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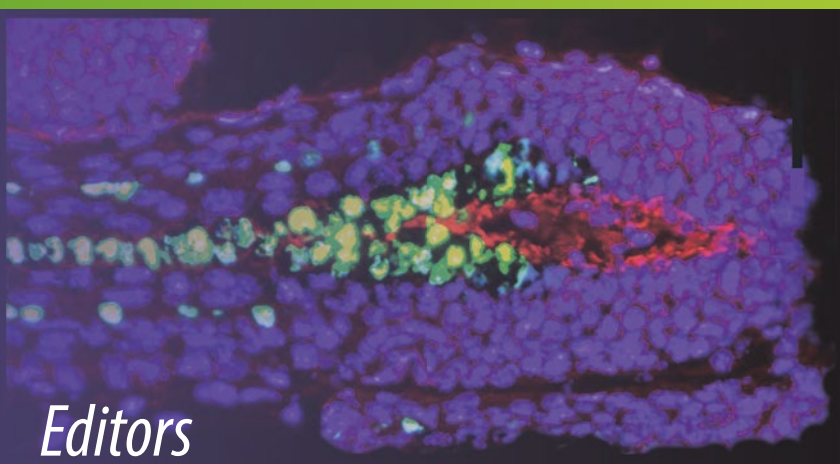

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Lawrence Lum *Editors*

Wnt Signaling

Methods and Protocols

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Preface

Secreted Wnt molecules are essential to the coordination of cell fate outcomes in developing and adult tissues and are thus indispensable to animal life. The past few decades of intense research have led to tremendous advances in the assembly of the genetic components necessary for the production of Wnt ligands and for eliciting cellular responses to Wnts. Having leveraged this knowledge to engineer the tools capable of achieving control of Wnt signaling in diverse adult animal tissues, we are now poised to evaluate the potential in modulating Wnt signaling for therapeutic agendas in cancer, wound healing, and degenerative disease.

This collection of protocols should facilitate these efforts by providing step-by-step guidance for successfully evaluating researcher-inspired hypotheses. Included are methods for using Wnt modulating chemicals in engineering tissues from induced pluripotent and embryonic stem cells, for monitoring Wnt transcriptional responses in diverse tissues such as bone and skin, and for using specific biochemical markers of Wnt signaling to either screen molecular libraries or evaluate novel reagents. These protocols also leverage unique experimental strengths from five different model organisms.

We also hope these selected protocols are representative of the diverse interests and awe-inspiring creativity that has placed a premium on understanding Wnt signal transduction in an effort to improve outcomes in regenerative medicine and cancer management.

Dallas, TX, USA

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Chapter 1

Visualizing Wnt Palmitoylation in Single Cells

Xinxin Gao and Rami N. Hannoush

Abstract

Wnt palmitoylation regulates its secretion and signaling activity in cells. Methods to monitor cellular Wnt palmitoylation are instrumental in investigating Wnt activity, secretion, and its interaction with cellular membrane compartments. This protocol describes a method we have recently developed to detect cellular Wnt palmitoylation. The method, combining click chemistry, bio-orthogonal fatty acid probes, and proximity ligation assay (PLA), provides high sensitivity and subcellular resolution for detection of Wnt palmitoylation. It is also compatible with multiple imaging platforms, and is applicable to detecting palmitoylated forms of other fatty acylated proteins.

Key words Wnt palmitoylation, Alkyne fatty acid, Click chemistry, Proximity ligation, Microscopy

1 Introduction

Protein palmitoylation, a posttranslational event involving the addition of a 16-carbon palmitic acid onto proteins, is an important modification that mediates protein functions, stability, subcellular localization, as well as protein–protein and protein–membrane interactions [1, 2]. One such protein, Wnt, is O-palmitoylated at a serine residue [3, 4], and this modification is critical in regulating Wnt activity, secretion, and its interaction with cellular membrane compartments [4–7]. Aberrant Wnt signaling promotes cancerous growth [8, 9], and represents a high-priority therapeutic target [10, 11]. Therefore, the development of novel tools and assays for monitoring the production of lipidated Wnt proteins could find great utility in drug discovery campaigns and diagnostic biomarker applications. Traditionally, detection of Wnt palmitoylation requires metabolic labeling of Wnt proteins with radioactive fatty acids (typically a palmitate which is converted to palmitoylate prior to attachment to Wnt proteins by the Wnt acyltransferase porcupine), followed by immunoprecipitation [12–14]. However, this method does not enable the in vivo characterization of the role lipidation plays in supporting Wnt production. To address the

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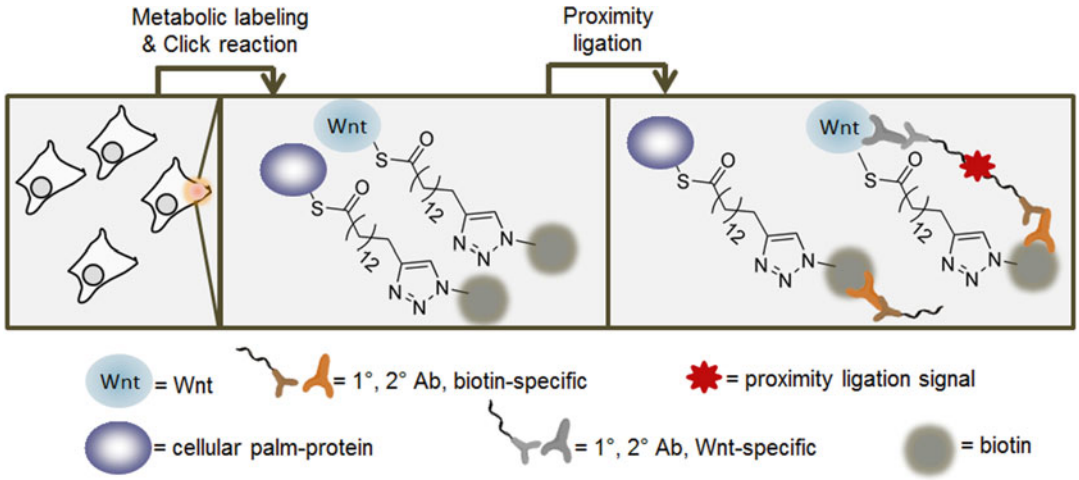

Fig. 1 Development of the imaging protocol for visualizing cellular palmitoylated Wnt proteins. Cells are metabolically labeled with Alk-C16, fixed, permeabilized, processed for click chemistry with biotin-azide, and then proximity ligated. Wnt palmitoylation can be visualized using a fluorescence microscope

unmet need for robust, sensitive, and non-radioactive methods for detecting Wnt palmitoylation, we have reported a click chemistry-proximity ligation-based method to track palmitoylated Wnt in single cells [3, 15, 16]. This imaging method provides high sensitivity and subcellular resolution for detection of Wnt palmitoylation, and has been used to reveal novel insights into Wnt trafficking, as well as its regulation by porcupine [3].

The imaging assay comprises metabolic labeling of the palmitoylated proteome with bio-orthogonal fatty acid probes [17–19] and click chemistry, followed by proximity ligation assay (PLA) [20–22] to detect the lipidated forms of Wnt proteins (Fig. 1). Using alkyne palmitic acid probe (Alk-C16) as an example, the fatty acid probe is incubated with live cells, and then the cells are fixed and permeabilized. The Alk-C16-labeled proteins are then conjugated to azide-tagged biotin or a fluorescent dye through a Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition reaction [17–19, 23]. Two primary antibodies are used to recognize Wnt and the biotin/dye on palmitoylated proteins. These two primary antibodies are in turn recognized by two different PLA secondary antibodies which are conjugated to unique oligonucleotides. When the two antibodies are in close proximity (<40 nm), the oligonucleotides can interact and form DNA circles upon addition of ligase. This circular DNA then is amplified through an enzyme-mediated rolling circle amplification (RCA) reaction, and the signal representing Wnt palmitoylation is produced by hybridization of fluorescently labeled oligonucleotides to the replicated DNA circle. Using this method, Wnt palmitoylation can be visualized using fluorescence microscopy.

The approach described here is compatible with multiple imaging platforms, and could be applied to detecting palmitoylated forms of other fatty acylated proteins [3, 15, 16]. For example, the method has been modified to image palmitoylation of Sonic Hedgehog, α-tubulin, and H-Ras in various cell lines [15]. In addition, it has been adapted to a 96-well plate format for potential use in the discovery of inhibitors of Wnt palmitoylation, and a confocal imaging format to co-image Wnt palmitoylation with a variety of cellular markers to study the subcellular trafficking routes of palmitoylated Wnt [3]. We envision that with the availability of click chemistry-based probes, the method could be adapted to detection of other protein modifications such as O-GlcNAcylation [24–26], acetylation [27], methylation [28, 29], cholesterylation [30], and sulfenation [31].

2 Materials

Prepare all solutions using Milli-Q-purified water or equivalent, and analytical grade reagents. Follow the material safety data sheet (MSDS) to use and properly dispose of reagents. Personal protective equipment (PPE) should be worn to minimize exposure to hazardous reagents.

2.1 Click Chemistry for Labeling Proteome Palmitoylation

1. 10% Triton X100 solution.
2. Bovine serum albumin.
3. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP).
4. Biotin-azide (Life Technologies, cat. no. B10184).
5. Dimethyl sulfoxide (DMSO).
6. Copper sulfate ($CuSO_4$, ≥99.99% purity).
7. Septihol Sterile Alcohol Solution.
8. L-Wnt3a cells (ATCC, cat. no. CRL-2647).
9. Dulbecco's modified Eagle's medium (DMEM).
10. Geneticin.
11. Fetal bovine serum (FBS).
12. GlutaMAX™ Supplement (Life Technologies, cat. no. 35050-061).
13. Fatty acid probe stock solution: Lyophilized Alk-C16 powder was synthesized as described by Hannoush and Arenas-Ramirez [19]. Dissolve lyophilized Alk-C16 powder in DMSO (50 mM). Sonicate the tube in an ultrasonic cleaner with water bath for 15–30 min. Aliquot (50 μl each tube) and store the tubes at −80 °C (*see* **Note 1**).

14. 5 mM Biotin-azide solution: Dissolve biotin-azide in DMSO. Sonicate the tube in an ultrasonic cleaner with water bath for 15–30 min. Aliquot the solution (10 μl each tube) and store the tubes at −20 °C (*see* **Note 1**).
15. TCEP solution and $CuSO_4$ solution: Dissolve TCEP or $CuSO_4$ in water to a final concentration of 50 mM. Vortex to dissolve (*see* **Note 2**).
16. L-Wnt3a cell culture medium: 10% (vol/vol) FBS, 0.4 g/l geneticin, and 2 mM GlutaMAX Supplement in DMEM.
17. L-Wnt3a cell culture medium without geneticin: 10% (vol/vol) FBS and 2 mM GlutaMAX Supplement in DMEM.
18. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.2.
19. Blocking buffer: 5% (wt/vol) BSA and 0.3% (vol/vol) Triton X-100 in PBS, pH 7.2.
20. Permeabilization buffer: 0.1% (vol/vol) Triton X100 in PBS.
21. PBS-T: 0.05% (vol/vol) Tween 20 in PBS.

2.2 Proximity Ligation Imaging Assay for Visualization of Palmitoylated Wnt3a

1. Anti-Wnt3a antibody (rabbit, Abcam, cat. no. ab28472).
2. Anti-biotin antibody (goat, Sigma-Aldrich, cat. no. B3640).
3. Duolink® in situ mounting medium with DAPI (Sigma-Aldrich, cat. no. DUO82040).
4. Duolink® in situ PLA® probe anti-rabbit PLUS (Sigma-Aldrich, cat. no. DUO92002).
5. Duolink® in situ PLA® probe anti-goat MINUS (Sigma-Aldrich, cat. no. DUO92006).
6. Duolink® in situ detection reagents orange (Sigma-Aldrich, cat. no. DUO92007).
7. 12-Well clear flat-bottom TC-treated multiwell cell culture plate.
8. Micro cover glasses, round, No. 1.
9. Superfrost plus microslide.
10. Leica TCS SPE system with an upright Leica DM 5500 Q microscope (Leica Microsystems).
11. Imaris software (Bitplane).
12. Leica Application Suite Advanced Fluorescence (LAS AF, Leica Microsystems).

3 Methods

3.1 Click Chemistry for Labeling Proteome Palmitoylation

1. Place three cover slips in a 60 mm tissue culture dish. Add 5–10 ml of Septihol Sterile Alcohol Solution to the dish and seal with Parafilm. The cover slips are kept in the solution for at least 18 h at room temperature to make sure that they are sterilized.

2. Place one cover slip in one well of a 12-well clear flat-bottom TC-treated multiwell cell culture plate using tweezers. Air-dry the cover slips for at least 10 min in the tissue culture hood (*see* **Note 3**).
3. Plate L-Wnt3a cells (maintained in L-Wnt3a cell culture medium) in each well (200,000 cells per well) in L-Wnt3a cell culture medium without geneticin. Make sure that cover slips are not floating in the medium and that cells settle evenly in each well. Incubate the cover slip containing plates in a 5% CO_2 tissue culture incubator for 24 h at 37 °C.
4. To treat cells for three cover slips, dissolve 3.2 μl Alk-C16 (50 mM in DMSO) in 1.6 ml L-Wnt3a cell culture medium without geneticin to a final concentration of 100 μM. Sonicate the mixture for 15 min in an ultrasonic cleaner with water bath. Incubate the mixture for 15 min at room temperature to allow the fatty acid to form complexes with albumin.
5. Aspirate media and then wash cells once with PBS. Aspirate PBS and then add Alk-C16-containing medium (500 μl each well) to label cells on three cover slips. Incubate cells in a 5% CO_2 tissue culture incubator for 18 h at 37 °C (*see* **Note 4**).
6. Wash cells three times with PBS. Fix cells by incubating cells with 1 ml pre-chilled (−20 °C) methanol for 10 min at −20 °C. Aspirate methanol and incubate the cells with 1 ml of 0.1% Triton X-100/PBS (vol/vol) for 5 min at room temperature.
7. Prepare a humidity chamber: Cover the bottom of a 150 mm tissue culture dish with Parafilm. Get rid of air bubbles by pressing the film against the bottom of the dish. Place dampened Kimwipes on both sides of the film and label sample numbers (1, 2, or 3) on the back of the dish. Transfer cover slips with tweezers carefully to the designated positions on the film in the dish (cell-attaching side facing upwards). Add 100 μl PBS on each cover slip without touching it.
8. Prepare 350 μl click reaction cocktail solution to treat three samples by sequentially adding 7 μl of 5 mM biotin-azide, 7 μl of 50 mM TCEP solution, and 7 μl of 50 mM $CuSO_4$ to 329 μl of PBS (*see* **Note 5**).
9. Vortex the mixture. Wash the cover slips with PBS (three times) and then incubate them with click reaction cocktail (100 μl each cover slip) for 1 h at room temperature.

3.2 Proximity Ligation Imaging Assay for Visualizing Palmitoylated Wnt3a

1. Wash cells six times with PBS and then incubate them with 100 μl of blocking buffer for 1 h at room temperature.
2. Incubate cells on cover slips 1 and 2 with 100 μl 10 μg/ml anti-Wnt3a antibody (rabbit) diluted in blocking buffer, or blocking buffer alone (for cells on cover slip 3) overnight at 4 °C.

3. Wash cells three times with PBS-T. Incubate cells on cover slips 1 and 3 with 100 μl of 3.3 μg/ml anti-biotin antibody (goat) diluted in blocking buffer, or blocking buffer alone (for cells on cover slip 2) for 1 h at room temperature.
4. Prepare 350 μl oligonucleotide-conjugated secondary antibody solution (for three cover slips) by mixing 70 μl of Duolink in situ PLA probe anti-rabbit PLUS (oligonucleotide-conjugated secondary antibody for anti-Wnt3a) and 70 μl of Duolink in situ PLA probe anti-goat MINUS (oligonucleotide-conjugated secondary antibody for anti-biotin) in 210 μl of blocking buffer. Wash cells three times with PBS-T and then incubate each cover slip with 100 μl oligonucleotide-conjugated secondary antibody solution for 1 h at 37 °C (*see* **Note 6**).
5. Prepare 350 μl ligation solution (for three cover slips) by mixing 70 μl ligation buffer and 8.8 μl ligase in 271.2 μl water. Wash cells three times with PBS-T and then incubate each cover slip with 100 μl of ligation solution for 30 min at 37 °C (*see* **Note 7**).
6. Prepare 350 μl amplification solution (for three cover slips) by mixing 70 μl amplification stock and 4.4 μl of polymerase in 275.6 μl water. Wash cells three times with PBS-T and incubate each cover slip with 100 μl amplification solution for 100 min in the dark at 37 °C (*see* **Note 8**).
7. Wash cells three times with PBS-T and once with PBS. Label two micro slides (two cover slips each slide) and spot ~20 μl (for each cover slip) of Duolink in situ mounting medium with DAPI on the designated positions on the micro slides. Place each cover slip to micro slides using tweezers, with the cell-attaching side facing the micro slide and in contact with the mounting medium.
8. Remove excess mounting medium around the cover slips carefully, by either vacuum suction or pipetting. Seal the cover slips by nail polish and leave the samples on a flat surface for 30–60 min in the dark at room temperature.

3.3 Imaging Palmitoylated Wnt3a with a Confocal Fluorescence Microscope

1. Image the samples using a confocal fluorescence microscope with a 63× objective (for example, Leica TCS SPE system with an upright Leica DM 5500 Q microscope) (*see* **Note 9**). Make sure that the microscope has the appropriate laser lines and filter settings. To image nuclei (DAPI), we recommend 405 nm laser excitation, 425–470 nm range for Acusto-Optical Tunable Filter (AOTF), and double-dichroic (DD) 405/532 beam splitter. To image palmitoylated Wnt3a, we recommend 532 nm laser excitation, 552–590 nm range for AOTF, and DD 405/532 beam splitter (*see* **Notes 9** and **10**). An example result is shown in Fig. 2. Acquire high-resolution single-plane images (1024 × 1024) and z-stack images (z-step size: 0.25 μm) (*see* **Note 10**).

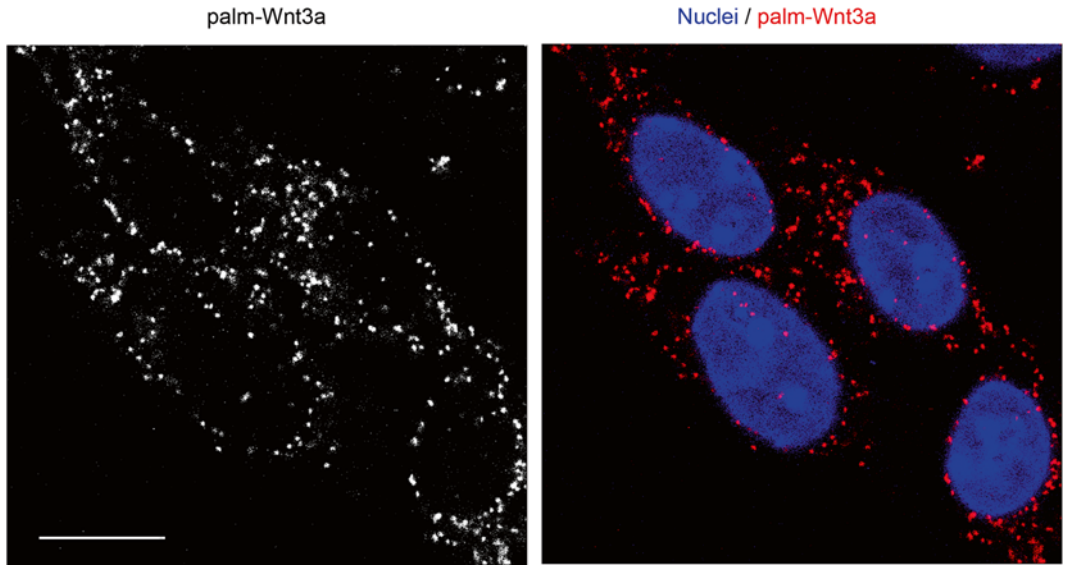

Fig. 2 An example of detecting palmitoylated Wnt3a in mouse fibroblast L cells stably overexpressing Wnt3a. Cells were imaged with Leica TCS SPE confocal imaging system using a 63× objective. Scale bar, 10 μm

2. Export high-resolution images from Leica Application Suite Advanced Fluorescence (LAS AF). Images can be processed with a standard image analysis software such as Imaris.

4 Notes

1. Avoid multiple freeze-thaw cycles of the reagents, as doing so may damage the quality of the reagent. Once frozen, the reagents are stable for at least 1 year.
2. Prepare TCEP solution and $CuSO_4$ solution fresh right before each experiment.
3. Cover slips, when wet, tend to stick together. Make sure that single cover slip is picked.
4. Carefully aspirate any liquid from wells without touching cells.
5. The order of addition of the reagents is critical and needs to be followed as described herein. Make sure that the solution covers the entire cover slip.
6. It is important to have one Duolink in situ PLA probe PLUS and one Duolink in situ PLA probe MINUS, against goat and rabbit, respectively. Using either two PLUS or two MINUS probes will lead to failed experiments.
7. Add the ligation buffer first and then vortex the solution. The ligase is added immediately before applying the mixture to cells.

8. Add the amplification stock first and then vortex the solution. The polymerase is added immediately before applying the mixture to cells. Make sure that the incubation time is 100 min, as longer incubation time could lead to bigger and/or merged dots and high background. The detection reagents are light sensitive and samples need to be protected from light.
9. Confocal microscopy is the recommended imaging platform because of better resolution of individual spots (signal corresponding to palmitoylated Wnt3a).
10. Optimal settings for imaging palmitoylated Wnt3a should be determined using all three samples. Adjust exposure settings according to control samples 2 and 3 (each treated with one antibody only) and the test sample 3 (treated with both antibodies) based on obtained fluorescence signal.

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Chapter 2

Monitoring Wnt Protein Acylation Using an In Vitro Cyclo-Addition Reaction

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Abstract

We describe here a technique for visualizing the lipidation status of Wnt proteins using azide-alkyne cycloaddition chemistry (click chemistry) and SDS-PAGE. This protocol incorporates in vivo labeling of a Wnt-IgG Fc fusion protein using an alkynylated palmitate probe but departs from a traditional approach by incorporating a secondary cycloaddition reaction performed on single-step purified Wnt protein immobilized on protein A resin. This approach mitigates experimental noise by decreasing the contribution of labeling from other palmitoylated proteins and by providing a robust method for normalizing labeling efficiency based on protein abundance.

Key words Wnt acylation, Click chemistry, Biotinylation, Palmitoleate, IgG fusion protein

1 Introduction

The advent of chemical probes that mimic endogenous metabolites has enabled the systematic quantification of biochemical events that interlink cellular metabolic and signaling machinery [1]. This effort has so far yielded a plethora of novel therapeutic intervention entry points. Whereas many of these posttranslational modifications can be readily inferred from mass spectrometric analysis of proteins isolated using the covalently attached probe as a handle, efforts focused on specific protein targets can be challenging due to limitations imposed by their abundance and their amenability to labeling [2]. In particular, secreted fatty acylated proteins such as the Wnt and Hedgehog proteins represent atypical subjects for this technique given their localization to the outer plasma membrane and the necessity of the fatty acyl donor (and thus the fatty acid probe) to traverse the plasma membrane twice prior to immobilization onto the substrate—once to be affixed to a CoA moiety and again to access the extracellular polypeptide likely by exiting a channel created by the Porcn acyltransferase [3].

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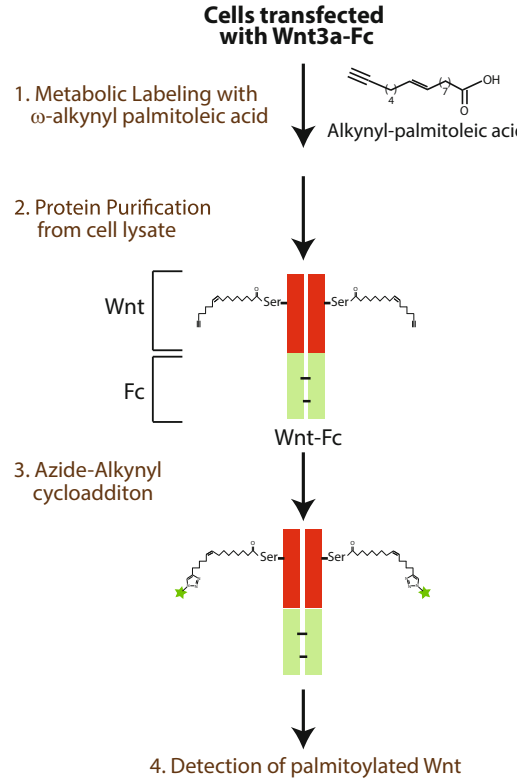

Fig. 1 Overview of modified Wnt labeling protocol using an alkynylated fatty acyl probe and copper-catalyzed alkyne-azide cycloaddition

To increase the efficiency of using fatty acid-like probes for labeling Wnt proteins, we have leveraged the amenability of secreted proteins to single-step purification using the IgG Fc fusion-protein A system and a modified in vitro secondary cycloaddition reaction for incorporating either a fluorescent or a biotin conjugate (Fig. 1). These fusion proteins serve as faithful substrates of the native Wnt production machinery given that (a) lipidation of Wnt-Fc fusion proteins in vivo is sensitive to inhibitors of the Wnt acyltransferase Porcupine [4], and (b) their lipidation status correlates with their ability to interact with the Wnt chaperone Wntless (WLS) [5]. This technique enables the in vivo evaluation of synthetic small molecules on Wnt lipidation, for example, and serves as a template for achieving similar experimental robustness for the study of other acylated secreted proteins.

2 Materials

2.1 Cell Labeling Components

1. Tissue cultureware: 60 mm^2 and 10 cm^2 tissue culture plates.
2. HEK293 cells.

3. DMEM culture medium: DMEM, 10% fetal bovine serum, 1% penicillin/streptomycin.
4. Serum-free DMEM: DMEM with no additives.
5. Cell trypsinization solution (0.25%).
6. Liposomal transfection reagent (i.e., Effectene, Qiagen).
7. Alkynyl palmitoleic acid: Prepare 50 mM stock solution in DMSO.

2.2 Protein Purification Reagents

1. Lysis buffer: 1% NP40 in PBS with protease inhibitors.
2. Protein A sepharose: Wash with 1% NP40/PBS, store as a slurry (1 volume beads: volume 1% NP40) at 4 °C until required.

2.3 Cycloaddition Reaction Reagents

1. Azide-biotin: Dissolve in DMSO to prepare 5 mM stock solution.
2. TCEP (Tris-2-carboxyethyl phosphine hydrochloride): Prepare a fresh 50 mM stock solution by dissolving in H_2O.
3. TBTA (tris((1-benzyl-1H-1,2,3-triazol-4-yl)methyl)amine): Dissolve in DMSO:t-butanol (1:4) to prepare 1.7 mM stock solution.
4. $CuSO_4$: Dissolve in H_2O to prepare 50 mM stock solution.

2.4 SDS-PAGE and Immunoblotting Components

1. 7.5% Acrylamide/bis-acrylamide gels: This can either be purchased from any number of vendors (Criterion Gels, Biorad) or prepared as described in [6].
2. SDS-PAGE running buffer: 0.025 M Tris–HCl, pH 8.3, 0.192 M glycine, 0.1% SDS.
3. Western blot transfer buffer: 0.025 M Tris, 0.192 M glycine, and 20% methanol.
4. Nitrocellulose membrane, 0.45 μm.
5. Diluent solution: PBS containing 0.05% Tween-20 (PBST).
6. Blocking solution: 5% Nonfat powdered milk dissolved in PBST.
7. Streptavidin cross-linked to infrared dye (Streptavidin IRDye).
8. Gel-imaging system or radiographic film.

3 Methods

3.1 Labeling Wnt Protein

1. Cell trypsinization: Wash 75% confluent HEK293 cells cultured in a 10 cm^2 culture dish with PBS and trypsinize the cells by adding 2 ml of trypsinization solution. Let cells incubate at 37 °C for 2 min. Neutralize the trypsin with DMEM culture medium and spin cells at 200 x *g* for 5 min. Resuspend the cells with DMEM culture medium and seed 1 x 10^6 cells in each 60 mm^2 culture dish.

2. Transiently transfect 60 mm plates of HEK293 cells with Wnt3a-Fc DNA before cells become adherent using a liposomal transfection reagent. Wait for 24 h before proceeding to the click chemistry reaction.
3. Dilute alkynyl palmitoleic acid in serum-free DMEM to the final concentration of 100 μM (*see* **Note 1**).
4. Sonicate the alkynyl palmitoleic acid containing DMEM for 15 min at room temperature.
5. Incubate the medium for 15 min at room temperature. This promotes formation of pre-complexes between alkynyl palmitoleic acid and medium.
6. Aspirate off the medium from cultured HEK293 cells.
7. Wash with PBS.
8. Add alkynyl palmitoleic acid containing medium to the cells.
9. Incubate for 6 h at 37 °C/5 % CO_2 (*see* **Note 2**).

3.2 Purifying Wnt Protein

1. Aspirate the alkynyl palmitoleic acid-containing media from the cells and wash with PBS. Repeat two times.
2. Lyse the cells using 500 μl of 1 % NP40 lysis buffer on ice.
3. Centrifuge at 16,000 x*g* for 10 min at 4 °C. Transfer the cell lysate to an eppendorf tube without disturbing the cell pellet.
4. Add 50 μl of protein A sepharose bead slurry to the cell lysate (*see* **Note 3**).
5. Rotate the samples minimally for 2 h at 4 °C. The Wnt-Fc protein in the cell lysis conditions described here appears to be stable with overnight incubations at 4 °C.
6. Centrifuge at 1000 x*g* for 5 min. Discard the supernatant.
7. Wash the beads by adding 1 % NP40 and rotating for 10 min at 4 °C.
8. Centrifuge at 1000 x*g* for 5 min. Discard the supernatant.
9. Repeat three times.

3.3 Azide Attack

1. Dilute azide-biotin in 100 μl of 1× PBS to the final concentration of 50 μM. A total volume of 90 μl of the azide-biotin-containing reaction buffer is added for each click chemistry reaction (*see* **Note 4**).
2. Add the azide-biotin mixture to Wnt3a-Fc protein immobilized on protein-A beads from Subheading 3.2 and mix gently.
3. Add 2 μl of TCEP (*see* **Note 5**).
4. Add 6 μl of TBTA solution and mix gently.
5. Add 2 μl of $CuSO_4$ solution. Mix gently.
6. Rotate the sample for 1 h at room temperature protected from light.

7. Centrifuge at 1000 x*g* for 5 min and aspirate the solution. Wash with cold lysis buffer. Repeat three times.
8. Add 60 μl sample-loading buffer to the protein-A bead slurry and heat at 95 °C for 2 min.
9. Centrifuge at 1000 x*g* again for 2 min to let the beads settle at the bottom and run the supernatant on SDS-PAGE.

3.4 SDS-PAGE and Immunoblotting

1. Prepare 7.5% acrylamide/bis-acrylamide gel by rinsing wells with running buffer and securing in gel-running chamber. Add running buffer to top and bottom chambers.
2. Load 30 μl of protein sample with gel-loading tips.
3. Run the gel for approximately 45 min at 250 mA.
4. Immediately following SDS-PAGE, transfer the gel to a nitrocellulose membrane for 1.5 h.
5. After the transfer is completed, cut excess membrane to smoothen edges.
6. Block the membrane with 5% milk solution for an hour at room temperature on a platform shaker.
7. Wash the membrane with PBST for 5 min. Repeat two times.
8. Incubate with Streptavidin IRDye for an hour (1:1000 dilution) at room temperature on a platform shaker (*see* **Note 6**).
9. Wash with PBST for 10 min. Repeat three times.

3.5 Detection

Scan the membrane with an Odyssey Fc imaging system using the 800 nm detection channel (*see* **Note 7**).

4 Notes

1. Instead of using alkynyl palmitoleic acid, you can label Wnt3a-Fc with azide palmitoleic acid.
2. Incubation of alkynyl palmitoleic acid for more than 6 h might be toxic to cells.
3. Protein A sepharose is supplied as a slurry that contains ethanol and/or sodium azide for preservation. It is important to wash the protein A sepharose with 1% NP40 several times before using for Wnt3a-Fc purification.
4. If azide palmitoleic acid is used for labeling Wnt3a-Fc (instead of alkynyl palmitoleic acid), you can use various alkyne-containing reagents for detection purposes.
5. A commercially available cycloaddition reaction kit can also be substituted starting from this point (for example, Click-iT Cell Protein Reaction Buffer Kit, Invitrogen).

6. If you prefer using chemiluminescent Western blotting detection system instead of infrared system, you can replace the Streptavidin IRDye with HRP-Streptavidin and use an ECL Western blotting substrate to detect the acylated Wnt3a-Fc proteins.
7. If the Streptavidin IRDye was substituted with a horseradish peroxidase (HRP)-coupled streptavidin protein, then expose the nitrocellulose film to chemiluminescence-compatible film.

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Chapter 3

Biochemical Methods to Analyze Wnt Protein Secretion

Kathrin Glaeser, Michael Boutros, and Julia Christina Gross

Abstract

Wnt proteins act as potent morphogens in various aspects of embryonic development and adult tissue homeostasis. However, in addition to its physiological importance, aberrant Wnt signaling has been linked to the onset and progression of different types of cancer. On the cellular level, the secretion of Wnt proteins involves trafficking of lipid-modified Wnts from the endoplasmic reticulum (ER) to Golgi and further compartments via the Wnt cargo receptor evenness interrupted. Others and we have recently shown that Wnt proteins are secreted on extracellular vesicles (EVs) such as microvesicles and exosomes. Although more details about specific regulation of Wnt secretion steps are emerging, it remains largely unknown how Wnt proteins are channeled into different release pathways such as lipoprotein particles, EVs and cytonemes. Here, we describe protocols to purify and quantify Wnts from the supernatant of cells by either assessing total Wnt proteins in the supernatant or monitoring Wnt proteins on EVs. Purified Wnts from the supernatant as well as total cellular protein content can be investigated by immunoblotting. Additionally, the relative activity of canonical Wnts in the supernatant can be assessed by a dual-luciferase Wnt reporter assay. Quantifying the amount of secreted Wnt proteins and their activity in the supernatant of cells allows the investigation of intracellular trafficking events that regulate Wnt secretion and the role of extracellular modulators of Wnt spreading.

Key words Wnt signaling activity, Hydrophobic proteins, Wnt secretion, Blue Sepharose, Purified Wnt, Wnts on extracellular vesicles

Abbreviations

EV	Extracellular vesicles
MV	Microvesicles
MVBs	Multivesicular bodies

1 Introduction

Wnt signaling is an important signaling pathway regulating developmental processes, stem cell regulation and adult tissue homeostasis. A physiological threshold of Wnt activity within the stem cell niche of the crypt is important to maintain a balanced gut epithelium. However, aberrant signaling caused by mutations

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within Wnt pathway components promotes stem cell progenitor proliferation and is thus linked to colorectal cancer [1]. An additional layer of regulation upstream of the Wnt-responsive cells is the secretion of Wnts from source cells. Understanding how the release of Wnt proteins from secreting cells and their subsequent spreading through the extracellular space is regulated can help to develop new strategies to interfere with aberrant Wnt signaling.

Secretion of newly synthesized Wnts starts with the attachment of palmitoleic acid to a conserved serine residue by the acyltransferase porcupine (Porcn) in the endoplasmic reticulum (ER) [2, 3]. This hydrophobic modification allows the Wnt proteins to interact with the transmembrane protein evenness interrupted (Evi/Wntless/Sprinter), which targets its cargo to the plasma membrane [4–6]. The importance of the Wnt lipid modification for another interaction, the Wnt-Frizzled receptor interaction, was shown in the recently resolved crystal structure of Xenopus Wnt8 in complex with the CRD domain of Frizzled 8 [7]. Thus, Wnts have to reach the receiving cell with the attached lipid modification to exert full activity. Different carriers were shown to assist Wnt proteins spreading through the extracellular space shielding their hydrophobicity: Lipoprotein particles (LPPs) were shown as long-range vehicles of Wnts in human cell culture and *Drosophila* [8, 9]. Other modes of Wnt transportation are locally more restricted by membrane protrusion such as cytonemes or actin-based filopodia [10, 11]. Additionally, others and we have shown that active Wnt proteins can travel on extracellular vesicles (EVs) such as microvesicles and exosomes [12–14]. EVs are a heterogeneous population of membrane particles released into the extracellular space in vitro and in vivo. Emerging data indicates that the content, size and membrane composition of EVs are highly dynamic and depend on the cellular source, state and environmental conditions (for review *see* ref. 15). Exosomes (50–100 nm) arise from intraluminal vesicles in multivesicular bodies (MVBs), which are released into the extracellular environment upon fusion of the MVBs with the plasma membrane. In contrast, microvesicles (MVs) (100–400 nm) are considered to originate directly from the plasma membrane by outward budding. Thus, there are likely differences in the cellular machineries generating and releasing EVs. In order to investigate how the release of Wnt proteins from the producing cells is regulated and which factors dictate the extracellular carrier of choice, reliable biochemical assays for monitoring Wnt secretion and Wnt activity are indispensable. To purify Wnts from conditioned medium, a fractionation protocol using Blue Sepharose (Cibracon Blue 3G coupled to Sepharose) was established [16]. In addition to its use in large-scale HP columns, Blue Sepharose can be used for batch purification to directly precipitate the Wnts from the supernatant of Wnt-producing cells. Applying this precipitation protocol (Subheading 3.2), presumably the total amount of secreted Wnt proteins can be assessed. To narrow the secreted Wnts

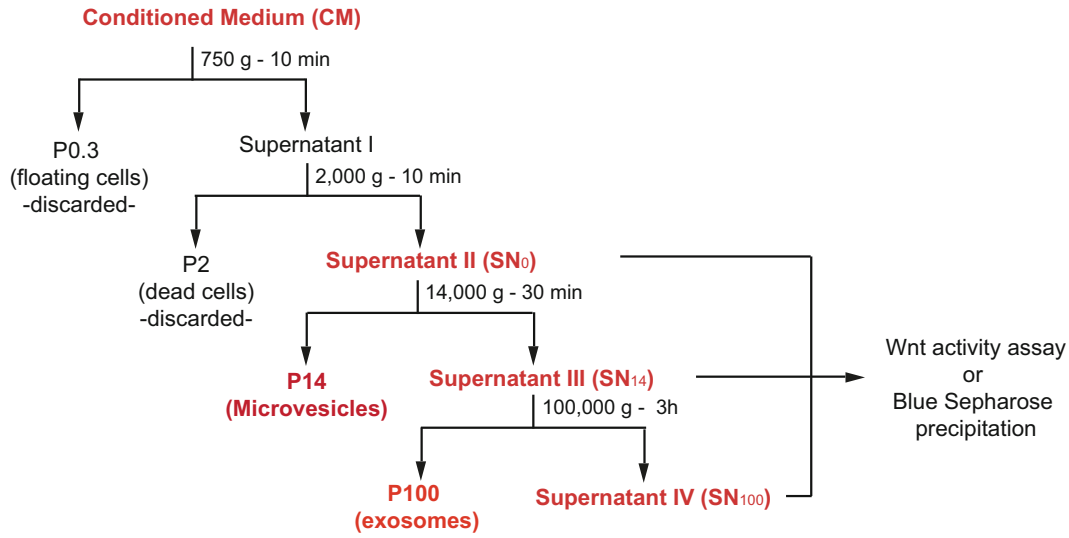

Fig. 1 Scheme of EV centrifugation or Blue Sepharose purification of Wnts. Conditioned medium (protocol 3.1) is subjected to a differential centrifugation protocol, pelleting cells and debris in the first steps. Subsequently, microvesicles are pelleted at 14,000 × *g* and exosomes at 100,000 × *g* (protocol 3.3). The respective supernatants can be used for Blue Sepharose precipitation or in Wnt activity assays as described in protocols 3.2 and 3.6

down to specific extracellular carriers such as EVs, a differential centrifugation protocol (Fig. 1) can be performed to purify Wnts on exosomes or microvesicles as described in Subheading 3.3. Additionally, a functional readout can be used to quantify the activity of secreted canonical Wnt proteins by performing a paracrine Wnt luciferase reporter assay (Subheading 3.6).

2 Materials

2.1 Production of Wnt Conditioned Medium (CM)

1. Cell line: Mouse L-Wnt3A, HEK293T (transiently) transfected with Wnt plasmid of choice or cell line with high endogenous Wnt levels.
2. Cell culture medium: Dulbecco's minimum essential medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS).
3. Dulbecco's phosphate-buffered saline (PBS).
4. 6-well plate, 10 cm dish, 15 cm dish or T75 tissue culture flask.
5. In case of transient transfection: transfection reagent.
6. In case of transient transfection: Wnt expression plasmid of choice (e.g. human Wnt3A or Wnt5A in pcDNA3 vector).
7. Bench-top centrifuge for 15 ml and 50 ml tubes.
8. 15 and 50 ml centrifuge tubes.
9. CO_2 incubator and laminar flow hood.

2.2 Blue Sepharose Purification of Wnts

1. At least 2 ml Wnt CM as prepared in Subheading 3.1.
2. 10% (v/v) Triton X-100.
3. Blue Sepharose 6 Fast Flow (GE Healthcare).
4. Binding and wash buffer: 150 mM KCl, 50 mM Tris–Cl, pH 7.5, 1% (w/v) CHAPS, or 1% (v/v) Triton X-100.
5. 2× SDS: 125 mM Tris–Cl, pH 6.8, 20% glycerol, 4% SDS, 10 μM TCEP, 0.04% bromophenol blue, 0.2 M DTT.
6. Eppendorf tubes for 1.5 ml.
7. Bench-top microcentrifuge for 1.5 ml tubes.

2.3 Purification of Extracellular Vesicles

1. At least 20 ml Wnt CM as prepared in Subheading 3.1.
2. 50 and 15 ml reaction tubes.
3. Ultracentrifugation tubes.
4. Dulbecco's phosphate-buffered saline (PBS).
5. 5× SDS: 312.5 mM Tris–Cl, pH 6.8, 50% glycerol, 10% SDS, 25 μM TCEP, 0.1% bromophenol blue, 0.5 M DTT.
6. Ultracentrifuge.
7. Swinging bucket rotor (SW40/41 Ti).

2.4 Preparation of Control Lysate

1. Dulbecco's phosphate-buffered saline (PBS).
2. Lysis buffer: 20 mM Tris–HCl, pH 7.4, 130 mM NaCl, 10% (w/v) glycerol, 2 mM EDTA, 1% (v/v) Triton X-100, supplemented with 1× protease inhibitor mix (e.g. complete mini EDTA-free protease-inhibitor cocktail from Roche); alternative lysis buffer: 8 M urea dissolved in PBS.
3. 5× SDS: 312.5 mM Tris–Cl, pH 6.8, 50% glycerol, 10% SDS, 25 μM TCEP, 0.1% bromophenol blue, 0.5 M DTT.
4. Eppendorf tubes for 1.5 ml.
5. Bench-top microcentrifuge for 1.5 ml tubes.

2.5 Immunoblotting

1. BCA protein concentration assay kit.
2. SDS-PAGE gel.
3. 1× MOPS SDS running buffer: 50 mM MOPS, 50 mM Trizma® Base, 0.1% [w/v] SDS, 0.03% [w/v] EDTA; pH 7.7.
4. 1× Transfer buffer: 25 mM Bicine, 25 mM Bis-Tris, 0.3% [w/v] EDTA, 10% [v/v] methanol.
5. Protein ladder.
6. Gel electrophoresis and blotting system.
7. Immuno-Blot PVDF membrane.
8. TBST: 137 mM NaCl, 20 mM Tris–HCl pH 7.6, 0.1% Tween20.

