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Galpha/LGN-mediated asymmetric spindle positioning does not lead to unequal cleavage of the mother cell in 3-D cultured MDCK cells

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Abstract

The position of the mitotic spindle plays a key role in spatial control of cell division. It is generally believed that when a spindle is positioned asymmetrically in a dividing cell, the resulting daughter cells are usually unequal in size due to eccentric cleavage of the mother cell. Molecular mechanisms underlying the generation of unequal sized daughter cells have been extensively studied in *Drosophila* neuroblast and *C. elegans* zygote where the Gα subunit of the heterotrimeric G proteins and its binding partner—Pins in *Drosophila* and GPR-1/2 in *C. elegans*—are shown to be critical in governing spindle positioning and asymmetric cleavage of the mother cell. In mammalian system, although Gα and LGN (mammalian Pins homologue) are also required for spindle orientation, whether they can mediate asymmetric spindle positioning or asymmetric cleavage of the mother cell is not known. Here, by artificially targeting Gαi to the apical cortex in 3-D cultured MDCK cells, we established a system where asymmetric spindle positioning can be consistently induced. Interestingly, this asymmetrically positioned spindle does not lead to asymmetric cleavage; instead it results in equal sized daughter cells. Live cell time-lapse analysis revealed that anaphase spindle elongation compensated the original asymmetric spindle positioning. Our findings demonstrate that asymmetric spindle positioning does not necessarily lead to unequal sized daughter cells in mammalian system. We discuss potential mechanisms in generating unequal sized daughter cells.

Keywords

Gα; LGN; cell division; asymmetric spindle positioning; cleavage furrow; cytokinesis

1. Introduction

Cells propagate their progenies through cell division. To generate identical daughter cells, the mother cells divide symmetrically by positioning the mitotic spindle at the center of the cell. The mitotic spindle then directs central cleavage furrow formation which leads to the production of two equal sized daughter cells that inherit identical set of genetic materials

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and cytoplasmic components. Another type of cell division, called asymmetric cell division, is usually utilized by stem/progenitor cells to generate different daughter cells with distinct cell fates [1]. Asymmetric cell division usually results in the generation of unequal sized daughter cells.

It is generally believed that the position of mitotic spindle dictates the relative size of the daughter cells: a centrally located spindle leads to an equal cleavage, whereas an eccentrically located spindle results in an unequal cleavage, therefore generates unequal sized daughter cells [2-4]. Spindle positioning is achieved through interactions between astral microtubules and the cell cortex [5]. Recent studies suggest that receptor-independent G-protein signaling is a major regulator of spindle positioning in model systems including *C. elegans* and *Drosophila* [3, 6]. Cortical G α and its binding partner GPR-1/2 (in *C. elegans*) and Pins (in *Drosophila*) are the key players for generating asymmetric spindle positioning and subsequent unequal sized daughter cells[6]. In *C. elegans* zygote, G α and GPR-1/2 are enriched at the posterior cell cortex where they serve as force generators, probably through cytoplasmic dynein, to produce stronger pulling forces on astral microtubules and lead to spindle displacement towards this side of the cell [7-9]. Such asymmetric spindle positioning leads to the production of a larger anterior and a smaller posterior daughter cell. In *Drosophila* neuroblast, apically localized G α and Pins are required for spindle positioning and the generation of unequal sized daughters—a larger apical daughter cell that remains to be a neuroblast and a smaller basal ganglion mother cell [10-13]. In mammalian system, G α and LGN (mammalian homologue of Pins) are also involved in regulating spindle orientation during neurogenesis, epidermal differentiation, epithelial morphogenesis and dermomyotome development [14-20]. However, whether G α /LGN complex can direct asymmetric spindle positioning and asymmetric cleavage of the mother cell in mammalian system is not known.

