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Megakaryocyte-Specific Knockout of the *Mir-99b/let7e/125a* Cluster Lowers Platelet Count without Altering Platelet Function

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Abstract

The purpose of this research was to assess the effects of a microRNA (miRNA) cluster on platelet production. Human chromosome 19q13.41 harbors an evolutionarily conserved cluster of three miRNA genes (*MIR99B, MIRLET7E, MIR125A*) within 727 base-pairs. We now report that levels of *miR-99b-5p, miR-let7e-5p* and *miR-125a-5p* are strongly correlated in human platelets, and all are positively associated with platelet count, but not white blood count or hemoglobin level. Although the cluster regulates hematopoietic stem cell proliferation, the function of this

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genomic locus in megakaryocyte (MK) differentiation and platelet production is unknown. Furthermore, studies of individual miRNAs do not represent broader effects in the context of a cluster. To address this possibility, MK/platelet lineage-specific Mir-99b/let7e/125a knockout mice were generated. Compared to wild type littermates, cluster knockout mice had significantly lower platelet counts and reduced MK proplatelet formation, but no differences in MK numbers, ploidy, maturation or ultra-structural morphology, and no differences in platelet function. Compared to wild type littermates, knockout mice showed similar survival after pulmonary embolism. The major conclusions are that the effect of the *Mir-99b/let7e/125a* cluster is confined to a late stage of thrombopoiesis, and this effect on platelet number is uncoupled from platelet function.

Keywords

Mir-99b/let7e/125a cluster; megakaryocyte; platelets; conditional knockout mice

Introduction

Megakaryopoiesis (MKpoiesis) and thrombopoiesis are complex biological processes wherein hematopoietic stem cells differentiate to megakaryocyte (MK) progenitors, undergo endomitosis, mature, and releases platelets into the circulation. We and others have shown that MKs and platelets carry a rich repertoire of miRNAs, a class of small non-coding RNAs that regulate expression of protein-coding genes critical for normal functioning of MKs and platelets [1]. Platelet *miR-125a-5p* is positively associated with human platelet number and enhances human MK proplatelet formation [2]. The mir-125a precursor is a part of an evolutionary conserved miRNA cluster (MIR-99b/let7e/125a) within 727 base-pairs on human chromosome 19. This cluster is known to regulate primitive hematopoietic cells[3], but whether it regulates MKpoiesis and thrombopoiesis remains unknown. *In vivo* systemic inhibitors of *miR-125a-5p* lower mouse platelet counts. However, because *miR-125a-5p* has a broad tissue distribution, it is not known whether this miRNA effect is mediated by MKs. The goal of this study was to determine whether the entire cluster of three miRNAs might regulate platelet production in a MK-specific manner. We now report generation of MK/platelet lineage specific Mir-99b/let7e/125a cluster knockout mice strain Mir-99b/let7e/125a$^{fl/fl}$ platelet factor 4 (PF4)-Cre, and investigate its contribution to platelet production and function. We show that loss of the Mir-99b/let7e/125a cluster in MKs reduces murine MK proplatelet formation and circulating platelet counts without altering MK differentiation or platelet function.

Material and Methods

Generation of Mir-99b/let7e/125a$^{fl/fl}$PF4-Cre mouse strain

Conditional floxed Mir-99b/let7e/125a allele (Mir-99b/let7e/125a$^{fl/fl}$) mice were custom generated by Biocytogen (Worcester, MA)[4] using homologous recombination in C57BL6 embryonic stem cells. Initially PCR products of desired locus in the C57BL/6N mouse were sequenced and confirmed as the same as those from the NCBI and Ensembl database. The ideal homologous arms for the target vector forced us to include exon 1 of the *Spaca6* gene within the loxP sites. *Spaca6* encodes Sperm acrosome associated 6, which

is not expressed in MKs or platelets. Cas9/sgRNA plasmids were generated and confirmed by DNA sequencing. Eight sets of sgRNAs were made and tested to select the most efficient set for CRISPR activity. Targeting vector with loxP flanking the three miRNA genes was constructed and verified by sequencing. RNA was prepared and mouse zygotes microinjected. Pup DNA was genotyped with PCR primers; seven pups had the targeted allele; pups were bred and seven confirmed by PCR as F1 heterozygotes. All animal studies were done under the approval by the Institutional Animal Care and Use Committee at the University of Utah.

