Maturation of Platelet Function During Murine Fetal Development In Vivo

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Objective—Platelet function has been intensively studied in the adult organism. However, little is known about the function and hemostatic capacity of platelets in the developing fetus as suitable in vivo models are lacking.

Approach and Results—To examine fetal platelet function in vivo, we generated a fetal thrombosis model and investigated light/dye-induced thrombus formation by intravital microscopy throughout gestation. We observed that significantly less and unstable thrombi were formed at embryonic day (E) 13.5 compared with E17.5. Flow cytometry revealed significantly lower platelet counts in E13.5 versus E17.5 fetuses versus adult controls. In addition, fetal platelets demonstrated changed activation responses of surface adhesion molecules and reduced P-selectin content and mobilization. Interestingly, we also measured reduced levels of the integrin-activating proteins Kindlin-3, Talin-1, and Rap1 during fetal development. Consistently, fetal platelets demonstrated diminished spreading capacity compared with adults. Transfusion of adult platelets into the fetal circulation led to rapid platelet aggregate formation even in young fetuses. Yet, retrospective data analysis of a neonatal cohort demonstrated no correlation of platelet transfusion with closure of a persistent ductus arteriosus, a process reported to be platelet dependent.

Conclusions—Taken together, we demonstrate an ontogenetic regulation of platelet function in vivo with physiologically low platelet numbers and hyporeactivity early during fetal development shedding new light on hemostatic function during fetal life.

Visual Overview—An online visual overview is available for this article. **(*Arterioscler Thromb Vasc Biol*. 2017;37:1076-1086. DOI: 10.1161/ATVBAHA.116.308464.)**

Key Words: blood platelets ■ fetal development ■ hemostasis ■ intravital microscopy ■ microcirculation ■ thrombosis

Platelets control primary hemostasis by constantly scanning the vasculature for vessel injuries. At sites of vascular lesions, components of the subendothelial matrix are recognized by various platelet receptors, which initiate intracellular signaling events leading to numerous platelet responses such as shape change, degranulation, and activation of platelet integrins.[1–3]

Although platelet function has been extensively studied in the adult mammalian organism, little is known about platelet function in the developing fetus. In vitro studies reported hyporeactive platelets in human newborns when stimulated with physiological agonists.[4,5] However, similar responses were observed in platelets from newborns and adults once stimulated with agonists bypassing the platelet-activating surface receptors (calcium ionophore/phorbol esters). Interestingly, platelet activity and platelet counts were found to correlate with gestational age.[6–9] At the same time, when comparing platelets from human neonates and adults, an age-related lack of conformational change of the integrin GPIIb/IIIa (CD41/CD61, $\alpha_{2b}\beta_3$) was revealed.[10]

In strong contrast, shorter bleeding times and enhanced ristocetin-induced agglutination were observed in term infants compared with adults,[11] which was explained by higher levels and enhanced activity of plasmatic von Willebrand factor along with an increased hematocrit. In addition, a predominance of unusually large von Willebrand Factor multimers was reported in term neonates, which most likely results from decreased activity of von Willebrand Factor–cleaving protease.[12]

Taken together, these studies provide evidence for significant differences of the hemostatic system in neonates

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Nonstandard Abbreviations and Acronyms	
HSPDA	hemodynamically significant patent ductus arteriosus
PDA	patent ductus arteriosus

compared with adults and suggest an ontogenetic regulation of platelet function during fetal life.

To date, in vivo studies investigating platelet function and thrombus formation in the developing mouse fetus are lacking, likely because of the difficult access to the fetal circulation for microscopic studies and the challenging experimental conditions related to the small fetal size in mice.

Therefore, it was our aim to generate an intravital fetal thrombosis model in the mouse and study the dynamic processes of platelet adhesion and thrombus formation throughout fetal development in vivo. Using this model, we found low to absent thrombus formation in yolk sac microvessels and reduced thrombus stability at early developmental stages compared with near-term fetuses. Concomitantly, we observed low platelet numbers, diminished activation responses of platelet surface receptors, and reduced expression of platelet integrin-adaptor molecules in fetuses compared with adults, suggesting an ontogenetically controlled development of platelet function during fetal life.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Model of In Vivo Thrombus Formation in the Murine Fetus

Using intravital epifluorescence and multiphoton microscopy, we set up a new phototoxic injury–induced thrombosis model in the living mouse fetus. This required microinjection of FITC (fluorescein isothiocyanate)-dextran into fetal yolk sac vessels leading to a local light/dye-induced microvascular injury (Movie I in the online-only Data Supplement).[13] Throughout the whole in vivo experiment, the fetus remained inside the yolk sac and attached to the placenta (Figure 1A through 1C). Using multiphoton laser-scanning microscopy, we also visualized the different vascular layers consisting of extraembryonic vessels of the yolk sac and intraembryonic vessels (Movie II in the online-only Data Supplement).

Impaired Thrombus Formation During Fetal Development

We first assessed platelet adhesion to the endothelium (onset). The percentage of vessels showing an onset of thrombus formation was significantly reduced in young fetuses (E13.5) compared with later developmental stages (E14.5–16.5; E17.5) with a mean onset rate of 17.7±9.5% (mean±SEM) compared with 65.6±8.5% and 75.0±16.4%, respectively (Figure 2A). In addition, initiation of thrombus formation was significantly delayed in young fetuses (Figure 2B), and primary vessel occlusion occurred only in 67% of E13.5 fetuses, whereas in older fetuses (E17.5), all vessels demonstrated a first occlusion (Figure 2C). Furthermore, thrombus growth and first vessel occlusion occurred significantly slower in E13.5 fetuses compared with older fetuses (Figure 2D).