9. Wnt primary antibodies e.g. directed against Wnt3A and Wnt5A.
10. Control primary antibodies directed against HSC70 and β-actin.
11. EV marker primary antibodies: CD81, Alix, CD147, Tsg101.
12. Anti-rabbit IgG horseradish peroxidase.
13. Anti-mouse IgG horseradish peroxidase.
14. Anti-goat IgG horseradish peroxidase.
15. Western blotting HRP substrate.
16. Amersham Hyperfilm ECL.

2.6 Wnt/TCF4 Reporter Assay

1. Cell line: HEK293T (transiently) transfected or stable reporter cells.
2. 384-well cell culture plates.
3. Cell culture medium: Dulbecco's minimum essential medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS).
4. Dulbecco's phosphate-buffered saline (PBS).
5. In case of transient transfection: transfection reagent.
6. In case of transient transfection: Wnt firefly luciferase and constitutive renilla luciferase reporter plasmids of choice (e.g. Wnt/TCF4 in pgl4.2 Vector [17]).
7. CO_2 incubator and laminar flow hood.

3 Methods

3.1 Production of Wnt Conditioned Medium (CM)

1. Plate ~4×10^5 cells in 2.5 ml DMEM (10% FBS) onto a 6-well plate, ~3.5×10^6 cells in 10 ml onto a 10 cm dish or 6×10^6 cells in 20 ml onto a 15 cm dish (*see* **Note 1**).
2. Incubate the cells at 37 °C and 5% CO_2.
3. In case of transient transfection: transfect the cells with the plasmids of choice according to the manufacturer's conditions.
4. Recover CM ~72 h after cell seeding and centrifuge at $2000 \times g$ for 10 min to remove cell debris.
5. Keep the supernatant and proceed to Subheading 3.2 **step 1** or 3.3 **step 3**. Alternatively, freeze the CM at −20 °C.

3.2 Blue Sepharose Purification of Wnts

The original protocol to purify Wnt proteins is a three-step fractionation protocol using Blue Sepharose, gel filtration and heparin cation-exchange columns in the presence of 1% CHAPS to maintain solubility of lipid-modified Wnt proteins [16], which has been used to compare the relative amount of Wnts secreted into cell culture supernatant [18]. In this protocol, we modified the washing buffer detergent from CHAPS to Triton X-100 in order to monitor other secreted proteins like HSC70 as loading control (Fig. 2).

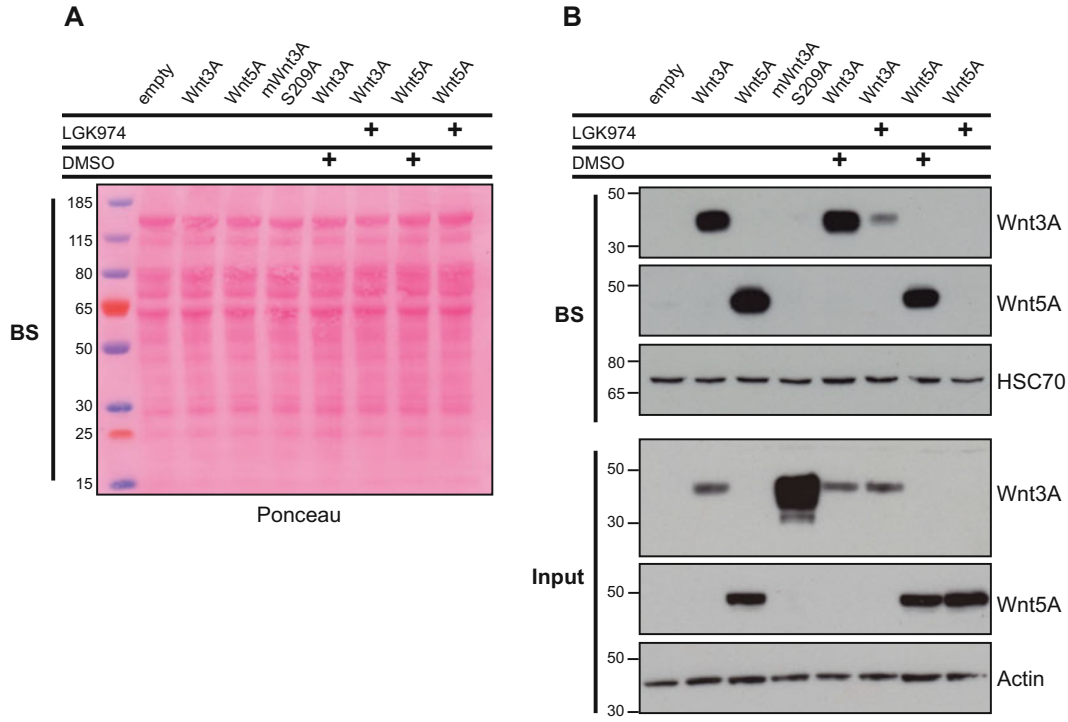

Fig. 2 Blue Sepharose purification was applied to analyze the secretion of Wnt3A and Wnt5A in dependency of Wnt palmitoylation. HEK293T cells were transiently transfected in 6-well plates with 700 ng human Wnt3A, human Wnt5A and mouse S209A Wnt3A overexpression plasmids. Treatment with 5 μM Porcn inhibitor LGK974 (6 and 30 h after transfection) [20] as well as mutation of the Wnt3A palmitoylation site S209 [3] was used to verify that Blue Sepharose can be applied to specifically purify lipid-modified and thus secreted Wnt proteins. 48 h after transfection, the conditioned medium of two 6-wells was combined and processed as described in protocols 3.1 and 3.2. (**a**) Ponceau staining of the Blue Sepharose (BS) samples shows that proteins other than Wnts were bound to Blue Sepharose if the beads were washed twice with washing buffer containing 1 % Triton X-100. These proteins could be used as loading or secretion controls. (**b**) Blotting the BS samples with Wnt3A and Wnt5A antibodies verifies that Blue Sepharose can be used to efficiently precipitate human Wnt3A and Wnt5A from conditioned medium (CM). Wnt secretion and thus BS precipitation of Wnts from CM were significantly reduced upon mutation of the Wnt3A palmitoylation site (S209A) and upon Porcn inhibition. In contrast, general secretion and loading were equal for all BS samples as indicated by blotting with the HSC70 antibody. Immunoblotting of control lysates (Input) shows increased levels of mouse S209A-Wnt3A, but equal expression of the analyzed human Wnt proteins

1. Supplement the CM prepared in Subheading 3.1 with Triton X-100 to a final concentration of 1 % (v/v).
2. Wash 40–50 μl Blue Sepharose Beads (per condition) by rotation in binding and wash buffer at 4 °C and subsequent centrifugation for 5 min at 2700 × *g*.
3. Transfer 50–100 μl binding and wash buffer to the pre-equilibrated beads and add the mixture to the CM (make sure to take up the beads by cutting the pipette tip).
4. Rotate for 2–5 h up to overnight at 4 °C.

5. Wash two times with binding and wash buffer by centrifugation at 2700 × *g* for 5 min at 4 °C (*see* **Note 2**).
6. Completely remove binding and wash buffer from the beads.
7. Add 25 µl binding and wash buffer as well as 25 µl 2× SDS buffer to the beads, boil for 5 min at 96 °C and centrifuge for 5 min at 2700 × *g*.
8. Load 25–50 µl of the supernatant onto a protein gel and continue with immunoblotting (**step 4** of Subheading 3.5, Fig. 2).

3.3 Purification of Extracellular Vesicles

1. Recover CM ~72 h after cell seeding and centrifuge in a 50 ml tube at 750 × *g* for 10 min to remove cells (*see* **Note 1**).
2. Transfer supernatant into a new 50 ml tube and centrifuge at 2000 × *g* for 10 min to remove cell debris.
3. Transfer supernatant into a new 50 ml tube and take an aliquot of supernatant (SN_0) for activity assay. Centrifuge at 14,000 × *g* for 30 min to pellet microvesicles. Resuspend pellet in 1 ml PBS and centrifuge at 14,000 × *g* for 30 min in a table-top centrifuge in a microcentrifuge tube. Resuspend in 50 µl of PBS.
4. Transfer supernatant (SN_{14}) into ultracentrifugation tubes and take an aliquot of supernatant (SN_{14}) for activity assay. Centrifuge in an ultracentrifuge at 100,000 × *g* for 3 h to pellet exosomes in a swinging bucket rotor (SW40/41 Ti) [19]. Take an aliquot of supernatant (SN_{100}) for activity assay. Wash pellet with 10 ml PBS and centrifuge at 100,000 × *g* for 3 h. Thoroughly resuspend exosomes at the bottom of the tube in 50 µl PBS.
6. Freeze the supernatants, microvesicles and exosomes or directly use for immunoblotting (Subheading 3.5, Fig. 3) or for activity assay (Subheading 3.6, Fig. 4, *see* **Note 3**).

3.4 Preparation of Control Lysate

1. In case of adherent cells, wash the cells once with PBS after recovering the CM.
2. Add 200 µl lysis buffer to a 6-well or 700 µl lysis buffer to a 10 cm dish and scrape the cells off with a cell scraper.
3. Transfer the cell lysates into Eppendorf tubes and incubate for 20 min on ice.
4. Centrifuge the lysates at full speed for 10 min at 4 °C.
5. Freeze the supernatant of the cell lysate or directly use for immunoblotting (Subheading 3.5).

3.5 Immunoblotting

1. Determine the protein concentration of the cell lysate (prepared in Subheading 3.4) by adding 1–2 µl of the lysate to 200 µl BCA reagent mix (4 µl reagent A + 196 µl reagent B) and incubate for 30 min at 37 °C. Compare the absorbance at 562 nm to an internal calibration curve generated with different BSA concentrations (0.5–4.0 µg/µl).

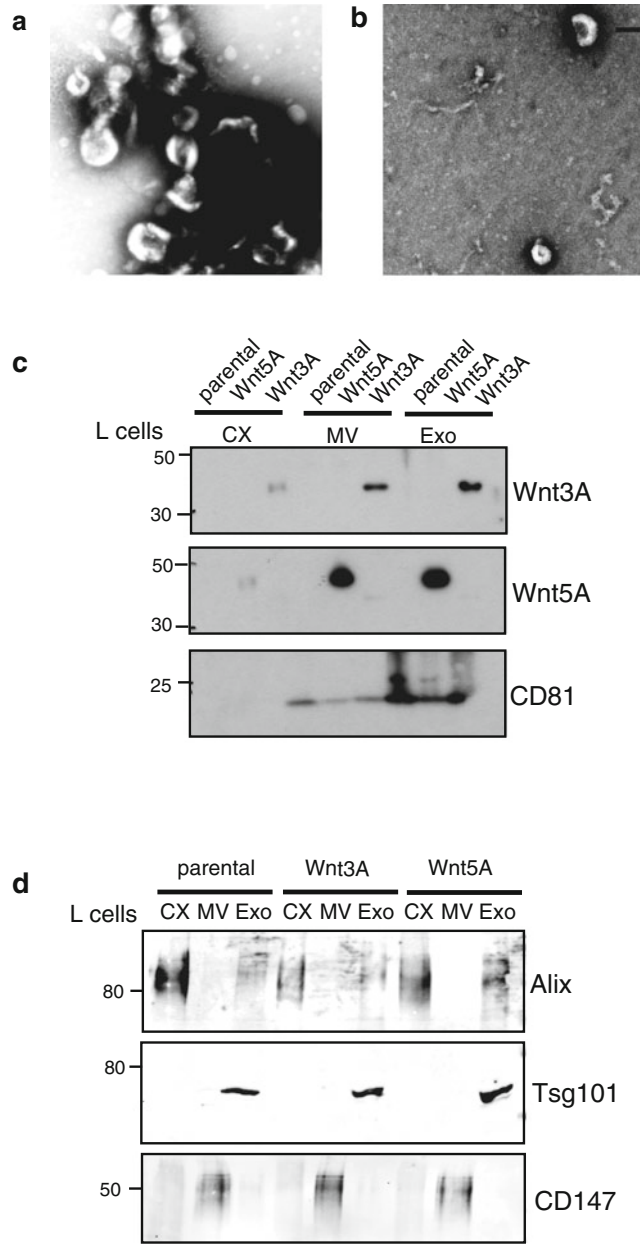

Fig. 3 Electron microscopy analysis of (**a**) microvesicle and (**b**) exosome fractions from the differential centrifugation protocol. Samples were contrast-stained with 4 % uranyl-acetate, scale bar 100 nm. (**c**) and (**d**) Purification and immunoblotting of microvesicles and exosomes from L-cell parental, Wnt3A and Wnt5A conditioned medium

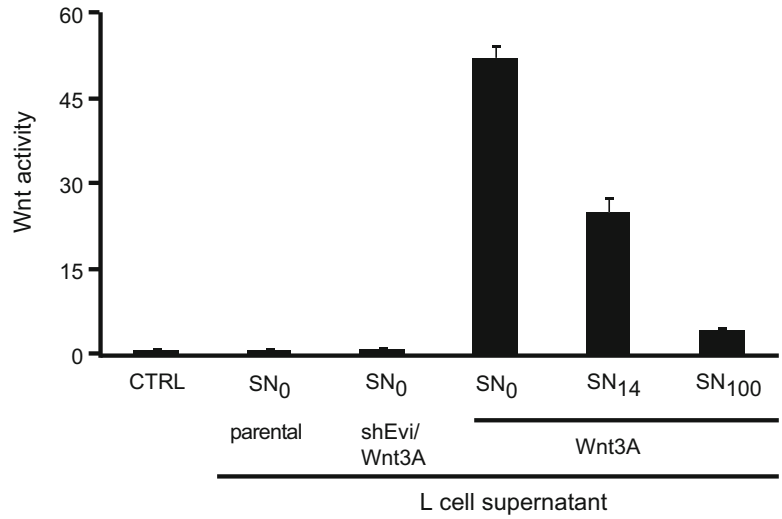

Fig. 4 Wnt activity was assessed by dual-luciferase assay 24 h after adding conditioned medium to reporter cells compared to basal reporter activity (CTRL) (protocol 3.6). Highest Wnt activity was found in the supernatant from L-cell Wnt3A before (SN_0) EV purification, with ~50 and 10 % activity in the supernatant after 14,000 × *g* (SN_{14}) and 100,000 × *g* (SN_{100}) ultracentrifugation steps, respectively. Parental L-cell and L-cell Wnt3A shEvi supernatants (SN_0) were used as control treatments

2. Supplement the protein lysates with SDS sample buffer and boil for 5 min at 96 °C.
3. Load 30–50 μg protein lysate and 25–50 μl of the purified Wnts (from Subheadings 3.2 and 3.3) on a 4–12 % gel.
4. Resolve proteins by electrophoresis in 1× MOPS SDS running buffer at 80–150 V.
5. Transfer the proteins onto a methanol-soaked PVDF membrane using 1× transfer buffer supplemented with 10 % methanol under constant voltage of 35 V for 1.5 h at room temperature.
6. Block the membrane in 5 % milk/TBST for 1 h with shaking at room temperature.
7. Incubate the membrane with the appropriate primary antibody (diluted in 5 % milk/TBST) overnight at 4 °C (*see* **Notes 4** and **5** for antibody dilutions).
8. Wash the membrane four times with TBST for 10 min at room temperature.
9. Incubate the membrane for 1 h at room temperature in secondary antibody, diluted 1:10,000 in a 1:1 mixture of 5 % milk and TBST.

10. Wash the membrane four times with TBST for 10 min at room temperature.
11. Incubate the membrane for 2 min with ECL substrate to detect the HRP-linked secondary antibodies.

3.6 Wnt/TCF4 Reporter Assay (Paracrine)

1. Plate 4×10^3 HEK293T cells in 30 µl/well in a 384-well plate.
2. Incubate the cells at 37 °C and 5 % CO_2.
3. Transfect the cells ~24 h after cell seeding with the Wnt reporter plasmids of choice according to manufacturer's conditions using the following amounts:
   (a) 10 ng of CMV renilla construct.
   (b) 10 ng of 6× KD-Luciferase construct.
   (c) 25 ng Empty plasmid.
4. Stimulate reporter cells with CM, purified EVs or 100 ng/ml recombinant Wnt3A in a total volume of 20 µl per well in a 384-well plate (in four replicates) ~72 h after cell seeding (*see* **Note 6**).
5. Perform dual-luciferase readout according to manufacturer's conditions ~96 h after cell seeding (Fig. 4).

4 Notes

1. In case the Blue Sepharose protocol is used to detect endogenous Wnt levels, at least 10 ml conditioned medium from a 10 cm dish or from a T75 tissue culture flask should be used. Upon Wnt overexpression, 2–4 ml conditioned medium should be sufficient. For purification of extracellular vesicles, at least 20 ml conditioned medium from two 15 cm dishes should be taken.
2. If the beads are washed twice with binding and wash buffer containing 1 % Triton X-100, proteins other than Wnts (such as HSC70) are bound to the beads which can be used as reference control for equal cell secretion and loading (Fig. 2).
3. Quality of EV purification can be assessed by whole-mount uranyl-acetate contrast-stained EVs monitored under the electron microscope (Fig. 3), where EVs appear as cup-shaped vesicles of 50–400 nm.
4. The Wnt3A antibody from Cell Signaling and the Wnt5A antibody from R&D Systems can be diluted 1:250–1:500. The HSC70, Alix and CD147 antibody from Santa Cruz, the CD81 from Immunotools and the TSG101 antibody from Sigma are diluted 1:1000 and the β-actin antibody from Abcam 1:20,000.
5. Specific and common markers for microvesicles and exosomes exist. While CD81 is found on both populations, CD147 is a

microvesicle-specific and Alix and Tsg101 are exosome-specific markers (Fig. 3).

6. Activity of microvesicles and exosomes is reduced if EVs clump together. For activity assay of pellets it is thus advised to centrifuge vesicles first on a 30% sucrose cushion in ultracentrifugation tubes, wash with 10 ml PBS, and resuspend in 50 µl PBS [19].

Acknowledgments

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Chapter 4

Methods for Studying Wnt Protein Modifications/ Inactivations by Extracellular Enzymes, Tiki and Notum

Xinjun Zhang and Xi He

Abstract

Wnt proteins are modified and inactivated by two extracellular enzymatic antagonists, Tiki and Notum. Tiki proteins act as membrane-tethered metalloproteases to cleave a fragment from the amino terminus of Wnt proteins. Notum is a Wnt deacylase that removes the lipid modification that is essential for Wnt activities. Here, we provide detailed procedures for preparing enzymatic active Tiki and Notum proteins and the in vitro enzymatic reactions. We also describe a metabolic labeling and click chemistry method for detection of Wnt protein acylation.

Key words Wnt, Tiki, Notum, Metalloprotease, Deacylase, Acylation, Click-chemistry

1 Introduction

Wnt signaling is tightly regulated by multiple extracellular inhibitors [1]. Most Wnt inhibitors, such as DKK1, SOST, and sFRPs, bind to Wnt receptors or ligands to prevent ligand–receptor complex formation. Tiki and Notum are enzymatic Wnt inhibitors that directly modify and inactivate Wnt proteins [2–4] (Fig. 1).

Tiki proteins act as novel metalloproteases that cleave a short fragment from the amino terminus of several Wnt proteins and inactivate them [4]. Tiki proteins have a large and conserved extracellular domain in the amino terminus and a predicted transmembrane domain near the C-terminus. We have shown that the extracellular domain of Tiki is sufficient to inactivate Wnt3a. To study Tiki cleavage of Wnt3a, we designed HA-Wnt3a, in which the HA tag is fused with the amino terminus of Wnt3a after the signal peptide. Tiki cleavage caused the removal of the HA tag from HA-Wnt3a as shown in an anti-HA immunoblot [4]. We purified TIKI2 extracellular domain (TIKI2N) from transfected HEK293T cells via a tandem affinity procedure. We also purified HA-Wnt3a from the conditioned medium (CM) of HEK293T cells stably expressing HA-Wnt3a. We successfully developed an

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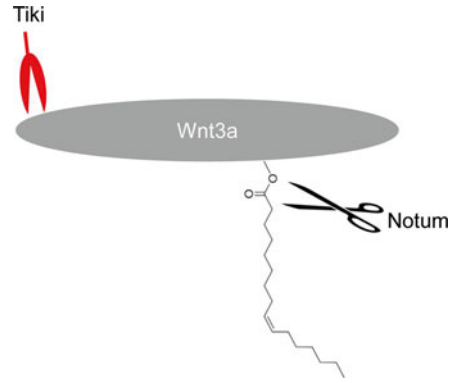

Fig. 1 Schemes of Wnt3a modifications/inactivations by Tiki and Notum. Tiki acts as a metalloprotease to cleave the amino terminus of Wnt3a and Notum acts as a deacylase to hydrolyze the palmitoleoylate adduct

in vitro enzymatic assay using recombinant TIKI2N and HA-Wnt3a. Using this assay, we have shown that TIKI2N, but not KRM2N (the extracellular domain of Krmen2 that was used as a negative control) or TIKI2NΔ (a deletion mutant that lacks a TIKI-domain segment), cleaved HA-Wnt3a (Fig. 2). This assay can be used to analyze TIKI enzyme activity and validate potential TIKI inhibitors.

Wnt proteins are lipid modified [5]. A mono-unsaturated palmitoleic acid is conjugated onto the hydroxyl group of a conserved serine residue (serine 209 in Wnt3a) and is essential for Wnt protein secretion and binding to Frizzled receptors [5–7]. Radiolabeled palmitic acids (^{3}H, ^{14}C and ^{125}I) have been used to label and monitor Wnt protein acylation [6, 8, 9]. These labeling approaches are in general insensitive and time consuming, and/or cumbersome for the lack of commercial reagents. A click chemistry-based metabolic labeling has recently been developed to detect lipid modifications of intracellular proteins [10, 11]. This method is based on the Cu^{1}-catalyzed azide-alkyne cycloaddition. We have successfully applied this approach to label Wnt3a lipidation [2, 4] (Figs. 3 and 4). This approach is composed of two steps. Firstly, an azide- or alkyne-conjugated palmitate analog is used to metabolically label Wnt3a in cells. Then, Wnt3a in CM is immunoprecipitated by antibody-conjugated agarose beads and a fluorescence molecule is added to the labeled Wnt3a via a click chemistry reaction. This click chemistry-based metabolic labeling approach is rapid and sensitive in detecting Wnt3a acylation.

Others and we have reported that Notum, a secreted Wnt signaling inhibitor of the α/β hydrolase superfamily, is a Wnt-specific deacylase [2, 3]. Using click chemistry-based metabolic labeling, we have shown that Notum diminished Wnt3a acylation when coexpressed with Wnt3a in cells [2]. To assess Notum enzymatic activity, we purified Notum protein from the CM of HEK293T cells

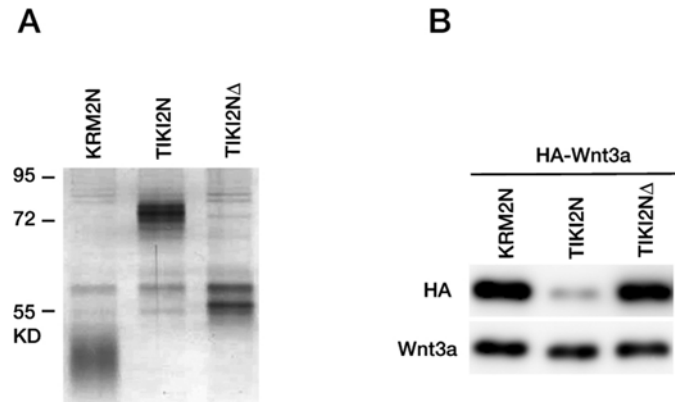

Fig. 2 Tiki cleavage of HA-Wnt3a in vitro. (**a**) Silver staining of purified KRM2N, TIKI2N, and TIKI2NΔ. (**b**) Purified TIKI2N, but neither KRM2N nor TIKI2NΔ, cleaved recombinant HA-Wnt3a in vitro (reproduced from [4] with permission from Elsevier)

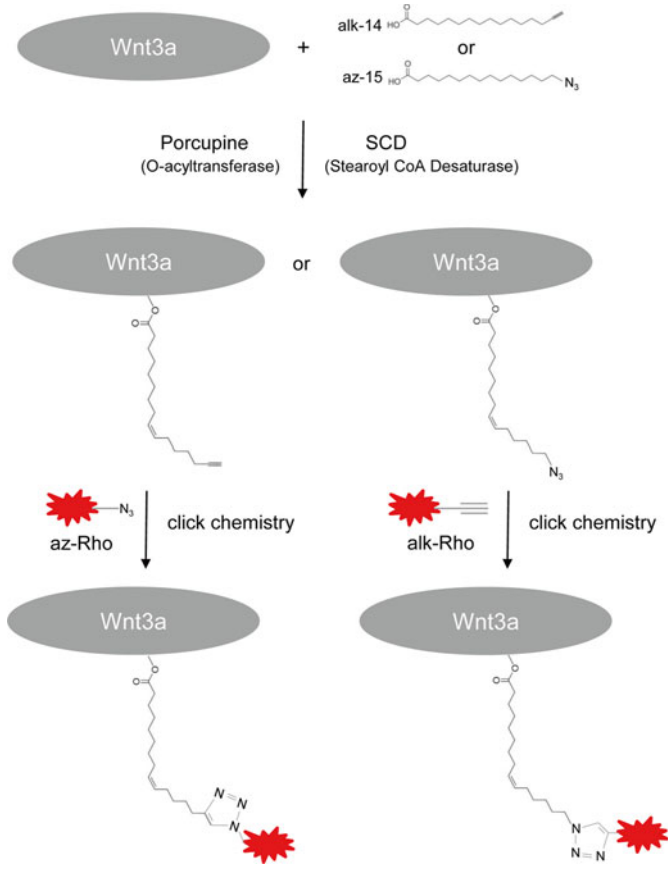

Fig. 3 Click chemistry-based metabolic labeling to detect Wnt3a acylation

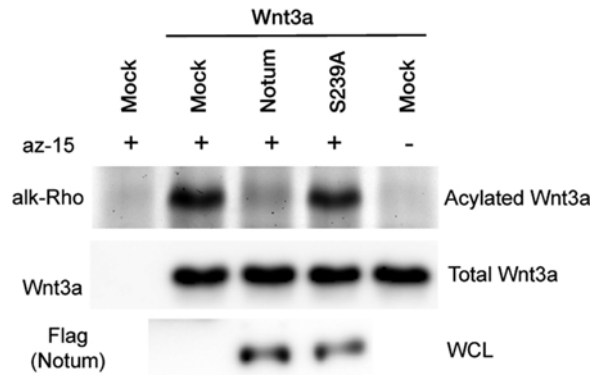

Fig. 4 Notum, but not Notum(S239A), reduced Wnt3a acylation when they were coexpressed in HEK293T cells (reproduced from [2] with permission from Elsevier)

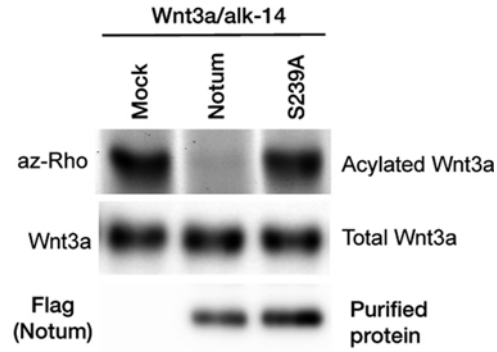

Fig. 5 Notum removed Wnt3a acylation in vitro. Purified metabolically labeled Wnt3a protein was incubated with control buffer (mock), purified Notum or Notum(S239A) proteins (reproduced from [2] with permission from Elsevier)

expressing Notum. We developed an in vitro assay to show that purified Notum, but not Notum(S239A) (an enzymatic dead mutant), can efficiently remove Wnt3a lipid labeling in vitro (Fig. 5).

In this chapter, we provide detailed protocols for purification of enzymatic active Tiki and Notum, in vitro enzymatic assays, as well as a click chemistry-based metabolic labeling approach for monitoring Wnt protein acylation.

2 Materials

2.1 Cell Culture and Transfection

1. Cell lines: HEK293T and HEK293T stably expressing HA-Wnt3a.
2. Expression vectors: KRM2N (the extracellular domain of Kremen 2), TIKI2N (the extracellular domain of TIKI2), and TIKI2NΔ (TIKI2N lacks a fragment from residue 223 to 349)

in pCS2+ fused with a C-terminal FLAG-6xHIS tag; Notum and Notum(S239A) in pCS2+ vector fused with a C-terminal FLAG tag.

3. Cell culture medium: DMEM supplemented with 10% FBS and 1% penicillin–streptomycin–glutamine.
4. Transfection reagent.

2.2 Protein Purification

1. Buffers: Cell lysis buffer (PBS containing 0.2% Triton X-100, 20 mM imidazole and a protease inhibitor cocktail); HIS tag elution buffer (PBS containing 0.2% Triton X-100 and 100 mM imidazole); wash buffer (PBS containing 0.2% Triton X-100); HEPES buffer (50 mM HEPES [pH 7.4], 100 mM NaCl).
2. Ni-NTA agarose.
3. FLAG antibody and HA antibody agarose.
4. 3xFLAG peptide stock: 2 mg/ml in 100 mM HEPES buffer (pH 7.4).
5. HA peptide stock: 5 mg/ml in water.
6. 3xFLAG peptide elution buffer: 50 μg/ml of 3xFLAG peptide in HEPES buffer (*see* **Note 1**).
7. HA peptide elution buffer: 250 μg/ml of HA peptide in HEPES buffer.

2.3 Metabolic Labeling of Wnt3a Acylation and Click Chemistry Reaction

1. Labeling medium: DMEM supplemented with 5% charcoal-stripped FBS and 1% penicillin–streptomycin–glutamine (*see* **Note 2**).
2. az-15 and alk-14 stock: 20 mM in DMSO (*see* **Note 3**).
3. az-Rho and alk-Rho stock: 5 mM in DMSO.
4. 0.5 M TCEP stock (in water) (*see* **Note 4**); 1 M $CuSO_4$ stock (in water); 10 mM Tris[(1-benzyl-1H-1,2,3-triazol-4-yl) methyl]amine (TBTA) (in 1:4 DMSO:t-butanol).

3 Methods

3.1 In Vitro Cleavage of Wnt3a by TIKI2

1. Transfect HEK293T cells in 100 mm dishes with expressing vectors (2.5 μg of expressing vector and 2.5 μg of empty vector for each dish).
2. 48 h after transfection, lyse the cells with ice-cold cell lysis buffer (1 ml buffer for each 100 mm dish).
3. Clear the whole-cell lysate (WCL) by centrifugation at top speed for 10 min at 4 °C on a bench-top centrifuge.
4. Wash Ni-NTA agarose beads with PBS three times, add 50 μl beads to each 1 ml of WCL, and incubate the mixture at 4 °C with rotation for 1 h.

5. Collect the beads by brief centrifugation and discard the supernatant. Add 1 ml lysis buffer and rotate for 10 min at 4 °C to wash. Repeat the wash step for total four times.
6. Elute the protein by adding 150 μl HIS tag elution buffer and rotate at 4 °C for 30 min.
7. Spin down the beads and collect the supernatant.
8. Repeat **steps 6** and **7** for another two times and pool the supernatants together (total volume should be around 450 μl).
9. Add 1 ml wash buffer to dilute the HIS tag elution buffer and add 20 μl of FLAG antibody agarose beads. Incubate the mixture at 4 °C with rotation for 2 h to overnight (*see* **Note 5**).
10. Spin down the beads and discard the supernatant. Add 1 ml wash buffer and rotate for 10 min to wash the beads. Wash three times.
11. Spin down the beads and discard the supernatant. Add 1 ml HEPES buffer to wash the beads.
12. Spin down the beads and discard the supernatant. Spin again and use a 25G needle syringe to remove the residue wash buffer (*see* **Note 6**). Add 50 μl of 3× FLAG peptide elution buffer and incubate at 4 °C with rotation for 4 h to overnight to elute protein.
13. Spin down the beads and move the supernatant to a new tube. The eluted protein can be quantified by SDS-PAGE followed with Western blot and Simple Blue staining. Store the purified protein at 4 °C (for several weeks) or −80 °C (for long term) (*see* **Note 7**).
14. Expand HEK293T cells stably expressing HA-Wnt3a and collect conditioned medium (HA-Wnt3a CM).
15. Clean the HA-Wnt3a CM by centrifugation at 10,000 × *g* for 10 min. Transfer the supernatant to a new tube and add 0.1% Triton X-100.
16. Add 50 μl of HA antibody agarose to 50 ml HA-Wnt3a CM and incubate the mixture at 4 °C overnight with rotation.
17. Spin down and transfer the beads to a new 1.5 ml Eppendorf tube with wash buffer. Wish the beads three times with wash buffer and one time with HEPES buffer.
18. Spin down the beads and discard the supernatant. Spin again and use a 25G needle syringe to remove the residue wash buffer. Add 100 μl of HA peptide elution buffer and incubate at 4 °C with rotation for 4 h to overnight to elute HA-Wnt3a protein.
19. Spin down the beads and transfer the supernatant to a new tube. HA-Wnt3a protein can be quantified by SDS-PAGE and Western blot. Store the purified protein at 4 °C (for several weeks) or −80 °C (for long term) (*see* **Note 8**).

20. Mix about 50 ng of purified TIKI2N, KRM2N, or TIKI2NΔ (from **step 13**) and about 10 ng of purified HA-Wnt3a (from **step 19**) in a total volume of 10 μl HEPES buffer, and incubate the mixture at 30 °C for 2 h to overnight (*see* **Note 9**).
21. Add 10 μl of 2× SDS-PAGE loading buffer and denature the proteins at 95 °C for 5 min.
22. Load 5–10 μl of denatured samples to SDS-PAGE and perform Western blot with anti-HA (detect the HA-Wnt3a), anti-Wnt3a (detect the total Wnt3a), and anti-FLAG (detect TIKI2N, KRM2N, and TIKI2NΔ) antibodies (Fig. 2).

3.2 Metabolic Labeling of Wnt3a Acylation

1. Plate HEK293T cells or HEK293T cells stably expressing HA-Wnt3a in 60 mm dishes.
2. When cells reach confluence (usually after 48 h), remove culture medium, carefully wash cells with warm serum-free DMEM once, and then add 3 ml of labeling medium containing 40 μM of az-15 or alk-14. Put the cells back in the incubator for 8–12 h.
3. Collect the CM and clear it by centrifugation at 10,000×*g* for 10 min. Add 0.1% Triton X-100 and 20 μl of HA antibody agarose beads. Incubate the mixture at 4 °C overnight with rotation.
4. Spin down the beads and discard the supernatant. Wash the beads with wash buffer three times and then with PBS one time.
5. Spin down the beads and discard the supernatant. Spin down again and use a 25G needle syringe to remove the residue buffer. Add 23 μl PBS to resuspend the beads.
6. Prepare fresh 50 mM TCEP solution (from 0.5 M stock) and 50 mM $CuSO_4$ solution (from 1 M stock).
7. Prepare click chemistry mixture. For each 25 μl reaction, mix 0.5 μl of TCEP, 0.5 μl of $CuSO_4$, 0.25 μl of TBTA, and 0.5 μl of alk-Rho (for az-15) or az-Rho (for alk-14) (*see* **Note 10**).
8. Add the click chemistry mixture to PBS-resuspended beads from **step 5** and mix it. Incubate the reaction mixture at room temperature in the dark for 1 h.
9. Add 10 μl of 4× SDS-PAGE loading buffer to the reaction mixture and denature the proteins at 95 °C for 5 min.
10. Load 1–2 μl of denatured samples to SDS-PAGE and perform Western blot with anti-Wnt3a antibody to detect the total Wnt3a protein.
11. Load 5–10 μl of denatured samples to SDS-PAGE. Wash the gel briefly with distilled water and then fix it in 40% methanol and 10% acetic acid with rotation for 1 h.
12. After fixation, wash the gel with distilled water three times, 5 min each time. Keep the gel in water.

13. Scan the gel on a fluorescent gel scanner (e.g., GE Healthcare Typhoon 9400 variable-mode imager) at excitation 532 nm/emission 580 nm (to detect rhodamine signal) (Figs. 3 and 4).

3.3 In Vitro Deacylation of Wnt3a by Notum

1. Transfect HEK293T cells in 100 mm dishes with expression vectors (5 μg for each dish).
2. 24 h after transfection, replace the medium with 6 ml fresh warm medium for each dish.
3. 48 h after transfection, collect the CM and clear it by centrifugation at 10,000 × *g* for 10 min. Add 0.1% Triton X-100 and 20 μl of FLAG antibody agarose beads. Incubate the mixture at 4 °C overnight with rotation.
4. Spin down the beads and discard the supernatant. Wash the beads with wish buffer three times and then with HEPES buffer one time.
5. Spin down the beads and discard the supernatant. Spin down again and use a 25G needle syringe to remove the residue buffer. Add 50 μl of 3× FLAG peptide elution buffer and incubate at 4 °C with rotation for 4 h to overnight to elute protein.
6. Spin down the beads and move the supernatant to a new tube. The eluted protein can be quantified by SDS-PAGE followed with Western blot. Store the purified protein at 4 °C (for several weeks) or −80 °C (for long term) (*see* **Note 11**).
7. Aliquot the HA antibody beads bound with labeled Wnt3a (from Subheading 3.2, **step 5**) into three 1.5 ml tubes, and carefully remove all the liquid with a 25G needle syringe.
8. Add 20 μl of purified Notum, Notum(S239A), or 3× FLAG elution buffer to each of the tubes and incubate them at 30 °C overnight in the dark.
9. Follow **steps 6–13** in Subheading 3.2 to analyze the labeled and total Wnt3a protein levels (Fig. 5).

4 Notes

1. 3× FLAG peptide is acidic. It is important to keep the 3× FLAG elution buffer at neutral pH in order for 3× FLAG peptide to efficiently compete and elute the FLAG fusion protein. We found that HEPES buffer works better than Tris–HCl buffer.
2. Wnt3a is hydrophobic in CM. Serum helps to keep Wnt3a protein soluble in CM. Charcoal-stripped FBS has less free fatty acid, and is helpful to increase the labeling efficiency.
3. We found that alk-14 (has 16 carbons including the 2 carbons in alkyne group) works much better than alk-16 (has totally 18 carbons) in labeling Wnt3a.

4. It is important to use TCEP-HCl power to make water stock solution. Some premade TCEP solutions do not work well in the click chemistry reaction.
5. We found that the incubation time between 2 h and overnight did not make much difference.
6. It is important to remove the residue wash buffer before applying elution buffer; otherwise the elution buffer will be diluted.
7. Purified TIKI2N protein is stable in elution buffer for several weeks at 4 °C. For long-term storage, we recommend to store at −80 °C.
8. Purified HA-Wnt3a protein is stable in elution buffer for several weeks at 4 °C. For long-term storage, we recommend to store at −80 °C.
9. After 2-h incubation, we started to observe significant cleavage of HA-Wnt3a, and overnight incubation resulted in nearly complete cleavage. Co^{2+} or Mn^{2+} can significantly increase the cleavage efficiency.
10. After mixing TCEP, $CuSO_4$, and TBTA, the solution should be in light yellow color that indicates the formation of Cu(I).
11. Purified Notum protein is stable in elution for several weeks at 4 °C. For long-term storage, we recommend to store at −80 °C.

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Chapter 5

Probing Wnt Receptor Turnover: A Critical Regulatory Point of Wnt Pathway

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Abstract

Wnt pathways are critical for embryonic development and adult tissue homeostasis in all multicellular animals. Many regulatory mechanisms exist to control proper signaling output. Recent studies suggest that cell surface Wnt receptor level is controlled by ubiquitination, and serve as a critical regulatory point of Wnt pathway activity as it determines the responsiveness of cells to Wnt signal. Here, we describe flow cytometry, cell surface protein biotinylation, and immunofluorescence pulse-chase methods to probe the surface expression, ubiquitination, and internalization of the Wnt receptors FZD and LRP6.

Key words Wnt, Receptor, FZD, LRP6, Flow cytometry, Cell surface protein biotinylation, Immunofluorescence, Pulse-chase

1 Introduction

Wnt proteins are secreted lipoglycoprotein ligands that control embryonic development, including cell proliferation, migration, cell fate specification, and polarity formation [1]. Regulation of Wnt signaling within the extracellular milieu comprises not just the regulation of Wnt concentration but also a complicated interplay between Wnt and multiple protein classes, including secreted inhibitory ligands and Wnt modifiers [2–6]. The responsiveness of Wnt-target cells to Wnt ligands is another critical regulatory point to control proper signaling output. Cell surface FZD level was shown to be regulated by deubiquitination possibly by deubiquitination enzyme UBPY/USP8 [7]. ZNRF3 and RNF43, two homologous cell-surface single-pass transmembrane E3 ubiquitin ligases, have emerged as the most potent negative feedback regulators of Wnt pathway identified so far. ZNRF3 and RNF43 are induced by Wnt/β-catenin signaling, which in turn shut off Wnt signaling by negatively controlling the cell surface level of Wnt receptors Frizzled (FZD) and LRP6 by promoting their ubiquitination and subsequent internalization and degradation [8, 9].

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The *Caenorhabditis elegans* ortholog of ZNRF3 and RNF43 (PLR-1) regulates Wnt receptor turnover, suggesting this function is evolutionarily conserved [10]. R-Spondin proteins (RSPO1-4) are stem cell growth factors that strongly potentiate Wnt signaling [11], and their function requires LGR4 and LGR5 [12–15]. Recent work suggests that R-Spondin sensitizes cells to Wnt signaling through binding to ZNRF3/RNF43 and LGR4/LGR5, and inducing membrane clearance of ZNRF3/RNF43 [8]. This hypothesis has been supported by co-crystal structures and functional analysis of R-Spondin-LGR4/5-ZNRF3/RNF43 complexes [16–22]. When both Znrf3 and Rnf43 are deleted in mouse intestinal epithelium, it causes unrestricted expansion of the intestinal stem cell zone and the rapid formation of adenoma [9]. Loss-of-function mutations of RNF43 and ZNRF3 [23–31], and overexpression of R-Spondin proteins through chromosomal translocation have been observed in human tumors [32], representing various mechanisms to escape from this negative feedback regulation to achieve high tumorigenic Wnt/β-catenin signaling.

Here, we describe three methods that have been used in published studies to monitor Wnt receptor cell surface level and internalization in mammalian cell culture systems. Flow cytometry is a straightforward method that can be used to monitor Wnt receptor level on the cell surface at any given steady state. Because ubiquitination and deubiquitination have key roles in controlling Wnt receptor internalization and degradation, FZD ubiquitination level can be monitored by Western blotting with a specific emphasis on cell surface FZD. Finally, using fluorescent labeling of cell surface FZD, we can monitor the internalization of surface FZD at different time points.

2 Materials

2.1 Cell Culture

1. Adherent mammalian cells such as HEK293T cells are used here as an example.
2. Standard tissue culture conditions. For HEK293T cells, use DMEM supplemented with Fetal Bovine Serum, and antibiotics (penicillin/streptomycin).

2.2 Flow Cytometry

1. Cell Dissociation Buffer (Life Technologies).
2. Flow Cytometry Staining Buffer: PBS, 1% BSA, or commercial Flow Cytometry Staining Buffer (e.g., from eBioscience).
3. Optional: Human Fc Receptor Binding Inhibitor Purified (eBioscience).
4. Antibodies for staining. Anti-pan-FZD antibody (clone 18R5) [33]. Anti-human-LRP6 (R&D systems AF1505). Appropriate secondary antibodies. For example, Allophycocyanin (APC) affiniPure F(ab')2 Fragment Goat Anti-Human IgG, Fcγ

Fragment Specific (Jackson ImmunoResearch), and Rabbit anti-Goat IgG (H + L) Secondary Antibody, Alexa Fluor® 647 conjugate (Life Technologies).