TaqMan advanced miRNA assay

Leukocyte depleted platelet (LDP) were prepared as described previously [5]. Total RNA was isolated using TRIzol method (Thermo Fisher Scientific, Waltham, MA). Mature platelet miRNA expression of *mmu-miR-99b-5p, mmu-let-7e-5p* and *mmu-miR-125a-5p* was detected using TaqMan advanced miRNA assay as per manufactures' protocol (Thermo Fisher Scientific, Waltham, MA).

MK quantification and characterization

Mouse bone marrow was isolated and MKs quantified as previously described [6]. For ploidy analysis, day 5 *ex vivo* generated MKs were stained with propidium iodide (50 μg/ml, Thermo Fisher Scientific, Waltham, MA) for 1 hour at room temperature in the dark, and CD41a positive MKs stained for propidium iodide were analyzed for ploidy analysis using a Cytoflex flow cytometer. Transmission electron microscopy of day 5 *ex vivo* MKs was performed as described previously[2] at the University of Utah, Electron Microscopy Core Facility.

Platelet functional characterization

Platelet cell counts were performed on blood collected from the retro-orbital plexus into tubes containing EDTA (Sarstedt, Inc. Newton, NC) using a Hemavet 950FS blood cell counter (Drew Scientific, Miami Lakes, FL). $2x10^8$ cells/ml washed murine platelets were activated with agonists, AYPGKF (50 μM, 100 μM and 150 μM) (GL Biochem (Shanghai) Ltd.) and collagen related peptide (CRP) (synthesized at Baylor College of Medicine and cross-linked with glutaraldehyde) (1 ng, 30 ng and 100 ng) or Tyrode's buffer (resting), as done previously [7]. For platelet aggregation, $2x10^8$ cells/ml washed platelets were stimulated with AYPGKF (30 μM, 150 μM) and CRP (30 ng, 100 ng), and change in the light transmission was measured by a lumi-aggregometer (Chrono-Log, Havertown, PA) at 37°C under stirring conditions.

For platelet spreading assays, washed platelets from knockout or littermates were plated on fibrinogen (100 μg/ml, Calbiochem-Novabiochem Corporation, San Diego, CA) or collagen (100 μg/ml, Chrono-Log, Havertown, PA) coated chamber slides for 45 minutes. Cells were fixed with 2% paraformaldehyde for 20 minutes on ice, and stained with Alexa Flour 488 Phalloidin (Thermo Fisher Scientific, Waltham, MA). Images were taken under 60x oil objective using a confocal microscope. Eight-week-old, gender matched knockout and littermates were used for platelet counts and functional assays, unless otherwise indicated.

Pulmonary embolism model

Collagen (0.2 mg/kg; Chronolog) and epinephrine (15 mg/kg; Sigma-Aldrich, St. Louis, MA) in 100 μL of PBS were administered through retro-orbital injection [8]. The time to death was defined as the time needed to the onset of respiratory arrest that lasted at least 2-10 minutes.

Statistics

Two-group comparisons of parametric data were done using a two-tailed Student t-test unless otherwise indicated. All statistical analyses were performed using Prism 9 software (GraphPad, La Jolla, CA). P value of 0.05 or less was considered as significant. All data are presented as mean ± standard error of the mean.

Results

miRNA-99b/let7e/125a cluster expression in differentiating human MKs and is positively correlated with platelet number

miR-125a-5p has been previously shown to correlate with human platelet number, so we initially tested whether *miR-99b-5p* and *miR-let-7e-5p* levels also correlated. Significant positive associations were observed between platelet count and each of the three miRNAs in 154 healthy donors, but not with white blood count or hemoglobin (Figure 1A), supporting the possibility that the whole cluster may impact plt numbers. Subsequent studies using real time PCR in human umbilical cord blood CD34 derived MK cultures showed the expression of *miR-99b-5p, miR-let-7e-5p* and *miR-125a-5p* increased from day 6 to 13 as MKs differentiate (Figure 1B). These results suggest the effect of this miRNA cluster may be at the level of platelet production by MKs.

