Chapter 9

Wnt Signaling During *Caenorhabditis elegans* Embryonic Development

Daniel J. Marston, Minna Roh, Amanda J. Mikels, Roel Nusse, and Bob Goldstein

Abstract

Wnt signaling has been demonstrated to regulate diverse cell processes throughout the development of the *Caenorhabditis elegans* embryo. This chapter describes methods that have been used to investigate some of these Wnt-dependent processes: endoderm specification, mitotic spindle orientation, and cell migration.

Key words: *C. elegans*, Wnt signaling, embryonic development.

1. Introduction

Wnt signaling has been demonstrated to regulate diverse cell processes throughout the development of the *Caenorhabditis elegans* embryo. A Wnt signal was identified as the signal produced by germ line 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)*. The following

Elizabeth Vincan (ed.), *Wnt Signaling, Volume II: Pathway Models, vol. 469*
© 2008 Humana Press, a part of Springer Science+Business Media, New York, NY
Book doi: 10.1007/978-1-60327-469-2

describes 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 cover slips #1.5 (Corning).
3. Glass slides (Gold-Seal).
4. Sterile egg buffer (EB): 118 mM NaCl, 48 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 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 (≥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 µg/mL in methanol (Sigma, store at –20°C).
25. Rabbit anti-P-Ser^{19}-MLC (Abcam).
26. Tyramide Signal Amplification Kit (TSA, Molecular Probes).
27. DAPI 1 µg/mL in PBS (Sigma, store at –20°C).
28. Prolong mounting media (Molecular Probes).
29. Isolated blastomeres (method from Edgar and Goldstein *[9]*)
30. Edgar's embryonic growth media (EGM; store at 4°C; ref. *10*).
31. Red sepharose beads (Amersham Biosciences) in Wnt Buffer: 1X PBS, 1 M NaCl, 1% (w/v) CHAPS.
32. 22 mm × 22 mm glass cover slips #1.5 (Corning).
33. 22.81 µm glass beads (Whitehouse Scientific) in ddH_20.
34. Purified Wnt protein in Wnt Buffer (see **item 31**; ref. *11*); mammalian Wnt proteins are also commercially available (R and D Systems) and the lyophilized protein can be resuspended in ddH_20 to a concentration of 50 µg/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 rhabditin 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 Rhabditin Granules

1. Coat 18 × 18 mm cover slips with poly-L-lysine by placing cover slips on 7 µL of poly-L-lysine on a slide and heating on hotplate until all liquid is evaporated.
2. Allow cover slip 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 cover slip. Embryos can be arranged by gently blowing EB out of mouth pipette before the embryos stick to the cover slip.
7. Invert the cover slip 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. Rhabditin 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 rhabditin granules will appear as bright spots (**Fig. 9.1**, compare wild-type with Wnt mutant embryos).

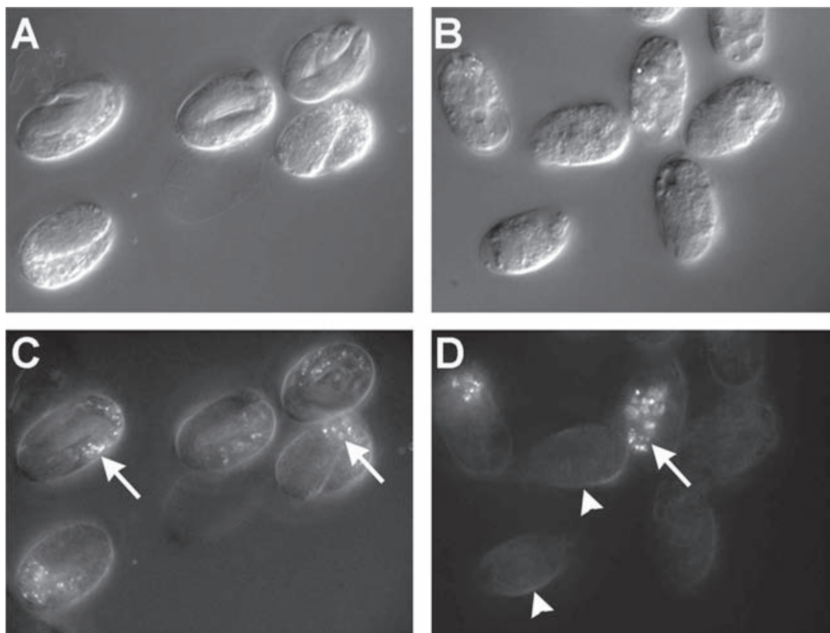

Fig. 9.1. Rhabditin granules under polarized light. Wild-type embryos (**A** and **C**) show the presence of birefringent rhabditin granules (arrows), marking the presence of endoderm. Compare with *mom-2*/Wnt mutant embryos **B** and **D**) where only 25 to 30% of embryos show the presence of rhabditin granules (arrows, arrowheads mark embryos with no granules). Figure shows embryos under DIC optics (**A** and **B**) and polarized light (**C** and **D**).