Next, we addressed thrombus stability and found that re-establishment of flow in primarily occluded vessels (reflow phenomenon) appeared more frequently in younger compared with older fetuses (Movie III in the online-only Data Supplement). Intriguingly, E13.5 fetuses were not able to mount a first stable vessel occluding thrombus as reflow occurred in all of the primarily occluded vessels. In contrast, in E17.5 fetuses, reflow was only seen in 33.3% of primarily occluded vessels (Figure 2E). Moreover, time until reflow occurred was shorter in E13.5 compared with E14.5 to 16.5 and E17.5 fetuses (Figure 2F). Finally, we investigated final vessel occlusion defined as permanent occlusion without any further reflow phenomenon throughout 60 minutes of observation. E13.5 fetuses exhibited a significantly lower rate of final vessel occlusion compared with E17.5 fetuses (Figure 2G). In addition, time until final vessel occlusion was delayed in E13.5 fetuses compared with older fetuses (Figure 2H).

Microvascular and hemodynamic parameters for the in vivo experiments are provided in Table 1.

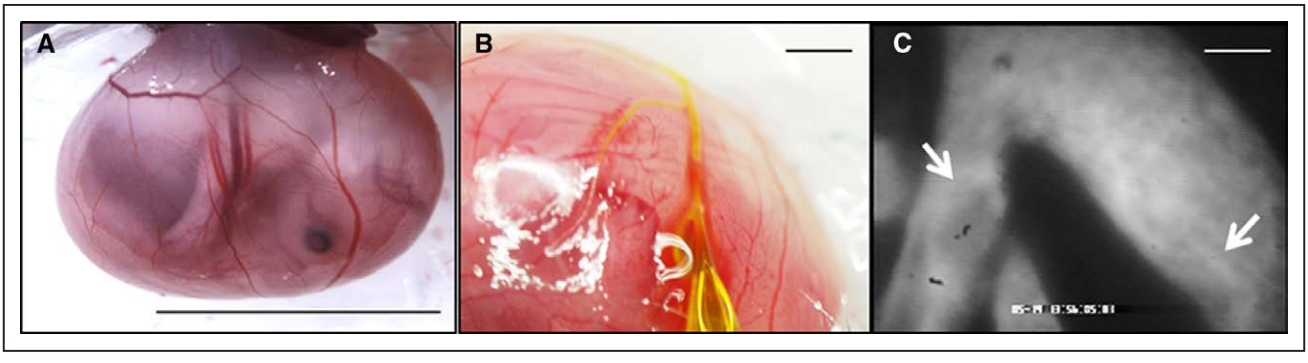

Figure 1. Model of in vivo thrombus formation in the murine fetus. **A**, To gain access to the yolk sac vasculature for microscopic studies, the fetus within the yolk sac is exteriorized from the uterus and placed in a modified Petri dish. The fetus remains attached to the placenta and thus the maternal circulation while protected inside the yolk sac (line=1 cm). **B**, Manually pulled and grinded microcapillaries are used to inject 10% fluorescein isothiocyanate (FITC)-dextran into fetal yolk sac vessels for later light/dye-induced thrombus formation (line=1 mm). **C**, Fluorescence microscopy of yolk sac vessels after injection of FITC-dextran (arrows show beginning thrombus formation, line=20 μm).

