



MALAT1 promotes platelet activity and thrombus formation through PI3k/Akt/GSK-3 β signalling pathwayYeying Sun ¹, Tao Wang,¹ Yan Lv,² Jiahua Li,¹ Xiaoli Jiang,¹ Jing Jiang,¹ Daolai Zhang,¹ Weihua Bian ¹, Chunxiang Zhang^{1,3}

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¹College of Pharmacy, Binzhou Medical University, Yantai, Shandong, China

²College of Life Sciences, Yantai University, Yantai, Shandong, China

³Department of Cardiology, Southwest Medical University, Luzhou, Sichuan, China

Correspondence to

Chunxiang Zhang;
bzmczcx@163.com

Dr Weihua Bian;
bian_1005@163.com

ABSTRACT

Background Ischaemic stroke and other cardiovascular illnesses are characterised by abnormalities in the processes of thrombosis and haemostasis, which rely on platelet activity. In platelets, a wide variety of microRNAs (long non-coding RNA, lncRNAs) is found. Due to the absence of nuclear DNA in platelets, lncRNAs may serve as critical post-transcriptional regulators of platelet activities. However, research into the roles of lncRNAs in platelets is limited.

Objective The purpose of this study is to learn more about the molecular mechanism by which MALAT1 affects platelet activity and thrombus formation.

Methods/results The CD34⁺ megakaryocytes used in this research as an in vitro model for human megakaryocytes and platelets. Cell adhesion and spreading are enhanced in the absence and presence of agonists in CD34⁺ megakaryocytes subjected to MALAT1 knockdown (KD). The adhesion and activity of platelet-like particles produced by MALAT1 KD cells are significantly enhanced at rest and after thrombin activation. Thrombus development on a collagen matrix is also greatly enhanced in the microfluidic whole-blood perfusion model: platelets lacking MALAT1 exhibit elevated accumulation, distributing area and activity. In addition, MALAT1-deficient mice bleed less and form a stable occlusive thrombus more quickly than wild-type mice. PTEN and PDK1 regulated the activity of MALAT1 in platelets to carry out its PI3k/Akt/GSK-3 β signalling pathway-related function.

Conclusion The suppression of MALAT1 expression significantly increases platelet adhesion, spreading, platelet activity, and thrombus formation. lncRNAs may constitute a unique class of platelet function modulators.

INTRODUCTION

Platelets are blood cells involved in a wide variety of both healthy and diseased processes. Platelets are vital to the processes of thrombosis and haemostasis, but now we believe that they have significant roles in tissue repair, immune defence, tumour metastasis and development, atherogenesis, and inflammation.^{1 2} Furthermore, platelet function is linked to incident cardiovascular morbidity and mortality.³ To some extent, the wide range in (cardiovascular disease) CVD risk observed among healthy people can be attributed to the wide range in platelet activity seen

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ A considerable amount of long non-coding RNAs are distributed in platelets. MALAT1 is one of the most abundant lncRNAs in platelets. However, the functional roles of MALAT1 in platelets are unexplored.

WHAT THIS STUDY ADDS

⇒ In this study, the function of MALAT1 in platelets was investigated in vitro and in vivo. Platelet deficiency of MALAT1 enhances platelet adhesion, spreading, platelet activity, Activation of GPIIb/IIIa and thrombus formation mediated by the PI3k/Akt/GSK-3 signalling pathway and its negative regulators PTEN and PDK1.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Platelet activity may be responsible for inducing dynamic changes in the expression patterns of lncRNAs. These changes have the potential to be used in the future, so opening up a new door for the treatment of thrombotic illnesses via RNA-based therapies.

among these people.^{4 5} Given that platelets only live for 8–9 days and lack nuclear DNAs, it is likely that post-transcriptional regulation plays a crucial role in their ability to retain a proteome and respond to changing environments. As such, platelets are similar to nucleated cells in that they can regulate RNA levels, translate, and splice posttranscriptionally. Non-coding RNAs made up a larger portion of the transcriptome in platelet than was seen in other cells, suggesting that post-transcriptional processes were relied on to regulate gene expression in the absence of transcription.⁶ It is true that platelets contain a high number of miRNAs, long non-coding RNAs (lncRNAs), and circRNAs.^{6–8} Only a few numbers of research have explored the impact of lncRNAs on platelets, and our understanding of the miRNAs that regulate platelet function is vague at best.^{9 10}

A lncRNA is one that is longer than 200 nucleotides yet can not encode for proteins.^{11 12} To date, over 10 000 lncRNAs have been found

in human cells, and this number is growing. In contrast to studies on other non-coding RNAs, such as miRNAs, biomedical research on lncRNAs is in its birth due to their complicated and diverse gene control methods.^{13 14} However, research suggests that lncRNAs have substantial biological activities and may be implicated in all normal and pathological events.^{15 16} MALAT1 is highly conserved compared with other lncRNAs and is widely expressed in a variety of tissues.^{17–20} MALAT1 regulates smooth muscle cell and endothelial functions and protects from atherosclerosis by regulating inflammation,²¹ and our high-throughput sequencing data (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE97348>) show that MALAT1 is listed as one of the most frequent lncRNAs in platelets. And MALAT1 is also one of the abundant genes in human cancers, CVDs and Alzheimer's disease.^{17 18} We investigated MALAT1's involvement in platelet activity and thrombus development to further understand the unique mechanisms for lncRNAs in platelet function. This work tested platelet activities and thrombogenesis in C57/Bl6 mice using the megakaryocytic cell line CD34+ megakaryocytes as a model of platelet-like activity.^{4 22} We found that decreased expression of MALAT1 in CD34+ megakaryocytes improved megakaryocyte adherence and dispersion. More importantly, platelet-like particles (PLPs) generated from MALAT1-deficient cells had significantly enhanced adherence and activities. Reduced MALAT1 expression dramatically boosted platelet adhesion, dispersion, activity and thrombus formation in the mice model. Our findings show that MALAT1 acts as a negative regulator of platelet activity by reducing platelet activity and thrombus formation.

MATERIALS AND METHODS

Platelet aggregation assay

All aggregation studies were finished within 2 hours following phlebotomy. Platelet aggregation was assessed according to Yee *et al* method.⁵ To obtain platelet-rich plasma, blood was centrifuged for 15 min at 150 g (PRP). However, platelet-poor plasma (PPP) was collected by centrifuging blood at 400 g for 5 min to remove platelets. A haemocytometer was used to determine how many platelets were in the PRP, and the concentration was then increased to between 200 000 and 250 000 platelets/ μ L by PPP. Epinephrine (0.4 μ M), bovine Achilles tendon collagen (20 μ g/mL), and adenosine diphosphate (1 μ M) were used to stimulate platelet aggregation, which was then evaluated using a Chrono-Log aggregometer (Chrono-Log, USA). The incubation temperature was 37°C, and the stir bar speed was 1200 revolutions per minute. The peak aggregation percentage was found 10 min after the agonists were added.

Platelet isolation, purification and RNA extraction

According to the findings of a previous study, density centrifugation and CD45+ cell depletion were used to isolate and purify platelets from whole blood.²³ Briefly,

peripheral blood samples were treated with citrate dextrose to obtain the plasma, which comprised 85 mM trisodium citrate, 78 mM citric acid, and 111 mM glucose. The samples were then centrifuged at 80×g for 10 min at 22°C. To collect the platelets, the PRP was centrifuged at 1000 × g for ten minutes at a temperature of 22°C. after being mixed with 2 mM of ethylenediaminetetraacetic acid. Bead buffer (0.02% KCl, 0.8% NaCl, 0.024% KH₂PO₄, 0.144% Na₂HPO₄, 0.5% bovine serum albumin, 2 mM ethylenediaminetetraacetic acid) and 40 μ L human CD45 MicroBeads reagent were added to the resuspended platelets in a volume of 3 mL. (Miltenyi Biotec, Germany). Using a MACS bead separation system (Miltenyi Biotec, Germany), we were able to isolate platelets free of leukocytes to greater than 99.99% after incubating them at room temperature for 45 min with gentle mixing. RNA from the platelets was isolated with the use of TRIzol (Life Technologies, USA). An Agilent Bioanalyzer was used throughout the process of quality control screening for each and every RNA sample.

