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**Loss of a Rho-Regulated Actin Nucleator, mDia2, Impairs Cytokinesis during Mouse Fetal Erythropoiesis**

Sadanori Watanabe, Tihana De Zan, Toshimasa Ishizaki, Shingo Yasuda, Hiroshi Kamijo, Daisuke Yamada, Tomohiro Aoki, Hiroshi Kiyonari, Hiroshi Kaneko, Ritsuko Shimizu, Masayuki Yamamoto, Gohta Goshima, and Shuh Narumiya

**INTRODUCTION**

In animal cytokinesis, actin filaments accumulate around the equator of a dividing cell in anaphase to form a ring-like apparatus, called the contractile ring, and myosin-based contraction of this ring physically separates a dividing cell into two in telophase (Green et al., 2012; Pollard, 2010). The small GTPase Rho functions as a molecular switch of cytokinesis by recruiting actin nucleators and preparing the contractile apparatus at the site of contraction (Jordan and Canman, 2012). Two types of actin filaments, branched and unbranched, are formed in cells through the action of actin nucleators, typically the Arp2/3 complex and the formin family proteins, respectively (Firat-Karalar and Welch, 2011). Involvement of Rho and the Rho-regulated formin family proteins in cytokinesis is conserved during evolution, Rho1p and Bnil1p in budding yeasts, Rho-1 and Cyk-1 in worms, and mDia2 functions critically in cytokinesis in vivo during mouse fetal erythropoiesis.

**RESULTS**

**Loss of mDia2 Leads to Embryonic Lethality**

To investigate in vivo functions of mDia2, we disrupted the murine mDia2 gene (Figures S1A–S1D). Whereas mDia2 heterozygous-deficient (mDia2+/-) mice were born healthy and fertile...
without any noticeable phenotype, all homozygous-deficient (mDia2\(^{-/-}\)) mice from mDia2\(^{+/+}\) intercrosses died in utero (Figure 1A), indicating that mDia2 is essential for embryonic development. Because mDia2 is essential for cytokinesis in cultured cells (Watanabe et al., 2009), we suspected that mDia2\(^{-/-}\) embryos die at an early developmental stage due to cytokinesis failure. However, we found that mDia2\(^{-/-}\) embryos survived without severe malformations in their body until embryonic day 10.5 (E10.5) after gestation (Figures 1B and S1E) and died in utero around E12.5 (Figure 1A). mDia2\(^{-/-}\) embryos were found viable at the expected Mendelian ratio at E11.5 but paler and smaller than their wild-type littermates (Figures 1A and 1B). Although the heart functioned normally, and the blood vessels in the yolk sacs were formed normally in mDia2\(^{-/-}\) embryos (Movies S1 and S2; Figure S1E), their yolk sacs appeared to have a less amount of blood than that of wild-type (Figure 1C), suggesting the presence of anemia in mDia2\(^{-/-}\) embryos.

**mDia2 Is Required for Fetal Erythropoiesis**

In mouse embryos, primitive erythropoiesis, which is characterized by the production of nucleated erythrocytes, is initiated in blood islands of the yolk sac at E7.5 (Palis et al., 2010). Although peripheral blood at E11.5 contains mostly nucleated primitive erythroblasts (Fraser et al., 2007), analysis of peripheral blood in E11.5 mDia2\(^{-/-}\) embryos revealed that their circulating blood cells were less in number than wild-type littermates, large, and frequently binuclear or multinuclear (Figures 2A–2C, S2A, and S2B), suggesting the primitive erythropoiesis failure in mDia2\(^{-/-}\) embryos. In addition to this primitive erythropoiesis, the definitive erythropoiesis starts around E11.5 in the fetal liver, in which a massive number of enucleated erythrocytes are produced from erythroblasts (Baron et al., 2012). Hematopoietic stem cells in fetal livers continuously replenish erythroid progenitors for the production of erythrocytes during late gestation. We therefore hypothesized that mDia2 is involved also in erythropoiesis in the fetal liver. Erythroid progenitors and erythroblasts in fetal liver can be distinguished by examining surface markers such as CD71 and Ter119 (Zhang et al., 2003) (Figures S2C and S2D). Flow cytometry of wild-type and mDia2\(^{-/-}\) fetal liver cells at E11.5 showed that mDia2\(^{-/-}\) fetal liver contained a significantly less number of Ter119\(^{+}\) erythrocytes and lower percentage of Ter119\(^{+}/CD71^{+}\) cells than wild-type control: 10.5% \(\pm\) 5.5% (n = 4) and 22.5% \(\pm\) 4.0% (n = 5) (p = 0.015), respectively (Figures S2E, 2D, and 2E). Probably reflecting this reduction of the erythroblast population, the percentages of Ter119\(^{+}/CD71^{+}\) erythroid populations were higher in the mDia2\(^{-/-}\) fetal liver than the wild-type control (Figure 2D). These results together suggest that mDia2 is required for both primitive and definitive erythropoiesis.