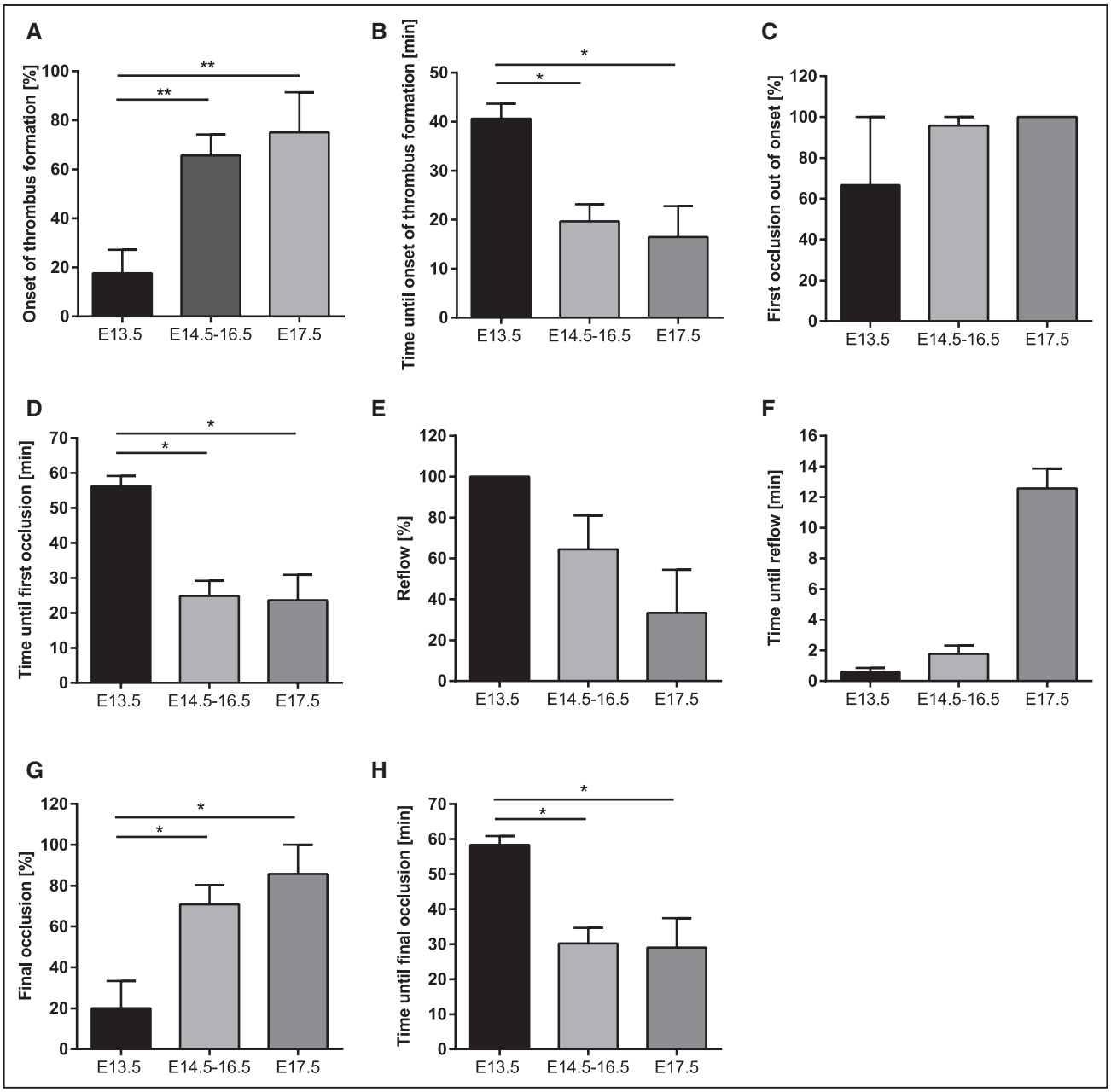

Figure 2. In vivo parameters of fetal platelet function and thrombus formation. Platelet adhesion, determined as onset of thrombus formation in E13.5 and older fetuses (**A** and **B**; n≥8 vessels per group). Rate of occurrence of first total vessel-occluding thrombi in fetal groups and time until first occlusion (**C** and **D**; n≥7 vessels). Re-establishment of flow and time until reflow in fetuses that exhibited a primary vessel occlusion (**E** and **F**; n≥2). Rate of final vessel-occluding thrombus formation without further reflow in the fetal groups and time until final vessel occlusion (**G** and **H**; n≥7). *P<0.05 and **P≤0.01. Data are presented as mean±SEM.

Significantly Diminished Platelet Counts and Altered Platelet Morphology in the Fetus

To determine reasons for reduced thrombus formation during fetal life, we analyzed platelet counts using flow cytometry. Fetal platelet counts were significantly reduced throughout development compared with adult values with lowest counts in the youngest fetuses (Figure 3A). Next, we studied platelet morphology using an Idexx hematology analyzer. Platelet distribution width, mean platelet volume, and platelet large cell ratio were found to be increased at early developmental (E13.5) compared with later time points (E17.5) and adult levels (Figure 3B through 3D), demonstrating that the existing low-numbered fetal platelets are significantly larger than adult control platelets. In addition, fetal erythrocyte counts were also found to depend on gestational age with significantly lower counts in E13.5 fetuses compared with adult controls (E13.5: 74±18×10^6/μL; E17.5: 239±30×10^6/μL; adult: 632±112×10^6/μL; P<0.001, data not shown).

Adhesion Molecule Expression on Platelets During Murine Fetal Development

Thrombus formation during primary hemostasis can be influenced not only by platelet numbers but also by expression of

Table 1. Microvascular and Hemodynamic Parameters of Intravital Microscopy Experiments

	Mother Animals, n	Fetuses, n	Vessels, n	Diameter, μm	Centerline Velocity, μm/s
E13.5	9	11	17	32±2	710±170
E14.5–16.5	12	14	32	31±1	780±90
E17.5	4	4	8	33±3	1740±740

Analysis of microvascular parameters showed no significant difference in vessel diameter and centerline velocity between the different groups.

adhesion molecules on platelets. Flow cytometric analysis of unstimulated platelets displayed lower surface levels of GPIb β and α (CD42c and b) in fetuses compared with adult mice (Figure 4A; Figures II through IV in the online-only Data Supplement). As expected,[14] we observed a decrease in GPIb surface expression after stimulation with thrombin (0.1 U/mL) in all groups. However, the decrease was more pronounced in fetal platelets leading to significantly lower stimulated levels of GPIb in fetal compared with adult platelets (Figure 4B; Figure II in the online-only Data Supplement). Next, we examined surface expression of GPIIb (CD41) on unstimulated platelets and found higher baseline values in young (E13.5) fetuses compared with adult controls (Figure 4C; Figures II through IV in the online-only Data Supplement). However, analysis of thrombin-stimulated platelets revealed that the shift toward a high-affinity conformation of GPIIb/IIIa (CD41/CD61) was significantly diminished in fetal platelets compared with adults, indicated as MFI-ratio relative to unstimulated baseline levels (Figure 4D; Figure II in the online-only Data Supplement). To exclude the possibility that the low response of fetal platelets toward thrombin is because of reduced thrombin receptor expression, Western blot analysis of platelet lysates was performed for protease-activated receptor 3 and 4 (PAR3 and PAR4). We found comparable PAR3 expression in E13.5, E17.5 fetuses and adult controls (Figure 4E, left), and even higher PAR4 levels in E13.5 fetuses compared with older fetuses and adults (Figure 4E, right). In addition, we repeated flow cytometric activation assays using ADP (10 μmol/L) as physiological weak agonist and calcium ionophore A23187 (3 μmol/L) as non-physiological agonist bypassing G-protein–coupled surface receptors. Although ADP elicited a mild activational upregulation of high-affinity GPIIb/IIIa in adult platelets, no such response was seen in fetal platelets. By contrast, after stimulation with calcium ionophore, we observed a clear increase in high-affinity GPIIb/IIIa even in platelets from E13.5 fetuses; however, levels were still significantly lower than that in adult controls (Figures III and IV in the online-only Data Supplement).

To better understand the reduced reactivity of fetal platelets, we next addressed integrin adaptor molecules Talin-1 and Kindlin-3 as well as small GTPase Rap1, which are essential for induction of the high-affinity conformation.[3,15,16] We found significantly lower levels of Kindlin-3, Talin-1, and Rap1 in fetal platelets (Figure 4F), which most likely contribute to impaired GPIIb/IIIa activation on platelets during fetal life.

