1A). These effects were also observed at the mRNA level (Supplemental Figure 1). VSMCs co-cultured with APs also exhibited reduced CCK-8 (Cell Counting Kit-8), BrdU incorporation (Figure 1B & C) and reduced expression of PCNA (Supplemental Figure 2), demonstrating decreased proliferation (another hallmark of VSMC differentiation). Taken together, activated platelet coculture appear to promote rather than inhibit, VSMC differentiation. This seemed paradoxical, as it is well recognized that platelet activation leads to rapid release of thromboxane and PDGF both of which reduces VSMC differentiation (repair process). Thus, activated platelets appear to both reduce, and promote, VSMC differentiation.

Important insights were gained when we co-cultured VSMCs with APs for 48 hours and performed confocal microscopy. We observed incorporation of whole APs by VSMCs and this incorporation progressively increased over time (Figure 1D). Notably, the APs localized to the cytoplasm at early time points (1-4 hours), but to the perinuclear region after 24 hours of co-culture (Figure 1D). Confocal laser scanning microscopy with three-dimensional reconstruction, supported that APs (green) were internalized into the VSMC, the internalized platelets (green) being on a similar level as the nucleus (blue) (Figure 1E). APs internalization by VSMCs
increased with time of co-culture and was observed in almost all VSMCs after 24 hours of co-culture. Transmission electron microscopy was performed to further confirm the process of platelet internalization in VSMCs. Key to the identification of platelets on electron microscopy is the presence of distinct granules. With activated platelets there is substantial granule release with associated changes in architecture, however some granules and architecture remain, allowing for precise platelet identification. Initially platelets are at the membrane of VSMCs (Figure 1F – red arrow). This is then followed by internalization where both the internalized VSMC plasma membrane and platelet plasma membrane are intact (Figure 1G – red arrow). Loss of plasma membranes likely occurs through a lysosomal mechanism, followed by incorporation into the vascular smooth muscle cell cytoplasm (Figure 1H & I – red arrow). The sources for each micrograph (lower magnification) are provided in Supplemental Figure 3.

To justify continuing the studies we wanted to determine whether activated platelets are taken up by injured vessels in vivo. We crossed the PF4-cre mice (platelet specific) with the mT/mG reporter line creating a mouse where platelets are permanently labeled with mGFP (green), while cells of all other lineages including VSMCs express mTomato (red). Thus, red VSMC would turn green from incorporation of mGFP, if platelets are incorporated. Femoral artery wire injury was performed to damage endothelium and activate platelets (an in vivo model for induction of VSMC dedifferentiation and repair). No platelets (green) were detected in the uninjured femoral arteries (Figure 1J). However, one week after injury, mGFP positive cells were readily found in the medial VSMC layer of the injured vessel (Figure 1J – arrows). Importantly, we demonstrated that the expression of cre recombinase can be detected in both resting and activated platelets isolated from PF4-mT/mG mice, but not in wildtype mice (Supplemental Figure 4). Platelets (no genomic DNA) and VSMCs were also isolated from PF4-mT/mG mice, and co-cultured for 48 hours. We detected genomic DNA recombination from co-cultured (48 hours) mice VSMCs with APs and cre groups in comparison with the resting platelet (RP) group (Supplemental Figure 5). Based on these initial in vitro and in vivo data we developed the hypothesis that upon arterial injury, activated platelets are internalized by VSMCs (hours to days), at sites of injury and may serve as a VSMC phenotypic switch.

Activated platelets transfer miRNAs into VSMCs to inhibit VSMC dedifferentiation. Incorporated platelet must contain potent agents to initiate the switch from VSMC dedifferentiation to differentiation. Platelets have been reported to contain a large number of
miRNAs (17-19). To initially determine whether platelet internalization could transfer miRNA we used a megakaryoblastic cell line MEG-01 to produce labeled platelets (15). Platelets were incorporated with 1) labeled RNA (assessed by EU incorporation) (Supplemental Figure 6A), 2) transfected synthetic exogenous miRNA syn-cel-miR-39 (Supplemental Figure 6B), and 3) transfected FITC-labeled scrambled miRNA (miR-Scr-FITC) (Supplemental Figure 6C). Each demonstrated incorporation of platelet derived miRNA in VSMCs. Based upon the EM (Figure 1F and Supplemental Figure 3) we hypothesize that the miRNA is initially located in the activated platelets but is released upon internalization and removal of platelet cell membrane. Taken together, these in vitro results supported transfer of miRNA from incorporated activated platelets into VSMCs.

To identify platelet miRNAs that may be involved in the horizontal transfer and inhibit VSMC dedifferentiation, we performed miRNA-seq on VSMCs co-cultured with APs (APs) or without APs (control) (Figure 2A). We identified a number of miRNAs that were differentially expressed in VSMCs when co-cultured with APs, including key miRNAs with established roles in regulating VSMC phenotype such as miR-223, as well as the VSMC master miRNA cluster miR-143/145. These changes were independently validated by qRT-PCR at various time points (2, 4, 8, 24 and 48 hours) following co-culturing with APs. We found significant upregulation of miRNA expression in APs group over control group, with expression peaking at 4 hours for miR-223 or 24 hours for miR-143/145 (Figure 2B). Since miRNAs are known to incorporate into Argonaute 2 (Ago2) effector complexes for the regulation of specific mRNAs through translational repression, we performed Ago2 immunoprecipitation, followed by qPCR detection of miR-223 and miR-143/145. Our results demonstrated Ago2•miR-143, miR-145 and miR-223 complexes in platelets, suggesting that the increased miRNAs were functional in VSMCs after co-culturing with APs (Supplemental Figure 7). Pretreatment with prostacyclin (prevents platelet activation) or RNase A (degrades the RNA) in platelets significantly attenuated miR-223 and miR-143/145 to VSMCs (Figure 2C), suggesting transfer of miRNA rather than induction in VSMC and was consistent with previous observations in endothelial cells and hepatocytes (14, 15). Importantly, pretreatment with these reagents (prostacyclin and RNase A) could significantly reduce the pro-differentiation effect induced by APs on VSMCs (Figure 2D). The observed changes were not due to an effect of thrombin itself (used to activate the platelets) on the endogenous expression of these three miRNAs in VSMCs (Supplemental Figure 8). Taken
together, these results support the uptake of activated platelets by VSMCs lead to horizontal transfer of platelet miRNA into VSMCs, resulting in a switch from VSMC dedifferentiation to VSMC differentiation.