5. Appropriate viability solutions, e.g., Propidium Iodide (Roche) or Fixable Viability Dyes.
6. Recombinant human R-Spondin2 (R&D systems) (*see* **Note 1**).

2.3 Surface Biotinylation Reagents

1. Mammalian expression plasmids. Myc-tagged FZD4: FZD4 wild type (WT) and FZD4 K0, in which all intracellular lysine residues of FZD4 are mutated to arginine. Note: make sure Myc tag is inserted between signal peptide and extracellular domain of FZD4 to ensure Myc tag is expressed on the extracellular side. HA-tagged Ubiquitin. Make sure antibody used for immunoprecipitation (mouse anti-Myc used here) is generated in a different species from antibody used for Western blotting (rabbit anti-Myc and rat anti-HA used here) to avoid high background (*see* **Note 2**).
2. Transfection reagent (for example, Fugene HD, Promega).
3. Any appropriate Lysis buffer. To achieve clean enrichment, perform cell lysis and pull down under denaturing conditions, for example by adding 1 % SDS to lysis buffer. Lysis buffer base can be PBS (phosphate-buffered saline), TBS (Tris Buffered Saline, 25 mM Tris–HCl, pH 7.2, 150 mM NaCl), RIPA buffer (25 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1 % NP-40, 1 % sodium deoxycholate, 0.1 % SDS), etc. Supplement lysis buffer with protease inhibitor cocktail (Roche).
4. *N*-ethylmaleimide (NEM) can be added to lysis buffer to inhibit de-ubiquitination enzymes. NEM is unstable in aqueous solution. NEM stock (1–2 M) can be prepared in ethanol (*see* **Note 3**).
5. Essential components from Cell surface protein isolation kit (Pierce): EZ-Link Sulfo-NHS-SS-Biotin for surface protein biotinylation, NeutrAvidin Agarose, Quenching Solution (can be substituted by TBS, or 100 mM Glycine in PBS), Wash Buffer (can be customized), Dithiothreitol (DTT).
6. Reagents to measure protein concentration, e.g., BCA protein assay kit (Pierce).
7. Anti-Myc antibody (mouse) conjugated sepharose beads (Cell Signaling Technology).

2.4 SDS-PAGE and Western Blot

1. SDS-PAGE Sample Buffer (e.g., 62.5 mM Tris–HCl, pH 6.8, 1 % SDS, 10 % glycerol, trace amount of bromophenol blue).
2. Tris-Glycine PAGE gel.
3. Gel running buffer compatible with your gel of choice.
4. Transfer system and Nitrocellulose membrane.

5. TBST (TBS + Tween 20) buffer.
6. Antibodies for Western blotting: anti-Myc and anti-HA.
7. ECL (enhanced chemiluminescence) reagents and film.

2.5 SNAP-tag, SNAP-tag Probe

1. Mammalian expression plasmids. SNAP-tagged FZD5. Make sure SNAP-tag is inserted between signal peptide and extracellular domain of FZD5. Expression plasmids for ZNRF3, RNF43, and empty vector.
2. Culture slides.
3. SNAP-tag substrate with fluorophore of choice (e.g., SNAP-surface549, NEB).
4. 4% paraformaldehyde in PBS.
5. Fluorescence microscope with suitable filter sets.

3 Methods

3.1 Flow Cytometry to Detect Cell Surface Wnt Receptors FZD and LRP6

The most straightforward and workhorse assay to measure cell surface Wnt receptor level is by flow cytometry for FZD and LRP6. This method measures a snapshot of cell surface protein at any given time under any given condition. For example, stem cell growth factor R-Spondin proteins function by antagonizing ZNRF3 and RNF43, therefore cells treated with R-Spondin have increased FZD and LRP6 level, and this can be measured a few hours (3–24 h) after R-Spondin treatment.

To measure cell surface level of FZD and LRP6, the following protocol may be used [8, 34].

1. Plate cells at an appropriate cell density on 6-well plates. Approximately 200,000 cells or more may be used for each staining sample.
2. After cells attach (usually the next day), perform appropriate treatment of interest (e.g., R-Spondin2 treatment at 200 ng/mL). Determine treatment length empirically. For example, for R-Spondin treatment, cell surface level FZD/LRP6 changes can be observed at 3–24 h post treatment.
3. At the end of treatment, wash cells once in PBS at room temperature.
4. Add Cell Dissociation Buffer (~0.5 mL/well) to cells to dissociate cells at room temperature for 1 min. Some cells types may take 5–10 min to dissociate. Do not leave cells at room temperature for too long to avoid changes of cell surface protein level. Other dissociation buffers such as Versene (Life Technologies) may be used (*see* **Note 4**).
5. After cells are dissociated, add to cells cold Flow Cytometry Staining Buffer. Transfer cells to 15 mL conical tubes and centrifuge at 400 × *g* for 3 min at 4 °C to wash.

6. Aspirate the supernatant and resuspend cells in 1 mL Flow Cytometry Staining Buffer. Count cells. 100,000 or more cells may be used for each staining sample.
7. [Optional] Block non-specific Fc-mediated interactions with Human Fc Receptor Binding Inhibitor for 10–20 min at 4 °C (*see* **Note 5**).
8. Add 0.1–1 million cells from each sample to a well of a 96-well round-bottom microtiter plate. Typical operation volume of 96-well plate is 100–250 μL/well. Centrifuge at 400 × *g* for 3 min at 4 °C. Flick the 96-well plate to remove supernatant.
9. Add 100 μL primary antibody at appropriate dilution to each well. Antibodies are diluted in Flow Cytometry Staining Buffer. Recommended dilution for pan-FZD antibody is 2.5 μg/mL; for anti-LRP6, use 2.5 μg/10^6 cells. Resuspend cells in primary antibody, and incubate for at least 1 h at 4 °C or on ice.
10. Wash cells by adding Flow Cytometry Staining Buffer at 200 μL/well for microtiter plates. Pellet cells by centrifugation at 400 × *g* for 3 min at 4 °C. Flick the 96-well plate to remove supernatant. Repeat once.
11. Add 100 μL appropriate fluorophore-conjugated secondary antibody to each well. Use anti-human antibody for anti-pan-FZD, and anti-goat antibody for anti-LRP6. Determine dilution empirically. For the two secondary antibodies listed in Materials, 1:200–1:1000 dilutions may be used. Resuspend cells in secondary antibody, and incubate for at least 30 min at 4 °C or on ice. Protect from light.
12. Wash cells by adding Flow Cytometry Staining Buffer. Use 200 μL/well for microtiter plates. Pellet cells by centrifugation at 400 × *g* for 3 min at 4 °C. Flick the 96-well plate to remove supernatant. Repeat once.
13. Stain samples with a compatible viability dye. For example, Propidium Iodide (1–10 μg/mL) is compatible with fluorophore Allophycocyanin (APC). Incubate for 5 min on ice before analyzing cells on a flow cytometer. Do not wash out Propidium Iodide because it must remain in the buffer during acquisition. Refer to specific product instructions if using other viability dye.
14. Acquire data with compatible Flow Cytometer, and analyze data with software platforms such as FACSDiva (BD Biosciences) or FlowJo (Treestar). Gate on total cell population. Then gate on cell singlets. Then gate out nonviable cells (Propidium Iodide positive).

3.2 FZD Ubiquitination

Because FZD surface level is controlled by its ubiquitination by E3 ligases ZNRF3 and RNF43, FZD ubiquitination level is a good indicator of ubiquitination-dependent FZD turnover process. Theoretically, under denaturing conditions, one can affinity purify

a specific protein from total cell lysate, and use SDS-PAGE and Western blotting to monitor any ubiquitin signal associated with this protein, which suggest covalent ubiquitination modification on this protein. In practice, due to the large abundance of various ubiquitinated protein species, one-step affinity-enrichment of your protein of interest may not be enough to generate clean results with ubiquitin Western blotting. To overcome this challenge, we take advantage of the fact that FZD is on the cell surface and that FZD is ubiquitinated by E3s on the cell surface. We first perform cell surface protein biotinylation and enrichment through NeutrAvidin pull-down, and then affinity purify FZD, before detecting ubiquitinated FZD by Western blotting. This two-step enrichment gives much cleaner results on ubiquitinated FZD.

To measure cell surface level of FZD ubiquitination, the following protocol may be used [8, 34].

3.2.1 Surface Labeling and Lysis

1. Plate cells at an appropriate cell density on appropriate plates. Depending on signal strength, one may need to adjust amount of starting material. One 10 cm^2 plates may be used to start with.
2. Transfect cells with Myc-tagged FZD4 and HA-tagged Ubiquitin. Myc-tagged FZD4 K0, in which all cytoplasmic lysine residues are mutated to arginine, resulting in loss of ubiquitination, may be used as a control. Determine the amount and ratio of plasmids empirically. To start with, use 1:1 ratio and 5–6 μg total plasmids for one 10 cm^2 plate. Wait for 24–36 h after transfection, before performing cell surface biotinylation. Cells may be 80–90 % confluent.
3. Remove media and wash cells with cold PBS 2–3 times. Wash thoroughly to remove serum proteins before biotinylation reaction, because Sulfo-NHS-SS-Biotin is an amine-reactive biotinylation reagent. Be careful not to detach cells.
4. Add 0.25 mg/mL Sulfo-NHS-SS-Biotin in cold PBS to cells. Incubate for 15–20 min at 4 °C with gentle agitation to ensure even coverage of cells with the labeling solution.
5. Quench the reaction by adding cold Quenching solution (e.g., TBS) to cells. Wash cells one more time with cold TBS. Gently scrape cells in 1–2 mL of TBS. Collect cells in a tube. Centrifuge at 400 × *g* for 5 min. Remove supernatant. Cell pellet can be frozen for future use.
6. Prepare lysis buffer by adding protease inhibitors and 10mM NEM to TBS or RIPA buffer with 1 % SDS, and add 0.2 mL of lysis buffer to cell pellet. Resuspend and lyse cells. Volumes noted here are only examples. Depending on the scale of the experiment, volumes can be adjusted.
7. Cell pellets resuspended in 1% SDS may be very viscous. Sonicate resuspended cell pellet briefly to solubilize. Be careful to avoid foaming.

8. Dilute lysates 10× (to 2 mL) to reach 0.1 % SDS concentration, suitable for pull-down.
9. Centrifuge cell lysate at 10,000–20,000 × *g* for 10–20 min at 4 °C. Take supernatant and transfer to new tube.
10. Measure protein concentration to make sure the same amounts of total proteins for all samples are used for subsequence steps.

3.2.2 NeutrAvidin Pull-down

1. Gently swirl NeutrAvidin Agarose beads to obtain an even suspension. Take aliquots of NeutrAvidin agarose, and wash with PBS, centrifuge at 300 × *g* for 1 min to remove supernatant, then resuspend in PBS, repeat 1–2 times. Use 30–50 μL NeutrAvidin Agarose slurry to each clarified cell lysate in appropriate tubes.
2. Rotate tubes containing lysate and NeutrAvidin beads end-over-end at 4 °C for 2–16 h.
3. Centrifuge at 300 × *g* for 1 min. Remove supernatant (unbound fraction).
4. Wash agarose beads with 1–2 mL lysis buffer (e.g., RIPA) supplemented with protease inhibitors and NEM, three times.
5. Elute bound protein by adding 0.2 mL buffer (e.g., PBS or TBS) containing 50 mM DTT (separates proteins from biotin label) and 1 % SDS (provides denaturing condition to achieve cleaner pull-down). Incubate at room temperature for 10 min.
6. Centrifuge at 1000 × *g* for 3 min. Collect supernatant.
7. Heat samples at 75 °C for 10 min. This helps further denature protein and remove nonspecific pull-down.
8. Dilute supernatant 10× (to 2 mL) to reach 0.1 % SDS concentration, suitable for pull-down. This is membrane protein fraction, which will be used as starting material for anti-Myc-immunoprecipitation. Save a small portion for Western blot analysis.

3.2.3 Myc Pull-down

1. Gently swirl mouse anti-Myc-antibody-conjugated sepharose beads. Take aliquots, wash 2–3 times with PBS. Use 30–50 μL anti-Myc-sepharose slurry to each sample.
2. Rotate tubes containing membrane protein fraction and anti-Myc-sepharose end-over-end at 4 °C for 2 h.
3. Centrifuge at 300 × *g* for 1 min. Remove supernatant (unbound fraction).
4. Wash anti-Myc-sepharose with lysis buffer (e.g., RIPA, PBS, or TBS) three times.
5. Elute with 50 μL SDS sample buffer. Heat sample at 95 °C for 5 min. Centrifuge at 10,000–20,000 × *g* for 3 min. Collect supernatant. This is membrane FZD fraction. Samples are then ready to be analyzed by Western blot for ubiquitinated-FZD.

6. For Western blot analysis, it is a good idea to include total cell lysate and membrane protein fraction samples to determine that total ubiquitination level, total FZD level, and membrane FZD level input are similar among samples to be compared. For final products (membrane FZD fraction), use part of the sample (1/10) for anti-Myc blot, to detect the amount of Myc-FZD immunoprecipitated to make sure they are similar among samples being compared. Use an antibody whose origin is different from the one used in anti-Myc-antibody beads to avoid heavy background. Use another part of the sample for anti-Ubiquitin blot, to detect amount of Ubiquitin (ubiquitinated FZD).

3.3 Surface FZD Labeling in Live Cells

While flow cytometry method measures FZD level on the cell surface, it only provides a static snapshot of how much FZD is on the cell surface. Cell surface FZD turnover is controlled by FZD ubiquitination and subsequent internalization and degradation. Therefore, it is informative to be able to monitor surface FZD and follow its trajectory over time. Here, we employ SNAP tag attached to the extracellular domain of an exogenously expressed FZD. The labeling of SNAP fusion proteins can be carried out either on fixed cells or in living cells. By attaching SNAP tag to the extracellular side of FZD, we can specifically label surface FZD with cell-impermeable substrates of SNAP tag in live cells and subsequently chase the labeled molecules. Live cell imaging permits the dynamic, real-time study of protein translocation.

Basal FZD internalization is relatively slow. However, when cells overexpress ZNRF3 or RNF43, surface FZD is constantly rapidly internalized. To monitor this process, the following protocol may be followed [9, 34].

1. Generate SNAP-FZD expressing stable cell line, with SNAP tag exposed to the extracellular space.
2. Express ZNRF3, RNF43, or empty vector in these cells. This can be achieved by either transient expression or stable expression. If transient expression is used, because transient expression may be heterogeneous, counter-staining of ZNRF3 or RNF43 would be required to identify cells with these proteins expressed.
3. Plate cells in culture slides at appropriate confluency. Allow ~24 h for cells to attach.
4. Prepare SNAP-surface549 (cell impermeable) stock solution (1 mM) in DMSO. Mix well. Prepare labeling medium by diluting SNAP-surface549 stock in complete medium to yield working concentration of 1–5 μM SNAP-tag substrates. Mix well.
5. Replace medium on cells with the labeling medium containing SNAP-tag substrates. Incubate at room temperature for 15 min to allow labeling of surface proteins.

6. Remove labeling media. Wash cells with normal media.
7. Cells can be observed with live cell fluorescence microscopy with appropriate filters. Or fixed at different time points after chasing, *see* below.
8. For time zero, cells can be immediately fixed.
9. To chase labeled surface proteins, incubate cells at 37 °C for 5 min (or other time points of interest) to allow surface proteins to internalize (*see* **Note 6**).
10. At desired time points, remove media and fix cells with 4% paraformaldehyde at room temperature for 10 min. Wash with PBS.
11. If needed, cells can also be counter-stained with other antibodies (e.g., tagged-ZNRF3 or RNF43, endosome and lysosome markers) and DNA dyes following appropriate staining procedures for simultaneous microscopic detection.
12. Mount slides with mounting media and coverslip. Examine by fluorescence microscopy.

4 Notes

1. Other R-Spondin proteins (e.g., R-Spondin1, 3, 4) can be used as well.
2. Endogenous Ubiquitin could also be detected, but signal may be much weaker than overexpressed tagged Ubiquitin.
3. Use freshly prepared NEM solution.
4. Enzyme-based dissociation methods are to be avoided in order to preserve cell surface proteins.
5. HEK293T cells do not express Fc receptor; therefore this step is optional.
6. In ZNRF3 or RNF43 expressing cells, surface FZD is internalized rapidly and can be observed with 5-min chase.

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Chapter 6

A Simple Method to Assess Abundance of the β-Catenin Signaling Pool in Cells

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Abstract

β-catenin (CTNNB1) is a dual-function cell–cell adhesion/transcriptional co-activator protein and an essential transducer of canonical Wnt signals. Although a number of established techniques and reagents are available to quantify the nuclear signaling activity of β-catenin (e.g., TCF-dependent reporter assays, nuclear accumulation of β-catenin, and generation of N-terminally hypophosphorylated β-catenin), there are cell-type and context-dependent limitations of these methods. Since the posttranscriptional stabilization of β-catenin outside of the cadherin complex appears universally required for β-catenin signaling, the following method allows for simple assessment of the cadherin-free fraction of β-catenin in cells, using a GST-tagged form of ICAT (*I*nhibitor of β-*Ca*tenin and *T*cf) as an affinity matrix. This method is more sensitive and quantitative than immunofluorescence and may be useful in studies that implicate TCF-independent signaling events.

Key words Wnt, β-catenin, ICAT, TCF (T-cell factor), GST (glutathione S transferase), Fusion proteins, Affinity precipitation, Western blot

1 Introduction

As a key subunit of the cadherin/catenin cell–cell adhesive complex, β-catenin is abundantly found at the plasma membrane and intercellular junctions (Fig. 1a, arrows). During Wnt signaling, modest fold changes in a small fraction of the total β-catenin pool are known to be biologically meaningful [1–3], but the magnitude of such changes can be challenging to quantify using immunofluorescence or detergent-free cell fractionation methods. While luciferase-based reporter assays are very sensitive, they are limited to cells that are easily transfected or infected by viruses and are unreliable in cell types, such as macrophages, that are notoriously difficult to transduce. Moreover, currently available reporters for β-catenin signaling rely exclusively on TCF-family consensus DNA binding sites, while other nuclear targets of β-catenin have been suggested [4–7]. Although nuclear fractionation techniques can be well applied to

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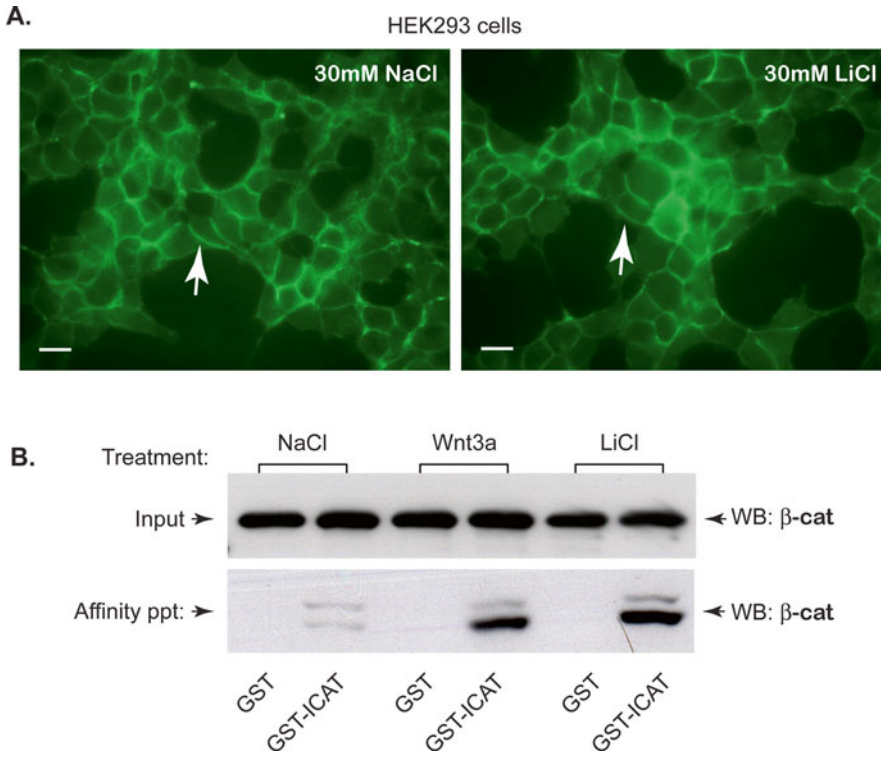

Fig. 1 Assessment of cadherin-free β-catenin by immunofluorescence and GST-ICAT affinity precipitation analysis. (**a**) Immunofluorescence images of HEK293 cells fixed and stained for β-catenin (*green*) after GSK3 inhibition by 30 mM LiCl (6 h). Media supplemented with 30 mM NaCl serves as a control. *Arrow* indicates β-catenin staining at cell–cell contacts. Scale bar = 10 μm. (**b**) Immunoblots from HEK293 cells (60 mm plate) treated 6 h with Wnt3a (R&D Biosystems), 30 mM LiCl or NaCl (control). Cells were solubilized, clarified and lysates were normalized by Bradford analysis. A small fraction was used for input lanes (*top blot*), while the remaining lysate was used for affinity precipitation by GST or GST-ICAT as described (*lower blot*)

the study of β-catenin signaling, this type of assay is more cumbersome than the method presented below, and is complicated by co-fractionation of cadherins with nuclei, greatly reducing sensitivity of the assay. Lastly, evidence that an antibody, which specifically recognizes an N-terminally unphosphorylated, "nuclear signaling" form of β-catenin [8, 9] also detects β-catenin associated with the cadherin complex [10, 11], indicates that interpreting changes in the abundance of hypophosphorylated β-catenin as a surrogate for changes in nuclear signaling should be considered carefully. Thus, there remains the need for a simple, sensitive method to assess changes in the signaling pool of β-catenin, where most of the total cellular β-catenin serves an important structural function as part of the cadherin complex.

The scale of the problem is visually depicted in Fig. 1, where stimulating β-catenin signaling in HEK293 cells with the GSK3 inhibitor, LiCl [12] or Wnt3a (not shown) more often than not fails to show visible differences in cytoplasmic/nuclear abundance

of β-catenin (Fig. 1a). However, when a parallel sample of cells are solubilized in detergent and the cadherin-free pool of β-catenin is affinity-captured through its ability to bind a GST-tagged version of ICAT (a 71 amino-acid negative regulator of β-catenin signaling [13, 14]), one can readily detect a Wnt3a- or LiCl-mediated increase in cellular β-catenin (Fig. 1b). As ICAT directly binds β-catenin [15, 16] and in a manner that is competitive with β-catenin binding to cadherins [17], the GST-tagged ICAT can be used in conjunction with glutathione-sepharose beads to perform an immunoprecipitation technique, which selectively extracts β-catenin that is not bound to cadherin at the membrane. The protein fraction enriched for soluble cadherin-free β-catenin is subsequently released from the GST beads and can then be detected for analysis as usual by Western blot using one of the many available antibodies to β-catenin (*see* Subheading 3 below).

Note that the GST-ICAT-precipitable form of β-catenin is minor relative to the total cellular pool of β-catenin, as input lanes for this pull-down show no quantifiable differences in total β-catenin (Fig. 1b). Thus, cellular β-catenin is mostly cadherin-bound, and Wnt or Wnt-like (e.g., LiCl) signals affect changes on a proportionally small fraction of total cellular β-catenin. Since β-catenin binds to cadherins with nanomolar to picomolar affinity [18] and removing β-catenin from the cadherin complex requires harsh denaturing conditions (e.g., urea [19]), the GST-ICAT affinity precipitation method largely captures the pool of β-catenin stabilized by Wnt or Wnt-like signals. While in principle, any high-affinity β-catenin-binding protein could be fused to GST and used similarly to ICAT [17], the latter's small size and structural stability make it well suited to amplification and purification from bacteria (Fig. 2), as well as storage at 4 °C for weeks.

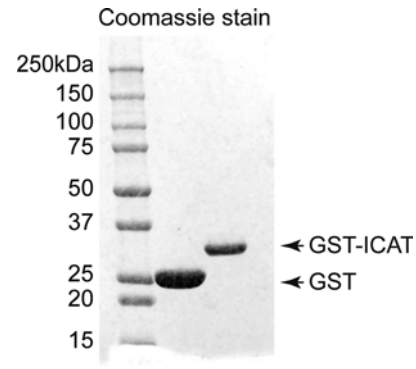

Fig. 2 Purification of GST-ICAT from bacteria. GST (control) and GST-ICAT were purified from bacteria as described and fractionated by standard SDS-PAGE. Gel was fixed in methanol/acetic acid and stained with Coomassie Brilliant Blue to visualize proteins. Molecular weight standards are shown

This method may not capture β-catenin already bound to components of the destruction complex (e.g., APC and Axin) or TCF-family members. However, a recent study suggests that stable accumulation of β-catenin within the destruction complex is unlikely [20]. Additionally, we and others (e.g., Xi He, Boston Children's Hospital, personal communication) have been unable to apply this technique to the quantification of β-catenin signaling from tissue homogenates, even using detergent washing conditions that increase stringency, for reasons that remain unknown. Thus, the method below is currently only validated for cells in culture.

2 Materials

All solutions should be prepared using ultrapure distilled deionized water (18 M Ω cm at 25 °C) and analytical grade reagents. Store and use all solutions at temperatures indicated. Dispose of waste materials according to local waste regulations.

2.1 Equipment and Disposables

1.5 mL microcentrifuge tubes, microcentrifuge tube rotator at 4 °C, refrigerated microcentrifuge, aspirator flask, pipettors and tips, cell scraper, sonicator, gel electrophoresis apparatus and power supply, electroblotter, blotting pads, nitrocellulose membrane, blot boxes, film cassette, film, film developer, heated water bath, petri dishes, 37 °C bacterial incubator, shaking incubator, 1000 mL flask, 15 and 50 mL sterile conical tubes, 17 × 100 mm sterile round-bottom tubes with cap, bacterial loop, cell spreader, 30 mL centrifuge tubes, spectrophotometer, 250 mL centrifuge bottles, refrigerated centrifuge, dialysis tubing or cartridges, stir bar, magnetic stir plate.

2.2 Reagents for GST-ICAT Fusion Protein Purification

1. pGEX GST-ICAT Plasmid DNA.
2. Glutathione sepharose beads.
3. BL21 (DE3pLysS) Competent Cells.
4. LB (Luria Broth) Miller.
5. LB Agar Miller.
6. Ampicillin (100 mg/mL stock in water).
7. SOC medium.
8. IPTG (100 mM stock in water).
9. Protease inhibitor tablets.
10. Lysis/Wash Buffer A (Non-ionic detergent buffer): 150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.5, 1% Triton X-100, 5% glycerol. For 500 mL: Add 400 mL water to 1 L beaker and add 15 mL of 5 M NaCl, 5 mL of 0.5 M EDTA, 25 mL of 1 M Tris pH 7.5 stock. Mix and adjust pH to 7.5 if necessary with HCl. Add 5 mL of 100% Triton-X100 and 25 mL of

100% glycerol. Bring final volume to 500 mL with water and transfer to storage bottle. Store at 4 °C.

11. Wash Buffer B (Non-ionic detergent "wash" buffer): same as Buffer A with the exception that the Triton X-100 concentration is reduced to 0.1% v/v (0.5 mL) (*see* **Note 1**).
12. Protease inhibitor tablets.
13. l-Glutathione.
14. Elution Buffer: 150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 8, 5% glycerol, 30 mM l-glutathione (*see* **Note 2**).
15. Dialysis Buffer: 15 mM Tris–HCl pH 7.5, 300 mM NaCl, 5% glycerol, store at 4 °C.
16. Protein Assay Reagent (BioRad Cat#500-006). Use as per manufacturer's instructions.
17. Coomassie Blue: 50 mL acetic acid (10% w/v), 150 mL methanol (30% w/v), 1.25 g Coomassie Brilliant Blue, stir to mix and filter through Whatman paper, store at room temperature.
18. Destain solution: Same as solution above without Coomassie Blue.

2.3 Reagents for GST-ICAT Pull-Down

1. GST-ICAT purified protein (*see* **Note 3**).
2. Glutathione Sepharose 4B beads.
3. PBS with calcium and magnesium.
4. Protease inhibitor tablets.
5. Lysis/Wash Buffer A (Non-ionic detergent buffer): 150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.5, 1% Triton X-100, 5% glycerol. For 500 mL: Add 400 mL water to 1 L beaker and add 15 mL of 5 M NaCl, 5 mL of 0.5 M EDTA, 25 mL of 1 M Tris pH 7.5 stock. Mix and adjust pH to 7.5 if necessary with HCl. Add 5 mL of 100% Triton-X100 and 25 mL of 100% glycerol. Bring final volume to 500 mL with water and transfer to storage bottle. Store at 4 °C.
6. Wash Buffer B (Non-ionic detergent "wash" buffer): Same as Buffer A with the exception that the Triton X-100 concentration is reduced to 0.1% v/v (0.5 mL) (*see* **Note 1**).
7. 2× SDS sample loading buffer: 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris–HCl pH 6.8. Store at room temperature.

2.4 Reagents for Western Blot

1. 10% Tris–HCl polyacrylamide gel.
2. 10× Tris-Glycine-SDS Running Buffer.
3. Protein Ladder.
4. Transfer Buffer: Add to beaker in this order: 1.6 L water, 28.8 g glycine, 6.06 g Tris Base, then stir solution to mix and

add 400 mL (20%) methanol (makes 2 L), store at room temperature.

5. TBS-Tween: Add to beaker 8 g NaCl, 0.2 g KCl, 3 g Tris Base, 800 mL water, adjust pH with HCl to 7.4, bring volume up to 1 L with water, add 1 mL/L Tween-20, store at room temperature.
6. 5% dry-milk TBS-Tween: Add to beaker 5 g dry milk per 100 mL TBS-T and stir to mix, store at 4 °C.
7. Ponceau S: Add to beaker 1 g Ponceau S (0.1% w/v), 50 mL acetic acid (5% w/v), and 950 mL water, mix with stir bar, store at room temperature.
8. C-terminal β-catenin mouse monoclonal antibody (BD Cat #610154) or N-terminal β-catenin rabbit polyclonal antibody (Millipore Cat #06-734).
9. ECL chemiluminescence reagent.

3 Methods

3.1 Transforming Protein-Competent Bacterial Cells with GST-ICAT

1. Thaw frozen BL21 cells on ice, flick tube gently to mix well.
2. Pipet 100 μL of BL21 cells to sterile pre-chilled 14 mL round-bottom tube on ice.
3. Add 2 μL (1–50 ng, volume not greater than 10 μL) pGEX GST-ICAT plasmid DNA to BL21 cells, gently flick tube to mix, and incubate on ice for 30 min.
4. Heat pulse transformation for 45 s at 42 °C in water bath.
5. Immediately place tube on ice for 2 min.
6. Add 900 μL pre-warmed SOC medium to the tube.
7. Incubate transformation at 37 °C in shaking incubator (225 rpm) for 1 h.
8. Pipet and spread 100 μL of transformation to LB/Agar plate (*see* **Note 4**).
9. Incubate inverted plate in bacterial culture incubator at 37 °C overnight (12–14 h).
10. Check plate for colonies (*see* **Note 5**), pick a single colony with a bacterial loop and inoculate 5 mL of LB containing 100 μg/mL of ampicillin, incubate culture with shaking at 37 °C overnight.
11. Inoculate 500 mL of LB containing 100 μg/mL of ampicillin in 1000 mL flask with the 5 mL overnight culture, incubate with shaking (225 rpm) for 2–3 h until OD reaches ~0.8 (*see* **Note 6**).
12. Add 500 μL (1 μL/mL) of stock IPTG to culture, continue incubation at 37 °C for additional 3 h.

13. Transfer culture to 2×250 mL centrifuge bottles and centrifuge to pellet bacteria at 1400×*g*/RCF (Floor Sorvall 3000rpm F14S rotor), 15 min, 4 °C. Decant supernatant. Bacterial pellet may be stored at –80 °C until further processing.

3.2 Purification of GST-ICAT Fusion Protein

Perform all procedures on ice with ice-cold reagents unless otherwise specified.

1. Add 10 mL Lysis Buffer A with protease inhibitor (1 tablet per 10 mL solution) to bacterial pellet and pipet up and down gently with serological pipet until pellet is fully resuspended (*see* **Note 7**).
2. Transfer suspension to a 50 mL conical tube on ice.
3. Sonicate lysate five times for 15 s each with rest period of 30 s between sonications (*see* **Note 8**).
4. Transfer lysate to 30 mL polypropylene centrifuge tube and centrifuge for 20 min at 12,000×*g*/RCF (Floor Sorvall 10000rpm SS-34 rotor) and at 4 °C.
5. Transfer cleared lysate (supernatant) to 15 mL conical centrifuge tube (*see* **Note 9**).
6. Prepare glutathione beads by washing three times in Lysis/Wash buffer A (*see* **Note 10**), and resuspend beads in Lysis/Wash buffer A to a 50:50 bead:buffer slurry.
7. Add 200 µL resuspended beads (100 µL of packed beads) to cleared lysate and tumble tube on tube rotator for 2 h at 4 °C.
8. Centrifuge tube of lysate for 5 min at 2100×*g*/RCF (Tabletop Beckman 3000rpm) and 4 °C to pellet beads.
9. Remove supernatant (*see* **Note 11**) and wash beads twice with ice-cold Lysis/Wash Buffer A (*see* **Note 12**) with centrifugations between as in **step 8**.
10. Repeat **step 9** with non-detergent Wash Buffer B.
11. Remove supernatant and add 1 mL elution buffer (*see* **Note 2**) to bead pellet, resuspend beads and transfer bead-buffer slurry to 1.5 mL microcentrifuge tube.
12. Tumble tube on tube rotator for 1 h at 4 °C.
13. Pre-equilibrate dialysis tubing or cassette in cold dialysis buffer at least 1 h before use.
14. Centrifuge tube 5 min at 11,000×*g*/RCF (Microcentrifuge 10,000rpm) and 4 °C to pellet beads.
15. Collect eluent protein supernatant and transfer to prepared dialysis tubing or cassette (*see* **Note 13**), place into beaker containing 2 L cold dialysis buffer and magnetic stir bar.
16. Place beaker on magnetic stir plate and dialyze protein overnight with stirring at 4 °C.

17. Repeat the dialysis the next morning with 2 L fresh dialysis buffer for an additional 4 h.
18. Collect eluent protein and aliquot 50 μL or smaller portions to microcentrifuge tubes (*see* **Notes 14** and **16**) and store at −80 °C.

3.3 Lysing Cells

Perform all procedures on ice with ice-cold reagents unless otherwise specified.

1. Aspirate media and rinse cells three times in PBS containing calcium and magnesium.
2. For a 100 mM culture dish of cells, add 0.5 mL Buffer A (1% TX-100) with protease inhibitor added (1 tablet per 10 mL solution), then use a cell scraper to detach the cells.
3. Transfer the cell suspension to a 1.5 mL microcentrifuge tube.
4. Sonicate cells on ice at the lowest power with pulse setting, ten pulses (*see* **Note 15**).
5. Centrifuge lysate at 21,000 × *g*/RCF (Microcentrifuge 14,000rpm) for 10 min at 4 °C.
6. Transfer supernatant to a fresh microcentrifuge tube.
7. Assay protein using standard Bradford Method to obtain concentration (*see* **Note 16**).

3.4 Preparing Glutathione-Sepharose Beads

Perform all procedures on ice with ice-cold reagents unless otherwise specified.

1. Use 50 μL of a 50:50 glutathione-sepharose bead:buffer slurry per sample. Pre-wash the required quantity of glutathione-sepharose beads to remove ethanol in 1 mL (or more) of Buffer B (0.1% TX-100), centrifuge at 21,000 × *g*/RCF (Microcentrifuge 14,000rpm) briefly to pellet the beads, aspirate the wash buffer and resuspend beads in required volume of Buffer B (*see* **Note 10**).

3.5 GST-ICAT Pull-Down

Perform all procedures on ice with ice-cold reagents unless otherwise specified.

1. Pipet 1000 μg cell lysate (*see* **Notes 17** and **18**) plus Buffer B (0.1% TX-100) up to 900 μL total volume into a 1.5 mL microcentrifuge tube.
2. Add 50 μL of glutathione-sepharose bead slurry per tube (*see* **Note 10**).
3. Add 10 μg GST-ICAT per tube (*see* **Notes 3** and **18**).
4. Tumble tubes on tube rotator at 4 °C, 2 h.
5. Centrifuge tubes at 4 °C to pellet beads at 21,000 × *g*/RCF (Microcentrifuge 14,000rpm), 2 min.

6. Aspirate supernatant (*see* **Note 19**).
7. Wash beads with 1 mL Buffer B (0.1% TX-100, centrifuge at 21,000×*g*/RCF (Microcentrifuge 14,000rpm), 2 min and aspirate off supernatant three times (*see* **Notes 12** and **20**).
8. Add 35 µL 2× SDS loading buffer to final bead pellet (*see* **Note 21**).
9. Heat protein/bead pellet at 95 °C for 5 min and centrifuge tubes to pellet beads before loading supernatant to acrylamide gel (*see* **Note 22**).

3.6 Western Blot for β-Catenin

1. Place gel into electrophoresis apparatus and add running buffer diluted to 1× in distilled deionized water, remove comb.
2. Load samples to 10% polyacrylamide-Tris-SDS gel with protein standard, run gel at 100 V until the lowest standard is at bottom of gel and/or dye front runs off.
3. Pre-soak gel, blotting pads and nitrocellulose membrane in transfer buffer for 15 min and assemble components according to the manufacturer's instructions and transblot proteins to nitrocellulose at 18 V for 1 h.
4. To view protein transfer quality, stain membrane in Ponceau S for 10 min at room temperature with rocking, rinse membrane in distilled H_2O.
5. Place membrane in blotting box and block membrane in 5% milk-TBS-T at room temperature for 1 h with rocking.
6. Remove blocking solution and replace with β-catenin antibody diluted at 1:1000 in 5% milk-TBS-T, incubate with rocking overnight at 4 °C.
7. Wash blot three times, 15 min each with TBS-T.
8. Dilute goat-anti-mouse-HRP secondary antibody 1:3000 in 5% milk-TBS-T, incubate with rocking at room temperature 1 h.
9. Wash blot three times, 15 min each with TBS-T.
10. Cover membrane with premixed ECL reagent for 1 min, wrap in plastic film and place in film cassette. Expose membrane to film up to 15 min and develop.

4 Notes

1. This buffer is used mainly for the final washes of immunoprecipitations. Note that excess non-ionic detergent binding to proteins is thought to inhibit SDS present in the sample buffer from binding/denaturing proteins for PAGE analysis.
2. Add glutathione just before use to an aliquot of non-detergent lysis buffer and adjust the pH back to 8. It will be too acidic if you do not re-pH and will not work.

3. The concentration of purified GST-ICAT protein will vary with preparation, assay for accuracy.
4. It may be helpful to also plate transformation reaction at dilutions of 1:10 and 1:100 to ensure individual colony growth. Spread solution evenly over agar surface using sterile cell spreader.
5. Agar plate with bacterial colonies may be wrapped with Parafilm and stored at 4 °C for later processing.
6. Set aside an aliquot of clean LB to serve as blank for spectrophotometer reading.
7. Allow cell pellet to thaw on ice if previously stored at –80 °C.
8. We use Branson Sonifier 250, at output level 2, constant duty cycle. Keep lysate tube on ice during sonication to prevent protein overheating and degradation.
9. A 20 μL aliquot of cleared lysate may be saved for gel analysis.
10. Glutathione-sepharose beads come as a suspension containing 20% ethanol, so calculate the amount of beads to wash to allow for 25 μL of packed beads per sample. A preparation for $n+1$ samples works best. Invert the vial of beads to resuspend before pipetting as they settle to the bottom. Resuspend packed beads in Buffer B (0.1% TX-100) to give a final slurry volume. Example: for ten samples, 350 μL beads equals ~280 μL packed beads, so wash and resuspend beads to 560 μL total slurry with buffer, and pipet 50 μL to each tube.
11. A 20 μL aliquot of unbound fraction supernatant may be saved for gel analysis.
12. Invert tube quickly 20 times to ensure adequate bead suspension and washing.
13. We have used SpectraPor dialysis membrane (MWCO 12-14,000) Cat # 132676, and Thermo Slide-A-Lyzer G2 dialysis cassette (10K MWCO) Cat # 88250.
14. Set aside two 10 μL aliquots of dialyzed protein for assay and gel analysis with BSA standard curve. Avoid multiple freeze/thaw of purified protein.
15. We use Branson Sonifier 250, at output level 1, 10% duty cycle. Keep lysate tube on ice during sonication to prevent protein overheating and degradation.
16. We used commercially available Bradford assay kit. Use as per manufacturer's instructions with BSA standard.
17. GST-ICAT pull-down may also be done proportionally without assay, saving 10% of input lysate to run on a duplicate gel.
18. The amount of protein needed to pull-down detectable β-catenin may vary greatly between cell types, so you may require much less than this. We have successfully used as little as 200 μg pro-

tein. The volume of GST-ICAT purified protein added will depend on its concentration. Use GST-ICAT at a minimum of 10% in proportion to the total quantity of protein.

19. Use an aspirator flask with the vacuum turned down to a low level, with a glass Pasteur pipet covered by a 200 µL plastic pipet tip so that the supernatant can be removed gently and slowly without losing beads. The 200 µL pipet tip can be quickly changed between samples. You may prefer to do this manually with a pipettor. The supernatant can be saved for future evaluation by gel electrophoresis of unbound fraction.
20. Invert tubes ten times to make sure beads have been resuspended in buffer and washed well before centrifuging.
21. Bead pellet may be stored at –20 °C until gel run.
22. Heating in sample loading buffer will release the protein from glutathione beads. Set pipettor to pull off volume of sample loading buffer added (35 µL). Do not load beads to gel wells.

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Chapter 7

Wnt-Dependent Control of Cell Polarity in Cultured Cells

Kristin B. Runkle and Eric S. Witze

Abstract

The secreted ligand Wnt5a regulates cell polarity and polarized cell movement during development by signaling through the poorly defined noncanonical Wnt pathway. Cell polarity regulates most aspects of cell behavior including the organization of apical/basolateral membrane domains of epithelial cells, polarized cell divisions along a directional plane, and front rear polarity during cell migration. These characteristics of cell polarity allow coordinated cell movements required for tissue formation and organogenesis during embryonic development. Genetic model organisms have been used to identify multiple signaling pathways including Wnt5a that are required to establish cell polarity and regulate polarized cell behavior. However, the downstream signaling events that regulate these complex cellular processes are still poorly understood. The methods below describe assays to study Wnt5a-induced cell polarity in cultured cells, which may facilitate our understanding of these complex signaling pathways.

Key words Wnt5a, Noncanonical Wnt, Cell polarity, Cell adhesion molecules, Cultured cells

1 Introduction

Inhibition of the noncanonical Wnt signaling pathway in the developmental model organisms, *Xenopus* and zebrafish, results in shortened anterior/posterior body axis due to failed polarized cell movements toward the midline of the embryo that drive axis elongation (convergent extension) [1]. Mouse embryos depleted of Wnt5a present a similar phenotype consisting of a shortened anterior–posterior axis, an underdeveloped posterior and shortened limbs with underdeveloped or missing distal structures [2]. In the Wnt5a knockout mouse, cell proliferation and cell survival appear normal and therefore the cause of the phenotype was initially unknown. Subsequent studies revealed that multiple polarized cell behaviors are disrupted in the limb of the Wnt5a knockout mouse. Live imaging studies showed Wnt5a-dependent directional migration of mesoderm cells into the developing limb bud. Furthermore, staining of the limb revealed that the Golgi apparatus orients distally in the limb of wild-type embryos whereas in Wnt5a −/− embryos

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the Golgi apparatus orients randomly [3]. The polarized directional orientation of the Golgi parallels the directionality of the cell division plane observed by live cell imaging. These Wnt5a-dependent polarized cell behaviors are thought to contribute to limb elongation and morphogenesis.

