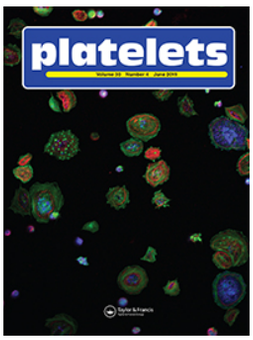

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ORIGINAL ARTICLE

Long non-coding RNA metallothionein 1 pseudogene 3 promotes p2y12 expression by sponging miR-126 to activate platelet in diabetic animal model

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Abstract

Platelet hyperaggregation and hypercoagulation are associated with increase of thrombogenic risk, especially in patients with type 2 diabetes (T2D). High activity of P2Y12 receptor is found in T2D patients, exposing such patients to a prothrombotic condition. P2Y12 is a promising target for antiplatelet, but due to P2Y12 receptor constitutive activation, the clinical practical phenomena such as "clopidogrel resistance" are commonly occurring. In this study, we investigate the role of lncRNA on platelet activation. By lncRNA array, we screened thousands of differentially expressed lncRNA in megakaryocytes from T2D patients and confirmed that lncRNA metallothionein 1 pseudogene 3 (MT1P3) was significantly upregulated in megakaryocytes from T2D patients than in healthy controls. And we further investigate the biofunction of MT1P3 on platelet activation and the regulatory mechanism on p2y12. MT1P3 was positively correlated with p2y12 mRNA levels and promoted p2y12 expression by sponging miR-126. Knockdown of MT1P3 by siRNA reduced p2y12 expression, inhibiting platelet activation and aggregation in diabetes animal model. In conclusion, our findings identify MT1P3 as a key regulator in platelet activation by increasing p2y12 expression through sponging miR-126 under T2D condition. These findings may provide a new insight for managing platelet hyperactivity-related diseases.

Keywords

Constitutive activation, diabetes, lncRNA, miR-126, platelet aggregation

History

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Introduction

Type 2 Diabetes (T2D), a metabolic disease with high mortality and morbidity, is increasing rapidly worldwide (1). T2D patients have high risk of microcirculation complications and thrombotic microangiopathies, resulting in cardiovascular disease (2). Platelet hyperaggregation and hypercoagulation are associated with increase of thrombogenic risk (3). T2D patients have higher mean platelet volume and platelet distribution width values (4). Increased *in vivo* platelet activation is also observed in patients with impairment of glucose metabolism and in the earlier stages of T2D (5). However, T2D patients frequently fail to respond to antiplatelet therapy, such as clopidogrel and Cangrelor, the most commonly used ADP receptor blocker (6). There is high residual platelet reactivity in T2D patients after ADP receptor blocker therapy, which may be a marker of vascular risk (7).

P2Y12 receptor is a Gi-coupled receptor predominantly expressed on platelets. ADP can physiologically activate P2Y12 receptor, decreasing cyclic AMP (cAMP) level, and then resulting in platelet aggregation (8). High activity of P2Y12 receptor is found in T2D patients, exposing such patients to a prothrombotic condition (9). P2Y12 is a promising target for antiplatelet, but due to P2Y12 receptor constitutive activation, the clinical practical phenomena such as "clopidogrel resistance" are commonly occurring. Recent study showed that inverse agonists of P2Y12 receptor have better effects on antiplatelet than neutral antagonists (10). Thus, decreasing P2Y12 receptor expression may be a potential strategy for inactivating the residual platelet reactivity.

Non-coding RNAs, including microRNA (miRNA) and long non-coding RNA (LncRNA), play an important role in cellular processes such as proliferation, differentiation, and metabolism. Emerging evidence also demonstrate that they play a role in the pathological processes of diabetes (11). MiR-126 has been found to be correlated with plasma levels of P-selectin, platelet factor 4 (12). Inhibition of miR-126 in mice reduced platelet aggregation, which was involved in regulation of P2Y12 receptor expression in direct or indirect manner (12). However, the role of lncRNA on platelet activation in T2D patients remains unknown.

Ming Zhou and Meng Gao contributed equally to this work.
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In this study, we investigate the role of lncRNA on platelet activation. By lncRNA array, we screened thousands of differentially expressed lncRNA in megakaryocytes from T2D patients, and confirmed that lncRNA metallothionein 1 pseudogene 3 (MT1P3, LOC103289737) was significantly upregulated in megakaryocytes from T2D patients than in healthy controls. We further investigate the biofunction of MT1P3 on platelet activation and the regulatory mechanism on p2y12.

Materials and methods

Study population

Whole-blood samples (100 ml for each sample) from healthy subjects ($n = 16$) and type-2 diabetes patients ($n = 16$) were collected from the first affiliated hospital of Hunan normal university. The information of these patients was obtained from the medical record. The clinical and laboratory characteristics of these individuals were shown in Table I. The present study was approved by the Ethics Committee of the first affiliated hospital of Hunan normal university (permit number: R15032). The inform consents were obtained from all the participants involved in this study.