**mDia2 Is Required for Expansion of Differentiating Erythroblasts**

The above results suggest that mDia2 plays critical roles in production of erythroid cells. To clarify at which step mDia2 regulates erythropoiesis, we first examined burst-forming unit erythroids (BFU-Es) and more mature erythroid colony-forming unit erythroids (CFU-Es) in the fetal liver at E11.5. Both types of colonies were formed even in the absence of mDia2. However, the colony numbers of BFU-Es and CFU-Es were significantly lower in mDia2\(^{-/-}\) cells than those of mDia2\(^{+/+}\) cells (Figure 3A), and CFU-E colony formation was more severely reduced in mDia2\(^{-/-}\) cells. These results suggest that mDia2 regulates erythropoiesis preferentially downstream of CFU-E (Figure S2C). We next used an in vitro culture system for erythroid cell differentiation (Zhang et al., 2003) to examine erythropoiesis failure in mDia2\(^{-/-}\) cells in more detail. Ter119\(^{+}\) cells were purified from fetal livers and cultured in the presence of erythropoietin (EPO). In this system, proliferation and differentiation of erythroid cells were examined by counting the cell number, Wright-Giemsa staining, and flow cytometry analysis. Counting the viable cell number revealed that proliferation was severely impaired in mDia2\(^{-/-}\) fetal liver cells compared to the mDia2\(^{+/+}\) cells (Figure 3B); the increase of the cell number was delayed already at 12 hr and significantly suppressed at 24 and 36 hr.
Concomitant with this suppression of cell proliferation, multinucleate cells increased in number in mDia2−/− cells from 12 to 36 hr of culture (Figures 3B–3D), suggesting that the proliferation defect was coupled with cytokinesis failure in mDia2−/− cells. We next treated the Ter119+ mDia2−/− cells with cytochalasin D for 12 hr and found that a much higher population of these cells (more than 90% of the cytochalasin D-treated cells) became multinucleated at 12 hr (Figure S3A) than mDia2+/+ cells (15.8% ± 3.1%) (Figure 3D). These findings demonstrate that cytokinesis occurs in the earlier stage of erythroid differentiation through control of F-actin assembly and that the cytokinesis at this stage is carried out without involvement of mDia2. We therefore next wondered whether an upstream molecule, Rho, is involved in the cytokinesis of the early stage of differentiation by introducing a Rho-specific inhibitor, C3, into the cells. Notably, 70% of the C3-treated cells became multinucleated at 12 hr (Figure S3A) than mDia2+/+ cells. We next treated the Ter119+ mDia2+/+ and mDia2−/− cells with cytochalasin D for 12 hr and found that a much higher population of these cells (more than 90% of the cytochalasin D-treated cells) became multinucleated at 12 hr (Figure S3A) than mDia2+/+ cells (15.8% ± 3.1%) (Figure 3D). These findings demonstrate that cytokinesis occurs in the earlier stage of erythroid differentiation through control of F-actin assembly and that the cytokinesis at this stage is carried out without involvement of mDia2. We therefore next wondered whether an upstream molecule, Rho, is involved in the cytokinesis of the early stage of differentiation by introducing a Rho-specific inhibitor, C3, into the cells. Notably, 70% of the C3-treated cells became multinucleated at 12 hr (Figure S3A). These results suggest that Rho controls cytokinesis from the earlier differentiation stage, whereas its effector, mDia2, becomes important for cytokinesis in the later stages of erythroid differentiation. We then used mDia2 siRNA. Depletion of mDia2 induced multinucleation of cultured cells, and Giemsa staining suggested that they were late-stage erythroblasts (Figure S3C), confirming the cytokinesis failure in mDia2−/− cells. Notably, the number of activated caspase-3+ cells increased time dependently in the mDia2−/− cell population (Figure 3E), suggesting that decreased proliferation and increased multinucleate cell number are associated with apoptotic cell death. Indeed, signals of activated caspase-3 were observed often in multinucleate erythroblasts (Figure S3D).