Within the developing embryo the concentration of Wnt5a and the influence of other soluble ligands are mostly unknown making it challenging to determine the molecular signaling events that regulate polarized cell processes. Our mechanistic understanding of other signaling pathways such as the MAP kinase pathway has been aided by tractable biochemical systems using cultured mammalian cells to acutely activate the pathway of interest. The signaling events downstream of Wnt5a could similarly benefit from a cultured cell system. Wnt5a is upregulated in melanoma cell lines that exhibit invasive behavior and immunohistochemistry staining of melanoma biopsies reveal high levels of Wnt5a in late-stage metastatic tumors compared to early nonmetastatic tumors [4]. Ectopic expression of Wnt5a also increases invasive behavior in melanoma cells that express low endogenous levels of Wnt5a [4]. This led us to utilize cultured melanoma cells to specifically study the regulation of cell polarity by Wnt5a signaling.

The use of Wnt5a-responsive cells has allowed the discovery of new signaling events that regulate polarized cell behavior in response to Wnt5a. The ability to activate Wnt5a signaling in cultured melanoma cells makes it possible to study dynamic polarized cell behavior in response to known amounts of active Wnt5a uniformly administered to cells for known lengths of time. Compared to other cell lines the melanoma cell line WM239A expresses relatively high levels of Wnt5a without secreting detectable levels into the medium [5]. These cells are highly responsive to stimulation with purified Wnt5a resulting in activation of protein kinase C and RhoB as well as inducing the formation of a polarized cytoskeletal structure [6]. The cells are also responsive to both the noncanonical and the canonical Wnt pathways. Stimulation of WM239A cells with purified Wnt3a activates a beta-catenin responsive luciferase reporter [6]. This method is amenable to studying the function of proteins on Wnt5a-induced cell polarity via transient or stable expression or by inhibition with shRNA in WM239A cells.

2 Materials

2.1 Measuring Wnt5a-Induced Polarization of Cells

1. Circular cover slips #1 thickness, 18 mm diameter (sterile).
2. 12-well tissue culture plate.
3. RPMI media.
4. Glutamine.
5. Fetal bovine serum.

6. Trypsin.
7. TBST (Tris-buffered salt solution with Tween): 0.3 M NaCl, 20 mM Tris-Cl, pH 8.0, 0.05% (v/v) 8. Tween-20.
8. Microscope slides.
9. Mounting media with DAPI.
10. Purified Wnt5a.
11. Primary antibodies to proteins of interest.
12. Fluorescently conjugated secondary antibodies.
13. Bovine serum albumin.
14. 4% formaldehyde, neutral buffered.
15. Triton X-100.

2.2 Live Cell Imaging of Wnt5a-Induced Polarity

1. RPMI media.
2. Glutamine.
3. Fetal bovine serum.
4. Trypsin.
5. 0.5 M HEPES, pH 7.4.
6. Hanks balanced salt solution.
7. Glass bottomed tissue culture dish.

2.3 Wnt5a-Mediated Polarization in Response to a Directional Cue

1. RPMI media.
2. Glutamine.
3. Fetal bovine serum.
4. Trypsin.
5. Circular cover slips #1 thickness, 18 mm diameter (sterile).
6. 12-well tissue culture plate.
7. Dunn chemotaxis chamber (Hawksley).
8. Purified SDF1/CXCL12.
9. Microscope slides.
10. Mounting media with DAPI.

3 Methods

3.1 Measuring Wnt5a-Induced Polarization of Cells

The ability to stimulate cultured cells with known amounts of Wnt5a makes it possible to study both the molecular mechanisms of signaling pathways downstream of Wnt5a as well as polarized cell behavior through immunofluorescence staining or live cell imaging. Uniform treatment of WM239A melanoma cells with 150 ng/mL of purified Wnt5a for 30 min induces the asymmetric localization of cell adhesion molecules like MCAM and CD44 to

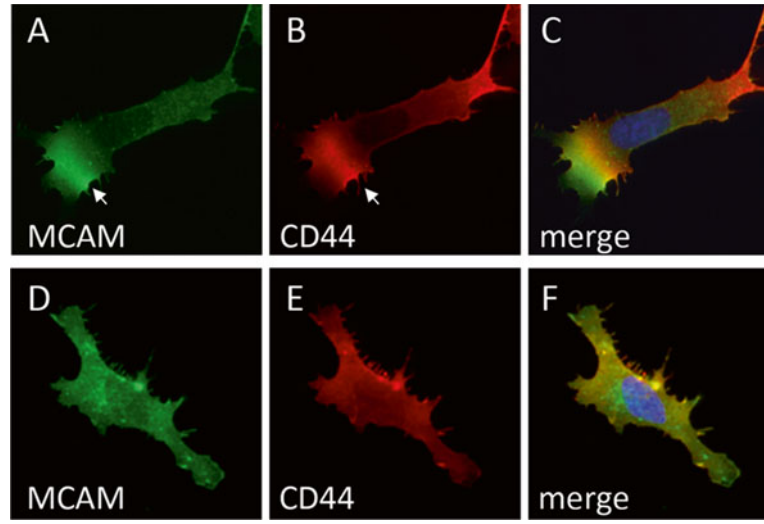

Fig. 1 WM239A cells were treated with purified Wnt5a or control buffer for 30 min, fixed and stained for MCAM and CD44. Wnt5a-treated cells show asymmetrically localized MCAM and CD44 at one end of the cell (*arrow*) (**a**–**c**). Without Wnt5a treatment the localization of MCAM and CD44 is uniformly distributed throughout the cell (**d**–**f**)

one end of the cell (Fig. 1a, b) [6, 7]. Wnt5a also induces the polarized organization of the actin cytoskeleton, myosin, and the Wnt receptor Frizzled3 [6]. The induction of polarized localization of cell adhesion molecules and cytoskeletal components occurs in the absence of any apparent external polarizing cues. The use of cultured cells allows pharmacologic inhibition of signaling components or inhibition by shRNA for unbiased or systematic screening using asymmetric protein localization as a read out. These methods were previously used to demonstrate that the asymmetric localization of MCAM requires known noncanonical Wnt components PKC and Dvl2 [6]. Biochemical analysis of Wnt5a-stimulated melanoma cells also revealed that the asymmetric localization of MCAM is mediated through a reversible lipid protein modification regulated by Wnt5a [7].

1. Place 1 coverslip/well in a 12-well plate and UV sterilize in a tissue culture hood for 15 min.
2. Remove media from the melanoma cell culture dish, wash 2 times with PBS and add trypsin for 1–2 min. Resuspend the cells at a density of 1×10^6 cells/mL and add 0.5 mL of cells to each well. Culture at 37 °C for 24 h.
3. Wash the coverslips once with 1 mL PBS and then incubate overnight in 1 mL of serum-free RPMI.

4. Remove media and add fresh serum-free RPMI containing approximately 150 ng/mL of Wnt5a or control purification buffer. Incubate at 37 °C for 30 min.
5. Fix the cells in 500 μL of 4% formaldehyde for 5 min at room temperature. Permeabilize in 500 μL of 0.1% Triton X-100 in TBST for 7 min, and then block in 5% BSA in TBST for 1 h.
6. Incubate overnight at 4 °C with the antibody to the protein of interest.
7. Wash 3 times with TBST for 10 min/wash.
8. Add fluorescently conjugated secondary antibody (2 μg/mL) in 5% BSA in TBST and incubate for 1 h at room temperature.
9. Wash 3 times with TBST for 10 min/wash.
10. Mount coverslips on microscope slides with 10 μL of preferred DAPI containing mounting media (i.e. Fluoromount G).
11. Image on a fluorescent microscope.

3.2 Live Cell Imaging of Wnt5a-Induced Polarity

The asymmetric localization of MCAM in fixed cells is static and is insufficient to capture the formation of the asymmetry. MCAM fused to GFP also localizes asymmetrically in response to Wnt5a allowing visualization of MCAM localization over time. Live imaging reveals dynamic movement of MCAM to one side of the cell followed by the transfer of MCAM to the opposite side. Translocation of MCAM across the cell occurs within approximately 20 min of Wnt5a stimulation in WM239A cells.

1. Grow cells in RPMI media supplemented with 10% FBS in glass-bottomed imaging dishes.
2. Wash the coverslips once with 1 mL PBS and incubate overnight in serum-free RPMI media.
3. Set the stage temperature to 35 °C. Wash cells in 1× HBSS and then add 2 mL of HBSS supplemented with 25 mM HEPES, pH 7.4. Capture images every 30–60 s on the GFP and bright field channels.
4. After 15 min of imaging, remove the media and add fresh media containing 150 ng/mL Wnt5a. Continue imaging every 30–60 s for an additional 15 min.

3.3 Wnt5a-Mediated Polarization in Response to a Directional Cue

The Golgi apparatus is often localized at the front of migrating cells in many cell types. Wnt5a stimulation by itself increases the percentage of cells with the Golgi distributed around the nucleus as opposed to localization on the opposite or same side as the asymmetrically localized MCAM (Fig. 2c). The Dunn chemotaxis chamber is used to expose cells to a linear gradient of CXCL12 with a uniform concentration of Wnt5a [8]. In the Dunn chamber Wnt5a-treated melanoma cells respond to the linear gradient of CXCL12 by orienting

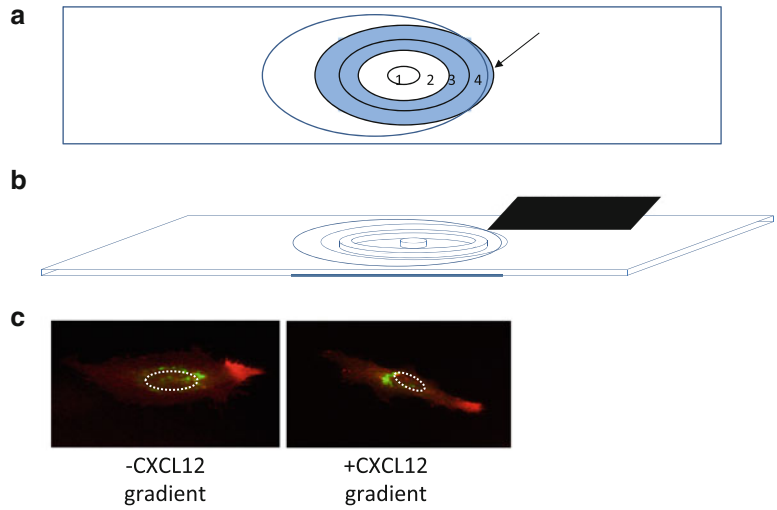

Fig. 2 Setting up the Dunn chemotaxis chamber. The Dunn chemotaxis chamber consists of an inner circular chamber "2" and an outer circular chamber "4" separated by a raised bridge "3". Both chambers are filled with media containing Wnt5a and a coverslip seeded with cells is placed over both chambers leaving a small gap on the edge of the outer chamber. A paper towel or filter paper is used to wick out the fluid in the outer chamber. A pipet with a gel loading tip is then used to fill the outer chamber with media containing Wnt5a and the chemoattractant. This establishes a gradient of the chemoattractant while maintaining a uniform concentration of Wnt5a

the Golgi apparatus to the side of the cell opposite of the asymmetrically localized MCAM (Fig. 2d) [6]. Subsequent studies revealed that Wnt5a is required for the coordinated orientation of the Golgi apparatus in cells of the mouse limb [3]. This simplified cell environment demonstrates that Wnt5a is required for cells to respond to a secondary directional signal, CXCL12.

1. Seed 500,000 cells onto 18 mm round coverslips in a 12-well plate and incubate at 37 °C for 24 h.
2. Wash once with 1 mL PBS and incubate with 1 mL of serum-free RPMI for 15 h.
3. Fill both the inner and outer chambers of the Dunn chemotaxis chamber (Hawksley) with serum-free RPMI containing either Wnt5a (150 ng/mL) or control buffer.
4. Place coverslips over both chambers leaving a small gap at the edge of the outer chamber (Fig. 2a, arrow).
5. Using a folded paper towel or filter paper wick the media from the outer chamber (region 4) (Fig. 2b).
6. Using a gel loading tip add serum-free RPMI containing CXCL12 (20 ng/mL)+Wnt5a (150 ng/mL) to the outer chamber (region 4).

7. Place the chamber in a 15 cm dish with a wet paper towel. Incubate the slide for 1 h at 37 °C.
8. Mark the center of the chamber on the coverslip with a permanent marker.
9. Remove coverslip and fix in formalin for 5 min.
10. Block in 5 % BSA in TBST for 1 h.
11. Incubate with antibodies to the rabbit 58-kD Golgi protein and mouse anti-MCAM diluted in 5 % BSA in TBST overnight at 4 °C.
12. Align the mark made on the coverslip with the center of the chamber. Using a pen, mark the back of the slide to show the area of the Dunn Chamber where the linear gradient formed. Cells lying in the area of the gradient of CXCL12 (region 3 in Fig. 2b) that have asymmetric MCAM staining are scored for Golgi orientation relative to the position of MCAM and to the CXCL12 source.

4 Notes

1. Wnt5a is purified in house using the protocol established by Willert et al. Nature 2003 [9]. The concentration of the purified Wnt5a is estimated by the band intensity on a coomassie or silver stained gel. In our hands the commercially available Wnt5a does not induce the asymmetry.
2. The yield of the purified ligand varies between purifications. Typically most batches of Wnt5a are diluted between 1:200 and 1:500 in serum-free media. The ligand can be titrated to find the minimum amount of Wnt5a required to induce the asymmetric MCAM localization. Typically, we observe around 15–20 % of the cells with asymmetric MCAM compared to around 5 % with control purification buffer alone.
3. The cover glasses are wiped with 70 % ethanol to remove any oil and glass dust. The cover glasses are then spread on a 15 cm dish and exposed to a UV lamp for at least 15 min in a laminar airflow hood. Alternatively, the cleaned cover glasses can be autoclaved.
4. The Wnt5a-induced asymmetric localization of MCAM is sensitive to media and substrate conditions. The asymmetric localization is inhibited by penicillin and streptomycin or insufficient fresh glutamine in the media. Glutamine (2 mM) is added to the media before cultivation, during serum deprivation and during Wnt5a stimulation. Poly-lysine-coated coverslips also inhibit the asymmetry.

5. WM239A cells are transfected using TransIT-LT1 Transfection Reagent as follows. Combine 200 μL of OptiMEM serum-free media, 2 μg of plasmid DNA and 6 μL of TransIT-LT1 transfection reagent. Incubate at room temperature for 30 min then add the transfection mixture dropwise to WM239A cells plated onto 6 cm dishes in RMPI + 10 % FBS. Incubate the cells at 37 °C for 24 h before plating onto cover glass or glass-bottomed imaging dishes. We have found transfecting onto glass substrates results in higher cell death than when transfected on plastic.

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Chapter 8

The Use of Chick Embryos to Study Wnt Activity Gradients

Lisa M. Galli, Tiffany Barnes, and Laura W. Burrus

Abstract

The chick spinal cord provides a valuable model for assessing Wnt signaling activity. Loss or gain of function constructs that are transfected by electroporation can be directed to a single side of the spinal cord, thus leaving the contralateral side as an internal control. Here, we describe a method for measuring Wnt signaling via the use of BAT-Gal, a β-catenin dependent Wnt reporter.

Key words WNT1, WNT3A, β-catenin, BAT-Gal, Electroporation, Gradient, Spinal cord, Chick, In ovo

1 Introduction

Wnts are secreted morphogens that act in a concentration-dependent manner [1, 2]. Disruption of Wnt gradients is implicated in developmental defects and in cancer [1, 2]. Thus, understanding the formation of vertebrate Wnt gradients is of great interest. We use the developing chick spinal cord as a biologically relevant three-dimensional tissue for the study of vertebrate Wnt gradient formation. Proper development of the spinal cord requires a dorsal to ventral gradient of WNT1 and WNT3A for outgrowth and patterning [3–5]. As it has not yet been possible to directly detect any Wnt protein gradient in vertebrates, researchers currently rely on visualization of the Wnt activity gradient [6]. Our lab has developed a number of protocols to assay for WNT1/3A activity and gradient formation [6–9]. These include measuring (1) the relative percent of Islet-1 positive DI3 interneurons and motor neurons [8, 10], (2) the relative percentage of cells in late G2/M phase as indicated by immunostaining with anti-phosphohistone H3 [6–9], (3) the expression domain of FZD10 [7, 11], and (4) activation of the β-catenin dependent BAT-Gal reporter [6, 12]. All of these assays take advantage of our ability to electroporate gain and loss of function constructs into the spinal

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cord after neurulation. However, the BAT-Gal reporter represents the most specific readout of β-catenin dependent Wnt signaling. A detailed protocol for electroporating DNA constructs, such as a WNT1 expression construct and the BAT-Gal reporter, into the spinal cord is presented below.

2 Materials

Prepare all solutions with ultrapure water. In our lab, we use distilled deionized water. These experiments require fertile eggs, purified DNA, a pneumatic injector, a micromanipulator, platinum electrodes, and a square pulse electroporator. Follow all waste disposal regulations when disposing of waste materials. Note that formaldehyde (the monomeric version of paraformaldehyde) and sodium azide are hazardous. Please check the Safety Data Sheet and handle with care. Disposal should be carried out according to institutional protocols.

2.1 Electroporation

1. Fertile chicken eggs are purchased from a local hatchery (Petaluma Farms) (*see* **Note 1**). If you are in a real pinch, it is possible to use fertile eggs from the grocery story so long as you buy them on the day they are delivered to the store. We find that storing eggs at room temperature is fine in the cool environment of San Francisco. In regions with warmer temperatures, it may be necessary to store them at 4 °C. It is best to use eggs within 1 week. It is not necessary to use pathogen-free eggs.
2. Tyrode's solution can be commercially purchased or prepared in the lab. To prepare Tyrode's, weigh 265 mg $CaCl_2{\cdot}2H_2O$, 214 mg $MgCl_2{\cdot}6H_2O$, 200 mg KCl, 1 g $NaHCO_3$, 8 g NaCl, 50 mg NaH_2PO_4 (anhydrous), 1 g d-glucose (dextrose). Add water to 800 ml and then adjust pH to 7.4 using 6 M HCl. Increase volume to 1 L, recheck pH, and adjust if necessary. Sterilize by filtration through a 0.2 μm filter; do not autoclave.
3. 5× Phosphate buffered saline, pH 7.4 (PBS): Weigh 40 g NaCl, 1 g KCl, 13.6 g $Na_2HPO_4{\cdot}7H_2O$, and 1.2 g KH_2PO_4. Add water up to a volume of 800 ml. Adjust pH with 7.4 using 5 M NaOH or 6 M HCl. Increase volume to 1 L, recheck pH and adjust if necessary. Sterilize by autoclaving. 5× PBS can be diluted to 1× PBS with water.
4. Pelikan India Ink (MisterArt): Dilute 1 ml of India ink to 50 ml using Tyrode's solution.
5. 1× Penicillin/Streptomycin (P/S): Dilute 0.5 ml of 100× P/S to a final volume of 50 ml using Tyrode's solution.
6. 6× Fast Green FCF (Fisher) is prepared at a concentration of 2 mg/ml in 1× PBS. Weigh 2 mg of Fast Green and dissolve in 1 ml 1× PBS.

7. 2% Carboxymethylcellulose sodium salt: Weigh 200 mg carboxymethylcellulose. Add water to a volume of 10 ml. Mix and store at room temperature.
8. 2:1 solution of Fast Green Dye and carboxymethylcellulose. Add 666 µl of 6× Fast Green Dye to 333 µl of 2% carboxymethylcellulose. Store at room temperature.
9. Purified DNA: The BAT-Gal reporter is used in combination with gain or loss of function constructs to specifically measure β-catenin dependent Wnt signaling (Fig. 1a, [12]). pCIG is used the base vector for most of our gain of function studies. In this vector, a β-actin promoter with a CMV enhancer drives the expression of a bicistronic transcript encoding a protein of choice and a nuclear variant of eGFP (Fig. 1b, [4]). A variant of psiSTRIKE in which hmgGFP has been replaced with a nuclear variant of eGFP is used for our loss-of-function studies (*see* **Note 2**).
10. DNA constructs can be purified using any of a variety of kits. We use a midi prep kit to purify DNA from 50 ml cultures using the instructions provided with the kit. Briefly, DNA pellets are resuspended in 30 µl of H_2O and incubated overnight at RT. The following day, the DNA solution is centrifuged at 15,000 × *g* before transferring supernatant to a new tube. We find that this extra step helps sediment any particulate matter and thus, prevents needles from clogging. The concentration is determined by spectrophotometry at 260 and 280 nm. Ideally, DNA should be between 4 and 9 mg/ml. DNA is stored in

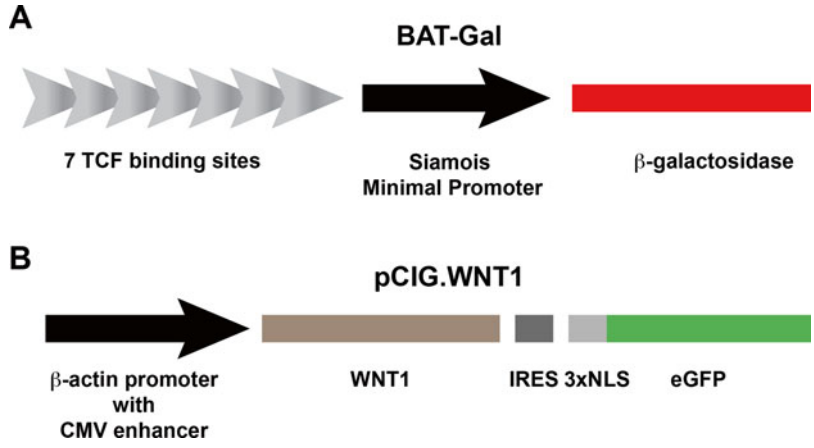

Fig. 1 Constructs used to detect Wnt signaling in the chick spinal cord. (**a**) 7 TCF sites positioned upstream of a minimal Siamois promoter are used to drive the expression of β-galactosidase [12]. (**b**) BAT-Gal can be co-electroporated in combination with any of a variety of loss or gain of function expression constructs. Here we depict the salient features of pCIG.WNT1 [4, 6]. Briefly, a β-actin promoter (with a CMV enhancer) is used to drive the expression of a bicistronic transcript encoding chick WNT1 and a nuclear variant of GFP (NLS = nuclear localization sequence). An internal ribosome entry site (IRES) allows the two proteins to be translated separately

2.5 μl aliquots at −80 °C. Immediately prior to electroporations, mix 1 μl of the 2:1 solution of 2% methylcellulose and 6× Fast Green (2 mg/ml in PBS) with 2.5 μl purified DNA.

11. Dissection tools—small dissection scissors and Student Dumont #5 forceps.
12. Syringes and needles—one 10 ml syringe with a 25 gauge needle; one 10 ml syringe with an 18 gauge needle.
13. Tungsten dissecting needle—We sharpen our dissecting needles using a modification of a technique first reported in 1965 [13]. A short piece of 0.13 mm diameter tungsten wire is connected to a toothpick and then electrolytically sharpened. To sharpen this blade, first connect the positive lead to the tungsten wire and then immerse it in 0.5 M KOH. The negative lead should be connected to a short copper wire, which is also submerged in the KOH solution. We gradually turn up the voltage to 5–10 V to sharpen the tungsten wire. Mild bubbling is to be expected, but really vigorous bubbling should be avoided.
14. Injection needles—We use a Sutter P-97 Micropipette needle puller to make needles for the injection of DNA into the spinal cord. We use thin wall borosilicate tubing with a filament and a 1.0 mm outside diameter (Sutter). The settings are variable depending on the filament in use. However, our typical settings are 75 Pull, 200 Velocity, and 80 Time. The "Heat" setting is determined by first running a ramp test. This program will generate a needle with a closed tip. The tip can be broken with forceps after loading with DNA. Needles can be pulled well in advance of an experiment and stored in a 150 mm petri dish with two strips of clay in the bottom. We use microloader tips to backfill needles with DNA solution.
15. Electrodes—While it is possible to purchase electrodes from commercial vendors, such as the Protech platinum z-shaped blunt needle electrode (CUY611 or 613) or BTX Genepaddles, it is much more economical to make your own. We construct our electrodes using a slight modification of the protocol outlined by Krull [14]. After pre-warming the soldering iron, we use lead-free solder to connect a 4–5 cm piece of platinum wire (Fig. 2c, 0.01 in. diameter platinum rod; A-M Systems, cat #711000) to the pin stamped brass (Fig. 2b, Digikey). It is important to use the minimal amount of solder. This process should be repeated to make a second electrode (Fig. 2d). Next, we solder red stranded 22 gauge wire (Newark Electronics) onto one gold-plated jack/socket (Newark Electronics) and black stranded 22 gauge wire (Fig. 2a; Newark Electronics) onto a second gold-plated jack/socket. The length of the red and black wire should be sufficient to comfortably span the distance from the electroporator to the egg. We then clip off

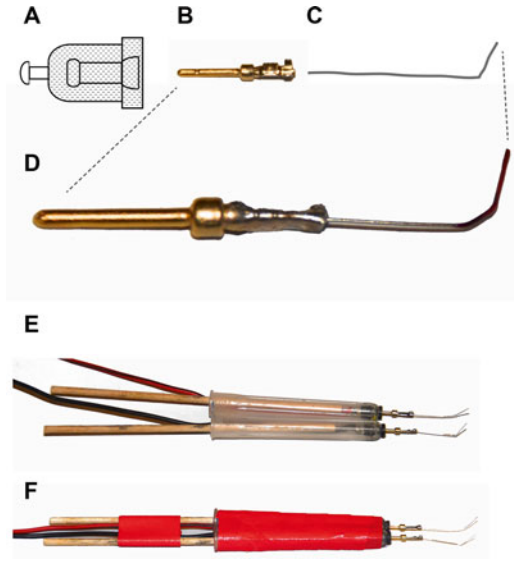

Fig. 2 Construction of electrodes. (**a**) Gold plated jack/socket. (**b**) Brass stamped pin. (**c**) Platinum wire (it can be bent before or after soldering). (**d**) A magnified image of the platinum wire that has been soldered to the brass stamped pin. The gold-plated jack/sockets are then soldered to the *black* and *red wires* (not shown). (**e**) This image shows how the gold plated jack/socket (*black*) is inserted into the cut end of a needle cover. A wooden handle is made by inserting the wood from a long cotton swab (with the cotton removed) into the opposite end of the needle cover. (**f**) The entire assembly is taped together with lab tape (*red*) to stabilize the configuration

the closed end of two needle covers (from 25 gauge needles). Take the free end of the red wire and pass it through the newly clipped end of the needle cover (from right to left in Fig. 2e) to secure the gold plated/socket in the end of the needle cover (only the lip of the gold plated/socket should remain outside the needle cover). This step is repeated for the black wire. We then make a handle by inserting two long sticks (from long cotton swabs) into the back end of the needle cover (Fig. 2e). We then use tape to seal the whole assembly (Fig. 2f). The red and black wires are then connected to a banana connectors with male ends (available at any electronics store). The soldered pin stamped brass/electrode assemblies should then be inserted into the gold plated jack/sockets. Use forceps to bend the ends of the electrodes (approximately 0.5 cm from the end); the electrodes are now ready for use.

16. Micromanipulator: We use a MM33 right micromanipulator (Sutter).
17. Injector: PV820 Pneumatic PicoPump with foot pedal World Precision Instruments hooked to a gas N_2 tank and needle holder is fitted with the green gasket (holds 1.0 mm needles) (*see* **Note 3**).

18. Electroporation system: There are many available square wave electroporation systems on the market. We use the ECM 830 Square Wave Electroporation system made by BTX (*see* **Notes 3** and **4**).

2.2 Embedding and Sectioning

1. 4% paraformaldehyde: 30 ml 1× PBS is added to 10 ml 16% paraformaldehyde and cooled on ice.
2. Embedding solutions: 15% sucrose in 1× PBS containing 0.01% NaN_3, 30% sucrose in 1× PBS containing 0.01% NaN_3, 60% sucrose in PBS:OCT (1:1) containing 0.01% NaN_3 and Tissue Tek OCT (VWR).
3. Histology molds: Peel-A-Way Disposable Embedding Molds Truncated 22 × 22 mm (VWR).
4. Glass slides and coverslips: VWR Superfrost Plus Microslide (VWR), Fisherfinest Premium Cover Glasses No. 1 (Fisher).
5. Mounting Solution: SlowFade Gold Antifade (Molecular Probes).
6. Cryostat: We use a Leica CM 1950 cryostat.

2.3 Immunostaining

1. Blocking buffer: 3% lamb serum in 1× PBS containing 0.1% Tween.
2. Antibodies—anti-β-galactosidase J1E7 (Developmental Studies Hybridoma Bank, supernatant diluted 1/100 in blocking buffer) and goat anti-mouse IgG(H + L)-Cy3 (Jackson ImmunoResearch, diluted 1/200 in blocking buffer).

3 Methods

All procedures should be carried out at room temperature unless otherwise specified.

3.1 Incubating Eggs

Eggs should be incubated on their side in a humidified egg chamber 39 °C. If available, it is preferable to use an incubator with egg turning capability as this will increase the viability of the embryos.

The Hamburger and Hamilton staging guide provides an estimate of the time needed for embryos to develop to certain stages [15]. Note that some seasonal variability is not unusual. Depending on the experiment, we electroporate embryos ranging from HH stage 12 to 17.

3.2 Loading Needles

1. Long microloader tips are used to backload needles with 3.5 µl of the DNA solution containing fast green dye and CM-cellulose.
2. After loading, the tip of the needle is clipped off using forceps.

3.3 Windowing Eggs

1. After the appropriate incubation time, eggs are removed from the incubator.
2. Small petri dishes can be modified with colored lab tape to make a suitable egg holder for injections.
3. Before windowing eggs, a small piece of packing tape should be adhered to the side of the egg that is facing up (Fig. 3a) (*see* **Note 5**). We use an 18 gauge needle to remove 5–7 ml of egg white from the large end of the egg. After gently penetrating the eggshell with the scissors, the needle should be angled down, bevel side down, to avoid puncturing the yolk.
4. Small dissection scissors are then used to cut a small circle in the eggshell on the taped side of the egg (Fig. 3b). The cut-out piece of shell can be discarded.

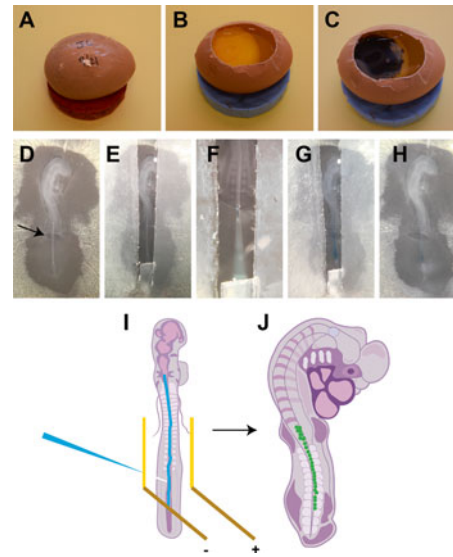

Fig. 3 Electroporation procedure: Here, we outline the basic steps of the electroporation. (**a**) The top of the egg is covered with packing tape to prevent the shell from cracking while cutting the window. A windowed egg is shown before (**b**) and after (**c**) the injection of India ink underneath the embryo. (**d**) An *arrow* demarcates the position of a small hole torn in the vitelline membrane. (**e**) The embryo is overlaid with a piece of Parafilm such that the spinal cord remains exposed. (**f**) The needle is oriented parallel to the spinal cord of the embryo and inserted into the spinal cord at the level of the segmented plate. (**g**) After injection of the DNA, the fast green dye is visible in the lumen of the spinal cord and extends up to at least the level of the heart. (**h**) After electroporating the embryo, the Parafilm is removed and the embryo is allowed to recover. In (**i**) and (**j**), we show a graphic of the overall electroporation procedure. (**i**) DNA (*blue*) is injected prior to the electroporation. (**j**) After 16–48 h of incubation, GFP is expressed on the right side of the spinal cord

3.4 Injecting and Electroporating

1. India ink diluted in 1× Tyrode's is then injected under the embryo using a 25 gauge needle connected to a 10 ml syringe. The needle should be inserted just outside the blood islands at a nearly horizontal angle, bevel side up. Ink should be injected until the embryo is clearly visible (Fig. 3c).
2. Stage embryos according to Hamburger and Hamilton [15].
3. We use an electrolytically sharpened tungsten needle to make a small tear in the vitelline membrane at the level of the segmental plate or most recently formed somites (Fig. 3d).
4. Parafilm cut in the shape of a "U" is first dipped in 1× P/S in Tyrode's solution and then placed over the embryo, leaving the spinal cord exposed. This protects the embryo from being scorched during the electroporation (Fig. 3d).
5. The embryo should be aligned such that the needle is oriented parallel with the spinal cord of the embryo (Fig. 3f).
6. The needle should be inserted into the posterior spinal cord where the vitelline membrane was torn (Fig. 3f). DNA should be injected until the entire lumen is full of DNA. The fast green dye in the DNA solution ensures that it is easily visible. The spinal cord should be filled from the posterior most end up to at least the level of the heart (*see* Fig. 3g). Once sufficiently full, retract the needle completely and rotate the egg 90° so that the tail of the embryo is orientated to the bottom of the stage.
7. Two to four drops of 1× P/S in Tryode's solution is added on top of the embryo and Parafilm.
8. Electrodes are then quickly placed on either side of the embryo on top of the Parafilm (Fig. 3i, *see* **Notes 6** and **7**). We use four pulses at voltages between 48 and 55 with a 50 ms pulse length (*see* **Note 8**). The voltage used depends on the stage of embryos and the electrodes used. Smaller embryos (HH stages 12–14) require lower voltage while larger embryos (HH stages 15–17) require higher voltage. We aim to use a voltage at which the majority of the embryos are successfully transfected and up to 25 % are dead (*see* **Note 9**).
9. After electroporation, the embryos are overlaid with an additional 1 ml of 1× P/S before removing the Parafilm (Fig. 3h) and resealing the eggs with packing tape. Eggs should be returned to the incubator with no turning (*see* **Note 10**).

3.5 Harvesting and Embedding Embryos

1. Between 16 and 48 h later, embryos are screened for GFP fluorescence using an Olympus SZX12 dissecting scope outfitted with a UV light source (in ovo). Successfully transfected embryos are then harvested for further analysis (Fig. 3j) (*see* **Note 10**). Note that embryos must be allowed to develop to at least HH stage 21 to effectively visualize the Wnt gradient

utilizing the Islet-1 or the phosphohistone H3 method for measurement. For the activation of the BAT-gal reporter HH stage 18 is sufficient.

2. When harvesting embryos, the extraembryonic tissue should be trimmed from embryo. However, it is not necessary to remove all of the tissue as it will not interfere with sectioning. The forebrain and hindbrain should be punctured with forceps to allow efficient infiltration of OCT. Alternatively, the entire head can be cut off.
3. Embryos are fixed in 4% paraformaldehyde prepared in 1× PBS for 1 h at 4 °C on a nutator.
4. Embryos are moved sequentially from 15% sucrose in PBS containing 0.01% NaN_3 to 60% sucrose in PBS:OCT (1:1) containing 0.01% NaN_3 at 4 °C on a nutator. Embryos are then equilibrated in OCT for 30 min at room temperature on a rotator prior to embedding.
5. Embryos are embedded in disposable histology molds. For transverse sections, we orient the embryo with its tail at the bottom and the head at the top. Once embryos are oriented, the block is frozen in a bath of dry ice and EtOH. Blocks can be stored at –80 °C.
6. We cut 10–20 μm sections using a Leica CM1950 Cryostat. We usually collect sections on 5–10 slides such that each slide has adjacent serial sections. For instance, section 1 goes on slide 1 of 10 while section 2 is placed on slide 2 of 10. We return to slide 1 of 10 for section 11. We usually make three rows of sections with 5–10 sections per row.

3.6 Immunostaining Embryos

1. Remove OCT from the slides by soaking in 1× PBS at room temperature for 10 min and then subsequently washed two additional times in 1× PBS (can be done a slide mailer).
2. Block slides (in blocking buffer) for 1 h in a humidified chamber (a plastic box with wet paper towels; the slides rest on two trimmed serological pipettes held in place by clay or tape).
3. Incubate slides in primary antibody diluted in blocking buffer overnight at 4 °C in a humidified chamber. The following day, slides are wash four times in 1× PBS containing 0.1% Tween in a quadriPERM dish (Four well plate, Greiner Bio-One).
4. Block slides (in blocking buffer) for 30 min at room temperature.
5. Incubate with secondary antibody for 2 h at room temperature. Protect from light from this point forward.
6. Wash four times with 1× PBS containing 0.1% Tween in a quadriPERM dish.

7. Post-fix slides in 4% paraformaldehyde diluted in 1× PBS in a slide mailer.
8. Wash in two times in 1× PBS containing 0.1% Tween in a quadriPERM dish.
9. Add 75–125 µl of SlowFade to the slide and carefully place coverslip on top.
10. Using a vacuum attached to a pipet tip to gently remove any excess SlowFade and then seal the edges of the coverslips with fingernail polish. Allow to dry before imaging.

3.7 Imaging Embryos

1. Confocal Microscopy: For confocal microscopy, specimens are excited using the 488-nm line of an argon ion laser for visualization of GFP and the 543-nm line of a green helium–neon laser for visualization of Cy3. We capture fluorescence emission on a Zeiss LSM710 confocal microscope with a 40× oil objective at 0.6× digital zoom (Fig. 4).
2. Image Processing: We use Adobe Photoshop for final image data processing.

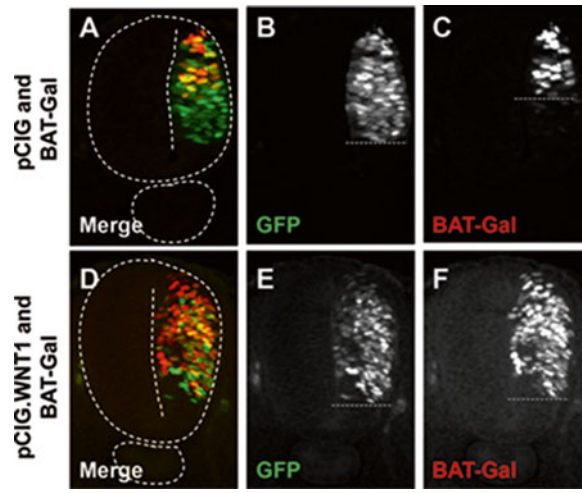

Fig. 4 Visualization of Wnt signaling in the developing chick spinal cord: HH stage 12–14 chick embryos were co-electroporated with pCIG (GFP alone) and BAT-Gal (**a**–**c**) or pCIG.WNT1 (GFP and WNT1) and BAT-Gal (**d**–**f**) and harvested at HH stage 18. The GFP expression (*green*) marks successfully electroporated cells. The activation of the BAT-Gal reporter is detected by immunostaining with anti-β-galactosidase antibody followed by anti-mouse secondary coupled with Cy3 (*red*). The spinal cord and notochord are outlined in (**a**) and (**d**). The *dashed lines* in (**b**–**c**, **e**–**f**) provide a comparison of the ventral most boundary of GFP and BAT-Gal. In (**a**–**c**), endogenous Wnt signaling is restricted to the dorsal half of the spinal cord while in (**d**–**f**), the overexpression of WNT1 causes the domain of Wnt signaling to fill the entire transfected region (as detected by GFP)

4 Notes

1. We use Rhode Island Red eggs for our electroporations (based on availability in our area); however, most chicken sequence information is derived from White Leghorns. While we would predict that small differences in sequence would have no significant effect on gain of function studies, it is possible that there could be ramifications for knockdown studies. Thus, if you choose to use a strain other than White Leghorn for knockdown studies, we recommend confirming the sequence of the gene you wish to target.
2. A variety of electroporation constructs are available that express a variety of fluorescent proteins. We find that the use of a nuclear localized variant of GFP (or whatever fluorescent protein you choose) is particularly useful as it allows us to use the NIH Image J Image Based Tool for Counting Nuclei to quantitate the total number of cells successfully transfected.
3. Foot pedals are highly useful for controlling the pneumatic injector and the electroporator. Using them frees up one hand for the micromanipulator and the other to hold the electrodes.
4. Though electroporations require some specialized equipment, the technique is accessible to all kinds of researchers, including undergraduate students. It typically requires 2–3 weeks for undergraduates in the lab to become proficient at electroporations.
5. The adhesive in packing tape can be toxic to the embryos. Therefore test the packing tape first on windowed eggs or use 3 M—Scotch® 3750 Packaging Tape—3″.
6. When electroporating embryos, depress the electrodes lightly to submerge the electrodes. By depressing electrodes equally, the DNA will be distributed along the entire dorsal/ventral extent of the spinal cord. By depressing one electrode more than the other, one can direct DNA to the dorsal or ventral spinal cord.
7. Electroporation should be carried out as quickly as possible after injecting DNA into the spinal cord.
8. Bubbles should be visible on the negative electrode after a successful electroporation. If no bubbles are observed, it is possible that no voltage was applied to the embryo. Ensure that electrodes are properly connected to power supply.
9. If electroporations are still not successful, we recommend starting with freshly prepared DNA and increasing the voltages used for the electroporation.
10. We find that although autoclaving egg waste is not required, it reduces unpleasant odors.

Acknowledgements

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Chapter 9

Monitoring Wnt Signaling in Zebrafish Using Fluorescent Biosensors

Nicola Facchinello, Marco Schiavone, Andrea Vettori, Francesco Argenton, and Natascia Tiso

Abstract

In this chapter, we are presenting methods to monitor and quantify in vivo canonical Wnt signaling activities at single-cell resolution in zebrafish. Our technology is based on artificial enhancers, obtained by polymerization of TCF binding elements, cloned upstream to ubiquitous or tissue-specific promoters. The different promoter/enhancer combinations are used to drive fluorescent protein reporter constructs integrated in the zebrafish germline by microinjection of fertilized zebrafish eggs. Fish with a single integration site are selected by Mendelian analysis of fluorescent carriers, and heterozygous offspring are used to monitor and quantify canonical Wnt activities. Open source public domain software such as ImageJ/Fiji is used to calculate the integrated densities in the region of interest and compare the effect of experimental conditions on control and treated animals.

Key words Zebrafish, Transgenic reporter, Transposase, Chemical biology

1 Introduction

Wnt/β-catenin signaling is initiated by the binding of a Wnt protein to a Frizzled seven-transmembrane receptor which in turn engages a Dishevelled cytoplasmic signal transducer to activate **β**-catenin/TCF/LEF transcriptional responses. In *H. sapiens* there are 19 Wnt ligands, 10 Frizzled receptors, 3 Dishevelled proteins, a single β-catenin protein, and 4 TCF/LEF DNA binding proteins, in addition to other mediators and regulators of the signaling cascade [1]. Despite the diverse signaling routes from receptor to transcriptional regulators that comprise Wnt cellular responses, all TCF/LEFs while differing in their tissue-specific gene expression share the same DNA sequence preferences. Using this common property of TCF/LEFs, we have prepared transgenic fluorescent reporter zebrafish that can be used to detect and quantify Wnt activity in vivo. This technique requires the preparation of artificial TCF/LEF/**β**-catenin-responsive reporters, zebrafish egg

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microinjection, and isolation of fish carrying a single integration with Mendelian characteristics. A simple protocol of fluorescent quantitative microscopy will allow detection and comparison of fluorescence levels between different treatments. The resulting fish strain with a stable genotype (single allele) can be used to screen embryos and larvae with bioactive libraries, with different treatments and mutations affecting the pathway [2]. Moreover, it is possible to detect potential cross-talk between canonical Wnt and other signaling pathways by simply crossing Wnt-responsive fish lines with other transgenic reporters that have different fluorescence spectra. Finally, a tissue-specific modification of this technique can be used to study Wnt activity in a single cell type. We describe here two methods: one for generating and validating ubiquitous Wnt signaling reporters and a second for the analysis of Wnt signals in pancreatic beta cells of living zebrafish embryos. Finally, we describe a simple protocol that uses publicly available software for the quantification of reporter fluorescence in living embryos.