**KLF4, KLF5 and PDGFRβ are the critical targets of miR-143, miR-145 and miR-223 for regulating VSMC dedifferentiation.** Bioinformatics analysis identified a conserved binding site for miR-145 in the 3’ untranslated region (UTR) of KLF4. For miR-143 and miR-145 putative binding sites were located at the 3’ UTR of KLF5 and for miR-143 and miR-223 putative sites at the 3’ UTR for PDGFRβ (*Supplemental Figure 9*). To determine whether miR-143 and miR-223 could bind to PDGFRβ and inhibit its expression directly, a fragment of the 3’-UTR of PDGFRβ was cloned into a luciferase reporter. Either adding miR-143 mimic or miR-223 mimic (*Supplemental Figure 10*) resulted in significant inhibition of luciferase activity, while in the mutated control groups the inhibitory effect of miR-143 mimic and miR-223 mimic on luciferase activity was abrogated (*Supplemental Figure 11A & B*). Our data demonstrate for the first time that miR-143 and miR-223 can directly target the 3’ UTR of PDGFRβ (*Supplemental Figure 11A & B*) and inhibit expression (*Supplemental Figure 11C*). Similarly, miR-143 could directly target the 3’ UTR of KLF5 (*Supplemental Figure 11D*) and inhibit its expression (*Supplemental Figure 11F*), This observation is supported by previous studies (20, 21). Our results also showed that miR-145 can directly bind to the 3’ UTR of KLF4 & KLF5 (*Supplemental Figure 11E & G*) and inhibit expression (*Supplemental Figure 11F & H*), and is also supported by previous reports (22). Taken together, these results indicate that miR-143, miR-145 and miR-223 can regulate VSMC dedifferentiation by targeting KLF4, KLF5, and PDGFRβ respectively, in VSMCs. Therefore, horizontal transfer of those miRNAs from activated platelets to VSMCs may contribute to VSMC phenotypic switching.

In order to further prove that externally derived (platelet) miR-143, miR-145 and miR-223 are indeed critical to VSMC phenotypic switching, rescue experiments are needed. We had found that expression of miR-143, miR-145 and miR-223 were significantly reduced in platelet-like particles (PLPs) derived from MEG-01 cells under high glucose (HG, 33 mM) compared with the levels of glucose found in the normal growth media (NG, 17.5 mM) (*Figure 3A*). To verify the importance of platelet-derived miRNAs in regulating VSMC phenotype, we cocultured VSMCs with PLPs derived from MEG-01 under normal glycemic conditions (NG PLPs) or high glucose (HG PLPs). As expected, NG PLPs could significantly inhibit the
expression of PDGFRβ, KLF4 and KLF5 (Figure 3B). However, HG PLPs with the reduced levels of miRNAs had no effect on regulation of these three proteins expression (Figure 3B). Next, lipofectamine transfection was performed to restore the expression levels of miR-143, miR-145 and miR-223 in HG PLPs (miRNA PLPs) (Supplemental Figure 12). We found that the expression of PDGFRβ, KLF4 and KLF5 were significantly inhibited in miRNA PLPs, resulting in VSMC differentiation (Figure 3C). Moreover, we found that the inhibitory effect of miR-223 is delayed compared to PDGF stimulation in vitro. At the early time points (6-12 hours) of co-culture, PDGF released from platelets promoted VSMC proliferation, and the platelet-derived miR-223 was accumulated in VSMCs (Figure 3D). At the later time points (24-48 hours), the platelet-derived miR-223 was utilized by VSMCs, resulting in inhibition of cell proliferation and its target PDGFRβ expression (Figure 3D). Taken together, these data demonstrate that miRNAs released by platelets are a major regulator of VSMC phenotype switch.

miR-223 modulates Pdgfrβ expression and intimal hyperplasia in vivo after femoral arterial injury. miR-143, miR-145 are native to both platelets and VSMC whereas miR-223 is specifically expressed in the hematopoietic system (23) (including platelets), with no significant VSMC expression. As proof of principle for our hypothesis that activated platelet uptake by VSMC transfers miRNA that leads to a phenotypic switch, we used a miR-223 platelet knockout and rescued with AgomiR-223 through local delivery at the site of femoral wire injury. We first determined the expression levels of Pdgfrβ (dedifferentiation) and Acta2 (differentiation) in femoral arteries with or without injury. As shown in Figure 4A, in uninjured vessels, the expression of Pdgfrβ was very low. At 4 weeks after injury, the expression of Pdgfrβ was upregulated particularly in the miR-223 KO mice treated with control miR-NC (Figure 4A & B). Treatment with AgomiR-223 significantly rescued the miR223 KO decreased the expression of Pdgfrβ (Figure 4A & B). These results suggest that Pdgfrβ is indeed a target gene of miR-223 in vivo. Phenotypic change of VSMC plays a critical role vascular neointimal development (intimal hyperplasia). We thus determined the effect of miR-223 on neointimal growth. After four weeks of femoral arterial injury, increased intimal size was observed in the miR-223 KO mice treated with AgomiR-NC (Figure 4C), as indicated by the significantly increased intima to media (I/M) ratio (Figure 4D). Restoration of the downregulated miR-223 in wire-injured femoral arteries significantly inhibited neointimal hyperplasia (Figure 4C & D). We additionally
performed an experiment demonstrating that the levels of internalized miR-223 in cocultured VSMCs with whole activated platelets is significantly higher than with activated platelet derived microparticles (Supplemental Figure 13). Together with the size and morphology of the internalized platelets (Figure 1F), support whole platelet uptake rather than internalization of platelet derived microparticle. Taken together these studies support a phenotypic switch provided by the transfer of miR-223 from activated whole platelets to VSMC.

