

LRRFIP1 expression triggers platelet agglutination by enhancing α IIB β 3 expression

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Abstract. Platelets primarily participate in hemostasis and antimicrobial host defense. The present study aimed to investigate the effects of leucine-rich repeat flightless-interacting protein-1 (LRRFIP1) on platelet agglutination. The bacterial strain of LRRFIP1 was used to synthesize the recombinant protein and a mouse model of LRRFIP1 gene knockout was established. Platelets were isolated from the mice and divided into the different trial groups according to their treatment with collagen, thrombin receptor SFLLRN, anti-wild-type (w) LRRFIP1 monoclonal antibodies and the model of LRRFIP1 gene knockout. The platelets were prepared and platelet agglutination was examined using platelet aggregation apparatus. The active α IIB β 3 integrin was examined by flow cytometry. The results revealed that the combined wLRRFIP1 protein was successfully expressed. wLRRFIP1 treatment significantly triggered platelet agglutination of collagen, thrombin and monoclonal antibody treated platelets. wLRRFIP1 knockout significantly decreased α IIB β 3 levels compared with the wild-type. Platelet agglutination was also significantly inhibited in the LRRFIP1^{-/-} mouse model compared with the wild-type. LRRFIP1 knockout significantly decreased the α IIB β 3 levels in platelets undergoing convulxin treatment. In conclusion, LRRFIP1 treatment triggered platelet agglutination and LRRFIP1 gene knockout inhibited platelet agglutination. In addition, LRRFIP1 gene knockout significantly decreased the levels of α IIB β 3. This suggests that LRRFIP1 may be applied to patients in a clinical setting to trigger platelet agglutination in inflammatory diseases and atherothrombotic diseases.

Introduction

Platelets primarily participate in hemostasis and antimicrobial host defense, however they also secrete cytokines, which induce inflammation and trigger tissue repair (1,2). It is common knowledge that platelets serve critical roles in the pathology of cardiovascular disorders, chronic inflammatory and atherothrombotic diseases (3). At injury sites platelets typically aggregate on vessel walls and prevent bleeding (4). Infections are associated with either the sudden increase or decrease in platelets (known as thrombocytosis and thrombocytopenia, respectively), which are also the biomarkers for disease progression or tissue healing (1). In a healthy individual platelets range between 150,000 and 400,000/ μ l of blood and there is a continual balance between platelet clearance and platelet production (1,5). This balance must be carefully maintained to avoid arterial occlusion, spontaneous bleeding and organ damage. However, understanding of this process is limited as has been poorly investigated.

Previous studies have reported a number of anti-coagulation drugs, including sodium citrate, heparin, vitamin K antagonist and nattokinase, all of which have been extensively utilized within a clinical setting (6-8). Physiological hemostasis has not been widely investigated in the past, however, hemostasis is important for the clearance of thrombus (9,10). Platelets serve a critical role in the process of physiological hemostasis, however their specific mechanisms and targets have not been fully clarified (9-11). The authors of the present study speculated that the identification of physiological hemostasis targets may be important for the prevention of thrombogenesis. Human platelets express a series of molecules, including immunoglobulin G immune complex receptor, glycoprotein VI (GPVI), Fc- γ -RIIa and C-type lectin-like receptor (12-14). The activation of these molecules contributes to physiological hemostasis or thrombosis (15).

The leucine-rich repeat flightless-interacting protein-1 (LRRFIP1) gene has previously been identified and revealed to serve a key role in the regulation of gene transcription (16). LRRFIP1 was originally derived as a GC-rich binding-protein that repressed the expression of platelet-derived growth factors (17). Silencing of the LRRFIP1 gene caused a notable decrease in thrombus formation and was positively correlated with levels of α IIB β 3 (18,19). Therefore, it was hypothesized that the LRRFIP1 gene may serve an important role in

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thrombus formation. The present study aimed to investigate the effects of LRRFIP1 on platelet agglutination.

Materials and methods

Bacterial strain and preparation of LRRFIP1 recombinant protein. The BL21 (DE3) *Escherichia coli* strain (cat. no. GSB013; ZonHon Biopharma Institute Inc., Changzhou, China) was cultured in lysogeny broth (LB) liquid medium (cat. no. L3152; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Full-length recombinant LRRFIP1 protein was purified from the total crude extract of BL21 as previously described (20). Briefly, the BL21 strain was cultured in the LB culture medium and then re-suspended in lysis liquid (cat. no. T9424; Sigma-Aldrich; Merck KGaA) to obtain total protein, which was purified using glutathione-S-transferase soluble protein (Sangon Biotech Co., Ltd., Shanghai, China). The prokaryotic-expressed proteins were extracted using glutathione sepharose 4B beads (GE Healthcare, Chicago, IL, USA) according to the manufacturer's protocol. The highly purified LRRFIP1 was identified as high-density bands on the SDS-PAGE images. Briefly, the concentration of the obtained recombinant LRRFIP1 was determined using a bicinchoninic acid protein assay kit according to the manufacturer's protocol. A total of 0.2 μ g protein lysates were separated with SDS-PAGE on a 15% gel.

Animals. A total of 20 BALB/C mice with 6-8 week-old (10 male and 10 female), weighting from 25 to 35 g were purchased from Beijing HFK Bioscience Co., Ltd. (Beijing, China). All mice used in the present study were housed in cages (5 mice/cage) under the same conditions, including a controlled environment at 22°C with 50% humidity and a 12 h light/dark cycle. The food, water and bedding were sterilized, and the mice had free access to food and water. All animal experiments were approved by the Ethics Committee of Daping Hospital (Chongqing, China) and all mice were handled in accordance with the Guidelines for Care and Use of Laboratory Animals by the National Institute of Health (21).

Establishment of plasmids and a mouse model of LRRFIP1 gene knockout. To create LRRFIP1 expression plasmids the targets of the LRRFIP1 gene were designed as detailed in Fig. 1A. Embryonic stem cells (cat. no. CRL-11379; American Type Culture Collection, Manassas, VA, USA) were used to clone the LRRFIP1 gene according to the previously published studies (16,17). Several strains of the LRRFIP1 gene are listed in Fig. 1B, and exon 2 was the most conservative. Therefore, the LRRFIP1 gene knockout mice were established by synthesizing the mutated exon 2 gene. Among the listed LRRFIP1 genes, three target gene sequences were selected and cloned into the px458 plasmid (cat. no. 3683466; BioVector NTCC Inc., Beijing, China) to construct LRRFIP1^{-/-} expression plasmids by employing the BamH I and EcoR I restriction enzymes (Fig. 1C); these plasmids were used to develop the LRRFIP1 knockout mouse model. Target gene 3 illustrated the highest-density band (Fig. 1D), which was selected to establish the LRRFIP1^{-/-} expression plasmid according to previous studies (16,17).

