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—Original Article—

The Dynamin 2 inhibitor Dynasore affects the actin filament distribution during mouse early embryo development

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Abstract. Dynamin 2 is a large GTPase notably involved in clathrin-mediated endocytosis, cell migration and cytokinesis in mitosis. Our previous study identified that Dynamin 2 regulated polar body extrusion in mammalian oocytes, but its roles in early embryo development, remain elusive. Here, we report the critical roles of Dynamin 2 in mouse early embryo development. Dynamin 2 accumulated at the periphery of the blastomere during embryonic development. When Dynamin 2 activity was inhibited by Dynasore, embryos failed to cleave to the 2-cell or 4-cell stage. Moreover, the actin filament distribution and relative amount were aberrant in the treatment group. Similar results were observed when embryos were cultured with Dynasore at the 8-cell stage; the embryos failed to undergo compaction and develop to the morula stage, indicating a role of Dynamin 2 in embryo cytokinesis. Therefore, our data indicate that Dynamin 2 might participate in the early embryonic development through an actin-based cytokinesis.
Key words: Actin filament, Cytokinesis, Dynamin, Embryo

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Dynamin has been known for its molecular motor-like properties [1]. Dynamin is a large multi-domain GTPase involved in diverse cellular processes, especially in clathrin-mediated endocytosis [2], cell migration [3] and cell division [4]. In mammals, there are three classical isoforms: Dynamin 1, Dynamin 2 and Dynamin 3. Different from Dynamin 1 and Dynamin 3, which are expressed in a tissue-specific manner, Dynamin 2 exhibits an ubiquitous expression [5, 6]. Structurally, a GTPase domain, a middle domain, a pleckstrin homology (PH) domain, a GTPase effector domain (GED) and a proline-rich domain (PRD) comprise the Dynamin 2 [7]. In addition to its critical roles in endocytosis, Dynamin 2 also binds with several proteins, such as Cortactin, Profilin, and Syndapin,to regulate the actin filaments [8–10].

Actin filaments exist in two forms in cells, the globular monomer (G-actin) or filamentous F-actin. The actin cytoskeleton is involved in cellular morphogenesis, motility, division, intracellular transport and other cellular processes [11]. Plenty of work has shown that actin dynamics are closely regulated by the actin regulatory proteins [12–14].

The mammalian embryo development process involves cell division, cell differentiation and cellular morphogenesis. After sperm penetration, actin is responsible for spindle rotation and pronuclear apposition as well as the cleavage and polarity of embryos in different species [15]. Our previous evidence shows that Dynamin 2 plays an important role in oocyte maturation through an actin-based pathway [16]; however, whether Dynamin 2 participates in embryo development remains elusive. In the present study, we focused on the relationship between Dynamin 2 and actin dynamics during embryonic development. Our results indicate that the Dynamin 2 inhibitor Dynasore affects actin assembly and subsequent mammalian embryo development.

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Materials and Methods

Antibodies and chemicals

Goat polyclonal anti-Dynamin 2 antibody was obtained form Santa Cruz Biotechnology (Santa Cruz, CA, USA), and Dynasore (Catalog number, 324410) was obtained from Calbiochem (Merck KGaA, Darmstadt, Germany). Alexa Fluor rabbit anti-goat 488 was obtained from Invitrogen (Carlsbad, CA, USA). Phalloidin-TRITC was purchased from Sigma-Aldrich (St Louis, MO, USA); we employed 1 μg/ml in the experiment.

Zygote collection and culture

All animal manipulations were conducted according to the guidelines of the Animal Research Committee of Nanjing Agricultural University, PR China. Mice were housed in a temperature-controlled room with proper light-dark cycles, fed a regular diet, and maintained under the care of the Laboratory Animal Unit, Nanjing Agricultural University, PR China. This study was specifically approved by the Committee of Animal Research Institute, Nanjing Agricultural University, PR China. Forty-eight hours after injection of pregnant mare serum gonadotrophin (PMSG), 6- to 8-week-old ICR mice were injected with human chorionic gonadotrophin (hCG) and immediately mated with male mice. After 18 h, zygotes were collected and cultured

in K modified simplex optimized medium (KSOM) (Chemicon, Billerica, MA, USA) covered with paraffin oil at 37 C in a 5% CO_2 atmosphere. Embryos were collected for immunofluorescence staining after different times in culture.

