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Loss of *GATA1* and gain of *FLI1* expression during thrombocyte maturation

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Abstract

In this paper we characterized expression of *GATA1* and *FLI1* gene promoters in thrombocytes of zebrafish transgenic lines, G1-GM2 and TG(fli1:EGFP)y1 that carry transgenes of *GATA1* and *FLI1* gene promoters driving GFP. We found two discrete populations of thrombocytes verified by morphology, labeled with GFP in both G1-GM2 and TG(fli1:EGFP)y1 lines: (1) the more intensely labeled GFP+ thrombocyte, and (2) the less intensely labeled GFP+ thrombocytes. The more intensely labeled GFP+ thrombocyte in G1-GM2 line and the less intensely labeled GFP+ thrombocytes in the TG(fli1:EGFP)y1 line corresponded to young thrombocytes. These results showed that young thrombocytes have higher *GATA1* promoter activity, while mature thrombocytes have more *FLI1* gene promoter transcription. This finding suggests that there is a gradual loss of *GATA1* and gain of *FLI1* expression as the thrombocytes mature, and this overexpression of *FLI1* may help maintain the thrombocyte lineage. Furthermore, the presence of transcriptional factors similar to those found in megakaryocytes raises the possibility that vertebrate thrombocytes may be the forerunners of mammalian megakaryocytes and, therefore, could serve as a model to study megakaryocyte maturation.

INTRODUCTION

Differentiation of megakaryocytes in mammals requires three transcription factors, namely *GATA1*, *FOG1* and *FLI1*^{1,2}. *GATA1* is a zinc-finger transcription factor that has been found in erythroid, megakaryocytic, mast and eosinophilic cells, and has been proposed to be involved in their maturation². *GATA1* interacts with a zinc finger cofactor called *FOG1* via zinc fingers³. *FLI1*, a member of the Ets family of transcription factors found in endothelial cells, has been shown to interact with *GATA1*, resulting in synergistic activation of megakaryocyte-specific genes⁴. *GATA1* and *FLI1* gene promoters have been used to drive the green fluorescent protein (GFP) gene in transgenic zebrafish lines: G1-GM2 and TG(fli1:EGFP)y1^{1,2}. These

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lines represented *GATA1* and *FLII* synthesis, and have been used to understand erythroid differentiation and vasculogenesis. In the TG(fli1:EGFP)y1 line, certain blood cells in embryonic and larval stages have been found to be labeled by GFP. It has been suggested that these blood cells could be circulating myeloid cells. However, it is difficult to rule out the possibility that these cells are megakaryocytes known to express *FLII*².

In this paper, we used G1-GM2 and TG(fli1:EGFP)y1 lines and found these gene promoters are active in thrombocytes making the thrombocytes GFP positive. We also found that there are two populations of thrombocytes in G1-GM2 and TG(fli1:EGFP)y1: the more intense GFP⁺ and less intense GFP⁺ thrombocytes. The more intense GFP⁺ thrombocytes in G1-GM2 and the less intense GFP⁺ thrombocytes in TG(fli1:EGFP)y1 corresponded to the young thrombocytes and vice versa⁷. These results suggest that *GATA1* is reduced in mature thrombocytes with increased *FLII* expression. Therefore, we propose that *GATA1* that has been shown to be required for erythroid synthesis slowly disappears as the thrombocytes mature, and the increased *FLII* compensates for the maintenance of thrombocyte lineage.

MATERIALS AND METHODS

Transgenic zebrafish

Generation of homozygous transgenic zebrafish containing *GATA1* gene promoter driving GFP and *FLII* gene promoter which drives EGFP were previously reported^{1,2}. The transgenic line TG(fli1:EGFP)^{y1} has a 5'-promoter containing *XbaI* fragment derived from a PAC clone with an EGFP inserted just upstream of the *FLII* start codon and contains >25 copies of the transgene. The transgenic line G1-GM2 contains a *GATA1* gene promoter followed by a modified GFP that was made by fusing the 5'-end of wild type GFP and 3'-end of GFP variant m2. This modified GFP has 30 fold greater fluorescence than the wild type GFP. The fish were kept in a water recirculation system and used for thrombocyte analysis. Anesthesia was performed as described earlier⁷.