**DM mice has reduced miR-223 and increased intimal hyperplasia.** It has been reported that the levels of miRNAs in platelets are altered under pathological conditions such as in DM (24,25). Assessment of both human and mouse DM platelets, demonstrated consistent reductions in miR-223 expression (Figure 5A). Femoral wire injury in DM mice demonstrated significant incorporation of platelets (green) into VSMC in the media as previously observed with WT mice (Figure 5B). Given that levels of miR-223 are reduced in DM platelets (Figure 5A), we set out to determine VSMC injury response, comparing DM to WT mice. We initially performed fluorescent in-situ hybridization (FISH) in femoral wire injury arteries to directly detect and visualize miR-223 uptake in VSMCs. One week after wire injury, the levels of miR-223 in mGFP-positive VSMCs (contributed by activated platelets) were notably higher in the non-DM than the STZ-DM mice (Figure 5C). Quantitation using image J demonstrated significant (p<0.01) reduction in the STZ-DM vs non-DM mice (Figure 5D). These in vivo results are supported by the in vitro mechanistic studies and are consistent with the low miRNAs observed in mouse STZ-DM platelets and human DM platelets. Moreover, concurrent staining for Acta2 was increased in the non-DM compared to the STZ-DM (lower miRNAs) also supporting the in vitro studies. Thus, under STZ-DM conditions, although platelets were incorporated into medial VSMCs, there is significantly reduced horizontal transfer of platelet miRNAs. These results provide the first direct visualization of platelet derived miRNA in vascular smooth muscle cells. However, although supported by the Acta2 staining, further proof was needed to demonstrate such incorporation has a biochemical and biological effect on vascular smooth muscle cells and injury repair.

We next set out to determine (using high resolution confocal microscopy) whether this translated to increased expression of their target genes in vivo (in single VSMC) that have taken up the activated platelets (green VSMCs). The basal expression of Pdgfrβ were undetectable by immunofluorescence in femoral arteries of non-DM and STZ-DM mice (Figure 6A). As
expected, one week after wire injury, Pdgfrβ expression was increased in non-DM group, but more-so in STZ-DM group (Figure 6A), reflecting the dedifferentiated state. We detected two populations of VSMCs in the media of injured tissues – VSMCs with internalized platelets (green with Dapi) and those without (Dapi alone) (Figure 6A). Media VSMCs with internalized platelets demonstrated significantly increased Pdgfrβ in the STZ-DM vs non-DM VSMC reflecting the reduced miR-223 in DM platelets (Figure 6B). VSMCs with internalized platelets also exhibited increased expression of Acta2 with reduced expression of Pdgfrβ (Figure 6A & B). These results are consistent with our in vitro findings that horizontal transfer of platelet-derived miR-223 into VSMCs inhibits VSMC dedifferentiation via the downregulation of Pdgfrβ. In their absence, such as under STZ-DM conditions or in miR-223 KO, the expression of Pdgfrβ were increased in the injured vessels, contributing to vascular smooth muscle cell proliferation and intimal hyperplasia.

We then used the miR-223 platelet knockout and rescue to assess whether DM leads to excess intimal hyperplasia (excess injury repair) and look for degree of rescue with AgomiR-223. In uninjured vessels, the expression of Acta2 was consistently high and Pdgfrβ was very low (Figure 6C & D). At 4 weeks after injury, the expression of Acta2 was very low in the STZ-DM treated with control and appeared to be rescued with the AgomiR-223 (Figure 6C & D). The converse was true with Pdgfrβ supporting the targeting of Pdgfrβ by miR-223. Moreover, when assessing for intimal hyperplasia, DM demonstrated a doubling of I/M ratio which was in part rescued by AgomiR-223 (Figure 6E & F). Taken together these studies support a phenotypic switch provided by the transfer of miR-223 from activated platelets to VSMC. DM demonstrates increased injury repair response which can be reduced by miR-223 mimics.

Model for platelet VSMC interaction (Figure 7). During normal physiology the endothelial barrier is intact, platelets are quiescent, exhibiting no interactions with VSMC. With injury (as with angioplasty), the endothelial barrier is compromised, exposing the subendothelium, leading to platelet activation. In addition to thrombosis there is release of agents (immediate) that initiate wound repair, including induction of VSMC dedifferentiation and proliferation. Activated platelets are then taken up by VSMC (hours to days) releasing miR-223 (and others such as miR143/145) which applies a brake on VSMC dedifferentiation, switching to VSMC differentiation. This second “wave” (delayed response) prevents excess VSMC repair by promoting injury response resolution and thus reduced intimal hyperplasia. With reduced miR-
223 in DM platelets, excess VSMC proliferation (reduced differentiation) leads to excess intimal hyperplasia.

**Discussion**

Upon vascular injury, the initial response is to maintain hemostasis with clot formation, vessel contraction and cellular proliferation. This immediate response is mediated largely by substances such as thromboxane and PDGF, which are released from activated platelets, promoting VSMC dedifferentiation in wound healing (26-28). If the response is left unabated, substantial intimal hyperplasia can occur. To prevent severe intimal hyperplasia, a brake needs to be applied relatively early to regulate the VSMC proliferative response (Figure 6).

**Direct visualization of platelet uptake**

We report that activated platelets are directly incorporated into VSMC at sites of injury to apply this needed brake. Our femoral artery wire injury model allowed for direct in vivo visualization of the interaction between circulating platelets and exposed VSMCs due to the denudation of the endothelium layer. To our knowledge, this is the first study to establish an in vivo model directly visualizing platelet (expressing PF4-icre driving mTmG recombination) internalization into recipient cells. In this powerful double-fluorescent system, mTomato labeled VSMCs (red) became mGFP-positive (green) once they are incorporated platelets. Recipient individual VSMCs can be precisely located in the injured artery, and biochemical changes followed. High expression of PDGFRβ in VSMCs exemplifies VSMC dedifferentiation, contributing to the VSMC proliferation and intimal hyperplasia (29). Distinct from the mTomato-positive VSMCs, the mGFP-expressing VSMCs (uptake of platelets) show reduced expression of PDGFRβ than the mTomato-expressing VSMCs (no platelet uptake), suggesting that platelet internalization can suppress VSMC dedifferentiation. We hypothesize that platelet internalization is likely followed by lysis of the platelet cell membrane, allowing for release of contents such as miRNA. Taken together, our study provides direct visual and biochemical evidence for platelets orchestrating VSMC response at the site of vascular injury.