2 Materials

2.1 Generation of Transgenic Lines: Cloning and Microinjection

1. Tol2 kit plasmids p5E-MCS (#228), pME-GFP (#385), p3E-polyA (#302), pDestTol2A2 (#394), pDestTol2CG2 (#395), pCS2FA-transposase (#396) are available upon request (kristen.kwan@genetics.utah.edu).
2. MultiSite Gateway Cloning Kit.
3. *SalI*, *NotI*, *EcoRI*, and *HindIII* restriction enzymes.
4. Alkaline Phosphatase, Calf Intestinal.
5. LR Clonase II Plus enzyme mix.
6. One Shot TOP10 *E. coli* cell strain or DH5α.
7. ApE software can be freely downloaded from en.bio-soft.net/plasmid/ApE.html.
8. TAE 50× solution: dissolve 242 g of Tris base, 18.6 g of EDTA sodium salt, and 57.1 mL of glacial acetic acid in 1 L of deionized water. Dilute 1:50 in deionized water before use.
9. 1–2 % Agarose gel in TAE 1× solution for plasmid analysis.
10. Gel and PCR Clean-Up System.
11. mMESSAGE mMACHINE SP6 transcription kit.
12. RNA Clean & Concentrator.
13. Danieau solution 10×: 580 mM NaCl, 7 mM KCl, 4 mM $MgSO_4$, 6 mM $Ca(NO_3)_2$, 50 mM HEPES, pH 7.6; filter-sterilized. Dilute 1:10 before use.
14. 0.5 % Phenol Red solution (#P5530, Sigma-Aldrich).

15. Injection solution: plasmids or mRNA at desired concentration, Danieau (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM HEPES pH 7.6) 1×, Phenol Red and sterile water RNAse/DNase free.
16. Breeding tanks with dividers.
17. Microinjection apparatus and injection needles (WPI); Petri dishes with glass slides, to hold embryos during microinjection.

2.2 Zebrafish Embryo Medium Preparation

1. Fish water 50× stock solution: 25 mM NaH_2PO_4; 25 mM Na_2HPO_4; 15 g/L Instant Ocean in deionized water. Dilute 1:50 before use and add 1 mg/L methylene blue to inhibit growth of contaminating fungi and other microorganisms.
2. 1-Phenyl-2-thiourea (PTU): Prepare 10× stock solution by adding 30 mg of PTU powder (#P7629, Sigma-Aldrich) in 100 mL of fish water. Store at 4 °C in the dark. Dilute 1:10 in fish water before use (*see* **Note 1**).

2.3 In Vivo Embryo Treatment

1. (2′Z,3′E)-6-Bromoindirubin-3′-oxime (BIO).
2. 3,5,7,8-Tetrahydro-2-[4-(trifluoromethyl)phenyl]-4*H*-thiopyrano[4,3-*d*]pyrimidin-4-one (XAV939).
3. Dimethyl Sulfoxide (DMSO).
4. Tricaine solution: prepare a 25× stock solution by adding 0.4 g Tricaine in 100 mL Tris–HCl 20 mM pH 9. Store at 4 °C in the dark. Dilute 1:25 in fish water 1× before use (*see* **Note 2**).
5. 1% Low melting agarose: 1 g low melting agarose in 100 mL fish water.
6. Epi-fluorescence stereomicroscope (Leica M165 FC) equipped with digital camera and GFP filter.

2.4 Image Analysis and Statistics

1. FiJi software for image analysis (*see* **Note 3**).
2. GraphPad or Microsoft Excel software for statistical analysis.

3 Methods

In the last few years much effort has been devoted to obtaining transgenic animals useful for monitoring in vivo activity of Wnt signaling. So far, at least three zebrafish Wnt-responsive transgenic lines have been generated and are available for the zebrafish scientific community [3–5]. These reporter lines are powerful tools for the study of Wnt signaling and have been extensively used in research laboratories. A straightforward approach to obtaining these transgenic fish lines is the generation of Tol2 plasmids and their injection into zebrafish embryos along with the transposase mRNA through a MultiSite Gateway system. The method to

analyze and quantify in vivo global and/or tissue-specific canonical Wnt activity requires: (Subheading 3.1) the preparation of a global and/or tissue-specific reporter plasmid; (Subheadings 3.2 and 3.3) its introduction in the zebrafish germline; (Subheading 3.4) the isolation of a single allele (insertion); (Subheading 3.5) its pharmacological (or genetic) validation; and finally (Subheading 3.6) the quantification of luminescence/fluorescence emitted by the region of interest (Fig. 1).

3.1 Generation of Wnt-Responsive Vectors Using the MultiSite Gateway System

The MultiSite Gateway technology is based on the lambda-phage-specific recombination system that allows the directional recombination in a unique plasmid of several DNA fragments containing specific *attL* and *attR* sites (LR recombination) [6]. The three-insert Multisite Gateway system has been optimized to combine, in a directional way, three vectors (named entry vectors) into a *Tol2* transposon backbone (destination vector) (Fig. 1). Generally, the three varieties of entry vectors are classified as: (i) *5′ clones* (p5E-7xTCF-promoter) that, for our specific purposes, will contain multimerized Wnt-responsive (7xTCF) sequences followed by a tissue-specific promoter; (ii) *middle clones* (pME-reporter), that usually contain a reporter gene; (iii) *3′ clones* (p3E-polyA), containing a polyA signal [7]. The Tol2 kit [6] (http://tol2kit.genetics.utah.edu/index.php/List_of_entry_and_destination_vectors) is a collection of plasmids optimized to perform recombination reactions. Here, we describe how to generate a Wnt-responsive Tol2 destination vector by using the MultiSite Gateway system:

1. Into the multiple cloning site of the p5E-MCS (#228 Tol2 kit) vector, insert the Wnt-responsive cassette named 7xTCF (Fig. 1). This cassette, harboring seven multimerized sequences recognized by the TCF transcription factor, has been successfully used to monitor Wnt activity in different animal models [4, 8]. Downstream to this responsive cassette, clone a minimal promoter (TATA box) or a tissue-specific promoter. This new plasmid (p5E-7xTCF-promoter) will represent the p5E vector to be used in the recombination reaction by Gateway system. The TATA box of Siamois gene (Fig. 2a) coupled with 7xTCF (*7xTCF-Xla.Siam*) has been successfully tested to analyze global Wnt activity in zebrafish [4] and is available upon request. On the other hand, to analyze the activation of Wnt signaling in a given cell type, a tissue-specific promoter can be cloned downstream of the 7xTCF cassette (Fig. 1). Figure 1b shows for example the p5E-7xTCF-1.2ins plasmid, used to analyze the activity of Wnt signaling in zebrafish pancreatic beta cells. To evaluate a potential Wnt-independent activity of the p5E-7xTCF-promoter reporter, generate also a negative control, represented by a p5E plasmid (p5E-7xTCF^{mut}-promoter) with mutated TCF binding sites (Fig. 1). For this point *see also* **Notes 4** and **5**.

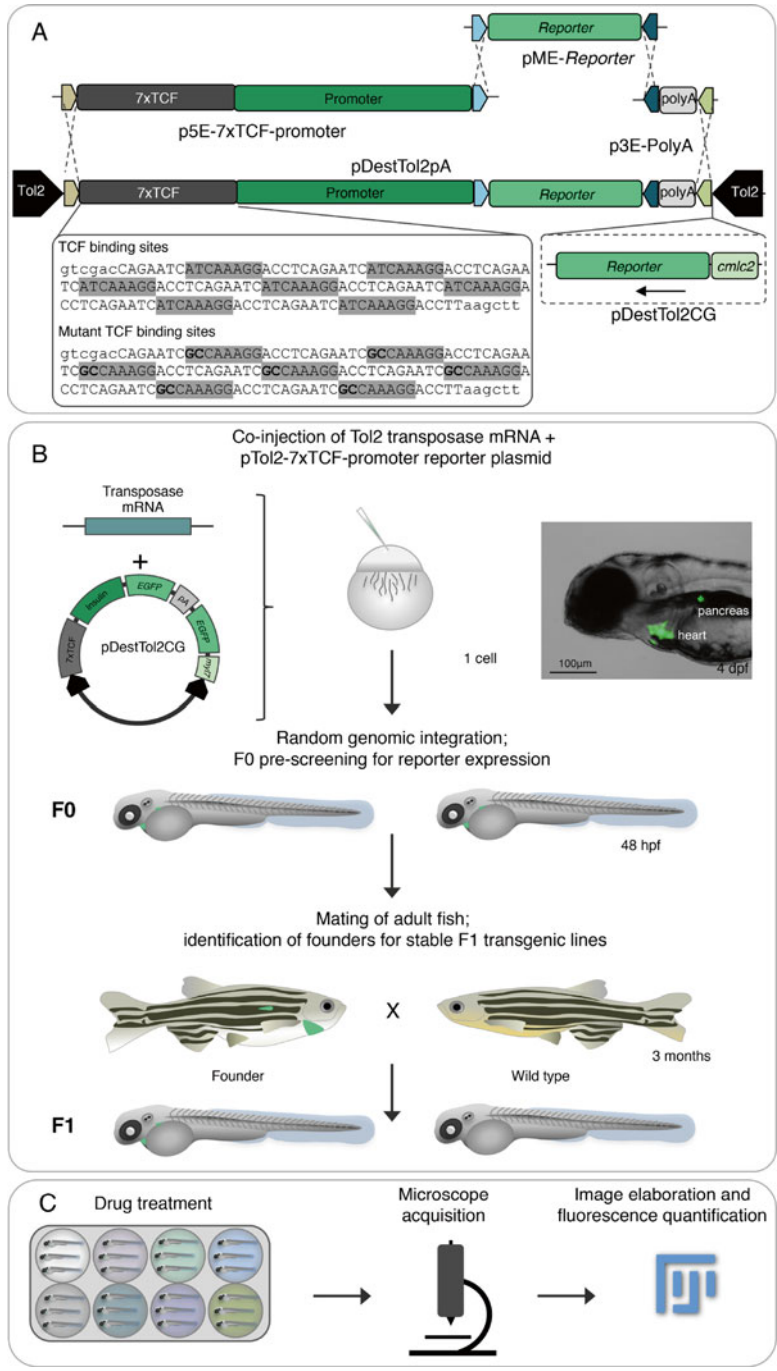

Fig. 1 Generation and analysis of promoter-directed Wnt reporter zebrafish lines. (**a**) Displays the LR cloning steps to produce Tol2-based destination (Dest) constructs harboring wild-type or mutant Wnt-responsive (TCF) elements, followed by a selected Promoter, a Reporter gene and a polyA sequence. The system may be implemented with a second reporter with known localized expression (e.g.: *cmlc2*:Reporter). TCF binding sites are *highlighted*; *bold letters* indicate the point mutations. For more details on the cloning steps, *see* Subheading 3.1. (**b**) Summarizes the steps for the generation of promoter-directed Wnt-responsive zebrafish lines. After transcription of Tol2 transposase mRNA (*see* Subheading 3.2) and its co-injection with the construct of interest (*see* Subheading 3.3), founders are pre-screened, raised to adulthood and crossed to wild types in order to identify stable transgenic lines (*see* Subheading 3.4). (**c**) Shows an example of reporter line validation, based on multi-well drug treatment of transgenic embryos and image acquisition (*see* Subheading 3.5), followed by software-based fluorescence analysis (*see* Subheading 3.6)

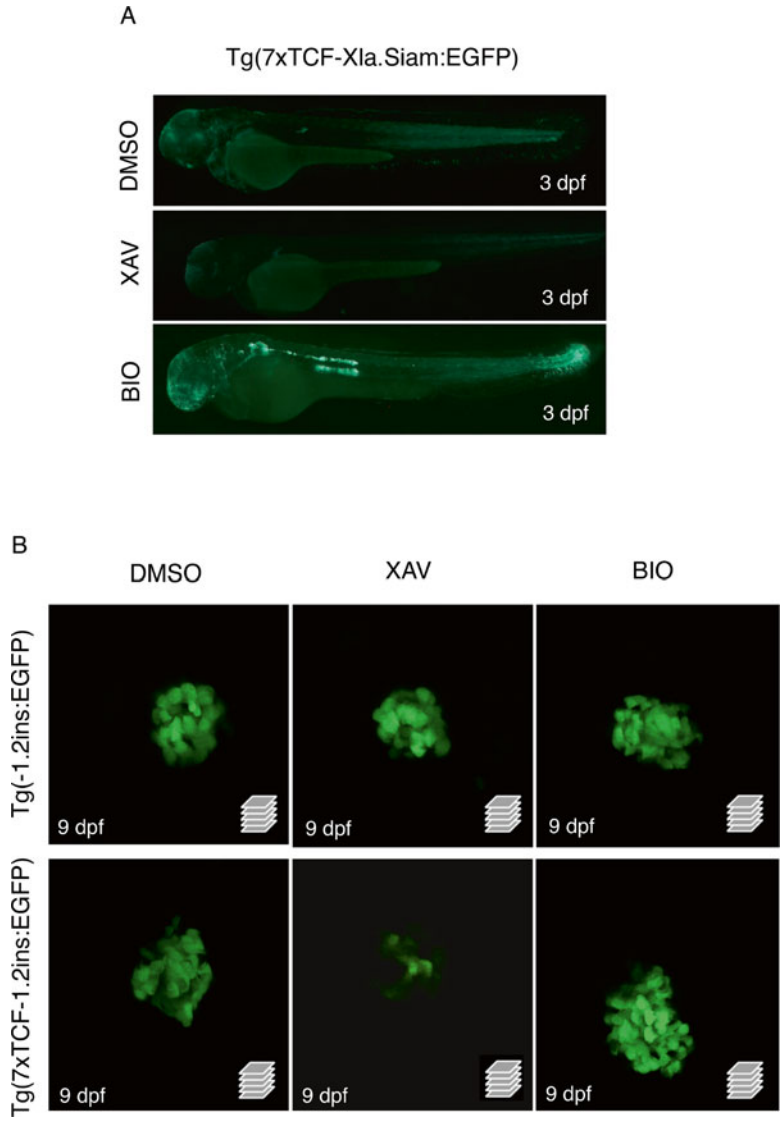

Fig. 2 Reporter responsiveness after chemical treatments. (**a**) Response of Tg(7xTCF-Xla.Siam:GFP)^{ia4} transgenic zebrafish to agonists and antagonists of Wnt signaling. Treatment with control DMSO does not alter reporter activity. Treatment of Tg(7xTCF-Xla.Siam:GFP)^{ia4} embryos with 5 mM XAV for 24 h dramatically reduces the expression of GFP compared with DMSO-treated embryos. Incubation of transgenic embryos with 5 mM BIO, from 24 to 48 hpf, induces a robust tissue-specific activation of the reporter. All embryos are displayed at 3 days post fertilization (dpf), in lateral view, anterior to the left. (**b**) Application of zebrafish tissue-directed reporter lines in chemical treatments. Comparison of tissue-specific Tg(-1.2ins:EGFP) and Wnt-responsive/tissue-specific Tg(7xTCF-1.2ins:EGFP) transgenic lines treated with DMSO, Wnt-inhibitor XAV or Wnt-agonist BIO. Pictures show confocal stack images of pancreatic islets (insulin-producing beta cells) in 9 dpf larvae treated for 96 h with DMSO, 4 μM XAV or 2 μM BIO. The number of fluorescent cells is globally unmodified in Tg(-1.2ins:EGFP), while reduction (with XAV) or increase (with BIO) of fluorescent signals are observed in drug-treated Tg(7xTCF-1.2ins:EGFP) larvae compared to DMSO-treated controls

2. Set up the LR plasmid recombination using the MultiSite Gateway system. In a 0.2 mL tube add 10 fmoles of p5E-7xTCF-promoter vector (described in **step 1**) containing the Wnt-responsive sequence 7xTCF associated with the driver promoter (e.g. p5E-7xTCF-Xla.Siam or p5E-7xTCF-1.2ins). Add 10 fmoles of a *middle vector* containing a reporter gene (*see* **Note 6**) and add 10 fmoles of p3E-polyA (#302) vector. Add 20 fmoles of a pDestination vector (#394 or #395) (*see* **Note 7**). Add TE buffer pH 8 up to a final reaction volume of 8 μL. Add 2 μL of LR Clonase II Plus enzyme mix. This reaction will generate the final Wnt-responsive pTol2-7xTCF-promoter vector.
3. With the same procedure described in **step 2**, arrange a second Gateway LR recombination using the p5E-7xTCF^{mut}-promoter entry plasmid. In a 0.2 mL tube mix 10 fmoles of p5E-7xTCF^{mut}-promoter, 10 fmoles of a *middle entry vector*, 10 fmoles of p3E-polyA (#302) vector and 20 fmoles of a pDestination vector. Add TE buffer pH 8 up to a final reaction volume of 8 μL. Add 2 μL of LR Clonase II Plus enzyme mix. This reaction will generate a pTol2-7xTCF^{mut}-promoter construct, subsequently used as negative control.
4. Incubate the reaction at 25 °C for 16 h.
5. Add 1 μL of Proteinase K solution provided with the Clonase II Plus enzyme. Incubate the reaction for 10 min at 37 °C.
6. Transform chemically competent cells (One Shot TOP10 or DH5α cells) with 3 μL of the LR reaction. The remaining recombination mixture can be stored at 4 °C for several days.
7. From each transformation vial, spread respectively 50 and 250 μL of bacteria on two pre-warmed LB agar plates containing 100 μg/mL ampicillin.
8. Invert the plates and incubate at 37 °C overnight (*see* **Note 8**).
9. Pick single colonies using toothpicks and place them into 5 mL of LB medium with ampicillin. Select 10–15 colonies and shake the vials at 200 rpm in a shaking incubator overnight.
10. Extract plasmid DNA from bacteria by commercial miniprep kits. The LR reactions can be tested via restriction digest. A useful tool to get the assembled sequence of the final vector after LR reaction is the ApE software that can calculate and visualize the Gateway recombination.

3.2 In Vitro Transcription of Capped Tol2 Transposase mRNA

1. Linearize 10 μg of pCS2FA-transposase plasmid DNA (# 396) in a total volume of 50 μL using *NotI*. Incubate the reaction mixture overnight at 37 °C.
2. Run 100 ng of the uncut vector DNA and 2 μL of cut DNA on a 1% w/v agarose gel to verify that the vector DNA is completely digested.

3. Purify linearized DNA and dissolve the template DNA with 20 μL of sterile water.
4. Set up in vitro transcription with the mMessage mMachine SP6 Kit using 2 μg of linearized pCS2FA-transposase DNA.
5. Incubate the reaction at 37 °C for at least 4 h. After incubation, add an additional μL of SP6 enzyme and incubate the reaction for 1 h.
6. Add 1 μL of DNase and incubate at 37 °C for 1 h.
7. Purify the in vitro transcribed RNA and resuspend the RNA in a final volume of 20 μL.
8. Run the transposase RNA on a 1% agarose gel to confirm its integrity and quantify its concentration.
9. Dilute the RNA at a final concentration of 250 μg/μL and aliquot 2 μL of RNA in 0.2 mL tubes. Store the labeled tube at −80 °C.

3.3 Injection of Tol2 Plasmid and Transposase mRNA into Zebrafish Embryos

1. The day before injection, prepare zebrafish couples for spawning. During the night, keep males and females separated, placing a divider in each breeding tank.
2. The day after, prepare the injection solution. In a 0.5 mL tube, mix 250 ng of transposase mRNA, 300–600 ng of pTol2-7xTCF-promoter vector (*see* Subheading 3.1, **step 4**), 1 μL of 0.5% Phenol Red solution, 1 μL of 10× Danieau solution, and water up to a total volume of 10 μL.
3. In a second 0.5 mL tube, mix 250 ng of transposase mRNA, 300–600 ng of Tol2-negative control pTol2-7xTCF^{mut}-promoter vector (*see* Subheading 3.1, **step 4**), 1 μL of 0.5% Phenol Red solution, 1 μL of 10× Danieau solution, and water up to a total volume of 10 μL (*see* **Note 9**).
4. Once the solutions are ready (*see* **Note 10**), rise the separator from the breeding tanks and wait for the fish to spawn.
5. Calibrate the injection needle by adding a drop of mineral oil on a cell counting chamber slide. In order to get an injection droplet of about 2–4 nL, adjust the injection parameters (pressure, injection time, dimension of the needle tip) to get a droplet of 0.16–0.20 mm.
6. From the breeding tanks, collect the embryos in fish water and align them alongside a microscope glass slide inside the lid of a 9-cm Petri dish. The solution should be injected in 1-cell stage embryos. It is crucial to inject the solution into the cytoplasm of the zebrafish embryos and not in the yolk to increase the chance of early integration. Inject at least 100 embryos with each solution containing respectively the pTol2-7xTCF-promoter and the pTol2-7xTCF^{mut}-promoter vector.

7. Incubate injected embryos at 28.5 °C overnight.
8. With an epi-fluorescent stereomicroscope analyze the injected fish at 24/48 h post-fertilization (hpf). Discard the non-fluorescent embryos while selecting and transferring in a new plate the fluorescent ones (*see* **Note 11**).
9. Raise to adulthood the fluorescent transgenic embryos (F0) injected with both constructs that develop normally.

3.4 Generation of Stable Wnt-Reporter Zebrafish Lines

1. Outcross fluorescent F0 adults with wild-type fish.
2. Screen the F0 offspring at 24–72 hpf to detect transgene expression. With this procedure F0 founders with transgene insertions in the germline can be identified and isolated.
3. Check the reporter activity in the F1 generation by fluorescence microscopy. If the transgene is expressed in more than 50% of the offspring, the integration of the Tol2 plasmid occurred in several points of the injected fish genome. The copy number of the integrated plasmid can be reduced performing further outcrosses with wild-type fish in order to obtain: (i) a stable transgenic line with one insertion of the transgene in the genome; (ii) offspring with homogeneous fluorescence and a 50:50 fluorescent-non fluorescent ratio.

3.5 Pharmacological Validation of Tissue-Specific Wnt Reporter Fish

The administration of agonist and antagonist chemical compounds to Wnt-responsive reporters can represent a straightforward and fast method to understand whether the transgenic line represents a *bona fide* reporter of Wnt activity. The Wnt activator BIO (6-bromoindirubin-3′-oxime), known to inhibit the glycogen-synthase-kinase, and the Wnt inhibitor XAV939 are highly recommended to perform this pharmacological validation [9, 10] (*see* **Note 12**) (Fig. 3).

1. Obtain heterozygous transgenic embryos by crossing stable Wnt reporters with wild-type individuals (*see* **Note 13**); sort fluorescent embryos under an epi-fluorescence stereo-microscope.
2. To improve image clarity, treat the embryos with PTU to prevent pigment formation (*see* **Note 1**).
3. To enhance drug absorption, remove the embryonic chorion, using thin needles or sharp forceps.
4. Dissolve BIO and XAV939 at 5 mM concentration in fish water containing 0.1% DMSO. Dilute BIO and XAV939 at a final concentration in a range between 2 and 5 μM in 3 mL of fresh fish water (*see* **Note 14**); dissolve an equal concentration of DMSO in 3 mL of fresh fish water (*see* **Note 15**).
5. Place transgenic embryos in 6- or 12-well plates at the desired stage; remove the growth medium and add BIO, XAV or vehicle solution; incubate the embryos at 28.5 °C for at least 24 h.

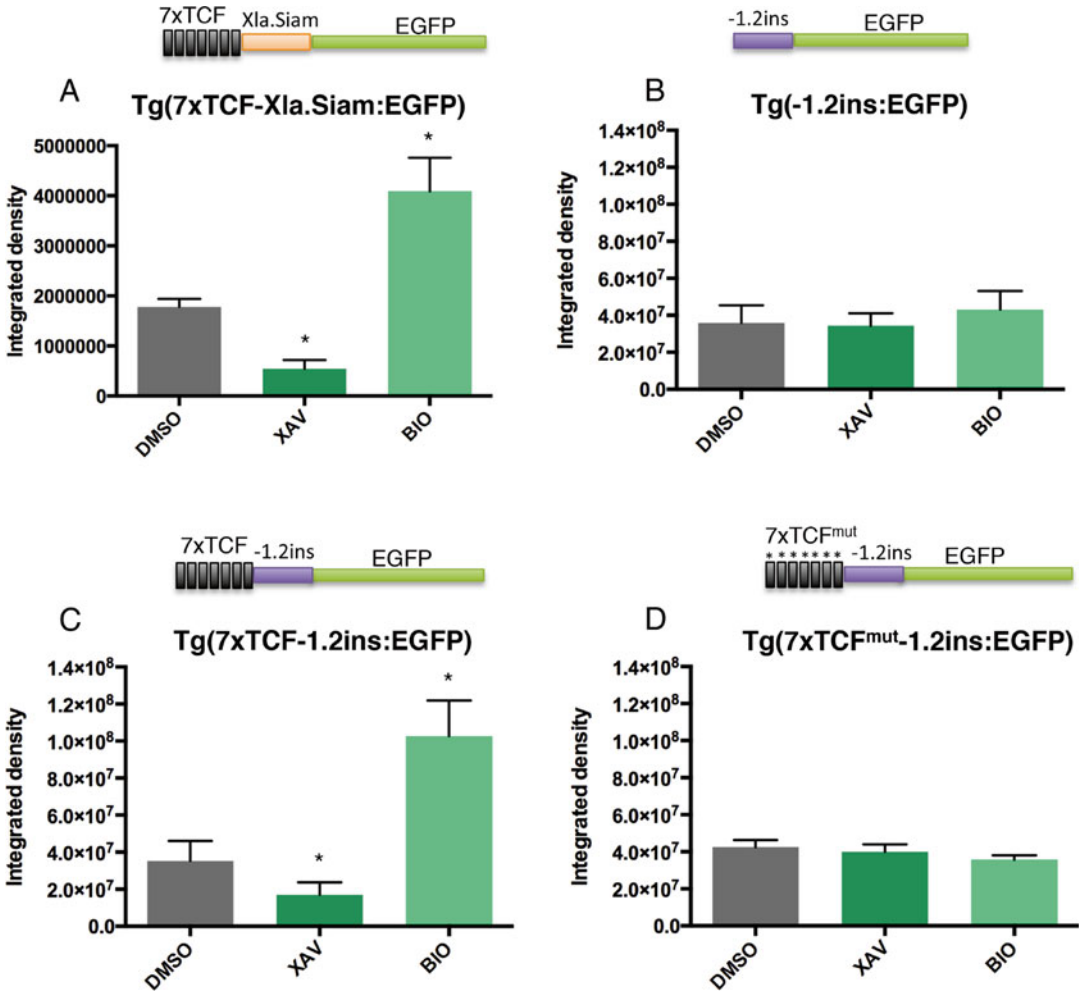

Fig. 3 Quantitative analysis of reporter fluorescence changes. Analysis of integrated density in the transgenic line Tg(7xTCF-Xla.Siam:GFP)[ia4] treated for 24 h with DMSO, XAV, or BIO (**a**). Analysis of integrated density in the transgenic lines Tg(ins:EGFP) (**b**), Tg(7xTCF-1.2ins:EGFP) (**c**), and Tg(7xTCF[mut]-1.2ins:EGFP) (**d**) treated for 96 h with DMSO, XAV or BIO. Drug effects on reporter are detected irrespective of the promoter type, ubiquitous (**a**) or tissue-specific (**c**), when the wild-type 7xTCF is used as an upstream enhancer. Conversely, fluorescence signals are not significantly affected when using TCF unresponsive promoters (**b**) or mutated versions of 7xTCF as upstream enhancer (**d**). Data were analyzed using Fiji software and represent mean ± s.d. ($n = 8$). Significance was determined by Student's *t*-test (*$p < 0.05$) forse manca da indicare n, il numero di embrioni analizzati - almeno mediamente...)

6. Collect BIO-, XAV939-, and vehicle-treated transgenic zebrafish embryos after treatment and proceed to the in vivo observation of organ/tissue of interest under a confocal microscope.
7. Anesthetize the embryos/larvae adding to the Petri dish 1 mL of Tricaine pH 7 (4% w/v).
8. Prepare a 1% (w/v) agarose low melting solution in fish water. The solid agar should be microwaved until becoming liquid (*see* **Note 16**).

9. With a Pasteur pipette, transfer anesthetized larvae into a shallow glass depression slide and add a small amount of agar to just fill the depression of the slide.
10. Move embryos into the preferred orientation using a tip and photograph under a fluorescence stereomicroscope or a confocal microscope.

3.6 Digital Quantification of Integrated Density Using Fiji Image Processing Software

1. Download and install Fiji software (*see* **Note 3**).
2. To enhance 3D capabilities of Fiji, install the 3D ImageJ Suite plugin [11] by following Fiji detailed instructions.
3. Use the image acquisition software coupled to the confocal microscopy device to obtain image stacks of fluorescence distribution covering the whole thickness of the organ/tissue of interest. During image acquisition try to use the full dynamic range; saturate the highest signal in your brighter sample but avoid over-saturation.
4. During acquisition use the same setting for all samples (control and treated).
5. Import the image stack or hyperstack with Fiji (*see* **Note 17**).
6. If more than a fluorochrome is analyze, use [Image → Color → Split Channel] to separate Red, Green and Blue, and proceed only with the channel of interest.
7. Optional (will save RAM for calculations): crop the arena of interest using the selection tool.
8. To remove the background from the image, select part of the image background using any selection tool and copy it to the ROI (Region Of Interest) manager by pressing "t". Repeat it for at least three representative regions. Measure the background value [Analyze → Measure]. Subtract from the whole image the "Mean Gray Value" [Process → Math → Subtract] of the most representative ROIs of your image backgrounds.
9. Set the threshold [Image → Adjust → Threshold] that best isolates positive regions from background. All voxels with intensities lower than threshold are discarded from calculations. You can use the "Set" button to type in values. "Set" is useful when you want to quickly threshold different images using exactly the same value. Click on "Apply" and select "Black background".
10. From the Plugins menu select [3D → 3D Manager Options]. Make sure you have "Integrated Density" and "Volume" selected; "Mean Gray Value" and "Maximum Gray Value" might also be of interest in signal quantification. Other options are morphometric parameters that require a different protocol for the preparation of the image.
11. To visualize the measurements of each image of interest use [Plugins → 3D → 3D Manager → "Add Image"] and click on

"Measure 3D" and "Quantify 3D": this creates two windows containing "Volumes" and "Integrated Densities".

12. Once you have finished, copy all data in the Results windows and paste them into a new spreadsheet or statistic program (*see* **Note 18**).
13. Compare the values of integrated density between different experimental groups and do the appropriated statistical analysis.
14. For the quantification of integrated density in images acquired with fluorescence stereomicroscope use **steps** from **4** to **9**. Go to [Analyze] [Set Measurements] and check "Limit to Threshold" to limit the area of calculation. Check "Display label" in order to have the name of file images listed in the "Results" window. Make the measure for each image by using [Analyze → Measure]. Then proceed to **steps 12** and **13**.

4 Notes

1. PTU affects early embryonic development: do not treat the embryos before the end of gastrulation (20 hpf). To prevent melanocytes development, do not treat embryos after 24/26 hpf.
2. Higher tricaine concentration will cause fish death.
3. Fiji can be freely downloaded from http://fiji.sc/Downloads.
4. From this control, you expect to get a final Tol2 plasmid insensitive to Wnt modulation. This negative control will be useful to confirm that transcription activity observed in the construct with the wild-type 7xTCF sequence reflects endogenous Wnt/β-catenin activity with the minimal promoter having no specific effect on modulation of transcription.
5. The cassette containing seven multimerized TCF/LEF binding sites was isolated from p5E-7xTCF-*Xla.Siam* [4] using the *EcoRI* restriction enzyme. After fill-in with the Klenow enzyme, the sequence was subcloned into the p5E-1.2ins [12] plasmid digested with *HindIII*, blunted with Klenow enzyme and dephosphorylated with alkaline phosphatase. The resulting plasmid was the p5E-7xTCF-1.2ins. To generate the mutant TCF binding sites 7xTCF^{mut}, we used the FOPflash sequence (#21-169, Upstate). This fragment, containing the restriction sites for *HindIII* and *SalI*, was delivered to us by Life Technologies in the pMA-RQ vector (Invitrogen). The sequence 7xTCF^{mut} was cut from pMA-RQ using *HindIII* and *SalI* and subcloned into p5E-1.2ins plasmid digested with the same enzymes and dephosphorylated with alkaline phosphatase. The resulting plasmid was p5E-7xTCF^{mut}-1.2ins. Positive

clones containing the wild-type/mutant TCF binding sites and insulin promoter were sequenced and recombined with the pME-eGFP and p3E-SV40 polyA vectors. Entry plasmids were finally recombined into the Tol2 destination vector (pTol2pa) as previously described [6]; *see also* Fig. 1a.

6. Several middle entry vectors containing the EGFP (nuclear (#383), cytoplasmic (#385) or membrane-localized (#384)), and the mCherry (nuclear (#233), cytoplasmic (#396) or membrane-localized (#550)) proteins are available in the Tol2 Kit. Other middle entry vectors containing reporter protein such as Kaede and Venus Pest have been already generated and can be requested.
7. The destination vectors pDestTol2pA2 (#394) and DestTol2CG2 (#395) are plasmids that contain two Tol2 sequences. The transposase is able to recognize these sequences and promote the integration of the transgene (cloned within the two Tol2 sequences) into the zebrafish genome. The pDestTol2CG2 vector includes also an extra *cmlc2:*EGFP-pA expression cassette that contains the cardiac myosin light chain gene (*cmlc2*) promoter. This promoter drives the expression of cytoplasmic EGFP in the heart. In zebrafish embryos, injected with Tol2 plasmids generated using pDestTol2CG2, the green fluorescent heart becomes an indicator for the insertion of the transgene. This cassette is designed to act as a transgenesis marker, and can be very useful when the injected transgene is non-fluorescent, or difficult to visualize.
8. You can find hundreds of colonies per plate. Remember to plate the reaction in the afternoon, to avoid that satellite colonies might obscure the results of the reaction.
9. Keep the injection solution on ice to prevent RNA degradation.
10. The injection solution should be freshly prepared. Do not re-use leftover injection solution.
11. If most of the injected embryos appear deformed or dead, repeat the injection procedure using a solution containing a lower quantity of plasmid. On the contrary, in some cases it is necessary to increase the concentration of Tol2 plasmid if the injected fishes do not exhibit the presence of fluorescent cells.
12. The procedure described below is essentially applicable to any compound to be tested for the modulation of Wnt activity.
13. The outcross of stable transgenic animals with wild-type individuals generates transgene carriers only in heterozygous (not homozygous) condition, thus ensuring homogeneous fluorescence level in all experimental animals before treatment.
14. The concentration depends on the toxicity of the chemical compound. For a 1-day treatment, use a concentration of 5 μM; for longer treatments use a lower concentration of 2 μM.

15. Important: use concentrations of DMSO lower than 0.5 %.
16. Let agarose cool down for sufficient amount of time (at least 15 min); the temperature of the solution should be warm enough that it can be touched, not overly hot.
17. Consider the bit depth of your images and check if 0 is black and (2bit depth-1) is white. If not, invert the image with [edit → invert].
18. To improve the protocol you can prepare a macro to automate repetitive tasks and share the procedure with colleagues.

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Chapter 10

Biochemical Analysis of Tankyrase Activity in Zebrafish In Vitro and In Vivo

Jesung Moon and James F. Amatruda

Abstract

The activity of tankyrase (Tnks) enzymes modulates the activity of the β-catenin destruction complex in the Wnt/β-catenin signaling pathway. Here, we describe a method for determining the accessibility of various zebrafish tissues in vivo and in vitro to small molecule inhibitors of Tankyrase enzymes. This biochemical assay will facilitate chemically based studies focused on understanding the role of Tankyrase in cell fate reprogramming and tissue homeostasis and provide insights into the potential role of Wnt/β-catenin signaling in these processes.

Key words Zebrafish, Wnt/β-catenin signaling, Wnt inhibitor, Tankyrase, Western blotting

1 Introduction

Tankyrase (Tnks) is a member of the poly ADP-ribose polymerase (PARP) protein superfamily. These PARP proteins catalyze the transfer of ADP-ribose units from NAD+ to the target protein to form a polymer of ADP-ribose. This modification, a process termed PARsylation, confers a negative charge to the target protein and alters its functional properties. The PARP protein superfamily has broad roles in cellular processes including cell proliferation, apoptosis, DNA repair, and Wnt signaling [1–3]. In the Wnt/β-catenin signaling pathway, Tnks enzymes regulate the turnover of Axin, a scaffolding protein in the β-catenin destruction complex. In the absence of Tnks activity, Axin proteins accumulate and accelerate the rate of β-catenin destruction thereby reducing the transcriptional activity of the TCF/LEF family of DNA-binding proteins. Recently identified Tnks inhibitors have been successfully used for interrogating roles of the Wnt/β-catenin signaling in embryonic development and adult tissue regeneration in lieu of genetic approaches [3–5]. In particular, chemically based strategies afford temporal and dose-dependent interrogation of complex biological phenomena [5–8].

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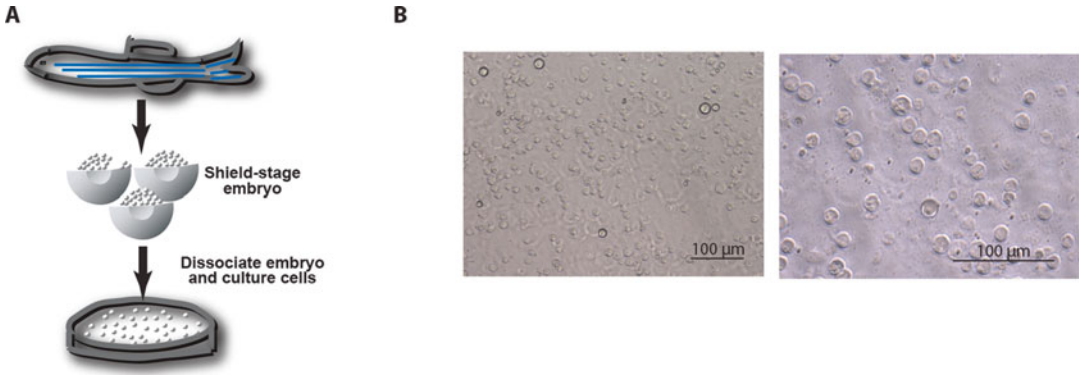

Fig. 1 Establishing an in vitro model of Wnt/β-catenin signaling in zebrafish embryonic cells. (**a**) In vitro culture of zebrafish embryonic cells. After dissociating embryos in the sphere stage, the cells were incubated at 25 °C. (**b**) Photographic images of zebrafish embryonic cells after 24 h of incubation

A universal consequence of inhibiting Tnks enzymes with directly binding antagonists is the accumulation of Tnks protein due to loss of auto-parsylation and destruction [3]. Thus, this phenomenon represents a hallmark of successful chemically mediated inhibition of Tnks activity. Previously, we demonstrated that a small-molecule inhibitor modulating the activity of Tnks enzymes (IWR-1) inhibits Wnt/β-catenin-dependent signaling in embryonic development and tissue regeneration in zebrafish [5, 6]. Here, we confirmed using Tnks accumulation as a read-out that IWR-1 retains activity in zebrafish with the help of cultured zebrafish fibroblasts isolated from late blastula-stage embryos (Fig. 1). We reasoned that homogenous cultured cell populations would render biochemical studies more accessible than in the case of whole tissue. Indeed, we observed accumulation of Tnks protein in response to IWR-1 cellular treatment thus signifying retention of IWR-1 on-target activity in zebrafish.

Here, we describe a biochemical assay for monitoring IWR-1/Tnks interactions in zebrafish tissue. In addition to presenting a protocol for evaluating other potential Tnks compounds using the in vitro cultured fibroblast technique as described above, we will also introduce how to evaluate bioavailability of IWR-1 in adult zebrafish. These techniques should enable researchers to corroborate phenotypic outcomes induced by Tnks inhibitors with biochemical evidence for on-target chemical activity.

2 Materials

2.1 Derivation of a Cell Culture from Zebrafish Embryos

1. Pronase solution.
2. Inverted microscope.
3. Trypsin/EDTA.

4. Culture medium (mixture of Leibowitz's L15, Dulbecco modified Eagle, and F12 media, 50:35:15) supplemented with 15 mM HEPES buffer, pH 7.2.
5. Fetal bovine serum (FBS).
6. 100 units/mL penicillin, and 100 μg/mL streptomycin.
7. Phosphate-buffered saline (PBS).
8. 70% Ethanol.
9. 35 mm tissue culture dish.
10. Cell strainer, 100 μm.
11. Breeding chamber with divider.
12. E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, pH 7.2).
13. Incubator.

2.2 Drug Treatment and Tissue Collection from Zebrafish Adult Fish

1. DMSO.
2. IWR-1.
3. Tricane (0.2%) solution.
4. Fish flake.
5. Fish net.
6. 500 mL transparent plastic bottles.
7. Forceps and scissors.

2.3 Western Blotting of Zebrafish Cell Cultures and Adult Tissues

1. NONIDET P-40.
2. Pre-cast SDS Polyacrylamide gel, 7.5%.
3. Nitrocellulose membrane.
4. Gel blotting paper.
5. PBS-Tween (PBST): Add Tween-20 to PBS to a final concentration of 0.1%.
6. Blocking buffer: Add nonfat milk to a final concentration of 5% in PBST.
7. Horseradish peroxidase (HRP) conjugated secondary antibodies.
8. Tankyrase (Santa Cruz Biotechnology) and β-actin antibodies.
9. Plastic tissue grinder.
10. Microcentrifuge.

3 Methods

3.1 Derivation of Embryonic Cell Culture from Zebrafish Embryos

1. Set up breeding chambers containing a male and a female fish.
2. Insert dividers into the chambers 1 day before embryo collection (*see* **Note 1**).

