The role of Tyro 3 subfamily receptors in the regulation of hemostasis and megakaryocytopoiesis

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ABSTRACT

Background and Objectives

The molecular mechanisms regulating megakaryocytopoiesis and hemostasis remain largely unknown. The Tyro 3 subfamily of receptor tyrosine kinases (RTK), which is composed of three members (Tyro 3, Axl and Mer), plays important roles in various tissues, such as those in the nervous, immune and reproductive systems. Here, we investigate the roles of the Tyro 3 RTK subfamily in regulating megakaryocytopoiesis and hemostasis.

Design and Methods

Single, double, and triple knock-out mice for the three Tyro 3 RTK were used in the study. Bleeding time, platelet count, megakaryocyte count, megakaryocyte ploidy, rate of proplatelet formation, platelet aggregation and ATP release were used as criteria to evaluate hemostasis, megakaryocytopoiesis and platelet function.

Results

Mice lacking all three receptors had impaired hemostasis and mild thrombocytopenia, which may be due to platelet dysfunction and defective megakaryocytopoiesis. Mice lacking different combinations of two receptors of the Tyro 3 RTK subfamily had normal platelet counts in peripheral blood, but exhibited impaired hemostasis and platelet function. Although knock-out mice for any single receptor had normal hemostasis and megakaryocytopoiesis, they exhibited a mild platelet dysfunction.

Interpretation and Conclusions

The Tyro 3 RTK subfamily plays important roles in regulating hemostasis, megakaryocytopoiesis and platelet function.

Key words: Tyro 3 RTK subfamily, hemostasis, megakaryocytopoiesis, platelet.

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Megakaryocytopoiesis is a complex and well-orchestrated cell differentiation process that involves the initial expansion of committed megakaryocytic progenitor cells, maturation of megakaryocytes (MK), and fragmentation into platelets.[1] Endomitosis is a unique event during this process, by which the usual tight coupling of DNA synthesis and cell division is dissociated in MK, resulting in a cell containing up to 128 times the normal chromosomal complement.[2] With expansion of the DNA, the MK undergo significant cytoplasmic maturation as demarcation membrane systems, granules and organelles are formed during their development.[3] Many factors and cellular interactions in bone marrow niches regulate MK maturation and platelet production. Thrombopoietin is thought to the primary regulator. Mice deficient in thrombopoietin (*Tpo*$^{-/-}$) or its receptors c-MPL (*Mpl*$^{-/-}$) have only 10% of the normal platelet count.[4,5] Other MK-active growth factors and chemokines such as interleukin (IL)-3, IL-6, IL-11, leukemia inhibitory factor, SDF-1 and FGF4 act to coordinate MK ontogeny in synergy with thrombopoietin.[6] Though MK have been studied for over a century, molecular mechanisms regulating megakaryocytopoiesis are still unclear.

Receptor tyrosine kinases (RTK), cell surface receptors with protein tyrosine kinase activity in their cytoplasmic regions, are responsible for transmembrane signal transduction after binding of the extracellular portion of the receptors to their ligands. They play critical roles in mediating cell-cell communication and interactions to regulate cell survival, proliferation and differentiation. The Tyro 3 RTK subfamily has three members, Tyro 3, Axl and Mer.[7] These three receptors share a distinctive structure of the extracellular regions composed of two immunoglobulin-related domains linked to two fibronectin type-III repeats and cytoplasmic regions that contain an intrinsic protein-tyrosine kinase domain. The product of growth arrest-specific gene 6 (Gas6) has been identified as the common ligand of the Tyro 3 subfamily.[8] Gas6 is composed of an N-terminal region containing 11 γ-carboxyglutamic acid residues (Gla domain), four EGF-like repeats, and a large C-terminal sex hormone-binding globulin (SHBG)-like structure. The SHBG domain is believed to interact directly with the receptors, while the Gla domain appears to facilitate this interaction in an indirect manner.[9] The Tyro 3 RTK subfamily can also be bound and activated by protein S, an anticoagulant in the blood coagulation cascade, whose structure is closely related to that of Gas6.[10]

Tyro 3 subfamily receptors are widely expressed in various mammalian tissues such as reproductive, lymphoid, vascular, neural and hematopoietic tissues, and in tumor cells derived from these sources.[11] Previous studies showed that the Tyro 3 subfamily receptors were involved in cell growth, differentiation, adhesion, migration and phagocytosis.[7] Genetic studies using gene knock-out models have provided direct insights into the physiological functions of the Tyro 3 subfamily receptors.[11-14] Mice lacking any single receptor or any combination of two receptors were viable and capable of producing apparently healthy offspring. However, the triple mutants displayed multiple major defects, including neurological abnormalities, severe lymphoproliferative disorders accompanied by autoimmunity, male sterility and impaired natural killer (NK) cell differentiation. The results of these studies suggested that the three members of the Tyro 3 RTK subfamily functioned in a redundant manner. Here, we report the functions of Tyro 3 subfamily receptors on regulation of megakaryocytopoiesis and hemostasis.