To further identify the differentiation step at which mDia2 deficiency impairs proliferation and induces apoptosis, we subjected cells of 0, 12, 24, and 36 hr of culture to flow cytometry analysis. In control mDia2+/+ cells, more than 90% of the population was CD71high Ter119+ at 12 hr of culture (Figure 3F), among which most appeared as c-Kit+ (Figure S3E), indicating that they represent proerythroblasts (Dev et al., 2010). mDia2−/− cells were then enriched in the Ter119+CD71+ c-Kit+ population in a time-dependent manner at 24 and 36 hr, indicating that they differentiated to polychromatic or orthochromat erythroblasts (Figures 3F and S3E) (Zhang et al., 2003). On the contrary, although mDia2−/− cells were enriched in the CD71high Ter119+ population at 12 hr, there was a significantly less accumulation of these cells in the Ter119+CD71+ population at 24 and 36 hr (Figure 3F). Because the appearance of apoptotic cells was associated with multinucleation and decreased viable cell number in mDia2−/− cells, a smaller accumulation of this genotype of cells in the polychromatic or orthochromatic erythroblast population suggests that multinucleation either in the transition from proerythroblast or during terminal differentiation induces apoptosis in these populations.

To examine whether the loss of mDia2 affects expression of erythroid differentiation characteristics in this process, we analyzed expression of several erythroid-related genes. We found no significant difference between mDia2+/+ or mDia2−/− and mDia2−/− cells in expression of Slc4a1, Spna1, EpoR, and Gata1 at 12 and 24 hr (Figure S3F). Interestingly, mDia2 expression increased during in vitro differentiation of erythroblasts, as reported previously by Ji et al. (2008); its expression more than
tripled in 24 hr (Figure S3G). These results together suggest that the loss of mDia2 does not directly block differentiation of erythroblasts but affects proliferation of differentiating erythroblasts possibly through cytokinesis failure.

**mDia2 Is Essential for Cytokinesis but Not for Enucleation of Erythroblasts**

In the terminal stage of erythroid differentiation in mouse, one proerythroblast cell undergoes three cycles of mitosis with decreasing its cell size and sequentially generating basophilic, polychromatic, and orthochromatic erythroblasts, of which the latter finally expel their nuclei to produce reticulocytes (Hattangadi et al., 2011; Liu et al., 2013). To analyze how the loss of mDia2 affects terminal erythroid differentiation, we monitored the process of cytokinesis during erythroid differentiation by live-cell imaging (Figure 4A). Cells from mDia2+/- as well as mDia2-/- fetal liver underwent rounds of successful cell divisions with decreasing their size (Figure 4A, top). On the other hand, mDia2-/- erythroblasts frequently failed in cytokinesis by exhibiting aberrant contraction (Figure 4A, bottom). The cleavage furrow was formed initially but got destabilized during contraction, and the ingestion was then fused back, and the cell could not complete cytokinesis (Figure 4A). Notably, smaller mDia2-/- cells failed in cytokinesis more frequently than larger mDia2-/- cells; the percentage of cytokinesis failure in mDia2-/- cells was 84.8% (n = 33) and 18.2% (n = 33) in smaller (<12.5 µm) and larger cells (≥12.5 µm), respectively. Given that erythroblasts decrease their size during differentiation, these results suggest that mDia2 plays an essential role in the cytokinesis of erythroblasts in the terminal stage of differentiation, i.e., of basophilic or polychromatic erythroblasts. Because mDia2 induces F-actin for the contractile ring in cultured mammalian cells (Watanabe et al., 2008), we stained for mDia2 and F-actin in differentiating erythroblasts. In the terminal stage of erythroid differentiation by live-cell imaging (Figure 4A). Cells from mDia2+/- as well as mDia2-/- fetal liver underwent rounds of successful cell divisions with decreasing their size (Figure 4A, top). 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Immunofluorescence shows enucleating erythroblasts from mDia2+/−/C0 and arrows indicate nuclei protruding from cells and accumulation of F-actin, respectively. Scale bars, 5 μm.