**Identification of critical platelet derived miRNAs that regulate the VSMC switch**
Using a genome-wide miRNA screening, we identified key platelet miRNAs (miR-223 and miR143-145) that target VSMC differentiation and proliferation genes. Platelet-derived miRNA is ideal as they can catalytically regulate many proteins and coordinately ensure return to a differentiated quiescent state. We demonstrate for the first time that platelet-derived miR-223 can downregulate PDGFRβ in VSMCs, in turn inhibiting PDGF-induced VSMC proliferation. Previous studies have demonstrated that PDGFRβ is primarily expressed in VSMCs (30), and inhibition of PDGFRβ suppressed VSMC proliferation in vitro and in the model of balloon-injured arteries (31). In addition, we also found that the platelet-derived miR-143 and miR-145 can downregulate KLF4 in VSMCs, corroborated by previous studies showing that endogenous VSMC miR-143 and miR-145 induces differentiation by directly targeting KLF4 and KLF5 (22, 32). KLF4 and KLF5 are the master regulators of VSMC dedifferentiation, and inhibition of KLF4 and KLF5 expression promotes VSMC differentiation. Taken together, we demonstrate both in vitro and in vivo, that platelet-derived miR-223 is incorporated into VSMCs, and directly target PDGFRβ respectively, resulting in inhibition of VSMC dedifferentiation and reduction of intimal hyperplasia. Thus, VSMC response to injury is a delicate balance between early dedifferentiation and late differentiation during recovery.

Diabetes and intimal hyperplasia

Key to our studies is the observation that DM platelets have reduced miR-223 expression and thus a reduced ability to apply a brake on the VSMC dedifferentiation. DM patients are recognized to be more prone to restenosis (intimal hyperplasia) and less responsive to drug eluting stent agents (33). We induced diabetes mellitus in the PF4-mTmG mice and performed femoral artery wire injury. Consistent with our hypothesis, the mGFP-VSMCs demonstrated higher expressions of PDGFRβ and KLF4 but lower levels of miR-223, compared to those in non-DM group, suggesting that DM platelets with deficiency of miR-223 infiltrating VSMCs failed to attenuate the increased expressions of PDGFRβ and KLF4. The present study sheds light on a possible cause for the severe intimal hyperplasia often observed with diabetes mellitus (29, 34). Our studies also provide some insights into additional miRNAs based therapeutic targets to prevent vascular disease, particularly in DM patients.

Implications for other vascular injuries.
Not only with mechanical injury repair such as balloon angioplasty (or femoral wire injury), but VSMC phenotypic switching also plays a crucial role in the pathogenesis of atherosclerosis. VSMCs residing in normal arteries exhibit a contractile phenotype with low proliferative rate (35, 36). However, atherosclerosis and vascular injury lead to VSMC migration and proliferation (37). Around the atherosclerotic regions, platelets are activated by the exposed collagen and shear stress (38, 39), resulting in the release of PDGF and other bioactive proteins, which induce VSMC dedifferentiation, at least in part leading to intimal hyperplasia. Targeting the mechanisms by which platelet miRNAs regulate the gene expression of VSMCs represents a potential therapeutic strategy for treatment of many vascular smooth muscle related vascular diseases.

Methods

Platelet purification and activation. Human platelets were isolated from venous blood of consenting volunteers (healthy and DM subjects) at Yale University School of Medicine (Human Investigation Committee No. 1005006865) (Table S1), and prepared as previously described (40). Briefly, platelet rich plasma (PRP) was obtained by centrifugation at 250 g for 15 minutes, and platelets were sedimented at 1000 g for 15 minutes and then resuspended at $10^8$ platelets/ml in HEPES-Tyrode’s buffer. Platelet activation were induced upon incubation with 0.1 U/ml thrombin (Sigma-Aldrich, USA) for 15 or 60 minutes at 37°C with gentle agitation. Platelet activation was inhibited by the addition of 20 mM EDTA, and platelets were pelleted by centrifugation at 3,200×g for 10 minutes. For murine platelets, blood was drawn from the right cardiac ventricle into 1.8% sodium citrate buffer (pH 7.4) and diluted with equal volume of HEPES/Tyrode’s buffer. PRP was prepared by centrifugation at 100 g for 10 mins and then washed platelets were prepared from PRP by centrifugation at 5000 g for 2 min. Platelet pellets were resuspended in HEPES/Tyrode’s buffer (41).

Cell culture. Primary human arterial smooth muscle cells (VSMCs) were obtained from Cell Applications (San Diego, CA) at passage 3. Cells were grown in smooth muscle cell media supplemented with 10% fetal bovine serum (FBS), smooth muscle cell growth supplement and 1% penicillin-streptomycin (GIBCO, USA). MEG-01 cells (ATCC, Georgetown, WA) were cultured in Dulbecco’s modified RPMI 1640 medium (GIBCO) supplemented with 4.5% (vol/vol) L-glutamine (GIBCO) and 10% (vol/vol) FBS (Invitrogen, USA). Primary mouse
VSMCs were isolated from wildtype and mTmG mice aorta by enzymatic digestion and cultured as described (42). VSMCs were co-cultured with or without resting platelets (RPs) or thrombin-activated platelets (APs). To determine the functional role of target miRNAs in VSMCs, VSMCs were transfected with miR-143 mimic, miR-145 mimic, miR-223 mimic or negative control (NC) (Gene-Pharma, Shanghai, China) at 100 nM by using lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer’s protocols. To restore the expression of miR-143, miR-145 and miR-223 in platelet-like particles (PLPs) derived from HG-treated MEG-01 cells, MEG-01 cells were simultaneously transfected with miR-143, miR-145 and miR-223 mimics at 100 nM by using lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer’s protocols. After that, PLPs were collected from Thrombopoietin (TPO, Life Technologies)-stimulated MEG-01 cells, and co-cultured with VSMCs. The proteins were collected after transfection for 48 hours. The sequences are listed in Table S2.