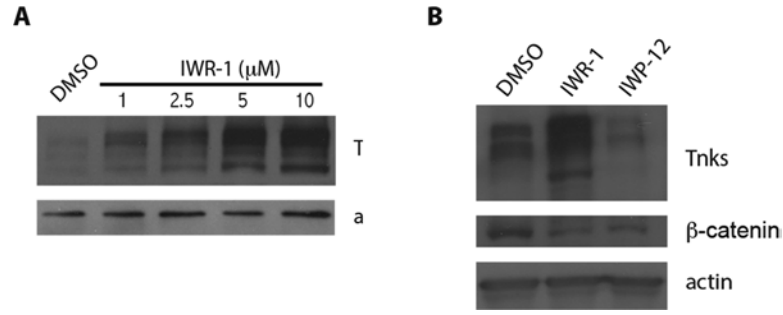

Fig. 2 Biochemical responsiveness of zebrafish embryonic cells after incubation of IWR-1. (**a**) Biochemical confirmation of IWR-1-mediated attack of tankyrase in zebrafish. Cultured cells were treated with increasing amount of IWR-1 and tankyrase proteins analyzed by Western blotting. Consistent with inhibition of autoparsylation activity that initiates its proteasome-mediated destruction, IWR-1 induced accumulation of Tnks protein in a dose-dependent manner. (**b**) Addition of the Tnks inhibitor (IWR-1) stabilizes Tnks protein and decreases β-catenin protein. In contrast, a porcupine inhibitor (IWP-12) [5] does not stabilize Tnks protein, but decreases β-catenin protein

3. On the morning of the next day, remove the dividers from chambers.
4. Collect 200 sphere-stage embryos (approximately 6 h post fertilization (hpf)) in a 35 mm dish.
5. Transfer embryos to a cell strainer or other fine-mesh strainer and clean embryos with PBS.
6. Immerse embryos in 70% EtOH for 10 s and rinse three times with PBS.
7. Transfer embryos to a clean 35 mm dish and add 300 μL of pronase to the dish and incubate approximately 5 min at room temperature (*see* **Note 2**).
8. Remove pronase solution and gently rinse three times with PBS.
9. Add 3 mL of Trypsin/EDTA solution into embryos and dissociate the embryos by pipetting up and down and add 10% (v/v) FBS to the dissociated embryos (Fig. 1).
10. Centrifuge the cells at 150 × *g* for 5 min.
11. Resuspend the cell pellet in 2 mL of culture medium [9].
12. Seed the cells into four wells of a 24-well tissue culture plate (*see* **Note 3**) (Fig. 1).
13. Add compounds to the wells at the desired concentration and incubate the cells at 28 °C for 24 h (Fig. 2).

3.2 Drug Treatment and Tissue Collection from Zebrafish Adult Fish

1. Add compound (IWR-1 at 10 μM or DMSO) into a plastic bottle containing 400 mL of E3 medium.
2. Shake well and add four adult fish to each bottle.

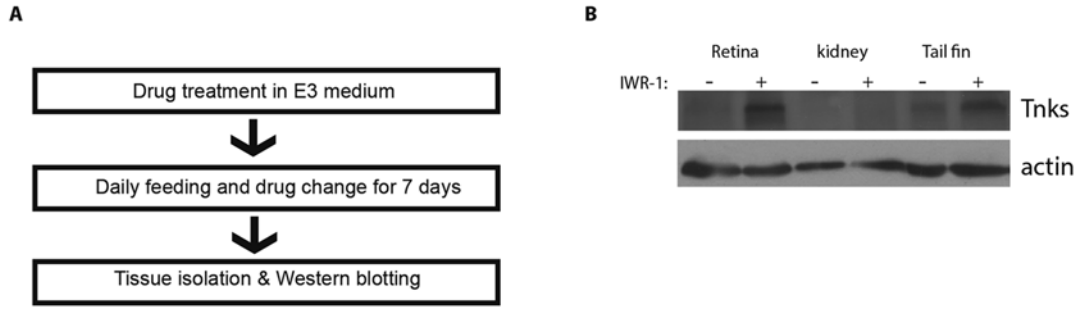

Fig. 3 Tankyrase responsiveness of adult zebrafish tissues after incubation of IWR-1. (**a**) A workflow for drug treatment in adult zebrafish. (**b**) Biochemical confirmation of tankyrase stabilization in zebrafish tissues after incubation of IWR-1 (10 μM) for 7 days

3. Feed fish daily with fish flake and change the water with a fresh compound (*see* **Note 4**) (Fig. 3).
4. After 7 days of drug treatment, euthanize fish with an excess of Tricane (0.1%).
5. Using a dissecting stereomicroscope, locate the target tissues.
6. Collect tissues with forceps and scissors.
7. Homogenize tissue samples in a 1.5 mL tube containing 1% NP 40 solution (*see* **Note 5**).
8. Centrifuge the tubes at 15,000 × *g*, 4 °C for 10 min.
9. Transfer supernatants into new tubes.

3.3 Western Blotting of Zebrafish Cell Cultures and Adult Tissues

1. Load protein lysates into 7.5% pre-cast polyacrylamide gel and perform electrophoresis, followed by transfer to nitrocellulose membrane.
2. Remove the membrane from the apparatus and incubate in blocking buffer for 30 min at room temperature to prevent nonspecific antibody binding.
3. Wash 3 × 10 min with PBST.
4. Incubate the nitrocellulose membranes with rabbit tankyrase antibody diluted to 1:1000 in PBST for 60 min at room temperature.
5. Wash 3 × 10 min with PBST.
6. Incubate with anti-rabbit secondary antibody conjugated with horseradish peroxidase (HRP) in PBST.
7. As a loading control, incubate the nitrocellulose membrane with mouse anti-β actin diluted to 1:2000 in PBST for 60 min at room temperature.
8. Wash 3 × 10 min with PBST.

9. Incubate with anti-mouse secondary antibody conjugated with horseradish peroxidase (HRP) in PBST.
10. Incubate the membrane with chemiluminescence detection reagents for 1 min to detect HRP-generated signal. Acquire HRP signals using a Li-COR Odyssey Fc instrument. A predefined optimal wavelength of excitation will be used to detect the respective infrared dye conjugated to the secondary antibody.

4 Notes

1. Sex ratio is male to female (1:1–2). Pull out dividers at the same time in order to synchronize the stage of embryonic development.
2. After adding pronase, monitor the loss of chorions and immediately transfer dechorionated embryos into a new 35 mm dish.
3. Most dissociated cells would not attach on the bottom of plate. Do not disturb the cells during incubation.
4. After feeding fish, prepare a fresh E3 medium and add IWR-1 compound (10 μM) or DMSO into the E3 medium.
5. Homogenize tissues in 100 μL of 1 % NP 40 solution with a plastic tissue grinder. Add an additional 400 μL of 1 % NP 40 solution into the tube.

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Chapter 11

Reconstitution of the Cytoplasmic Regulation of the Wnt Signaling Pathway Using Xenopus Egg Extracts

Annastasia Simone Hyde, Brian I. Hang, and Ethan Lee

Abstract

The regulation of β-catenin turnover is the central mechanism governing activation of the Wnt signaling pathway. All components of the pathway are present in the early embryo of *Xenopus laevis*, and *Xenopus* egg extracts have been used to recapitulate complex biological reactions such as microtubule dynamics, DNA replication, chromatin assembly, and phases of the cell cycle. Herein, we describe a biochemical method for analyzing β-catenin degradation using radiolabeled and luciferase-fusion proteins in *Xenopus* egg extracts. We show that in such a biochemical system, cytoplasmic β-catenin degradation is regulated by soluble components of the Wnt pathway as well as small molecules.

Key words *Xenopus laevis*, Ubiquitin, Proteasome, Egg extract, Axin, β-Catenin destruction complex

1 Introduction

1.1 The Wnt Signaling Pathway

Wnt signaling is a highly conserved pathway critical for metazoan development and tissue homeostasis in adult organisms. Dysregulation of Wnt signaling has been associated with several types of cancers in humans, including colorectal cancer [1]. In the absence of a Wnt ligand, β-catenin is maintained at low levels via the assembly of a destruction complex comprised of the scaffold protein, Axin, the tumor suppressor adenomatous polyposis coli (APC), and the kinases glycogen synthase kinase-3 (GSK3) and casein kinase 1α (CK1α) [2]. This complex acts to phosphorylate β-catenin at specific sites and target it for polyubiquitination by the $SCF^{\beta\text{-}TRCP}$ complex and subsequent degradation by the proteasome. Wnt signaling is activated when Wnt ligands bind to the receptors Frizzled (Fz) and low-density lipoprotein-related receptor 5/6 (LRP 5/6), thereby triggering a cascade that leads to inhibition of β-catenin degradation. Stabilized β-catenin accumulates in the cytoplasm and subsequently translocates into the nucleus to initiate a Wnt-specific transcriptional program. Thus, the

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regulation of β-catenin turnover is critical for the downstream activation of Wnt target genes.

The importance of β-catenin degradation is highlighted by the fact that mutations that inhibit β-catenin degradation by affecting the stability of this complex (e.g., APC and β-catenin) are found in ~90% of nonhereditary cases of colorectal cancer [3]. Several decades of research have highlighted the critical role of Wnt signaling in the earliest events of embryonic development of the African clawed frog, *Xenopus laevis*. Not surprisingly, *Xenopus* egg extract has been shown to contain all the essential components of the β-catenin destruction complex, and it offers a robust in vitro system for studying the biochemistry of cytoplasmic Wnt regulation [4–12].

1.2 Use of Xenopus Egg Extracts to Study the Wnt Signaling Pathway

Extracts are a cell-free, biologically active cytoplasm that mimic the cellular environment. *Xenopus* eggs are collected, and through a series of centrifugation steps, the cytoplasmic fraction is isolated in its undiluted form. *Xenopus* egg extract contains all of the cytoplasmic proteins, organelles, and other components at or near physiological levels, such that complex cellular pathways often remain intact.

An advantage of *Xenopus* egg extract is that β-catenin levels can be readily measured without interference from changes in its steady-state levels because these extracts lack the capacity to support transcription or translation in the absence of supplementation. Conversely, components of the pathway (e.g., Axin) can be added as recombinant proteins into the system to influence the kinetics of β-catenin degradation [4, 7]. In addition, components can be immunodepleted using antibodies to conduct loss-of-function studies. Accordingly, *Xenopus* egg extracts have provided important insight into mechanistic details of Wnt signaling.

1.3 Xenopus Egg Extract as a Tool for Drug Discovery

Studies have successfully used *Xenopus* egg extracts as a tool to identify small-molecule effectors [8, 13–16]. This system is ideal for small molecule discovery as it contains native forms of all of the relevant protein components of the Wnt pathway. Extract-based assays targeting the Wnt pathway have identified molecules such as pyrvinium, a potent Wnt inhibitor that promotes β-catenin degradation via enhancement of CK1α activity [13]. Thus, *Xenopus* egg extracts provide a novel and useful tool for the discovery of modulators of the Wnt pathway.

2 Materials

1. Pregnant mare serum gonadotropin (PMSG): 250 U/ml stock.
2. 20× Marc's Modified Ringer solution (MMR): 2 M Sodium chloride, 40 mM potassium chloride, 40 mM calcium chloride,

20 mM magnesium chloride, and 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4.

3. Human chorionic gonadotropin (HCG): 1000 U/ml.
4. 2%l-Cysteine, pH 8.1.
5. Leupeptin, pepstatin, aprotinin mixture (LPA).
6. Cytochalasin D.
7. Anti-Axin antibody.
8. Protein A beads.
9. [^{35}S] Methionine.
10. In vitro translation kit.
11. 20× Energy regeneration mix: 150 mM Creatine phosphate, 20 mM ATP, 600 μg/ml creatine phosphokinase, 20 mM $MgCl_2$.

3 Methods

3.1 Harvesting Xenopus Eggs

3.1.1 Inject Female Xenopus laevis

1. Hold the female *Xenopus* frog firmly with one hand, with its head facing towards your wrist and one leg between your thumb and index finger, and the other leg between your index and middle fingers.
2. Locate the site of injection, the dorsal lymph sac, which is adjacent to the lateral line and around 1 cm from the midline.
3. Using a hypodermic syringe with a 25 G needle, inject 100 U of freshly made PMSG subcutaneously into the frog. Be sure to insert the needle with the bevel side up, from the rear, with the tip of the needle pointing towards the head of the frog. Once the puncture is made with the needle, insert it about 1 cm forward from the puncture site between the skin and the muscle. Gently expel the PMSG solution. Once injection is complete, slowly pull out the needle. Inject 10–20 female frogs.
4. After injection, return the primed frogs to 16 °C water for 5–10 days. In the meantime, make fresh 0.5× MMR solution from a 20× stock. Prepare 4 L tanks filled with 16 °C 0.5× MMR. At the end of priming with PMSG, place one frog into each of the 4 L tanks. Do not place more than one frog per tank to eliminate the time-consuming task of removing poor-quality eggs in case one of the frogs should lay a bad batch.
5. After 5–10 days, inject 1000 U of HCG in the same manner as done for the PMSG. Place the frogs in a 16 °C incubator and wait for 16 h before the females start laying eggs directly into the tank water. In order to obtain large amounts of eggs, gently massage the abdomen and sides of the frogs to expel more eggs.

3.1.2 Harvest Xenopus Eggs

1. After the frogs have laid the eggs, remove the eggs and most of the MMR from the tank, leaving approximately 1–200 ml of MMR plus the eggs in the tank (*see* **Note 1**).
2. Evaluate the quality of the eggs and remove any poor-quality ones with a transfer pipette. In general, discard the entire batch if poor-quality eggs exceed 10% of the total. After discarding bad eggs from each of the 4 L tanks, collect all remaining good eggs and combine them in a 500 ml beaker. Remove as much MMR as possible while at the same time keeping the eggs fully submerged. To wash the eggs, add two egg volumes of 0.5× MMR and swirl the beaker, removing any debris and remaining poor-quality eggs. Repeat this washing step at least once.
3. To de-jelly the eggs, pour out as much MMR as possible and add 100 ml of 2% cysteine, pH 8.1 solution to the beaker, mix by swirling, and allow the eggs to settle for 1–2 min. The detached jelly will float to the top of the eggs and make the eggs more compact. Pour off the cysteine solution with the detached jelly. Repeat the process of adding the cysteine solution, allowing the eggs to de-jelly, and pouring off the solution until the eggs have become tightly packed. This usually happens at the end of the third cysteine treatment.
4. Wash the eggs with 100 ml 1× MMR a total of ten times. The solution should become increasingly clear, typically at the end of the second wash. Finally, wash with 30 ml of 0.1× MMR and pour off the MMR solution. Maintain the eggs in 0.1× MMR.

3.2 Preparation of Xenopus Egg Extract

A summary of *Xenopus* egg extract preparation is shown in Fig. 1.

3.2.1 Centrifugation of Xenopus Eggs

1. Add the LPA protease inhibitor at 10 μg/ml and Cytochalasin D at 20 μg/ml into 20 ml of 0.1× MMR. Add this 0.1× MMR solution directly to the eggs, swirl, and incubate at 16 °C for 5 min.
2. Use a transfer pipette to transfer the eggs to pre-chilled 50 ml centrifuge tubes at 4 °C. Wait for the eggs to settle and then remove any extra buffer from the top. Repeat the process by adding additional eggs to the centrifuge tube until it is filled.
3. For the packing spin, spin the centrifuge tubes at 400 × *g* for 30 s at 4 °C. After spinning, remove any extra buffer from the top of the centrifuge tubes.
4. For the crushing spin, spin the centrifuge tubes at 15,000 × *g* for 5 min at 4 °C.

3.2.2 Collect and Prepare the Cytoplasmic Layer of Extract

1. To collect the cytoplasmic layer of the extract, use a P1000 pipette tip to puncture through the lipid layer, which is the topmost layer in the centrifuge tube. Then, using a new P1000

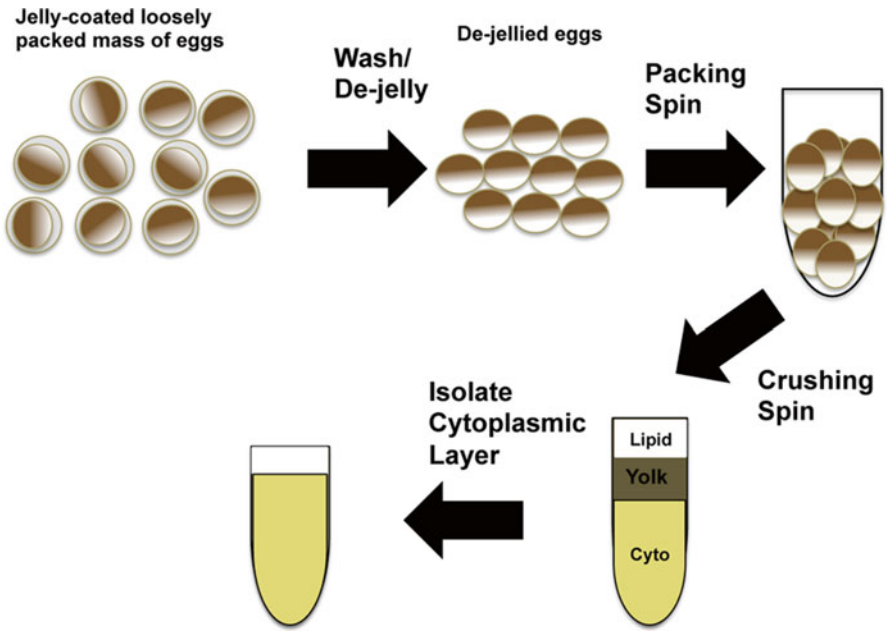

Fig. 1 Preparation of *Xenopus* egg extract. Flow chart indicating individual steps to prepare *Xenopus* egg extract. Frog eggs are covered with a sticky jelly coat that must be removed prior to extract preparation. Harvested eggs are washed and de-jellied with cysteine before performing a packing spin to remove residual buffer. The crushing spin separates the egg extract into a cytoplasmic, yolk, and lipid layers. The cytoplasmic layer is isolated and used to perform β-catenin degradation assays

pipette tip, proceed through the punctured lipid layer to reach the cytoplasmic layer and begin withdrawing.

2. Collect the cytoplasmic layer in a pre-chilled centrifuge tube at 4 °C.
3. Spin the tubes containing the cytoplasmic layer at 15,000 × *g* for 10 min at 4 °C.
4. Using the same method, collect the cytoplasmic layer again and repeat the spinning step to further isolate the layer. At the end of this step, the cytoplasmic extract should be straw colored and uniform (*see* **Note 2**).
5. Add LPA and Cytochalasin D to the cytoplasmic extract with a final concentration of 10 μg/ml for each.
6. Snap freeze the tubes in liquid nitrogen and transfer to a −80 °C freezer for long-term storage.

3.3 Depletion of Extracts

In order to experimentally control perturbations of the Wnt signaling pathway, *Xenopus* egg extracts can be depleted of individual protein components. This depletion allows the investigator to add back known amounts of the component in order to determine its kinetic effect on β-catenin degradation.

3.3.1 Deplete Xenopus Egg Extracts of Endogenous Axin

1. Conjugate anti-Axin (rabbit IgG) to protein A beads as per the manufacturer's protocol.
2. Wash conjugated beads 3× with compatible wash buffer (e.g., 50 mM Tris pH 7.4, 150 mM NaCl, 0.1% Triton X-100).
3. Thaw previously prepared *Xenopus* egg extracts on ice.
4. Discard wash buffer after the last spin. Add extract directly to washed beads in a 1:10 ratio.
5. Incubate the extract with the beads on a rotating platform for 1 h at 4 °C.
6. After incubation, spin the extract-bead mixture at 12,000 × *g* for at least 1 min at 4 °C.
7. The supernatant contains extract depleted of endogenous Axin protein. Transfer this depleted extract to a new microcentrifuge tube on ice and confirm depletion of Axin by immunoblotting using an appropriate anti-Axin antibody. Depleted extract may be aliquoted and snap frozen prior to storage at −80 °C.

3.4 Degradation Assay

The use of extracts to determine the kinetics of β-catenin degradation has played an important role in our understanding of Wnt signaling under normal and pathological conditions. Further, depletion of extracts to determine the effects of individual components of the pathway on β-catenin degradation has provided many mechanistic insights into the pathway.

3.4.1 Prepare Radiolabeled β-catenin

1. Prepare [^{35}S]β-catenin protein using a commercially available in vitro translation kit as per the manufacturer's protocol.
2. Confirm successful translation of radiolabeled protein by performing autoradiography. Subject the in vitro-translated (IVT) protein (0.5–1 μl) to SDS-PAGE gel. Fix the gel in 50% methanol/20% acetic acid, and dry the gel on Whatman paper. Perform autoradiography to assess successful incorporation of [^{35}S] into the IVT protein.
3. The IVT-protein may be stored at −80 °C for up to 2 months without significant loss of radioactivity.

3.4.2 Prepare Egg Xenopus Extract for Degradation Assay

1. Thaw *Xenopus* egg extracts quickly in hands, and then place immediately on ice. Thaw 20× energy regeneration mix and store on ice.
2. Add 10 μl of 20× ER per 200 μl *Xenopus* egg extract. Mix thoroughly. Spin down the extract quickly and place on ice until ready for use in the degradation assay. Aliquots can also be stored at −80 °C and thawed immediately before use.

3.4.3 Perform Degradation Assay

1. Add 1–5 μl of [^{35}S] radiolabeled β-catenin, depending on radiolabeling efficiency, to 20 μl of *Xenopus* egg extract (containing energy regeneration mix). If small molecules or

proteins are being tested, add appropriate volumes such that the volume of such components is less than 10% of the total reaction volume.

2. Mix the reaction mixture by vortexing in short bursts (2–3×) and pulse spinning. Place mixture on ice.
3. Begin degradation assay by incubating tubes at room temperature.
4. Perform time points by removing 1–5 μl of the mixture and stopping the reaction with SDS sample buffer. Vortex the tubes vigorously and boil the samples at 95 °C.
5. Run degradation assay samples on SDS-PAGE gels and perform autoradiography with suitable film (Fig. 2). Results may be quantified using imaging software such as ImageJ.

3.4.4 Perform β-Catenin Luciferase Degradation Assay

1. Make IVT luciferase-tagged β-catenin as per the manufacturer's protocol.
2. Confirm successful translation of non-radiolabeled, luciferase-tagged β-catenin by performing a luciferase assay to measure activity. As controls, perform the luciferase assay using translated luciferase protein or untranslated IVT reaction mixture.

   Commercial kits are available for measuring luciferase activity.
3. Add luciferase-tagged β-catenin to 20 μl of *Xenopus* egg extract (containing energy regeneration mix) such that the luciferase activity is ~50,000 relative luciferase units (RLU)/μl of extract as measured by the luciferase assay in Subheading 3.4.4, **step 2**. Mix well mixture by vortexing in short bursts (2–3×) and pulse spinning.
4. Perform the degradation assay at room temperature and remove aliquots of the reaction at desired time points. The volume of aliquots removed is typically calculated as the

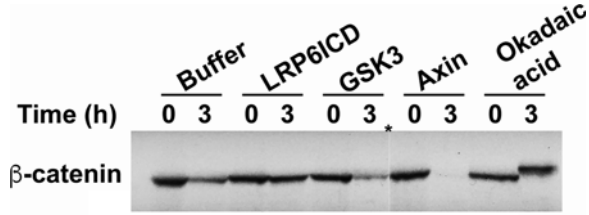

Fig. 2 Reconstitution of radiolabeled β-catenin degradation in *Xenopus* egg extracts. LRP6ICD inhibits, whereas GSK3 and Axin stimulate, the turnover of radiolabeled β-catenin in *Xenopus* egg extracts. In vitro-translated [^{35}S]β-catenin was added to *Xenopus* egg extracts and incubated with buffer, LRP6ICD (1.5 μM), GSK3 (1 μM), Axin (50 nM), and okadaic acid (1 μM). At the indicated times, aliquots were removed and subjected to SDS-PAGE/autoradiography. LRP6ICD is the intracellular domain of the Wnt co-receptor, LRP6. *, Indicate intervening lanes removed

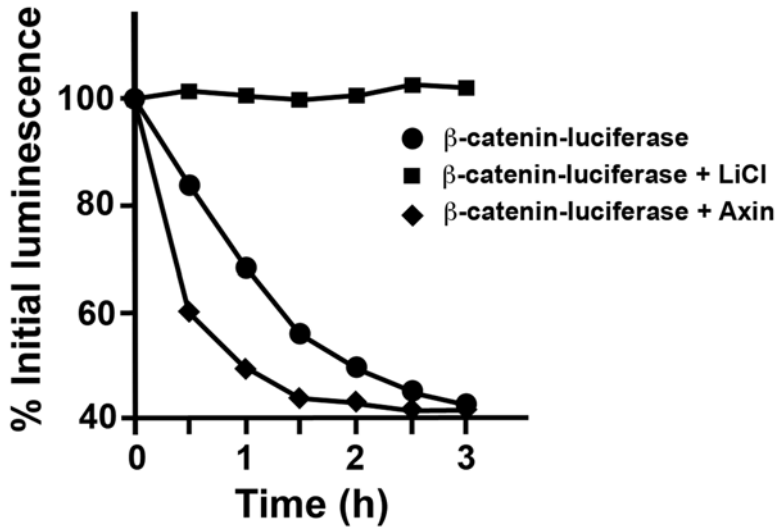

Fig. 3 Reconstitution of β-catenin-luciferase degradation in *Xenopus* egg extracts**.** In vitro-translated β-catenin-luciferase fusion was incubated in *Xenopus* egg extracts in the presence of buffer, Axin (50 nM), or lithium chloride (20 mM; inhibitor of GSK3). At the indicated times, an aliquot was removed and luciferase activity measured. Background signal observed in the β-catenin-luciferase degradation assay is due to free luciferase protein, which degrades slowly

volume that is sufficient to yield at least 5000 RLU at the start of the reaction. Snap freeze these aliquots in liquid nitrogen for storage at −80 °C.

5. To analyze samples, thaw the samples on ice, and immediately measure luciferase activity as described in Subheading 3.4.4, **step 2** (Fig. 3).

4 Notes

1. High-quality eggs should have a clear separation between the darkly pigmented animal hemisphere and the lightly pigmented vegetal hemisphere. Poor-quality eggs are blotchy, and puffy, and/or are part of a large, stringy mass of eggs.
2. If contamination from the lightly colored lipid layer or the darkly colored pigmented layer should occur repeat the spinning and extraction protocol.

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Chapter 12

Delivery of the Porcupine Inhibitor WNT974 in Mice

Li-shu Zhang and Lawrence Lum

Abstract

We describe here a technique for delivering the porcupine inhibitor WNT974 (formerly LGK974) in mice. The protocol entails once-a-day oral delivery of WNT974 for up to 3 months at a concentration sufficient to achieve systemic Wnt pathway inhibition with limited toxicity as measured by weight change. This route of delivery enables extended durations of Wnt signaling inhibition in a mammalian model organism.

Key words Porcupine, Wnt974/LGK974, Mice, Drug delivery

1 Introduction

WNT974 (also known as LGK974) is a first-in-class picomolar inhibitor of the Wnt acyltransferase porcupine (Porcn) (Fig. 1) [1]. The compound is currently being evaluated as an anticancer agent against a broad range of diseases associated with deviant Wnt signaling. Although some cancer-associated mutations such as loss of the Wnt receptor ubiquitin ligase RNF43 are anticipated to promote Wnt ligand-mediated signaling in certain types of cancers [1–5], an incomplete inventory of Wnt pathway regulators suggests that genetic mutations yet to be unidentified may give rise to tumors that would be responsive to Porcn inhibitors. At the same time, regenerative medicine goals may see advances with the help of Wnt pathway modulators such as WNT974 by facilitating wound healing [6]. Thus, preclinical testing of compounds such as WNT974 in mouse models of disease is critical to a broader understanding of chemical utility.

A longstanding concern associated with chemically disabling Wnt signaling is the compromising of the intestinal epithelial lining that depends on Wnt ligands for renewal [7]. Although suppression of Wnt pathway activity with inhibitors targeting the Tankyrase enzymes which indirectly control β-catenin abundance

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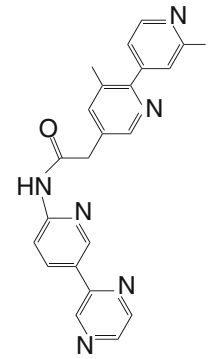

Fig. 1 The chemical structure of WNT974

by regulating the stability of its destruction complex is anticipated to have a poor therapeutic index, porcupine inhibitors appear to be relatively well tolerated [8, 9]. Thus, Porcn inhibitors such as WNT974 enable a variety of preclinical studies focused on Wnt signaling while limiting the potential influence of unwanted side effects on test outcomes. This protocol will provide an accessible approach for delivering WNT974 in animals by gavage and should be useful for a broad range of preclinical studies in mice that require short- or long-term suppression of Wnt-mediated signaling.

2 Materials

2.1 Drug Preparation

1. WNT974 (*see* **Note 1**). Store at –20 °C.
2. Dimethylacetamide (DMA).
3. Cremophor EL.
4. Tween 80.
5. Dextrose solution: 5% Dextrose (w/v) in distilled water. Store at 4 °C.
6. Carboxymethylcellulose solution: 0.5% Carboxymethylcellulose (w/v) added to the dextrose solution. Store at 4 °C.
7. 50 ml Conical tubes.
8. 3.7 ml Glass vials with screw thread.
9. Analytical balance, readability 0.01 mg.
10. Vortex mixer.

2.2 Drug Delivery

1. Mice, normal or disease models (*see* **Note 2**).
2. Feeding needles, plastic or metal.
3. 1 ml Disposable syringes.
4. Soap solution: Add 1 ml hand soap to 49 ml distilled water, and mix well.
5. Ethanol solution: Add 35 ml 100% ethanol to 15 ml distilled water, and mix well.

3 Methods

An animal protocol must be approved by the IACUC prior to the start of WNT974 delivery in vivo. Animal research training and badge access to the animal facility are also required. For this protocol, we will use as an example 20 mice randomly divided into two cohorts henceforth referred to as control or WNT974 group. In addition, we will be dosing animals at 5 mg/kg.

3.1 Drug Preparation

1. Randomly group animals into two cohorts. Weigh each animal and calculate the average body weight (BW) for each group one day before dosing begins. For example, we will use an average body weight of 25 g for each group.
2. Calculate the amount of WNT974 required for one dose using the average body weight: 5 mg/kg × (25 g/1000) = 0.125 mg. The dosing volume for each mouse is 200 μl. Keep in mind that animal weight may change, thus necessitating modulation of daily total compound needs as the study progresses.
3. Remove WNT974 from a −20 °C freezer and wait until the vial reaches room temperature. Weigh sufficient amount of compound for the total number of animals to be injected plus one extra dose in case of drug loss using the calculation from "2." Place compound into a glass vial (*see* **Note 3**). For the example used in this protocol, this would be 0.125 mg × 11 = 1.375 mg.
4. Dropwise add DMA with gentle vortexing to achieve a final concentration of 5%. In this case, since we dissolve 1.375 mg of WNT974 into a total volume of 2.2 ml, this would be equal to 5% × 2.2 ml = 121 μl. The solution should be clear.
5. Dropwise add Cremophor EL with gentle vortexing to a final concentration of 9% to the WNT974 in DMA. In this case, that would be 217.8 μl.
6. Dropwise add Tween 80 with gentle vortexing to the WNT974/DMA/Cremophor EL solution to achieve a final 1% concentration. In this case, this would be 24.2 μl.
7. Dropwise add carboxymethylcellulose solution with gentle vortexing to the WNT974/DMA/Cremophor EL/Tween solution to reach a final 85% concentration. In our example, this would be 2057 μl. The WNT974 solution should be used within 2 h of preparation.
8. Prepare the vehicle solution (without WNT974) for the control group similarly. The vehicle solution should be used within 2 h of preparation.

3.2 Drug Delivery

1. Don appropriate personal protective apparel as required by the animal facility prior to entering the facility (facemask, gown, booties, gloves, etc.).

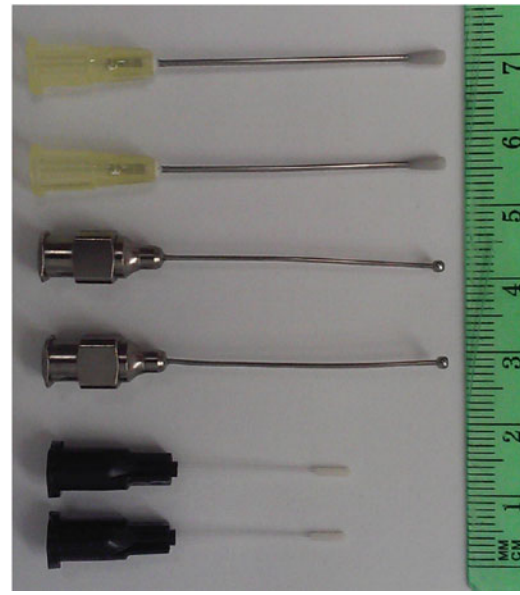

Fig. 2 Feeding needles. Three different types of feeding needles are shown. The *upper* two needles are disposable and the *middle* two are reusable. Both are suitable for dosing mice older than 6 weeks old. The *lower* two needles are disposable and suitable for dosing mice younger than 6 weeks old

2. Disinfect both gloved hands and the surface area one will work on.
3. Draw 1 ml of the WNT974 or vehicle solution into a 1 ml syringe attached to a feeding needle (Fig. 2). Dislodge the air bubbles by gently tapping the side of the syringe with the feeding needle pointing upwards.
4. Take one mouse out of the cage. Hold the mouse upright with one hand (Fig. 3). Insert the feeding needle gently into the mouth with the other hand. Allow the needle to slide into the esophagus and the opening of the stomach (*see* **Note 4**).
5. Push the plunger with the forefinger slowly but firmly to inject 200 μl of the solution into the stomach.
6. Remove the needle and release the animal back into its cage.
7. Wash the needles after all of the mice have been gavaged. Flush the needles four to six times each in the following sequence: 2% soap solution, distilled water, and 70% ethanol. Store the needles in clean 50 ml conical tubes for future use.
8. Repeat this procedure for the total number of days desired. We have dosed mice daily (6 days a week) with 5 mg/kg WNT974 for up to 3 months without noticeable toxicity as measured by weight loss.
9. Weigh mice three times a week. Do not forget to adjust the amount of WNT974 required per dose according to weight change in animals.

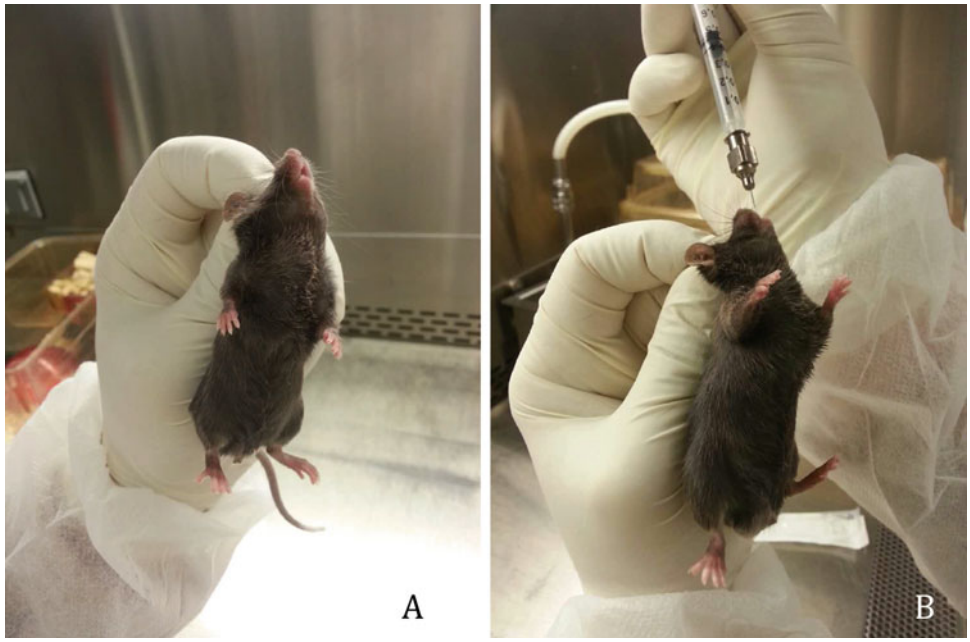

Fig. 3 Oral gavaging. Holding mouse firmly (**a**) is the most important step for oral gavage. If the mouse's head or body moves freely, one will have to release and hold the mouse again. Next, allow the needle to slide into the esophagus and the opening of the stomach (**b**). Stop immediately if one feels blockage from the front end of the needle and re-try. In some cases, one will need to release the mouse and start again

4 Notes

1. When planning the experiment with the compound WNT974, it is advisable to purchase enough compound for the entire experiment to avoid the batch-to-batch differences. The activity of the compound should be tested in vitro before in vivo administration to ensure activity. Two routine methods can be used to evaluate WNT974 activity: detecting Wnt-induced transcriptional responses using culture cells expressing Wnt3a and harboring the Wnt-specific reporter known as Super Top Flash (STF reporter assay) [10] or monitoring levels of phospho-Dvl2 protein in a cell line such as HeLa cells that have cell-autonomous Wnt signaling [11].
2. For gavaging 4- to 6-week-old mice, one should use smaller feeding needles (*see* Fig. 2) to avoid possible trauma to the esophagus. For gavaging mice younger than 4 weeks old, one may expect loss of some animals.
3. After calculating the amount of WNT974 for each group per dosing, we measure out the total WNT974 powder directly into a glass vial placed on an analytical balance. Of course, zero the balance with the empty glass vial first before adding compound.

For convenience, prepare 12 aliquots each sufficient for a day's worth of dosing. The aliquots can be stored at −20 °C. Reserve an extra amount of WNT974 for each dose in order to compensate for drug loss and changes in average animal weight. Typically, we include additional compound sufficient for two extra animals per aliquot.

4. Take the feeding needle out of the mouth if one feels blockage. Release the mouse and hold it again to let the needle slide into the mouse. It is strongly advised to practice gavaging prior to initiating an experiment. Holding the mouse upright firmly but not too tightly is key to successful gavaging.

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Chapter 13

Use of Primary Calvarial Osteoblasts to Evaluate the Function of Wnt Signaling in Osteogenesis

Zhendong A. Zhong, Nicole J. Ethen, and Bart O. Williams

Abstract

In vitro culture and genetic manipulation of primary calvarial cell cultures is a convenient and robust system to investigate gene function in osteoblast differentiation. We have used this system to study the functions of many genes in the Wnt signaling pathway within osteoblasts. Here, we describe a detailed protocol outlining the establishment and characterization of primary calvarial cells from mice carrying a conditionally inactivatable allele of the Wntless (Wls) gene ($Wls^{flox/flox}$). We previously used this approach to delete the Wntless gene by infecting with a Cre-expressing adenovirus, and to evaluate the effects of Wnt signaling loss on osteogenic potential in osteogenic medium with ascorbic acid. This detailed protocol is adaptable to use with any floxed allele.

Key words Calvarial cell, Osteogenic differentiation, Adenovirus, Alkaline phosphatase, Alizarin red

1 Introduction

The past decade has seen the accumulation of evidence supporting a role of Wnt signaling in regulating bone development and homeostasis [1–3]. Therefore, understanding how the Wnt signaling pathway regulates skeletal homeostasis is of great value for human skeletal health. Although genetically engineered mouse models (GEMMs) have been and are being utilized to discover mechanisms and etiology of bone diseases in the context of Wnt signaling, cell in vitro osteogenic differentiation is routinely used to further investigate the molecular mechanism [4–6].

Use of Cre-expressing adenovirus (Ad-Cre) to transiently express Cre in cells homozygous for a floxed allele of a gene cells can permanently delete the gene. Calvarial cells can be very efficiently infected with adenovirus, providing a very useful system to assess gene function within osteoblasts. Our laboratory has used this system to study the roles of numerous genes in the Wnt pathway over the last decade [5–7] and provide a detailed protocol to facilitate the use of this system by other laboratories.

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2 Materials

These procedures have been adapted from previous reports [7, 8].

2.1 Calvarial Cell Isolation

1. Sterile 1× phosphate-buffered saline (PBS), pre-warmed to 37 °C in a water bath.
2. Minimum essential medium alpha + 10% fetal bovine serum +1% penicillin/streptomycin (P/S0 (*see* **Note 1**).
3. Type I Collagenase at ~200 U/mL.

   Dissolve 180 mg of collagenase in 100 mL of 1× PBS, filter with 0.2 μM filter, and warm to 37 °C in a water bath.
4. 70 μM Cell strainer.
5. Dissection scissors and forceps.
6. Temperature-controlled shaker.

2.2 Gene Knockout with Adenoviral Infection

1. Ad-Cre and Ad-GFP stocks are diluted to a concentration of 1.0×10^7 PFU/μL [6].

2.3 Osteogenic Differentiation

1. L-Ascorbic acid (*see* **Note 2**).
2. β-Glycerophosphate disodium salt hydrate (βGP).
3. Minimum essential medium alpha + 10% fetal bovine serum +1% P/S.
4. Osteogenic medium: αMEM, 10% FBS, 1% P/S, 50 μg/mL ascorbic acid, 10 mM β-glycerol phosphate (βGP). Filter it through a 0.2 μM filter before use.

2.4 Alkaline Phosphatase Quantification

1. 1-Step™ NBT/BCIP Substrate Solution (ThermoFisher Scientific, Cat # 34042).
2. Formalin, RIPA cell lysate buffer, protein quantification reagents.
3. A visible absorbance microplate reader.

2.5 Alizarin Red Staining

1. Alizarin Red S solution (40 mM solution prepared in dH2O, pH adjusted to 4.1 with 10% (v/v) ammonium hydroxide).
2. Ethanol.

2.6 Von Kossa Staining

1. Von Kossa solution: 3% Silver nitrate in distilled water.
2. Formalin.

3 Methods

We used Cre-expressing adenovirus (Ad-Cre) to infect *Wls*$^{flox/flox}$ calvarial cells to delete the *Wls* gene, while GFP-expressing adenovirus (Ad-GFP) was used as a control to infect cells of the same

genotype. Alkaline phosphatase (ALP) is one of the early markers of osteoblast activity. ALP staining could be performed on culture plates for imaging, or cell lysate for quantification. Alizarin Red Staining is used for detecting calcium deposition on extracellular matrix, which usually can be performed after 14 days of osteogenic differentiation. Von Kossa Staining is used for detecting phosphate deposition on extracellular matrix, which usually can be performed after 14 days of osteogenic differentiation.

3.1 Calvarial Cell Isolation

1. Set up matings to generate neonatal mice homozygous for the floxed allele of interest.
2. Euthanize a 1–3-day-old neonatal mouse by decapitation and sterilize the head by soaking in 70% ethanol for 2 s.
3. Isolate calvarium by slipping scissors under the skin at the back of the neck and moving forward to break the skin and reveal the calvarium. Puncture the sagittal sutures and cut along the calvarium. Transfer to a 100 mm Petri dish containing ice-cold PBS with fine forceps and clean off any associated connective tissue or brain (*see* **Note 3**) (Fig. 1).
4. When all calvaria are collected into ice-cold PBS, cut many slits into individual calvarium and place it in a 50 mL Erlenmeyer flask with 10 mL of pre-warmed PBS (*see* **Note 4**).
5. Shake at 37 °C for 10 min at ~90 oscillations/min.
6. Carefully aspirate the PBS and repeat two times.
7. Add 10 mL of collagenase solution and shake for 10 min at 37 °C (*see* **Note 5**).
8. Allow the calvarium to settle and then carefully remove the collagenase solution.
9. Repeat **steps 6** and 7. The first two cell populations are not enriched for (pre-) osteoblasts, so they can be discarded.
10. Add 10 mL of collagenase solution and shake for 15 min at 37 °C.

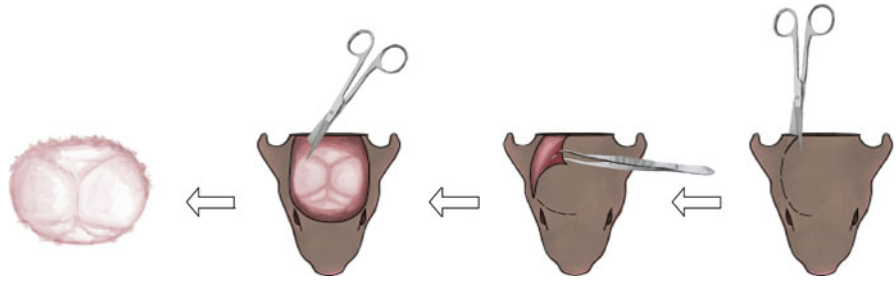

Fig. 1 Neonatal calvarial dissection**.** The calvarium is exposed by slipping scissors under the skin at the back of the neck and moving forward to break the skin. Puncture the sagittal sutures and cut along the calvarium to isolate for digestion and establishment of cultures

11. Collect the supernatant collagenase solution, and pass it through a cell strainer into a 50 mL conical to collect population 3. Place on ice.
12. Repeat **steps 9** and **10** twice to collect populations 4 and 5.
13. Centrifuge the pooled digestions (populations 3, 4, and 5) for 10 min at 500 × *g*.
14. Resuspend the pellets in αMEM + FBS + P/S and plate to 100 mm tissue culture plates (*see* **Note 6**).