Cell and cell culture

Allcells Biotechnology (Shanghai, China) was the supplier of the Cord Blood-Stem/Progenitor (CD34⁺) cells that were used in this study. Serum-free expansion medium (StemCell Technologies, Canada) was used in the cultivation of CD34⁺ cells. To facilitate megakaryocyte differentiation, CD34⁺ cells were seeded at a density of 1×10⁴ cells/mL in a volume of 1 mL each well in a 12-well plate. The medium used was SFEM II, and StemCell Technologies' StemSpan Megakaryocyte Expansion Supplement was added.

Fresh megakaryocyte growth medium in the amount of 500 μ L was added once every 2 days. Every 7 days, the cells were pelleted and then resuspended in fresh megakaryocyte growth media. At days 14 and 28, the cells were examined to determine the degree to which they expressed the megakaryocyte lineage markers CD41a and CD42b. Human megakaryoblastic leukaemia cell line MEG-01 was obtained through a request to the American Type Culture Collection. MEG-01 cells were cultivated at a temperature of 37°C in an atmosphere that was humidified and contained 5% carbon dioxide. The media the cells were grown in was RPMI 1640 medium (Thermo Fisher, USA), which was augmented with 10% fetal bovine serum (Biological Industries, Israel).

Plasmid construction and shRNA transfection

The pLKO.3G cloning vector was used to construct the plasmids of a scramble and anti-MALAT1 shRNA-targeted at human MALAT1 and mouse MALAT1. The sequence of the siRNA designed to target MALAT1 in humans was 5'-GGCTGAGTGTTGAGGAAATTT-3', while the sequence designed to target MALAT1 in mice was 5'-GCCTTAACCTTGCTAGCTTAATT-3'. As a non-target control, we employed a sequence that had its bases mixed together (5'-AATTCTCCGAACGTGTCACGT-3'). The scramble plasmid and MALAT1 knockdown (KD) shRNA

plasmids were packaged using 293 T cells with pMD2.G and psPAX2, respectively, plasmids to generate MALAT1 KD lentivirus. The lentivirus was concentrated in the supernatants. After that, the lentivirus was introduced to the CD34⁺ megakaryocytes cell culture medium at a concentration of 4×10^5 cells per mL, while polybrene was present at a concentration of 10 µg/mL and the mixture was allowed to incubate for 24 hours. Lentiviral particles were transduced at MOI of 5, according to a protocol reported previously.²⁴ The efficacy of the shRNA KD was determined using real-time PCR.

Bone marrow transplantation

All mouse strains were bred from C57/BL6 mice to ensure consistency. Bone marrow transplants were performed on 5 animals of equal age, weight and sex ratios (1:1). National Institutes of Health guidelines for the care and use of laboratory animals and the recommendations of the ** laboratory animal ethics committee were followed throughout all animal research. The MACS lineage depletion kit (Miltenyi Biotec GmbH, Germany) was used to separate bone marrow cells from the femur and tibia bone marrow of an 8-week-old C57/BL6 mice. The procedure was carried out in accordance with the guidelines provided by the manufacturer. Using a Lenti-XTM concentrator (Clontech, Canada), we infected the recovered stem cells twice with either a highly purified form of the mice MALAT1 shRNA lentivirus or a scramble lentivirus.²⁵ To test the effects of a high dosage of gamma radiation, 9.6 Gy was administered to C57/BL6 mice that were around 8 weeks old. One million infected cells were injected retro-orbitally into the irradiated mice. Platelets were extracted from recipient mice 4–6 weeks after transplantation.

Cell and platelet spreading

Twenty-four well plates were coated with collagen or fibrinogen overnight at 4°C and then placed in the refrigerator. After discarding the excess liquid, the coverslips were washed three times in PBS and then blocked with bovine serum albumin at a concentration of 1% for 15 min. Twenty minutes of staining with 1 M DiOC6 using collagen-coated coverslips in the presence or absence of 1 U/mL thrombin for 24 hours at 37°C was performed on CD34⁺ megakaryocytes cells and PLPs. Bone marrow transplant recipient mice had their platelets cultured on fibrinogen-coated coverslips for 1 hour at 37°C. Excess platelets were removed after incubation, and three washes with PBS were performed. Adherent platelets were fixed with 4% paraformaldehyde and permeabilised with 0.2% Triton X-100 solution. Then, the platelets were stained for 1 hour at room temperature in the dark with Rhodamine-conjugated phalloidin. The images of CD34⁺ megakaryocytes, PLPs, and platelets were captured using confocal microscopy (ZEISS LSM800, Germany), and the spreading areas were quantified using Image J software.

Flow cytometry

Fluorescein isothiocyanate (FITC)-conjugated anti-CD41b and anti-61 antibodies (BD Biosciences, USA) were used to label 3×10^5 MEG-01 cells for 30 min in the dark. Isolated PLPs were stained for 30 min at 4°C with APC-CD62P ((P-selectin, CD62 Antigen-like Family Member P) and FITC-CD61 antibodies (BD Biosciences, USA) after being extracted from cultivated MEG-01 cells. Paraformaldehyde (1%) was used to fix all conjugated cells before flow cytometric analysis (BD FACSaria, USA). Two-colour analysis was used to assess mouse platelet activation by measuring the expression of P-selectin (Wug. E9-FITC, D200, Emfret Analytics, Germany) and the active form of α IIb β 3 integrin (JON/A-PE, D200, Emfret Analytics, Germany), as described before.

Fluorescence in situ hybridisation and preparation of cytoplasmic and nuclear RNA

Cultured MEG01 cells and platelet samples were used for fluorescence in situ hybridisation (FISH). The cells were stained with fluorescent probe hybridisation solution and incubated overnight at 37°C after being fixed with 4% paraformaldehyde and permeabilised with 0.5% TritonX-100; the nuclei were labelled with DAPI. Laser confocal microscopy (TCS SPE, Leica, Germany) was used to take the photos. Nuclear/Cytosolic Fractionation Kit (Cell Biolabs, USA) was used to isolate nuclear extract from the cytoplasmic fraction of MEG01 cells. RNA from each subcellular fraction was analysed by qPCR to determine the distribution of MALAT1.

Real-time reverse transcription PCR

Extracted total RNAs were reverse-transcribed to cDNA using the Evo M-MLV RT Kit (Accurate Biotechnology(Hunan), China). This study's internal control was GAPDH.

By comparing threshold cycle values, we were able to determine MALAT1's relative gene expression (CT). Primer sequences for quantitative reverse transcription PCR (qRT-PCR) are as follows: GAPDH sense: 5'-GGAGCGAGATCCCTCCAAAT-3', antisense: 5'-GGCTGTTGTCATACTTCTCATGG-3'; MALAT1-mouse sense: 5'-GGCGGAATTGCTGGTAGTTT-3', antisense: 5'-AGCATAGCAGTACACGCCTT-3'; MALAT1-human sense: 5'-CTAAACGCAGACGAAATGGA-3', antisense: 5'-CCCGTACTTCTGTCTTCCAGT-3'.

Western blot

A proteinase inhibitor cocktail (RIPA) lysis buffer (1% protease inhibitors) was used to lyse the cells (Absin, China). Antibodies against GAPDH, PDK1, PTEN, p-Akt, Akt, and p-GSK-3 were used to probe proteins separated by SDS-polyacrylamide gel electrophoresis and transferred using a Wet Transfer System (Cell Signaling Technology, USA).