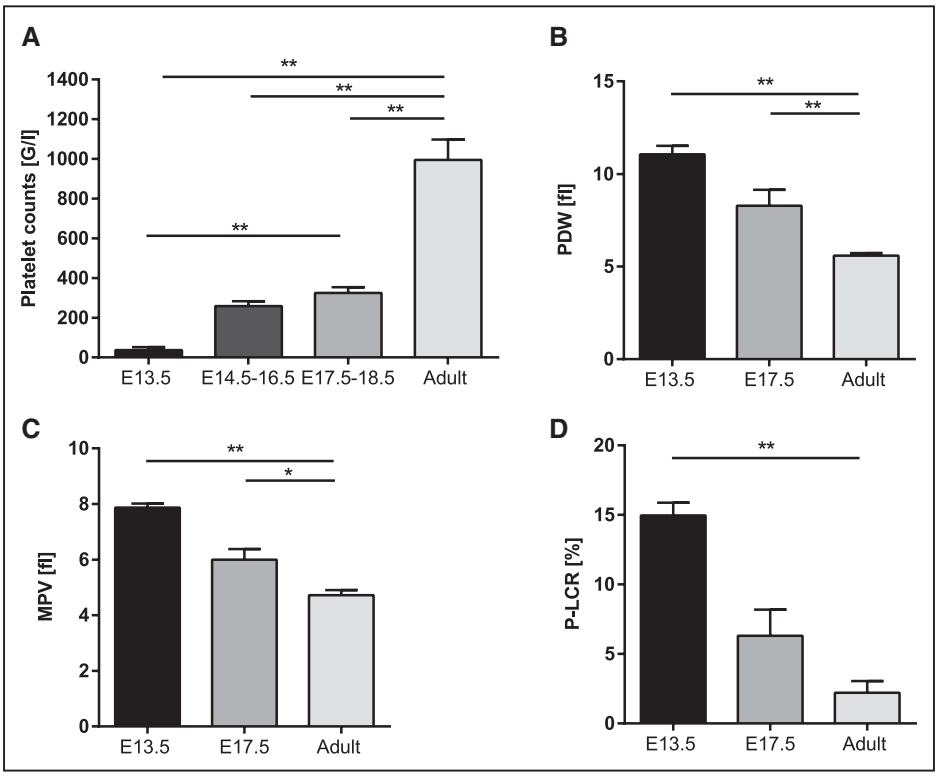

Figure 3. Platelet counts during fetal development. Flow cytometry was performed to investigate platelet counts in fetuses compared with adults (**A**; n≥5). In addition, platelet distribution width (PDW; **B**), mean platelet volume (MPV; **C**) and platelet large cell ratio (P-LRC; **D**) were determined using an Idexx hematology analyzer (n≥9). *P<0.05 and **P≤0.01. Data are presented as mean±SEM.

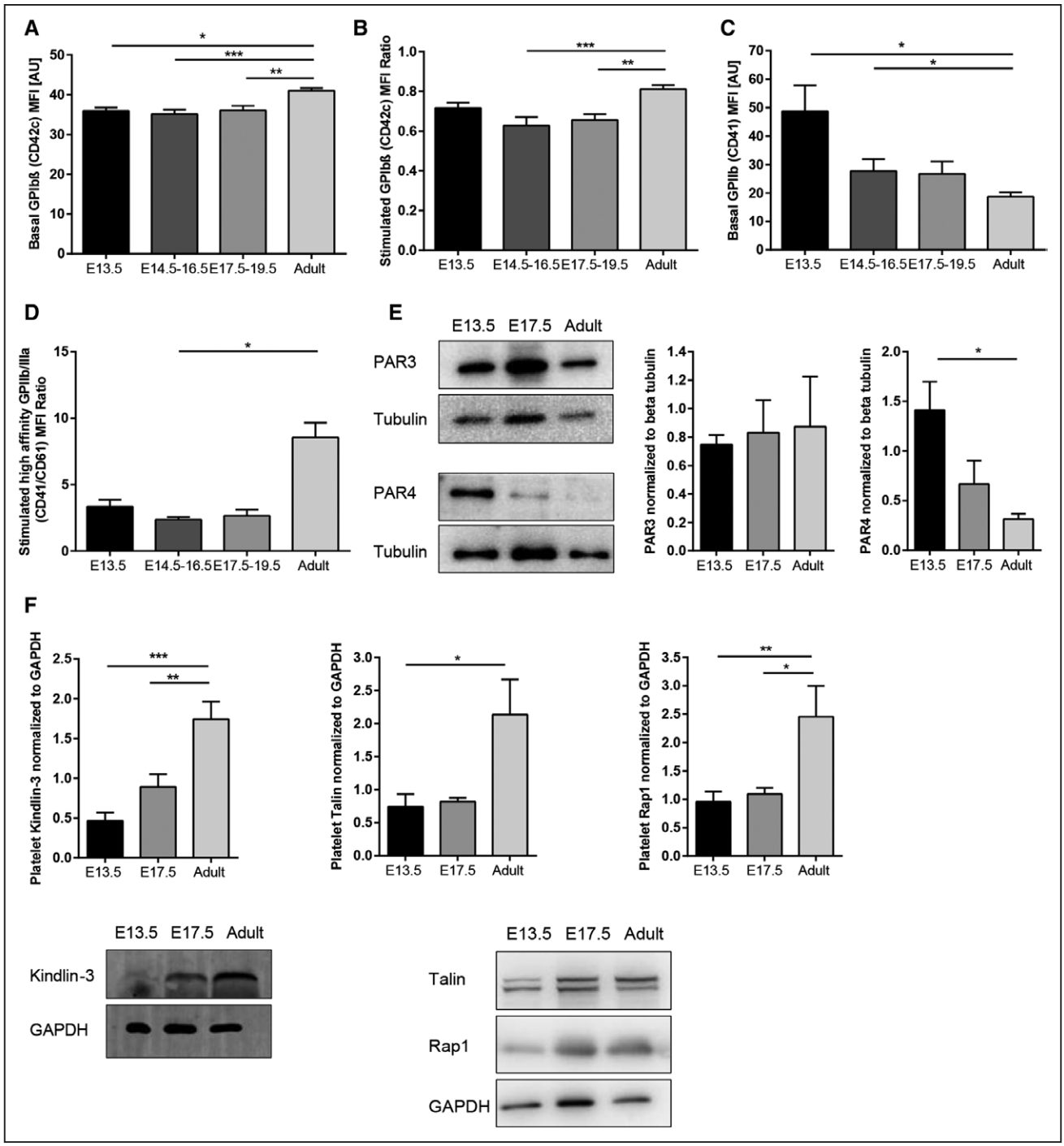

Figure 4. Expression levels of platelet surface receptors during fetal development. Flow cytometry was used to investigate platelet surface receptor expression on fetal and adult platelets. **A**, Basal and (**B**) thrombin-stimulated expression (relative to respective basal values) of GPIb β (CD42c). **C**, Baseline expression of GPIIb (CD41) and (**D**) change in expression of high-affinity GPIIb/IIIa (CD41/CD61) after thrombin stimulation (relative to respective basal values). Western blot analysis of platelet lysates for (**E**) levels of thrombin receptor protease-activated receptor (PAR)-3 and PAR4 and (**F**) integrin adaptor molecules Kindlin-3, Talin-1, and small GTPase Rap1 in fetal and adult platelets (n≥3 blots). *P<0.05, **P≤0.01, ***P≤0.001. Data are presented as mean±SEM.

