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Platelets activated during myocardial infarction release functional miRNA which can be taken up by endothelial cells and regulate ICAM1 expression

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Key Points:

1. Myocardial infarction patients have altered platelet miRNA profiles
2. Activated platelets release miRNAs that can be taken up by endothelial cells and regulate ICAM1 gene expression

Abstract

Platelets play a crucial role in the molecular mechanism of myocardial infarction, adhering to the site of a ruptured atherosclerotic plaque and releasing a multitude of pro-inflammatory mediators. The aim of this study was to screen for differences in miRNA content of platelets from patients with ST-elevation myocardial infarction (STEMI) and controls, to investigate a possible release of miRNAs from activated platelets and to elucidate whether platelet-derived miRNAs could act as paracrine regulators of endothelial cell gene expression. Using RNA-seq, we found 9 differentially expressed miRNAs in patients compared to healthy controls, of which 8 were decreased in patients. Of these, miR-22, -185, -320b and -423-5p increased in the supernatant of platelets after aggregation and were depleted in platelet-containing thrombi aspirated from STEMI patients, indicating release of certain miRNAs from activated platelets. To confirm that endothelial cells could take up the released platelet miRNAs, transfer of both fluorescently labeled miRNA and exogenous cel-miR-39 from activated platelets to endothelial cells was shown. Lastly, a possible paracrine role of released platelet miR-320b on endothelial cell ICAM1 expression was shown. Thus, platelets from STEMI patients exhibit loss of specific miRNAs and activated platelets shed miRNAs that can regulate endothelial cell gene expression.

Introduction

Acute coronary syndrome (ACS), including acute myocardial infarction (AMI) and unstable angina, is the result of destabilization of an atherosclerotic plaque in a coronary artery[1]. The subsequent exposure and release of sub-endothelial factors (e.g. collagen and von Willebrand Factor) trigger activation of platelets in the vicinity of the lesion[2]. Activated platelets in turn release a multitude of substances affecting the chemotactic, adhesive and proteolytic properties of endothelial cells[3,4].

Although platelets lack nuclei, they do retain a specific set of megakaryocyte mRNAs[5,6] and are capable of *de novo* protein synthesis[7-9]. Protein translation in platelets has been shown to be under posttranscriptional control[9,10]. In addition to having protein coding transcripts a recent report revealed that platelets contain more than 170 different microRNA (miRNA) species as well as the enzymes required to convert precursor miRNA into mature miRNA[11]. miRNAs are short (19-25 nucleotides), non-coding RNA species which play a crucial role in regulating post transcriptional gene expression through binding to the 3' untranslated region (3'-UTR) of target mRNAs[12]. miR-96 has been shown to regulate the expression of VAMP8, a critical v-SNARE involved in platelet granule secretion[13], indicating a functional role of miRNA in regulating platelet mRNA translation.

The concept of exovesicle mediated transfer of miRNA to the endothelium has been explored in recent studies. For example, transfer of miR-126 between endothelial cells via apoptotic bodies was shown to limit atherosclerosis, promote incorporation of progenitor cells and increase plaque stability in a mouse model through targeting RGS16 and increasing production of CXCL12[14].

Moreover, a recent study[15] demonstrated that monocyte-derived exosomes carrying miR-150 were taken up by endothelial cells enhancing cell migration. The aim of this work was (1) to screen for differences in miRNA content in platelets of patients diagnosed with ST-elevation myocardial infarction (STEMI) and healthy individuals, (2) to investigate the possible release and transfer of miRNAs from activated platelets to endothelial cells and (3) to assess the role of platelet-derived miRNAs in the endothelium.

Methods

Patients and samples

Patients diagnosed with STEMI were recruited during 2010 at the coronary care unit at Skane University Hospital. Diagnoses were based on ECG criteria.

Isolation and preparation of platelets, plasma and thrombi

The procedure for platelet preparation has been described in detail elsewhere[6]. Briefly, 100 ml of blood was drawn by self-propagated flow, platelet poor plasma was passed through a Pall Autostop Leukocyte removal filter (Pall Incorporated, NY, USA) and depleted of additional leukocytes and erythrocytes using dynabeads (Dynal, Oslo, Norway) conjugated with anti-CD235a and anti-CD45 (Becton Dickinson, Franklin Lakes, NJ, USA). Plasma was prepared by centrifugation of whole blood for 15 minutes at 1600*g. For a detailed protocol of platelet and thrombi preparation, see Supplemental Methods.

RNA preparation

Cells and plasma/cell supernatants were mixed with Qiazol and TRIzol LS (Life Technologies (Carlsbad, CA, USA), respectively, and RNA was prepared using the miRNeasy mini kit (Qiagen, Hilden, Germany). For miRNA and mRNA analysis, cDNA was synthesized using the miRCURY LNA Universal RT microRNA cDNA kit (Exiqon, Vedbaek, Denmark) and the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA), respectively.

RNA-seq

Platelet RNA samples from two male STEMI patients and two age and sex matched healthy controls were prepared using the Small RNA Sample Preparation Kit (Illumina, CA, USA). Sequencing was performed on the Illumina GAIIx with a read length of 36nt. miRNA sequences iwere dentified using miRanalyzer[16] based on the bowtie architecture. The number of unique reads were counted, normalized to transcript size and expressed relative to the total number of reads.

qRT-PCR

miRNA levels were assessed by qRT-PCR using microRNA LNA Primer sets (Exiqon) specific for hsa-miR-16, -22, -126, -185, -320b, 423-5p, U6 snRNA and cel-miR-39-5p. mRNA levels for ICAM1 and Cyclophilin were analyzed with TaqMan Assays (Life Technologies). See Supplemental Methods for details.

Confirmation of deep sequencing results by qRT-PCR

Platelet RNA was prepared as described above from STEMI patients (n=10) and healthy controls (n=15). The level of U6 RNA was unaltered in STEMI patients compared to controls (CV=8.02%, Supplemental Figure 1) and was therefore considered reliable for normalization of platelet miRNA data.

Assessment of miRNA release from aggregated platelets

Platelets were preincubated 5 minutes with 0,25 mg/ml fibrinogen before addition of 1 U/ml of thrombin. Platelets were allowed to aggregate for 5 minutes, then centrifuged and an aliquot of the supernatant was taken. Before cDNA synthesis an exogenous miRNA spike-in (Exiqon) was added to the RNA

preparations. The levels of the different miRNAs in the supernatant was normalized to the exogenous RNA spike-in.

Immunofluorescence staining of coronary thrombi

See Supplemental Methods.

Cell culture

See Supplemental Methods.

Transfer of miRNA between platelets and endothelial cells

The procedure for transfecting platelets were based on the protocol by Hong et al (2011)[17] (See Supplemental Methods for a detailed description). Platelets were transfected with 40 pM synthetic C. Elegans miRNA miR-39 (syn-cel-miR-39) or 400 nM of fluorescently labeled scrambled miRNA (miR-Scr-FITC) using 6 ul Lipofectamine LTX (Life Technologies) per transfection. Transfection efficiency was determined after 24 hours by flow cytometry as the proportion of FITC-positive events in the platelet gate (mean transfection efficiency=17,4%, SEM=3,3). The effects of transfection on platelet morphology, activation and apoptosis were deemed minor (Supplemental Figure 2). Untreated platelets were completely negative for cel-miR-39 expression (data not shown). To confirm that miRNA was taken up in the platelet cytoplasm, transfected platelets were treated with 20 ug/ml RNase A (Thermo Scientific) and 2 U/ul RNase T1 (Thermo Scientific) for 30 minutes at 37 °C (Supplemental Figure 3). As a negative control, platelets were transfected with cel-miR-39 pre-treated with RNase. The level of cel-miR-39 in transfected platelets was unaffected by RNase-

treatment, whereas platelets transfected with degraded cel-miR-39 was completely devoid of cel-miR-39. Moreover, the fluorescence signal from platelets transfected with miR-FITC was unaltered by RNase-treatment. Taken together, this provides evidence that the transfected miRNA is taken up into the cytoplasm of the platelets.