To establish a mouse model of LRRFIP1 gene knockout, LRRFIP1 genes were synthesized by Western Biotech, Inc.

(Chongqing, China). The metaphase II oocytes of fertilized embryos were cultured in M2 medium (cat. no. M8410) for 24 h at 37°C and treated with acidic tyrode's solution (cat. no. P4417; both Sigma-Aldrich; Merck KGaA) for 60 sec at 37°C. The cells were subsequently cultured with Opti-minimal essential medium (MEM; cat. no. 31985-070; Life Technologies; Thermo Fisher Scientific, Inc.) for 24 h at 37°C. For Cas9 plasmid (cat. no. 44758; Addgene, Inc., Cambridge, MA, USA) transfection, 500 ng purified Cas9 plasmid was added to 25 μ l Opti-MEM, followed by the addition of 100 ng guide RNA (gRNA; GenScript Corporation, Piscataway, NJ, USA). A total of 2 μ l Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) was diluted into 25 μ l opti-MEM and mixed with the Cas9 plasmid/gRNA sample. The mixture was incubated at 37°C for 10 min prior to the addition of the fertilized embryos. The oviger stage fertilized embryos were subsequently transplanted into the mice by injecting the embryos into the uterus in a specific pathogenic and virus antigen free environment. The mice were monitored for breathing problems, marasmus, roughened hair and fever to ensure that the specific pathogenic and virus antigen free environment was maintained. DNA was extracted from the blood of the BALB/C mice using the commercial RNA extraction kit (cat. no. DP432; Tiangen Biotech Co., Ltd., Beijing, China) and the first strand cDNA synthesis kit (cat. no. K1612; Thermo Fisher Scientific, Inc.). The PCR assay was conducted using the SYBR PCR system (cat. no. DRR820A; Takara Biotechnology Co., Ltd., Dalian, China) to confirm that the mice harbored the desired LRRFIP1 mutation. The primers were as follows: forward, 5'-CTAAGC CGGGCACAGTAACA-3' and reverse, 5'-TAAAGGGCA AGCTCTCAGGC-3'. Amplification conditions for PCR were as follows: 94°C for 4 min, followed by 35 cycles of 95°C for 20 sec, 60°C for 30 sec and 72°C for 30 sec, and terminated at 72°C for 10 min.

The following humane endpoint was established according to a previous study to ensure that the welfare of the mice was protected (22): A body weight loss of >15% compared with the weight at the time of experiment onset, accompanied by hunched posture, lethargy and poor grooming. A total of 10% (2/20) of the mice were euthanized as a result of reaching the established humane endpoint. This percentage of mortality was expected in the present study based on prior-experiments by our group. At 1 day following the end of the study, all remaining mice were sacrificed via 150 mg/kg body weight intraperitoneal injection of thiopental (Altana AG, Wesel, Germany).

Platelet preparation and platelet agglutination assay. The platelet agglutination assay was performed and evaluated as previously described with several modifications (18). Briefly, 1 week after the establishment of the model, whole blood (0.1 ml) was collected from the caudal vein and added into glass evacuated tubes containing 1.5 ml 3.28% poncirin acid sodium salt (BD Biosciences, Franklin Lakes, NJ, USA). The platelet-rich plasma (PRP) was obtained from whole blood by centrifuging at 1,000 \times g for 10 min at room temperature. The PRP was used immediately or was stored without agitation at 4°C for 48 h. The PRP was centrifuged at 3,000 \times g for 10 min at room temperature to form a pellet of platelets. The platelet poor plasma was decanted and the platelets were suspended in