Dynasore treatment

A 50 mM solution of Dynasore (diluted with DMSO) was diluted with KSOM to 200 μM. After collection, the mouse embryos were cultured in KSOM medium (the volume of each drop was 50 μl in a 35 mm Corning dish containing 200 μM Dynasore for different periods of time to examine the effects of Dynasore on embryo development.

Confocal microscopy

For immunofluorescence staining, zygotes were fixed in 4% paraformaldehyde in PBS at room temperature for 30 min and then transferred to a membrane permeabilization solution (0.5% Triton X-100) for 20 min. After 1 h in blocking buffer (1% BSA in PBS), zygotes were incubated with goat anti-Dynamin 2 (1:25) antibody overnight at 4 C. After washing three times (2 minutes each time) in wash buffer (0.1% Tween 20 and 0.01% Triton X-100 in PBS), zygotes were labeled with FITC-anti-goat IgG (1:100) at room temperature for 1 h. For Phalloidin-TRITC staining, after fixation, permeabilization and blocking as described above, zygotes were incubated with Phalloidin-TRITC (1 μg/ml) for 1 h and then washed three times (2 minutes each time) in wash buffer. The samples were then co-stained with Hoechst 33342 (10 μg/ml in PBS) for 10 min. A confocal laser-scanning microscope (Zeiss LSM 700 META, Germany) was employed to examine the samples mounted on glass slides.

Images were taken at 10 (eye lens) × 40 (objective) × 1.8 (scan zoom)-fold magnification in an optical cross section through the center of the oocyte at its largest nuclear diameter. The micrographs were analyzed using the ImageJ 1.44P software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA), to measure the fluorescence intensity of embryos.

Fluorescence intensity analysis

Fluorescence intensity statistics were evaluated using the ImageJ (NIH) software. To analyze actin fluorescence intensity, samples of control embryos and treated embryos were mounted on the same glass slide. The ImageJ software was used to define a region of interest (ROI), and the average fluorescence intensity per unit area within the ROI was determined. Independent measurements using identically sized ROIs were made for the cell membrane and cytoplasm. The average values of all measurements were used to compare the final average intensities between control and treated embryos. The intensity curves and the histograms were produced by the ZEN 2009 software (Zeiss, Jena, Germany).

Statistical analysis

At least three replicates were performed for each experiment, and the results were expressed as means ± SEM's. Statistical comparisons between groups were made by analysis of variance (ANOVA) followed by Duncan's multiple comparisons test. A P-value of < 0.05 was considered significant. Fluorescence was measured by confocal microscope (Zeiss, LSM 700).

Results

Disruption of Dynamin 2 arrests mouse early embryonic development

Before we investigated the role of Dynamin 2 in mouse embryo development, its localization was confirmed by immunofluorescent staining. As shown in Fig. 1A, during the whole process of embryo development, Dynamin accumulated around the periphery of each blastomere. For co-staining of Dynamin 2 and actin, Dynamin 2 showed a localization pattern similar to actin at the periphery of blastomeres (Fig. 1B). To further explore the function of Dynamin 2 in mouse embryonic development, we cultured the embryo at the zygote and 2-cell stage with Dynasore for 24 h, respectively. The results revealed a significant phenomenon: when inhibition began at the zygote stage, none of the zygotes could develop to 2-cell stage (Supplementary Fig. 1A: on-line only). When the inhibition began at the 2-cell stage, the embryos failed to develop to the 4-cell stage: 88 ± 3% (n = 178) of the embryos in the control group developed to the 4-cell stage (Fig. 2A) compared with 10 ± 5% (n = 175) in the treatment group (Fig. 2B) ($P<0.05$). Thus, the results reveal that the Dynamin inhibitor affected mouse early embryo cleavage.

Dynasore treatment causes failure of blastocyst formation

In mice, compaction begins at the 8-cell stage, and embryos are subjected to morphogenetic changes to prepare for implantation. Since we observed the change in Dynamin 2 localization at the 8-cell stage, we then tested the effect of Dynasore on 8-cell embryo development. After 24 h culture, we observed the failure of blastocyst formation (Fig. 3A) in the Dynasore-treated embryos; 39 ± 10% (n = 88) of the embryos in the control group developed to blastocysts or hatched blastocysts, while only 2 ± 2% (n = 94) developed normally in the treatment group (Fig. 3B) ($P<0.05$). Therefore, the results further demonstrate that Dynasore could inhibit mouse morula/blastocyst formation.