Design and Methods

Mice

Mice lacking Tyro 3 subfamily receptors were kindly provided by Dr. Lemke (Salk Institute for Biological Studies, La Jolla, CA, USA). All the genotypically different mice were produced by cross-mating single, double and triple mutant mice. The normal mice used as controls were also obtained from the mating for maintenance of similar genetic backgrounds. Mice were bred and housed in an air-conditioned, pathogen-free animal room. All animals were handled in compliance with the guidelines for the care and use of laboratory animals established by the Chinese Council on Animal Care. The mice used in this study were 10-week old females.

Antibodies and reagents

Rat anti-mouse Tyro 3 monoclonal antibody was purchased from R&D Systems, Inc. (Minneapolis, MN, USA) and goat anti-mouse Axl and Mer polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Fluorescein isothiocyanate (FITC)-rat anti-mouse CD41 antibody and Mouse BD Fc Block™ were obtained from BD Pharmingen Inc. (San Jose, CA, USA). Acetylthiocholine iodide and propidium iodide (PI) were purchased from Sigma Inc (St. Louis, MO, USA). Equine type I collagen, ADP, ATP standard and Chronolume reagent were purchased from Chrono-log Co. (Havertown, PA, USA).

Analysis of hemostasis

Bleeding time was assessed using a previously described method.[15] In brief, mice were anesthetized with a mixture of ketamine and xylazine (100 mg/kg ketamine plus 20 mg/kg xylazine, administered intraperitoneally). The tail was amputated 2 mm from the tip. A stopwatch was started immediately upon transection to determine the time to cessation of bleeding. Blood drops were removed every 30 seconds using a filter paper. If bleeding did not recur within 60 seconds of cessation, bleeding was considered to have stopped. Any continued bleeding was stopped by cauterization at 15 minutes to prevent hypovolemic shock in cases.

Blood analysis

Peripheral blood was collected into K_3EDTA-containing

tubes via the vena cava under inhalation anesthesia (isoflurane). The blood samples (100 μL) were immediately diluted in 300 μL of phosphate-buffered saline (PBS) containing K_3EDTA and analyzed on an ADVIA 2120 hematology system blood cell analyzer (Bayer Diagnostics, Germany).

Ploidy analysis

Ploidy profiles were analyzed as previously reported.[16] Briefly, bone marrow cells were labeled with a saturating concentration of FITC–rat anti-mouse CD41 antibody for 60 minutes on ice after preincubattion with Mouse BD Fc Block™ for 10 minutes. The cells were then washed, and incubated in a hypotonic citrate solution containing 50 mg/mL PI (Sigma) for 2 hours at 4°C. The cells were treated with 50 mg/mL RNase (Sigma) for 30 minutes followed by filtering through a 200-mesh filter, and analyzed on a FACScan system (BD Biosciences, San Diego, CA, USA). Splenocytes were isolated by grinding spleen fragments which were then filtered through a 200-mesh sieve. To enrich splenic MK, splenocytes were centrifuged in a 50% Percoll/CATCH solution as previous described.[17] The intermediate layer was recovered and placed on top of a bovine serum albumin (BSA) density gradient. Following the effect of gravity for 40 minutes at room temperature, enriched MK were collected from the bottom and ploidy analysis was performed as above.

Proplatelet formation assay

Bone marrow cells from both femora were collected in CATCH buffer (1×Hanks balance salt solution, 5% fetal bovine serum, 0.38% sodium citrate, 1 mM adenosine, 2 mM theophylline) and filtered through a 200-mesh filter. MK were enriched by a modified two-step separation technique.[18] Briefly, the cell suspension was adjusted to 2×10^7 cells/mL, and overlaid on a discontinuous BSA (Sigma) density gradient (0%/1.5%/3.0% BSA in CATCH buffer) in a 50 mL centrifuge tube. The tube was stood for 40 minutes at room temperature. MK were collected from the bottom of the tube. Proplatelet formation assays were performed as described previously.[19] Briefly, the enriched MK were cultured in serum-free medium (S-clone; Sanko) containing 1% BSA. Four thousands cells in 200 μL of medium were plated per well in a 96-well plate. Each condition was replicated in three wells. At 6, 12 and 24 hours, the number of MK forming proplatelets was counted by phase contrast microscopy.