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 cover slip. Heat on hotplate until all liquid is evaporated, rinse well with ddH_20, 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 four-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 minutes in humidified chamber.
6. Use narrow bore capillary to move embryos to fresh well containing 10% sodium hypochlorite in EB and incubate for 3 minutes.
7. Wash embryos by moving through three fresh wells containing EB.
8. Place embryos in a fresh well and remove all excess EB. Add 15 µL chitinase/chymotrypsin mix and incubate at room temperature for 4 minutes (*see* **Note 3**).
9. Add 10 µL room temperature 10 mg/mL L- -lyso-phosphatidyl choline to 990 µL room temperature FB. Add 200 µL of this to embryos for 2 minutes to fix and permeabilize.
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 minutes.
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 µL of PBS on 8-well slides coated with poly-L-lysine.
13. Extract embryos with PBS/0.2% Triton X-100 for 5 minutes.
14. Wash with PBS by removing all liquid and replacing with PBS. Repeat wash three times.
15. Block for 1 hour 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 hour.

17. Wash three times, 5 minutes each with PBS.
18. Add goat anti-rabbit HRP (from TSA kit) 1/300 in 0.1% block in PBS and incubate for 1 hour in humidified chamber.
19. Wash six times, 5 minutes each with PBS.
20. Treat with tyramide reagent for 3 minutes as per TSA kit instructions.
21. Wash three times with PBS.
22. Add DAPI solution and incubate for 10 minutes.
23. Remove all liquid, add 7 μL of prolong mounting medium per well, cover with a cover slip, 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 to 2 days. Store at 4°C.

1. Rock 50 μL of protein in Wnt Buffer with 10 μL of red sepharose beads at 4°C for at least 1 hour.
2. Remove unbound protein by washing the Wnt-coated beads twice with Wnt buffer. To do this, transfer 2 μL of coated beads to 98 μL 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 μL of coated beads into another 98 μL 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 μL of beads to 98 μL 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 P_1 cells with blastomere isolation techniques *(9)*. Keep P_1 isolates in a humid chamber to prevent evaporation while preparing the cover slips. 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 μL of the suspension onto a cover slip as five drops: one on each corner, and one in the middle (**Fig. 9.2A**, *see* **Note 5**).
3. Wick up all the excess water around the glass beads.
4. Pipette 2.5–3 μL of the Wnt-coated bead/EGM suspension onto the center of the cover slip (**Fig. 9.2A**).
5. Using the same needle as was used for blastomere isolation, mouth pipette the isolated P_1 cell (before it has divided into EMS and P_2) into the center of the EGM suspension and allow it to settle.
6. Gently place a glass slide over the face-up cover slip. Once the cover slip and slide come in contact, the media will spread