For assessment of miRNA transfer, HMEC-1 were seeded 24 hours before co-culture. 0,5-1*10^7 platelets transfected either with syn-cel-miR-39 or Scr-miR-FITC were added per well in the presence or absence of 1 U/ml thrombin and/or 10 ug/ml of Brefeldin A. Presence of syn-cel-miR-39 or Scr-miR-FITC in HMEC-1 were assessed with qRT-PCR and confocal microscopy, respectively. A detailed protocol can be found in Supplemental Methods.

miRNA levels in HMEC-1 co-cultured with platelets

HMEC-1 were seeded in 96-well plates and co-cultured with washed platelets as described. HMEC-1 were harvested at 0, 1, 3, 8 and 24 hours after addition of thrombin. RNA was prepared and the levels of each miRNA relative to miR-16 was determined. miR-16 was unaffected by thrombin during the time course (CV=11,09 %).

Assessment of platelet microparticles

Platelets transfected with miR-Scr-FITC were seeded in TAB in a 96-well plate at ~1*10^5 platelets/well. 10 ug/ml brefeldin A was added and the platelets were incubated for 30 minutes. 1 U/ml thrombin was added and the platelets were incubated for 20 minutes. Platelets were stained with CD42a-PE, fixed with 0,5 % PFA and run on a Accuri C6 flow cytometer. The microparticle population was

defined first on size using 800 nm latex beads (Sigma-Aldrich, St Louise, MO, USA) and subsequently on the expression of CD42a. A PE-conjugated isotype control antibody was used to set the CD42a-gate. Mean fluorescence in FL-1 within the microparticle gate was used to assess FITC^{+} microparticles.

A screening was performed on three compounds reported to inhibit microparticle or exosome release: Brefeldin A[18], Cyclosporin A[19] and caspase inhibitor[20]. 10 ug/ml of Brefeldin A (Sigma), 10 uM Cyclosporin A (Sigma) and 100 uM caspase inhibitor (Ac-DEVD-CHO, Promega) were added to platelet rich plasma for 30 minutes at 37 °C before the addition of 1 U/ml Thrombin for 20 minutes and microparticles were detected by flow cytometry.

3'-UTR Target Plasmid Reporter Assay

miTarget miRNA Target Sequence 3'-UTR Expression Clones (GeneCopoeia, Rockville, MD, USA) was used for validation of predicted mRNA targets. See Supplemental Methods for a detailed protocol.

Stimulation of HMEC-1 with platelet releasate

Platelets were incubated in 25 μM N-terminal thrombin receptor fragment SFLLRN (Bachem, Bubendorf, Swizerland) for 20 minutes at room temperature. Aggregated platelets were pelleted and the platelet releasate was collected and stored at -80° C.

HMEC-1 were seeded in 12-well plates and after 24 hours, fresh medium with or without 20% platelet releasate containing 0 or 100 nM of Anti-miR miRNA Inhibitor (Life Technologies) corresponding to miR-22, -185, -320b or -423-5p,

was added. After another 24 hours, the cells were harvested by addition of Qiazol.

Overexpression of miR-320b in HMEC-1

See Supplemental Methods.

Statistical analysis

All statistical analyses were performed in Prism v. 4.0b (GraphPad Software Inc., CA, USA). Data represent the mean ± SEM and were analyzed by Student's *t* test, one or two way ANOVA with Tukey *post hoc* analysis as appropriate. Statistical significance was considered where $p<0.05$.

Ethics

This study was conducted according to the principles of the Declaration of Helsinki and was approved by the local ethics commitee of Skåne University Hospital. All patients gave their written approval before participation.

Results

Altered platelet miRNA profile in STEMI patients

We performed RNA-seq of platelet miRNA in two STEMI patients as well as in two age and sex matched healthy individuals. The mean number of total transcripts detected was 136 670 and mean of 109 different miRNA species were detected in the samples. The twenty most highly expressed miRNAs in each individual is presented in Table 2. The complete miRNA profiles can be found in Supplemental Table 1. The levels of nine different miRNA species were significantly altered between patients and controls (Supplemental Figure 5). Of these, miR-320a was upregulated whereas all the other miRNAs were lower in patients than in controls. Possible mRNA targets of these miRNAs were assessed with TargetScan (v. 5.1) and four candidate miRNAs; miR-22, -185, -320b and -423-5p were selected for further evaluation (Table 1). The criteria for selection were based on: (i) the potential relevance to atherosclerosis or inflammation (ii) the relative abundance in platelets and (iii) the magnitude of the difference between patients and controls. To validate the findings indicated by RNA-seq the levels of the four selected miRNAs were determined in the platelets from STEMI patients (n=10) and from healthy controls (n=15) (Figure 1a). The results demonstrated an approximately 70-90% decrease of all the four miRNAs in STEMI patients ($p<0.01$), thus confirming the results of the RNA-seq.

Platelets release miRNAs upon aggregation

The fact that out of the nine differentially expressed miRNAs, eight showed decreased levels in patients led us to hypothesize that the higher level of platelet activation in STEMI patients resulted in release of platelet miRNA. To examine

this, we used qRT-PCR to measure the levels of the four candidate miRNAs in the supernatant of resting platelets and platelets aggregated with 1 U/ml thrombin with qRT-PCR (Figure 1b). Following aggregation the levels of all four miRNAs increased in the supernatant. Although the magnitude of the increase varied between donors, from 3.1-fold (SEM=0.49) to 21-fold (SEM=7.9), the increase was significant in all of them.

To further investigate the idea of miRNA release from activated platelets, we compared the miRNA profiles of platelets from healthy individuals, STEMI patients and from coronary thrombi retrieved from the occlusion causing the STEMI (Figure 1c). The results confirmed the previous findings as the levels of all four miRNAs were even lower in thrombi than in STEMI platelets indicating an activation-generated depletion of certain miRNAs. The presence of platelets in thrombi was confirmed by confocal microscopy (Supplemental Figure 6).

The levels of these miRNAs were also analyzed in the circulation of a subset of patients. miR-22 and miR-423-5p were undetectable, miR-185 was unaltered and miR-320b was slightly elevated (p=0.31, Supplemental Figure 7).

miRNA is transfered from platelets to endothelial cells

We hypothesized that the miRNAs released from activated platelets could be taken up by endothelial cells. To test this idea, platelets were transfected with a synthetic exogenous miRNA, syn-cel-miR-39. The efficiency of the transfection was determined by absolute qRT-PCR and while untransfected platelets were completely negative for cel-miR-39 expression transfected platelets contained ~2000 copies/platelet. Activation of transfected platelets with 1 U/ml Thrombin resulted in a 21-fold increase of cel-miR-39 in the supernatant and a

corresponding 60% decrease in intracellular cel-miR-39 (Figure 2a). In the next experiment transfected platelets were co-cultured with HMEC-1 cells for either 1 or 3 hours, in the presence or absence of 1 U/ml thrombin. After removing the platelets and washing the monolayer, cel-miR-39 levels were assessed with qRT-PCR (Figure 2b). It was found that while Cel-miR-39 was undetectable in HMEC-1 after one hour of co-culture (data not shown), 3 hours of co-culture produced levels that were readily detectable in HMEC-1. Furthermore, the transfer was clearly dependant on the platelet activation status as cells co-cultured with activated platelets contained 80-fold more cel-miR-39 than cells co-cultured with resting platelets.