Reduced Total P-Selectin Expression in Fetal Platelets

Thrombin-induced upregulation of P-selectin (CD62P) surface expression is prominent on adult platelets and used as a platelet activation marker.[17] Flow cytometry revealed that P-selectin surface expression on unstimulated platelets was low without significant difference between fetal and adult platelets (Figure 5A; Figures III and IV in the online-only Data Supplement). Intriguingly, upregulation of P-selectin surface expression following stimulation with thrombin and calcium ionophore was almost completely absent in fetal platelets (Figure 5B; Figure III and IV in the online-only Data

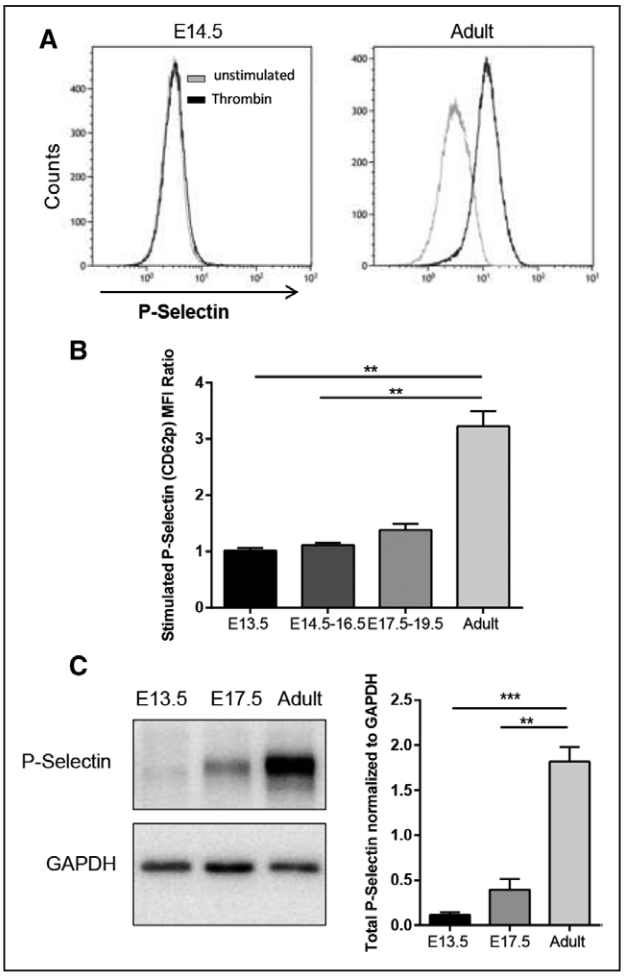

Figure 5. P-selectin levels of fetal platelets. Flow cytometry was used to measure P-selectin surface levels on unstimulated and thrombin-stimulated platelets. Representative flow cytometry measurements of E14.5 (**left**) and adult platelets (**right**) are shown (**A**). Upregulation of P-selectin surface levels after thrombin stimulation (relative to basal values) in fetal platelets compared with adults (**B**; n≥3). In addition, total P-selectin content of platelet lysates was measured by Western blot in fetuses compared with adults (**C**; n≥4). Images are representative for indicated n-number of blots. **P*<0.05 and ***P*≤0.01 (n≥3). Data are presented as mean±SEM.

Supplement). However, with advancing gestational age, a progressive increase in P-selectin upregulation could be observed. Yet, all fetal values still remained significantly below adult values (Figure 5B).

To investigate whether low P-selectin surface levels upon stimulation were because of decreased degranulation or diminished platelet P-selectin content, Western blot analysis of platelet lysates was performed. Our results revealed severely reduced total platelet P-selectin levels during fetal life with lowest content in the youngest fetuses (Figure 5C).

Reduced Spreading Capacity of Fetal Platelets

To analyze whether the observed differences in the levels of integrin-activating proteins and high-affinity GPIIb/IIIa surface expression on fetal platelets have functional consequences, we investigated platelet spreading on fibrinogen. Again, platelets from E13.5 fetuses were found to be