Signal was detected using an ECL luminescence reagent and horseradish peroxidase-conjugated secondary antibodies specific to the target species (Absin, China).

Thrombus formation under flow conditions

The Bioflux-200 system was used to carry out the *in vitro* examination of the development of a thrombus (Fluxion, USA). At the same time platelets in whole mouse blood were labelled with calcein (10 μ M) for 1 hour, bioflux plates were coated with collagen at a concentration of 100 mg/mL for the same period of time. The collagen-coated plates were subjected to a shear force of 40 dynes/cm² for 5 min, during which time the tagged blood was perfused through the plates. Adherent platelets were visualised with an $\times 20$ objective lens using an inverted fluorescence microscope (Axio Vert.A1, ZEISS, Germany). The thrombus formation area was measured using ImageJ software.

Ferric chloride-induced thrombosis mouse model

Ferric chloride was used to produce thrombosis in a mouse model.²⁶ Briefly, after administering 4% chloralhydrate to anaesthetise the mice, a catheter was used to inject calcein (10 μ M) -labelled platelets into the jugular veins. When applied to the mesentery, filter paper that was saturated with a solution containing 10% FeCl₃ externalises the vascular bed and causes thrombosis in the mesenteric arterioles, which range in diameter from 60 to 80 μ m. Fluorescence microscopy was used to continuously monitor the thrombosis of arterioles.

Identification of the expression profiles of MALAT1 KD cells and control

To determine the expression profiles of mRNAs in MEG-01 cells, RNAs from MALAT1 KD cells and control were used. After DNase I treatment and ribosomal RNA (rRNA) depletion, 3 μ g of total RNA was employed as the starting material for each RNA sample preparation.

Deep sequencing using Illumina platforms was used to assess mRNA expression profiles. To create the 90 bp paired-end reads, Anoroad Genome (Beijing, China) used an Illumina Hiseq 2000 platform to sequence the libraries.

Significantly differentially expressed mRNAs were determined to have a log₂Ratio ≥ 1 , a $p < 0.05$, and a false discovery rate (FDR) < 0.1 . The R software's clusterprofiler package was used to conduct GO and KEGG pathway studies for functional enrichment.

Statistical analysis

Mean \pm SD (for deep sequencing data) or an SE was used to represent all data (for qRT-PCR data). The mean value of the vehicle control group is set as 1, or 100% when referring to relative gene expression. The study of the data consisted of employing student t-tests and analysis of variance on the unpaired, two-tailed data. The analysis of the data was performed by using SPSS V.17.0. The value of $p < 0.05$ was chosen as the threshold for a significant difference.

RESULTS

MALAT1 is abundant in the cytoplasm of MEG01 and downregulated in activated MEG-01 cells

MEG-01 cells were used as a source of human platelet precursors. In a process that consumed nearly the entire

cytoplasmic complement of membranes, including mRNAs and non-coding RNAs, we hypothesised that platelet MALAT1 was from the cytoplasm of megakaryocyte because platelets form by the fragmentation of megakaryocyte membrane extensions. In contrast to previous reports on MALAT1 being located exclusively in the nucleus, the amount of MALAT1 transcripts in MEG-01 cells resides in the cytoplasm (figure 1A). We confirmed the cytoplasm localisation using quantitative PCR with RT-qPCR (figure 1B). The expression levels of platelet MALAT1 were also investigated using FISH and RT-qPCR (online supplemental figure S1). In both human and mouse platelets, MALAT1 expression was shown to be comparable to that of the housekeeping gene GAPDH.

The transcription of platelets in individuals who have a hyperreactive platelet phenotype was simulated using activated MEG-01 cells as a model.⁴ We observed an over 50% decrease in MALAT1 transcript levels at 12 and 24 hours after MEG-01 cell stimulation with thrombin on collagen-coated coverslips (figure 1C). This finding suggests that MALAT1 is of great importance in platelet activity.

MALAT1 represses CD34⁺ megakaryocytes adhesion, spreading and PLP activity

MALAT1 KD, control cells and their corresponding PLPs were generated by transducing CD34⁺ megakaryocytes with a lentivirus expressing MALAT1-specific or scrambled shRNA (online supplemental figure S2). We examined the adherence of resting and activated megakaryocytes to collagen-coated coverslips to ascertain whether or not MALAT1 downregulation impacts megakaryocyte function. The results showed that there was an increase in the adhesion of MALAT1 KD megakaryocytes from an average of 3.9% to 11.5% in the resting state up to 22.5% following thrombin activation compared with the control CD34⁺ megakaryocytes (figure 2A,B). Furthermore, MALAT1 KD cells were more spread and extended than the control cells in the presence or absence of thrombin (figure 2C,D). MEG-01 cells could generate PLPs, which had similar characteristics to human platelets. To evaluate the effect of MALAT1 on PLP activity, we compared the adhesion and spreading of PLPs between those produced by MALAT1 KD cells and the control cells. The adherence of PLPs produced by MALAT1 KD CD34⁺ megakaryocytes was significantly higher than that of control cells (1.45% \pm 0.07% vs 1.111% \pm 0.06%, $p < 0.05$) (figure 3A,B). In addition, there was an increase in the spreading of PLPs that were formed by the KD cells in a manner analogous to that of MALAT1 KD cells. Flow cytometry was used to compare the levels of PLPs expressed by MALAT1 KD cells to those expressed by control cells to determine whether or not MALAT1 has a definitive impact on PLP activity. MALAT1 KD CD34⁺ megakaryocyte cells expressed greater levels of CD62P/CD61 in PLPs after 7 days in culture than control cells (figure 3E,F). These findings show that MALAT1 KD cells have improved megakaryocyte adhesion, spreading and PLP activities.