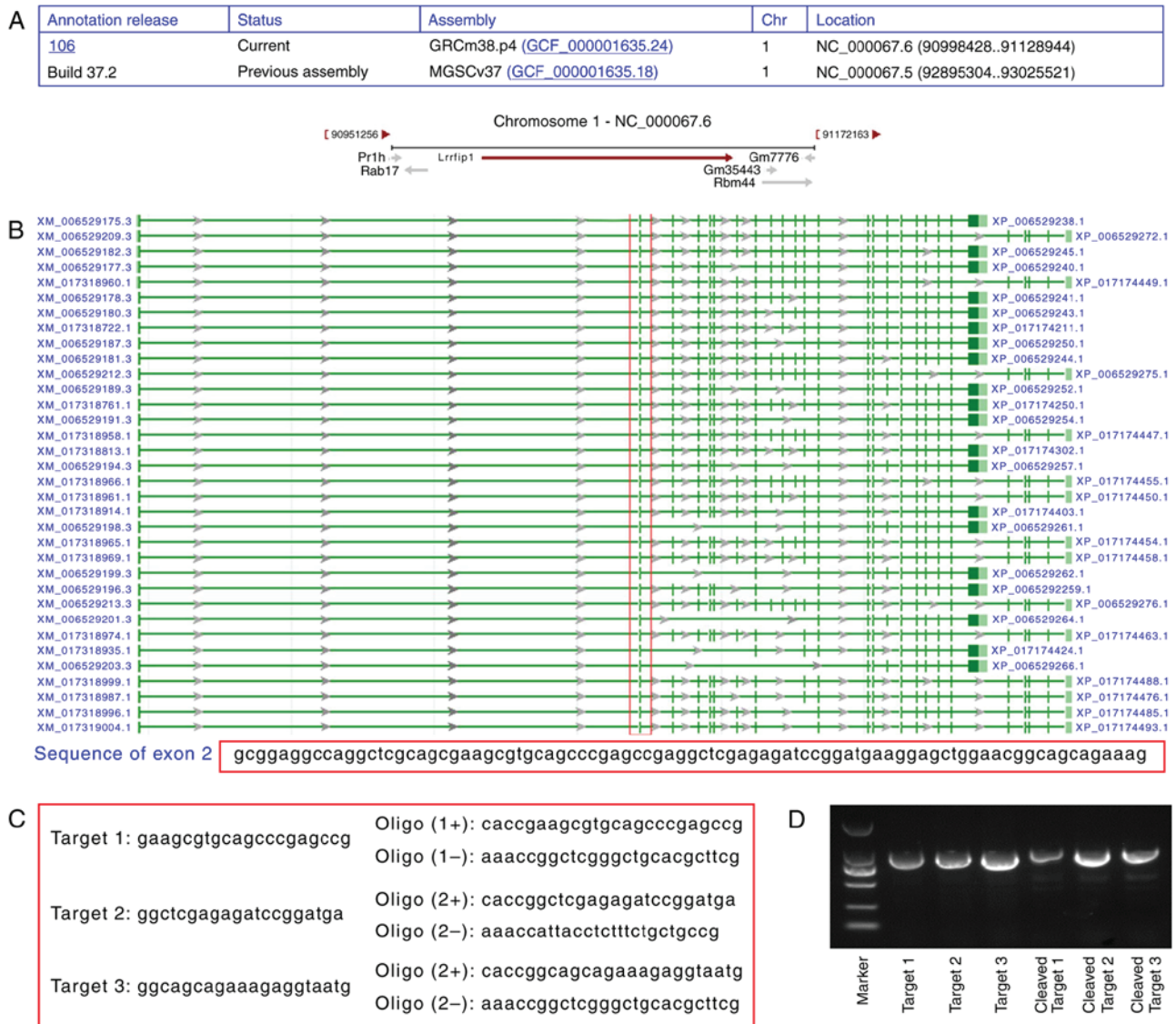


Figure 1. Processes of gene selection and plasmid construction and the establishment for a mouse model of LRRFIP1 gene knockout. (A) The targeting gene for LRRFIP1. (B) Several strains of the targeting LRRFIP1 genes. (C) Targeting gene sequences for LRRFIP1 amplification. (D) The confirmed 3 amplified targeting genes illustrating the LRRFIP1 bands. LRRFIP1, leucine-rich repeat flightless-interacting protein-1.

2 ml HEPES/Tyrod's buffer (Gibco; Thermo Fisher Scientific, Inc.) without Ca^{2+} or Mg^{2+} . Platelet agglutination rate maximum [PAG (M)] and PAG at 1 min [PAG (I)] were examined and an agglutination assay was conducted using a platelet aggregation apparatus (Order no. H79194; Shanghai Huanxi Medical Devices Co., Ltd., Shanghai, China) according to the manufacturer's protocol. Additionally, a type of agglutination index (%) was evaluated as shown in a previous study (5).

Trial groupings. All treatments were added directly to the platelets. For the collagen protein-induced platelet agglutination assay the following groups were used: i) Platelet control group, which did not receive treatment; ii) collagen (0.07 $\mu\text{g}/\text{ml}$) and low-molecular heparin (LMH; 1.2 IU/ml; both Sigma-Aldrich; Merck KGaA) treated platelet group (P+C+LMH); iii) collagen (0.07 $\mu\text{g}/\text{ml}$), LMH (1.2 IU/ml) and wild-type LRRFIP1 (wLRRFIP1; 50 nM) treated platelet group (P+C+LMH+wLRRFIP150); and iv) collagen (0.07 $\mu\text{g}/\text{ml}$), LMH (1.2 IU/ml) and wLRRFIP1 (100 nM) treated platelet

group (P+C+LMH+wLRRFIP1100). A total of 24 h after the platelet treatment, the agglutination assay was conducted.

For the thrombin receptor-mediated platelet agglutination assay the following groups were used: i) Thrombin receptor activator (SFLLRN; 2 μM ; cat. no. S1820; Sigma-Aldrich; Merck KGaA) treated platelet group (P+SFLLRN); ii) LMH (1.2 IU/ml) and SFLLRN (2 μM) treated platelet group (P+LMH+SFLLRN); iii) wLRRFIP1 (100 nM) and SFLLRN (2 μM) treated platelet group (P+wLRRFIP1100+SFLLRN); and iv) wLRRFIP1 (200 nM) and SFLLRN (2 μM) treated platelet group (P+wLRRFIP1200+SFLLRN).

For the monoclonal antibody regulated platelet agglutination assay, the following groups were used (n=4/group): i) Platelet control group; ii) collagen (0.07 $\mu\text{g}/\text{ml}$) treated platelet group (P+C); iii) collagen (0.07 $\mu\text{g}/\text{ml}$) and anti-wLRRFIP1 antibody (30 $\mu\text{g}/\text{ml}$; cat. no. sc-515571; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) treated platelet group (P+C+anti-wLRRFIP1); iv) SFLLRN (2 μM) treated platelet group (P+SFLLRN); and v) SFLLRN (2 μM) and

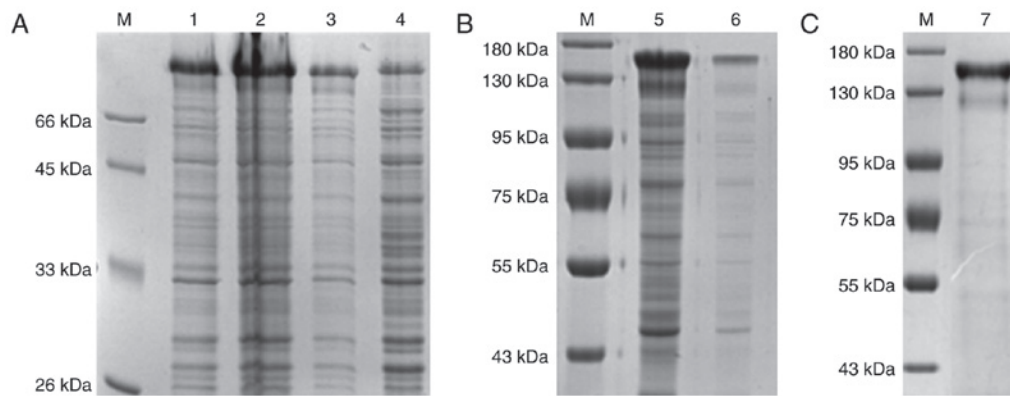


Figure 2. Observation of wild-type LRRFIP1 protein expression using SDS-PAGE. The gel bands of proteins expressed at (A) low levels, (B) high levels and (C) high levels following purification. The DNA markers are listed at the left of the images. The numbers 1 to 7 represent the expressed wild-type LRRFIP1 protein. M, DNA marker. LRRFIP1, leucine-rich repeat flightless-interacting protein-1.

anti-wLRRFIP1 antibody (30 μ g/ml) treated platelet group (P+SFLLRN+anti-wLRRFIP1).