Histology of thrombocytes

Blood collected from G1-GM2 and TG(fli1:EGFP)y1 zebrafish was smeared on microscopic slides. Bright field images and GFP fluorescent images excited at 450–490 nm were acquired using a Nikon E995 Coolpix digital camera mounted on a fluorescent microscope. After noting the coordinates for the position of the slide on the microscope, it was then stained with Wright-Giemsa staining as described earlier⁸. The slide was mounted back on the microscope at the previously noted coordinates, and the Wright-Giemsa stained image was obtained using the bright field view.

Fluorescence microscopy and flow cytometry

Thrombocytes were labeled *in vivo*⁹ by injecting G1-GM2 and TG(fli1:EGFP)y1 zebrafish with 10 µl of the diluted solutions of DiI-C₁₈ (DiI) (10 µM) prepared from 10 mM DiI (in DMF) stock solution. After 20 minutes, blood was collected⁸ into an equal amount of heparin solution (20 mg/ml in PBS), and cells were either smeared or kept under a cover slip for observations under a fluorescent microscope. The DiI labeled thrombocytes were observed by fluorescence with excitation at 510–560 nm⁹. GFP labeled thrombocytes and dually labeled thrombocytes were observed by fluorescence with excitation at 450–490 nm⁹. Thrombocytes were sorted by FACS as described earlier⁷.

In vitro thrombocyte aggregation and *in vivo* arterial thrombosis

Whole adult blood aggregation was induced by adenosine diphosphate (ADP) as described earlier⁷. For arterial thrombosis experiment, five day old TG(fli1:EGFP)y1 zebrafish larvae

were placed on a microscopic slide under a fluorescent microscope after anesthesia with Tricaine (10 μ M) solution⁸. Arterial thrombosis was induced in the caudal artery (between the fifth and seventh somite) of the zebrafish larva using a nitrogen pulsed laser passed through coumarin 440 dye (445 nm) (MicroPoint Laser system, Photonic Instruments Inc. IL) for 5 seconds at 15 pulses/second with a laser intensity of setting 10. The thrombus formation was recorded using a Nikon E995 Coolpix digital camera attached to a VHS recorder and a monitor¹⁰.

RT-PCR

The blood from TG(fli1:EGFP)y1 zebrafish was diluted and placed on the microscopic slide so that thrombocytes were well separated from other cells. The less intense and more intense GFP thrombocytes were pipetted separately using a Nanoject II micropipette (Drummond Scientific Company, Broomall, PA) under Nikon Eclipse 80i (with excitation at 450–490 nm) and were used in cell to cDNA kit (Agilent Technologies, LaJolla, CA) to amplify the *GATA1*, *FLI1* and Elongation Factor 1 α control (EF1- α) mRNA. We designed forward 5'-ATGAACCTTTCTACTCAAGCT-3' and reverse 5'-GCTGCTTCCACTTCCACTCAT-3' primers for *GATA1*, forward 5'-CACAAAATCAACCCCATTC-3' and reverse 5'-ATGGCCCAGTCTAACCAGT-3' primers for *FLI1*, and forward 5'-CGGTGACAACATGCTGGAGG-3' and reverse 5'-ACCAGTCTCCACACGACCCA-3' primers for *EF1- α* respectively; these were synthesized by Biosynthesis, Lewisville, TX. They were used to simultaneously amplify the 410 bp product for *GATA1*, 299 bp product for *FLI1* and 220 bp product for the *EF1- α* control. The RT-PCR products were resolved on 1.5% agarose gels and their DNA sequences were determined using sequencing service by Lone Star Labs, Houston, TX. The density of RT-PCR products was measured by Quantity One software from Bio-Rad Laboratories, Inc. Hercules, CA.