Using an apically located protein Crumb3 (Crb3) as a vehicle, we have successfully targeted ectopically expressed G α i1 and endogenous LGN to the apical cell membrane in 3-D cultured MDCK epithelial cells [20]. Here, we report that the apical G α i1/LGN complex not only redirects the mitotic spindle orientation from perpendicular to parallel to the apical-basal axis, but also consistently positioned the metaphase spindle asymmetrically toward the apical cell membrane. Such a reproducible and biologically relevant system allowed us to test whether asymmetric spindle positioning leads to the generation of unequal sized daughter cells. By analyzing fixed cysts samples and live cell imaging of the cell division process, surprisingly, we found that G α i1/LGN-mediated asymmetric spindle positioning does not result in asymmetric cleavage of the mother cell; instead, the mother cell eventually generates two equal sized daughter cells. Our findings challenge the general opinion that asymmetric spindle positioning leads to unequal sized daughter cells.

2. Materials and methods

2.1. Antibodies

The following antibodies were used: mouse anti- α -tubulin (Sigma-Aldrich), rabbit anti- γ -tubulin (Invitrogen), mouse anti- β -catenin (BD); and secondary Alexa 488, Alexa 594, Alexa 680 (Invitrogen), and IRDye800 (Rockland) conjugated goat anti-mouse or rabbit antibodies. Hoechst 33342 (Invitrogen) was used for DNA staining.

2.2. Cell culture and stable cell lines

MDCK cells were cultured in DMEM supplemented with 10% fetal calf serum and penicillin/streptomycin (100 IU/ml and 100 mg/ml, respectively) at 37 °C in a humidified 5% CO₂ atmosphere. Stable Tet-Off inducible MDCK Crb3-Venus-G α i1 cell line was

generated as described previously [20]. Briefly, Crb3 cDNA was first cloned in pTRE2Hyg vector. Venus was then cloned downstream of, and in frame with Crb3 to make pTRE2Crb3-Venus. cDNA encoding Gai1 were inserted in pTRE2Crb3-Venus to generate pTRE2Crb3-Venus-Gai1, and the plasmid was transfected into MDCK T23 cells and stable clones were selected using hygromycin. For the Crb3-Venus-Gai1/mCherry- α -tubulin cell line, the mCherry- α -tubulin cassette was released from pmCherry- α -tubulin-IRES-puro2 plasmid (Addgene plasmid 21043) and cloned into pcDNA3.1/Zeo (Invitrogen) to generate pcDNA3.1-mCherry- α -tubulin-zoe. The plasmid was transfected into Crb3-Venus-Gai1 cell line in the presence of doxycycline and selected by Zeocin (Invitrogen).

2.3. 3-D culture

The 3-D culture of MDCK cells in matrigel was performed as previously described [20]. Briefly, cells were trypsinized and resuspended to single cell suspension of 4×10^4 cells/ml in 2% matrigel (BD). 400 μ l of cells were plated in each well of 8-well Lab-Tek II chamber slides (Thermo Fisher Scientific) precoated with 30 μ l of matrigel. Cells were grown for 3-4 days.

2.4. Western blot analysis

Cells were washed with cold PBS and collected in cell lysis buffer (25 mM Hepes, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 0.5 mM EDTA, 5 mM MgCl₂, 1 mM DTT, 1 mM PMSF, 10 μ g/ml leupeptin, and 20 μ g/ml aprotinin). Cell debris was removed by centrifugation at 14,000 rpm for 20 min at 4°C. SDS sample buffer was added to equal amounts of cell lysate and proteins were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and analyzed with anti- α -tubulin antibody.

2.5. Immunofluorescence microscopy

Cysts images were captured on a confocal microscope (510 LSM; Carl Zeiss, Inc.) using a Plan Apochromat 63 $\times$ /1.4 NA oil objectives (Carl Zeiss, Inc.) and analyzed using the LSM Image Examiner and Image J software. To quantitate spindle and spindle pole to cell cortex distances, γ -tubulin staining was used to visualize spindle poles. To quantitate the areas of daughter cells, β -catenin staining was used to mark the cell border. Standard deviation was calculated, and statistical significance was determined by Student's t test.