Inhibition of Dynamin 2 activity disrupts the actin filament distribution

The failure of early embryonic development led us to explore the mechanism during this process. We employed fluorescent staining to observe the dynamics of actin filaments. The results showed that the distribution of actin filaments in the cortex of embryos (2-cell stage) increased significantly in the presence of Dynasore compared with the control embryos, which developed to 4-cell stage (cultured for 24 h, mounted on the same glass) (Fig. 4A). Furthermore, the fluorescent intensity curve (Fig. 4B) and histogram (Fig. 4C) also revealed that the amount of actin in the treatment group was significantly higher than that in the control group. There were no other differences when the embryos were treated with 50 μM Dynasore with respect to the actin filaments (Supplementary Fig 1B and C: on-line only). To further confirm this, we cultured the 2-cell embryos for 12 h (when all of the control embryos and treated embryos remained at the 2-cell stage), and similar results were found in this case: the actin filaments were also enriched in the cortex of Dynasore-treated 2-cell embryos compared with the control embryos (Fig. 4D and E).

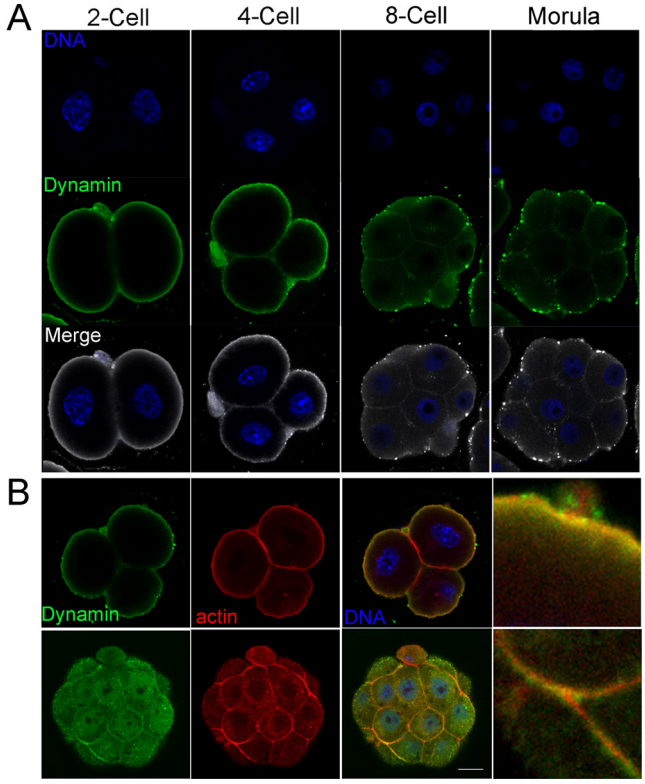

Fig. 1. Dynamin 2 expression and localization in mouse embryos. (A) Dynamin localized at the periphery of blastomeres during the 2-cell, 4-cell, morula and blastocyst stages of mouse embryos. Green, Dynamin 2; blue, chromatin. (B) Co-staining of Dynamin 2 and actin. Dynamin co-localized with actin, and the signals were overlapped. Green, Dynamin 2; blue, chromatin; red, actin; yellow, overlap of green and red. Bar=20 μm.