For the LRRFIP1 gene knockout associated platelet agglutination assay the following groups were used: i) Platelets in wLRRFIP1^{+/+} mice group (P+LRRFIP1^{+/+}); ii) platelets in wLRRFIP1^{-/-} mice group (P+LRRFIP1^{-/-}); iii) collagen (0.07 μ g/ml) treated platelets in wLRRFIP1^{+/+} mice group (P+C+LRRFIP1^{+/+}); iv) collagen (0.07 μ g/ml) treated platelets in wLRRFIP1^{-/-} mice group (P+C+LRRFIP1^{-/-}); v) SFLLRN (2 μ M) treated platelets in wLRRFIP1^{+/+} mice group (P+SFLLRN+LRRFIP1^{+/+}); vi) SFLLRN (2 μ M) treated platelets in wLRRFIP1^{-/-} mice group (P+SFLLRN+LRRFIP1^{-/-}).

Flow cytometry for α IIB β 3 examination. α IIB β 3 was examined in the LRRFIP1^{+/+}, LRRFIP1^{-/-}, LMH, LRRFIP1^{+/+} + Convulxin and LRRFIP1^{-/-} + Convulxin groups. Convulxin (0.2 nmol/l; Sigma-Aldrich; Merck KGaA) was added to the platelets. Then active α IIB β 3 integrin was examined by flow cytometry as previously described (23). Briefly, the activated integrin α IIB β 3 was quantified by binding to the FITC-labeled rabbit anti-mouse α IIB β 3 monoclonal antibodies (1:1,000; cat. no. 340507; BD Biosciences). A BD FACSVantage flow cytometer (BD Biosciences) was used to quantify the data. CELIQUEST software (version 5.1; BD Biosciences) was used to analysis the flow cytometry data.

Statistical analysis. All data in the present study are presented as the mean \pm standard deviation and were analyzed using SPSS 20.0 software (IBM Corp., Armonk, NY, USA). The data was obtained from a minimum of three independent experiments. One-way analysis of variance was used to compare multiple groups followed by Tukey's Honest Significant Difference post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

wLRRFIP1 was successfully expressed. The wLRRFIP1 protein was successfully expressed as determined by SDS-PAGE analysis (Fig. 2). The SDS-PAGE images illustrated that the wLRRFIP1 protein was highly expressed in the ultrasonic precipitations.

wLRRFIP1 treatment triggers platelet agglutination of collagen treated platelets. A low dose of wLRRFIP1 (P+C+LMH+wLRRFIP150 group) and a high-dose of wLRRFIP1 (P+C+LMH+wLRRFIP1100 group) significantly increased the levels of adenosine diphosphate-PAG (M), PAG (1) and significantly decreased the I% values compared with the platelet group (all $P < 0.05$; Fig. 3A). Low- and high-dose wLRRFIP1 significantly decreased the time for maximum PAG [T(M)] values (Fig. 3B) and high dose wLRRFIP1 significantly decreased the PRP values (Fig. 3C) compared with the platelet group (all $P < 0.05$), demonstrating that platelet agglutination increased. Additionally, the P+C+LMH+wLRRFIP1100 group had a decreased PAG (1) compared with the P+C+LMH+wLRRFIP150 group.

wLRRFIP1 treatment causes platelet agglutination of thrombin receptor treated platelets. The levels of PAG (M) and PAG (1) were significantly increased, and I% values were significantly decreased in the P+wLRRFIP1200+SFLLRN group compared with the P+LMH+SFLLRN group (all $P < 0.05$; Fig. 4A). PAG (M) and PAG (1) were slightly increased, and I% values were significantly decreased in the P+wLRRFIP1100+SFLLRN group compared with the P+SFLLRN group ($P < 0.05$). In addition, wLRRFIP1 significantly decreased the T(M) values (Fig. 4B) and significantly increased the PRP values (Fig. 4C) compared with the P+SFLLRN group (all $P < 0.05$), demonstrating that platelet agglutination increased.

wLRRFIP1 treatment triggers platelet agglutination of monoclonal antibody incubated platelets. The levels of PAG (M) and PAG (1) were significantly decreased and I% values were significantly increased in P+C+anti-wLRRFIP1 group compared with the P+C group ($P < 0.05$; Fig. 5A). Anti-wLRRFIP1 treatment also significantly affected the T (M) (Fig. 5B) and PRP values (Fig. 5C) in the P+C+anti-wLRRFIP1 group when compared with that in the P+C group. In addition, the levels of PAG (M) and PAG (1) were significantly decreased and the I% values significantly increased in the P+SFLLRN+anti-wLRRFIP1 group compared with the P+SFLLRN group ($P < 0.05$; Fig. 5A). Anti-wLRRFIP1 treatment also significantly affected the T (M) (Fig. 5B) and