Cell culture

The CD34+ cells were isolated from whole-blood using a human CD34+ selection kit (StemSep, Cat. 18056, StemCell Technologies,Vancouver, BC, Canada) via a magnetic separation column as per manufacturer's instructions. These cells were cultured in Phase I medium (StemSpan medium supplemented with stem cell factor (SCF) 100 ng/ml, thrombopoietin (TPO) 30 ng/ml, Erythropoietin (EPO) 0.5 U/ml) for 6 days and cultured in Phase II medium (SCF 50 ng/ml, TPO 50 ng/ml, EPO 0.5 U/ml) for 4 days, finally cultured in Phase III medium (TPO 100 ng/ml) for 4 days. After 14-day culture, these cells differentiate to mature megakaryocytes (MKs). CD41+ cells were collected by flow cytometry sorting using Hu CD41a PE HIP8 antibody (cat no. 555467, BD Pharmingen, United States). After 14 days of culture, >90% of the cells were megakaryocytes (CD41+) (supplemental Figure 1). About 3×10^5 MKs could be isolated from 100 ml peripheral blood.

Megakaryocytic cell line, Dami cells were obtained from ATCC. Cells were cultured with RPMI-1640 containing 10% fetal bovine serum (FBS, Gibco-BRL) and maintained in a humidified atmosphere of 5% CO_2 in air at 37°C.

Antibodies

The following antibodies were used in this study: GAPDH from Santa Cruz Biotechnology (Dallas, TX, USA); c-AMP from Abnova (Taipei City, Taiwan); VASP (phospho Ser157) from Abbkine (Wuhan, China); p2y12 from Abcam (Cambridge, UK).

Table I. Characteristics of study population.

	Healthy ($n = 16$)	T2DM ($n = 16$)
Male	9	10
Female	7	6
Age	55 ± 3.2	58 ± 5.6
HbA1c (%)	5.6 ± 0.4	9.0 ± 0.9*
Fasting plasma glucose (mmol/L)	5.1 ± 0.3	12.1 ± 0.7*
Anti-platelet drugs (yes/no)	0/16	0/16
Hypertension (yes/no)	0/16	6/10
Coronary heart disease (yes/no)	0/16	5/11
Diabetic nephropathy (yes/no)	0/16	4/12

*$p < 0.05$ compared with the healthy. T2DM: type 2 diabetes mellitus.

Cell treatment

The lentivirus containing MT1P3 siRNA or MT1P3 expressed plasmid and empty control were purchased from Genepharma Company (Beijing, China). The Dami cells were infected with these lentiviruses for 14 days for further analysis. To ectopic express miR-126, miR-126 mimics (RiboBio Co., Ltd, Guangzhou, China) were transfected into Dami cells by using Lip3000 (Life technologies) according to the manufacturers' instructions.

Long non-coding RNA array

Arraystar Human LncRNA Microarray v4.0 was used for global scanning of lncRNA expression in total RNA samples from megakaryocytes of healthy subjects and T2D patients. Total RNA was sent to the Kangchen Biotech Inc. (Shanghai, China), and analyzed on LncRNA Microarray v4.0 (Affymetrix, Santa Clara, CA). Six hundred ng of total RNA from each sample were used. Sample labeling, microarray hybridization, and washing were performed based on the manufacturer's standard protocols. The raw data were normalized with the quantile algorithm. Differentially expressed lncRNAs were then identified through fold change as well as the p-value. The threshold set for significantly up- and down-regulated genes was a fold change >2.0 and a p-value <0.05.

Quantitative PCR

Total RNA was extracted from cells using Trizol reagent (Invitrogen, CA, USA). The expression of HIPK1-AS1, LANCL1-AS1, TIPARP-AS1, MT1P3, and p2y12 was measured by OneStep RT-PCR Kit (QIAGEN, USA) according to manufacturers' instructions. Expression of β-actin was used as an endogenous control. MiR-126-3p (miR-126) expression in cells was detected using a Hairpin-it TM miRNAs qPCR kit (Genepharma, Shanghai, China) according to manufacturers' instructions. Expression of RNU6B was used as an endogenous control. The primer sequences were shown in Table II. QPCR was performed at the condition: 95.0°C for 3 min, and 39 circles of 95.0°C for 10 s and 60°C for 30 s. Data were processed using $2^{-\Delta\Delta CT}$ method.

Western blotting

The total protein was extracted by using cold RIPA (Radio-Immunoprecipitation Assay) lysis buffer. The protein was separated with 10% SDS-PAGE, which was then transferred to polyvinylidene fluoride (PVDF) membrane (Thermo Fisher). After that, the membrane was incubated in phosphate buffer saline (PBS) with 5% nonfat dried milk (Mengniu, Hohhot, China) for 3 h at 4°C. Then, the membrane was incubated with primary antibodies overnight at 4°C, and then with appropriate secondary antibody for 1 h at 37°C. The immune complexes were detected using ECL Western Blotting Kit (Millipore). The relative protein expression was analyzed using Image-Pro plus software 6.0, and GAPDH was used as the internal reference.