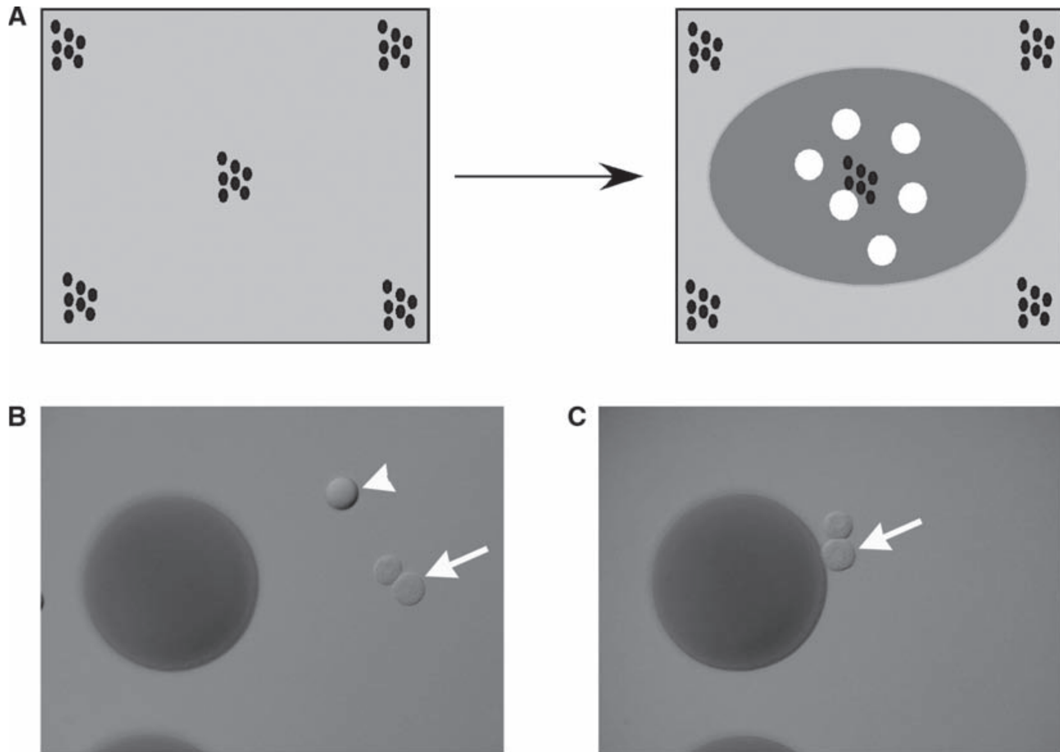

Fig. 9.2. Placing Wnt-coated beads in contact with isolated cells. (**A**) A cartoon depiction of the glass bead approach. Left: glass beads (black spots) in water are pipetted onto cover slips in 5 drops. Right: once the excess water is wicked away, the Wnt-coated beads (white circles) in EGM (dark gray) are pipetted onto the center of the cover slip. (**B**) Cells are placed in between a cover slip and slide, using glass beads (arrowhead) as a spacer. P_1 has divided into P_2 and EMS (arrow) near a Wnt-coated bead (large bead). (**C**) Using the glass beads as supporting rollers, the cover slip is manually pushed over the slide, and the cells (arrow) are placed in contact with the nearby Wnt-coated bead.

out, but it should not reach the edges of the cover slip (*see* **Note 6**).

7. The slides can now be flipped over such that the cover slip is on top of the slide.
8. Place the slides in a humid chamber until P_1 has divided into P_2 and EMS (**Fig. 9.2B**).
9. When EMS is born, gently roll the cover slip over the glass slide, using the glass beads as rollers: The glass beads will allow the cover slip 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 5 minutes of being born (**Fig. 9.2C**, *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 cover slip. 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 (e.g., 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 three-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 ~19–25 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 seconds 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 cover slip because if the liquid reaches the edges of the cover slip, 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 P_1 cell at the two-cell stage and allow P_1 to divide into EMS and P_2, EMS and P_2 cells can also be isolated at the four-cell stage after they are born. Isolating cells at the P_1 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)*.

Reference

1. Thorpe, C. J., et al. (1997) Wnt signaling polarizes an early C. elegans blastomere to distinguish endoderm from mesoderm. *Cell* 90, 695–705.
2. Rocheleau, C. E., et al. (1997) Wnt signaling and an APC-related gene specify endoderm in early C. elegans embryos. *Cell* 90, 707–716.
3. Korswagen, H. C. (2002) Canonical and non-canonical Wnt signaling pathways in Caenorhabditis elegans: variations on a common signaling theme. *Bioessays* 24, 801–810.
4. Lee, J. Y., et al. (2006) Wnt/Frizzled signaling controls C. elegans gastrulation by activating actomyosin contractility. *Curr Biol* 16, 1986–1997.
5. Walston, T., et al. (2004) Multiple Wnt signaling pathways converge to orient the mitotic spindle in early C. elegans embryos. *Dev Cell* 7, 831–841.
6. Thorpe, C. J., Schlesinger, A., Bowerman, B. (2000) Wnt signalling in Caenorhabditis elegans: regulating repressors and polarizing the cytoskeleton. *Trends Cell Biol* 10, 10–17.
7. Goldstein, B., et al. (2006) Wnt signals can function as positional cues in establishing cell polarity. *Dev Cell* 10, 391–396.
8. Schlesinger, A., et al. (1999) Wnt pathway components orient a mitotic spindle in the early Caenorhabditis elegans embryo without requiring gene transcription in the responding cell. *Genes Dev* 13, 2028–2038.
9. Edgar, L.G., Goldstein B. (2007) *Methods in Cell Biol, in press.*
10. Edgar, L. G. (1995) Blastomere culture and analysis. *Methods Cell Biol* 48, 303–321.
11. Willert, K., et al. (2003) Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* 423, 448–452.
12. Laufer, J. S., Bazzicalupo, P., Wood, W. B. (1980) Segregation of developmental potential in early embryos of Caenorhabditis elegans. *Cell* 19, 569–577.
13. Costa, M., Draper, B. W., Priess, J.R. (1997) The role of actin filaments in patterning the Caenorhabditis elegans cuticle. *Dev Biol* 184, 373–384.
14. Goldstein, B. (1995) Cell contacts orient some cell division axes in the Caenorhabditis elegans embryo. *J Cell Biol* 129, 1071–1080.