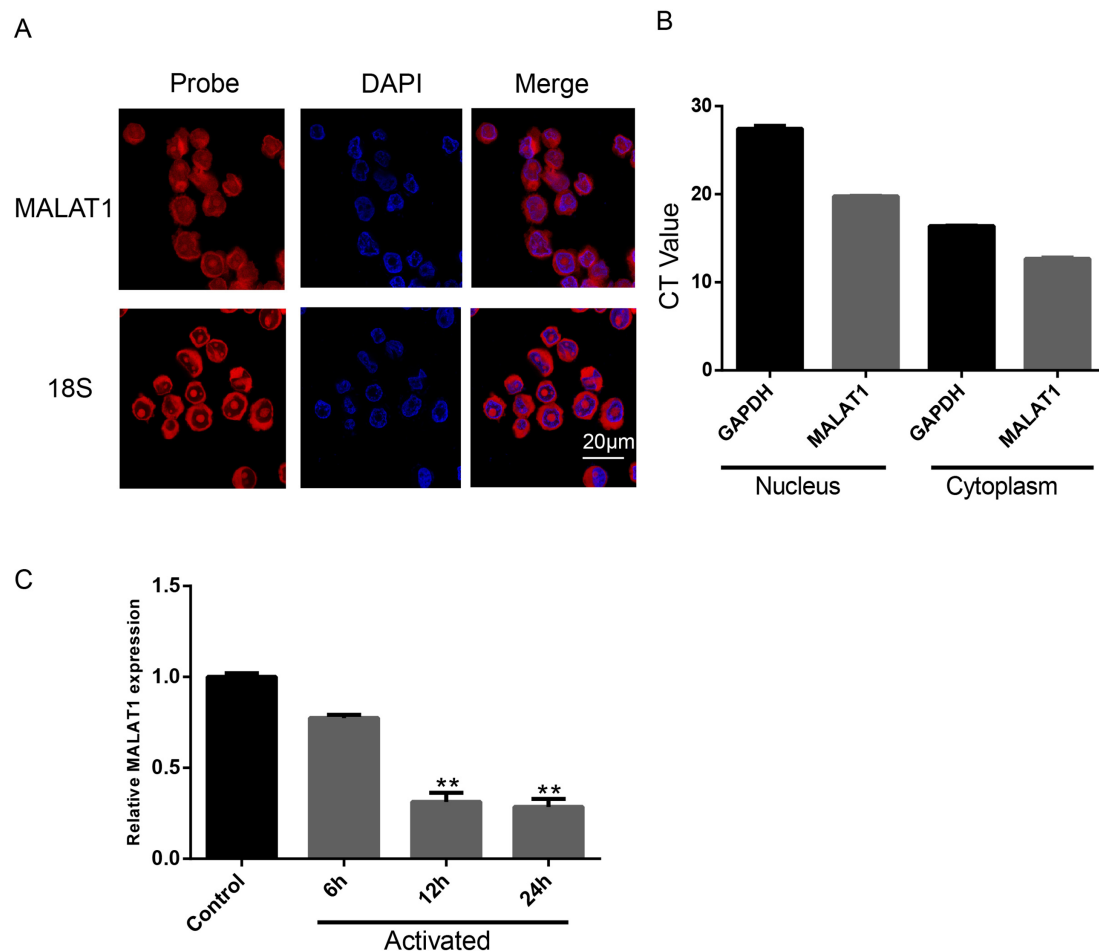


Figure 1 MALAT1 is present in the cytoplasm of MEG01 and downregulated in activated MEG01 cells. (A) RNA fluorescence in situ hybridisation (FISH) of the MALAT1 and 18S in MEG01 cells. Scale bar, 20 μ m. There were three separate tests carried out, and each image represents a different one of those experiments. (B) MALAT1 distribution in the nucleus and cytoplasm of MEG01 cells by RT-PCR. Y-axis represents the mean of CT value. GAPDH is used as a cytoplasmic reference. (C) After treating MEG-01 cells with thrombin at a concentration of 1 U/mL for 6, 12, and 24 hours, relative MALAT1 expression was evaluated (** $p < 0.01$) in these cells on collagen-coated coverslips. RT-PCR, reverse transcription PCR.

MALAT1 regulates platelet spreading, aggregation and activity

Bone marrow stem cells transfected with either a scramble or MALAT1 KD gene were transplanted into irradiated mice to test for an effect on platelet activity. There was no difference between MALAT1 KD and WT mice in terms of WBCs count, RBCs count, platelet count, platelet, corpuscular volume or other parameters (online supplemental table S1). MALAT1 KD platelets adherent to fibrinogen were well spread, showing more pseudopod formation or a flattened morphology than platelets from the scramble groups in the presence or absence of thrombin (figure 4A). Spreading was quantified as a percentage of the area covered by the spreading platelets, with the area covered by the MALAT1 KD platelets being significantly greater than that of the control ($9.25\% \pm 0.41\%$ vs $13.73\% \pm 0.46$, $p < 0.01$, no agonist; $12.15\% \pm 0.56\%$ vs $21.81\% \pm 2.33\%$, $p < 0.01$, thrombin) (figure 4B). The high-affinity conformation of mouse integrin α IIb3 is targeted by the JON/A antibody.

In addition, the Wug.E9 antibody identifies mouse P-selectin, which is a single-chain polypeptide with a

molecular weight of 140 kDa and belongs to the selectin family of adherence molecules. We tested the functionality of MALAT1 KD platelets using PE-labelled JON/A and FITC-labelled Wug.E9 antibodies. The MALAT1 KD platelets were more active than the scrambling groups whether exposed to thrombin or not (figure 4C,E). Furthermore, the maximum aggregation of MALAT1 KD platelets was much higher than that of WT platelets (figure 4D,F). These findings suggest that MALAT1 acts as a suppressor of platelet reactivity, hence boosting platelet spreading and aggregation via activation of integrin outside-in signalling.

MALAT1 is a critical regulator of thrombus formation in vitro and in vivo

To investigate whether the MALAT KD platelets accelerated thrombus formation, we analysed thrombus formation in vitro using a microfluidic whole-blood perfusion assay and in vivo FeCl_3 -induced damage of the endothelium in mesenteric arterioles. Thrombus formation by MALAT1 KD platelets was larger than that by WT platelets

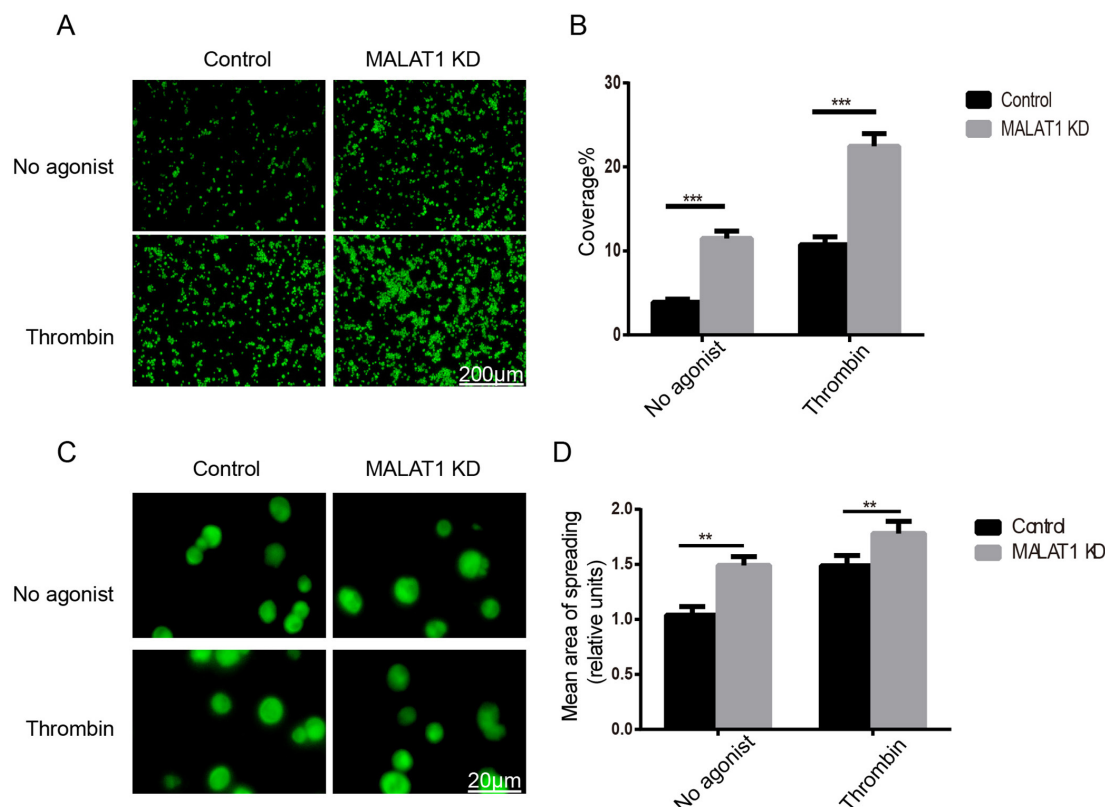


Figure 2 MALAT1 knockdown (KD) in CD34⁺ megakaryocytes represses adhesion and spreading. Each experiment was performed in triplicate. (A) Comparison of the capacity of MALAT1 KD and control CD34⁺ megakaryocytes (green) to adhere to collagen-coated coverslips following stimulation with or without thrombin 1 U/mL (scale bar, 200 μ m). (B) Statistical analysis of the adhesion area of CD34⁺ megakaryocytes in collagen-coated coverslips. Values are mean \pm SD (*** p <0.001). (C) Comparison of CD34⁺ megakaryocyte spreading potential on collagen-coated coverslips between MALAT1 KD and control groups without and with 1 U/mL thrombin (scale bar, 20 μ m). (D) Spreading area was compared with that of platelets of the wild type (** p <0.01), and the mean \pm SEM was plotted. Around 30 platelets from each genotype were counted on average.

at different perfusion times (figure 5A,B). In vivo, MALAT1 knockout mice (KD) had significantly shorter times to establish a stable occlusive thrombus in mesenteric arterioles compared with controls (figure 5C,D). These results indicated that reducing MALAT1 in platelets accelerated arterial thrombus formation. Similarly, the time is taken to form a haemostatic plug and stop bleeding ($216.0\text{S}\pm 8.040\text{S}$, $n=9$ in control vs $181.9\text{S}\pm 6.168\text{S}$, $n=9$ in MALAT1 KD mice; ** p <0.01) was shorter in MALAT1 KD mice in a tail bleeding time model (figure 5E). Based on these findings, MALAT1 seems to be a key player in thrombus generation.