2.6. Time-lapse analysis

Time-lapse analysis was performed on a LSM 7 Live laser-scanning confocal microscope (Carl Zeiss, Inc), with a plan-Apochromat 63 $\times$ /1.4 oil objective. Since the mCherry- α -tubulin forms mitotic spindle only during mitosis, it is easy to identify mitotic cells in the cysts. The images were acquired every 10 seconds with ZEN 2009 software (Carl Zeiss, Inc).

3. Results

3.1. Apical targeting of Gai1/LGN leads to asymmetric spindle positioning during cystogenesis of MDCK cells

Organotypic 3-D culture of MDCK cells phenocopies many aspects of *in vivo* cystogenesis and has been used as a model system to study epithelial morphogenesis *in vitro*. Using such a system, we showed previously that LGN plays a critical role in directing spindle orientation during epithelial morphogenesis [20]. During cystogenesis, atypical protein kinase C-mediated apical exclusion leads to restricted lateral cortical localization of LGN which directs spindle orientation to be perpendicular to the apical-basal axis [20, 21]. To test the effect of apical mistargeting of LGN on spindle orientation and cystogenesis, we

generated inducible MDCK cell lines expressing a Crb3-fused wild type Gai1 (Crb3-Venus-Gai1). Ectopic expression of Crb3-Venus-Gai1 resulted in apical localization of the fusion protein which in turn recruits endogenous LGN to the apical cell cortex during cystogenesis [20]. Such artificial apical targeting of endogenous LGN led to a complete reversion of the mitotic spindle axis—from perpendicular to parallel to the apical-basal axis—and profound cystogenesis defects [20]. Interestingly, when carefully examining Crb3-Venus-Gai1 cysts stained with anti- α -tubulin antibody, we noticed that except for reorientation, the spindle appeared to be always positioned asymmetrically towards the apical cell cortex (Fig. 1B). To confirm such observation, we stained the cysts with anti- γ -tubulin antibody (Fig. 1C) and measured the distances between spindle poles to the apical or basal cell membrane, respectively, as depicted in Fig. 1D. Indeed, the distance from the apical spindle pole to apical cell membrane is significant shorter than that from the basal spindle pole to the basal membrane (Fig. 1E). When doxycycline was present in the medium to suppress the expression of Crb3-Venus-Gai1, the mitotic spindle positioned perpendicular to the apical-basal axis and in the center of the cell (Figure 1A). Importantly, in dividing Crb3-Venus-Gai1 cells, besides spindle misorientation and mispositioning, we did not observe obvious defect in spindle organization and chromosome alignment (Fig. 1B). Thus, apical targeting of Gai1/LGN complex led to asymmetric spindle positioning; further support the hypothesis that this complex could generate pulling forces on astral microtubules.

3.2. Asymmetric spindle positioning generates equal sized daughter cells

During asymmetric cell division of *Drosophila* neuroblast, apically localized G α and Pins are involved in generating unequally sized apical and basal spindles as well as astral microtubules [10, 13]. We wondered whether similar outcome could be observed in our system. We carefully compared the apical and basal spindle length by measuring the distances from the apical or basal spindle poles to the center of aligned chromosome (Fig. 1D). No obvious difference in length between apical and basal spindles was observed (Fig. 1E). We were not able to analyze astral MTs due to the limited image resolution in 3-D cysts.

As asymmetrically localized G α /Pins or G α /GPR-1,2 led to asymmetric cleavage of *Drosophila* neuroblast and *C. elegans* zygote, we wondered whether Gai1/LGN-mediated asymmetric spindle positioning also led to asymmetric cleavage of the mother cell in our system. Surprisingly, when we viewed telophase daughter cells, we did not observe obvious size difference between apical and basal daughter cells (Fig. 2A). To more precisely compare daughter cell size, we stained the cysts with anti- β -catenin antibody to mark the cell border (Fig. 2B). Again, the metaphase spindle is apparently asymmetrically positioned towards the apical cell cortex (Fig. 2B). We then measured and compared the area of telophase daughter cells. Indeed, there is no significant difference in daughter cell sizes (Fig. 2C; the area ratio of apical to basal cell is 1.05 ± 0.08 , $n=20$).