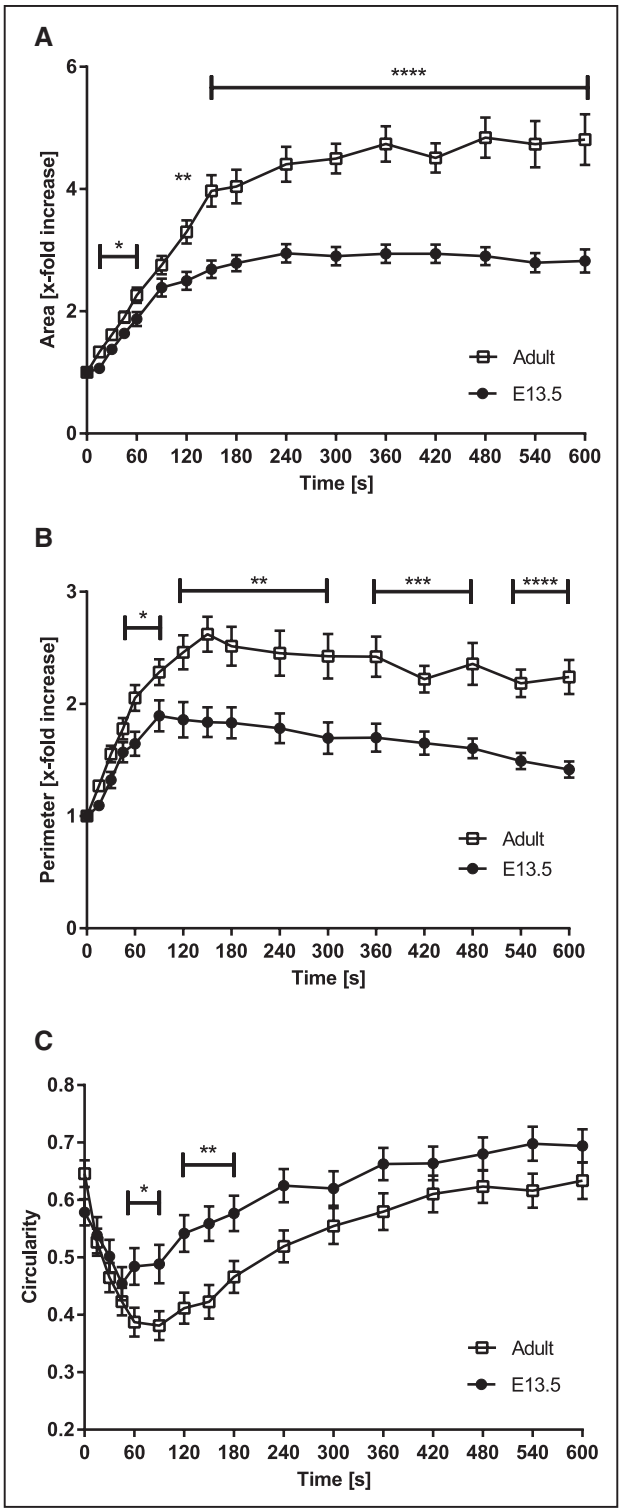

Figure 6. Platelet spreading on fibrinogen. Isolated platelets from E13.5 fetuses (n=5) and adult mice (n=5) were stimulated with 0.1 U/mL thrombin, and spreading of platelets in µ-slides coated with 1 mg/mL fibrinogen was recorded over 10 min. **A**, Change in platelet area relative to baseline (t0). **B**, Change in platelet perimeter relative to baseline (t0). **C**, Change in platelet circularity. A value of 1 indicates perfect circularity. **P*<0.05, ***P*<0.01, ****P*<0.001, and *****P*<0.0001. Data are presented as mean±SEM.

significantly larger at baseline compared with adult platelets (11.7±0.6 versus 6.2±0.3 μm² [mean±SEM], respectively; $P<0.001$). Therefore, changes in platelet area and perimeter were calculated as ratio relative to baseline. In line with the activation assays, platelets from E13.5 fetuses displayed a significantly reduced capability to spread on fibrinogen-coated surface after stimulation with thrombin compared with adult platelets (Figure 6A and 6B). Interestingly, spreading morphology between adult and fetal platelets also differed significantly. Although adult platelets showed normal stages of spreading including spindle-like forms and extrusion of long filopodia (decrease in circularity) followed by expansion of lamellipodia leading to the fully spread fried egg form (increase in circularity),[18] the majority of E13.5 platelets only formed short filopodia and spread in a circular fashion by primary expansion of lamellipodia (Figure 6C; Figure V in the online-only Data Supplement).

Transfusion of Adult Platelets Into E14.5 Fetuses Leads to Platelet Aggregate Formation But Does Not Promote Adhesion of Fetal Leukocytes

On the basis of our findings of a functional and numeric defect in the fetal platelet population, we wanted to assess how adult platelets behave in the fetal yolk sac circulation and whether they are able to form thrombi in E14.5 fetuses, indicating that reduced platelet interaction and thrombus formation are because of a platelet-intrinsic maturation defect. To this end, labeled platelets from adult donor animals were injected into yolk sac vessels of E14.5 fetuses (200 000 platelets/fetus). Rapid spontaneous platelet adhesion at multiple sites of the yolk sac vasculature could be noted together with platelet aggregate formation (Movie IV in the online-only Data Supplement). Interestingly, the forming thrombi exhibited a tendency to release emboli into the fetal circulation. By contrast, transfusion of age-matched E14.5 platelets into E14.5 fetuses (200 000 platelets/fetus) lead to platelet aggregate formation in only 33.3% of experiments compared with 100% after transfusion of adult platelets (n=3 per group). To exclude that the propensity of adult platelets to form spontaneous aggregates in the fetal circulation is merely because of platelet activation by the isolation and staining procedure, we investigated behavior of transfused adult platelets in mouse cremaster muscle vessels. In this setting, despite increasing the amount of transfused platelets to 40 million (≈ 3% to 5% of total platelet count), which equals roughly the concentration of platelets transfused into the fetal circulation, we did not observe any spontaneous platelet aggregation or thrombus formation (data not shown).

Apart from their role in primary hemostasis, platelets have been shown to be important regulators in inflammation and to facilitate leukocyte recruitment.[19,20] As we have recently demonstrated that fetal leukocytes at early developmental stages (<E15) are not able to adhere to the yolk sac vasculature in a model of traumatic inflammation,[21] we were interested whether transfusion of adult platelets is able to promote adhesion of fetal leukocytes.

For this purpose, isolated rhodamine 6G-labeled adult platelets were transfused into LysM-EGFP (enhanced green fluorescent protein) E14.5 fetuses displaying fluorescent neutrophils. Interestingly, despite rapid platelet adhesion, we did not observe interactions between adult platelets and fetal EGFP⁺ cells and saw no incorporation of EGFP⁺ leukocytes into the forming thrombus (Movies V and VI in the online-only Data Supplement).