3.2 Gene Knockout with Adenoviral Infection

1. Dilute Ad-Cre and Ad-GFP viral stocks to achieve the determined optimal MOI in 3 mL of PBS. The optimal multiplicity of infection (MOI) of adenovirus should be determined in advance for the most effective deletion. Calvarial cells obtained from the mT/mG cre reporter mouse [9] are very useful for this assessment. Typically MOIs of 25, 50, or 100 worked well for most of our experiments. We typically start seeing GFP-positive cells in Ad-GFP-infected cells 12 h after infection (Fig. 2a). Genomic DNA and protein were collected to assess the *wls* gene deletion by PCR and Western blot analysis (Fig. 2b, c—adapted from [6]).
2. Wash isolated calvarial cells in 100 mm plates (Passage 0) at 70–80% confluency (~2×10^6 osteoblasts) with 10 mL of PBS to remove serum proteins (*see* **Note 7**).
3. Add the Ad-Cre or Ad-GFP in 3 mL of PBS solution to the corresponding plates, and incubate at 37 °C for 60 min. Shake the plates gently by hand every 15 min to ensure complete coverage of plate with solution during incubation (*see* **Note 8**).
4. Add 10 mL of culture media directly to the virus-laden PBS solution for each plate. Serum proteins will stop the viral infection (*see* **Note 9**).
5. Allow the cells to recover for 48 h, and to express the CRE or GFP transgene and remove the floxed gene (*see* **Note 10**).
6. Subculture for assessment of confirmation of gene deletion and osteogenic differentiation.

3.3 Osteogenic Differentiation

1. Actively dividing cells (48 h after the adenovirus infection) are seeded at ~10,000 cells/cm^2 with normal culture medium (αMEM + FBS + P/S), and allowed to adhere overnight.
2. Replace culture medium with osteogenic medium.
3. Osteogenic medium should be replaced every 2–3 days (*see* **Note 11**). Osteogenesis can be monitored using RT-PCR strategies targeting known bone markers (Fig. 2d).

3.4 Alkaline Phosphatase Quantification

1. Wash cultures with PBS and fix 10 min with 10% formalin in PH 7.0 PBS.
2. Wash with distilled water once.

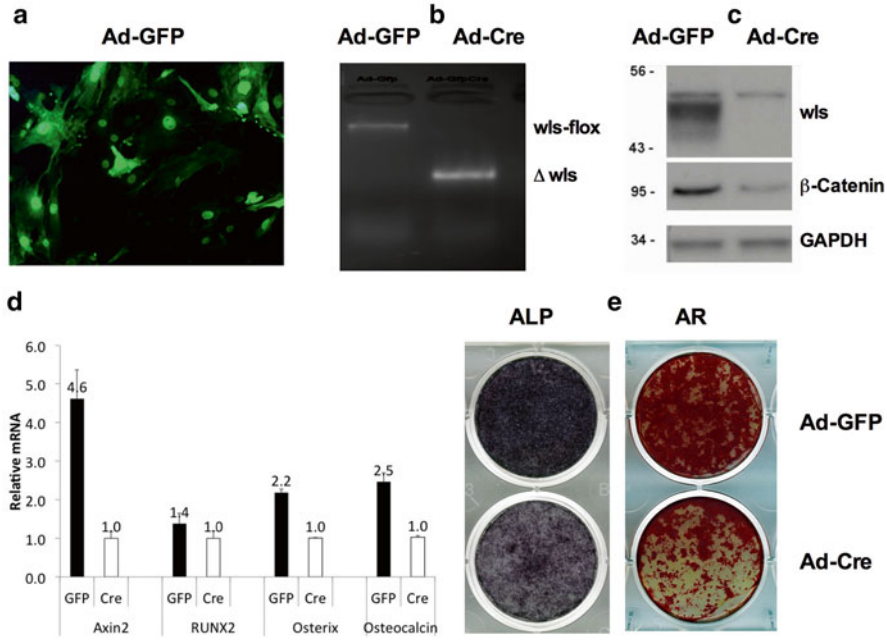

Fig. 2 Adenovirus infection and osteogenic assay on *Wlsflox/flox* calvarial cells (adapted from [6]). (**a**) Calvarial cells were infected with GFP-expressing adenovirus (Ad-GFP). The picture was taken 48 h after infection. (**b**) Genomic DNA was isolated from *wls*-flox/flox calvarial cells with Ad-GFP or Ad-Cre infection. Allele-specific PCR was performed to identify the deletion in the floxed *wls* gene. (**c**) Whole-cell lysates from the calvarial cells were collected 48 h after Ad-GFP or Ad-Cre infection. Immunoblotting was performed with anti-Gpr177 (protein product of the gene *wls*) and anti-β-catenin antibodies; GAPDH was the loading control. The Gpr177 protein was almost absent in Cre-infected cells (a nonspecific band was observed above Gpr177 band). β-Catenin was down-regulated dramatically in Cre-infected cells. Axin2 is a downstream target of Wnt/ β-Catenin signaling, while RUNX2, osterix and osteocalcin are osteoblastic differentiation markers. (**d**) Real-time PCR using the RNA from the *wls*-flox/flox calvarial cells infected with either Ad-GFP or Ad-Cre and differentiated in osteogenic medium for 14 days. (**e**) Representative images of alkaline phosphatase staining (ALP) and alizarin red staining (AR) after 14 days of osteogenic differentiation

3. Add enough NBT-BCIP to each well (1–2 mL for a well of 6-well plates).
4. Incubate at 37 °C for 30 min for reaction (*see* **Note 12**).
5. Wash with distilled water.
6. Dry and scan the plate on a visible absorbance microplate reader.
7. Alkaline phosphatase can also be quantified from cell lyste (*see* **Note 13**). Sample results are provided in Fig. 2e.

3.5 Alizarin Red Staining

1. Rinse cultures with PBS once, and fix with 100% ethanol for 10 min at room temperature.
2. Rinse cultures with dH_2O once.
3. Add 1 mL/well Alizarin Red Solution, and shake very slowly for 30 min at room temperature (*see* **Note 14**).

4. Rinse cultures with tap water until clear, then dry, and scan on a visible absorbance microplate reader. Sample results are provided in Fig. 2e.

3.6 Von Kossa Staining

1. Wash cultures with PBS once, and fix cultures for 10 min with 10% formalin at room temperature;
2. Wash five times with distilled water over 30 min to remove any trace of phosphate from PBS.
3. Stain with Von Kossa solution for 30 min under direct light. UV light in a cell culture hood or other source may speed up the reaction.
4. Wash with distilled water, dry, and scan using a visible absorbance microplate reader. Plates should be stored in the dark.

4 Notes

1. For optimal results, culture media containing no ascorbic acid should be used, such as ThermoFisher Scientific #A10490-01.
2. The half-life of L-ascorbic acid in culture medium was determined to be around 1 h, and 4% of ascorbic acid in medium would be lost every day when stored at 4 °C [10]. Therefore, it is best to prepare fresh stocks of ascorbic acid and βGP with each medium change to prevent breakdown.
3. To enrich for pre-osteoblast cells, it is important to remove the connective tissues at the back of head.
4. Up to 5 calvaria from the same group can be combined in one flask with 20 mL solution for wash or digestion.
5. Cells remain viable for hours in this concentration of collagenase I.
6. As a general rule, seed the cells from 1 to 1.5 calvaria into a 100 mm plate, or cells can be counted and seeded at a density of 2500–5000 cells/cm^2, so that the cells reach 70–80% confluency in 3–4 days.
7. Actively dividing cells are optimal for adenovirus infection.
8. Some cells will detach from the plate during the 1-h incubation in PBS due to the low calcium concentration of calcium. These cells will often re-attach upon the addition of culture media.
9. We recommend adding the culture media to the virus-laden PBS solution rather than removing the virus laden PBS solution to allow cells to re-attach after viral infection.
10. The infection efficiency may be evaluated by observing GFP-positive cells if the mTmG reporter allele has been crossed into mouse strain.

11. Use care not to disrupt the cell monolayer, especially during the later time points while matrix is being deposited and mineralized. Alkaline phosphatase staining is best at days 7 and 12. Mineralization is best at days 14–21. RNA can be isolated as desired. To evaluate all three aspects simultaneously, a single time point at day 14 is best.
12. Usually 30 min is sufficient for ALP staining on wild-type cells after 7 days of osteogenic differentiation. For later time points, the reaction time can be shortened, performed at room temperature.
13. Cells on plates are washed with cold pH 7.0 PBS, and lysed with RIPA cell lysis buffer. More differentiated cells are harder to be lysed. Putting enough RIPA buffer and shaking the plate on ice for up to 1 h will usually give us satisfactory amounts of protein. Add 50 μL per well cell lysate to a 96-well plate, and then add NBT-BCIP solution (5 μL per well). Incubate at 37 °C for 30 min. Read optical density (OD) 570 with a plate reader, and normalize data to the protein content of the corresponding cell lysate.
14. Cells at high density will detach from plates more easily after longer than 2 weeks of culture. Reduce or eliminate shaking the plate to minimize this if necessary.

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Chapter 14

Monitoring Wnt/β-Catenin Signaling in Skin

Amy T. Ku, Qi Miao, and Hoang Nguyen

Abstract

Wnt signaling through β-catenin plays a crucial role in skin development and homeostasis. Disruption or hyperactivation of this pathway results in skin defects and diseases (Lim and Nusse, Cold Spring Harb Perspect Biol 5(2), 2013). Monitoring Wnt signaling in skin under normal and abnormal conditions is therefore critical to understand the role of this pathway in development and homeostasis.

In this chapter, we provide methods to detect Wnt/β-catenin (canonical) signaling in the skin. We present a comprehensive list of Wnt reporter mice and detail the processing of skin tissue to detect reporter genes. From this list, we focus on the three most recent lines that, according to reports, are the most sensitive in skin. Additionally, we describe a protocol to detect nuclear β-catenin, a hallmark of active Wnt signaling, although this technique should be used with caution due to its limited sensitivity. The techniques outlined below will be useful for detecting active Wnt signaling in skin.

Key words Skin, Epidermis, Hair follicles, Wnt reporter mice

1 Introduction

The skin is a self-renewing tissue made up of the stratified epidermis, its appendages and the underlying dermis [1]. The stratified epidermis, which provides protective barrier function of the skin, continually sheds its outermost layer. Cells in the basal layer proliferate, move upward and differentiate to replenish the outermost layer. Whereas the stratified epidermis self-renews continuously, hair follicles undergo periodic degeneration and regeneration, depending on signals from a specific dermal niche, the dermal papilla [2]. In response to wounding, stem cells from both the stratified epidermis and hair follicles contribute to repair of the epidermis [3–9].

Canonical Wnt signaling plays a critical role in skin epidermal development and homeostasis [10]. During morphogenesis, Wnt/β-catenin signaling is required in both dermal and epidermal cells to specify formation of the hair placode and the dermal papilla precursor, the dermal condensate [11]. Subsequently, Wnt/β-catenin signaling is essential in both hair follicular progenitor

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cells and the dermal papilla for hair regeneration to occur [12–15]. Under homeostatic conditions, Wnt/β-catenin controls normal proliferation of the basal cells of the stratified epidermis [16, 17].

Given the crucial role of Wnt/β-catenin signaling in skin biology, monitoring Wnt signaling in the skin is of vital interest to skin researchers. Because canonical Wnt signaling results in the translocation of β-catenin into the nucleus, detection of nuclear β-catenin has been used to demonstrate active Wnt signaling. However, due to the low sensitivity of the assay, nuclear β-catenin may reflect only very high Wnt activity. A more sensitive method is based on the detection of Wnt target genes. Wnt target genes are transcribed when nuclear β-catenin binds to TCF/LEF, which bind to TCF/LEF binding sites of the promoters of Wnt target genes, and transactivate their transcription. In order to detect Wnt activity, a series of Wnt reporter mice have been developed. These mice can be categorized into two groups according to the types of promoter. One group expresses reporter gene *lacZ* or green fluorescent protein (GFP) or its variants under the promoter containing multimerized TCF/LEF binding sites [18–25]. The other group contains the reporter gene knock-in under the control of the endogenous promoter of the Wnt target gene *Axin2*, or inducible cre-recombinase (creERT2) knocked into the *Axin2* locus and used with Rosa26 reporter lines [17, 26–30].

In this chapter, we summarize the current Wnt reporter strains used to monitor canonical Wnt signaling and provide methods for detecting different reporter genes in the skin. We also provide an immunostaining protocol for detecting nuclear β-catenin.

2 Materials

2.1 Wnt Reporter Mice

See Table 1. We used wild-type C57BL/6 mice, TCF/LEF:H2B-GFP mice [25], BAT-GAL mice [20], and *Axin2*^{lacZ} mice [27] as specific examples in this chapter. Low Wnt activity is detected in basal cells of the epidermis only in TCF/LEF:H2B-GFP mice and Wnt reporter mice with *Axin2* promoter [16, 17].

2.2 Tissue Embedding Components

1. Electric clipper.
2. 4-in. scissors.
3. 4-in. dissecting forceps.
4. Razor blade.
5. Brown paper towels.
6. Cryomolds 25 x 20 mm.
7. O.C.T. compound.
8. Dry ice block.

Table 1
Wnt reporter mice

Transgenic reporter mice with promoter containing multimerized TCF/LEF binding sites		
	Promoter	**References**
TOP-GAL	3 TCF/LEF binding sites with c-fos minimal promoter	DasGupta and Fuchs, 1999 [18]
TOP-lacZ	3 TCF/LEF binding sites with TK minimal promoter	Staal et al., 2001 [19]
BAT-GAL	7 TCF/LEF binding sites with siamois minimal promoter	Maretto et al., 2003 [20]
TCF/LEF-lacZ	6 TCF/LEF binding sites with hsp68 minimal promoter	Mohamed et al., 2004 [21]
BAT-lacZ	8 TCF/LEF binding sites with minimal siamois promoter	Nakaya et al., 2005 [22]
Ins-TOPGAL	6 TCF/LEF binding sites with TK minimal promoter	Moriyama et al., 2007 [23]
Ins-TOPEGFP	6 TCF/LEF binding sites with TK minimal promoter	Moriyama et al., 2007 [23]
LEF-EGFP	7 TCF/LEF binding sites with c-fos minimal promoter	Currier et al., 2010 [24]
TCF/LEF:H2B-GFP	6 TCF/LEF binding sites with hsp68 minimal promoter	Ferrer-Vaquer et al., 2010 [25]
Reporter mice with Wnt target gene promoter (Axin2)		
Axin2-EGFP	5.6 kb genomic fragment containing promoter and first intron of Axin2	Jho et al., 2002 [26]
$Axin2^{lacZ}$	Endogenous Axin2 promoter	Lustig et al., 2002 [27]
$Axin2^{creERT2}$;$Rosa26R^{mTmG}$	Endogenous Axin2 promoter	van Amerongen et al., 2012; Muzumdar et al., 2007 [28, 29]
$Axin2^{creERT2}$;Rosa26RlacZ	Endogenous Axin2 promoter	van Amerongen et al., 2012; Soriano 1999 [28, 30]
$Axin2^{creERT/tdT}$;$Rosa26R^{mTmG}$	Endogenous Axin2 promoter	Choi et al., 2013; Muzumdar et al., 2007 [17, 29]

2.3 Tissue Fixation Reagents and Components

1. 30% sucrose solution. Filtrate with 0.45 um filter. Store at 4 °C.
2. 10% sucrose solution. Filtrate with 0.45 um filter. Store at 4 °C.
3. Phosphate-buffered saline (PBS).
4. EMS 16% paraformaldehyde (PFA) in 10 mL glass ampules.
5. Falcon 5 mL 12×75 mm snap-cap polypropylene tubes.
6. Aluminum foil.
7. Shaker.

2.4 Tissue Sectioning and Staining Components

1. Cryostat.
2. Adhesion Micro Slides.
3. Microscope cover glass.
4. Hematoxylin 2.
5. Glass staining jar.
6. 10% Triton X-100 solution.
7. 20% bovine serum albumin (BSA). Filtrate with 0.22 um filter, aliquot, and store at −20 °C.
8. Gelatin from cold water fish skin (Sigma).
9. 10% normal donkey serum (NDS).
10. PBSGT buffer: 2% fish gelatin, 0.2% Triton, in PBS.
11. Blocking buffer: PBSGT buffer with 2% BSA with 10% NDS.
12. Staining buffer: PBSGT buffer with 1% BSA with 5% NDS.
13. Hoechst 33342.
14. ProLong Gold Antifade (Molecular Probes, Thermofisher).
15. Nail polish.

Additional components for immunohistochemistry staining of β-catenin using M.O.M kit

1. Citrus clearing solvent as xylene substitute.
2. Ethanol series: 100, 95, 85, 70, 50%.
3. 10 mM citric acid buffer, pH 6.0.
4. 0.3% Hydrogen peroxidase (H_2O_2). Prepare fresh from 30% hydrogen peroxide.
5. Mouse on Mouse (M.O.M.™) basic kit (Vector).
6. Avidin/Biotin blocking kit.
7. M.O.M blocking buffer with Avidin: Add 45 μL of IgG reagent (from M.O.M. kit), four drops of avidin solution (from Avidin/Biotin Blocking kit) to 1 mL PBSGT buffer.
8. M.O.M. Diluent Buffer with Biotin: Add 100 μL of Protein Concentrate reagent (from M.O.M. kit), four drops of biotin solution (from Avidin/Biotin Blocking kit) to 1 mL PBSGT buffer.
9. M.O.M. Diluent Buffer without Biotin: Add 80 μL of Protein Concentrate reagent (from M.O.M. kit) to 1 mL PBSGT buffer.
10. Vectastain Elite ABC reagent: Prepare fresh. Add one drop of Reagent A to 1.25 mL PBS, mixing well, then adding one drop of Reagent B, then mixing well again. Allow ABC mixture to stand for 30 min at room temperature before use.
11. ImmPACT DAB Peroxidase (HRP) substrate: Prepare fresh. Add one drop of concentrated DAB to 1 mL of the provided diluent buffer.

12. Clarifier 2.
13. Bluing reagent.
14. Permount Mounting Medium.
15. Coplin staining jar.
16. Hybridization oven.
17. Microwave oven.

2.5 Primary and Secondary Antibodies

1. Rabbit α GFP (Molecular Probes, G10362, dilution 1:250).
2. Rabbit α mouse keratin 5 primary antibody.
3. Rat α β-4 integrin (BD, 553745, dilution 1:200).
4. Mouse α β-catenin monoclonal antibody (Sigma, 15B8, dilution 1:500).
5. AF488-conjugated donkey α rabbit secondary antibody.
6. RRX-conjugated donkey α rabbit secondary antibody (2° Abs: affinity-purified whole IgG, cross-adsorbed).
7. RRX-conjugated donkey α rat secondary antibody (2° Abs: affinity-purified whole IgG, cross-adsorbed).

2.6 X-gal Staining

1. 25% glutaraldehyde solution.
2. VECTOR Nuclear Fast Red solution (Vector Lab).
3. 20 mg/mL X gal stock: Dissolve X-gal in N′N′ dimethylformamide (DMF). Store at −20 °C protect from light.
4. 1 M Na-phosphate solution.
5. 1 M $MgCl_2$.
6. 50 mM $K_3Fe(CN)_6$. Store at 4 °C, protect from light.
7. 50 mM $K_4Fe(CN)_6$. Store at 4 °C, protect from light.
8. 10% Na deoxycholate.
9. 10% NP40.
10. 80% Glycerol.
11. X-gal staining solution: 100 mM Na-phosphate (pH 7.3), 1.3 mM $MgCl_2$, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$, 0.01% Na deoxycholate, 0.2% NP40, 2 mg/mL X-Gal.

2.7 Microscopy and Imaging Setup

1. Transmitted light microscope.
2. Fluorescence microscope.

3 Methods

We describe here the use of two types of Wnt reporter mice to monitor the activity of Wnt/β-catenin signaling: TCF/LEF:H2B-GFP [25] and BAT-GAL [20] or *Axin2*^{lacZ} [27]. The reporter genes are either GFP- or β-galactosidase-based. TCF/LEF:H2B-GFP is a

transgenic line expressing histone H2B fused with GFP under a promoter containing 6 TCF/LEF binding sites [25]. BAT-GAL is a transgenic line expressing *lacZ* reporter gene under a promoter containing 6 TCF/LEF binding sites [20]. *Axin2*^{lacZ} line contains the *lacZ* gene inserted into the *Axin2* locus [27]. Immunofluorescent detection of GFP and immunohistochemical detection of β-galactosidase activity are both performed on fresh cryosections. To visualize GFP signal in situ, pre-fixation (fixation before tissue embedding) is recommended to preserve the GFP signal.

3.1 Harvesting Backskin Tissue

1. Sacrifice the mice according to institutional guidelines (*see* **Note 1**) and closely shave all hair on the back with an electric shaver.
2. Mark the midline from neck to tail with a marker. Lift the backskin at the back of the neck with the fine forceps and make an incision. Insert the scissors underneath the skin and cut a rectangle shape from upper back to the lower back. Gently peel the entire backskin away from the body (Fig. 1).
3. Place the skin onto a paper towel with the epidermis side up and the dermis on top of the towel. Use forceps to spread the skin so that midline is straight. When working with multiple mice, align all pieces of skin in the same head-to-tail orientation (*see* **Note 2**).
4. Use a razor blade to cut the skin attached to the paper towel at the midline and on two sides parallel to the midline to yield two strips of skin 0.5 cm × 2.5 cm (*see* **Note 3**).
5. With GFP reporter mice proceed to the tissue processing with prefixation Subheading 3.2. When using *lacZ* reporter mice proceed to the embedding Subheading 3.3.

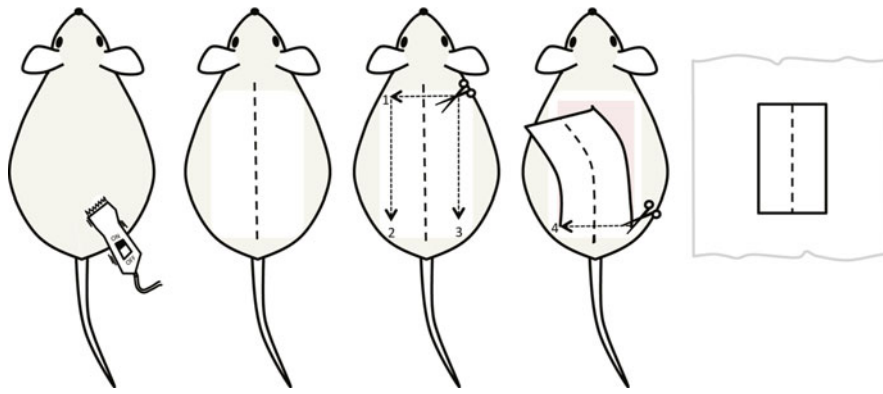

Fig. 1 Stepwise illustration of backskin tissue harvesting. The mouse backskin is closely shaved before being marked at the midline from the neck to the base of the tail. An incision is made across the upper back and then along the two sides parallel to the midline. The skin is gently peeled off with the final cut across the back above the hind legs. The skin is spread on a brown paper towel with the midline aligned straight. The skin then can be cut into strips for processing

3.2 Prefixation of Tissue for GFP Preservation

For GFP reporter mice, prefixation of tissue prevents GFP signal loss. However, in the TCF/LEF: H2B-GFP [25] the GFP is fused with histone H2B, which renders the GFP much more stable, and therefore with this mouse line the prefixation step is optional. Prefixation is required for all other GFP reporter lines.

1. Transfer the skin strips to an aluminum foil-wrapped Falcon 5 mL tube to protect it from light throughout the whole procedure. All incubation steps below are performed with gentle rocking on the shaker.
2. Fix with fresh 4% PFA for 2 h. Ensure the strip is fully immersed (*see* **Note 4**). Discard PFA as hazardous waste.
3. Wash with PBS 2–3 times, 5 min each wash.
4. Soak tissues in 10% sucrose and incubate for 2 h. Aspirate.
5. Soak tissues in 30% sucrose and incubate overnight at 4 °C in the dark.
6. The next day, equilibrate with 1:1 O.C.T. slurry (30% sucrose: O.C.T. = 1:1) for 6 h. O.C.T. slurry can be made by damping out half of the sucrose and adding O.C.T. for 1:1 mixture. Proceed to embedding backskin in O.C.T. for cryosectioning (Subheading 3.3).

3.3 Embedding Backskin in OCT Block

1. Fill the cryomold with O.C.T.
2. Hold both ends of the skin strip with fine forceps, and place the skin strip with the midline touching the bottom of O.C.T.-filled cryomold (Fig 2a). Use fine forceps to hold the skin straight and to make sure the skin freezes exactly perpendicular

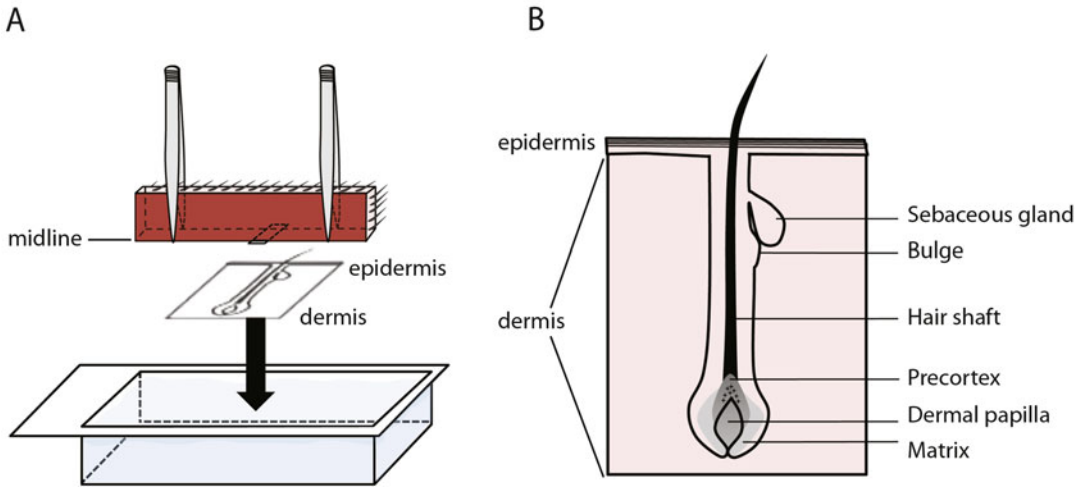

Fig. 2 Illustration of skin embedding to acquire longitudinal sections of hair follicles. (**a**) Skin strip is placed into O.C.T. filled cryomold with the midline facing and touching the bottom of the cryomold. (**b**) Cartoon depicting a good day-28 skin section with an intact hair follicle

to the bottom of the block (*see* **Note 5**). Hold the skin straight while placing the O.C.T. block on the dry ice to freeze (*see* **Note 6**).

3. Transfer the frozen O.C.T. block to prechilled cryostat for sectioning or store at −80 °C.

3.4 Cryosectioning

1. Follow cryostat instruction (*see* **Note 7**) to section tissue in 8 μm (range: 6–12 μm), two sections per slide.
2. Air-dry the first collected section on the slide and counterstain with hematoxylin solution for 1 min.
3. Gently wash off excess staining solution with tap water. Visualize hair follicles under a light microscope and adjust block angle until intact hair follicles can be detected (Fig. 2b, *see* **Note 8**).
4. Collect sections, air dry for 20 min (range: 15–30 min), and freeze at −80 °C for long-term storage.

3.5 Visualizing GFP Signal in GFP Reporter Mouse Skin

1. GFP can be visualized directly under a fluorescence microscope (*see* **Note 9**). If stored at −80 °C, thaw at room temperature for 10 min before use. To colocalize GFP signal with epidermal markers (or other markers), proceed to the next step (*see* **Note 10**). If GFP signal is weak, proceed to next step and immunostain for GFP expression (Fig. 3a).
2. Use a pap pen to draw a circle around the skin tissue to create a hydrophobic barrier.
3. Transfer the slide to an aluminum foil-wrapped, humidified staining chamber (*see* **Note 11**) and incubate with blocking buffer (150–200 μL/section in general) for 1 h.
4. Add primary antibody (α β-4 integrin 1:200 and optional α GFP 1:250 diluted in staining buffer) and incubate for 2 h at room temperature or overnight (16–24 h) at 4 °C in humidified chamber.
5. The next day, wash with PBS in the staining jar, three times, 5 min each time.
6. Add fluorophore-conjugated secondary antibody (AF488 1:150 and RRX 1:200 diluted in staining buffer) for 45 min in the humidified chamber.
7. Wash in PBS briefly.
8. Counterstain in Hoechst 33342 (1:2000 dilution in PBS) at room temperature for 2 min.
9. Wash with PBS three times, 10 min total.
10. Aspirate excess buffer and mount the section with ProLong Gold antifade and cure overnight at room temperature in the dark.
11. Seal the slide with nail polish and air-dry for 30 min before imaging with a fluorescence microscope. If immediate imaging is not possible, slides can be stored at −20 °C (*see* **Note 9**).

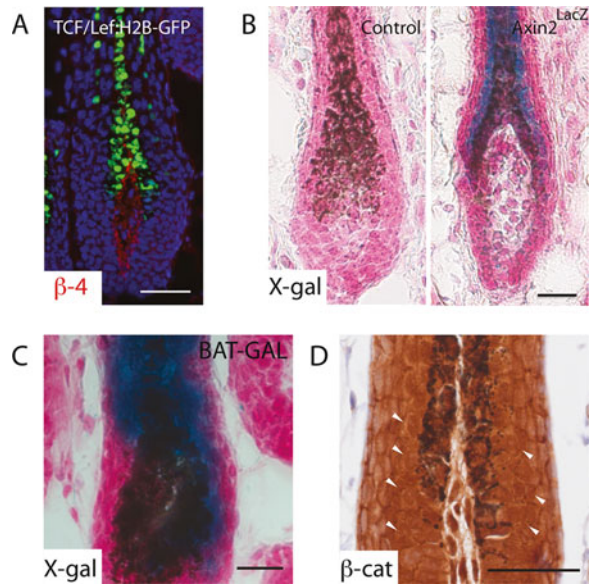

Fig. 3 Detecting Wnt/β-catenin signaling. (**a**) Fluorescence image of endogenous GFP signal in the hair follicle of 28-day-old TCF/LEF:H2B-GFP mouse, with nuclei counterstained with Hoechst. β4-integrin co-staining in red labels the basement membrane, delineating the interface between epithelial cells and the dermal papilla. X-Gal staining of 28-day-old (**b**) wild-type or *Axin2*^{lacZ} and (**c**) BAT-GAL mice. Skins were counterstained with Nuclear Fast Red to mark nuclei. *Blue* signal in the precortex of hair follicle indicates Wnt activity. (**d**) Immunostaining of β-catenin in 28-day-old wild-type mouse skin. *Arrowheads* point to nuclear β-catenin. Scale bar denotes 50 μm

3.6 X-gal Staining of lacZ Reporter Mouse Skin

1. Thaw slides from −80 °C for 10 min at room temperature (*see* **Note 12**).
2. Fix in freshly prepared 0.1% glutaraldehyde in PBS for 2 min.
3. Wash with PBS in the staining jar at least five times, 5 min per wash.
4. Prepare X-Gal staining solution fresh by adding X-Gal (2 mg/mL) to pre-warmed base solution.
5. Incubate the slides at 37 °C in the hybridization oven. Cover the jar with slides with aluminum foil. Active β-galactosidase digests X-Gal as a substrate and converts it into a blue precipitate. Check the color development from 2 to 3 h of staining and let it go overnight if necessary.
6. Wash with PBS three times, 5 min each.
7. Rinse with distilled water.
8. Counterstain with Nuclear Fast Red for 5 min.

9. Wash with running water for 10 min.
10. Mount slides with 80% glycerol and seal with nail polish.
11. Acquire images with a light microscope (Fig. 3b, c).

3.7 Immunostaining for Nuclear β-catenin

Use 5 μm skin sections from a formaldehyde-fixed paraffin-embedded (FFPE) block (*see* **Note 13**).

3.7.1 Dehydration and Antigen Retrieval of FFPE Sample

1. Dewax paraffin sections by placing slides in a 60 °C prewarmed citrus solvent and incubate in the hybridization oven at 60 °C for 10 min.
2. Rehydrate the slides by sequential incubation in an alcohol series, 3 min each step: 100% ethanol, 100% ethanol, 95% ethanol, 85% ethanol, 70% ethanol, 50% ethanol.
3. Rinse the slides twice briefly in distilled water and transfer to the Coplin jar.
4. Antigen unmasking is achieved using the heat-induced epitope retrieval (HIER) method:
   - Fill the jar to the top with citric acid buffer (pH 6.0).
   - Boil in a microwave at 500 W (50% power in regular microwave) for three rounds, 5 min each (15 min in total).
   - Between each round, refill citric buffer to the top to prevent the sections from drying. Set the jar on the benchtop and chill for 20 min until it reaches room temperature.

3.7.2 Blocking and Antibody Staining with M.O.M Kit

1. Briefly wash slides with distilled water.
2. Block endogenous peroxidase activity by incubating slides in 0.3% H_2O_2 solution for 15 min in the glass staining jar (*see* **Note 14**).
3. Wash with PBS three times, 5 min each.
4. Aspirate residual PBS without drying the tissue.
5. Draw a circle around the skin tissue with a Pap pen to create a hydrophobic border.
6. To block nonspecific antibody binding and endogenous biotin, add 100–200 μL M.O.M blocking buffer with Avidin per section and incubate for 1 h in the humidified staining chamber (*see* **Note 15**).
7. Remove the blocking buffer and rinse with PBS.
8. Add 100–200 μL M.O.M diluent buffer per section and incubate for 5 min.
9. Aspirate the diluent.
10. Add 100–200 μL of primary antibody (Mouse α β-catenin, 1:500) in M.O.M diluent buffer with biotin onto the skin section and incubate overnight in the humidified staining chamber at 4 °C.
11. The next day, wash with PBS three times, 5 min each.

3.7.3 Signal Amplification and Development

1. Aspirate excess PBS buffer around the tissue after the last wash.
2. Prepare secondary antibody by diluting α Mouse IgG Biotinylated antibody (1:200, from the M.O.M. kit) in M.O.M. diluent buffer without biotin.
3. Add 100–200 μL of secondary antibody solution per section and incubate for 30 min (*see* **Note 16**). At the same time, prepare Vectastain Elite ABC reagent.
4. Wash with PBS three times, 5 min each.
5. Add 100–200 μL ABC reagent mixture onto the skin section and let incubate for 30 min.
6. Wash with PBS two times, 5 min each.
7. For color development, prepare DAB solution and add 100–200 μL DAB substrate to the section. Monitor DAB colorization (brown) under the light microscope until the intensity of the color is satisfactory (between 3 and 5 min, *see* **Note 17**) and wash with tap water for 5 min (Fig. 3d).

3.7.4 Hematoxylin Counterstain and Imaging

Hematoxylin counterstain marks the cell's nucleus to reveal histology and also enhances the contrast from DAB development.

1. Stain the slides in hematoxylin for 30 s.
2. Wash with tap water for 1 min.
3. Incubate in clarifier for 1 min.
4. Wash with tap water for 1 min.
5. Incubate in bluing reagent for 1 min.
6. Wash with tap water for 1 min.
7. Wash four times total with increasing concentrations of Ethanol: 70% ethanol, 95% ethanol, 100% ethanol, 100% ethanol, then two times with citrus solvent, 1 min each time.
8. Allow evaporation of citrus solvent in the chemical hood for 2–5 min.
9. Mount the slide with a drop (~50 μL) of Permount solution and let solidify overnight.
10. The next day, image slides or store slides at room temperature for future imaging.

4 Notes

1. CO_2 euthanasia of mice is performed according to the American Veterinary Medical Association (AVMA) guidelines (CO_2: 3 min on and 2 min off at a defined flow rate in closed chamber).
2. When harvesting skins of multiple mice, keep the skins moist with PBS until all the skins are processed. Maximum five mice per batch are recommended.

3. The phases of the hair cycle may differ in different regions of the backskin. Therefore, always collect skin from the same region of the back.
4. During tissue harvesting and fixation, ensure the skin strip is fully immersed in PFA without air bubbles. Since the skin will become more rigid after fixation, avoid tissue distortion or bending during PFA incubation.
5. If multiple skin pieces are embedded in one cryomold, stack them on top of each other with O.C.T. compound squeezed between them. Make sure that the midlines of all the skin strips are facing and touching the bottom of the cryomold. A maximum of three skin strips in one cryomold is strongly recommended.
6. Use dry ice blocks with a flat surface to ensure direct contact and immediate freezing of the O.C.T. compound.
7. The cryostat setting for skin sectioning is CT = –24 °C, OT = –27 °C (CT = chamber temperature, OT = object temperature).
8. A good embedding minimizes angle adjustment during cryostat sectioning. A good section should include a couple of hair follicles that are contiguous to the basal layer of the epidermis at the top and has contact with the dermal papilla at the bottom.
9. The GFP signal in TCF/LEF:H2B-GFP reporter mice is relatively stable compared with other GFP-based Wnt reporter mice. Unfixed TCF/LEF:H2B-GFP skin in O.C.T. retains GFP signal for at least 2 years.
10. Co-immunostaining of structural protein such as β4-integrin or Keratin 5 can help define the structure of hair follicles. β4-integrin antibody labels the basement membrane, which marks the interface between epidermal and dermal compartments. Keratin 5 antibody marks keratinocytes in the basal layer of the interfollicular epidermis and the outer root sheath of the hair follicle.
11. A humidified chamber is assembled with a homemade slide rack and PBS-soaked paper towels underneath to minimize reagent evaporation during the incubation step. To create a homemade slide rack, we align two serological pipettes (10 mL) that have been shortened to fit into a BioAssay Dish. The pipettes are attached by tape to the base of the dish to form two parallel tubes where the slides can be placed on top. To minimize light exposure during staining, the lid and bottom dish of BioAssay Dish are wrapped with aluminum foil.
12. The best X-gal staining result is obtained with freshly embedded/sectioned skin of *Axin2*^{lacZ} reporter mice.
13. Detection of nuclear β-catenin in skin is best achieved by immunohistochemistry with paraffin embedded skin.

14. H_2O_2 in 0.3% solution should be prepared fresh from a 30% stock with tap water in a glass jar.
15. The M.O.M kit is used in conjunction with mouse-raised primary antibody in mouse tissue.
16. The ABC reagent doesn't spread because it has no serum or protein to reduce surface tension. Ensure the complete coverage of specimen by gently adding reagent directly onto the specimen.
17. A satisfactory level for DAB development is defined by the balance between the intensity of the brown signal in the nucleus and the overall background signal.

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Chapter 15

The Generation of Organoids for Studying Wnt Signaling

Jarno Drost, Benedetta Artegiani, and Hans Clevers

Abstract

We established an in vitro culture model in which intestinal epithelial stem cells can grow into three-dimensional, ever-expanding epithelial organoids that retain their original organ identity and genetic stability. Moreover, organoids can easily be genetically modified using different genome modification strategies, including viral delivery of transgenes and CRISPR/Cas9 technology. These combined characteristics make them a useful in vitro model system to study many biological processes including the contribution of cellular signaling pathways to tissue homeostasis and disease. Here we describe our current laboratory protocols to establish human intestinal organoids and how to genetically modify both mouse and human intestinal organoids to study cellular signaling pathways, specifically Wnt signaling. Moreover, we provide a detailed protocol for lentiviral transduction and CRISPR/Cas9-mediated genome modification of organoid cultures.

Key words Organoids, Intestine, Wnt signaling, Lentivirus, CRISPR/Cas9

1 Introduction

The Wnt signaling pathway plays an essential role during embryonic development, cell proliferation, differentiation and cell-fate determination. The Wnt pathway regulates, through a core set of evolutionarily highly conserved proteins, the stability of the multifunctional protein ß-catenin which can activate the transcription of specific Wnt target genes. Deregulation of this pathway may cause disease, including cancer [1].

Transgenic mouse models are widely used to study Wnt signaling and its role in disease. The most commonly used transgenic models inactivate the APC tumor suppressor gene. APC is a negative regulator of Wnt signaling and inactivating mutations therefore result in constitutive activation of the Wnt signaling pathway. One of the consequences of loss of APC function is the formation of intestinal benign polyps. The $APC^{MIN/+}$ mouse model (MIN, multiple intestinal neoplasia), for example, models Familial Adenomatous Polyposis (FAP), a syndrome in which patients develop multiple colorectal polyps [2, 3]. Although genetically modified mice

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provide important insights in the Wnt signaling pathway, drawbacks include the long generation time (1–2 years) and low throughput.

Until recently, it seemed impossible to establish long-term cultures from primary adult tissues. In vitro model systems encompassed in vitro immortalized primary material and cell lines derived from progressed cancers, both poor representatives of the in vivo situation. We recently established an in vitro culture system to grow primary epithelium from mouse small intestine. These organoids structurally mimic the tissue of origin with proliferative crypt and differentiated villus compartments [4]. Since then, similar organoid culture systems have been established for mouse and human colon, stomach, pancreas, liver and prostate [5–11]. Furthermore, we and others have succeeded in genetically modifying these organoids, by either viral delivery of transgenes (overexpression or knockdown) or CRISPR/Cas9-mediated genome editing [12–15], making the organoids a state-of-the-art tool to study cellular signaling pathways, tissue homeostasis, and disease.

Here we describe our current laboratory protocols to establish organoids from human intestinal tissue and how these can be genetically modified using lentiviral transduction and CRISPR/Cas9 technology. We describe how to design and clone single guided RNAs (sgRNAs) and the analysis of the genotype of the engineered mutant organoids. Specifically, we describe how to generate and maintain organoid cultures derived from human intestinal tissue, transduce organoids with lentivirus, and use CRISPR/Cas9 to genome edit in intestinal organoids.

2 Materials

2.1 Generation and Maintenance of Organoid Cultures Derived from Human Intestinal Tissue

2.1.1 Isolation of Crypts from Human Intestinal Tissue

1. Basal culture medium: Advanced DMEM/F12, 2 mM GlutaMax 100, 10 mM HEPES, 100 U/ml penicillin/100 mg/ml streptomycin, 10 μM Rho kinase inhibitor Y-27683 (only for tissue collection).
2. Chelating stock solution (5×): 28 mM Na_2HPO_4, 40 mM KH_2PO_4, 480 mM NaCl, 8 mM KCl, 220 mM sucrose, 274 mM d-sorbitol in MilliQ H_2O.
3. Crypt isolation medium: 4 ml chelating stock solution (5×), 16 ml MilliQ H_2O, 1.6 mg DTT, 80 μl 0.5 M EDTA (final concentration 2 mM, add just before incubation).
4. Fetal bovine serum (FBS).

2.1.2 Culturing of Human Intestinal Crypts

1. Matrigel™ (BD Biosciences), Basement Membrane Matrix, Growth Factor Reduced (GFR), Phenol Red-free. Thaw overnight on ice and store 1 ml aliquots at −20 °C. Thaw the required amount of 1 ml aliquots of Matrigel™ overnight on ice before use.