Luciferase reporter assay

Cells were plated in 96-well clusters (5×10^3 cells/well) for 24 h, and then cotransfected with luciferase reporter with a mixture of 50 ng firefly luciferase (FL) reporter vectors, 5 ng RL reporter vectors, and miRNA mimics for 48 h. The 200 miRNA mimics were obtained from Life Technologies. The luciferase activity was measured with a dual luciferase reporter assay system (Promega, Madison, WI) and normalized to Renilla activity. In the luciferase screening assay, we used one internal control (Renilla luciferase

Table II. The primer sequences used in qPCR.

Gene	Sense (5′-3′)	Anti-sense (5′-3′)
p2y12	TCCAGGGTCAGATTACAAGAGC	CAGTGGTCCTGTTCCCAGTT
HIPK1-AS1	GCCTCTACCAGAAGGAAGGC	CCAGCACTTGTGGGATGGAA
LANCL1-AS1	ACTGTCTAAACACTGGCGCA	GCTGTACAGGGTTTCTGGCT
TIPARP-AS1	ACTAAATCCTTCGGCGCCTC	CCTGTCGTCCGTTCTCTCAG
MT1P3	ACTGTAAACGCCTGGCTCAA	GAGGTGCATGTGCAGTCTTG
miR-126	GCTGGCGACGGGACATTAT	CGGCGCATTATTACTCACGG
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
β-actin	GCCCTATAAAACCCAGCGGC	TCGATGGGGTACTTCAGGGT

(RL) reporter) and negative control. Each miRNA or negative control RNA was co-transfected with RL reporter and FL reporter with or without the lncRNA MT1P3. The foldchange was calculated by each miRNA compared with negative control. The mutant type of MT1P3 (LUC-MT1P3 mutant) with a mutant seed sequence of miR-126, let-7, miR-103, miR-223, and miR-1 was synthesized by Genepharma Co. Ltd. (Shanghai, China).

Animals

Animal experiments were approved by the Institutional Animal Care and Use Committee of the first affiliated hospital of Hunan normal university in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The 8 weeks old C57BL/6J ($n = 8$) and ob/ob ($n = 8$) mouse, and Wistar ($n = 8$) and Goto-Kakizaki (GK, $n = 18$) rats were purchased from Silaike Experimental Animal Co. Ltd (Changsha, China). To knockdown MT1P3 in GK rats, the lentivirus containing MT1P3 siRNA (250 μl per rat, 10^9 U/ml) were intra-bone marrow injected into tibia of posterior limb of GK rats ($n = 5$) for 14 days. Briefly, we first slowly rotated the 1-ml syringe needle to make a hole in the center of tibial plateau, and then injected lentivirus along the tunnel using a sterile micro syringe for 2 min, finally pressured the puncture point for 30 s using a cotton swab after slowly pulling the needle. The rats injected with empty lentivirus were used as negative control. Fourteen days after injection, the rats were isofluorane-anesthetized and were removed the blood and bone marrow. The CD41+ cells and platelet were isolated for analysis.

Rat and mice bone marrow isolation and CD41+ megakaryocyte preparation

Bone marrow aspiration samples of rats or mice were collected in tubes containing ethylene diamine tetraacetic acid (EDTA). Mononuclear cells were isolated from bone marrow samples as previously reported (13). After removal of adherent cells, CD41+ megakaryocytes population was enriched with PE Mouse Anti-Human CD41a (cat no. 560979, BD Pharmingen, United States) according to the manufacturer's instruction. Flow cytometry was performed to verify the purity of the megakaryocyte sample. After the purification of megakaryocytes, cells were collected and processed for RNA and protein extraction.

Preparation of washed platelets

Blood was collected from the abdominal aorta of isofluorane-anesthetized mice or rats using 1⁄6 volume of trisodium citrate as anticoagulant. The platelets were then washed twice with CGS (0.12 M sodium chloride, 0.0129 M trisodium citrate, 0.03 M D-glucose, pH 6.5), and resuspended in modified Tyrode's buffer (cat no. BCZ0061, BioRike, Changsha, China) and incubated for 1 h at 22°C before use.

Platelet aggregation

Platelet aggregation at 37°C was measured by sequential platelet counting method using platelet functions analyzer (PL-12, Sinnowa, Nanjing, China). ATP release was measured by adding luciferin/luciferase reagent (5 μl) to 250 μl of a washed platelet suspension 1 min before stimulation.

Statistical analysis

Data are expressed as the mean ± S.E.M. SPSS 18.0 software package (SPSS, Chicago, IL, USA) was used to perform statistical analysis. Difference between two groups was compared by independent-samples *t*-test. Difference among three or more groups was compared by One-Way ANOVA with post hoc Bonferroni test. The *p*-value less than 0.05 was considered statistically significant.