To further confirm miRNA transfer from platelets to endothelial cells, platelets were transfected with a scrambled, fluorescently labeled miRNA (miR-Scr-FITC). Transfected platelets were co-cultured with HMEC-1 for three hours in the presence of 1 U/ml of thrombin. After removal of the platelets, the HMEC-1 cells were analyzed with confocal microscopy. The cytoplasm of HMEC-1 was clearly fluorescent, indicating uptake of miRNA from activated platelets (Figure 2c and Supplemental Figure 8).

Following these proof-of-concept experiments, we wanted to examine if there was an endothelial uptake of the four miRNAs that were shown to be released from platelets (miR-22, -185, -320b and -423-5). Platelets and endothelial cells were again co-cultured in the presence or absence of thrombin and the timecourse for the uptake process was measured using qRT-PCR (Figure 3). In the presence of thrombin-activated platelets, there was a distinct but transient increase of all four miRNAs in the endothelial cells, peaking at either 3 or 8 hours

depending on the miRNA species. However, when the experiment was repeated using resting platelets, the miRNA levels were unaffected. Expression of the endothelial cell-enriched miRNA miR-126[21] was unaffected by the presence of activated platelets (Supplemental Figure 9). Thrombin itself did not have any effect on miRNA levels in the endothelial cells (Supplemental Figure 10).

Platelet miRNA is released and transfered by a vesicle-dependent mechanism

Considering the recent reports of microvesicle-dependent cell-to-cell miRNA transfer and the well established notion of microparticle release from platelets, we hypothesized that the platelet miRNAs were released in microparticles. After a screening of several compounds reported to inhibit microparticle formation, we found that Brefeldin A was the most effective microparticle inhibitor (Figure 4a and Supplemental Figure 11). We then transfected platelets with fluorescently labeled miR-Scr-FITC and stimulated with thrombin in the presence or absence of brefeldin A. Fluorescent labeling of the generated microparticle fraction was then assessed with flow cytometry. There was a significant enrichment of miR-Scr-FITC in the CD42a^{+} microparticle population after stimulation with thrombin. This effect was partly inhibited with the addition of 10 ug/ml of brefeldin A (Figure 4b). There was also a small but significant decrease in fluorescence intensity in transfected platelets, indicating release of miR-FITC upon activation (Suplemental Figure 12).

To further test this, we co-cultured miR-Scr-FITC transfected platelets with HMEC-1 and activated the platelets with thrombin in the presence or absence of brefeldin A. After 3 hours of co-culture, the proportion of FITC^{+} HMEC-1 was then assessed with confocal microscopy (Figure 4c). In the presence of activated

platelets, ~50% of the cells were FITC$^+$ (a 1.6-fold increase compared to cells cultured with untreated platelets, $p<0.05$). The addition of Brefeldin A significantly decreased the proportion of FITC$^+$ HMEC below the baseline, indicating that vesicle formation is required for an efficient release and transfer of miRNA from platelets to endothelial cells.

Endothelial cell gene expression is altered by activated platelets via a miRNA-dependent mechanism

Finally, our aim was to investigate if the miRNAs released from activated platelets could confer any effect on target gene expression in the endothelial cells. The first step was to confirm the predicted mRNA targets of miR-22, -185, -320b and -423-5p (see Table 2). HEK293 cells were co-transfected with luciferase reporter plasmids containing the 3'-UTR of ICAM-1, eNOS, VEGFA and VEGFB and pre-miRNA corresponding to each of the four miRNA candidates. Over expression of miR-22 and miR-320b caused significant dose-dependent quenching of the ICAM1 reporter signal compared to cells transfected with a scrambled pre-miR, indicating interaction of miRNA and mRNA (Figure 5a). The remaining miRNA-target interactions could not be confirmed (data not shown) so we chose to focus the rest of the study on the interaction of miR-22 and miR-320b with ICAM1.

To investigate whether activated platelets could affect ICAM1 gene expression, HMEC-1 cells were cultured in the presence of platelet releasate. After 24 hours ICAM1 expression was downregulated by ~30% ($p<0.05$, Figure 5b), supporting the hypothesis that the effect is mediated by miRNA.

To further confirm the interaction between miR-22/-320b and ICAM1 mRNA, we knocked down endogenous miRNA using anti-miRNAs corresponding to miR-22 and miR-320b and measured ICAM1 gene expression using qRT-PCR (Figure 5c). As expected, knock down of these miRNAs caused a significant increase in target mRNA, by 2- ($p<0.05$) and 4-fold($p<0.001$), respectively, compared to cells treated with a scrambled Anti-miRNA. Then, our aim was to examine whether the effect on ICAM1 expression seen when adding platelet releasate was caused by miRNAs. Platelet releasate was added to HMEC-1 cells where miR-22 and miR-320b had been suppressed using anti-miRNA. Interestingly, the effect of anti-miR-320b, but not anti-miR-22, on ICAM-1 expression was rescued by the addition of platelet releasate to the endothelial cells ($p<0.01$). This indicates an efficient transfer of specific functional miRNA species from the platelet releasate to the endothelial cells.

miR-320b regulates cell surface ICAM-1 expression on endothelial cells

To confirm that miR-320b can confer an effect on ICAM1 also on the protein level, HMEC-1 were transfected with synthetic precursor miRNA or scrambled pre-miRNA and cell surface bound ICAM-1 protein was quantified with flow cytometry after 48 hours. Overexpression of miR-320b was confirmed by qRT-PCR (Supplemental Figure 13). The ICAM1 mean fluorescence intensity decreased 7% ($p<0.001$) in cells overexpressing miR-320b as compared to scrambled control (Figure 5d and e), indicating a functional role of miR-320b on ICAM-1 in endothelial cells.

Discussion

The discovery of miRNA[22,23] and the enzymatic infrastructure required to convert precursor-miRNA into mature miRNA[11] in platelets was reported recently. A few studies mapping the full platelet miRNA repertoire have been conducted since then [24,25] but to our knowledge, no one has studied the platelet miRNA transcriptome in patients with myocardial infarction. In an effort to address this question we conducted RNA-seq of highly purified platelet preparations from patients with STEMI and from healthy individuals. The dominating miRNA species in both patients and controls were miR-320a, comprising >80% of total miRNA transcripts in healthy individuals. miR-320a has been detected in platelets in previous studies but not in the same proportions as here. Overall, the platelet miRNA profiles reported so far are quite disparate. Apart from differences in method used (microarray, qRT-PCR, RNA-seq), this might relate to differences in platelet preparation, varying degrees of platelet activation or presence of contaminating leukocyte or erythrocyte miRNAs. It is worth mentioning that RNA-seq, unlike for example microarray, is not a hybridization-based technique and the results are therefore considered more unbiased[26].

We found nine differentially expressed miRNAs in patients compared to controls of which eight were downregulated in patients. This prompted us to investigate whether general platelet activation following myocardial infarction leads to a release of miRNA from platelets. The notion of miRNA release from activated or apoptotic cells have been proposed by several groups recently. Exosome-mediated transfer of miRNA have been shown in activated mast cells[27] and from T cells to antigen-presenting cells[18]. In a recent report, apoptotic bodies carrying

miR-126 between endothelial cells caused upregulation of CXCL12 in recipient cells[14]. A very recent report shows that smooth muscle cells provide an atheroprotective signal to endothelial cells through vesicular transfer of miR-143/145[28].