Table 2. Clinical Parameters of Premature Infants With HSPDA and Indomethacin Therapy With or Without Platelet Transfusion

		No Platelet Transfusion		
	Platelet Transfusion	Thrombocytopenia	Normal Platelets	*P* Value
No. of patients, n	8	17	172	
Gestational age, wk, range	25 (22–27)	26 (23–30)	26 (23–32)	NS
Birth weight, g, range	598 (440–940)*	780 (320–1450)	822 (370–1480)	0.048
SGA, n (%)	2 (25)*	4 (24)*	9 (5)	0.004
Platelet count, G/L, SD	89.9±27.5*	101±22.6*	276.5±99.3	<0.0001
Hematocrit, %, SD	30±5†	39±7	36±6	0.011
Surgical ligation, n (%)	6 (75)*†	3 (17.6)	57 (33.1)	0.017
Death, n (%)	1 (12.5)	3 (17.6)*	4 (2.3)	0.004
Maternal diagnoses				
HELLP syndrome, %	0	12	4	NS
Preeclampsia, %	13	6	4	NS
Placental insufficiency, %	38*	6	6	0.005
Amnioninfection, %	25	12	16	NS

Platelet counts and hematocrit were obtained at the time PDA treatment was started. HSPDA indicates hemodynamically significant patent ductus arteriosus; HELLP, hemolysis, elevated liver enzymes, and low platelets; PDA, patent ductus arteriosus; and SGA, small for gestational age.

*Significance vs infants with normal platelet counts.

†Significance vs nontransfused thrombocytopenic infants.

Transfusion of Adult Platelets Into Thrombocytopenic Premature Infants Does Not Promote Pharmacological Closure of a Patent Ductus Arteriosus

It has been demonstrated that postnatal closure of a patent ductus arteriosus in term infants is associated with the formation of a stable thrombus and subsequent clot consolidation.[22] Preterm infants, however, frequently experience hemodynamically significant patent ductus arteriosus (HSPDA), and medical or surgical patent ductus arteriosus (PDA) closure is eventually needed to prevent organ failure or progressive heart failure.

On the basis of our finding of excessive in vivo aggregate formation after transfusion of adult platelets into fetuses, we became interested whether platelet transfusion impacts HSPDA closure in thrombocytopenic premature infants. To this end, we performed a retrospective analysis of 718 children born <1500 g and ≤32 weeks of gestation at the tertiary neonatal intensive care unit at the University Children's Hospital Munich (Table 2). A total of 197 infants (27.4%) developed a HSPDA requiring medical treatment with indomethacin and subsequent surgical ligation, if pharmacological treatment failed. Of those, 25 neonates (12.7%) exhibited thrombocytopenia (platelet count <150 G/L) and 8 infants (32% of thrombocytopenic infants) received at least one platelet transfusion at the time of HSPDA treatment. When focusing on the need for surgical closure of HSPDA and previous platelet transfusion, we found that surgical closure rate was significantly higher in infants transfused with adult platelets compared with nontransfused thrombocytopenic infants and infants with normal platelet counts (75% versus 17.6% and 33.1%, respectively; P=0.017; Table 2), suggesting that platelet transfusion does not demonstrate a beneficial effect on pharmacological closure of a hemodynamically relevant PDA in thrombocytopenic premature neonates. Finally, we analyzed mortality in our neonatal cohort and found a higher mortality in thrombocytopenic premature infants compared with infants with normal platelet counts. However, this was independent of platelet transfusions (Table 2).

Discussion

In the present study, we investigated in vivo thrombus formation at different developmental stages of the mouse fetus using a novel light/dye-induced fetal thrombosis model featuring an intact fetomaternal placental unit. Although light/dye-induced injury results in platelet adhesion and thrombus formation in cremaster muscle venules of adult mice within a few minutes,[23] we observed significantly decreased and delayed rates of functional thrombus formation in the developing fetus and a dependency on fetal maturation. Thus, our findings reveal an ontogenetic regulation of platelet function, impacting in vivo clot formation. This is in accordance with previous studies showing that bleeding time in human premature infants <33 weeks of gestation is about twice that of mature neonates.[24] Intriguingly, not only the clot-forming capacity seemed to depend on gestational age but also clot stability as evidenced by the reflow phenomenon. In this context, a recent study looking at clot formation in human neonates using rotational thromboelastometry found that clot firmness correlated with gestational age and was significantly reduced in premature newborns.[25]

To uncover reasons for reduced fetal in vivo thrombus formation, we investigated platelet counts and found strongly reduced platelet numbers in murine fetuses compared with adults and a correlation with gestational age. A multicenter study in >47 000 neonates reported lower platelet counts for premature infants <32 weeks of gestation compared with older ones.[26] In addition, as in murine fetuses, the platelet distribution width was found to be increased in preterm compared with term neonates.[27] Whether the changes in platelet indices are merely a result of the reduced platelet count[28] or possibly represent a mechanism to compensate for altered platelet function[29] deserves further investigation. In vivo experiments using antibodies to reduce murine platelet counts to numbers in the range we observed in the fetuses show that, depending on the model, counts as low as 25 G/L are sufficient to promote thrombus formation.[30] Thus, the reduced platelet count is likely to contribute to defective thrombus formation especially in the E13.5 fetuses but cannot fully explain our findings.

Our further in vitro analyses revealed altered integrin expression and a hyporeactive phenotype of fetal platelets. Different mechanisms have been discussed regarding the nature of platelet hyporeactivity in premature and mature infants, including impairment of calcium mobilization and altered intracellular signaling.[5,31] Our results support the concept that hyporesponsiveness of fetal platelets is multifaceted including altered signal transduction of surface receptors (which can be bypassed using calcium ionophore) but also an altered intracellular machinery necessary for integrin activation. In line with this notion, we were able to demonstrate that expression of Talin-1, Kindlin-3, and Rap1, which have been shown to be essential for inside-out mediated integrin activation and platelet function,[3,15,16] were significantly reduced in fetal platelets. It is plausible that these findings directly relate to the reduced capacity of fetal platelets to spread on fibrinogen and to stabilize forming thrombi in vivo, as both processes critically depend on GPIIb/IIIa integrin–mediated signaling (outside-in) upon substrate ligation.[32] The altered spreading morphology of fetal platelets with reduced filopodia formation is of interest with respect to thrombus stability, as filopodia enable binding to fibrin strands and other platelets to form a 3-dimensional thrombus.[33] In Wistar Furth rats, which display hereditary macrothrombocytopenia, a similar spreading pattern has been described and linked to the higher thrombus fragility found in these animals.[18]