Mir-99b/let7e/125a$^{fl/fl}$PF4-Cre mice are thrombocytopenic

Conditional floxed Mir-99b/let7e/125a allele (Mir-99b/let7e/125a$^{fl/fl}$) mice were custom generated by Biocytogen (Worcester, MA)[4] using homologous recombination in C57BL6 embryonic stem cells using the strategy shown in Figure 2A (see Methods for more details). Germ line transmission of genetic recombination in F1 animals was confirmed with long fragment PCR and sequencing analysis. Genotyping primers used in PCR analyses are shown in Supplemental Figure 1 (red arrows). No random insertions were observed by Southern blotting using an internal probe (Supplemental Figure 2). Mir-99b/let7e/125a$^{fl/fl}$ mice were crossed with C57BL/6 mice expressing platelet factor 4 promoter-driven Cre-recombinase (PF4-Cre)[9] to generate mice lacking expression of Mir-99b/let-7e/125a in MKs and platelets (Mir-99b/let-7e/125a$^{fl/fl}$PF4-Cre). Throughout the manuscript these knockout mice are referred as ‘Mir-99b/let7e/125a −/−’, and their littermate control mice are referred as ‘WT’.

Mir-99b/let7e/125a−/− mice did not display any overt physical abnormalities, were fertile, and bred normally. The expression of *miR-99b-5p, miR-let-7e-5p* and *miR-125a-5p* was abolished in Mir-99b/let7e/125a −/− purified platelets, confirming successful deletion of the cluster (Figure 2B). A modest but significant (p=0.02) reduction in peripheral blood platelet

count was observed in *Mir-99b/let-7e/125a* −/− compared to wild type littermates. (Figure 2C).

Mir-99b/let7e/125a$^{fl/fl}$PF4-Cre mice have defective thrombopoiesis

Since this cluster is known to regulate HSC proliferation, we assessed whether MK-specific deletion would affect early stages of MKpoiesis, but observed no differences between knockout mice and wild type littermates in the number of mature MKs (Figure 3A), the levels of MK polyploidy (Figure 3B) or MK ultra-structural morphology (Figure 3C). However, compared to MKs from wild-type littermates, MKs from *Mir-99b/let7e/125a* −/− mice displayed a 71.4% reduction (p=0.01) in MK proplatelet formation (Figure 3D). These results suggested the effect of the miRNA cluster on platelet number may be at the late stages of thrombopoiesis.

Mir-99b/let7e/125a −/− mice has no detectable platelet function defects

In additional to the quantitative effect on platelet numbers, it was of interest to assess whether MK/platelet-specific expression of Mir-99b/let7e/125a alters platelet function. Compared to platelets from wild type littermates, platelets from *Mir-99b/let-7e/125a* −/− mice showed no differences in measures of platelet agonist-induced αIIbβ3 integrin activation (Figure 4A), alpha-granule release (Figure 4B) or platelet aggregation (Figure 4C). In addition, platelets from the knockout mice showed no differences in ultra-structural morphology (Figure 4D) or spreading on fibrinogen or collagen (Figure 4E–F). There was also no difference in mean platelet volume (data not shown). Finally, compared to platelets from wild type littermates, platelets from *Mir-99b/let-7e/125a* −/− mice showed no significant difference in survival in a pulmonary thromboembolism model (Figure 4G). Thus, Mir-99b/let7e/125a cluster is dispensable for murine platelet *function*.

Discussion

Prior work has shown diverse roles of miRNAs in MK and platelet physiology [10–18]. *miR-125a-5p* regulates MK proplatelet formation via targeting actin bundling protein, L-plastin [2]. In humans, about 40% of miRNA genes localize as clusters in the genome [19]. miRNA clusters are regulated by genetic and epigenetic modifications, and such clusters regulate many cellular processes, including proliferation, differentiation, immunity, apoptosis, DNA repair and self-renewal [20]. Aberrant expression of miRNA clusters can impact the pathogenesis of many diseases, including carcinogenesis [21]. The Mir-99b/let7e/miR125a cluster has been linked to oncogenesis[22], tumor metastasis [23], inflammation [24–26] and mechanisms leading to cystic fibrosis pathogenesis [27]. This is the first report of the generation of a MK/platelet specific *Mir-99b/let-7e/125*−/− mice strain and its characterization in MK-platelet function. The major findings in this report are that expression of this cluster in murine MKs is required for normal MK proplatelet formations and normal platelet production. Prior studies overexpressing the miR-99b/let7e/125a cluster have suggested that *miR-125a-5p* may account for the majority of effects on murine hematopoiesis [3], consistent with our MK-specific findings in this report and prior reports in cultured human MKs [2].