We next examined enucleation in mDia2−/− erythroblasts. Enucleation occurs in two steps: the first is protrusion of the nucleus, and the second is pinching off the protruded nucleus (Palis, 2012). We found that multinucleate mDia2−/− erythroblasts frequently showed protrusion of their nuclei (Figures 4F–4H and S4A; Movie S3). Consistent with the notion that the nuclear protrusion is carried out by the F-actin assembly, F-actin accumulated at the boundary between the cytoplasm and the protruded nuclei in mDia2−/− erythroblasts as seen in mDia2 wild-type and mDia2+/− cells (Figure 4G). We then wondered if mDia2−/− multinucleate erythroblasts could complete enucleation and found that there are big enucleated mDia2−/− erythroblasts, though with reduced frequency compared to that of mDia2+/− erythroblasts (Figures 4F and 4G). These results suggest that mDia2 is not essential for the enucleation process at least in vitro. In addition to mDia2−/− cells, Rho-inactivated multinucleate erythroblasts also showed nuclear protrusion in enucleation (data not shown), suggesting that F-actin assembly pathways other than Rho-mDia2 function in enucleation. We therefore examined the effects of inhibitors of actin polymerization and other small GTPases regulating F-actin assembly (Ji et al., 2008; Konstantinidis et al., 2012; Wang et al., 2012). The protrusion of nuclei and F-actin accumulation in multinucleate erythroblasts was either seen at aberrant sites or was severely impaired at the proper position of the cleavage furrow in erythroblasts to perform persistent contraction of the contractile ring during cytokinesis.

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mDia2−/− cells were inhibited by treatment of the cells with cytochalasin D, latrunculin A, and a Rac1 inhibitor (Figures S4B–S4D; Movie S4). These results suggest that the Rac-dependent F-actin assembly pathway functions in erythroblasts and that Rho and mDia2 are dispensable in this process, indicating the cooperation of multiple Rho GTPase signaling pathways to ensure the successful production of erythrocytes (Ji et al., 2008) (Figure S4E).

**DISCUSSION**

Here, we have revealed the essential role of mDia2 for cytokinesis of erythroblasts during mouse development. Our main findings are discussed below.

First, our study demonstrated that the Rho–mDia2 signaling found in cultured cells (Watanabe et al., 2008) indeed functions in cytokinesis in vivo and plays an essential role in development. Our findings have thus revealed the involvement of an actin nucleator in mammalian somatic cytokinesis in vivo. Previous findings on the involvement of actin nucleators in cytokinesis were limited to meiosis (Leader et al., 2002; Pfender et al., 2011; Yi et al., 2011). Importantly, the cytokinesis failure phenotype of mDia2-deficient mice was most evident during erythropoiesis. Such erythropoiesis failure was not found in either mDia1-deficient (Peng et al., 2007; Sakata et al., 2007) or mDia3-deficient mice, although immunofluorescence signal for mDia1 is observed in the cleavage furrow and midbody of erythroblasts (S.W., unpublished data). These results indicate that mDia2 rather than mDia1 and mDia3 functions critically in erythroblast cytokinesis. Why the phenotype of mDia2-deficient mice, the cytokinesis failure, is mainly observed in erythropoiesis remains to be investigated. Because similar erythropoiesis-specific cytokinesis failure is observed in patients with congenital dyserythropoietic anemia, who have a mutation in KIF23 (Lijeholm et al., 2013), one of the important factors controlling RhoA in cytokinesis, these results might reflect the high susceptibility of erythroid cells to the defect in Rho signaling. We further identified the point of the mDia2 action in cytokinesis at the stage of basophilic and polychromatic erythroblasts. Because cell death is frequently observed in multinucleate erythroblasts, the proliferation defect is most probably due to the cytokinesis failure of erythroblasts, though we could not exclude additional mechanisms. The impaired cytokinesis during erythroid differentiation in mDia2−/− cells might also delay the differentiation of erythroblasts. These results taken together suggest that the cytokinesis failure in erythroblasts is the major cause of anemia in mDia2−/− mice, which finally results in the embryonic lethality in the mice.