Transmission electron microscopy

Bone marrow flushed from the femoral cavity was fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.6) for 2 hours at 4°C and post-fixed in osmium tetroxide for 60 minutes at 4°C. The samples were then dehydrated in alcohol at progressively higher concentrations and embedded in Epon 812 resin (Polysciences, Warrington, PA, USA). Consecutive thin and ultrathin sections were cut using a Reichert ultramicrotome. Ultrathin sections were collected on 100-mesh copper grids, counterstained with uranyl acetate and lead citrate and observed under an EM 109 Zeiss electron microscope. Ultrastructure analysis was performed on samples from three mice.

Statistical analysis

Results are presented as means ± SD. The data were analyzed using ANOVA test or Student's t test statistics with SPSS 10.0 software. In the statistic analysis, the level of significance of hypothesis testing (α) was 0.05.

Results

Analysis of hemostasis

We noticed that mice mutant for all the three *Tyro 3*, *Axl* and *Mer* genes exhibited impaired hemostasis of blood following tail-tip transection. We, therefore, analyzed hemostasis in single, double and triple mutant mice. The results are shown in Figure 1. The mean bleeding time in control mice was 4.18 minutes. Mice lacking a single Tyro 3 subfamily receptor (*Tyro 3*$^{-/-}$ [T], *Axl*$^{-/-}$ [A] or *Mer*$^{-/-}$ [M]), had normal bleeding times compared to control mice (on average, 4.33, 4.55, 4.08 minutes, respectively). However, mice with double or triple mutations of Tyro 3 subfamily receptors had prolonged bleeding times. The mean bleeding times of double null mutant mice, *Tyro 3*$^{-/-}$ *Axl*$^{-/-}$ (TA), *Tyro 3*$^{-/-}$ *Mer*$^{-/-}$ (TM) and *Axl*$^{-/-}$*Mer*$^{-/-}$ (AM), were 8.91, 8.6, 10.48 minutes, respectively. The triple null mutant, *Tyro 3*$^{-/-}$ *Axl*$^{-/-}$ *Mer*$^{-/-}$ (TAM), displayed the most severely impaired hemostasis. In our study, bleeding did not stop within 15 minutes in 70% of TAM mice (the bleeding was arbitrarily stopped at this time point to avoid possible death from hypovolemic shock). These data suggested that Tyro 3 subfamily receptors play important roles in regulating hemostasis.

Thrombocytopenia in TAM mice

To investigate the factors contributing to the impaired hemostasis in mice lacking Tyro 3 subfamily receptors, we further analyzed peripheral blood samples of mutant mice with different genotypes and normal littermates for their hematologic profile. The mean values of eight female mice for each genotype are presented in *Supplementary Table 1*. The numbers of white blood cells, lymphocytes, neutrophils and monocytes in the peripheral blood were slightly increased in MT, TA and TAM mice. The red cell count and hemoglobin concentration were comparable in the mice of each genotype. Only triple mutant mice displayed mild thrombocytopenia. The platelet count of TAM mice was less than 50% that of control mice (*Supplementary Table 1 online*). This decreased number of platelets was not observed in any single or double mutant mice. These results indicate that Tyro 3 subfamily receptors could regulate platelet biogenesis in a redundant manner.