miRNAs in genomic clusters are often transcribed into a common precursor pri-miRNA,, e.g. the miR-17/92 cluster and its paralogues[28], and there is RT-PCR evidence for a single transcript in HeLa cells that contains each of the three miRNAs in the miR-99b/let7e/125a cluster.[27] The strong correlations we observed in human platelets support a similar mechanism of transcriptional regulation of a polycistronic RNA containing all three genes.

Conclusions

This is the first report of generating MK/platelet specific miRNA cluster knockout mice strain and its characterization in MK-platelet physiology. The MK-specific expression of Mir-99b/let7e/125a positively regulates murine platelet numbers without any deleterious effects on MK differentiation or platelet activation/function. This discovery has potential implications for several translational applications including hematologic malignancies, *in vitro* platelet production and transfusion medicine. Lastly, numerous studies have linked each of the miRNAs of the cluster individually to a different condition. But since each miRNA can have numerous targets, over-expressing or silencing these miRNAs one at a time shows their individual roles in a specific physiology or pathophysiology. Deleting a cluster of miRNAs asks a different question altogether - that of “synergy.” This is only the second example we are aware of in which an entire cluster of miRNAs has been deleted, and the only MK-specific miRNA cluster deletion. As such, our flowed mice may prove instructive for assessing function in other cell types.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

MK	megakaryocyte
Mkpoiesis	megakaryopoiesis
PF4	platelet factor 4

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Highlights:

- The Mir-99b/let7e/125a cluster regulates late thrombopoiesis and platelet count
- First generation of megakaryocyte-platelet-specific miRNA knockout

A

	hsa-miR-99b-5p		*hsa-let7e-5p*		*hsa-miR-125a-5p*	
	r	P value	r	P value	r	P value
Platelet count	0.17	0.03	0.22	0.007	0.16	0.02
White Blood Count	0.03	0.71	0.06	0.46	0.05	0.55
Hemoglobin	-0.04	0.59	-0.05	0.54	-0.05	0.54