Besides altered integrin expression and activation, we also found decreased P-selectin upregulation on fetal platelets. This is in accordance with recent findings in human neonates,[7,34] and likely related to the strongly reduced total P-selectin content we discovered in murine fetal platelets. P-selectin has been demonstrated to be instrumental in promoting platelet–leukocyte interactions.[20,35] Remarkably, in the fetal yolk sac, fetal leukocytes did not interact with fully mature transfused adult platelets (with presumably normal P-selectin expression). This might be because of reduced levels of PSGL-1, the major P-selectin ligand on

fetal leukocytes, as demonstrated earlier.[21,36] Although our findings elicit an important role of modulated platelet function and numbers, other factors are also likely to influence hemostasis during fetal development.[37] These include a lower erythrocyte count found in young fetuses, changes in the level and activity of plasmatic coagulation factors, and altered endothelial cell function. In addition, the release of second wave agonists such as ADP and thromboxane A2 has also been shown to be reduced in platelets from premature human neonates.[38] Because of the limited amount of blood, and the low platelet numbers in the murine fetus, it is at present not possible to perform reliable measurements of blood coagulation parameters and agonist release from murine fetal platelets.

The reduced capacity to form a stable clot, particularly during early fetal life, might be a protective factor against vascular thrombotic injury with its detrimental consequences for normal development. Studies in mice deficient in the transcription factor NF-E2 demonstrated a maturation arrest of megakaryocytes with loss of platelets. Yet, NF-E2–deficient embryos developed to term without major impairment, suggesting that strongly reduced platelet function and number do not necessarily impact fetal development in utero.[39] These results seem to conflict with studies demonstrating an essential role of platelets in lymph vessel development by interaction of platelet C-type lectin-like receptor with podoplanin on lymphatic endothelial cells.[40] However, the authors argue that for normal lymph vessel development, the presence of few platelets, which can still be found in NF-E2–deficient mice, is sufficient.

It is tempting to speculate that the maturation of fetal platelets during gestation reflects developmental differences in gene expression and function of hematopoietic stem cells as hematopoiesis moves from the yolk sac and aorta-gonad-mesonephros region to the fetal liver and finally the bone marrow. Megakaryopoiesis is differentially regulated during primitive (c-myb independent) and definitive hematopoiesis (c-myb dependent) with primitive megakaryocyte progenitors likely giving rise to the first megakaryocytes present in the yolk sac and large highly nucleated platelets found in the embryonic circulation by E10.5. Around that time, hematopoiesis moves to the fetal liver and by E12.5, the liver becomes the main hematopoietic niche for stem cell expansion and differentiation.[41,42] It is conceivable that early platelets derived from primitive progenitors exhibit distinct functional characteristics as compared with platelets from definitive progenitors. In E13.5 fetuses, both types of platelets likely are present concurrently, whereas the number of early primitive platelets should decrease with advancing gestation.

The ontogenetic regulation of platelet function becomes problematic in the setting of premature birth, where the fetus is suddenly exposed to the threats of extrauterine life. Indeed, one of the major complications in preterm infants is intraventricular hemorrhage and its incidence is inversely correlated with gestational age.[43] Thus, the otherwise physiological platelet hyporeactivity found in premature infants may, together with other factors, such as an immature vascular system and changing blood flow properties, contribute to the development of intraventricular hemorrhage. Furthermore, in a mouse study of PDA, it was suggested that platelets contribute to the closure of the ductus arteriosus following birth, and thrombocytopenia was proven to be a predictor for PDA in preterm infants.[22]

Based on our observation of platelet aggregate formation in fetuses following transfusion of adult platelets, we performed a retrospective data analysis investigating the impact of adult platelet transfusion on pharmacological PDA closure and mortality. Unexpectedly, we found that administration of adult platelets had no beneficial effects on PDA closure in thrombocytopenic premature infants treated with indomethacin. However, factors such as ductal size, treatment timing, differences in hematocrit and possibly coagulation parameters, and accompanying comorbidities may confound these results. Therefore, these findings need to be interpreted with caution and investigated by larger prospective studies. This is undermined by the existing conflicting clinical data on the role of platelets and ductal closure.[22,44,45] Interestingly, when looking for underlying maternal conditions affecting neonatal platelet counts, we observed a significantly higher rate of placental insufficiency in mothers from neonates requiring transfusion than in the other groups. Even though this observation does not allow concluding on a causal relationship because of the retrospective approach and the limited number of patients, we think that it is still noteworthy and deserves further evaluation. Finally, we observed similar overall mortality in transfused versus nontransfused thrombocytopenic premature infants. However, the decision for platelet transfusion should be considered carefully in view of possible adverse effects after transfusion of inadequately active platelets in neonates.[46] In this context, Ferrer-Marin et al[47] could elegantly show that adult platelets transfused into fetal blood in vitro exhibit an increased aggregatory and hemostatic performance compared with neonatal platelets pointing toward the existence of a developmental mismatch in platelet transfusion. In line with this notion, we observed rapid and spontaneous clot formation after transfusion of adult platelets into the fetal circulation, whereas transfusion of age-matched platelets did not result in thrombus formation. Furthermore, adult platelets transfused into the adult circulation did not exhibit any propensity to form spontaneous aggregates. These observations support the concept that the fetal plasma exerts some prothrombotic activity (eg, through increased levels and presence of ultralarge von Willebrand Factor),[11,12] possibly to compensate for reduced platelet numbers and function.

In summary, using a novel murine fetal thrombosis model, we demonstrate a developmental regulation of platelet function and thrombus formation. This involves platelet numbers and key molecules of platelet integrin signaling and surface receptors relevant for platelet adhesion and aggregation. Furthermore, we show that transfusion of adult platelets can override reduced clot-forming capacity in young fetuses. These findings do not only provide new valuable insights into the regulation of primary hemostasis during fetal life but also demand future clinical studies to further evaluate the benefits and risks of adult platelet transfusion in premature infants.

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Disclosures

None.

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Highlights

- A novel murine fetal thrombosis model is introduced to study platelet function in vivo at different time points during fetal life.
- In vivo platelet adhesion, aggregation, and thrombus stability are ontogenetically regulated and severely reduced in mid-gestation murine fetuses.
- Besides reduced platelet counts, diminished activational responses and reduced integrin adaptor molecule levels contribute to the reduced fetal thrombus formation.