3.3. Anaphase spindle elongation compensates the original asymmetric spindle positioning

How could apparent asymmetric spindle positioning end up with equal sized daughter cells? The best way to answer this question is to monitor the detailed process of cell division in our system. We then generated double stable cell lines by introducing mCherry- α -tubulin into Crb3-Venus-Gai1 cell line, so we can monitor the mitotic spindle movement in live cells. Western blot analysis indicated that mCherry- α -tubulin was expressed at a level that is comparable to endogenous α -tubulin in the cell line we generated (Figure 3A).

We performed time-lapse analysis of mitotic cells in 3-D cultured cysts. First, we grew Crb3-Venus-Gai1/mCherry- α -tubulin cells in 3-D culture in the presence of doxycycline to inhibit the ectopic expression of Crb3-Venus-Gai1. The cells propagated nicely into single-lumened cysts, just like control MDCK cells (data not shown). Live cell analysis confirmed previous results that the mitotic spindle aligned predominantly perpendicular to the apical basal axis of the cell [20]. Although slight oscillation of the spindle axis was observed, the cell eventually divided along the planar axis. During anaphase when spindle elongation occurs, the two spindle poles move at similar speed towards the lateral cell cortex (Figure 3B, Movie 1). Next, we grew Crb3-Venus-Gai1/mCherry- α -tubulin cells in 3-D culture in the absence of doxycycline to allow the expression of Crb3-Venus-Gai1. Not surprisingly, the mitotic spindle rotated from perpendicular to parallel to the apical basal axis of the cell, and always positioned asymmetrically towards apically localized Crb3-Venus-Gai1 (Fig. 3C). When the cells entered anaphase, apical and basal spindle elongated at similar speed. Strikingly, accompanying spindle elongation, the basal spindle pole appeared to be pushed towards the basal cortex while the apical spindle pole did not show apparent movement (Fig. 3C, Movie 2). When the basal spindle pole almost reached the cell cortex, cleavage furrow started to form in the middle of the cell and eventually led to the production of equal sized daughter cells (Fig. 3C, Movie 2). Such pattern of spindle pole movement was observed in multiple cells and quantitation of the distance from spindle poles to cell cortex at different time points revealed that the basal spindle pole was initially far away from the basal cortex, and this distance was consistent through metaphase; when cell entered anaphase, this distance was shortened gradually until it was close to that of from apical spindle pole to the apical cell cortex (Figure 3D). We conclude that anaphase spindle elongation compensates the original asymmetric spindle positioning in our system.

4. Discussion

Mechanisms of asymmetric spindle positioning and cell division have been intensely studied in the *C. elegans* zygote and *Drosophila* neuroblast [1, 3, 22]. Although most of the identified molecules that are involved in the regulation of asymmetric cell division are conserved in the two systems, there are obvious differences between the two types of cells in the ways of producing unequal sized daughter cells. In *Drosophila* neuroblast, asymmetric cell division involves differential spindle length regulation—the apical spindle is longer than the basal spindle during anaphase [13]. It also involves asymmetric spindle positioning—the spindle is positioned closer to the basal cell cortex, probably due to asymmetric astral microtubule nucleation [10]. Such asymmetry in spindle length and overall spindle positioning lead to the asymmetric cleavage and appear to rely on redundant Par3/Insc and Pins/G α pathways [10, 12]. In *C. elegans* zygote, right after metaphase onset, a so-called spindle displacement, featured by the movement of the entire spindle towards the posterior cell cortex in a G α /GPR-1,2 dependent manner, results in the asymmetric positioning of the spindle and subsequent asymmetric cleavage of the zygote [22]. No obvious difference in terms of the anterior and posterior spindle length is observed, although the length difference between anterior and posterior astral MTs is apparent [23].