Second, and equally importantly, we discovered that mDia2 is not always essential for cytokinesis. Indeed, mDia2−/− embryos developed until E11.5. We also found that cytokinesis of erythroid progenitors is not sensitive to the loss of mDia2 but fails upon Rho inactivation and treatment with actin polymerization inhibitors. It is noteworthy that the frequency of cytokinesis failure increased during erythroblast differentiation, and although the full ingress of cleavage furrow was often observed in mDia2−/− erythroblasts of a relatively bigger size in the early stage, no or little ingress was observed in more differentiated, smaller erythroblasts. These results implicate an alternative actin nucleator or F-actin assembly pathway downstream of Rho during cytokinesis of erythroid progenitor cells and suggest that mDia2 replaces their role during erythroblast differentiation. It is interesting in this respect that expression of mDia2 more than tripled during this time.

Finally, we have revealed that cytokinesis and enucleation are driven by different Rho-GTPase signaling mechanisms. Previously, Ji et al. (2008) reported that the percentage of enucleated erythroblasts in mDia2 shRNA-treated cells was decreased and suggested the involvement of mDia2 in enucleation. We also found the similar decrease of the number of enucleated cells from mDia2−/− cells. However, we suggest that this is due to the cytokinesis failure during erythropoiesis, which resulted in the decrease of orthochromatic erythroblasts and increase in multinucleated cells. The multinucleation itself might also affect the efficiency of enucleation due to the increase in the number of nuclei in one cell, although multinucleate mDia2−/− erythroblasts could extrude their nuclei with F-actin accumulation, albeit lower frequency, to give rise to big enucleated cells. On the other hand, the nuclear protrusion in mDia2−/− erythroblasts was blocked by inhibition of Rac or F-actin depolymerization, which is in accordance with the previous reports by Ji et al. (2008) and Konstantinidis et al. (2012). These results suggest the presence of other characterized F-actin assembly mechanisms downstream of Rac in the enucleation process.

In summary, we have identified the Rho–mDia2 pathway as the conserved Rho-formin family protein axis in cytokinesis of erythroblasts in vivo. Our study has further demonstrated that mDia2 is not critical in cytokinesis of every type of cell and that Rac utilizes yet unidentified actin-dependent mechanisms in enucleation.

**EXPERIMENTAL PROCEDURES**

**Hematological Analysis and In Vitro Differentiation of Erythroid Cells**

We have obtained all the necessary permissions from boards of animal and DNA experiments in Kyoto University and RIKEN CDB for the mice used in our research. Fetal livers were isolated from E11.5–E12 embryos and mechanically dissociated by pipetting with a 25G needle in Iscove’s Modified Dulbecco’s Medium (IMDM) containing 2% FBS (STEMCELL Technologies). Single-cell suspensions were prepared by passing the dissociated cells through a 25 μm cell strainer (BD Falcon). In vitro erythroid differentiation was performed according to Zhang et al. (2003). In brief, fetal liver cells were labeled with biotin-conjugated Ter119 antibody (BD Pharmingen), and Ter119+ cells were purified through MS column and Streptavidin MicroBeads (Milenyi Biotec). Purified cells were seeded with IMDM GlutaMAX (Invitrogen), 15% FBS (STEMCELL Technologies), 200 μg/ml holo-transferrin, 10 μg/ml recombinant human insulin, 0.1 mM l-mercaptoethanol (Invitrogen), 1× penicillin-streptomycin mixed solution (PS) (Nacalai Tesque), and 2 U/ml EPO (Roche) into a dish precoated with 50 μg/ml fibronectin (Sigma-Aldrich). The cells were maintained at 37°C with an atmosphere containing 5% CO2. For Figures 4F–4H and S4, the medium was replaced at 24 hr culture with IMDM GlutaMAX containing 20% FBS, 1× PS, 0.1 mM l-mercaptoethanol and further cultured for 12–36 hr.

**Statistical Analysis**

Data are presented as mean ± SD or SEM and were evaluated using indicated statistical tests on Prism 4.0 software (GraphPad). A value of p < 0.05 was considered statistically significant.
SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.10.021.

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