RESULTS

We previously described two distinct populations of thrombocytes that exist in zebrafish: one that was labeled by DiI (DiI+) and the other not labeled by this dye (DiI-). These DiI+ and DiI- thrombocytes have been classified as young and mature thrombocytes respectively corresponding to young and mature platelets. Even though thrombocytes are classified as platelet equivalents, they have nuclei and probably have transcriptional machinery similar to that operating in megakaryocytes. Megakaryocytes are known to be regulated by *GATA1* and *FLI1*; since the transgenic G1-GM2 and TG(fli1:EGFP)y1 lines represent *GATA1* and *FLI1* synthesis, we hypothesized that examining these fish for GFP expression in thrombocytes may provide insight into expression of these genes in megakaryocytes. Therefore, we characterized G1-GM2 and TG(fli1:EGFP)y1 transgenic zebrafish lines for expression of GFP in thrombocytes.

To identify whether thrombocytes were labeled in G1-GM2 and TG(fli1:EGFP)y1 lines, the fluorescent images of the blood smears from adult G1-GM2 and TG(fli1:EGFP)y1 fish were taken; the slides were then stained with Wright-Giemsa staining and photographed again using the same coordinates where the GFP pictures were taken. This modification was needed upon the realization that Wright-Giemsa staining quenched the GFP fluorescence. The results revealed that all GFP+ cells in TG(fli1:EGFP)y1 fish had thrombocyte morphology similar to earlier reports in which the thrombocytes were distinguishable because they were the smallest of the blood cells, mostly filled with nucleus, and had a scanty cytoplasm (Fig. 1). In G1-GM2 fish, all the red cells, leucocytes, and thrombocytes were labeled (Fig. 1). We also noted that within the two populations of the thrombocytes, in both G1-GM2 and TG(fli1:EGFP)y1 lines, one was more intensely labeled with GFP and the other that was less intensely labeled with GFP. To identify whether the two populations of thrombocytes with different intensities

observed in G1-GM2 and TG(fli1:EGFP)y1 lines corresponded to young and mature thrombocytes, we labeled the young thrombocytes with DiI in these two lines. We observed that in the TG(fli1:EGFP)y1 line, the less intense GFP+ thrombocytes were labeled with DiI, whereas in the G1-GM2 line, the more intense GFP+ thrombocytes were labeled (Fig. 2).

The TG(fli1:EGFP)y1 line was further tested as to whether the GFP+ thrombocytes aggregate in response to ADP. The results confirmed that they do aggregate as described earlier (Fig. 3). This provided additional evidence that the GFP+ blood cells are in fact functionally thrombocytes. In addition, we observed clustering of two populations of thrombocytes as noted earlier. The G1-GM2 line revealed aggregates of thrombocytes over the background of red cells and leukocytes, however, it was difficult to detect the clustering within this background (data not shown). As with our earlier report that the young thrombocytes come to the wounding site first followed by mature thrombocytes, we predicted that the less intense GFP+ thrombocytes in G1-GM2 line would come to the site of laser injury first followed by more intense GFP+ thrombocytes. When we performed the laser injury to the zebrafish larval artery, as predicted, the less intense young GFP+ thrombocytes arrived at the site of injury confirming the above finding that young thrombocytes correspond to the less intense GFP+ thrombocytes (Fig. 4). Furthermore, we observed clustering of young thrombocytes followed by the accumulation of mature thrombocyte clusters. Considering the parameters of thrombocytes in the maturation process were unknown, we determined the relative sizes of the young and mature thrombocytes by flow cytometry. We found that the young thrombocytes are smaller in size by their position in side scattering on scatter plots (Fig. 5). To provide evidence that less intense and more intense GFP+ thrombocytes have higher and lower levels of GATA1 mRNA respectively and quite the opposite levels of FLI1 mRNA, we prepared RNA from these two cell types and conducted RT-PCR. The results showed reduced levels of GATA1 and increased levels of FLI1 transcripts in more intense GFP+ thrombocytes cells and quite the contrary in the less intense GFP+ thrombocytes (Fig. 6).