MALAT1 regulates platelet activity by activating PI3k/Akt/GSK-3 β signalling pathway

To identify the potential links between the function of platelets and the expression of MALAT1, first, the expression profiles of MALAT1 KD MEG-01 cells and controls were compared. Interestingly, differential transcriptional expression was shown to be a striking feature of MALAT1 KD MEG-01 cells when compared with controls by deep sequencing analysis (online supplemental figure S3A). KEGG analysis was carried out to determine the biological processes in which the differentially expressed mRNAs were significantly enriched. Platelet activation,

the extracellular matrix receptor interaction, and the PI3K-Akt signalling pathway were all high-scoring pathways related to platelet activities and platelet-mediated biological effects (online supplemental figure S3B), as shown by the KEGG enrichment score graph.^{27–29} We analysed the signalling pathway in platelets mediated by MALAT1 and found that MALAT1 regulates platelet activity through the PI3k/Akt/GSK-3 β signalling pathway. When it comes to platelet activation, stable adhesion, and integrin outside-in signalling, the PI3k/Akt/GSK-3 β signalling pathway is crucial.²⁸ Our data revealed that MALAT1-deficient platelets had significantly elevated phosphorylation of Akt(Thr³⁰⁸), Akt(Ser⁴⁷³) and GSK-3 β (figure 6A,B). However, the expression of these phosphorylated proteins was inhibited by the PI3k inhibitor, LY294002 (figure 6A,B). PDK1 and PTEN are important upstream regulators of the PI3k/Akt signalling pathway.^{30,31} We investigated the expression of PDK1 and PTEN in MALAT1-deficient platelets and controls to see if these proteins play a role in MALAT1-mediated platelet regulation. Figure 6C,D show that PDK1 phosphorylation was highly elevated, but this elevation was suppressed by LY294002 treatment. However, phosphorylation of PDK1 was notably increased in MALAT1-deficient platelets,

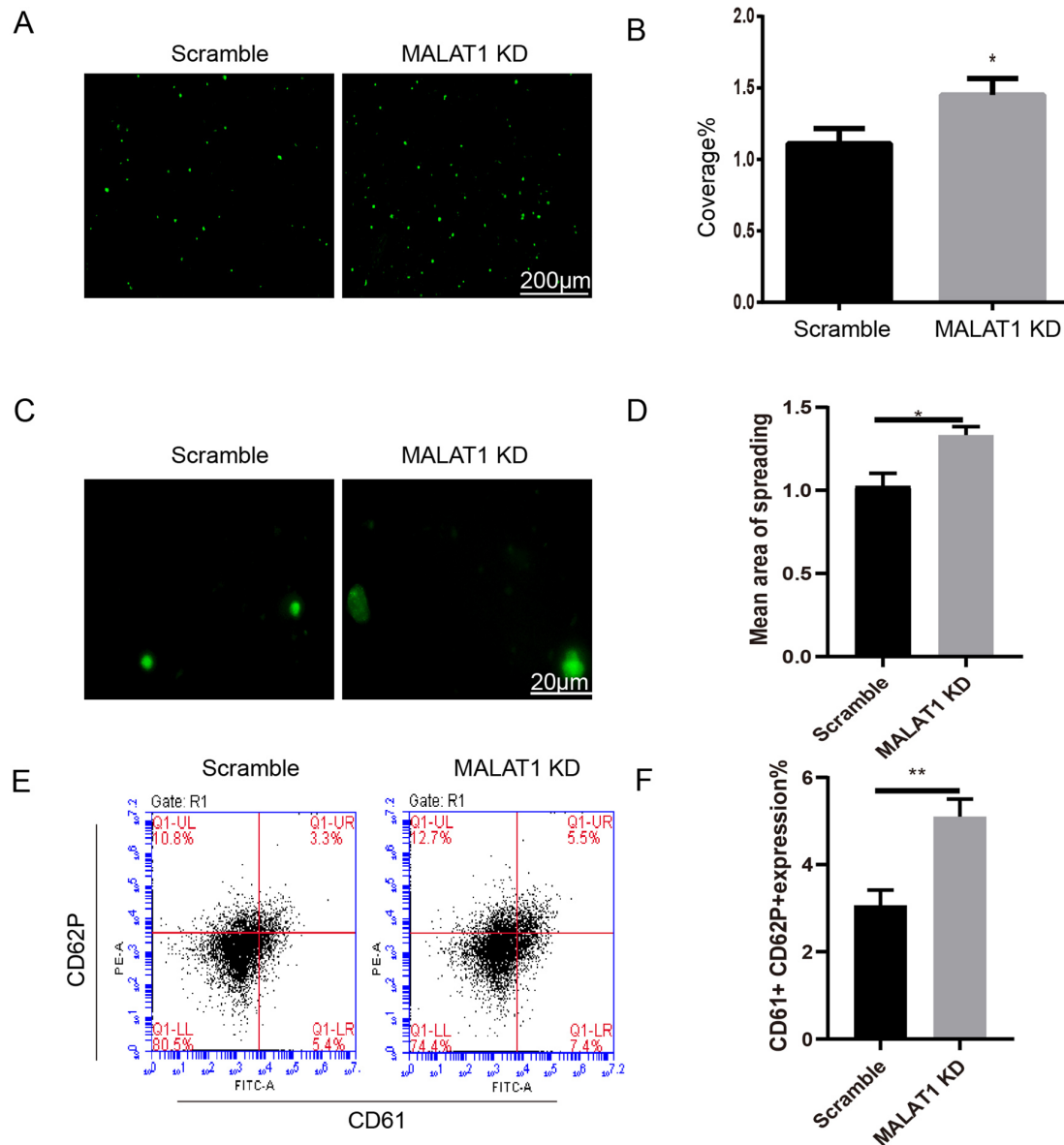


Figure 3 MALAT1 represses PLP adhesion, spreading and activity. (A) Adhesion of PLPs (green) from MALAT1 KD and control CD34⁺ megakaryocytes after treatment by thrombin 1 U/mL (scale bar, 200 μ m). Three separate sets of data were collected for each experiment. (B) A statistical examination of the adhesion area of PLPs in coverslips that were coated with collagen. The values are shown as the mean \pm SD (* p < 0.05). (C) A comparison of the capacity of PLPs to spread derived from MALAT1 kD CD34⁺ megakaryocytes and the scramble in the presence of thrombin at a concentration of 1 U/mL on coverslips coated with collagen (scale bar, 20 μ m). (D) Histogram showing the relative area of PLPs for MALAT1 KD and scramble. Data are from three independent experiments. Error bars denote SD * p < 0.05. (E) Flow cytometric analysis of CD61 and CD62P expressions on the PLPs isolated from MALAT1 kD megakaryocytes and scramble. (F) Histogram showing the proportions of CD61⁺CD62P⁺ cells for MALAT1 kD megakaryocytes and scramble group. Data are from three independent experiments. Error bars denote SD ** p < 0.01. KD, knockdown; PLPs, platelet-like particles.

and its expression was partly recovered by LY294002 treatment. The effect of the PI3K inhibitor LY294002 on MALAT1-regulated platelet response to thrombin was also examined using flow cytometry. LY294002 inhibited thrombin-stimulated platelet activity in both wild-type and MALAT1-deficient platelets (figure 6E,F). These data show that MALAT1 acts upstream of PDK1 and PTEN to regulate integrin α IIb β 3-mediated outside-in signalling via the PI3k/Akt/GSK-3 β signalling pathway.