We could confirm release of miR-22, -185, 320b and -423-5p from *ex vivo* aggregated platelets and also noticed a depletion of these miRNAs in thrombi aspirated from STEMI patients. The release of platelet miRNAs was not reflected in increased overall levels in the circulation of patients. It could be argued that the release of miRNAs from activated platelets is likely to be a local event at the site of the ruptured plaque and would therefore not be detectable in the periphery. Regarding the results from the thrombi, it is possible that the relative decrease in platelet miRNAs is partly due to the dilution of platelets with other cell types in the thrombus. This may exaggerate the decline in platelet miRNAs somewhat but does not account for the total lack of miR-22, for example.

We were able to demostrate efficient transfer of both fluorescently labeled miRNA and exogenous C. Elegans miRNA from activated platelets to an endothelial cell line. Co-culture of activated platelets and endothelial cells caused a transient increase of all four platelet miRNAs, but not the endothelial cell-enriched miR-126, in HMEC-1 cells. Considering that the miRNA transfer could be completely abolished with the addition of Brefeldin A, which we show to be an inhibitor of platelet microparticle release, it is likely that the miRNA release is vesicle-dependent. The effect of Brefeldin A is probably best explained by its well documented ability to disrupt vesicular transport processes[29]. Brefeldin A has recently been reported to inhibit release of exosome-like vesicles from cells[30] and to suppress exosome-dependent transfer of miRNA between immune cells[18].

We can however not rule out that Brefeldin A also affects the endothelial uptake of miRNA. In a recent report, Diehl et al showed that platelet microparticles were enriched for distinct miRNA species[31], which suggests that secretion of miRNAs from platelets is a selective process. Although the exact mechanism of release and uptake requires further research, recent reports on exovesicle/microparticle-mediated cell-to-cell transfer of miRNA and the well-researched mechanism of platelet microparticle release, do strengthen this hypothesis.

We observed a decreased expression of ICAM-1 in the presence of platelet releasate and hypothesized that this effect might be mediated by released miRNA. De-repression of ICAM-1 mediated by anti-miR-320b, but not anti-miR-22, was almost completely abolished by the addition of platelet releasate. This selective effect rules against the posibility that some factor in the platelet releasate causes a general effect on the endothelium, independent of miRNA.

In contrast to the results presented herein, previous studies have reported increased endothelial ICAM-1 expression in the presence of platelet releasate through a IL-1 dependent mechanism[32,33]. The discrepancy might be attributed to differences in the dynamics of ICAM-1 regulation. Gawaz et al (2000) reported a maximal ICAM-1 expression after 16 hours. In the present study, ICAM-1 expression was evaluated after 24 hours. It is possible that regulation of ICAM-1 expression by external miRNAs might represent an additional, regulatory mechanism, counteracting that of IL-1 in the long-term. One might speculate that the role of platelet-derived miRNA in the endothelium might be to regulate and fine tune the effects of the pro-inflammatory mediators

in the releasate. Indeed, several recent papers have proposed miRNAs as fine tuners of the inflammatory response[34,35].

In conclusion, we report that the platelet miRNA content of STEMI patients is distinctly different to that of healthy individuals, that miRNAs are shed from activated platelets and that platelet-derived miRNA can affect endothelial cell gene expression.

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Authorship contributions

OG: designed and performed experiments, analysed data, wrote the manuscript

MvB: designed and performed experiments, analysed data, edited the manuscript

JÖ: designed and performed experiments, analysed data, edited the manuscript

PA: provided vital samples, edited the manuscript

BO: designed experiments, edited the manuscript

CW: designed the study, edited the manuscript

DE: designed the study, edited the manuscript

Conflicts of interest

None

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Figure legends

Figure 1. miRNA is released from activated platelets

A)Platelet miRNA levels in STEMI patients (n=10) and controls (n=15) are expressed relative to U6 snRNA and normalized to the mean of the control samples. **p<0.01, ***p<0.001.

B) miRNA levels in the supernatant of pelleted platelets from three individual donors before and after aggregation with 1 U/ml thrombin (n=3). Data are expressed relative to an exogenous RNA spike-in and normalized against the control samples (before aggregation).

C) Levels of miR-22, -185, -320b and -423-5p in platelets of healthy individuals (n=15), STEMI patients (n=10) and thrombi aspirated from STEMI patients (n=2) relative to U6 RNA. N.D., not detected.

Figure 2. Activated platelets transfer miRNA to endothelial cells in vitro

A) Relative quantity of intracellular and secreted cel-miR-39 from resting and activated platelets. Expression data were normalized to an exogenous miRNA spike in for the supernatant and to U6 RNA for the intracellular levels (n=3).

B) Relative quantity of cel-miR-39 in HMEC-1 co-cultured with transfected platelets after 3 hours of co-culture in the presence or absence of 1 U/ml thrombin (n=3). Levels are presented relative to miR-16 and normalized against the mean of the cells without thrombin.

C) Representative confocal image of HMEC-1 co-cultured with miR-Scr-FITC transfected platelets in the presence of 1 u/ml thrombin. Nuclei are stained red.

Figure 3. Relative miRNA levels in endothelial cells co-cultured with platelets

Levels of miR-22, -185, -320b and 423-5p in HMEC-1 co-cultured with platelets in the presence or absence of 1 U/ml thrombin at indicated time points. Levels are expressed relative to miR-16 and normalized to the mean baseline expression in each group (n=3). Two-way ANOVA with Bonferroni *post hoc* test was used to assess the effect of platelet activation on the levels of miRNA in HMEC-1 (*p<0.05, **p<0.01, ***p<0.001).

Figure 4. miRNA is transfered to endothelial cells by a vesicle-dependent mechanism

A) The effect of Brefeldin A on platelet microparticle release was assessed with flow cytometry. 10 ug/ml of Brefeldin A was added to platelet rich plasma 30 minutes before addition of 1 U/ml Thrombin. The microparticle population was defined based on size and expression of CD42a. **p<0.01 comparing untreated with thrombin-stimulated, +p<0.05 comparing thrombin versus thrombin+brefeldin A, n=3.

B) Mean fluorescence in the FL1 channel within the microparticle population was used to assess miR-Scr-FITC content (***p<0.001 comparing no thrombin versus thrombin-stimulated, +p<0.05 comparing thrombin versus thrombin+brefeldin A, n=6).

C) Quantification of FITC-labeled miRNA in HMEC-1 cells. Pixels of intensity higher than background staining were pseudocolored white. In each image the percentage of cells positive for white pixels were counted manually (*p<0.05 comparing HMEC-1 co-cultured with platelets in the presence or absence of thrombin, ++p<0.01 comparing HMEC-1 co-cultured with platelets in the presence of thrombin alone or with brefeldin A).

Figure 5. Function of miR-320b in endothelial cells

A) Reporter gene signal from HEK293 cells transfected with 10 ng of a reporter plasmid containing the 3'-UTR of ICAM1 and 20 or 100 nM precursor miRNA corresponding to miR-22 or miR-320b and 10 ng of a reporter plasmid containing the 3'-UTR of ICAM1. The reporter signal (Firefly luciferase) was normalized to Renilla luciferase to account for differences in transfection efficiency and expressed relative to the mean of the cells transfected with plasmid alone. Data come from three separate experiments with each sample run in triplicates. **$p<0.01$, ***$p<0.001$.

B) The level of ICAM-1 expression in HMEC-1 cultured for 24 hours in the presence of 20% platelet releasate. Gene expression was measured by qRT-PCR and is presented relative to the expression of cyclophilin A and normalized against the mean of the untreated control samples. Data from three separate experiments using triplicates are presented. *$p<0.05$.