B

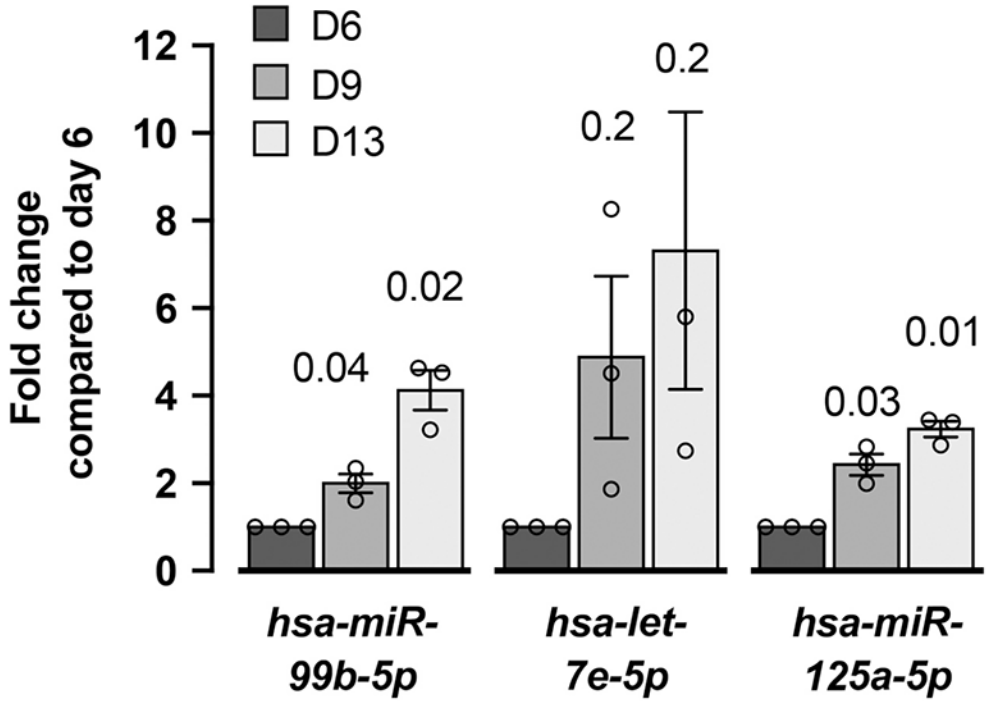

Figure 1. The expression of *miR-99b-5p, miR-let7e-5p* and *miR-125a-5p* increases with human MK differentiation and associates positively with human platelet numbers.

A. Correlations between miRNAs and blood platelet count, white blood count or hemoglobin level in 154 healthy donors from the Platelet RNA and Expression 1 data set. r, Pearson correlation coefficient. **B.** Human umbilical cord blood derived, CD61-purified MK cultures were assessed for the expression of mature miRNAs, **miR-99b-5p, let-7e-5p** and **miR-125a-5p** by real time PCR analysis at days 6, 9 and 13. Fold changes were calculated for their expression at days 9 and 13 compared to day 6. n=3 different cords (=independent experiments).