DISCUSSION

Hyperreactivity of platelets is a known risk factor for a wide variety of human disorders.^{5 32} An atherosclerotic lesion increase was observed in mice with a haematopoietic MALAT1 deletion, according to a recent study. In addition, symptomatic individuals' atherosclerotic lesions showed decreased MALAT1 levels.²¹ Our results showed that haematopoietic deficiency of MALAT1 could increase platelet activity and thrombus formation.

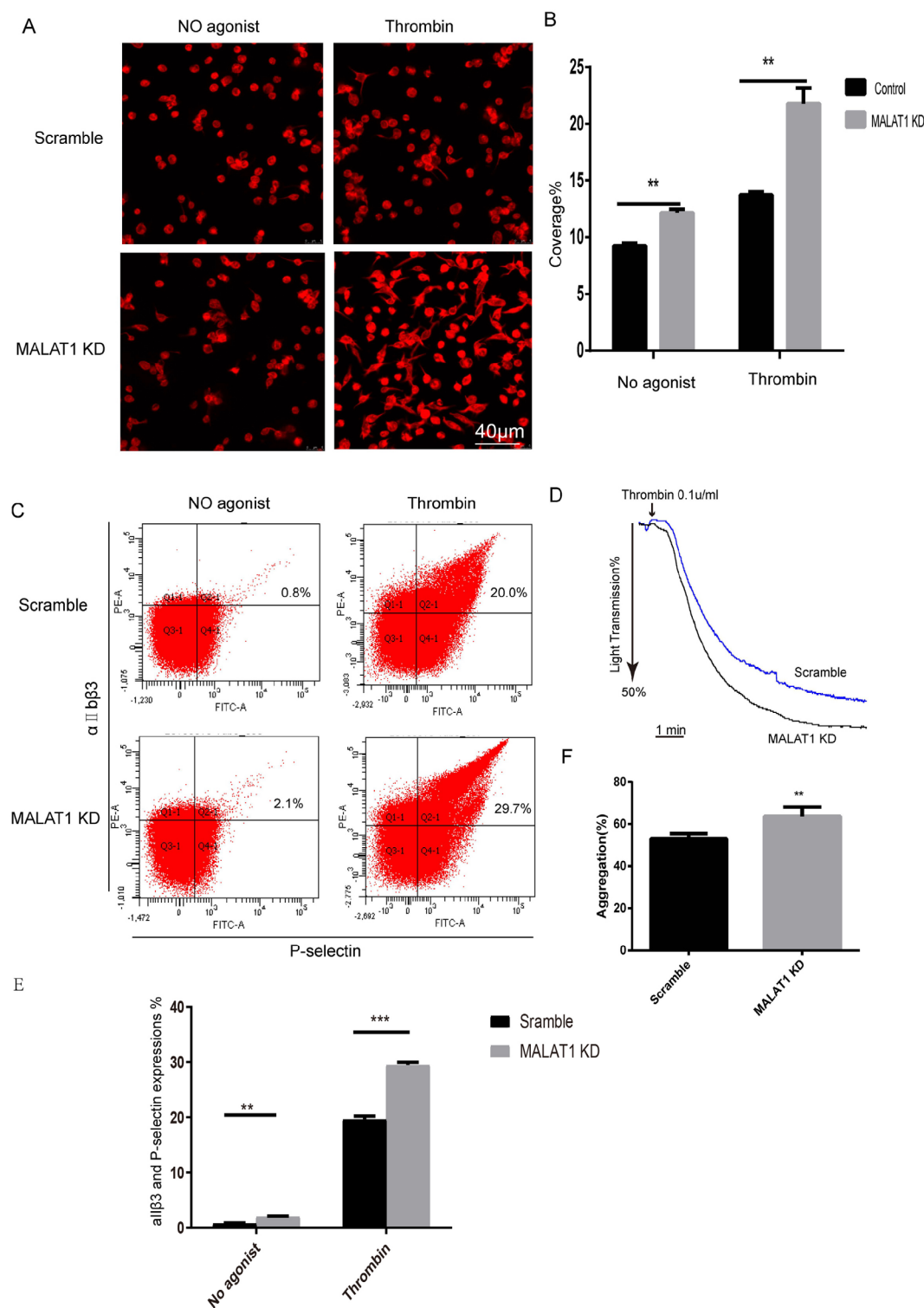


Figure 4 MALAT1 regulates platelet spreading, aggregation, and activity. (A) Platelets with a deficiency in MALAT1 and platelets with a wild-type genotype (WT) were spread out over fibrinogen-coated coverslips with or without thrombin (0.1 U/mL). (B) Quantification analysis of the spreading area of MALAT1-deficient and WT platelets. Values are mean±SD (**p<0.001). (C) Flow cytometric analysis of integrin αIIbβ3 and P-selectin expressions of MALAT1-deficient and WT platelets in the absence or presence of thrombin (0.1 U/mL), using JON/A PE-labelled JON/A antibody and FITC-labelled Wug.E9 antibody (n=6). (D) Aggregation of washed MALAT1-deficient and WT platelets using thrombin (0.1 U/mL). (E) Histogram showing the proportions of αIIbβ3 and P-selectin cells for MALAT1 KD megakaryocytes and scramble group. Data are from three independent experiments. Error bars denote SD **p<0.01, ***p<0.001. (F) Panel D depicts the percentage of platelet maximum aggregation based on the findings of at least five separate studies, and the data are presented as the mean±SD (**p<0.01). FITC, fluorescein isothiocyanate; KD, knockdown.