DISCUSSION

In this paper we characterized transgenic lines of zebrafish, which express GFP driven by two different gene promoters GATA1 and FLI1 for the promoter expression in thrombocytes. The fact that in both G1-GM2 and TG(fli1:EGFP)y1 lines thrombocytes were GFP+ suggests that GATA1 and FLI1 gene promoters are active in thrombocytes. In the G1-GM2 line we have shown the more intense GFP+ thrombocytes are young thrombocytes and that the mature thrombocytes do not express GFP very well. Sometimes it was difficult to observe any GFP fluorescence in certain thrombocytes. Thus, from these results it is evident that the maturation of thrombocytes appears to coincide with the loss of activity of the GATA1 gene promoter, and suggests loss of *GATA1*. In contrast, in the TG(fli1:EGFP)y1 line the young thrombocytes maintain low levels of GFP expression, whereas the mature thrombocytes have high levels of GFP expression suggesting that there is enhancement of FLI1 gene promoter expression resulting in increased expression of *FLI1*. These transgene expression results are consistent with RT-PCR results in which we have observed reduced levels of endogenous GATA1 mRNA and increased levels of FLI1 mRNA in mature thrombocytes and vice versa in young thrombocytes. These findings are especially interesting due to the fact that *GATA1*, along with *FLI1*, has been shown to synergistically activate genes in megakaryocytes. Thus, since both *GATA1* and *FLI1* are present in young thrombocytes, even though *FLI1* is at low levels, this will still confer thrombocyte-specific gene expression. However, as the thrombocytes mature, the loss of *GATA1* may be compensated by the over expression of *FLI1*. It is not yet clear whether such altered expression of these factors will result in alterations in gene expression between young and mature thrombocytes. Nevertheless, the current finding that thrombocyte maturation involves transcription factors that are involved in controlling megakaryocytes is important because understanding regulation in zebrafish thrombocyte maturation might

provide insight into the gene control in megakaryocyte maturation. In addition, the observation of selective labeling of thrombocytes in TG(fli1:EGFP)y1, similar to the recently developed transgenic line where thrombocytes are labeled by GFP using GPIIb gene promoter, provides additional transgenic line for studies on thrombocyte biology.

The current work has demonstrated that young thrombocytes first appear at the site of injury. Also, the results showed clustering of young thrombocytes is followed by clustering of mature thrombocytes. These results are similar to the earlier findings using the dye labeling methods. The current results have confirmed previous findings and offer significance due to the fact that previous studies used dye labeling methods which begged the question of whether the observed thrombus formation could be due to dye effects despite the documentation that DiI labeling did not alter thrombocyte function. The current study eliminates such concerns and provides additional proof for the earlier observations.

The present work also establishes that the size of the thrombocyte is increased when a young thrombocyte matures. It is interesting to note in this context that in platelet maturation it has been claimed that the young platelets are in fact larger than mature platelets. Thus, fish thrombocyte departs from the mammalian platelet in this regard. However, it resembles the megakaryocyte maturation with respect to increases in size although the polyploidy does not appear to exist in fish thrombocytes.