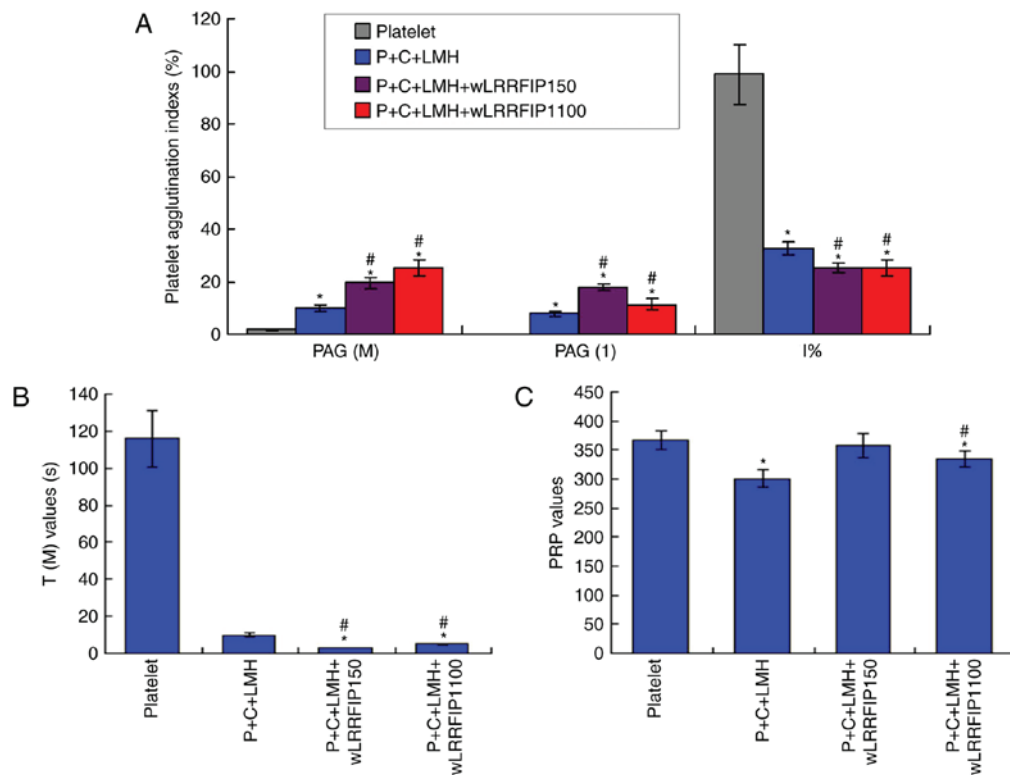


Figure 3. Platelet agglutination of collagen treated platelets undergoing wLRRFIP1 treatment. (A) Platelet agglutination indexes of PAG (M), PAG (1) and I% of platelet agglutination. (B) T (M) and (C) PRP values of platelet agglutination. *P<0.05 vs. the platelet group; #P<0.05 vs. the P+C+LMH group. PAG, adenosine diphosphate-platelet agglutination rate; PRP, platelet-rich plasma; PAG (M), adenosine diphosphate-platelet agglutination rate maximum; PAG (1), PAG at 1 min; T (M), the time for maximum PAG; LMH, low-molecular heparin; wLRRFIP1, wild-type leucine-rich repeat flightless-interacting protein-1; P, platelets; C, collagen.

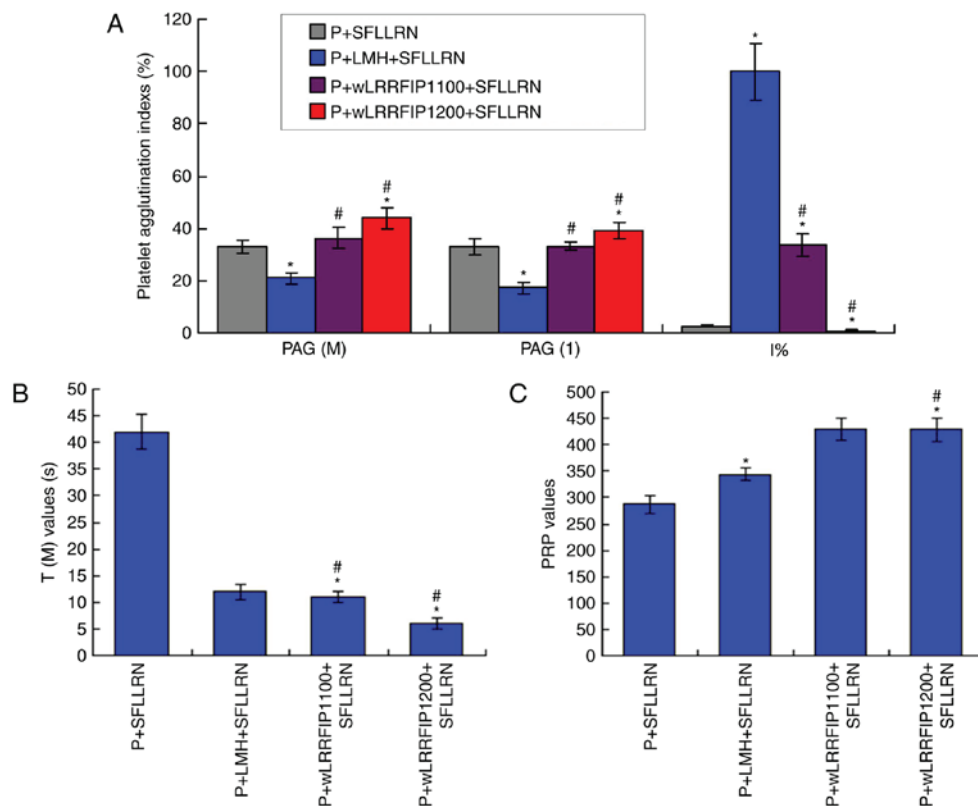


Figure 4. Platelet agglutination of thrombin receptor treated platelet undergoing wLRRFIP1 treatment. (A) Platelet agglutination indexes of PAG (M), PAG (1) and I% of platelet agglutination. (B) T (M) and (C) PRP values of platelet agglutination. *P<0.05 vs. the P+SFLLRN group; #P<0.05 vs. the P+LMH+SFLLRN group. PAG, adenosine diphosphate-platelet agglutination rate; PRP, platelet-rich plasma; PAG (M), adenosine diphosphate-platelet agglutination rate maximum; PAG (1), PAG at 1 min; T (M), the time for maximum PAG; SFLLRN, thrombin receptor activator; wLRRFIP1, wild-type leucine-rich repeat flightless-interacting protein-1; P, platelets; LMH, low-molecular heparin.