Impaired platelet function in mice lacking Tyro 3 RTK subfamily receptors

Since a reduction of half the number of platelets in the triple knock-out mice did not account for the impaired hemostasis in in these animals, we analyzed platelet function by platelet aggregation and ATP release assays. As shown in *Supplementary Figure 1A and B*, platelets from control mice achieved 61.5% maximal aggregation in response to a low concentration of ADP (2 μM). In contrast, platelets from single mutant mice displayed mild decreases in aggregation rates (about 75% of normal control). Platelets from double and triple mutant mice exhibited evident decreased aggregation rates (about 50% and 28% of normal control rates, respectively). We also used a higher concentration of ADP (5 μM) as the platelet stimulant. Platelets from normal mice were induced into irreversible aggregation, and exhibited 71% maximal aggregation. However, this concentration of ADP failed to induce irreversible aggregation of platelets from Tyro 3 RTKs knockout mice and the extent of platelet aggregation reduced significantly (about 84%, 52% and 49% of normal control aggregation on average for single, double and triple mutant mice, respectively). Type I collagen, another agonist, elicited clear shape changes in normal platelets followed by 60.5% maximal aggregation at a concentration of 4 μg/mL (*Supplementary Fig. 1 C, D online*). In contrast, platelets of single mutant mice displayed 36.6-42.2% maximal aggregation. However, platelets of double mutants exhibited very low aggregation rates (less than 8%) after stimulation, and platelets of TAM mice only showed shape changes. A lower concentration of collagen (2 μg/mL) was only able to induce shape changes in platelets from double or triple mutant mice (*data not shown*). Consistent with the results of platelet aggregation, secretion of dense granule stores (evaluated by measuring the release of ATP) was mildly impaired in platelets from single mutant mice and severely impaired in platelets from double and triple mutant mice. With 5 μM ADP, wild-type platelets released 0.56 nmoles of ATP, while the platelets of single, double and triple mutant released, on average, 0.43, 0.12 and 0.09 nmoles of ATP, respectively (*Supplementary Figures 1 E, F online*). Taken together, the results show a mild defect of platelet function in the single knockout mice, an intermediate phenotype in the double mutants and most severe dysfunction in TAM mice, suggesting that the prolonged bleeding time in double and triple mutant mice should be due to severe platelet dysfunction.

Expression of Tyro 3 subfamily receptors in MK

A previous study showed that Tyro 3 subfamily receptors are expressed in platelets,[20] as argued by another study.[21] Here, we examined the pattern of expression of Tyro 3, Mer, and Axl proteins in MK, precursors of platelets. As shown in *Supplementary Figure 2A*, MK displayed strong positive immunostaining of all three receptors, while neutrophil cells and lymphocytes showed no staining. Besides MK, some unidentified cells in the bone marrow were also positively stained for the receptors. Based on earlier studies,[14,22] these cells could be monocytes or $CD34^+$ hematopoietic stem cells. The expression of the three receptors in MK was confirmed by RT-PCR. The specific bands for all three receptor genes were strongly detected in the RNA from enriched MK (*Supplementary Figure 2B online*).

Decreased MK numbers in bone marrow and elevated splenic megakaryocytopoiesis in AM and TAM mice

One of the direct causes of thrombocytopenia is a reduction in the number of MK in bone marrow. We therefore examined MK counts in femora after staining for acetylcholine/esterase, a marker of murine MK.[23] As shown in *Supplementary Figure 3*, the number of MK per femur in single mutant mice (T, A, M), as well as in TA and TM mice did not differ significantly from that in control mice (n=5), being about 8730 MK per femur. However, the number of MK per femur in TAM mice was significantly lower, being only 60% of that in control mice. Surprisingly, although platelet counts in AM mice were normal, the number of MK per femur was decreased, being 65% of that in control mice. These results suggest that the combination of Axl and Mer might play more important roles in megakaryocytopoiesis compared to other two combinations of Tyro 3 RTK: Tyro 3 plus Axl and Tyro 3 plus Mer.

MK rarely appeared in the spleen of normal adult mice, but were often found in the spleen when megakaryocytopoiesis in bone marrow was disrupted.[24,25] Thus we also examined the number of splenic MK in mutant mice. The spleens of AM and TAM mice contained a significantly increased number of MK (*Supplementary Figure 4A,B*), with MK counts being approximately 8 and 9 times, respectively, higher than those of the 10-week old control mice, indicating that megakaryocytopoiesis happened in the spleens of AM and TAM mice. By contrast, the splenic MK counts of any single mutant mice (T, A, M), as well as the double mutant TA and TM mice were not significantly different from those of control mice.

