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Wnt signaling during *C. elegans* embryonic development

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Abstract

Wnt signaling has been demonstrated to regulate diverse cell processes throughout the development of the *C. elegans* embryo. Here we describe methods that have been used to investigate some of these Wnt dependent processes; endoderm specification, mitotic spindle orientation and cell migration.

Keywords

C.elegans; Wnt signaling; embryonic development

1. Introduction

Wnt signaling has been demonstrated to regulate diverse cell processes throughout the development of the *C. elegans* embryo. A Wnt signal was identified as the signal produced by germline precursors required to induce endoderm from mesendoderm precursors at the four-cell stage (1,2). Wnt signaling has been shown to regulate cell fate specification at many other points during *C. elegans* embryonic development (3). More recently Wnt signals have been shown to regulate other cell biological processes such as orientating mitotic spindles and thereby cell division axes, and regulating cell migrations (4–8). Below we describe cell biological techniques used to investigate how Wnts regulate these cellular processes in *C. elegans*.

2. Materials

1. 0.1% (w/v) Poly-L-lysine in water (Sigma)
2. 18 mm × 18 mm glass coverslips #1.5 (Corning)
3. Glass slides (Gold-Seal)
4. Sterile egg buffer (EB): 118 mM NaCl, 48 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 25 mM Hepes, pH 7.3 (store at room temperature)
5. 3% (w/v) Agar in EB, melted and kept covered at 60°C
6. Dissecting microscope.
7. Depression slides.
8. Needles: 25G 5/8.
9. *Caenorhabditis elegans* adult hermaphrodites.
10. Borosilicate capillaries pulled to narrow and wide bore sizes (1 mm inner diameter with filament, World Precision Instruments, see **Note 1**)

11. Mouth pipettes (Sigma Aspirator Tube Assembly)
12. Vaseline (melted in an eppendorf tube at 60°C)
13. Small paintbrush
14. Polarized light microscope with DIC optics.
15. 8-well slides (MP Biomedicals).
16. Humid chamber (clear plastic box with damp paper and supports for slides).
17. Sodium hypochlorite ($\geq 4\%$ Cl, Sigma).
18. Chitinase/chymotrypsin mix: 3 U/mL chitinase (Sigma), 10 U/mL chymotrypsin (Sigma) in EB (store at -20°C).
19. Fix buffer (FB): 10 mM MES, pH 6.1, 125 mM KCl, 3 mM MgCl, 2 mM EGTa, 10% (w/v) sucrose, 4% (w/v) formaldehyde (store at 4°C for up to a month, but allow to warm to room temp before use).
20. 10 mg/mL L- α -lyso-phosphatidyl choline in water (store at -20°C , but allow to warm to room temp before use).
21. Agar/FB: 0.8% (w/v) Agar in FB without sucrose and formaldehyde.
22. Agar/PBS: 0.8% (w/v) Agar in PBS.
23. 10% (v/v) Triton X-100 (Sigma) in PBS.
24. TRITC phalloidin 50 $\mu\text{g/mL}$ in methanol (Sigma, store at -20°C).
25. Rabbit anti-P-Ser¹⁹-MLC (Abcam)
26. Tyramide Signal Amplification Kit (TSA, Molecular Probes).
27. DAPI 1 $\mu\text{g/mL}$ in PBS (Sigma, store at -20°C).
28. Prolong mounting media (Molecular Probes).
29. Isolated blastomeres (method from Edgar and Goldstein,(9)).
30. EGM - Edgar's embryonic growth media (10) (store at 4°C).
31. Red sepharose beads (Amersham Biosciences) in Wnt Buffer: 1X PBS, 1 M NaCl, 1% (w/v) CHAPS.
32. 22 mm $\times$ 22 mm glass coverslips #1.5 (Corning).
33. 22.81 μm glass beads (Whitehouse Scientific) in ddH₂O.
34. Purified Wnt protein in Wnt Buffer (*see Materials #31*) (11) - mammalian Wnt proteins are also commercially available (R and D Systems) and the lyophilized protein can be resuspended in ddH₂O to a concentration of 50 $\mu\text{g/mL}$.

3. Methods

In the following methods we describe techniques to assay downstream effects of Wnt signaling on cell fate and the cytoskeleton during *C. elegans* development. The methods include (1) mounting embryos for 4D-videomicroscopy and visualization of rhabditi granules, which are the easiest markers of endoderm specification (12), (2) fixing and staining gastrulation stage embryos to detect myosin activation (4), and (3) a new method we have developed for placing isolated embryonic cells in contact with a purified signaling molecule, for studying cell polarization in response to an intercellular signal.