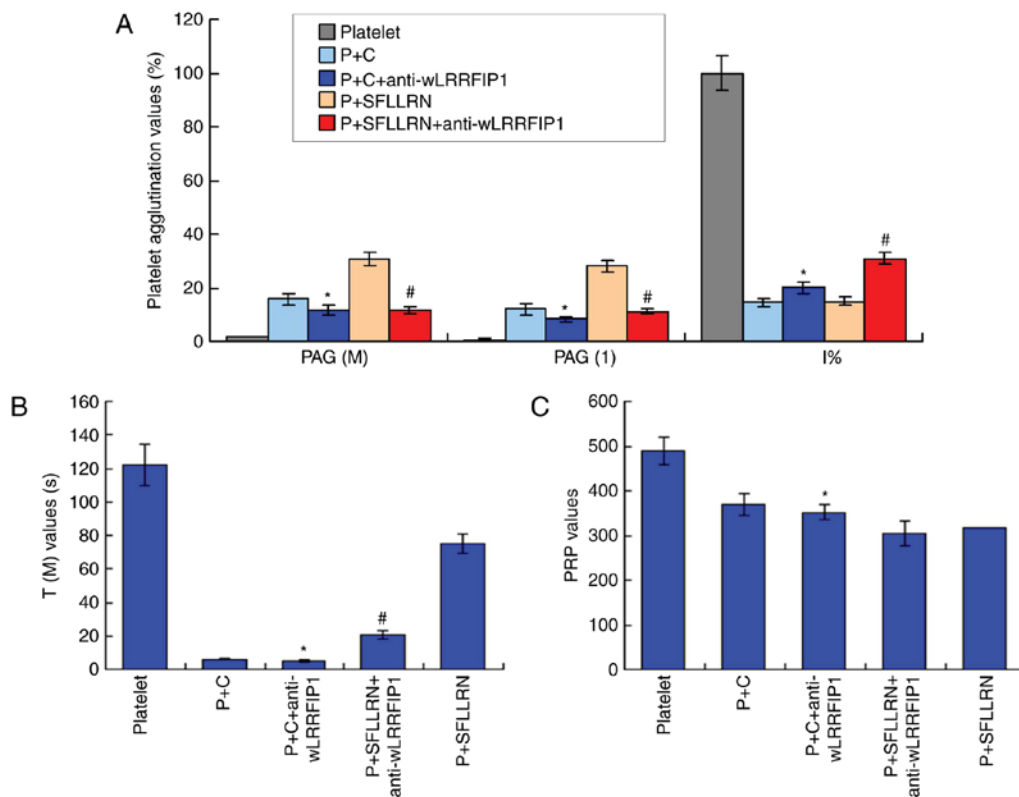


Figure 5. Platelet agglutination of monoclonal antibody incubated platelet undergoing wLRRFIP1 treatment. (A) Platelet agglutination indexes of PAG (M), PAG (1) and I% of platelet agglutination. (B) T (M) and (C) PRP values of platelet agglutination. * $P < 0.05$ vs. the P+C group; # $P < 0.05$ vs. the P+SFLLRN group. PAG, adenosine diphosphate-platelet agglutination rate; PRP, platelet-rich plasma; PAG (M), adenosine diphosphate-platelet agglutination rate maximum; PAG (1), PAG at 1 min; T (M), the time for maximum PAG; P, platelets; C, collagen; SFLLRN, thrombin receptor activator; wLRRFIP1; wild-type leucine-rich repeat flightless-interacting protein-1.

PRP values (Fig. 5C) in the P+SFLLRN+anti-LRRFIP1 group compared with that in the P+SFLLRN group. Therefore, wLRRFIP1 triggered platelet agglutination.

wLRRFIP1 knockout decreases α IIB β 3 levels. The α IIB β 3 integrin levels were examined by flow cytometry. The results indicated that the levels of α IIB β 3 in the LRRFIP1^{-/-} group were significantly decreased compared with the LRRFIP1^{+/+} group ($P < 0.05$; Fig. 6). The levels of α IIB β 3 in the LMH group were also significantly decreased compared with the LRRFIP1^{+/+} group ($P < 0.05$).

Platelet agglutination is significantly inhibited in a mouse model of LRRFIP1^{-/-}. The results demonstrated that PAG (M), PAG (1) and I% values in the P+LRRFIP1^{-/-}, P+C+LRRFIP1^{-/-} and P+SFLLRN+LRRFIP1^{-/-} groups were significantly decreased compared with that in the P+LRRFIP1^{+/+}, P+C+LRRFIP1^{+/+} and P+SFLLRN+LRRFIP1^{+/+} groups, respectively ($P < 0.05$; Fig. 7A). Platelet agglutination was significantly inhibited in the P+SFLLRN+LRRFIP1^{-/-} group compared with the P+SFLLRN+LRRFIP1^{+/+} group as indicated by changes in the levels of PAG (M), PAG (1) and I%. The T (M) values in the P+LRRFIP1^{-/-} and P+C+LRRFIP1^{-/-} groups were significantly decreased compared with that in the P+LRRFIP1^{+/+} and P+C+LRRFIP1^{+/+} groups, respectively ($P < 0.05$; Fig. 7B). The T (M) values in the P+SFLLRN+LRRFIP1^{-/-} group were significantly increased compared with that in the P+SFLLRN+LRRFIP1^{+/+} group ($P < 0.05$). The PRP

values in the P+LRRFIP1^{-/-}, P+C+LRRFIP1^{-/-} and P+SFLLRN+LRRFIP1^{-/-} groups were significantly increased compared with that in the P+LRRFIP1^{+/+}, P+C+LRRFIP1^{+/+} and P+SFLLRN+LRRFIP1^{+/+} groups, respectively ($P < 0.05$; Fig. 7C). Therefore, the changes observed in the T (M) and PRP values also illustrated the inhibition of platelet agglutination in the LRRFIP1^{-/-} mouse model compared with the LRRFIP1^{+/+} mouse model.

wLRRFIP1 knockout decreases α IIB β 3 levels in platelets treated with Convulxin. The α IIB β 3 integrin levels were examined by flow cytometry. The results revealed that levels of α IIB β 3 in the LRRFIP1^{-/-} group were significantly decreased compared with the LRRFIP1^{+/+} group ($P < 0.05$; Fig. 8). The levels of α IIB β 3 in the LRRFIP1^{-/-} + Convulxin group were also significantly decreased compared with the LRRFIP1^{+/+} + Convulxin group ($P < 0.05$; Fig. 8). This result suggests that knocking out wLRRFIP1 reduced α IIB β 3 levels in the platelets treated with Convulxin.