Ploidy profiles of MK

DNA ploidy can reflect rates of megakaryocytopoiesis, so we next examined DNA content and ploidy frequency distributions of MK in the genotypically different mice. Representative DNA content distributions of bone marrow MK in TAM and control mice are shown in Figure 2 *and Supplementary Table 2.* The major polyploid DNA content of MK from control mice was 16N, occurring in 48.6% of all MK. Other majority populations of MK were 2N (14.3%), 4N (7.7%), 8N (11.6%) and 32N (14.3%). The 64N and 128N populations of MK accounted for less than 2% in control mice. This ploidy profile was disrupted in TAM mice. The frequency of MK with 2N and 4N DNA content in TAM mice increased markedly, reaching 29.6% and 14.8%, respectively, while the proportion of MK with 16N DNA content decreased to 28.5%. The percentage of 32N or higher level ploidy in TAM mice did not alter significantly. The ploidy profile in AM mice was similar to

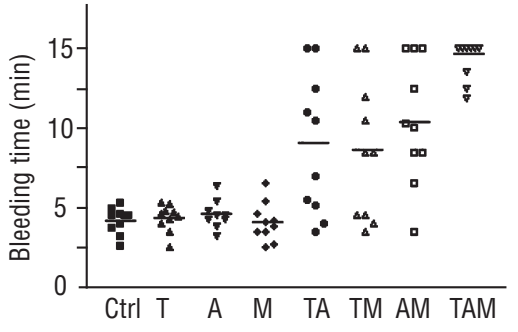

Figure 1. Tail-bleeding times. Bleeding time was determined using a standardized model (*see Design and Methods*). Each symbol represents the bleeding time of one mouse and mean values (n=10) are indicated by bars. Single mutant mice (T, A, M) had normal bleeding times (4.33, 4.55, 4.08 minutes respectively) similar to that of the control (Ctrl) mice (4.18 minutes), while prolonged bleeding time was observed in TA, TM, AM and TAM mice. TAM mice had the most severe hemostatic impairment bleeding failed to stop within 15 minutes in 70% of these animals.

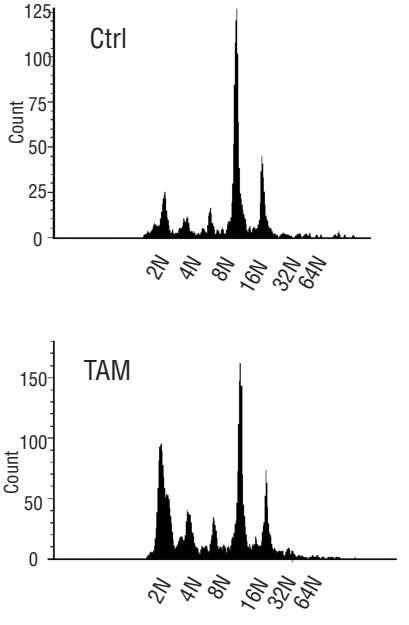

Figure 2. DNA contents of bone marrow MK. Bone marrow MK from control and TAM mice were collected from femora and stained with the FITC-anti-CD41 antibody and propidium iodide. The proportion of cells in each ploidy class was analyzed by flow cytometry. There was an increase in MK with 2N/4N DNA contents and a decrease of MK with 8N/16N DNA contents in TAM mice compared in controls (Ctrl).

that of TAM mice, exhibiting a significantly lower level of polyploidy compared to that of control mice. DNA ploidy in single mutant (T, A, M), and TA and TM mice was not significantly different from that in control mice (*Supplementary Table 2*).

We also enriched splenic MK and detected DNA ploidy classes. Due to the rarity of the MK, we could not analyze DNA contents of MK from control mice, single mutant and AT mice by flow cytometry. As illustrated in *Supplementary Figure 5*, most splenic MK from MT mice were predominately 2N and 4N (61.5% and 20.7%, respectively), and the frequency of 16N and 32N was less than 10%. However, the splenic MK of AM and TAM mice showed marked increases in the proportion of cells with a DNA content greater than 8N (23% and 46.2% in AM and TAM mice, respectively), indicating that more mature MK were present in the spleen of AM and TAM mice. Morphological observation of MK after staining for acetylcholine/esterase revealed comparable numbers of mature MK in TAM spleen and control bone marrow (*Supplementary Figure 5B*). These data indicate effective megakaryocytopoiesis in spleens of TAM mice.

Defective proplatelet formation by MK from AM and TAM mice

To further study the characteristics of MK from TAM mice, we examined the rate of proplatelet formation by MK *in vitro*. Enriched MK were cultured in serum-free medium containing 1% BSA. At 6 hours after culture, a proportion of control MK formed long and beaded proplatelets (Figures 3A and C), and the rate of proplatelet formation reached a peak at 24 hours. Single mutant mice (T, A, M), as well as TA and TM mice displayed normal rates of proplatelet formation (Figure 3E). In contrast, very few MK from AM and TAM mice could develop proplatelets and the majority of them were still round after 24 hours of culture (Figures 3 B,D). The percentage of proplatelet formation by AM and TAM MK was only 50% and 20%, respectively, of that by control MK (Figure 3E), indicating impaired terminal maturation of MK from AM and TAM mice.