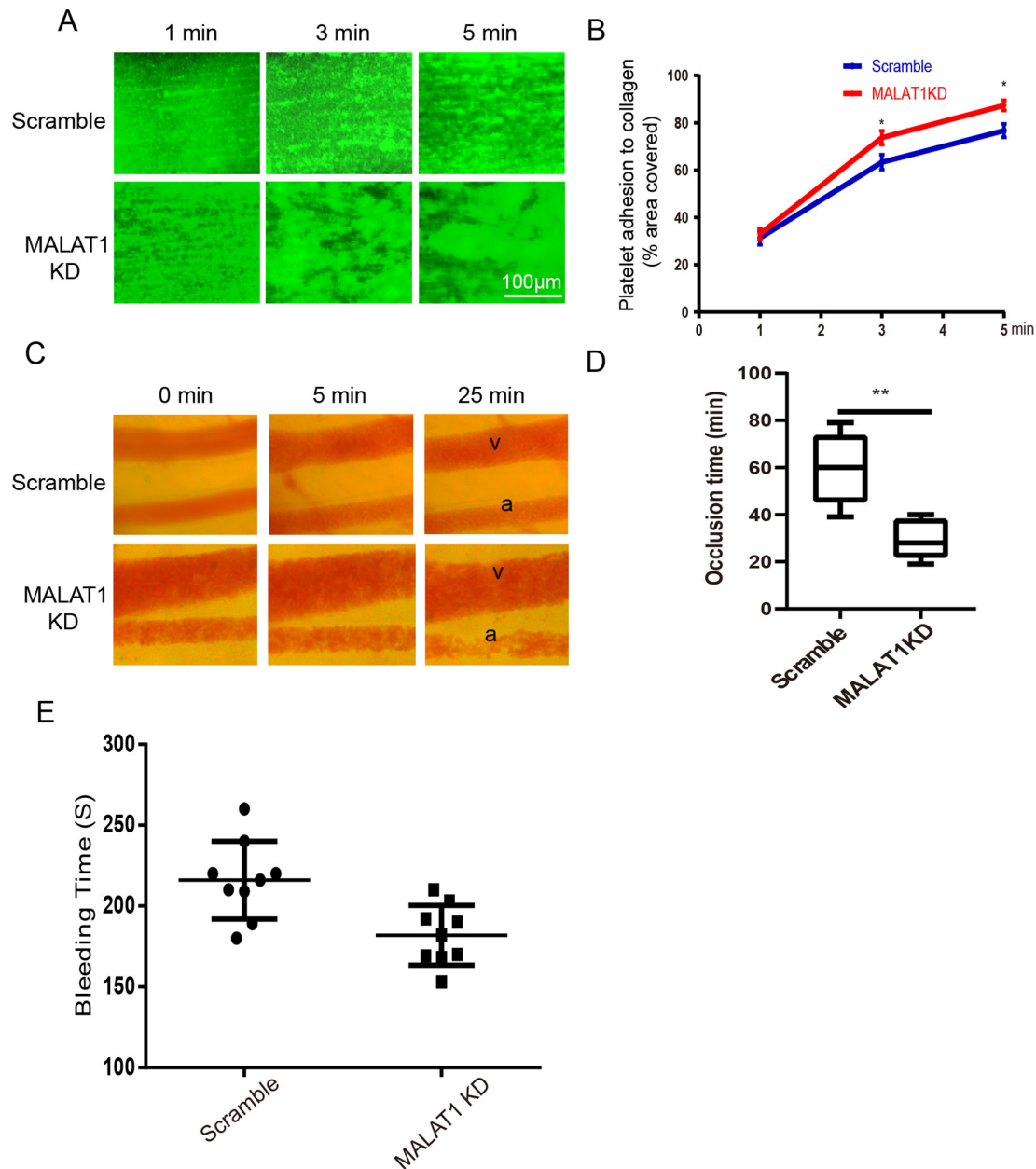


Figure 5 Downregulated MALAT1 in platelets increases thrombus formation in vitro and in vivo. (A) Photomicrographs that show the progression of platelet adhesion from MALAT1 KD and scramble mice on collagen. Blood from MALAT1 KD and scrambling animals was collected in sodium citrate (3.8% w/v) and fluorescently tagged with calcein (1 μ M) for 1 hour before being perfused through fibrillar collagen-coated bioflux plates at a shear rate of 40 dynes/cm² for 5 min. (B) Dot plot comparing the area coverage of platelets derived from scramble mice and MALAT1 knockout mice. There were a total of three separate trials carried out. * p <0.05 (C) Images showing representative stages of thrombus formation at 0 min, 5 min, and 25 min after an injury caused by FeCl₃ to mesenteric arterioles in MALAT1 KD mice and scrambling mice. A, arteriole; V, venule. (D) The occlusion periods for arteriole thrombosis produced by FeCl₃ in scramble and MALAT1 KD mice are displayed as box plots (n =5). ** p <0.01. (E) MALAT1 KD accelerates haemostatic plug formation in mice. horizontal lines represent the mean (eight individuals in each group). KD, knockdown.

Platelets have no nuclear DNA and can maintain their proteome, which response very well to many environmental factors over their lifespan. The molecular mechanisms of the important feature of the anucleated blood cells have not been uncovered until recently. Platelets, which lack nuclei, are still able to regulate RNA levels through post-transcriptional mechanisms such as miRNAs. Through this investigation, we identify lncRNAs

as a newly unrecognised group of gene regulators in platelets. Since there are no nuclear DNAs in the non-nucleated platelets, the significance of these lncRNAs in platelet cellular processes may be much more significant than in nucleated cells. Some of the platelets may depend on certain lncRNAs, and it is important to learn more about biological processes and their functions in the future. Nevertheless, platelet lncRNAs may represent