Discussion

Platelets are critical for atherothrombosis, hemostasis and wound healing (24,25). The platelet count and volume and the response of platelets are independent risk factors for myocardial infarction; anti-platelet therapy cannot reverse the effects of hyperactive platelets (26). Although a number of regulatory factors/proteins of platelet function have been

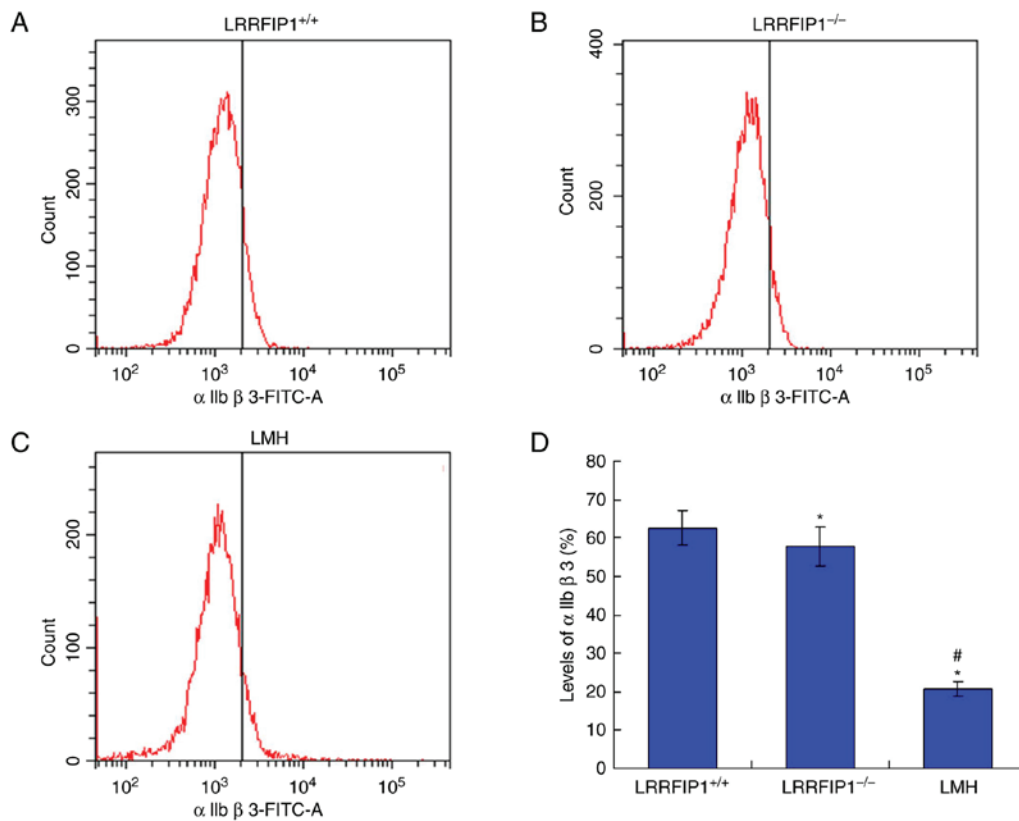


Figure 6. α IIb β 3 integrin levels in an LRRFIP1^{-/-} or LRRFIP1^{+/+} mouse model. α IIb β 3 integrin levels in the (A) LRRFIP1^{+/+}, (B) LRRFIP1^{-/-} and (C) LMH group. (D) Statistical analysis of the α IIb β 3 integrin levels. *P<0.05 vs. the LRRFIP1^{+/+} group; #P<0.05 vs. the LRRFIP1^{-/-} group. LRRFIP1^{+/+}, leucine-rich repeat flightless-interacting protein-1 positive mice; LRRFIP1^{-/-}, leucine-rich repeat flightless-interacting protein-1 knockout mice; LMH, low-molecular heparin.

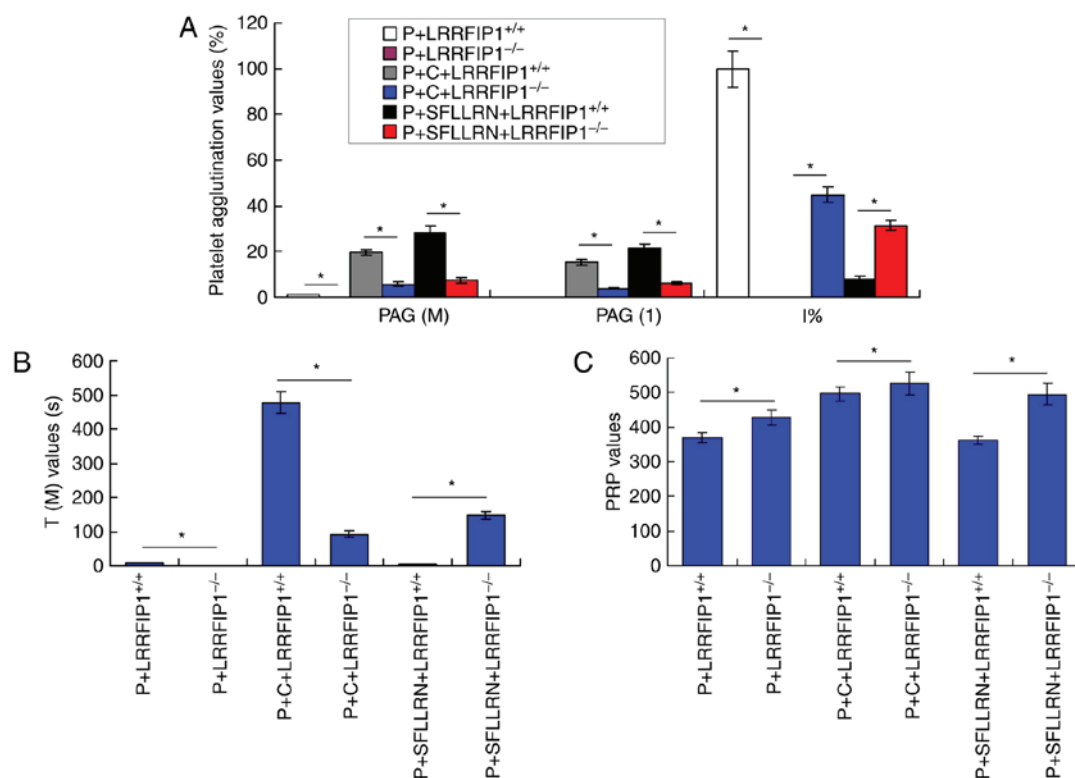


Figure 7. Observation of the platelet agglutination in a mouse model of LRRFIP1^{-/-}. (A) Platelet agglutination indexes of PAG (M), PAG (1) and I% of platelet agglutination. (B) T (M) and (C) PRP values of platelet agglutination. *P<0.05. PAG, adenosine diphosphate-platelet agglutination rate; PRP, platelet-rich plasma; PAG (M), adenosine diphosphate-platelet agglutination rate maximum; PAG (1), PAG at 1 min; T (M), the time for maximum PAG; P, platelets; C, collagen; SFLLRN, thrombin receptor activator; LRRFIP1^{-/-}, leucine-rich repeat flightless-interacting protein-1 knockout mice; LRRFIP1^{+/+}, leucine-rich repeat flightless-interacting protein-1 positive mice.