Changes in the intracellular demarcation membrane system of TAM MK

To further characterize morphologic changes of MK from TAM mice, we performed ultrastructural analysis on MK by transmission electron microscopy. As shown in Figure 4, the majority of control mature MK exhibited well-demarcated cytoplasmic territories with extensive dilation of the demarcation membrane system and numerous organelles (Figures 4A,C). The demarcation membrane system of MK, which has been described as a membrane reservoir for proplatelet formation,[3] was dilated and regularly scattered in the cytoplasm of control MK. However, most of the TAM MK exhibited abnormal cytoplasmic territories with a constricted and parallel membrane system (Figure 4B, D). In the cytoplasm of TAM MK, we found many vacuoles that were not present in control MK. These results indicate that TAM MK had disordered cytoplasmic maturation, which could result in low efficiency of platelet formation.

Discussion

The three members of the Tyro 3 RTK subfamily (Tyro 3, Axl, and Mer) are preferentially expressed in the nervous, immune and reproductive systems. Single, double and triple knockout mice for Tyro 3, Axl and Mer have demonstrated that these three receptors play essential roles in these locations.[11-14] It was recently reported that loss of Gas6, a common ligand of Tyro 3 subfamily receptors, protected mice against thrombosis.[20] The mice lacking any one

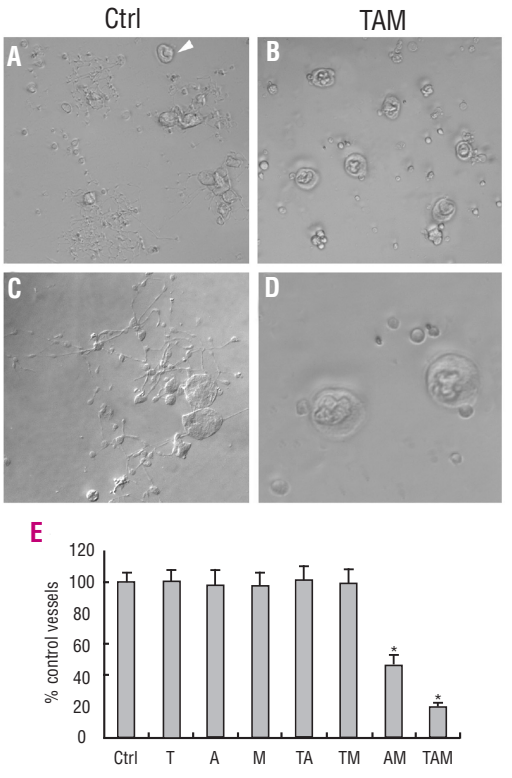

Figure 3. Proplatelet formation assays of MK. MK fractions (enriched by a BSA density gradient) were incubated in S-clone supplemented with 1% BSA and subjected to examination by phase contrast microscopy at different times. A and C show proplatelet-forming MK from control (Ctrl) mice after culture *in vitro* for 24 hours. The arrowhead shows a MK without proplatelet formation. B and D indicate that most MK from TAM mice do not form proplatelets at 24 hours after culture. E shows the rate of proplatelet formation by MK from genotypically different mice at 24 hours after culture (*$p<0.05$). The data are averages ± SD for three mice. Original magnification, ×200 for panels A and B and ×400 for panels C, D.