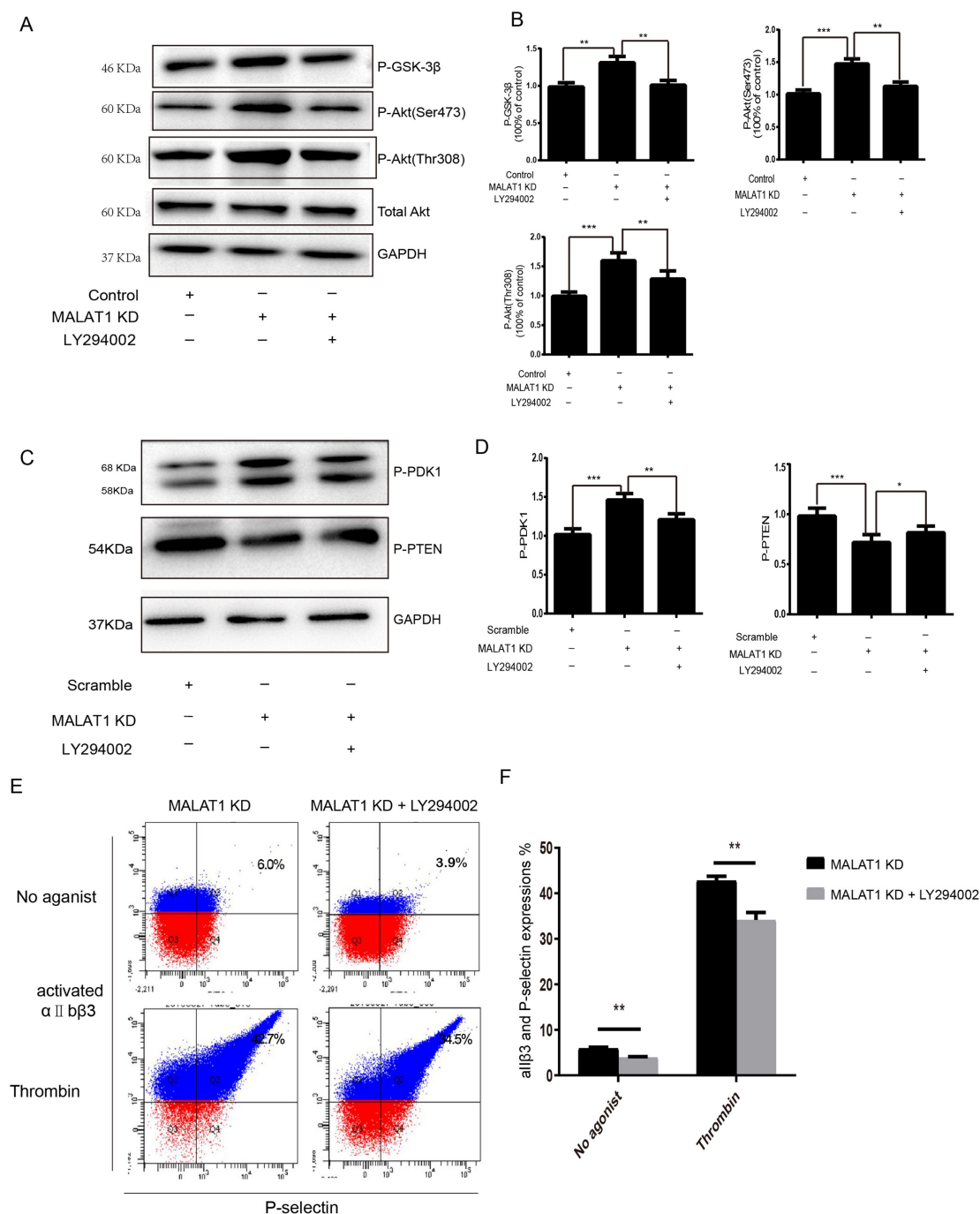


Figure 6 MALAT1 deficiency in platelets activates PI3k/Akt signal pathway. (A) Western blot analysis of WT and MALAT1-deficient platelets in the absence or presence of LY294002 (50 nmol/kg) with antibodies that recognise phosphorylated GSK-3 β , phosphorylated Akt Thr308, phosphorylated Akt Ser473, and total Akt. (B) Western blot results were quantified and presented as mean \pm SD (** p <0.01, *** p <0.001). (C) Analysis of phosphorylated PDK1 and PTEN by Western blotting in MALAT1-deficient and WT platelets in the absence or presence of LY294002 (50 nmol/kg), respectively. (D) The findings of the Western blot were analysed quantitatively and displayed as the mean \pm SD (* p <0.05, ** p <0.01, *** p <0.001). (E) Flow cytometric examination of integrin α IIb β 3 and P-selectin expressions with JON/A PE-labelled JON/A antibody and FITC-labelled Wug.E9 antibody (n=6). This was done in the absence or presence of the PI3K inhibitor LY294002. (F) A histogram depicting the percentages of cells expressing α IIb β 3 and P-selectin with or without the PI3K inhibitor LY294002. Data are from three independent experiments. Error bars denote SD ** p <0.01. WT, wild-type.

a novel research field. Interestingly, our study showed that MALAT1, which is normally found in the nucleus, is expressed in anucleated platelets and serves as a crucial regulator of platelet activity.

Based on our findings, it is now abundantly obvious that MALAT1 controls megakaryocytes' cellular activity, PLPs and platelets, thereby affecting their functions. Thus, reduced MALAT1 in CD34⁺ megakaryocytes cells

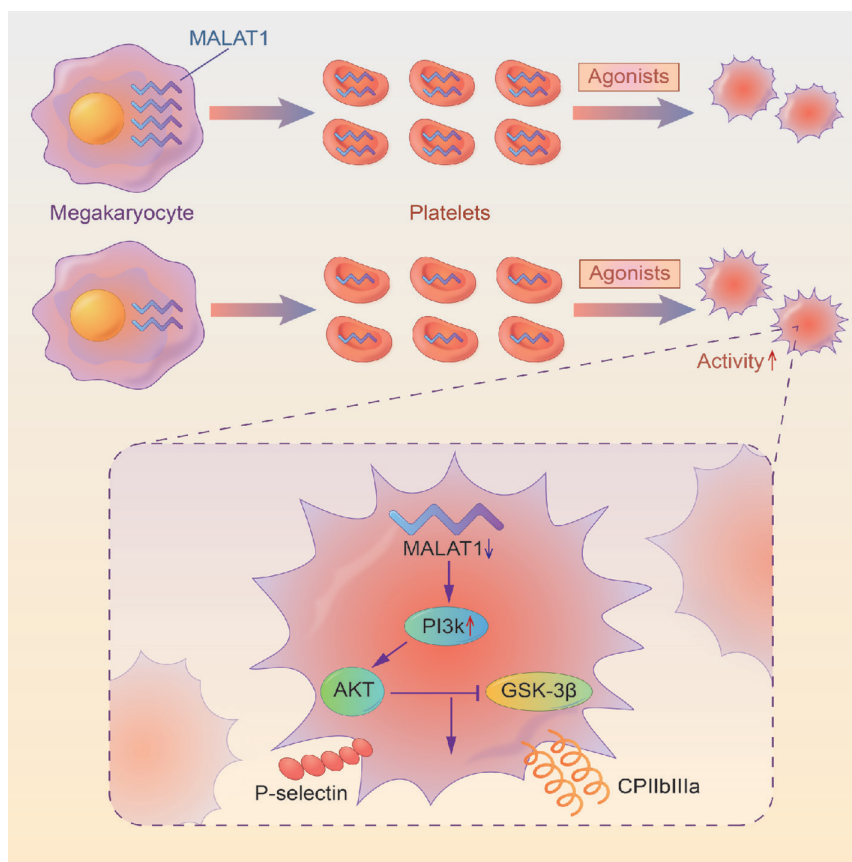


Figure 7 MALAT1 regulates platelet activity through PI3k/Akt/GSK-3 β pathway. MALAT1 is a potent negative regulator of platelet activity. In the absence of this lncRNA, integrin outside-in signalling can be activated by PI3k/Akt/GSK-3 β pathway.

increases adhesion, spreading, and PLPs activity. After bone marrow transplantation, haematopoietic MALAT1 KD mice exhibited enhanced platelet adhesion, aggregation and activity compared with WT mice. We concluded that a lack of MALAT1 in mouse platelets makes the production of thrombi more severe both in vitro and in vivo. MALAT1 may play a significant role in platelet function and thrombus development.

Our findings show that MALAT1 influences platelet activity by influencing the PI3k/Akt/GSK-3 β signalling pathway (figure 7), with regard to the mechanism. Important steps in platelet granule release, activation, adhesion and integrin outside-in signalling all occur via the PI3K-Akt signalling pathway.^{33 34} Tumour cells and other cell lines have been studied extensively to explain MALAT1's modes of action, and these studies have shown that MALAT1 can exert its effects via the PI3K/Akt pathway. Knocking down MALAT1 in breast cancer cell lines activates the PI3K/Akt pathway and causes the cells to undergo an epithelial-mesenchymal transition.³⁵ It was shown in another study that MALAT1 increases ox-LDL-induced autophagy in HUVECs via the PI3K/AKT signalling pathway.³⁶ As MALAT1 was found to be significantly expressed in the cytoplasm of both MEG-01 cells and platelets, we set out to determine lncRNA functions in platelets. Our data showed that the downregulation of MALAT1 in platelets can activate the PI3K/AKT/

GSK-3 β pathway. Our findings found a new way to regulate the critical PI3K/AKT/GSK-3 β signalling pathway of platelet activation. The PDK1 and PTEN are critical regulators of the PI3K/Akt pathway. Our results demonstrated that MALAT1 controls the expression of PDK1 and PTEN. However, until today, it was unclear exactly how MALAT1 controls the expression of PDK1 and PTEN. By capturing target miRNAs and preventing them from influencing downstream gene expression, MALAT1 can serve as a miRNA sponge. It was shown that microRNA-503 blocks the PDK1/PI3K/AKT pathway, which is responsible for the advancement of non-small cell lung cancer (NSCLC).³⁷ By binding to miR-503, MALAT1 controls cell growth and death in ovarian cancer.^{38 39} According to another study's findings, MALAT1 may contribute as a ceRNA for miR-205-3 p in order to regulate PTEN expression and, as a result, may inhibit apoptosis in ischaemic stroke.⁴⁰ Based on these findings, it seems likely that MALAT1 targets miRNAs to trigger the PI3k/Akt/GSK-3 β signalling pathway.

In summary, we identify that MALAT1 is an essential factor in platelet activity and thrombosis based on the results of our investigation. Through the PI3K/AKT/GSK-3 β signalling pathway, MALAT1 stimulates platelet spreading and aggregation by activating integrin outside-in signalling. Perhaps lncRNAs could be

used as a novel therapeutic target for treating thrombus generation.

Contributors YS and JL designed, performed the research and analysed the results; YS and TW wrote the manuscript; YL cultured the MEG-01 cells; JJ and DZ helped to generate the MALAT1 deficient mice; and CZ and WB designed the research, revised the manuscript and acted as guarantors.

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Competing interests None declared.

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ORCID iDs

Yeying Sun <http://orcid.org/0000-0003-0397-1083>

Weihua Bian <http://orcid.org/0000-0003-2369-2073>

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