In mammalian system, the G α /LGN complex is also required for directing spindle orientation during asymmetric cell division of neuronal and epidermal progenitor cells [16-18]. However, asymmetric spindle positioning and asymmetric cleavage of the mother cell have not been reported in both systems, probably due to technical difficulties for *in vivo* analysis in mammals. So far, there is no mammalian system that can consistently produce asymmetric spindle positioning without compromising normal spindle organization and subsequent cell division. We have now, for the first time, established such a system. Artificially targeting of Gai1 to the apical cell membrane led to apical recruitment of endogenous LGN, which mimics the asymmetric localization of this protein complex in

Drosophila neuroblast and *C. elegans* zygote. Indeed, apically localized Gai1/LGN can redirect spindle orientation from perpendicular to parallel to the apical-basal axis during cystogenesis, suggesting a conserved function for this complex in directing spindle orientation. Intriguingly, asymmetric localization of Gai1/LGN complex also leads to asymmetric spindle positioning within the cell—the spindle is always positioned towards the apical cell cortex, which is similar to the spindle displacement in *C. elegans* zygote. Unexpectedly, however, Gai1/LGN-mediated asymmetric spindle positioning in our system does not lead to asymmetric cleavage of the mother cell; instead, it generates two equal sized daughter cells. Careful time-lapse analysis revealed that anaphase spindle elongation gradually compensates the initial asymmetric localization of the metaphase spindle.

We believe that our system is different from *Drosophila* neuroblast, although the role of asymmetrically localized Gai1/LGN in directing spindle orientation is conserved. First, we did not observe differential spindle lengthening in our system either during metaphase or anaphase. Second, we did not see the robust apical astral microtubule nucleation as observed in anaphase neuroblast. In *Drosophila* neuroblast, the asymmetric spindle lengthening and astral microtubule nucleation is regulated by redundant Gai/Pins and Par3/Insc pathways [12]. In our system, Insc may not be expressed (data not shown); and Par3 also localizes differently—at tight junctions rather than at the apical membrane. Par3 and Insc may modulate the interaction between Pins and other proteins [24] which may contribute to spindle length regulation. Alternatively, in our system, Gai1 is fused with Crb3-Venus which may interfere with the normal signaling property of Gai1 that is required for spindle length control or astral microtubule nucleation. It is also possible that some basally localized proteins, which are absent in our system, are involved in shortening the basal spindle in neuroblast. Further studies are needed to distinguish these possibilities.

Our system is more like *C. elegans* zygote where mitotic spindle is displaced towards asymmetrically localized Gai/GPR-1,2. One obvious difference, however, is that *C. elegans* zygote is almost 3 times larger than MDCK cells. Such size provides more than enough space for anaphase spindle elongation in *C. elegans*, and the two spindle poles are far away from the cell cortex when cytokinesis is initiated. In most mammalian cells, especially for those that round up during mitosis, anaphase spindle elongation pushes the spindle poles towards the cell cortex and the maximum spindle length is almost equal to the length of the cell body. We speculate that if the mitotic cells in our system were larger than the maximum length of the anaphase spindle, we would predict asymmetric cleavage and the production of unequal sized daughter cells, as the central spindle, which plays a dominant role in dictating cleavage furrow formation in mammalian cells, would still be eccentrically positioned when cleavage furrow starts to form.

Our study also suggests that the timing of cleavage furrow formation must be tightly regulated in our system—the cleavage furrow starts to form only when the basal spindle pole has moved to be close enough to the basal cell cortex. If the cleavage furrow started to form before the basal spindle pole reached the cell cortex, it might also end up with asymmetric cleavage. Such timing may involve the interaction between astral microtubules and cortical cues.