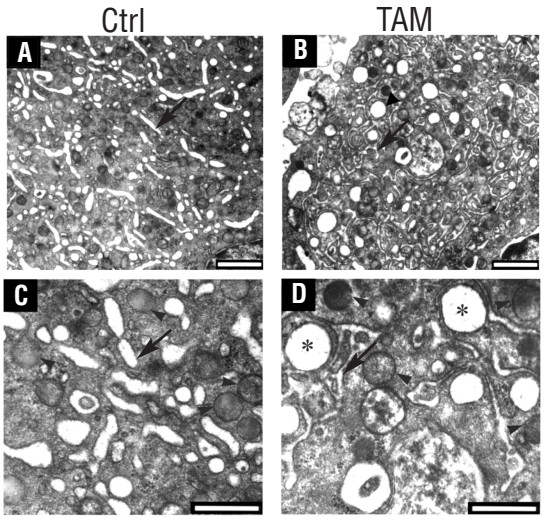

Figure 4. Ultrastructure of MK. Bone marrow from mouse femora was visualized under electron microscopy. The upper and lower panels represent the same cells from control (Ctrl) (A,C) and TAM (B,D) mice at different magnifications (original magnification, ×5000 for the upper panels and ×15,000 for the lower panels). Control MK show well-formed platelet territories with extensive dilation of the demarcation membranes (arrows in A,C), while MK derived from TAM exhibit poorly developed and disordered demarcation membrane systems (arrows in B,D). Moreover, many vacuoles (asterisks) are present in the cytoplasm of TAM MK. Arrowheads indicate α-granules. The bars of the upper and lower panels represent 1 μm and 0.5 μm, respectively.

of these receptors had a phenotype similar to that of Gas6$^{-/-}$ mice, protecting the animals against pathological thrombosis *in vivo* by preventing the formation of stable platelet plugs.[15] These mice had normal bleeding times, platelet counts, and megakaryocytopoiesis. In this study, we found that Tyro 3 subfamily receptors are co-expressed in MK and regulate hemostasis and megakaryocytopoiesis in a redundant manner.

The RTK are a large family of transmembrane proteins with great diversity in their extracellular regions and a common highly conserved intracellular tyrosine kinase domain.[7] Each member of the Tyro 3 subfamily possesses distinct expression profiles as well as discrete functions.[7] Tyro 3 is a neurotrophic factor receptor.[26,27] Axl preferentially regulates cell survival, proliferation and migration.[28,29] Mer mediates ingestion of apoptotic cells by phagocytes, such as macrophages[30] and retinal pigment epithelial cells.[31] In this study, we showed that the three receptors are coexpressed in MK. Mice lacking any single receptor had mild platelet dysfunction, but normal hemostasis and megakaryocytopoiesis. However, double and triple mutant mice had severe platelet dysfunction and impaired hemostasis. Apparent defective megakaryocytopoiesis was only observed in TAM and AM mice. These results suggest that the three receptors play different roles in regulating these processes. The collaborative functions of Tyro 3 subfamily receptors have been reported with respect to regulation of spermatogenesis, natural killer (NK) cell differentiation and immune homeostasis.[11,12,14] Consistent with the functional collaboration of Tyro 3 subfamily receptors, co-expression of the three receptors is frequently observed within the same cell, such as Sertoli cells, dendritic cells, monocytes, NK cells as well as MK in this study, which facilitates their funtions.

A previous study demonstrated that the Tyro 3 RTK subfamily is involved in pathological thrombosis by regulating the formation of stable platelet plugs, and the deficiency of any one of the receptors protected mice against thrombosis to the same significant extent, which suggested that each of these receptors had a comparable role in platelet aggregation.[15] In the present study, although mice lacking any one of the three receptors (Tyro 3, Axl, or Mer) had normal bleeding times, the mice lacking any combination of two receptors of the Tyro 3 subfamily had prolonged bleeding times. All single and double mutant mice had normal platelet counts in peripheral blood. These results suggest that prolonged bleeding times are not due to impaired platelet biogenesis but are due, at least in part, to platelet dysfunction. In order to evaluate the platelet function, we performed platelet aggregation and ATP release assays. The results showed a mild defect of platelet function in the single knockout mice, an intermediate phenotype in the double mutants and the most severe dysfunction in the TAM

mice. The increased bleeding time was probably due to the severe platelet function abnormality in TAM mice, although mild thrombocytopenia was observed in these triple mutant mice. A 50% decreased in platelet count normally has no effect on hemostatic capabilities. These observations indicate that Tyro 3 RTK co-operatively regulate platelet function, and are, thus, involved in hemostasis. Notably, though mice double mutant for different combinations of two receptors (TA, AM, or TM) had normal platelet levels, they displayed different megakaryocytopoiesis phenotypes.

Megakaryocytopoiesis was impaired in AM mice, but less severely than in TAM mice. By contrast, both TA and TM mice had normal megkaryocytopoiesis. These results suggest that the three members of the Tyro 3 RTK subfamily play different roles in regulating megakaryocytopoiesis, and that the combination of Axl and Mer could play a more critical role in megakaryocytopoiesis than that of the other two combinations (TA and TM).