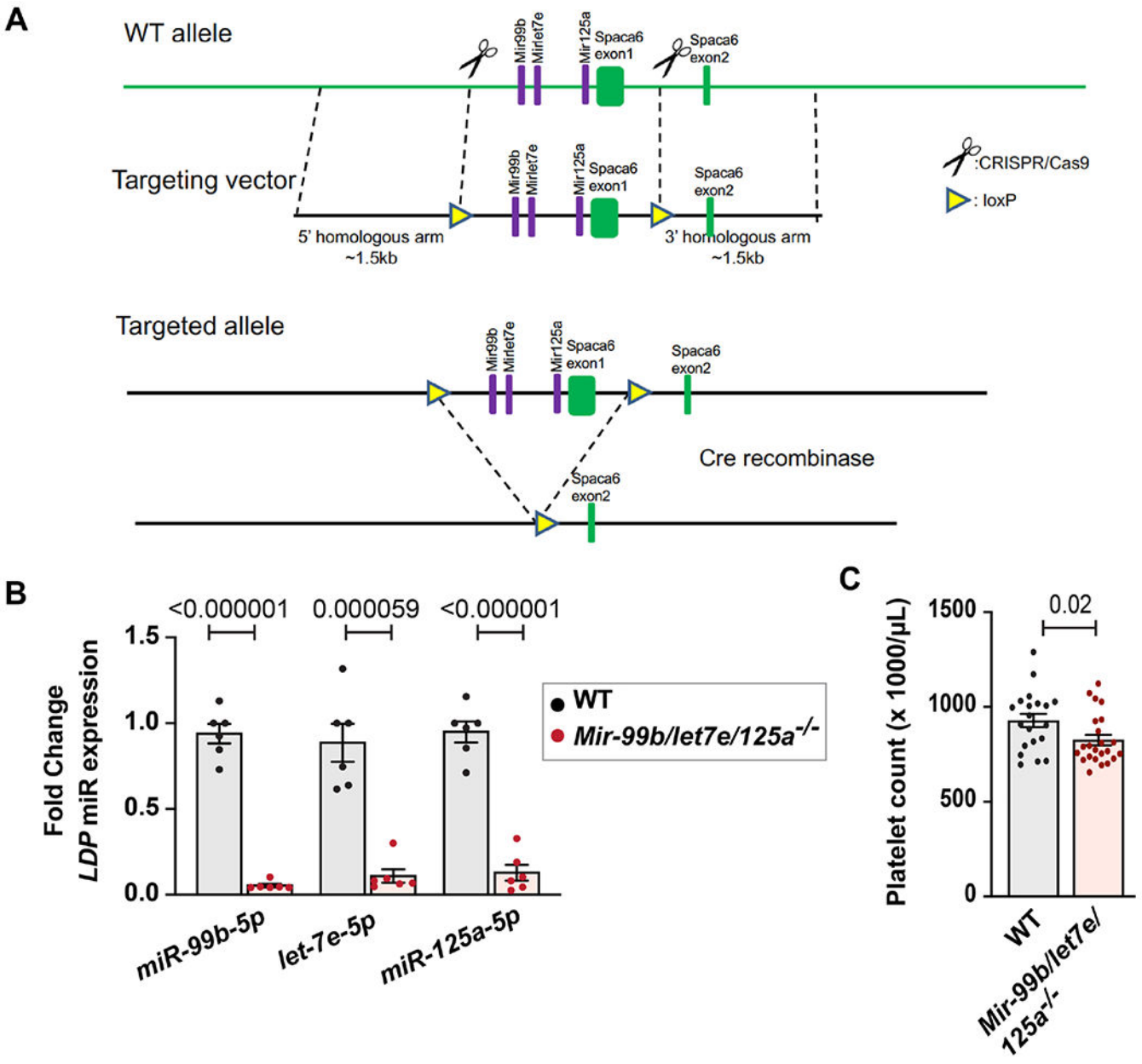

Figure 2. Deletion of the Mir-99b/let7e/125a cluster lower blood platelet counts.

A. Schema showing experimental design. Mice were generated with the targeted allele containing loxP sites flanking the Mir-99b/let7e/125a cluster. These mice were bred with PFA-Cre mice resulting in mice with MKs lacking the Mir-99b/let7e/125a cluster. **B.** Fold change of *mmu-miR-99b-5p, mmu-let-7e-5p* and *mmu-miR-125a-5p* in leukocyte depleted platelets (LDP) isolated from *Mir-99b/let-7e/125a −/− mice* (n=6) compared to their littermates (n=6), as assessed by TaqMan advance miRNA assay. *miR-320a-5p* (expression did not change in knockout vs littermates, data not shown) was used as a housekeeping gene for fold change calculation. **C.** Peripheral blood platelet count measured by Hemavet for *Mir-99b/let7e/125a*−/− mice (n=23) or littermates (n=20) (8 weeks old, and gender matched).