Results

Differentially expressed lncRNAs in megakaryocytes from type 2 diabetes patients

To investigate the differentially expressed lncRNAs in megakaryocytes, we isolated the CD34+ cells to generate CD41+ megakaryocytes. By lncRNA array of three cases of healthy controls and three cases of T2D patients, we screened thousands of differentially expressed lncRNAs, including 1259 downregulated lncRNAs and 2473 upregulated lncRNAs (Figure 1A, B). Then we confirmed the expression of four upregulated lncRNAs in 16 cases of healthy controls and T2D patients. We found that HIPK3-AS1 ($p = 0.043$) and MT1P3 ($p < 0.001$) were significantly upregulated in T2D patients compared to healthy controls (Figure 1C, F), while the expression of LANCL1-AS1 and TIPARP-AS1 was comparable in healthy controls and T2D patients (Figure 1D, E). To investigate the relationship between the constitutive activity of p2y12 and lncRNA MT1P3, we also measured the mRNA and protein levels of p2y12 in megakaryocytes. We found that p2y12 was significantly upregulated in mRNA and protein levels in T2D compared with healthy controls (Figure 2A, B). Correlation analysis for the expression of p2y12 mRNA and MT1P3 showed that there was a positive correlation between them (Figure 2C), while there was no significant correlation between HIPK3-AS1 and p2y12 in MKs (data not shown). Intriguingly, MT1P3 also positively correlated with HbA1c levels in diabetes patients, indicating that the expression of MT1P3 may be correlated with the severity of DM2 (Figure 2D).

MT1P3 positively regulates p2y12 by sponging miR-126 in Dami cells

To investigate whether MT1P3 can regulate p2y12 expression, we knocked down MT1P3 and overexpressed MT1P3 in Dami cells (Figure 3A). We found that p2y12 mRNA level was reduced by

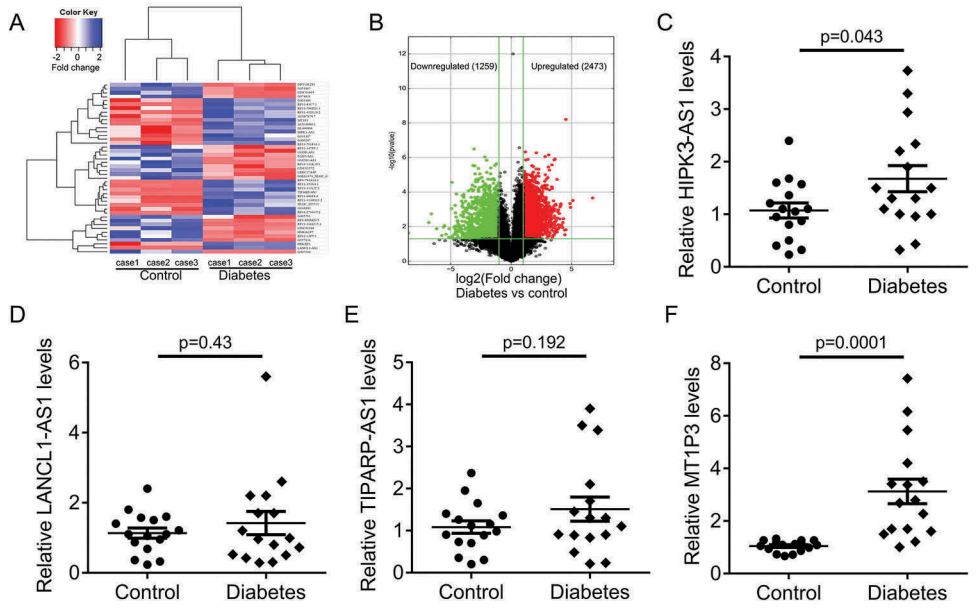
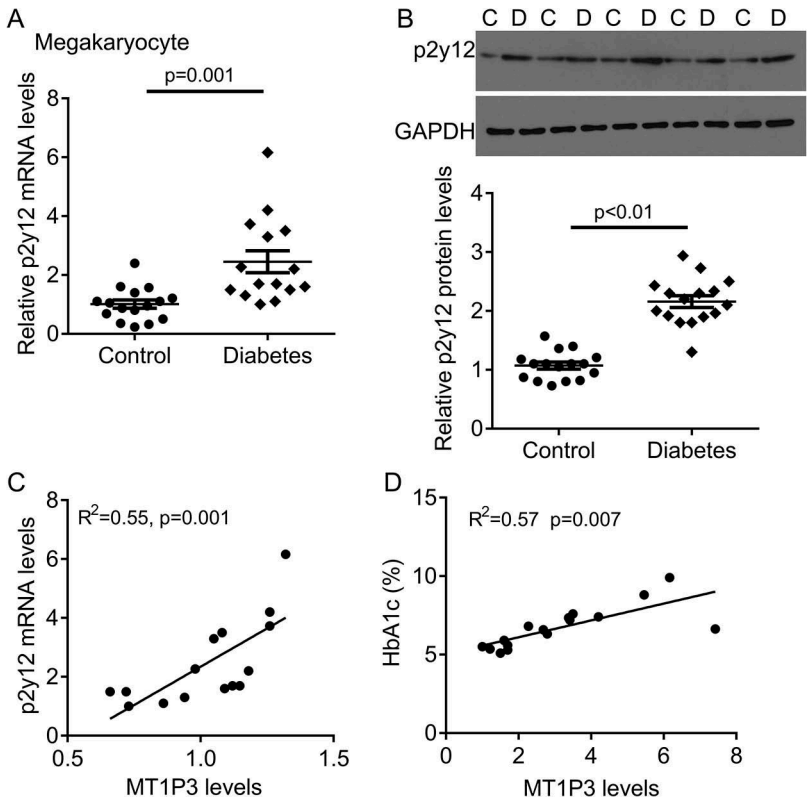