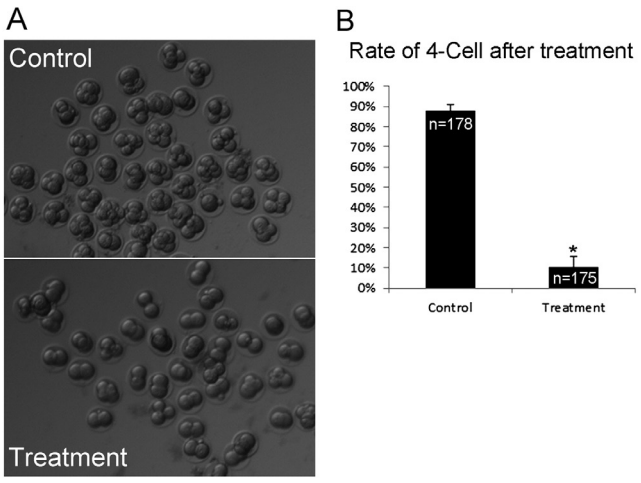

Fig. 2. Effects of the Dynamin 2 inhibitor Dynasore effects early mouse embryo cleavage. (A) After culture in Dynasore for 24 h at the 2-cell stage, the embryos failed to cleave and arrested at the 2-cell stage, while the control embryos developed to the 4-cell stage. (B) Rate of embryo development after Dynasore treatment. * Significantly different ($P<0.05$).

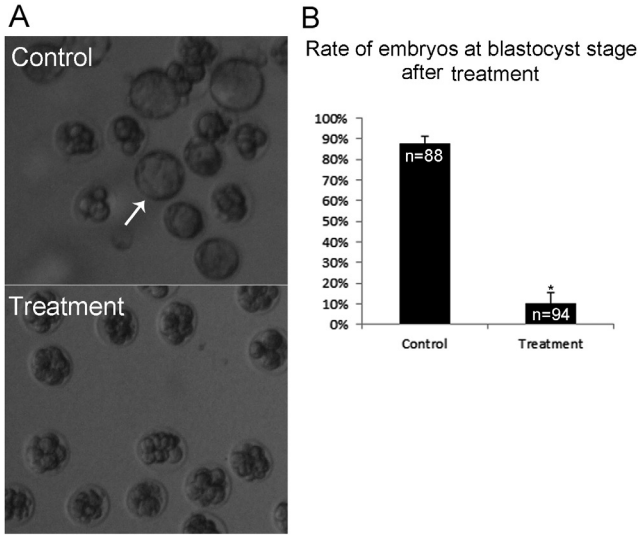

Fig. 3. Effects of the Dynamin 2 inhibitor Dynasore on blastocyst formation during mouse embryo development. (A) After Dynasore treatment at the 8-cell stage, embryos failed to develop to the blastocyst, while the control embryos developed normally to the blastocyst stage (arrow). (B) Rate of blastocyst formation after Dynasore treatment. * Significantly different ($P<0.05$).

Discussion

Our previous work confirmed the involvement of Dynamin 2 in meiosis by regulating oocyte polar body extrusion [16]. In the present study, we preliminarily explored the possible function of Dynamin 2 in embryo development. The results demonstrate that the large GTPase Dynamin 2 takes part in embryo cleavage through an actin-based pathway.

We first examined the localization of Dynamin 2 during embryonic development. From the 2-cell to morula stage, Dynamin 2 accumulated at the periphery of blastomeres, and the localization pattern of Dynamin 2 was similar to that of actin. The localization pattern was similar to that in previous work with a somatic cell model and our findings in mouse oocytes [4, 16]. The special localization of Dynamin 2 prompted us to further explore the possible roles of Dynamin 2 during early embryo development. Inhibition of Dynamin 2 activity with Dynasore caused the failure of embryo cleavage. We cultured embryos from the zygote stage or 2-cell stage, and most of the Dynasore-treated embryos could not develop to the subsequent stage. Also, inhibition of Dynamin 2 from the 8-cell stage caused the failure of compaction. Since compaction of embryos occur at the 8-cell stage and a previous study demonstrated that molecular disruption of actin prevents embryo compaction [17], we reasoned that the failure of blastocyst formation results from the failure of compaction. Furthermore, previous studies together with our results have proved that Dynamin is required for cytokinesis in cancer cells [18], in zebrafish embryos [19] and in mammalian oocytes maturation [16, 20]. Thus, we infer that the function of Dynamin