3.1. Mounting embryos for 4D videomicroscopy and visualization of rhabditi granules

1. Coat 18 × 18 mm coverslips with poly-L-lysine by placing coverslips on 7 µL of poly-L-lysine on a slide and heating on hotplate until all liquid is evaporated.
2. Allow coverslip to cool, then turn it over and place 10 µL of EB on coated side.
3. Make agar pad by placing a drop of 3% agar/EB on a slide, then place another slide on top (*see Note 2*).
4. Place young adult *C. elegans* in a depression slide with 200 µL of EB.
5. Cut adult worms in half by shearing across the worm's midline using two 25G needles.
6. Select early embryos under the dissecting microscope using a narrow bore capillary and mouth pipette, and transfer to the drop of EB on the coated coverslip. Embryos can be arranged by gently blowing EB out of mouth pipette before the embryos stick to the coverslip.
7. Invert the coverslip onto the 3% agar pad, and seal the preparation by painting around the edges of the slide with Vaseline. The Vaseline allows gas exchange but prevents desiccation.
8. Embryonic cell movements and division can now be visualized using DIC optics.
9. Rhabditi granules, which mark differentiated endoderm, can be visualized by looking at the embryos under polarized light. If analyzer and polarizer are set perpendicular to each other, to give maximum extinction, birefringent rhabditi granules will appear as bright spots (Fig. 1, compare wild-type with Wnt mutant embryos).

3.2. Fixing and staining gastrulation stage embryos for active myosin and filamentous actin

This protocol is adapted from reference (13).

1. Coat 8-well slide by placing 3 µL of poly-L-lysine in each well and covering with a coverslip. Heat on hotplate until all liquid is evaporated, rinse well with ddH₂O, and allow to dry.
2. Place 10 young adult N2 worms in 200 µL of EB in a depression slide.
3. Cut adult worms in half by shearing across the worm's midline using two 25G needles.
4. Select embryos at 4-cell stage using narrow bore capillary and mouth pipette, and transfer to a fresh depression slide well containing 200 µL of EB.
5. Leave to age at room temperature for 40 min in humidified chamber.
6. Use narrow bore capillary to move embryos to fresh well containing 10% sodium hypochlorite in EB and incubate for 3 min.
7. Wash embryos by moving through 3 fresh wells containing EB.
8. Place embryos in a fresh well and remove all excess EB. Add 15 µL of chitinase/chymotrypsin mix and incubate at room temperature for 4 min (*see Note 3*).
9. Add 10 µL room temperature 10 mg/mL L- α -lyso-phosphatidyl choline to 990 µL room temperature EB. Add 200 µL of this to embryos for 2 min to fix and permeabilise.
10. Transfer embryos, using a wide bore needle, to a fresh well that has been coated with agar/FB to prevent embryos from sticking, containing 200 µL of FB for 10 min.

11. Use wide bore capillary to move embryos to a fresh well coated with agar/PBS containing PBS and repeat three times.
12. Place embryos in 10 μ L of PBS on 8 well slides coated with poly-L-lysine.
13. Extract embryos with PBS/0.2% Triton X-100 for 5min.
14. Wash with PBS by removing all liquid and replacing with PBS. Repeat wash three times.
15. Block for 1 hr in humidified chamber using 1% block from TSA kit in PBS.
16. Add Phalloidin 1/400 and Rabbit anti-P-MLC 1/100 in 1% block in PBS and incubate in humidified chamber for 1 hr.
17. Wash three times, 5min each with PBS.
18. Add goat anti-rabbit HRP (from TSA kit) 1/300 in 0.1% block in PBS and incubate for 1 hr in humidified chamber.
19. Wash six times, 5 min each with PBS.
20. Treat with tyramide reagent for 3 min as per TSA kit instructions.
21. Wash three times with PBS.
22. Add DAPI solution and incubate for 10 min.
23. Remove all liquid, add 7 μ L of prolong mounting medium per well, cover with a coverslip, and allow to set overnight.

3.3. Method for investigating Wnt/MOM-2 induced cell polarity in vitro

3.3.1. Preparing Wnt coated beads—Wnt coated beads can generally be used for 1 – 2 days. Store at 4°C.

1. Rock 50 μ L of protein in Wnt Buffer with 10 μ L of red sepharose beads at 4°C for at least one hour.
2. Remove unbound protein by washing the Wnt-coated beads twice with Wnt Buffer. To do this, transfer 2 μ L of coated beads to 98 μ L of Wnt Buffer alone and invert the tube gently to wash the beads. Allow the beads to settle to the bottom of the tube and repeat wash by transferring 2 μ L of coated beads into another 98 μ L of Wnt Buffer alone. Rock the tube gently again and allow the beads to settle (*see Note 4*).
3. Transfer the coated beads to EGM by again transferring 2 μ L of beads to 98 μ L of EGM. The Wnt Buffer contains a high amount of salt and detergent, so the amount of Wnt Buffer that is added to EGM is minimized.