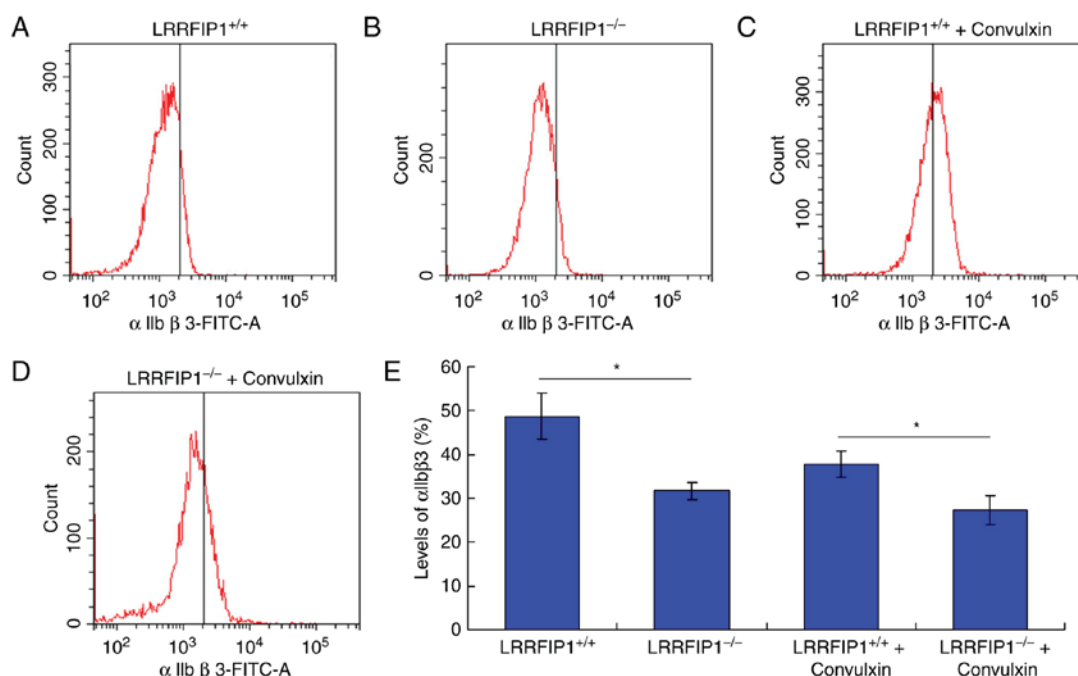


Figure 8. α IIb β 3 integrin levels in an LRRFIP1^{-/-} or LRRFIP1^{+/+} mouse model or Convulxin treated groups. (A) The α IIb β 3 integrin levels in the (A) LRRFIP1^{+/+}, (B) LRRFIP1^{-/-}, (C) LRRFIP1^{+/+} + Convulxin and (D) LRRFIP1^{-/-} + Convulxin groups. (E) Statistical analysis of the α IIb β 3 integrin levels. *P<0.05. LRRFIP1^{-/-}, leucine-rich repeat flightless-interacting protein-1 knockout mice; LRRFIP1^{+/+}, leucine-rich repeat flightless-interacting protein-1 positive mice; FITC, fluorescein isothiocyanate.

identified, the transcription profile of platelets is incomplete. Goodall *et al* (18) identified the nonsynonymous single nucleotide polymorphism (SNP; SNP site: rs3739038) in the LRRFIP1 as a putative and novel signal correlated with myocardial infarction. Therefore, the present study investigated the effects of the LRRFIP1 on platelet agglutination.

The LRRFIP1 gene is correlated with the regulation of gene transcription, however its function in platelet regulation has not been fully elucidated (20). LRRFIP1 has also been identified as a repressor for the transcription of genes, including platelet-derived growth factor subunit A, epidermal growth factor receptor and tumor necrosis factor α , by closely binding with their promoter regions (16). Previous studies have suggested that LRRFIP1 serves as an important role in platelet activation, while also regulating platelet agglutination (20,27).

The results of the present study indicated that while cells were undergoing treatment with collagen, LMH and SFLRN, LRRFIP1 significantly increased the PAG (M), PAG (I) and PRP values and significantly decreased the I% and T (M) values of platelets compared with the controls. In addition, when the platelets were treated with anti-wLRRFIP1 antibodies the PAG (M), PAG (I) and PRP values were significantly decreased and the I% and T (M) values were significantly increased compared with the untreated groups. A mouse model of LRRFIP1 gene knockout was established and the results demonstrated that knockout of the LRRFIP1 gene clearly increased the PAG (M), PAG (I) and PRP values and significantly decreased the I% and T (M) values compared with the wLRRFIP1^{+/+} mice. These results suggest that LRRFIP1 may cause platelet agglutination and LRRFIP1 inhibition may alleviate the platelet agglutination process. These findings are consistent with a previously published study, which reported that LRRFIP1 acts as a component of the platelet cytoskeleton and regulates platelet responses (18).

A previous study reported that LRRFIP1 is correlated with the interaction between fibrinogen and α IIb β 3 (28). Platelet aggregation and activation are complex processes and involve a number of protein interactions (29). Aggregation processes are initiated by the activation of α IIb β 3, which binds to fibrinogen and adjacent platelets (30,31). Chen *et al* (32) previously reported that anti- α IIb β 3 complex antibodies inhibited the aggregation of platelets. The present study examined the levels of α IIb β 3 to identify the effects of the LRRFIP1 on platelet agglutination. The results demonstrated that the level of α IIb β 3 in the LRRFIP1^{-/-} group was significantly decreased compared with the LRRFIP1^{+/+} group. This suggests that LRRFIP1 gene knockout inhibits the expression of α IIb β 3.

In conclusion, LRRFIP1 treatment induces platelet agglutination and LRRFIP1 gene knockout inhibits platelet agglutination. In addition, LRRFIP1 gene knockout significantly decreases the levels of α IIb β 3. Therefore, LRRFIP1 triggers platelet agglutination by enhancing the expression of α IIb β 3.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

XY, PL, YYL, MYL and WLF performed the agglutination assay. BYL conducted the SDS-PAGE. XY, PL and YYL performed the preparation of LRRFIP1 recombinant protein and established the LRRFIP1 knockout models. XY and JHZ wrote the manuscript and were the primary designers of the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by Ethics Committee of Daping Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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