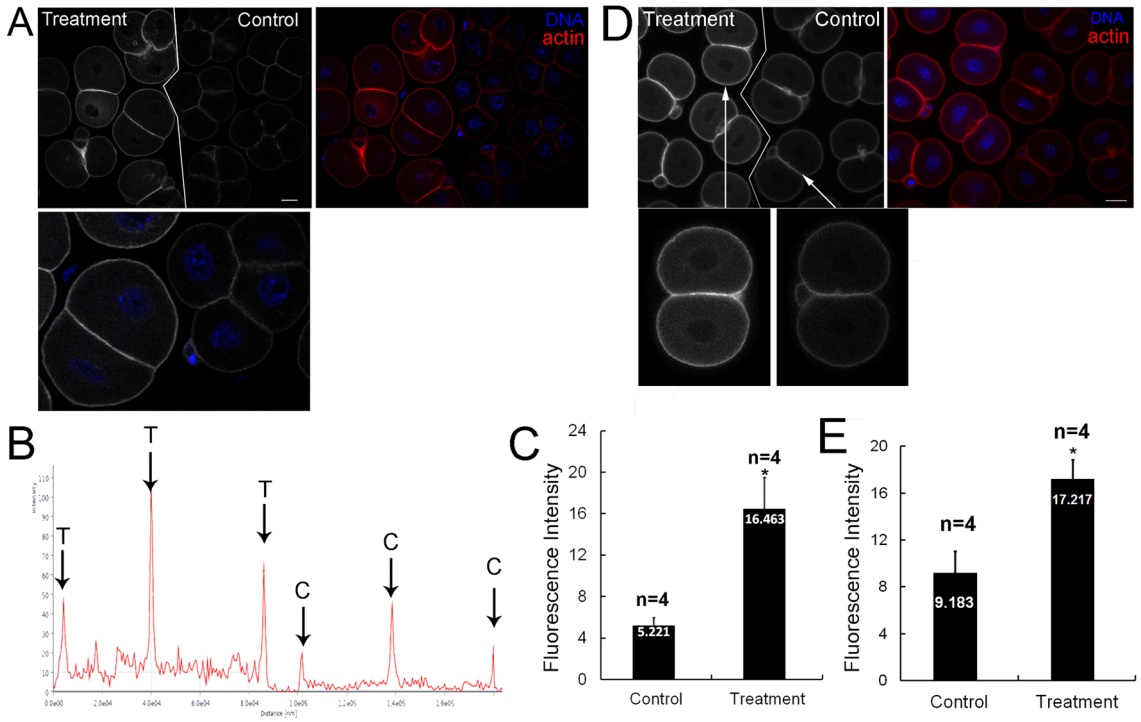

Fig. 4. Changes in actin expression after suppressing Dynamin 2 activity by Dynasore treatment. (A) After culture for 24 h, the control group developed to the 4-cell stage, while the treatment group arrested at the 2-cell stage; its fluorescence signal was also higher than that in the control group. Red, actin; blue, chromatin. Bar=20 μm. (B) The fluorescence intensity curve shows a comparison of the actin fluorescence signals of representative embryos for Fig. 4A. The peaks (arrows) indicate the actin signal at the membrane of the blastomere in mouse embryos. C, control; T, treatment. (C) The measurements of the fluorescence intensity of actin after 24 h of treatment. * Significantly different ($P<0.05$). (D) After 12 h of culture, both the control and treatment group were at the 2-cell stage, and the fluorescence signal of actin in the treatment group was higher than that in the control group. (E) The measurements of the fluorescence intensity of actin after 12 h of treatment. * Significantly different ($P<0.05$). Red, actin; blue, chromatin. Bar=20 μm.

in the embryo is conserved and that the failure of embryo cleavage is possibly caused by a deficiency of cytokinesis.

Accumulating work has shown that Dynamin regulates actin polymerization in various models [16, 20–22], which led us to explore the relationship between Dynamin and actin in mouse embryos. The co-localization of Dynamin and actin indicated a relationship between them. This hypothesis was also confirmed in our study, and inhibition of Dynamin by Dynasore caused an aberrant distribution of actin filaments, which was consistent with previous work showing that a low concentration of Dynamin 2 enhanced actin nucleation via Arp2/3 complex and cortactin [23]. Decreased actin assembly as a result of high inhibition of Dyanmin 2 activity is an interesting phenotype and indicates that Dyanmin 2 plays a dynamic and balanced regulatory role in actin assembly and disassembly. Our results, together with those of previous studies, shows a conserved role of Dynamin 2 in actin nucleation or assembly. Therefore, our study reveals that Dynamin regulates mouse embryo cleavage via its effect on the actin filament distribution.

Taken together, our preliminary results indicate that Dynamin 2 is essential for mouse early embryo cleavage, and this regulation might be mediated in an actin-based way.

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