**CCK-8 assay.** The ability of cell proliferation was analyzed via Cell Counting (CCK8) Kit-8 (Dojindo, Japan). After treatment as described above, VSMCs were incubated with 10 μl WST-8 in 100 μl culture medium for 2 hours at 37 °C. The absorbance of each sample was measured at 450 nm with a microplate reader (Thermo Scientific).

**BrdU incorporation assay.** VSMCs were cultured (10^4 per well) in 96-well plates overnight, followed by serum starvation for 24 hours. Starved VSMCs were treated as described as above for 48 hours. Cell proliferation was also evaluated using Bromodeoxyuridine (BrdU) Cell Proliferation Assay Kit (Millipore, USA) according to the manufacturer's instructions. Briefly, cells were incubated with BrdU for 12 hours before measurement. The cells were fixed and denatured the DNA using the Fixing Solution. After fixation, cells were labelled with a peroxidase-conjugated BrdU antibody, followed by incubation with the peroxidase substrate. Finally, the acid stop solution was added into each well to stop the reaction. The absorbance of the samples was measured by a microplate reader at 450 nm (OD450) with reference measurement at 550 nm (OD550). Absorbance (A450nm-A550nm) values representing cell proliferation ability were compared between treatments.

**miRNA sequencing and data analysis.** Total RNA was extracted using a miRNeasy mini kit (QIAGEN, GmBH, Germany) according to the manufacturer’s protocol. The RNA molecules (18 ~30 nt) were enriched by polyacrylamide gel electrophoresis (PAGE). The 3’ adapters were then added, and the RNA molecules (36 ~44 nt) were enriched. The 5’ adapters were then
ligated to the RNAs as well. The ligation products were amplified by RT-PCR. The PCR products with 140-160 bp were enriched to generate a cDNA library, and sequenced using Illumina HiSeqTM 2500 by Gene Denovo Biotechnology Co. (Guangzhou, China). The raw data were processed using the Illumina Genome Analyzer Pipeline software and submitted to data filtration. After filtering the low-quality reads and trimming the adaptor sequences, clean reads were obtained. Total miRNA consists of existing miRNA, known miRNA and novel miRNA, based on their expression in each sample, the miRNA expression level was calculated and normalized to transcripts per million (TPM). The heatmaps of existing miRNA, known miRNA and novel miRNA were drawn to display miRNA expression levels in different samples and to cluster miRNAs with similar expression pattern. Those miRNAs with a fold change greater than 1.5 and P value higher than 0.05 were regarded as significant differentially expressed miRNAs. Results from the sequencing was deposited in the NCBI database (https://www.ncbi.nlm.nih.gov/Traces/study/?acc=SRP135734).

**Isolation of RNA and miRNA for quantitative RT-PCR.** Total RNA, including miRNA, was isolated from cells using the miRNeasy RNA isolation kit (QIAGEN). RNA and miRNA were reverse-transcribed using the PrimeScrip™ RT reagent Kit (TAKARA, Japan) and the Hairpin-it™ microRNA & U6 snRNA Normalization RT-PCR Quantitation Kit (GenePharma) respectively. RNA was extracted from plasma and serum using the miRNeasy Serum/Plasma kit (QIAGEN). The C. elegans cel-miR-39 (GenePharma) was used as the spike-in control. The transcript levels were analyzed by quantitative RT-PCR using the $2^{-\Delta\Delta Ct}$ method. Primer sequences are listed in Table S3 and S4.

**Labeling of RNA and miRNA in PLPs.** MEG-01 cells were incubated with 0.5 mM 5-ethynyluridine (EU) (Life Technologies) in combination with 100 ng/ml TPO (Life Technologies) for 48 hours (15) to produce platelet-like particles (PLPs) that incorporated EU into their RNA. Isolated PLPs were washed twice in RPMI 1640 medium (GIBCO). EU was visualized using the Click-iT RNA Alexa Fluor 488 Imaging Kit (Life Technologies). For labeling miRNA, platelets were transfected with synthetic cel-miR-39 or fluorescently labeled scrambled miRNA (miR-Src-FITC) using lipofectamine RNAiMAX reagent, and then washed twice to remove the transfection reagent. After transfection, the platelets were activated by 0.1 U/ml thrombin, and then co-cultured with VSMCs. The RNAs were harvested from VSMCs after 4 hours of co-culture, and the level of miR-39 transfer was determined by qRT-PCR.
**Western blot analysis.** The protein was extracted from cells using protein lysis buffer and separated using 12% SDS-PAGE gels. Primary antibodies for PCNA (Abcam, catalog ab29), KLF4 (GeneTex, GTX 101508), KLF5 (Abcam, catalog ab137676), Osteopontin (OPN, Abcam, catalog ab91655), α-smooth muscle actin (ACTA2, Abcam, catalog ab119952), transgelin (TAGLN, Abcam, catalog ab14106), smooth muscle calponin (CNN1, Abcam, catalog ab46794), and PDGFRβ (Abcam, catalog ab32570), were purchased from Abcam. GAPDH (Abcam, catalog ab125247) or α-tubulin (Sigma-aldrich, catalog T8203, Clone AA13) were used as the loading control for normalization. Densitometry of the bands was quantified using the Image Lab software (USA). All antibodies were used at a dilution of 1:1,000 unless specified otherwise. Full, uncut gels for all the Western blots are shown in the supplemental material.

**Immunofluorescence.** Cells and tissues were fixed and stained according to the standard protocols (42). Primary antibodies include anti-rabbit PDGFRβ antibody (1:200, Santa Cruz Biotechnology, catalog sc-374573), anti-Goat KLF4 antibody (1:200, R&D Systems, catalog AF3158), and Alexa Fluor®594-conjugated anti-ACTA2 (1:200, Abcam, catalog ab202510). DAPI staining was performed to visualize the nuclei. Images were taken on the Leica SP8 (Leica Instruments) confocal microscope with 100X oil immersion lens. The mean fluorescent intensity (MFI) was calculated by ImageJ software and subjected to statistical analysis for significance.