Figure 1. Differentially expressed lncRNA in megakaryocytes from type 2 diabetes.(A) Heat map for differentially expressed lncRNA in megakaryocytes from healthy controls and diabetes. (B) Volcano Plots show the upregulated (green) and downregulated (red) lncRNAs in megakaryocytes from diabetes compared with healthy controls. (C–F) QPCR was performed to measure the expression of HIPK3-AS1 (C), LANCL1-AS1 (D), TIPARP-AS1 (E), and MT1P3 (F) in megakaryocytes from 16 cases of healthy controls and diabetes.

Figure 2. MT1P3 positively correlates with p2y12 levels. (A) QPCR was performed to measure the mRNA expression of p2y12 in megakaryocytes from 16 cases of healthy controls and diabetes. (B) Representative western blot bands for p2y12 in megakaryocytes from 16 cases of healthy controls and diabetes, and quantification (right). C, control; D, diabetes. (C) MT1P3 positively correlates with p2y12 mRNA levels in megakaryocytes from diabetes. (D) MT1P3 positively correlates with HbA1c levels in diabetes patients.



MT1P3 downregulation and increased by MT1P3 upregulation (Figure 3B). These results were also confirmed in p2y12 protein levels (Figure 3C). LncRNAs function their regulatory effects on genes commonly through sponging miRNAs. We screened the putative sponging miRNAs by luciferase reporter assay in miRNAs library. We screened five miRNAs (miR-126, let-7, miR-103, miR-223, and miR-1) with significantly low luciferase activity (Figure 3D). We further confirmed their target relationship and found that wild-type MT1P3 significantly inhibited the luciferase activity of miR-126 but not in other miRNAs, and its mutant type abolished this inhibition (Figure 3E). We treated the Dami cells with high-glucose to mimic the DM2 condition and found that the expression of MT1P3 and p2y12 was induced by high-glucose, and miR-126 levels was reduced by high-glucose compared with the controls (Figure 3F). In addition, we treated Dami cells with MT1P3 together with miR-126 mimics, and found that miR-126 decreased p2y12 mRNA expression, and miR-126 mimics reversed MT1P3-mediated upregulation of