C) The levels of ICAM-1 in HMEC-1 upon treatment with 100 nM of scrambled anti-miRNA (anti-miR-Scr), anti-miR-22 or -320b in the presence or absence of 20% platelet releasate. Expression data are handled as mentioned above (n=6). *$p<0.05$, ***$p<0.001$ comparing cells treated with anti-miR-scr and anti-miR in the absence of platelet releasate. ##$p<0.01$, comparing cells treated with anti-miR in the absence of releasate with cells treated with a combination of anti-miR and releasate.

D) Representative FACS plot of HMEC-1 cells transfected with 100 nM pre-miR-320b (black line) or scrambled control pre-miRNA (grey line) and stained with a FITC-conjugated monoclonal antibody to ICAM1. FL-1 fluorescence intensity reflects the surface expression of ICAM1.

E) Surface expression of ICAM-1 in HMEC-1 (n=3) transfected with 100 nM pre-miR-320b or scrambled control pre-miRNA assessed with flow cytometry. ICAM-1 levels are expressed as the mean fluorescence intensity (MFI).***$p<0.001$.

Table 1. Putative mRNA targets of candidate miRNAs

miRNA	Target
miR-22	ICAM1
miR-185	eNOS VEGFA
miR-320b	ICAM1
miR-423-5p	VEGFB eNOS

Possbible targets were analyzed using TargetScan v. 5.1.

Table 2a. Twenty most highly expressed miRNAs in STEMI Patients platelets

Patient 1				Patient 2			
miRNA ID	Transcripts	Normalized[1]	% of total	miRNA ID	Transcripts	Normalized[1]	% of total
miR-320a	44170	42043,45	97,77	miR-320a	15711	14927,68	97,97
miR-423-5p	180	169,43	0,40	miR-423-5p	93	87,74	0,58
miR-320b	139	127,00	0,31	miR-320b	49	44,14	0,31
miR-1274a	74	73,94	0,16	miR-185	24	22,77	0,15
miR-1908	60	55,48	0,13	miR-1274a	19	19,00	0,12
miR-185	57	54,41	0,13	miR-378	13	11,29	0,08
let-7b	43	40,05	0,10	let-7b	11	10,36	0,07
miR-320c	34	31,55	0,08	miR-1908	11	9,95	0,07
let-7c	33	31,23	0,07	miR-320c	11	9,85	0,07
let-7a	26	24,64	0,06	miR-1274b	7	7,00	0,04
miR-378	28	23,86	0,06	miR-1	7	6,68	0,04
let-7f	25	23,55	0,06	let-7a	7	6,64	0,04
miR-103	23	21,87	0,05	miR-1307	7	6,14	0,04
miR-221	16	14,57	0,04	miR-422a	6	5,73	0,04
miR-1290	14	13,26	0,03	miR-33a	5	4,76	0,03
miR-1	14	13,09	0,03	miR-375	4	3,82	0,02
miR-382	14	13,05	0,03	miR-765	4	3,81	0,02
let-7e	13	12,27	0,03	miR-107	4	3,70	0,02
miR-483-5p	13	11,91	0,03	miR-576-3p	4	3,68	0,02
miR-1224-3p	11	8,95	0,02	miR-1308	3	2,83	0,02

1. Normalized to transcript size

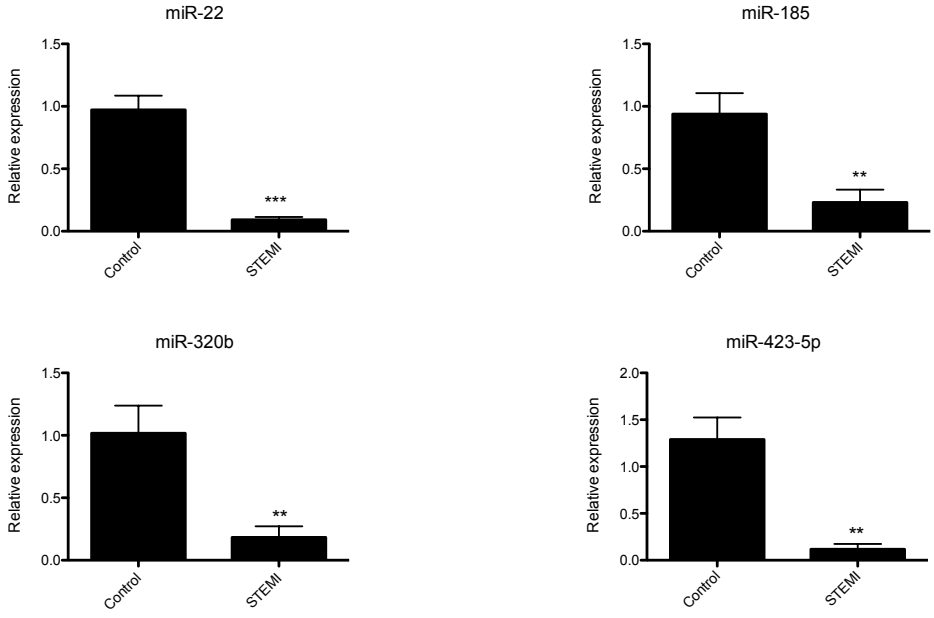
Table 2b. Twenty most highly expressed miRNAs in healthy individuals

Control 1				Control 2			
miRNA ID	Transcripts	Normalized[1]	% of total	miRNA ID	Transcripts	Normalized[1]	% of total
miR-320a	19496	18526,55	82,81	miR-320a	467302	444454,86	88,44
miR-423-5p	2020	1903,52	8,58	miR-423-5p	41477	39257,65	7,85
miR-185	285	270,82	1,21	miR-185	6134	5839,23	1,16
let-7a	191	180,86	0,81	miR-320b	1591	1451,23	0,30
let-7c	188	177,64	0,80	miR-221	1524	1416,91	0,29
let-7f	184	174,77	0,78	let-7b	695	653,77	0,13
let-7b	112	104,32	0,48	miR-378	639	570,71	0,12
miR-221	102	93,61	0,43	miR-22	587	558,23	0,11
let-7e	85	80,59	0,36	let-7f	568	538,18	0,11
miR-320b	71	64,32	0,30	miR-1307	546	505,05	0,10
miR-21	57	54,14	0,24	let-7a	531	504,32	0,10
miR-103	44	41,57	0,19	miR-1274a	498	497,89	0,09
miR-378	40	36,24	0,17	miR-320c	478	441,20	0,09
miR-1274a	32	32,00	0,14	miR-423-3p	279	261,52	0,05
let-7d	34	32,00	0,14	miR-103	253	239,17	0,05
miR-1307	32	29,77	0,14	miR-744	251	234,59	0,05
miR-22	30	28,41	0,13	miR-139-3p	229	226,64	0,04
miR-423-3p	27	25,65	0,11	miR-1908	233	215,14	0,04
let-7g	24	22,64	0,10	miR-584	233	211,86	0,04