3.3.2. Placing isolated cells in contact with Wnt-coated beads

1. Isolate P₁ cells with blastomere isolation techniques (9). Keep P₁ isolates in a humid chamber to prevent evaporation while preparing the coverslips. Cells that have not been isolated properly will bleb or will not continue to divide.
2. Resuspend the glass beads in water by flicking the tube and pipette 5 μ L of the suspension onto a coverslip as 5 drops: one on each corner, and one in the middle (Fig. 2a, *see Note 5*).
3. Wick up all the excess water around the glass beads.
4. Pipette 2.5 – 3 μ L of the Wnt-coated bead/EGM suspension onto the centre of the coverslip (Fig. 2a).

5. Using the same needle as was used for blastomere isolation, mouth pipette the isolated P₁ cell (before it has divided into EMS and P₂) into the centre of the EGM suspension and allow it to settle.
6. Gently place a glass slide over the face-up coverslip. Once the coverslip and slide come in contact, the media will spread out, but it should not reach the edges of the coverslip (*see Note 6*).
7. The slides can now be flipped over such that the coverslip is on top of the slide.
8. Place the slides in a humid chamber until P₁ has divided into P₂ and EMS (Fig. 2b).
9. When EMS is born, gently roll the coverslip over the glass slide, using the glass beads as rollers: The glass beads will allow the coverslip to move along the glass slide, but prevent the cells from being compressed. The EMS cells must be in contact with the Wnt-coated beads within five minutes of being born (Fig. 2c, *see Note 7*).
10. Once EMS is in contact with a Wnt-coated bead, completely seal the slide by dipping a paintbrush into melted Vaseline and painting the Vaseline around the edges of the coverslip. Cells can now be visualized using DIC optics.

4. Notes

1. Borosilicate needles can be hand pulled over a flame or using a needle puller, for example the Sutter P-97 Flaming/Brown micropipette puller. The end is then broken using a razor blade or a microforge (Narishige) to give a bore size of ~50 µm for narrow needles or 80 – 100 µm for wide bore needles.
2. Agar pads of the correct thickness are obtained by using slides coated with lab tape as spacers. Place two slides, to which two strips of tape have been applied to each, on either side of a clean slide. Add a drop of 60°C agar to the central slide, and immediately place another clean slide on top, resting on the tape strips and the liquid agar.
3. New batches of chitinase need to be tested. This is done by treating embryos with chitinase/chymotrypsin and determining how long it takes until 3-cell embryos are found with the two anterior AB daughters appearing symmetrical, bulging the softened eggshell. This generally takes 3 to 8 minutes.
4. Be sure to be gentle with the suspension as the Wnt protein attaches to the red sepharose beads through non-covalent interactions and aggressive shaking and vortexing of the suspension may disturb these interactions.
5. Although most of the glass beads will be approximately 22 µm, there will also be bead sizes that range from ~19 µm to ~25 µm. Larger glass beads make it harder to manipulate the position of the cells and the Wnt-coated beads, so to preferentially select for the smaller glass bead populations, allow the glass beads to settle for 5–10 sec after flicking the tube. The larger beads will settle faster than the smaller beads. Then, pipette from the upper region of the solution, which should contain the smaller bead population.
6. It is best to minimize the volume pipetted onto the coverslip because if the liquid reaches the edges of the coverslip, it will be difficult to seal the slide without moving the cells and potentially disturbing the contact between the bead and the cells.
7. Although it is easier to remove the P₁ cell at the 2-cell stage and allow P₁ to divide into EMS and P₂, EMS and P₂ cells can also be isolated at the 4-cell stage after they are born. Isolating cells at the P₁ stage allows for more time to place EMS in contact

with the Wnt-coated bead during the critical period, as EMS is only responsive to spindle positioning cues for approximately 5 minutes after EMS is born (14).

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Fig. 1. Rhabditin granules under polarized light

Wild-type embryos (A and C) show the presence of birefringent rhabditin granules (arrows) marking the presence of endoderm. Compare with *mom-2/Wnt* mutant embryos (B and D) where only 25–30% of embryos show the presence of rhabditin granules (arrows, arrowheads mark embryos with no granules). Figure shows embryos under DIC optics (A and B) and polarized light (C and D).

Fig. 2. Placing Wnt-coated beads in contact with isolated cells

(A) A cartoon depiction of the glass bead approach. Left: glass beads (black spots) in water are pipetted onto coverslips in 5 drops. Right: once the excess water is wicked away, the Wnt-coated beads (white circles) in EGM (dark grey) are pipetted onto the centre of the coverslip. (B) Cells are placed in between a coverslip and slide, using glass beads (arrowhead) as a spacer. P₁ has divided into P₂ and EMS (arrow) near a Wnt-coated bead (large bead). (C) Using the glass beads as supporting rollers, the coverslip is manually pushed over the slide, and the cells (arrow) are placed in contact with the nearby Wnt-coated bead.