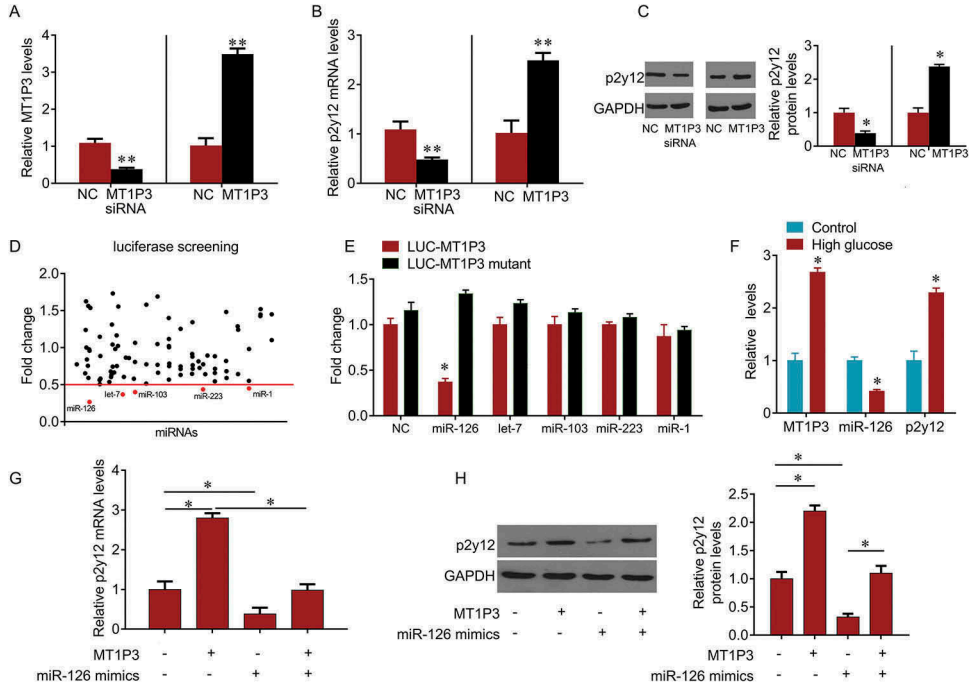


Figure 3. MT1P3 increases p2y12 expression by sponging miR-126 in Dami cells. (A) QPCR was performed to measure the expression of MT1P3 in Dami cells after indicated transfection. (B) QPCR was performed to measure the expression of p2y12 in Dami cells after indicated transfection. (C) Representative western blot bands for p2y12 in Dami cells after indicated transfection (left), and quantification (right). (D) Luciferase reporter gene assay in miRNAs library to screen the potential MT1P3 targets. (E) Daul luciferase reporter gene assay for miR-126, let-7, miR-223, miR-103, and miR-1. (F) QPCR was performed to measure the expression of MT1P3, miR-126, and p2y12 in Dami cells under high glucose stimulation. (G) QPCR was performed to measure the expression of p2y12 in Dami cells after MT1P3 or/and miR-126 mimics transfection. (H) Representative western blot bands for p2y12 in Dami cells after MT1P3 or/and miR-126 mimics transfection (left), and quantification (right). *$p < 0.05$, **$p < 0.01$.

p2y12 (Figure 3G). These effects were also observed in p2y12 protein levels (Figure 3H). Thus, these results demonstrated that MT1P3 increased p2y12 expression by sponging miR-126.

The expression of MT1P3 and miR-126 in diabetes animal model

To investigate the role of MT1P3 *in vivo*, we measured its expression in two common diabetes animal models, ob/ob mice and GK rats. We found that MT1P3 was significantly upregulated in megakaryocytes from ob/ob mice compared with that from C57BL/6J mice, while miR-126 expression was significantly reduced in diabetes mice compared with health mice, and upregulation of p2y12 was observed in diabetes mice (Figure 4A). Upregulation of MT1P3 and p2y12, and downregulation of miR-126 were also observed in GK rats (Figure 4B). In addition, the protein expression of p2y12 was increased and c-AMP and p-VASP were decreased in platelet from diabetes animals (Figure 4C). These results indicate that MT1P3 positively correlated with p2y12 expression.

Knockdown of MT1P3 reduces platelet aggregation in GK rats

To further confirm the role of MT1P3 on platelet aggregation *in vivo*, we injected lentivirus containing MT1P3 siRNA into bone marrow of posterior limb of GK rats, and found that the expression of MT1P3 and p2y12 was significantly decreased, while miR-126 was dramatically increased in megakaryocytes (Figure 5A). There were no significant alterations on the counts of RBC, WBC, and platelet (Supplemental Figure 2). Platelet aggregation and ATP secretion elicited by low-dose agonists ADP were reduced by MT1P3 downregulation. At high concentrations, ADP elicited similar levels of platelet aggregation and ATP release in MT1P3 siRNA and control platelets (Figure 5B, C). The protein expression of p2y12 was decreased, and c-AMP and p-VASP expression were elevated in platelet compared with negative control (Figure 5D). These results indicate that MT1P3 downregulation inhibits platelet activation and aggregation by decreasing p2y12 expression.

Discussion

In this study, we screened thousands of differentially expressed lncRNAs in megakaryocytes from T2D patients, and further confirmed that lncRNA MT1P3 upregulated in megakaryocytes from T2D patients. MT1P3 was positively correlated with p2y12 mRNA levels, and regulated p2y12 expression by sponging miR-126. Knockdown of MT1P3 by siRNA reduced p2y12 expression, inhibiting platelet activation.

MT1P3 was located on chromosome 20. The metallothionein-1 (MT-1) genes comprise a multigene family. Three MT-1 pseudogenes (MT1P1, MT1P2, and MT1P3) arise by reverse transcription of processed mRNA transcripts. MT1P3 is a retrogene which derive from the MT-1 mRNA (14). There is another pseudogene of the human MT multigene family, MT-like gene that is not expressed in response to metal induction in two human cell lines (15). LncRNA, Metallothionein 1D, Pseudogene (MT1DP), a member of MT multigene family, was found to act as a tumor suppressor in liver cancer by inhibiting Forkhead box A1, and MT1DP was negatively regulated by Runt-related transcription factor 2 (Runx2) and Yes-associated protein (YAP) (16). Many studies have indicated that Metallothionein plays an important role in diabetes. Metallothionein is a metal-binding protein. Metallothionein can be induced by a variety of conditions including high glucose (17,18), and plays a crucial role in mediating anti-apoptosis and anti-inflammation. By modulating stress-