**Luciferase reporter assay.** psi-CHECK2-KLF4, -KLF5 or -PDGFRβ 3’untranslated region (UTR) plasmid was obtained from Addgene (plasmid #31882). HEK293 cells were plated in a 24-well plate and transfected with 1 μg plasmid and 50 nM miRNA mimics (miR-143, miR-145 or miR-223 mimics) by using lipofectamine RNAiMAX reagent. Dual luciferase assay was performed after 48 hours to measure the firefly luciferase conjugated to the 3’UTR normalized to Renilla luciferase activity followed the instructions of manufacturer (Promega, USA).

**Development of PF4-cre:mT/mG Mouse model.** Eight to twelve-week-old mice were used for all studies. Procedures were approved by the Institutional Animal Care and Use Committee of Yale University. The PF4-cre mice (C57BL/6-Tg(PF4-icre)Q3Rsko/J, stock No. 008535) and the mT/mG mice (B6.129(Cg)-Gt(ROSA)26Sor^{m4[ACTB-tdTomato,-EGFP]Luo}/J, stock No. 007676) were purchased from Jackson Laboratory and crossed to generate the PF4-cre:mT/mG mice. Genotyping was performed via PCR using standard methods. We used PF4-icre×Gt (ROSA) 26 mTmG (here after called PF4/mTmG) reporter mice, in which PF4 (platelet factor 4)-icre drives membrane GFP expression in megakaryocytes and platelets, while all other cells were labeled.
with membrane tomato (43). To induce diabetes mellitus, 8-week old mice were injected with streptozotocin (STZ; 50 mg/kg) intraperitoneally for 5 days.

Detection of cre-recombination of the ROSA26mTmG allele. The cre-recombination of the ROSA26mTmG allele was detected according to Hann S et al. (44). Briefly, total DNA was extracted from individual tissues using the DNeasy blood and tissue extraction kit (QIAGEN). One primer pair (mT-F gccgctgtcttgatg and mT-R tgtctcctcctcctg) was designed to generate a 200 bp amplimer from the non-recombined ROSA26mTmG allele, one primer pair (mT-F gccgctgtcttgatg and mG-R tgtctcctcctcctg) was designed to generate a 293 bp amplimer from the recombining ROSA26mTmG allele and another primer pair (mG-F gtctcctcctcctcctg and mG-R tgtctcctcctcctg) was designed to generate a 376 bp amplimer from the cre-recombined ROSA26mTmG allele. The sensitivity of the latter primer pair for detecting cre-recombination of the ROSA26mTmG allele was determined by PCR using template genomic DNA from non-recombined mice that contained serial diluted genomic DNA from mice and VSMCs that had completely recombined alleles. The unmixed genomic DNAs were used as negative and positive controls for each primer pair, and the recombination assay was performed using 200 ng of template DNA.

Femoral artery wire injury model. Endoluminal injury to the common femoral artery was produced by three passages of a 0.15-mm-diameter angioplasty guidewire (Advanced Cardiovascular Systems). Non-operated contralateral femoral arteries were used as controls. Arterial specimens were blindly analyzed by computerized morphometry (NIH Image 1.60 software). Neointima formation was assessed by hematoxylin-eosin (HE) staining and sections were analyzed and percentage of luminal narrowing and the intima-to-media (I/M) ratio were calculated as previously described (45). Intraobserver and interobserver variation coefficients for serial measurements of morphometric parameters were <0.5%. Arteries with occlusive thrombi were not included in the morphometric analysis.

Animal care and use was in accordance with institutional guidelines. The global miR-223 knockout (KO) mice were purchased from Jackson Laboratory (Bar Harbor, USA) and backcrossed to C57BL/6 mice for ≥5 generations. In this strain, the allele replaces the entire coding region of the microRNA-223 (Mir223) gene with a ffr-flanked neomycin (neo) resistance cassette, abolishing gene function. These mice also harbor the CD45.1 (Ly5.1 or Ptprc) allele rather than the CD45.2 (Ly5.2 or Ptprcb) allele normally present in C57BL/6 mice. For
perivascular delivery of miR-223, wire-injured femoral arteries were randomly assigned miR-223 or miR-NC agomiRs group and applied with pluronic gel as described in previous studies (46). Briefly, after wire injury, 100 μl of 30% pluronic gel containing chemically modified and cholesterol-conjugated 2.5 nmol miR-223 or scrambled (miR-NC) agomiRs was applied perivascularly to injured femoral arteries. The micrON™ miRNA agomiRs were purchased from GenePharma (Shanghai GenePharma Co., Ltd., China).

**Fluorescent in situ hybridization (FISH).** FISH was performed according to the manufacturer’s protocol. Briefly, frozen sections were fixed with 4% paraformaldehyde for 30 min and washed with PBS (GIBCO). Fixed sections were treated with proteinase K at 37°C for 10 min, followed by dehydration in ethanol for 5 min. The probe targeting miR-143-3p, miR-145-5p or miR-223-3p (BioSense, China) was then added to the sections respectively. Hybridization was performed in a humid chamber at 42°C overnight. Post-hybridization wash was first performed with 50% formamide with 2×saline sodium citrate at 42°C, and then washed by 2×saline sodium citrate at room temperature to remove non-specific and repetitive RNA hybridization. Slides were counterstained with DAPI (Vector Laboratories, Inc.) and examined with a Leica SP8 (Leica) confocal microscopy. The mean fluorescent intensity (MFI) was calculated by ImageJ software and subjected to statistical analysis for significance.

**Electron microscopy.** For ultrastructural analysis, VSMCs were co-cultured with activated platelets for 4, 24, 48 hours, and then fixed with 2.5% glutaraldehyde for more than 2 hours. The fixed cells were then rinsed with 0.1 M sodium cacodylate buffer for 6 times (30 minutes each) and post fixed with 1% osmium tetroxide for 1 hour at 4°C. Cells were washed 3 times in 0.1 M sodium cacodylate buffer and dehydrated with a graded series of ethanol (30%, 50%, 70%, 90% and 100%; 5 minutes each) followed by embedding in Epon812 and ultrathin sectioning. After uranyl acetate and lead citrate staining, ultrathin sections were examined by the transmission electron microscope FEI CM100 (Japan Electron Optics Laboratory, Tokyo, Japan). Images were captured with an Advantage CCD camera using iTEM software (Olympus, Tokyo, Japan).