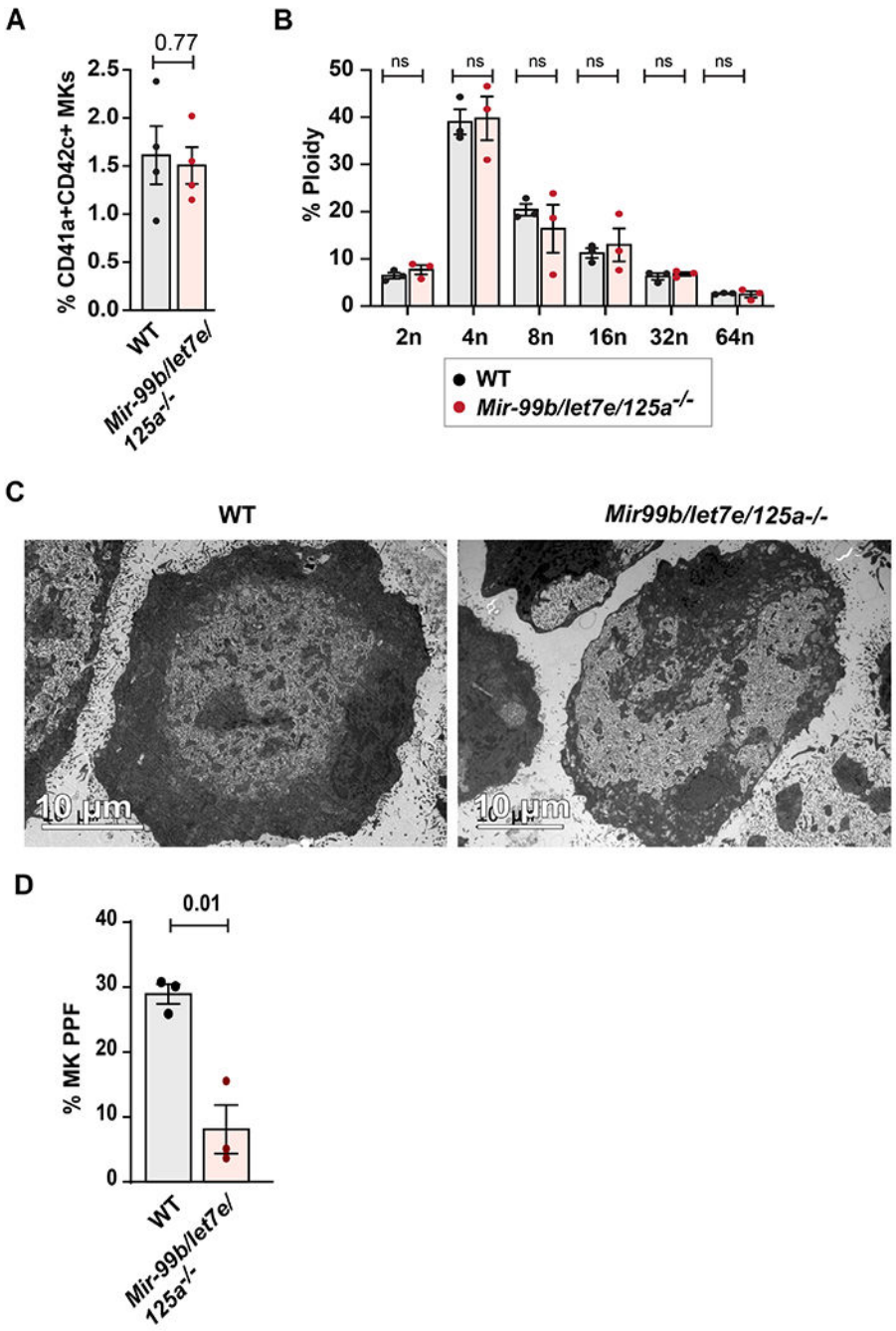

Figure 3. The Mir-99b/let7e/125a cluster is required for normal MK proplatelet formation, but not for differentiation, ploidy or cytoplasmic maturation.

A. The percentage of CD41a+ CD42c+ bone marrow MKs were measured by flow cytometric analysis in *Mir-99b/let-7e/125a* −/− or littermates (n=4 each group). **B.** Ploidy analysis of *ex vivo* cultured bone marrow MKs at day 5, detected by propidium iodide staining. Total percentage of CD41a+ propidium iodide positive MKs representing 2n, 4n, 8n, 16n, 32n and 64n ploidy were plotted for *Mir-99b/let-7e/125a* −/− or littermate mice (n=3 each). Sub-2n and apoptotic cells were gated out in this analysis. **C.** Representative

transmission electron microscopic images showing *ex vivo* cultured bone marrow derived MK for *Mir-99b/let-7e/125a* −/− or littermate mice (n=3). **D.** Proplatelet formation assay for *ex vivo* cultured MKs isolated from femurs of *Mir-99b/let7e/125a* −/− or littermates (n=3 each). Quantification was done blinded to the treatment. Schematic representation shows experimental design for generating Mir-99b/let7e/125a cluster mice that were flanked with loxP sites.