Platelets are formed and released into the bloodstream by MK in bone marrow. Each MK has been estimated to generate and release thousands of platelets.[32] Before a MK has the capacity to release platelets, it undergoes significant maturation as internal membrane systems, granules and organelles are assembled. In humans, thrombocytopenia (platelet count less than 150,000/μL) can lead to inadequate clot formation and increase bleeding times, while thrombocythemia (platelet count greater than 600,000/μL) can increase the risk of thrombotic events, including stroke, peripheral ischemia and myocardial infarction.[33] There are different mechanisms involved in platelet biogenesis. The majority of experimental evidence supports a proplatelet-based mechanism. In this model, platelets are assembled along essential intermediate cytoplasmic extensions, called proplatelets.[33] In the absence of such extensions, only a few platelets are released.[34,35] In this study, proplatelet formation by MK from TAM mice was substantially less than that by MK from control mice, which may be a cause of the thrombocytopenia in TAM mice. In agreement with this finding, it was observed, by electron microscopy, that TAM MK have a poorly developed demarcation membrane system, which has been described to be the origin of platelets, reflecting defects in MK differentiation in these mice. Notably, the marked defect in proplatelet formation is inconsistent with the mild decrease in peripheral blood platelet count in TAM mice. Particularly, the defect of proplatelet formation does not decrease the platelet numbers in AM mice. This inconsistency may reflect the importance of the microenvironment in megakaryocytopoiesis. The proplatelet formation of MK from TAM mice may be more severely defective *in vitro*. Although apparent defects of megakaryocytopoiesis were observed in bone marrows from TAM and AM mice, evident splenic megakaryocytopoiesis occurs in these two types of mice. Moreover, a large number of splenic MK can develop into mature MK with 16N and 32N DNA in TAM and AM mice. Therefore, the splenic megakaryocytopoiesis may compensate for the lowered peripheral blood platelet count. This speculation needs to be further investigated.

The mechanisms by which the triple mutation of Tyro 3 RTK causes impaired megakaryocytopoiesis are unknown. The fact that the three members of the Tyro 3 RTK subfamily are co-expressed in MK suggests an autonomous defect of MK differentiation due to mutation of the genes. However, the fact that mature MK can be formed in spleens of mutant mice does not support this suggestion. Most likely, impaired megakaryocytopoiesis is due to a disruption of the marrow microenvironment, hampering MK development. Dormady *et al*. showed that Gas6-transfected 3T3 cells could support hematopoiesis, but control 3T3 cells or soluble Gas6 (added to cultures of 3T3 cells) had no effect on supporting hematopoiesis. It was postulated that membrane-associated Gas6 could act on progenitor cells facilitating their adhesion to the stromal cells or serve as a ligand to promote cell-cell contacts between stem/progenitor cell and stromal cells.[36] Tyro 3 subfamily receptors contain both immunoglobulin and fibronectin III repeats reminiscent of cell adhesion molecules such as NCAM and L1. Several studies have demonstrated that Gas6 and Tyro 3 subfamily receptors can mediate adhesion of cells.[37,38] It has been reported that endothelial cells and marrow stromal cells express Tyro 3 RTK subfamily receptors and their ligand Gas6.[22,39,40] It is possible that Gas6 and Tyro 3 subfamily receptors promote intimate contacts between MK/MK progenitors and stromal cells/or bone marrow endothelial cells during megakaryocytopoiesis, and that loss of Tyro 3 subfamily receptors would disrupt this supportive effect to megakaryocytopoiesis. We are now investigating this possibility.

In summary, we here show that double mutant mice for Tyro 3 subfamily receptors have a prolonged bleeding time, and that mice lacking all three members of the subfamily have mild thrombocytopenia and more severe impaired hemostasis. The decreased platelet count could be related to a severe defect in megakaryocytopoiesis. The impaired hemostasis is most likely due to platelet dysfunction. The results suggest that Tyro 3 subfamily receptors play important roles in regulating megakaryocytopoiesis and platelet function. Moreover, the mutant mice for the Tyro 3 RTK subfamily receptors may provide a useful model for studying the microenvironment of megakaryocytopoiesis.

Authors' Contributions

HW, DH conceived the study. The study was performed by HW and SC's group, who also performed all the pilot experiments and most of the analyses. YC performed the ultrastructural analysis on megakaryocytes. HW, HW, HT, WX, JM, YG, and QL performed the bench work and obtained all data for this study. HW and DH wrote the manuscript.

Conflict of Interest

The authors reported no potential conflicts of interest.

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