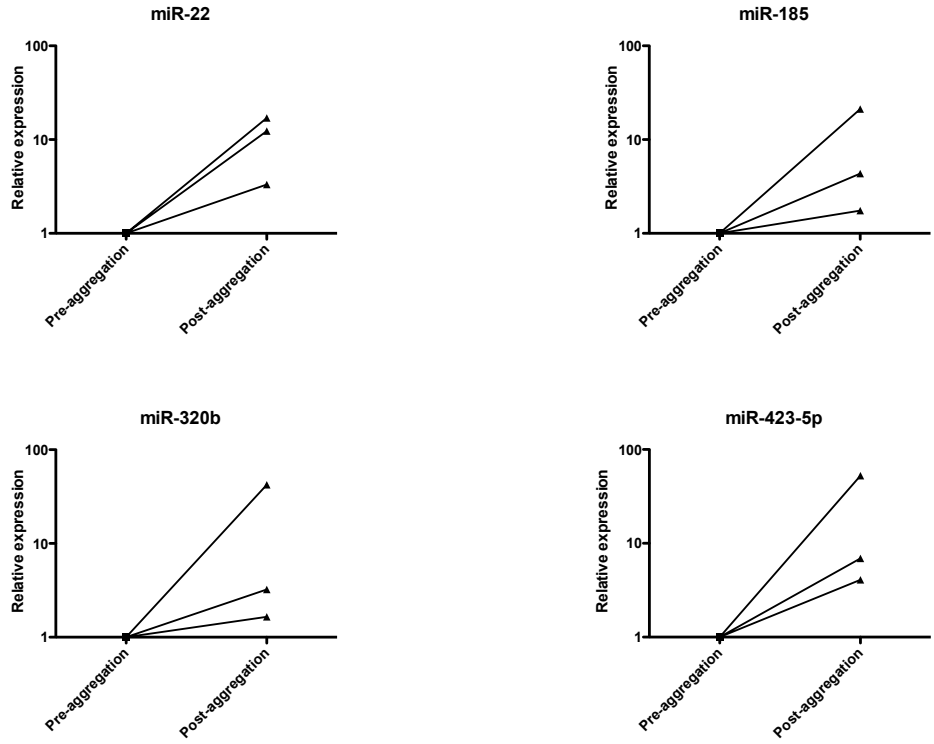
1. Normalized to transcript size

Figure 1

a)


miR-22
miR-185
miR-320b
miR-423-5p
Relative expression
Control
STEMI
***
**

b)


miR-22
miR-185
miR-320b
miR-423-5p
Relative expression
Pre-aggregation
Post-aggregation

c)

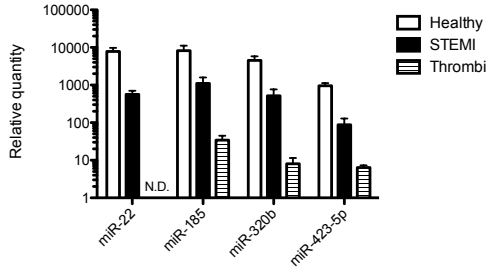
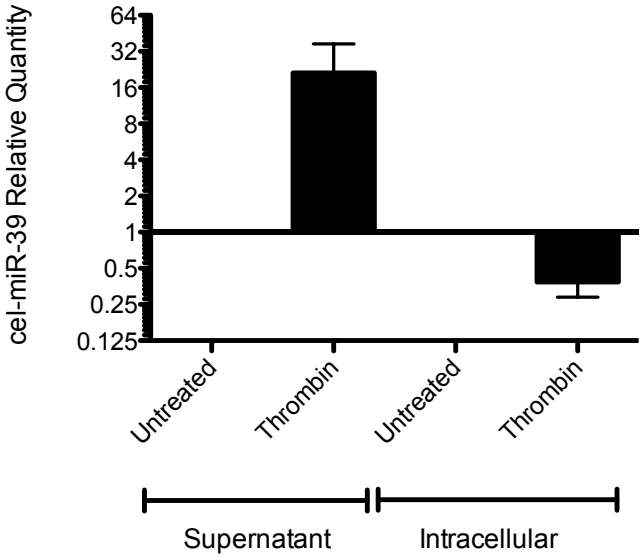

Relative quantity
Healthy
STEMI
Thrombi
N.D.
miR-22
miR-185
miR-320b
miR-423-5p

Figure 2

a)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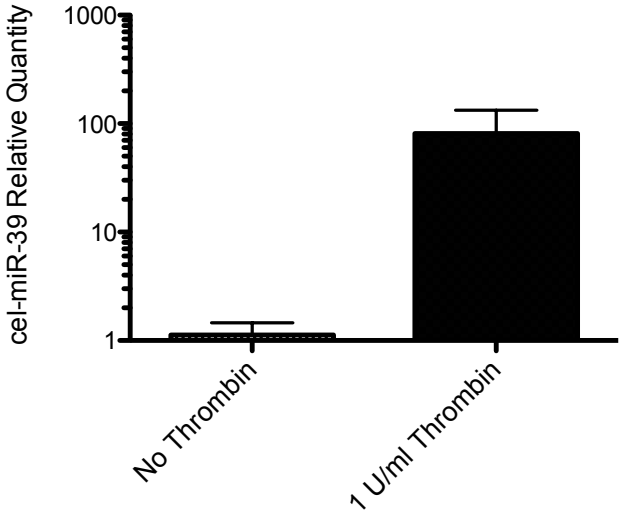
64
32
16
8
4
2
1
0.5
0.25
0.125
Untreated
Thrombin
Untreated
Thrombin
Supernatant
Intracellular

b)


cel-miR-39 Relative Quantity
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100
10
1
No Thrombin
1 U/ml Thrombin

c)