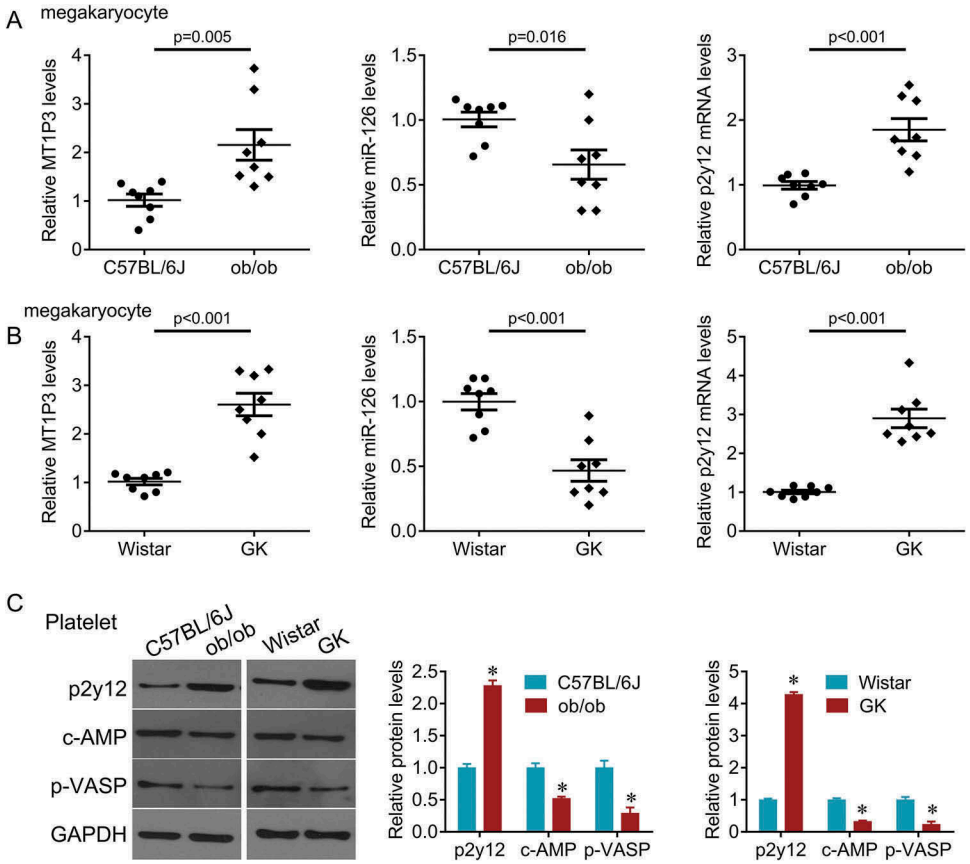
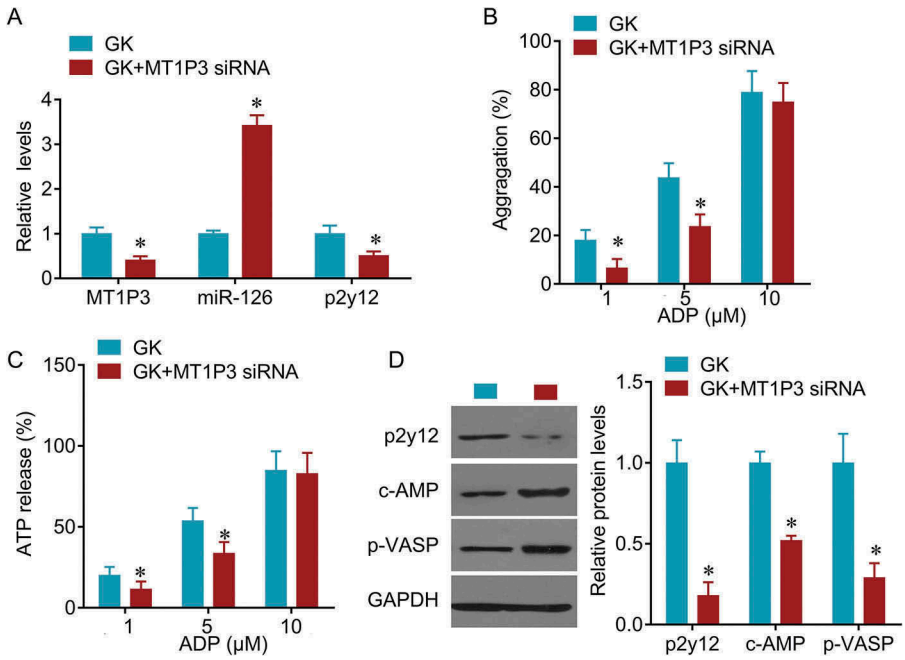


Figure 4. The expression of MT1P3, miR-126, and p2y12 in diabetes animal models. (A) QPCR was performed to measure the expression of MT1P3, miR-126, and p2y12 in megakaryocytes from C57BL/6J and ob/ob mice. (B) QPCR was performed to measure the expression of MT1P3, miR-126, and p2y12 in megakaryocytes from Wistar and GK rats. (C) Representative western blot bands for p2y12, c-AMP, and p-VASP in platelets from C57BL/6J and ob/ob mice, and Wistar and GK rats, and quantification of the bands. $N = 8$ for each group. $^*p < 0.05$.