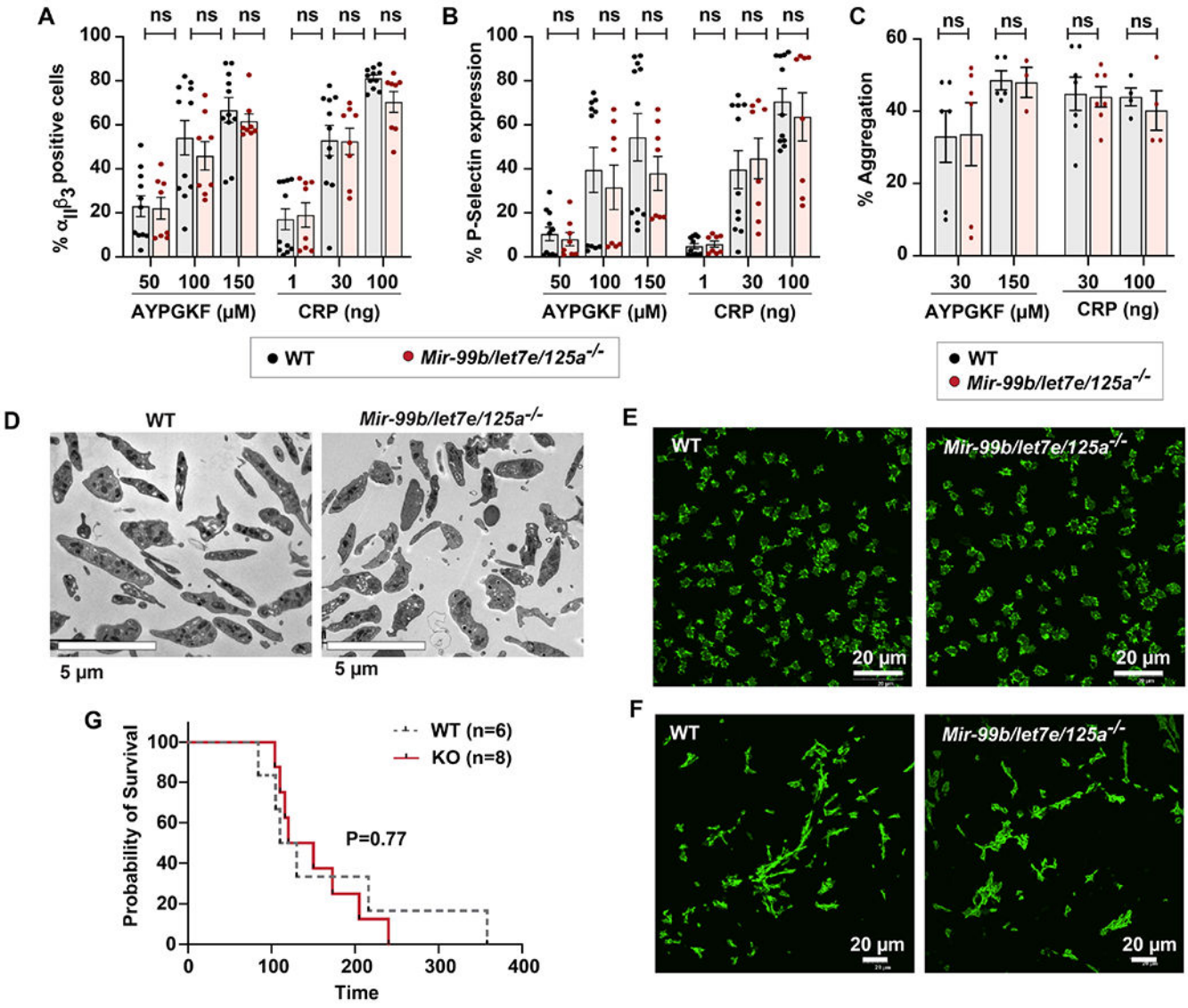

Figure 4. MK/platelet specific loss of Mir-99b/let7e/125a does not alter platelet function or *in vivo* thrombosis.

A-B. Washed platelets from *Mir-99b/let7e/125a* −/− (n=8) or littermates (n=11) were activated with 50, 100 or 150 μM AYPGKF and 1, 30 or 100 ng/ml collagen receptor peptide (CRP). **A.** JON/A binding and **B.** P-selectin was measured by flow cytometry. **C.** Washed platelets from *Mir-99b/let7e/125a* −/− (n=8) or littermates (n=11) were activated with AYPGKF (30 and 100 μM) or collagen (30 and 100 ng). Aggregation was measured using Chronolog aggregometer and plotted (n=3 to 7). **D-E.** Phalloidin staining of washed platelets isolated from *Mir-99b/let7e/125a* −/− or littermates, that were adhered on **D.** immobilized fibrinogen or **E.** collagen (n=3 each). **F.** Pulmonary thromboembolism was induced by injecting *Mir-99b/let7e/125a* −/− mice or littermates with collagen and epinephrine (n=10 each). Time to death was monitored and plotted.