**Statistical Analysis.** For normally distributed data, values are expressed as mean ± SD. Parametric t-test was performed for comparing two groups. 1-way ANOVA followed by Tukey-Kramer multiple comparisons test was performed for comparing three groups or more within the same study. For data that were not distributed normally, values were expressed as medians and interquartile ranges (IQR). The Mann-Whitney test was used to analyze differences between
indicated groups. All the statistical analysis was performed using Prism 7 software (GraphPad). A difference of \( P<0.05 \) was considered significant.

Study approval. The animals were housed at the Yale Animal facility 300 George St. New Haven, CT, under the supervision of YARC and animal faculty manager. All procedures were approved under animal protocol #11413 (Yale IACUC) and the Institutional Animal Care and Use Committee of Guangzhou Medical University (No. SYXK2016-0168). All human blood studies were approved by the Yale Human Investigation Committee (protocol# 1005006865). Informed consent was obtained from each subject and conform to the principles set out in the WMA Declaration of Helsinki and the Belmont Report.

Authorship contributions
Conceptualization, ZZ, JH and WHT; Methodology, ZZ, LX XF, ACO, TY, MS, YZ, XP, YX, LP, XG, SKC, and WHT; Investigation, ZZ, LX, XF, MS, RL, JH, and WHT Writing – Original Draft, ZZ, LX, and WHT; Writing – Review and editing, KAM, RL, JH, and WHT; Funding Acquisition, RL, JH, and WHT; Resources, JH, and WHT; Supervision, JH, and WHT.

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References


Figure 1. Platelets were internalized by VSMCs and induced VSMC differentiation. (A) The expressions of markers for differentiation (ACTA2, CNN1, TAGLN) and dedifferentiation (KLF4, KLF5, OPN) in VSMCs (Ctrl) and in VSMCs after co-cultured with resting platelets (RPs) or activated platelets (APs) were determined by Western blot (n = 4). Cell proliferation was assessed by CCK-8 assays (n = 7) (B) and BrdU incorporation assays (n = 4) (C). Data are presented as mean ± SD. *P<0.05, **P<0.01, ***P<0.001 vs Ctrl; #P<0.05, ##P<0.01, ###P<0.001 vs RPs. (D) Representative images of VSMCs co-cultured with CMFDA-labeled platelets (green) for 1, 2, 4 or 24 h in the presence of thrombin. VSMCs were stained with ACTA2 (red) and nuclei visualized with DAPI (n = 6). Scale bars: 2.5 μm. (E) Three-dimensional reconstruction of confocal Z-stack images of the whole VSMC co-cultured with CMFDA-labeled platelets (green). (F, G, H, I) Transmission electron microscopy imaging of VSMCs co-cultured with activated platelets. Panels provide sequence of platelet internalization and incorporation into VSMC. The red arrow indicates the entering and internalized platelet. Lower magnification source electron micrographs are in Supplemental Figure 3. Scale bar: 1 μm. (J) Representative images of the sections from uninjured and injured femoral arteries of PF4-cre:mT/mG WT mice (n = 7). Arrows indicated multiple VSMCs with incorporated green platelets. The injured femoral arteries were harvested at 7th day post-injury. Scale bar: 20 μm. Statistical significance was determined using 1-way ANOVA followed by Tukey-Kramer multiple comparisons test (A, B and C).
Figure 2

A. Heatmap showing expression levels of various miRNAs in Control and APs conditions.

B. Graphs depicting relative expression levels of specific miRNAs over time in Ctrl and APs conditions.

C. Graph showing relative miRNA levels normalized to GAPDH for miR-143, miR-145, and miR-223 in HASMC under various conditions.

D. Graphs showing arbitrary units for ACTA2 and KLF4 expression under different treatments, with corresponding Western blot images.
Figure 2. Activated platelets transferred miR-143, miR-145, and miR-223 into VSMCs, and promoted VSMC differentiation. (A) Heatmap of the top 10 differentially expressed miRNAs in VSMCs co-cultured with or without APs for 24 hours (n = 3). (B) The expression of miR-143, miR-145, and miR-223 in VSMCs (Ctrl) and VSMCs co-cultured with APs (n = 4). (C) The expression of miR-143, miR-145, and miR-223 in VSMCs (Ctrl) and VSMCs co-cultured with RPs or APs after pretreatment with prostacyclin, RNase A or brefeldin A (n = 4). (D) The expression of ACTA2 and KLF4 in VSMCs (Ctrl) and VSMCs co-cultured with RPs or APs after pretreatment with prostacyclin, RNase A or brefeldin A (n = 4). Data are presented as mean ± SD. *P<0.05, **P<0.01, ***P<0.001 vs. Ctrl; &P<0.05, &&P<0.01 vs. RPs; #P<0.05, ##P<0.01 vs APs. Statistical significance was determined using parametric t-test (B), and 1-way ANOVA followed by Tukey-Kramer multiple comparisons test (C and D).
Figure 3