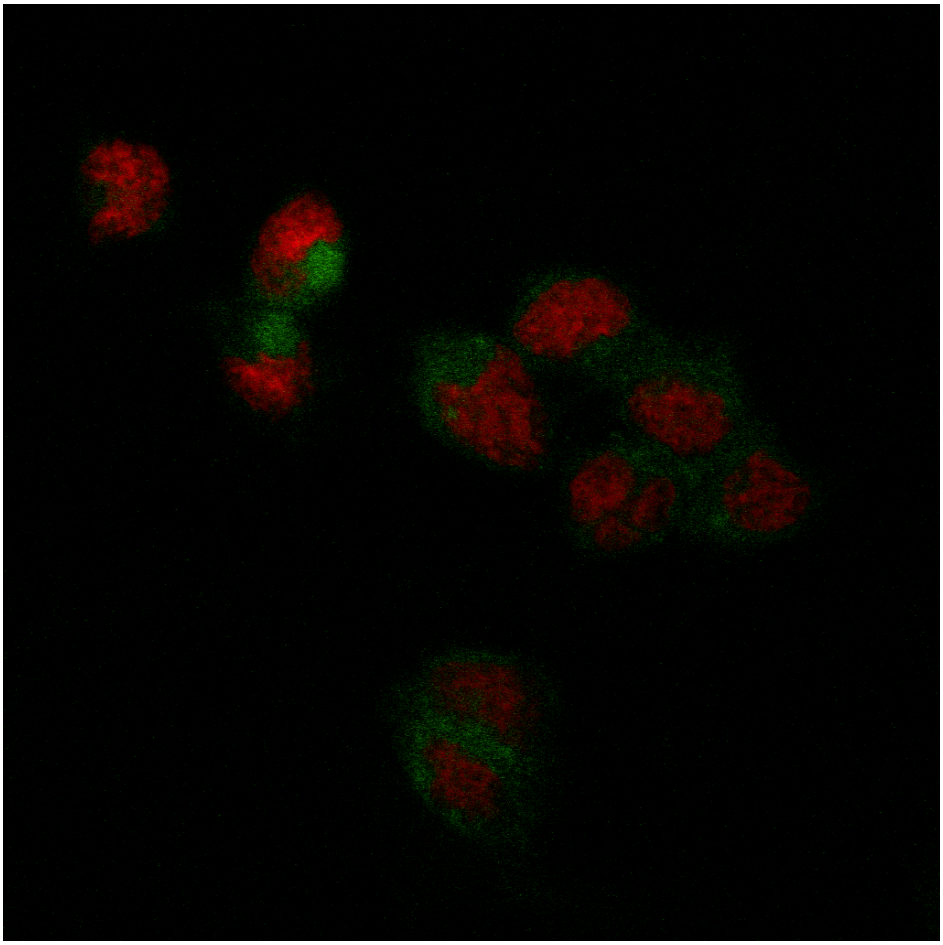


Figure 3

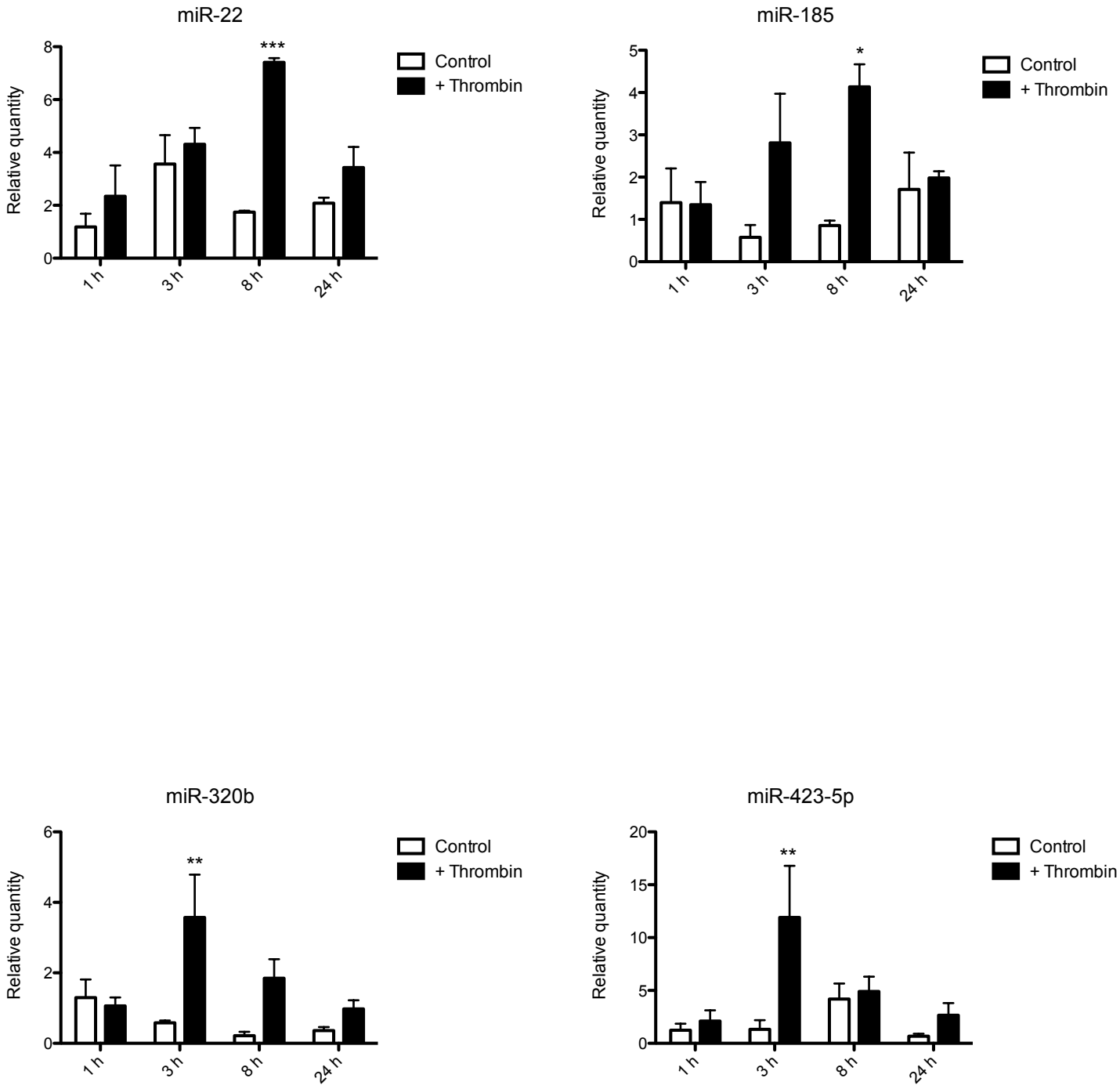
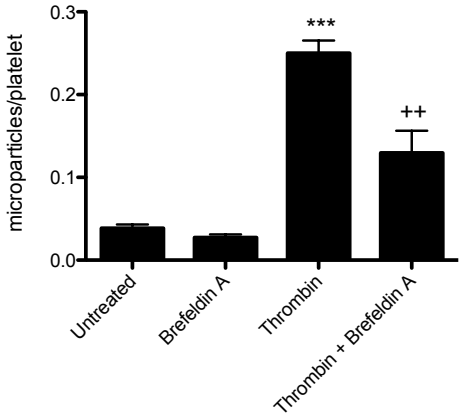

miR-22
Relative quantity
0
2
4
6
8
***
1 h
3 h
8 h
24 h
Control
+ Thrombin
miR-185
Relative quantity
0
1
2
3
4
5
*
1 h
3 h
8 h
24 h
Control
+ Thrombin
miR-320b
Relative quantity
0
2
4
6
**
1 h
3 h
8 h
24 h
Control
+ Thrombin
miR-423-5p
Relative quantity
0
5
10
15
20
**
1 h
3 h
8 h
24 h
Control
+ Thrombin

Figure 4

a)



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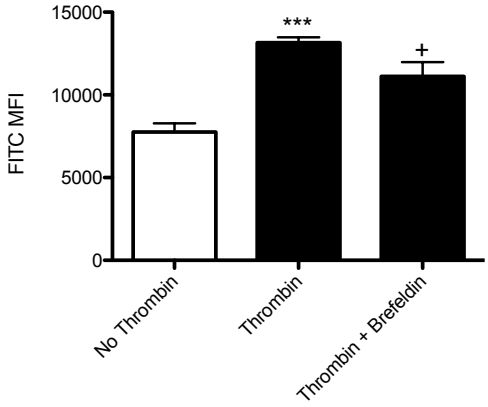
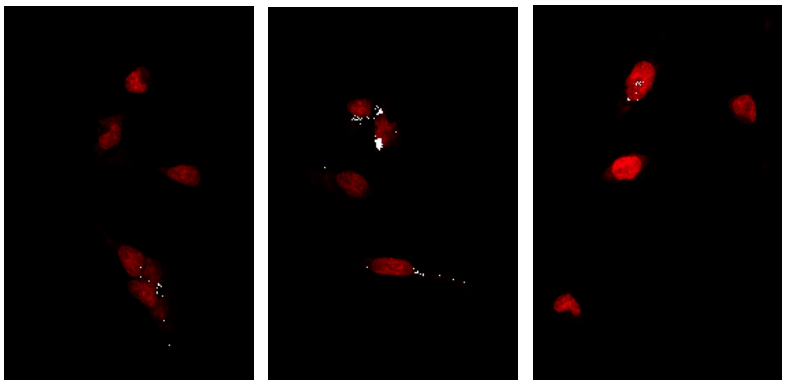


c)