Figure 5. Knockdown of MT1P3 reduces platelet aggregation in GK rats. (A) QPCR was performed to measure the expression of MT1P3, miR-126, and p2y12 in megakaryocytes from GK rats 14 days after lentivirus intra-bone marrow injection. (B) Decreased platelet aggregation in response to ADP in diabetic GK rats with MT1P3 siRNA compared with control GK rats. (C) Decreased ATP release in response to ADP in diabetic GK rats with MT1P3 siRNA compared with control GK rats. (D) Representative western blot bands for p2y12, c-AMP, and p-VASP in platelets from GK rats 14 days after lentivirus intra-bone marrow injection (left), and quantification (right). $N = 5$ for each group. $^*p < 0.05$.



induced signaling pathways of Wnt, NF-kB, and PI3K, Metallothionein can alleviate diabetes and diabetic complications (19). Fasting resulted in a significant increase in metallothionein 1 gene expressions in retroperitoneal, epididymal, and inguinal white adipose tissue of rats, and this effect was reversed by refeeding. Altered expressions of metallothionein 1 gene were reflected by changes in serum metallothionein 1 levels. Insulin addition resulted in a significant decrease in metallothionein 1 gene expression in isolated adipocytes (20). Here, we demonstrated that MT1P3 was upregulated in human T2D patients and

diabetes animal model megakaryocytes. Since previous studies supported that metallothionein 1 has a protective role on diabetes-induced cardiac pathological changes, diabetic nephropathy (21–23), the link of MT1P3 and metallothionein 1 needs to be further investigated. Recently, the drugs most commonly used to antiplatelet therapy, such as clopidogrel and Cangrelor, target the ADP receptor in mature platelet. However, megakaryocytes may continuously produce new pathological platelets under diabetic conditions. Dysmaturity and dysplasia of megakaryocytes were found in many conditions. Developing approaches for regulating function and proliferation of megakaryocytes would control the platelet dysfunction-induced diseases at the source. However, this treatment is not specific to megakaryocytes.

We further illustrated the role of MT1P3 on platelet activation. Knockdown of MT1P3 did not alter the counts of RBC, WBC, and platelet, but inhibit the platelet aggregation and activation. MT1P3 downregulation by siRNA decreased the expression of p2y12 by sponging miR-126. Through the luciferase screening, we identified that the luciferase activity of miR-126, miR-223, let-7, miR-103, and miR-1 was significantly reduced by MT1P3. The confirmation experiments showed that miR-126 directly targeted MT1P3. MiRNAs derived from platelets seem to participate in platelet function, hemostasis, thrombosis, and unstable coronary syndromes (24–26). Let-7 levels are decreased in diabetic human carotid plaques and diabetic ApoE-/- mouse atherosclerosis. Ectopic overexpression of let-7 in vascular smooth muscle cells inhibited inflammatory responses (27). let-7b was upregulated during platelet storage, and let-7b influenced Bcl-xL and Bak protein expression at posttranscriptional levels (28). MiR-223 was the most abundant in platelets and has previously been implicated in myeloid lineage development and demonstrated to have anti-inflammatory effects (29–31). ADP was shown to play an important role in the release of microRNAs from platelets (32,33). miR-223 and miR-126 were significantly reduced in platelets and megakaryocytes in T2D, which were induced by hyperglycemia, causing upregulation of P2RY12 and p-selectin mRNAs that may contribute to adverse platelet function (34). We here also found that miR-126 mimics inhibited p2y12 mRNA and protein expression, which reversed MT1P3-mediated upregulation of p2y12. MiR-126 is the second abundant miRNA in platelets and has been implicated in vascular homeostasis and inflammation (35,36). MiR-126 expression was decreased in plasma response to platelet inhibition (37). MiR-126 was reduced in the rabbit atherosclerotic plaque model group, which was inversely correlated with the expression of p2y12 and VCAM-1 (38). Calpeptin pretreatment to inhibit Dicer activity restored miRNA levels including miR-126 in hyperglycemic MKs. Treating diabetic mice with a calpain inhibitor prevented loss of platelet dicer as well as the diabetes-mellitus-induced decrease in platelet miRNA levels and the upregulation of miRNAs target proteins, such as p2y12 (39). In addition, the deletion of Dicer1 resulted in increased surface expression of integrins αIIb and β3, enhanced platelet binding to fibrinogen, heightened platelet reactivity, shortened tail-bleeding time (40). Thus, the function of miRNAs may be Dicer1-dependent MKs and platelets. In recent study, we demonstrated that MT1P3 downregulated miR-126 expression, and subsequent upregulation of p2y12. It is not yet clear whether MT1P3 regulating miR-126 is dependent on Dicer. Our recent results indicated a positive correlation between the expression of MT1P3 and HbA1c levels on T2D patients. However, limited by the small number patients enrolled in this study, this correlation should be confirmed in a larger cohort with more detailed information, such as HbA1c levels and the diabetic complications (diabetic nephropathy and diabetic retinopathy, etc.), so that we can confirm that the expression of MT1P3 is correlated with the severity of DM2.

In conclusion, our findings identify MT1P3 as a key regulator in platelet activation by increasing p2y12 expression through sponging miR-126 under T2D condition. These findings may provide a new insight for managing platelet hyperactivity-related diseases.

Declaration of interest

The authors state no conflict of interests.

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Supplemental data

Supplemental data for this article can be accessed here.

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