A

B

C

D

3 h 6 h 12 h 24 h 48 h

PDGFRβ

α-TUBULIN
Figure 3. Incorporation of platelet-derived miRNAs is required for inhibiting VSMC dedifferentiation. (A) The levels of miR-143/145 and miR-223 in platelet-like particles (PLPs) derived from MEG-01 cells under normal glucose (NG, 17.5 mM) or high glucose (HG, 35 mM) for 0, 24 hours, 1 week or 2 weeks. Data are presented as mean ± SD (n = 6). **P<0.01, ***P<0.001 vs NG. (B) The expressions of PDGFRβ, KLF4, KLF5 in VSMCs co-cultured with or without PLPs derived from MEG-01 under NG (NG PLPs) or HG (HG PLPs). Data are presented as mean ± SD (n = 6). ***P<0.001 vs Ctrl; ###P<0.001 vs NG PLPs. (C) The expressions of PDGFRβ, KLF4, KLF5 in VSMCs co-cultured with or without PLPs derived from MEG-01 under HG (HG PLPs) or HG PLPs transfected with miR-143, miR-145 and miR-223 mimics (miRNA PLPs). Data are presented as mean ± SD (n = 6). **P<0.01, ***P<0.001 vs HG PLP. (D) VSMCs were co-cultured with activated platelets (APs) for 3, 6, 12, 24 and 48 hours. The PDGFRβ expression (red line), cell proliferation rate (blue line), and the miR-223 level (black line) were detected by Western blot, BrdU incorporation assay, and qRT-PCR, respectively. Data are presented as mean ± SD (n = 4). Statistical significance was determined using 1-way ANOVA followed by Tukey-Kramer multiple comparisons test (A, B, C and D).
Figure 4. miR-223 modulates Pdgfrβ expression and intimal hyperplasia in vivo after femoral arterial injury. (A) Representative immunofluorescence of Pdgfrβ in uninjured or injured femoral arteries from WT, miR-223 KO mice treated with AgomiR-NC or AgomiR-223 at 4 weeks after wire-injury (n = 5). Note: Green (Pdgfrβ) Blue (Dapi nuclear staining) and Red (Acta2 in VSMCs). Scale bar: 20 μm. (B) Quantification of the Pdgfrβ expression in VSMCs in the injured femoral arteries. Data are presented as mean ± SD (n = 5). ***P<0.001 vs WT; ###P<0.001 vs miR-223 KO mice treated with AgomiR-NC. (C) Hematoxylin and eosin staining of serial cross sections from femoral arteries from WT, miR-223 KO mice treated with AgomiR-NC or AgomiR-223 at 4 weeks post-injury (n = 5). Scale bar: 100 μm. (D) Morphometric measurements of the ratio of intimal and media area (I/M ratio) in the injured femoral arterial sections. Data are presented as mean ± SD of I/M ratio (n = 5). **P<0.01 vs WT; ##P<0.01 vs miR-223 KO mice treated with AgomiR-NC. Statistical significance was determined using 1-way ANOVA followed by Tukey-Kramer multiple comparisons test (B and D).
Figure 5. The miR-223 level was reduced in DM platelets and VSMCs in injured femoral arteries. (A) The expression of miR-223 in platelets from healthy subjects (HS, n = 8) and DM patients (DM, n = 13), non-DM murine (non-DM, n = 10) and STZ-induced DM mice (STZ-DM, n = 11). Data were presented as medians and interquartile ranges (IQR). *P<0.05 vs HS or Non-DM. (B) Representative images of the sections from uninjured and injured femoral arteries of PF4-cre:mT/mG STZ-DM mice (n = 7). Arrows indicated VSMCs with incorporated platelets. The injured femoral arteries were harvested at 7th day post-injury. Scale bar: 20 μm. (C) Representative images of miR-223 expressions in injured femoral arteries of PF4-cre:mT/mG mice under non-DM or STZ-DM condition (n = 5). The injured femoral arteries were harvested at 7th day post-injury. Acta2 (red), mGFP (green), and Dapi (white). Scale bar: 20 μm. (D) Quantification of the expression of miR-223 in mGFP-positive VSMCs in the injured femoral arteries (n = 5). Data were presented as mean ± SD. *P<0.05 vs Non-DM. Statistical significance was determined using the Mann-Whitney test (A), and parametric t-test (D).
Figure 6. Platelets-derived miR-223 is responsible for inhibition of neointima formation in diabetic mice after femoral artery wire injury. (A) Representative images of Pdgfrβ expression in uninjured and injured femoral arteries of PF4-cre:mT/mG mice under non-DM or STZ-DM condition (n = 5). The injured femoral arteries were harvested at 7th day post-injury. Scale bar: 20 μm. (B) Quantification of the Pdgfrβ expression in mGFP-positive VSMCs in the injured femoral arteries (n = 5). Data were presented as mean ± SD. *P<0.05 vs Non-DM. (C) Representative immunofluorescence of Pdgfrβ in uninjured or injured femoral arteries from Non-DM, STZ-DM mice treated with AgomiR-NC or AgomiR-223 at 4 weeks after wire-injury (n = 5). Note: Green (Pdgfrβ), Blue (Dapi that reflects total cells), Red (Acta2 in VSMCs). Scale bar: 20 μm. (D) Quantification of the Pdgfrβ expression in VSMCs in the injured femoral arteries (n = 5). Data were presented as mean ± SD. **P<0.01 vs Non-DM; ###P<0.001 vs STZ-DM mice treated with AgomiR-NC. (E) Hematoxylin and eosin staining of serial cross sections from femoral arteries from Non-DM, STZ-DM mice treated with AgomiR-NC or AgomiR-223 at 4 weeks post-injury (n = 5). Scale bar: 100 μm. (F) Morphometric measurements of the ratio of intimal and media area (I/M ratio) in the injured femoral arterial sections (n = 5). Data are presented as mean ± SD of I/M ratio. ***P<0.001 vs Non-DM; #P<0.05 vs STZ-DM mice treated with AgomiR-NC. Statistical significance was determined using parametric t-test (B), and 1-way ANOVA followed by Tukey-Kramer multiple comparisons test (D and F).
Figure 7

1. PLT activation release (PDGF, TXA2)

2. PLT uptake (miRNA)

↑ miR-223→PDGFRβ
↑ DIFFERENTIATION
↓ INTIMAL HYPERPLASIA

↓ miR-223→PDGFRβ
↓ DIFFERENTIATION
↑ INTIMAL HYPERPLASIA
Figure 7. Proposed model for miRNAs transfer in normal and DM platelets into VSMCs in injured vessel. With no vessel injury the endothelial barrier is intact and platelets are quiescent. With injury (as with angioplasty), the endothelial barrier is compromised, platelets activated and 1), release agents that promote VSMC dedifferentiation, and 2), are taken up by VSMC and release miR-223 which apply a brake on VSMC, preventing excess VSMC repair, reducing intimal hyperplasia. DM platelets have reduced miR-223, resulting in excess VSMC proliferation upon injury. The inset panels indicate biochemical target for miR-223 including PDGFRβ. The levels of miR-223 is significantly reduced in DM platelets resulting in up-regulation of PDGFRβ, promoting aberrant VSMC dedifferentiation, proliferation and intimal hyperplasia.