In light of the above finding, as well as the previous studies, it is important to address the role of thrombocytes. Are they platelet equivalents or megakaryocyte forerunners? On one side, even though nucleated, they are found in circulation, aggregate in response to platelet agonists, and play a central role in arterial thrombus formation. Thus, physiologically, with respect to hemostatic function, one could acknowledge that they are equivalent to platelets. However, by the fact the thrombocytes are nucleated and express transcription factors that are present in megakaryocytes, in transcriptional sense they have similarities to megakaryocytes. Moreover, the young thrombocytes appear to increase in size similar to increases in megakaryocytes, although at present, the mechanism for the increases in size of thrombocytes is not known. Furthermore, thrombocytes are produced in kidney marrow in fish but they are synthesized in bone marrow in birds. Since they are roughly half the size of the mammalian red cells, they could exit the marrow easily. However, during evolution, at the time of avian and mammalian radiation, a mutation probably created polyploidization of thrombocytes, resulting in a mammalian megakaryocyte that is so large it would explain entrapment within marrow. Considering the role required for hemostasis, apoptosis of the megakaryocyte results in the release of platelets into the blood stream in mammals. As platelets are megakaryocyte vesicles, megakaryocytes should have functions that platelets possess. In fact, Shattil and coworkers have used megakaryocytes to understand platelet functions¹¹. It makes teleological sense that initially thrombocytes, the forerunners of megakaryocytes, were performing the hemostatic function and evolved to reside in the bone marrow of mammals releasing their vesicles as platelets. Based on transcriptional machinery of thrombocytes reported here, and the previous reports claiming that thrombocytes are platelet equivalents, thrombocytes provide a model system to study both megakaryocyte and platelet functions.

In summary, we have provided evidence that there is a gradual loss of *GATA1* and gain of *FLII* expression during maturation of thrombocytes therefore, making thrombocytes a novel model for studying certain aspects of megakaryocyte maturation in consideration that the thrombocytes may be the forerunners of the megakaryocytes. In addition, the finding that the circulating thrombocytes are selectively labeled in TG(fli1:EGFP)y1 fish will provide a tool in understanding thrombocyte development and differentiation in zebrafish.

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Figure 1.

Identification of thrombocytes in TG(fli1:EGFP)y1 and G1-GM2 lines. Top and bottom panels, from left to right are images of thrombocytes, under bright field, fluorescence with excitation at 450–490nm (GFP fluorescence) and stained with Wright-Giemsa stain on blood smears obtained from TG(fli1:EGFP)y1 and G1-GM2 lines, respectively. Black arrows show thrombocytes.

Figure 2.

Identification of young and mature thrombocytes in TG(fli1:EGFP)y1 and G1-GM2 lines. Top and bottom panels, from left to right show representative images of thrombocytes under bright field, fluorescence with excitation at 450–490nm and fluorescence with excitation at 510–560 nm on blood cells under a cover slip obtained from TG(fli1:EGFP)y1 and G1-GM2 lines respectively. Orange fluorescence is due to dual labeling of young thrombocyte by GFP and DiI.

Figure 3. ADP induced thrombocyte aggregates in TG(fli1:EGFP)y1 line. Left and right panels are images of thrombocyte cluster under bright field and fluorescence with excitation at 450–490nm (GFP fluorescence).

Figure 4.

Thrombus growth in laser induced arterial thrombosis. Top and bottom panels, from left to right show growth of thrombus in arteries of two independent larvae with initiating young thrombocyte cluster (less intense thrombocytes) at the site of laser injury shown by arrow in TG(fli1:EGFP)y1 line. The mature more intense thrombocytes appear later. The rest of the area of green fluorescence is the blood vessels that are positive for GFP.

Figure 5.

Analysis of young and mature thrombocytes by flow cytometry. Representative dot plots demonstrating presence of GFP and DiI positive cells in whole blood of TG(fli1:EGFP)y1 line. All cell populations were included in the gate. Top panels from left to right show unstained control and GFP+ thrombocytes. Bottom panels from left to right show DiI labeled thrombocytes (fluorescence was measured in FL2 channel shown on Y-axis) and GFP+ thrombocytes labeled with DiI.

Figure 6.

Simultaneous amplification of GATA1, FLI1 and EF1- α mRNA by RT-PCR. **A.** Agarose gel showing the 410 bp, 299 bp and 220 bp RT-PCR amplified bands (shown by arrows) corresponding GATA1, FLI1 and EF1- α mRNA respectively in young and mature thrombocytes. 2-log DNA Ladder (New England Biolabs, Ipswich, MA) used as DNA size markers (Marker) are in left lane. **B.** Table showing the quantitation of the above bands. The numbers are the actual intensities measured by Quantity One software