Control Thrombin Thrombin + Bref A

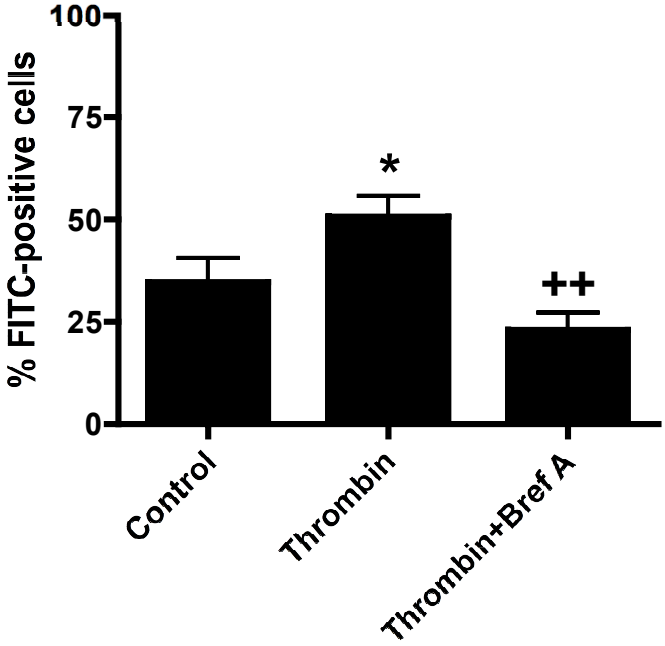
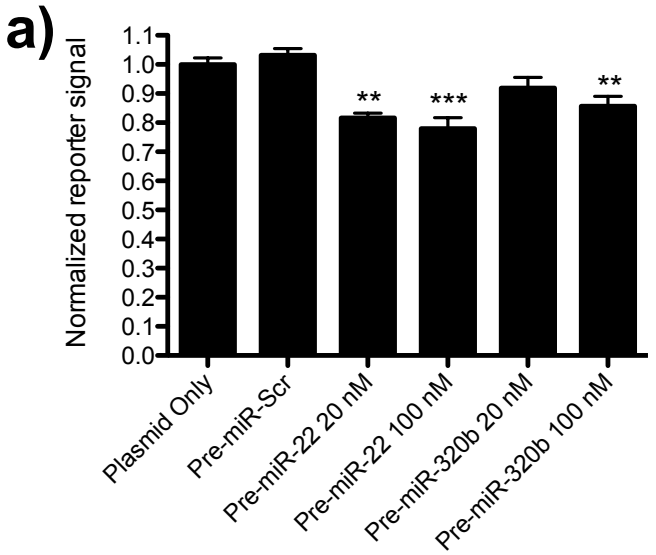
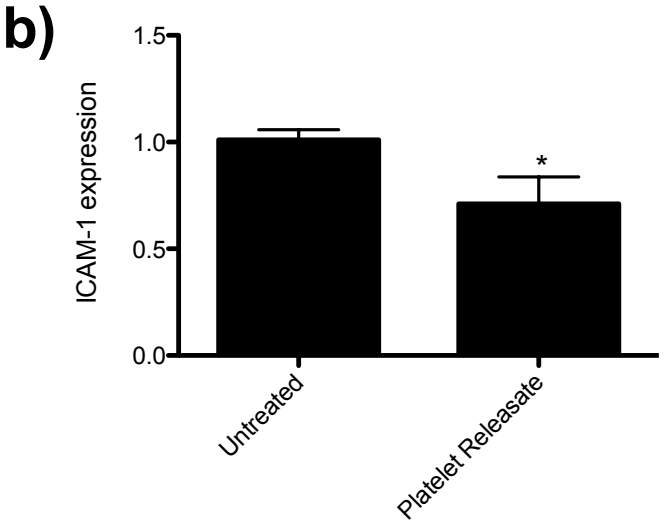
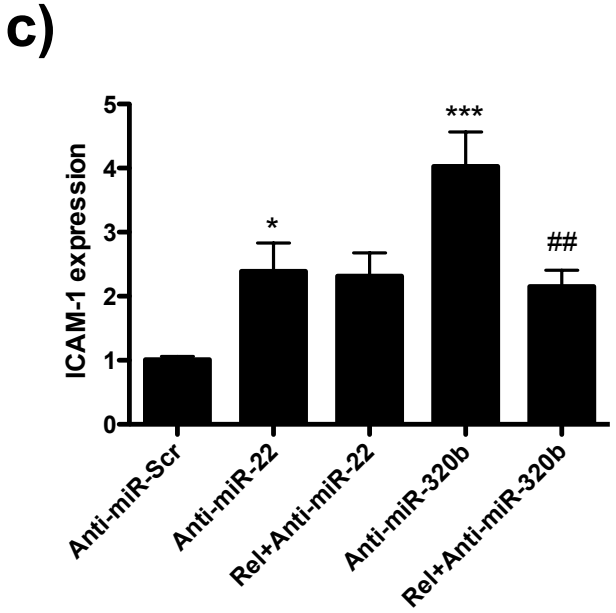
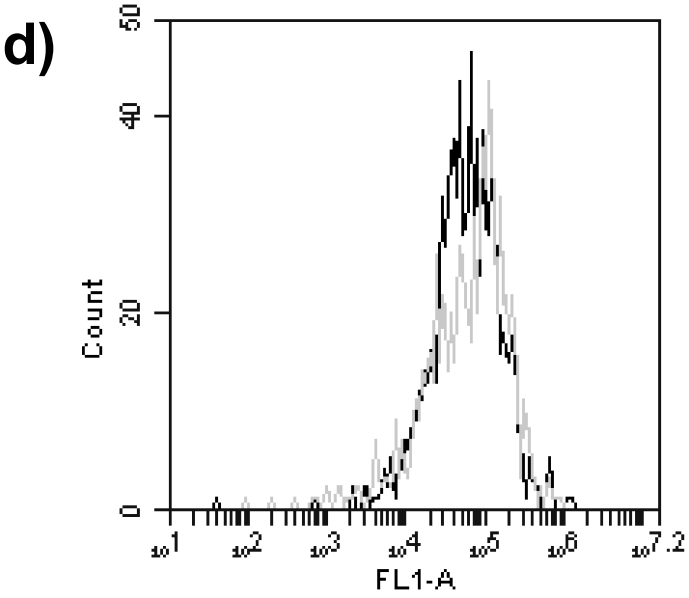


Figure 5


a)
Normalized reporter signal
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
**
***
**
Plasmid Only
Pre-miR-Scr
Pre-miR-22 20 nM
Pre-miR-22 100 nM
Pre-miR-320b 20 nM
Pre-miR-320b 100 nM


b)
ICAM-1 expression
1.5
1.0
0.5
0.0
*
Untreated
Platelet Releasate


c)
ICAM-1 expression
5
4
3
2
1
0
*
***
##
Anti-miR-Scr
Anti-miR-22
Rel+Anti-miR-22
Anti-miR-320b
Rel+Anti-miR-320b


d)
Count
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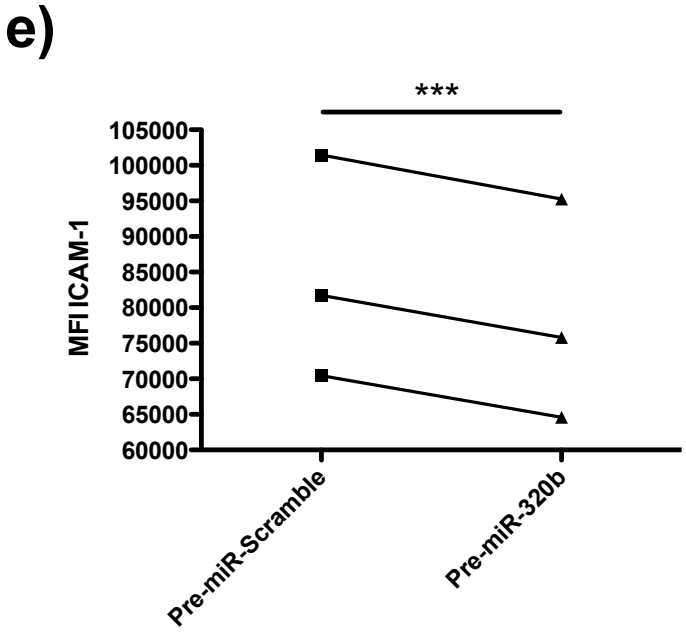
40
20
0
FL1-A


e)
***
MFI ICAM-1
105000
100000
95000
90000
85000
80000
75000
70000
65000
60000
Pre-miR-Scramble
Pre-miR-320b