Taken together, we propose that the lack of differential spindle lengthening, the limited size of the mother cell and the regulated timing of cleavage furrow formation may underlie the eventual symmetric cleavage and the generation of equal sized daughter cells in our system, although the metaphase spindle was initially asymmetrically positioned. Our study suggests that asymmetric spindle positioning does not necessarily lead to the production of unequal sized daughter cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviation

Gα	the alpha subunit of heterotrimeric G proteins
Pins	Partner of Inscuteable
LGN	mammalian homologue of Pins
3-D	three dimensional
MDCK	Madin Darby canine kidney
Crb3	Crumb3

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Highlights

1. Apical targeting of Gai1/LGN leads to asymmetric spindle positioning in 3-D cultured MDCK cells.
2. We established a system where asymmetric spindle positioning can be consistently induced.
3. Live cell time-lapse analysis of cell division in 3-D cultured MDCK cells.
4. Asymmetric spindle positioning does not lead to the generation of unequal sized daughter cells.

Figure 1.

Apical targeting of Gai1 results in asymmetric spindle positioning. Stable Tet-Off MDCK cells expressing Crb3-Venus-Gai1 were cultured in the presence (A. +Dox) or absence (B, C. -Dox) of doxycycline in matrigel for 3 days to allow 3-D cystogenesis. (A). Cysts were fixed and stained with anti- α -tubulin (red) and anti- γ -tubulin (green) antibodies. DNA was stained with Hoechst 33342. (B). Cysts were fixed and stained with anti- α -tubulin (red) antibody. DNA was stained with Hoechst 33342. (C). Cysts were fixed and stained with anti- γ -tubulin (red) antibody. DNA was stained with Hoechst 33342. Single confocal images were shown in A, B and C, white lines were drawn to outline the mitotic cell in A, B and C. (D). Schematic illustration for the measurement of relative spindle length and spindle positioning. Apical cell surface is facing the lumen. For each metaphase cell, two lines were drawn, one crossing the two spindle poles and the other along the long axis of the chromosome. The crossing point of the two lines was defined as "O". The distance from apical spindle pole to "O" was defined as "A" while the distance from basal spindle pole to "O" as "B". Similarly, the distance from apical spindle pole to apical cell cortex is defined as "a" while the distance from the basal spindle pole to basal cell cortex as "b". (E). Quantitation of A/B and a/b ratio as indicated in (D). n=30. Error bars represent SD (Standard Deviation).

Figure 2.

Asymmetric spindle positioning does not lead to the production of unequally sized daughter cells. (A), (B). Stable Tet-Off MDCK cells expressing Crb3-Venus-Gα1 were cultured in the absence of doxycycline in matrigel for 3 days to allow 3-D cystogenesis. Cysts were fixed and stained with anti- α -tubulin antibody (A, red) or anti- α -tubulin plus anti- β -catenin antibodies (B, red). DNA was stained with Hoechst 33342. Single confocal images were shown, white lines were drawn to outline the mitotic cell in A. (C). Quantitation of daughter cell area. **, $P < 0.05$.

Figure 3.

Timelapse analysis of spindle movement in 3-D cultured cysts. (A). Western blot analysis of mCherry- α -tubulin expression. Equal amounts of cell lysates from parental MDCK T23, MDCK T23 Crb3-Venus-Gai1 and MDCK T23 Crb3-Venus-Gai1/mCherry- α -tubulin cells were subjected to western blot analysis using anti- α -tubulin antibody. (B). Fluorescent images from time-lapse analysis of mitotic Crb3-Venus-Gai1/mCherry- α -tubulin cell in 3-D cyst in the presence of doxycycline. Time points are shown as seconds. (C). Fluorescent images from time-lapse analysis of mitotic Crb3-Venus-Gai1/mCherry- α -tubulin cell in 3-D cyst in the absence of doxycycline. Time points are shown as seconds. (D). Quantitative analysis of fluorescent images in C. Distances from apical spindle pole to the apical cortex and distances from basal spindle pole to basal cortex were measured at different time points during cell division. Results from three different cells are marked by different colors.