2. Basal culture medium: *see* Subheading 2.1.1.
3. Wnt3A-conditioned medium (*see* **Note 1**).
4. B27 supplement, 50×, 400 µl aliquots are stored at −20 °C.
5. N-Acetylcysteine, 400× stock: 500 mM in distilled H_2O, 125 µl aliquots are stored at −20 °C.
6. 0.1% BSA/PBS filter sterilized (0.2 µm).
7. Recombinant human EGF, 10,000× stock: 500 µg/ml in 0.1% BSA/PBS. 5 µl aliquots are stored at −20 °C.
8. Recombinant human Noggin, 1000× stock: 100 µg/ml in 0.1% BSA/PBS. 20 µl aliquots are stored at −20 °C.
9. Recombinant human R-spondin 1, 500× stock: 500 µg/ml in 0.1% BSA/PBS. 100 µl aliquots are stored at −20 °C (*see* **Note 2**).
10. A83-01, 50,000× stock: 25 mM in DMSO, 5 µl aliquots are stored at −20 °C.
11. SB202190, 10,000× stock: 30 mM in DMSO, 5 µl aliquots are stored at −20 °C.
12. Y-27632 dihydrochloride, 10,000× stock: 100 mM in distilled H_2O, 50 µl aliquots are stored at −20 °C.
13. Primocin, 500×, stored at −20 °C.
14. Human intestinal organoid culture medium: 50% Wnt3A-conditioned medium, 1× B27 supplement, 1.25 mM N-acetylcysteine, 50 ng/ml recombinant human EGF, 100 ng/ml recombinant human Noggin, 1 µg/ml recombinant human R-spondin 1, 500 nM A83-01, 3 µM SB202190, 1× Primocin, and 10 µM Y-27632 dihydrochloride (only after first plating or passaging) in basal culture medium (*see* **Note 3**).

2.1.3 Passaging of Human Intestinal Organoids

1. Matrigel™: *see* Subheading 2.1.2.
2. Glass pasteur pipettes, fire-polished to narrow opening (approximately 0.5–1 mm).
3. Basal culture medium: *see* Subheading 2.1.1.
4. Human intestinal organoid culture medium: *see* Subheading 2.1.2.

2.2 Lentiviral Transduction of Mouse and Human Intestinal Organoids

2.2.1 Production of Lentiviruses

1. HEK293T cells.
2. Polyethylenimine (PEI): 1 mg/ml in H_2O.
3. Opti-MEM.
4. DMEM.
5. Lentiviral plasmid containing the gene of interest flanked by long terminal repeats.
6. Packaging plasmid(s) encoding the Gag, Pol, Rev, and Tat genes. The number of plasmids encoding these genes depends on the lentiviral system used.

7. Envelope (Env)-encoding plasmid.
8. Polybrene
9. Infection medium: mouse intestinal organoid culture medium (*see* Subheading 2.2.2) plus 50% Wnt-conditioned medium, 10 mM nicotinamide, 10 μM Y-27632, and 8 μg/ml polybrene; human intestinal organoid culture medium (*see* Subheading 2.1.2) plus 10 μM Y-27632 dihydrochloride and 8 μg/ml polybrene.

2.2.2 Lentiviral Transduction of Intestinal Organoids

1. Lentiviruses: *see* Subheading 2.2.1.
2. Intestinal organoids: for human *see* Subheading 2.1.
3. TrypLE Express (Life Technologies).
4. Basal culture medium: *see* Subheading 2.1.1.
5. Matrigel™: *see* Subheading 2.1.2.
6. Mouse intestinal organoid culture medium: 1× B27 supplement, 1.25 mM N-acetylcysteine, 50 ng/ml recombinant mouse EGF (Peprotech), 100 ng/ml recombinant murine Noggin, 500 ng/ml recombinant human R-spondin 1, 1× Primocin, and 10 μM Y-27632 dihydrochloride (only after first plating or passaging) in basal culture medium (*see* **Note 3**).
7. Human intestinal organoid culture medium plus 10 μM Y-27632 dihydrochloride: *see* Subheading 2.1.2.
8. Nicotinamide.
9. Infection medium: *see* Subheading 2.2.1.

2.3 CRISPR/Cas9-mediated Genome Editing in Mouse and Human Intestinal Organoids

2.3.1 Design and Cloning of the Cas9 and sgRNA Expression Construct

1. Top and bottom oligo strands:

   APC_gRNA1,

   top 5′-CACCGTTTGAGCTGTTTGAGGAGG-3′

   bottom 5′-AAACCCTCCTCAAACAGCTCAAAC -3

   or oligo strands for any desired Wnt-pathway component.
2. ATP (10 mM).
3. DTT (10 mM).
4. FastDigest BpiI 10 U/μl (Thermo Scientific).
5. MilliQ H_2O.
6. Plasmid Safe ATP-dependent DNase 10 U/μl.
7. PlasmidSafe buffer 10×.
8. pSpCas9 (BB)-2A-GFP (Addgene).
9. Tango buffer 10×.
10. T4 PNK 10 U/μl.
11. PNK buffer 10×.
12. T7 DNA Ligase 3000 U/μl.

2.3.2 Bacteria Transformation with sgRNA Constructs

1. Chemically competent bacteria.
2. Incubator at 37 °C.
3. LB agar with ampicillin plates.
4. LB medium.
5. Shaking incubator at 37 °C.
6. Water bath at 42 °C.

2.3.3 Plasmid Purification and Sequencing

1. LB medium.
2. Ampicillin.
3. Plasmid DNA Mini-prep kit.
4. Plasmid DNA Maxi- or Midi-prep kit.
5. Sequencing primer:

   U6-Fw 5′-GAGGGCCTATTTCCCATGATTCC-3′ for pSpCas9 (BB)-2A-GFP.

2.3.4 Preparation of Lipofection Mixes

1. pSpCas9 (BB)-2A-GFP-APC sgRNA or any other plasmid expressing Cas9 endonuclease and sgRNA(s): *see* Subheading 2.3.1.
2. Opti-MEM.
3. Transfection reagent (for example Lipofectamine).

2.3.5 Preparation and Lipofection of Mouse and Human Intestinal Organoids

1. Intestinal organoids: for human *see* Subheading 2.1.
2. TrypLE Express.
3. Basal culture medium: *see* Subheading 2.1.1.
4. Matrigel™: *see* Subheading 2.1.2.
5. Human intestinal organoid culture medium plus 10 μM Y-27632 dihydrochloride: *see* Subheading 2.1.2.

2.3.6 Genotyping Organoids: Genomic DNA Extraction from Organoid Culture

1 PBS 1×.

2 Lysis buffer (for 500 ml: 50 ml 1 M Tris–HCl pH 8.5, 20 ml 5 M NaCl, 10 ml 10% SDS, 5 ml 0.5 M EDTA, 415 ml H_2O).

3 Proteinase K (20 mg/ml).

4 70% ethanol.

5 Isopropanol.

6 Elution buffer (EB) (50 ml: 0.5 ml 1 M Tris–HCl pH 8, 20 μl 0.5 M EDTA, 49.48 ml H_2O).

7 DNA loading dye 10×.

8 TAE buffer 50× (242 g Tris base in 750 ml H_2O, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA (pH 8.0) and adjust the solution to a final volume of 1 l. pH should be around 8.5).

9 1.5% agarose gel in TAE buffer 1×.

10 Ethidium bromide (10 mg/ml).

2.3.7 Genotyping Organoids: PCR Amplification of the Targeted Region

1. Template genomic DNA (*see* Subheading 2.3.6).
2. DNA polymerase and accompanying buffers.
3. APC forward primer 5′-TGTAATCAGACGACACAGGAA GCAGA-3′.
4. APC reverse primer 5′-TGGACCCTCTGAACTGCAGCAT-3′.
5. MilliQ H_2O.
6. DNA loading dye 10×.
7. TAE buffer 50× (242 g Tris base in 750 ml H_2O, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA (pH 8.0) and adjust the solution to a final volume of 1 l. pH should be around 8.5).
8. 1.5% agarose gel in TAE buffer 1×.
9. Ethidium bromide (10 mg/ml).
10. Agarose gel extraction kit.

2.3.8 Genotyping Organoids: Cloning of the PCR Product and Sequencing of the Cloned Fragment

1. Plasmid vector backbone.
2. DNA ligase buffer.
3. T4 DNA ligase.
4. Chemically competent bacteria.
5. X-gal.
6. LB with ampicillin and X-gal plates.
7. Plasmid DNA Mini-prep kit.
8. Sequencing primer Sp6: 5′-ATTTAGGTGACACTATAG-3′.
9. Sequencing primer T7: 5′-AATACGACTCACTATAG-3′.

3 Methods

3.1 Generation and Maintenance of Organoid Cultures Derived from Human Intestinal Tissue

We have previously described an "indefinite" 3D intestinal stem cell culture system allowing growth of single stem cells derived from mouse small intestine. In this culture system, the ever-expanding "organoids" structurally and functionally resemble in vivo intestinal epithelium, while remaining phenotypically and genetically stable [3, 16]. This makes the organoid culture system an excellent system to study the signaling pathways involved in tissue homeostasis and disease. Since then, our lab has developed similar organoid cultures from mouse and human intestine, colon, stomach, pancreas, liver, and prostate [5–10]. A detailed protocol for the generation and maintenance of organoid cultures derived from mouse small intestine has been reported repeatedly [16, 17]. Here we provide the detailed protocol for the establishment and maintenance of human intestinal organoids.

3.1.1 Isolation of Crypts from Human Intestinal Tissue

1. Collect human colon tissue in 50 ml conical tubes with 10–15 ml basal culture medium with Y-27632.
2. Remove muscle layer and fat using surgical scissors and forceps under a dissection microscope.
3. Cut thin strips of ~ 1–2 mm.
4. Wash three times with fresh crypt isolation medium.
5. Add EDTA (final concentration 2 mM).
6. Incubate 30 min at 4 °C on rocking tube platform or roller.
7. Shake vigorously and check under a light microscope if crypts detached from the mesenchyme. If not, repeat incubation with crypt isolation medium plus 2 mM EDTA.
8. Remove supernatant with crypts.
9. Add 5–10 ml FBS and spin down the crypts at 130×*g* for 5 min.

3.1.2 Culturing of Human Intestinal Crypts

1. Before starting:

   Thaw the required amount of 1 ml Matrigel™ aliquots on ice.

   Pre-warm (overnight) a 24-well plate in a CO_2 incubator (5% CO_2, 37 °C).

   Prepare human intestinal organoid culture medium.
2. Wash the crypts (*see* Subheading 3.1.1) three times with basal culture medium.
3. Resuspend the crypts in the desired volume of Matrigel™. For 1 well of a 24-well cell culture plate, take up approximately 1000 crypts in 50 µl of Matrigel™ resuspend by gently pipetting up and down using a 200 µl tip. Ensure that the crypt–Matrigel™ mixture remains cold.
4. Plate 50 µl of the resuspended crypt–Matrigel™ mixture without bubbles into the pre-warmed 24-well plate.
5. Transfer the 24-well plate into a CO_2 incubator (5% CO_2, 37 °C) for approximately 15 min to allow the Matrigel™ to solidify.
6. Add 500 µl of human intestinal organoid culture medium plus 10 µM Y-27632 dihydrochloride per well of the 24-well plate.
7. Incubate the organoids in a CO_2 incubator (5% CO_2, 37 °C).
8. Cystic organoids will appear 2–3 days after plating.
9. Exchange culture medium plus 10 µM Y-27632 dihydrochloride with human intestinal organoid culture medium 2 days after plating. Refresh every 2–3 days.
10. Organoids should be passaged every 7–10 days.

3.1.3 Passaging of Human Intestinal Organoids

1. Before starting:

   Fire-polish glass pipettes and make opening of about 0.5–1 mm.

   Thaw the required amount of 1 ml MatrigelTM aliquots on ice.

   Pre-warm (overnight) a 24-well plate in a CO2 incubator (5% CO2, 37 °C).

   Prepare human intestinal organoid culture medium.
2. Split ratio is usually 1:4.
3. Aspirate the medium from the well.
4. Break up the Matrigel™ in 1 ml ice-cold basal culture medium using a P1000 tip.
5. Transfer the suspension to a conical 15 ml tube.
6. Pre-wet the glass pipet by pipetting up and down basal culture medium to prevent organoids from sticking to the glass.
7. Pipette up and down the organoid suspension 10–15 times.
8. Add 3 ml of ice-cold basal culture medium and centrifuge at 200 × *g* for 5 min at 4 °C.
9. Aspirate supernatant and add 200 μl of Matrigel™. Mix the suspension well by pipetting up and down, but prevent the appearance of air bubbles.
10. Plate four times 50 μl of the resuspended crypt–Matrigel™ mixture without bubbles into a pre-warmed 24-well plate.
11. Transfer the 24-well plate into a CO_2 incubator (5% CO_2, 37 °C) for approximately 15 min to allow the Matrigel™ to solidify.
12. Add 500 μl of human intestinal organoid culture medium per well of the 24-well plate.
13. Incubate the organoids in a CO_2 incubator (5% CO_2, 37 °C).
14. Refresh medium every 2–3 days.
15. Continue passaging the organoids until enough organoids are obtained for performing the desired experiment(s).
16. To study signaling pathways (e.g., Wnt signaling), the established organoids can be modified using multiple techniques; including lentiviral transductions (*see* Subheading 3.2) and CRISPR/Cas9 (*see* Subheading 3.3).

3.2 Lentiviral Transduction of Intestinal Organoids

To utilize the organoid culture system to study the Wnt signaling pathway, one can establish organoid cultures from preexisting transgenic mouse models. Additionally, when these models are lacking or when the use of a human model system is preferred, the organoids can also be genetically modified by viral delivery of transgenes, either overexpression or knockdown [12]. This method can be efficiently used to study signaling pathways in the organoid system.

3.2.1 Production of Lentiviruses for the Transduction of Intestinal Organoids

1. Before starting:

   Passage HEK293T cells in a 15 cm culture dish in such a way that they have reached a confluency of approximately 75% at the day of transfection.

   Prepare infection medium.

2. Per 15 cm culture dish, mix the following DNA plasmids in a 15 ml tube:
   (a) 3.6 μg of plasmid encoding Gag and Pol genes.
   (b) 3.6 μg of plasmid encoding Rev gene.
   (c) 3.6 μg of plasmid encoding Tat gene.
   (d) 7.2 μg of plasmid encoding Env gene.
   (e) 45 μg of lentiviral plasmid containing the gene of interest flanked by long terminal repeats.
3. Add 3 ml of Opti-MEM per tube containing the mixed DNA.
4. Per transfection reaction, mix 315 μl PEI (Polyethylenimine) (5 μl of PEI per μg DNA) with 2 ml Opti-MEM. Mix by vortexing for 1 s and incubate at room temperature for 5 min.
5. Dropwise add PEI/Opti-MEM mix to DNA/Opti-MEM mix by vortexing for 1 s and incubate at room temperature for 15 min.
6. Pipette the PEI–DNA mixture into the 15 cm culture dish containing the HEK293T cells. Incubate in a CO_2 incubator (5% CO_2, 37 °C).
7. Change the medium the day after transfection.
8. Filter (0.45 μm) the medium 2–3 days after transfection to remove any residual HEK293T cells.
9. Collect the filtered medium in an ultracentrifuge tube and centrifuge at 20,000 × *g* for 1:30 h at 4 °C in an ultracentrifuge.
10. Remove the supernatant and resuspend the pellet in 500 μl infection medium.

3.2.2 Lentiviral Transduction of Mouse and Human Intestinal Organoids

1. Before starting: In contrast to human intestinal organoids, mouse intestinal organoids are grown in culture medium without Wnt-conditioned medium. To obtain a higher percentage of stem cells per organoid culture and, as a result, a higher transduction efficiency, mouse intestinal organoids are grown in culture medium containing 50% Wnt-conditioned medium from approximately 1 week before the transduction. For similar reasons, 10 mM nicotinamide is added to the Wnt-conditioned medium-containing culture medium 2–3 days before transduction.

   Passage the organoids as described in Subheading 3.1.3 ~ 3 days before the transduction. Approximately 50 μl of Matrigel containing the organoids should be used per transduction.

Thaw the required amount of 1 ml Matrigel™ aliquots on ice.

Prepare mouse or human intestinal organoid culture medium.

Pre-warm (overnight) a 24-well plate in a CO_2 incubator (5% CO_2, 37 °C).

2. Aspirate the medium from the well.
3. Break up the Matrigel™ in 1 ml ice-cold basal culture medium using a P1000 tip.
4. Transfer the suspension to a conical 15 ml tube and add 3 ml of ice-cold basal culture medium. Centrifuge at 200 × *g* for 5 min at 4 °C.
5. Aspirate the supernatant and resuspend the pellet in 500 μl TrypLE Express plus 10 μM Y-27632 dihydrochloride to obtain single cells.
6. Incubate at 37 °C for 5–10 min and subsequently pipette up and down with a P1000 tip 5–10 times.
7. Add 10 ml of ice-cold basal culture medium and centrifuge at 200 × *g* for 5 min at 4 °C.
8. Aspirate the supernatant and resuspend the pellet in a small volume of infection medium (~25 μl per transduction).
9. Combine ~25 μl of cells with 250 μl of viral suspension (*see* Subheading 3.2.1, **step 9**) in a well of a 48-well culture plate and mix by pipetting up and down 2–3 times with a P1000 tip.
10. Wrap the plate with Parafilm and centrifuge at 600 × *g* for 60 min at 32 °C.
11. Remove Parafilm and incubate the plate for 6 h in a CO_2 incubator (5% CO_2, 37 °C).
12. Collect the cells in a 1.5 ml microcentrifuge tube and centrifuge for 5 min at 845 × *g* in a microcentrifuge.
13. Discard the supernatant and resuspend the cells in 100 μl Matrigel™ (approximately two times the volume of Matrigel™ used for the transduction).
14. Plate two times 50 μl of the resuspended mixture without bubbles into a pre-warmed 24-well plate.
15. Transfer the 24-well plate into a CO_2 incubator (5% CO_2, 37 °C) for approximately 15 min to allow the Matrigel™ to solidify.
16. Add 500 μl infection medium without polybrene.
17. After 2–3 days, start the antibiotic selection by replacing the infection medium with mouse (without Wnt-conditioned medium and nicotinamide) or human organoid culture medium containing the antibiotic that is encoded in the used lentiviral plasmid.

18. After approximately 1 week, passage surviving organoids as described in Subheading 3.1.3 and [17]. The split ratio is dependent on the number of surviving organoids.

3.3 CRISPR/Cas9-mediated Genome Editing in Mouse and Human Intestinal Organoids

Another strategy to study gene function in organoids is to perform gene editing using CRISPR/Cas9 technology [18]. The CRISPR/Cas9 system allows efficient genome editing through the selective recruitment of the Cas9 nuclease on specific DNA sequences by a single guide RNA (sgRNA) [19]. In principle, sgRNAs can be designed to target any 20 bp-long genomic locus that proceeds an "-NGG" sequence, the so-called PAM sequence. Then, the RNA-guided Cas9 nuclease induces double strand brakes (DSB) that can be exploited for genome editing either via nonhomologous end joining (NHEJ) or homology direct repair (HDR). NHEJ is error prone and often leads to indel mutations that can result in a frameshift within the coding sequence. In contrast, HDR can, in the presence of the appropriate DNA template, mediate events of recombination, to generate, for example, point mutations or insertion of resistance genes. Therefore, by using the CRISPR/Cas9 technology in organoid cultures, components of the Wnt signaling pathways can be mutated or knocked-out in order to further study their functions. We and others have successfully performed gene correction and introduced common colorectal cancer mutations in both mouse and human intestinal organoids [13–15]. Introduction of the mutations is accomplished by transient transfection of plasmids expressing Cas9 endonuclease and sgRNAs that target the gene of interest [20]. The sgRNAs are designed using online tools such as http://crispr.mit.edu or http://cas9.cbi.pku.edu.cn, which identify and rank suitable target sites within the selected genomic region and also predict possible off-target sites. They can be designed in such a way that they target a specific region in the gene body (e.g., a mutation hotspot region in a specific genetic disease). In the case of generation of knockout mutants, it is preferable to target the gene within the first exon(s) or preceding important functional domains. In case one wishes to introduce a mutation at a specific location, the sgRNAs should be designed to target in close proximity of the desired location. Plasmid delivery into the organoids is enabled using a lipid-based transfection method [13, 14], but also other transfection methods have been successfully used [15]. A functional selection strategy can be used to select for organoids harboring inactivating mutations in the Wnt signaling pathway. For instance, loss of APC function enables organoids to grow in the absence of Wnt-conditioned medium and R-spondin 1, which are indispensable for wild-type intestinal organoids, providing a clean selection strategy for loss-of-function mutants.

3.3.1 Design and Cloning of the Cas9 and sgRNA Expression Construct

1. Select the region to target, and provide it as the input sequence in the online design tool. Select the sgRNA based on ranking and low number of potential off-targets. The sgRNA can target both the plus and the minus strand.
2. Add the CACCg sequence to the 5′-end of the sgRNA sequence (omit the "G" in case the designed sgRNA already starts with a guanine). This sequence constitutes the top primer. For the APC sgRNA, 5′-CACCGTTTGAGCTGTTTGAGGAGG-3′. The bottom primer is simply obtained by reversing and complementing the sgRNA sequence and adding AAAC to its 5′- and add a C to its 3′-end, in case a guanine has been added to the 5′-end of the top primer). For the APC sgRNA, 5′-AAACCCTCCTCAAACAGCTCAAAC-3′.
3. Resuspend the top and the bottom primers to a final concentration of 100 μM in H_2O.

   Anneal and phosphorylate the primers by setting up the following reaction:

TOP primer (100 μM)	1 μl
BOTTOM primer (100 μM)	1 μl
PNK buffer 10×	1 μl
T4 PNK 10 U/μl	1 μl
H_2O	6 μl

   Incubate the reaction following these conditions:

   37 °C 30 min.

   95 °C 5 min.

   Ramp down to 25 °C by decreasing 5 °C per min.
4. Dilute the annealed and phosphorylated primers 1:200 in H_2O.
5. Clone the annealed and phosphorylated primers into the pSpCas9 (BB)-2A-GFP plasmid by setting up the following reaction:

pSpCas9 (BB)-2A-GFP (100 ng total)	×μl
Primers from **step 4**	2 μl
Tango buffer 10×	2 μl
DTT 10 mM	1 μl
ATP 10 mM	1 μl
FastDigest BpiI 10 U/μl	1 μl
T7 DNA Ligase 3000 U/μl	0.5 μl
H_2O	up to 20 μl

Incubate the reaction following these conditions:

37 °C 5 min

21 °C 5 min

Repeat this cycle for six times (total time: 1 h)

6. In order to remove any residual linearized plasmid, set up this reaction with the PlasmidSafe exonuclease:

Ligation reaction from **step 5**	11 µl
PlasmidSafe buffer 10×	1.5 µl
ATP 10 mM	1.5 µl
PlasmidSafe exonuclease	1 µl

Incubate the reaction following these conditions:

37 °C 30 min.

21 °C 30 min.

3.3.2 Bacteria Transformation with sgRNA Constructs

1. Add 2 µl of the ligated sgRNA plasmid from Subheading 3.3.1, **step 6** to 50 µl of competent *E. coli* cells (DH5α or Stbl3 strain) and mix gently.
2. Incubate on ice for 20–30 min.
3. Heat-shock the cells by incubating 1 min at 42 °C in a water bath.
4. Immediately transfer on ice and incubate for 5 min.
5. Add 500 µl of LB medium to the vial and shake the vial at 37 °C for 1 h at 150 rpm using a shaking incubator.
6. Spread 200 µl of the transformation from **step 5** on a pre-warmed LB agar/ampicillin plate. Invert the plate and incubate overnight at 37 °C.
7. The next day, check the plate for colonies growth.

3.3.3 Plasmid Purification and Sequencing

1. Pick 2–5 single bacterial colonies and grow each of them in 5 ml of LB medium containing 100 µg/ml of ampicillin at 37 °C overnight.
2. Purify the DNA using a Plasmid DNA Mini-prep kit according to the manufacturer's instruction.
3. Check each plasmid preparation by sequencing using the U6-fw primer. Verify that the 20-nt sgRNA has been inserted correctly in the BpiI site within the sgRNA scaffold.
4. Bacteria transformed with the corrected pSpCas9(BB)-2A-GFP-gRNA plasmid can be incubated in larger volume of LB/ampicillin medium (from 50 to 500 ml) at 37 °C overnight and used to purify larger amount of the sgRNA plasmid by using either Plasmid DNA Maxi- or Midi-prep kit.

3.3.4 Preparation of Lipofection Mixes

1. Pipet 4.0 µl of Lipofectamine 2000 in 50 µl of Opti-MEM and incubate for 5 min at room temperature.
2. Add a total of 1.5 µg of pSpCas9-2A-GFP-APC sgRNA to 50 µl of Opti-MEM.
3. Pool both mixes and incubate for 15 min at room temperature.

3.3.5 Preparation and Lipofection of Mouse and Human Intestinal Organoids

1. For the same reasons explained in Subheading 3.2.2, **step 1**, mouse intestinal organoids are grown in culture medium containing 50% Wnt-conditioned medium from approximately 1 week before transfection, and 10 mM nicotinamide is added 2–3 days before transfection.

   Passage the organoids as described in Subheading 3.1.3 ~ 3 days before the transduction. Approximately 100 µl of Matrigel (containing the organoids) should be used per transfection.

   Thaw Matrigel on ice.

   Prepare mouse or human intestinal organoid culture medium.

   Pre-warm (overnight) a 24-well plate in a CO_2 incubator (5% CO_2, 37 °C).
2. Aspirate the medium from the wells.
3. Break up the Matrigel™ in 1 ml ice-cold basal culture medium using a P1000 tip.
4. Transfer the suspension to a conical 15 ml tube and add 3 ml of ice-cold basal culture medium. Centrifuge at 200 × *g* for 5 min at 4 °C.
5. Aspirate the supernatant and resuspend the pellet in 500 µl TrypLE Express plus 10 µM Y-27632 dihydrochloride to obtain single cells.
6. Incubate at 37 °C for 5–10 min and subsequently pipette up and down with a P1000 tip 5–10 times.
7. Add 10 ml of ice-cold basal culture medium and centrifuge at 200 × *g* for 5 min at 4 °C.
8. Resuspend cells in 450 µl mouse intestinal organoid culture medium containing 50% Wnt-conditioned medium and 10 mM nicotinamide, or human intestinal organoid culture medium.
9. Plate cells in a well of a 48-well culture plate (80–90% confluency) and add 50 µl of the DNA–Lipofectamine 2000 mix (Subheading 3.3.4, **step 3**) to the cells.
10. Wrap the plate with Parafilm and centrifuge at 600 × *g* for 60 min at 32 °C.
11. Remove Parafilm and incubate the plate for 4–6 h in a CO_2 incubator (5% CO_2, 37 °C).

12. Collect the cells in a 1.5 ml microcentrifuge tube and centrifuge for 5 min at 845 × *g* in a microcentrifuge.
13. Discard the supernatant and resuspend the cells in 100 µl Matrigel™ (approximately the same volume of Matrigel™ used for the lipofection).
14. Plate two times 50 µl of the resuspended mixture without bubbles into a pre-warmed 24-well plate.
15. Transfer the 24-well plate into a CO_2 incubator (5% CO_2, 37 °C) for approximately 15 min to allow the Matrigel™ to solidify.
16. Add 500 µl mouse intestinal organoid culture medium containing 50% Wnt-conditioned medium (without nicotinamide), or human intestinal organoid culture medium.
17. After 2–3 days, start the functional selection by replacing the mouse or human intestinal organoid culture medium for intestinal organoid culture medium without Wnt-conditioned medium and R-spondin 1 to select for organoids that have mutated APC.
18. After approximately 2 weeks, pick single surviving organoids and disrupt by pipetting up and down ~75 times. Since single cells were used for the lipofection, most of the outgrowing organoids have originated from a single cell. Picking and expanding of a single organoid will therefore result in a clonal organoid line.
19. Plate disrupted organoids separately in ~20 µl Matrigel™ in a pre-warmed 24-well plate.
20. Transfer the 24-well plate into a CO_2 incubator (5% CO_2, 37 °C) for approximately 15 min to allow the Matrigel™ to solidify.
21. Add 500 µl mouse or human intestinal organoid culture medium without 50% Wnt-conditioned medium and R-spondin 1.
22. After approximately 1 week, passage clonal organoids as described in Subheading 3.1.3 and [17].
23. Continue passaging the clonal organoids until enough material is obtained to perform genotyping (*see* Subheading 3.3.6).

3.3.6 Genotyping Organoids: Genomic DNA Extraction from Organoid Culture

1. From a 48-well plate, select one confluent well for each of the clones that have to be genotyped. Aspirate the medium and add 1 ml of ice-cold PBS 1×.
2. Collect the Matrigel™ drop containing the organoids (from a single clone) using a P1000 pipet and transfer it in a 15 ml tube. Add 10 ml of cold PBS 1× and incubate 5 min on ice in order to dissolve the Matrigel™.

3. Centrifuge 5 min at 300 × *g* at 4 °C.
4. Remove the supernatant without disturbing the cell pellet.
5. Resuspend the cell pellet in 200 μl of complete lysis buffer (196 μl lysis buffer + 4 μl of Proteinase K).
6. Incubate overnight in a 55 °C incubator.
7. Briefly spin and add 200 μl of isopropanol.
8. Mix by tapping gently; DNA could be visible as a white precipitate.
9. Centrifuge at maximum speed for 5 min at 4 °C.
10. Carefully remove the supernatant.
11. Wash the pellet with 500 μl of 70 % ethanol, spin again at maximum speed for 5 min.
12. Discard the supernatant and air-dry the pellet.
13. Add 100 μl of EB to the DNA and dissolve the pellet at 55 °C for 15 min in a water bath.
14. Use 3 μl of the isolated DNA from each clone to PCR-amplify the sequence of interest by using primers flanking the targeted region.

3.3.7 Genotyping Organoids: PCR Amplification of the Targeted Region

1. Set up the PCR reaction for the amplification of the targeted region:

Genomic DNA	3 μl
GoTaq Green Master Mix	25 μl
Primer fw	2.5 μl
Primer rev	2.5 μl
H_2O	17 μl

2. Perform the PCR reaction by following these cycling conditions:

   94 °C 2 min (initial denaturation) 1 cycle.

   94 °C 20 s (denaturation)/ 58 °C 30 s (annealing)/ 72 °C 1 min (elongation) 30 cycles.

   72 °C 7 min (final elongation) 1 cycle.

   4 °C until removal.

3. Once the PCR reaction is completed, run the samples on a 1.5 % agarose gel in TAE buffer, containing 0.25 μg/ml Ethidium Bromide, for 45 min at 70 V.
4. Check the samples using a UV trans-illuminator. Only one band is supposed to be present, or two, in case the mutation causes a size shift that is possible to visualize by electrophoresis.

5. Excise the correct band(s) from the agarose gel using a scalpel. Proceed to DNA extraction by using the QIAquick Gel extraction kit according to manufacturer's instruction.

3.3.8 Genotyping Organoids: Cloning of the PCR Product and Sequencing of the Cloned Fragment

The PCR product(s) obtained as described in Subheading 3.3.7 represent(s) a mixture of amplicons derived from both alleles of the gene of interest. In order to sequence both the alleles, the PCR product is cloned in the pGEM-T Easy vector and plasmid DNA extracted from single colonies is sequenced.

1. Set up the ligation reaction as described below:

2× T4 Rapid Ligation buffer	5 µl
pGEM-T easy vector (50 ng/µl)	1 µl
PCR product	3 µl
T4 DNA ligase	1 µl

2. Mix by pipetting and incubate for 1 h at room temperature (alternatively, the reaction can be incubated overnight at 4 °C).
3. Use 2 µl of the reaction to transform 50 µl of competent *E. coli* cells (DH5α or Stbl3 strain) as described in Subheading 3.3.2.
4. Plate the bacteria on LB agar/ampicillin/X-gal plates (*see* **Note 4**) and incubate overnight at 37 °C.
5. Pick 5–10 white colonies (blue/white colony screening is used to recognize recombinant (white) from non-recombinant (blue) colonies) and purify the DNA as described in Subheading 3.3.3.
6. Check each plasmid preparation by sequencing using either the T7 or the Sp6 primer.
7. Analyze the result by annealing the obtained sequences with the sequence of the wild-type gene, and determine if the desired mutation(s) is present.

4 Notes

1. Homemade Wnt3A-conditioned medium is used in the protocol. Recombinant Wnt3A is commercially available, but might not sustain growth as efficiently as homemade medium. We recommend using conditioned medium. Wnt3A is produced from L cells. This cell line can be requested from our lab.
2. Instead of commercial R-spondin 1, it is also possible to use 10% R-spondin 1-conditioned medium derived from R-spondin1-FC producing cell lines [21].
3. Organoid culture medium can be stored at 4 °C for up to 1 week.

4. LB agar/ampicillin/X-gal plates are prepared by plating 30 μl of X-gal solution (50 mg/ml in dimethylformamide) on a LB agar/ampicillin plate. Dry them at 37 °C.

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Chapter 16

Methods to Manipulate and Monitor Wnt Signaling in Human Pluripotent Stem Cells

Ian J. Huggins, David Brafman, and Karl Willert

Abstract

Human pluripotent stem cells (hPSCs) may revolutionize medical practice by providing: (a) a renewable source of cells for tissue replacement therapies, (b) a powerful system to model human diseases in a dish, and (c) a platform for examining efficacy and safety of novel drugs. Furthermore, these cells offer a unique opportunity to study early human development in vitro, in particular, the process by which a seemingly uniform cell population interacts to give rise to the three main embryonic lineages: ectoderm, endoderm. and mesoderm. This process of lineage allocation is regulated by a number of inductive signals that are mediated by growth factors, including FGF, TGFβ, and Wnt. In this book chapter, we introduce a set of tools, methods, and protocols to specifically manipulate the Wnt signaling pathway with the intention of altering the cell fate outcome of hPSCs.

Key words Wnt, Human pluripotent stem cell (hPSC), Human embryonic stem cell (hESC), Induced pluripotent stem cell (iPSC), Embryonic development

1 Introduction

From their first isolation and extended culture [1], human embryonic stem cells (hESCs) have been the subject of intense scrutiny and experimentation. Being capable of differentiation to cellular derivatives of all three germ layers and amenable to genetic engineering and genomic editing, hESCs have become the model system of choice for genetic and biochemical studies of early human development. The discovery that somatic cells can be readily reprogrammed to induced pluripotent stem cells (iPSCs) [2] further expanded the utility and application of these cells and provided a cell-based system to interrogate and model the development and progression of complex human disorders, such as Alzheimer's' disease, diabetes, and heart disease [3, 4].

Embryogenesis is a complex and meticulously orchestrated process that involves the synergistic activities of multiple signaling pathways, cell types, and physiological responses. A handful of

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signaling modules, including fibroblast growth factors (FGF), Hedgehog (Hh), Notch, transforming growth factors beta (TGFβ), and wingless-type MMTV integration site family (Wnt), have emerged as crucial regulators of embryonic development. Among these is the *Wnt* gene family, which encodes secreted lipid-modified growth factors [5] with alterations in expression of individual *Wnt* genes producing a spectrum of phenotypes. For example, mutations in *Wnt3* prevent the mouse embryo from completing gastrulation [6], whereas ectopic expression of *XWnt8* in Xenopus has axis-inducing activity [7]. In later stages of development and during adulthood, Wnt is critically important for tissue and organ development and homeostasis and for maintenance of stem cell populations of the blood, intestine, skin, and liver.

Given the many roles Wnt proteins in development, it is not surprising that it plays an equally critical role in hPSCs (*see* **Note 1**). Several studies, including from our laboratory, indicate that the amount of Wnt signaling activity is critical to the response of hPSCs, with low levels being critical for the maintenance of pluripotency and high levels promoting differentiation into mesendodermal lineages [8–11]. Ectopic activation of Wnt signaling in undifferentiated hPSCs is widely used to promote differentiation into definitive endoderm [12, 13], which is a precursor cell population to derive such tissues and organs as thymus, lung, liver, pancreas, stomach, and intestine. Furthermore, we recently demonstrated that prolonged Wnt activation yields an expandable progenitor cell population restricted to intermediate mesodermal fates, such as the renal lineage [14].

In this book chapter, we discuss and describe several of the methods used to manipulate Wnt signaling in hPSCs. To monitor Wnt signaling activity in hPSCs, we describe short-term readouts (24–72 h), including changes in cell morphology, gene expression and activation of reporter genes. Even though several distinct Wnt signaling pathways have been described, we focus our attention on the Wnt/β-catenin (aka. canonical) pathway (Fig. 1). The reader should consult the literature for methods to stimulate and assay the various noncanonical Wnt pathways (*see* **Note 2**).

Wnt signaling can be activated at multiple points along its transduction pathway (Fig. 1 and Table 1). The most direct way to stimulate Wnt signaling is through the addition of recombinant Wnt proteins, which engage the Wnt receptor complex comprising a FRIZZLED (FZD) and the low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6) (reviewed in ref. 15). To date, two recombinant Wnt proteins, Wnt3a and Wnt5a, have been successfully purified to near homogeneity [16–18] (*see* **Note 3**). While Wnt3a produces readily apparent changes in hPSC morphology and gene expression, the effects of Wnt5a application on hPSCs are less well characterized and documented. A common and more cost-effective alternative to purified Wnt proteins is the use of conditioned media (CM) from cells engineered to overexpress a *Wnt*

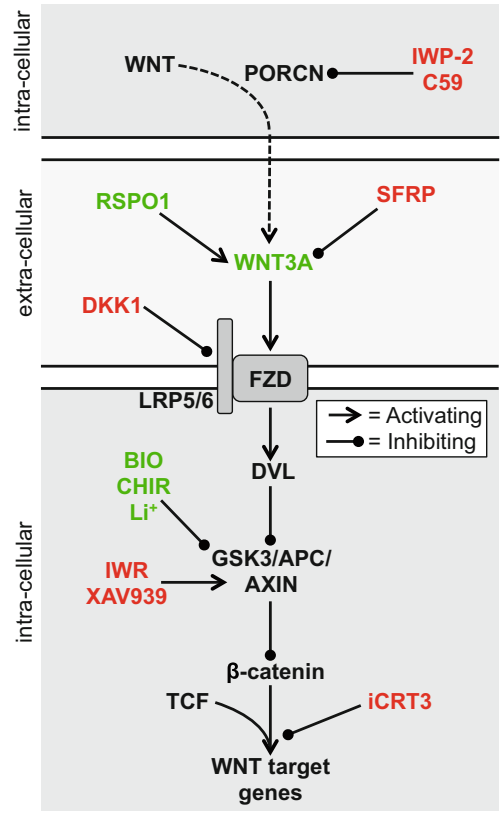

Fig. 1 Schematic of Wnt signaling pathway**.** Small molecules and recombinant proteins commonly used to manipulate Wnt signaling in cell culture are indicated in *green* (activating) and *red* (inhibiting). The mode of action of these agonists and antagonists is described in the text and outlined in Table 1

gene. Such Wnt CM is easy to generate, however, CM is rich with many other proteins (e.g., components of fetal bovine serum and factors secreted from the engineered cells) that may have undesired effects on hPSC self-renewal, proliferation, and differentiation.

Another potent recombinant protein used to augment Wnt signaling is R-Spondin-1 (RSPO1) [19]. RSPO1 by itself does not activate Wnt/β-catenin signaling but rather requires either an endogenously expressed or exogenously added Wnt to exhibit activating activity. This property has been particularly useful in the growth of gut and liver organoids [20–22] where it potently augments the low level of endogenous Wnt signaling activity. Since a low level of Wnt signaling activity is present in undifferentiated hPSCs [10, 11, 23, 24], addition of RSPO1 may act to promote their signal. RSPO1 enhances Wnt signaling through its interaction with its receptors LGR4/5, which leads to sequestration of the transmembrane ubiquitin ligases RNF43 and ZNRF3, thereby preventing FZD ubiquitination and subsequent degradation [25–29].

Table 1
List of commonly used Wnt agonists and antagonists

Name	Mode of action	Stock concentration	Working concentration	References
Small molecules				
6-bromoindirubin-3′-oxime (BIO)	*Activator* GSK3β inhibitor	10 mM in DMSO	1–10 μM	[32]
C59	*Inhibitor* Inhibitor of PORCN to block Wnt secretion	50 mM in DMSO	10–1000 nM	Reported in U.S. patent 8,546,396
CHIR 98014	*Activator* GSK3β inhibitor	25 mM in DMSO	10–1000 nM	[31]
iCRT3	*Inhibitor* Disrupts β-catenin/TCF interaction	10 mM in DMSO	1–100 μM	[42]
IWP-2	*Inhibitor* Inhibitor of PORCN to block Wnt secretion	2.5 mM in DMSO	10–1000 nM	[37]
IWR-1	*Inhibitor* Stabilizes Axin	50 mM in DMSO	10–1000 nM	[37]
LiCl	*Activator* GSK3β inhibitor	1 M in water	10–50 mM	[30]
XAV939	*Inhibitor* Tankyrase inhibitor	10 mM in DMSO	1–100 μM	[41]
Recombinant proteins				
DKK1	*Inhibitor* Binds to LRP5/6 and prevents receptor complex formation	100 μg/mL in PBS	10–100 ng/mL	[34]
R-Spondin 1	*Activator* Prevents FZD downregulation by RNF43 and ZNRF3 (Note: augments Wnt, inactive by itself)	2.5 μM (~100 μg/mL) in PBS	1–100 nM	[19]
SFRP1	*Inhibitor* Binds and sequesters Wnt [a]	250 μg/mL in PBS	10–1000 ng/mL	[33]
Wnt3a	*Activator* Binds FZD to activate signaling	1 μM (~40 μg/mL) in PBS [b]	1–10 nM	[17, 18]

A more comprehensive list of small molecule inhibitors and activators is available at http://web.stanford.edu/group/nusselab/cgi-bin/Wnt/smallmolecules

[a] In certain contexts, SFRPs promote Wnt signaling (for examples, *see* [35, 36])

[b] Commercially available Wnt proteins are reconstituted in PBS. However, purified Wnt protein when purified as previously described is stored in Buffer (PBS, 1% CHAPS, 1 M NaCl, pH7.5). When stimulating cells with high doses of Wnt protein, it is advisable to use to Buffer alone as a negative control

Wnt/β-catenin signaling can also be activated with small molecules, many of which inhibit GSK3β (including LiCl, BIO, and CHIR 98014) [30–32], thus preventing β-catenin phosphorylation followed by proteasome-mediated degradation. This mode of Wnt pathway activation is quite potent, however, GSK3β's functions in multiple signaling pathways, and hence its inhibition may have effects that are independent of Wnt signaling. Nonetheless, for several hPSC protocols, such as mesendodermal differentiation, GSK3β inhibitors are an appropriate substitute for purified Wnt3a protein. In our experience, the GSK3β inhibitor CHIR 98014 is highly active with little toxicity at low concentrations.

Wnt signaling can be blocked at multiple levels of the pathway (Fig. 1 and Table 1). Extracellular Wnt signals can be blocked with recombinant proteins that block Wnts' interactions with FZD receptors, such as Secreted Frizzled-Related Proteins (SFRPs) [33], or with recombinant proteins that prevent interactions between the co-receptors LRP5/6 and FZD, such as DKK1 [34]. However, it is unclear whether these methods block the activity of all Wnt proteins. For example, SFRPs do not only exert antagonistic effects on Wnt signaling (for examples, *see* [35, 36]. In several settings, addition of recombinant SFRPs has been found to promote Wnt signaling. It is therefore important to assess to what extent a recombinant SFRP antagonizes or promotes Wnt signaling.

Arguably the most effective method to block endogenous Wnt signaling is with small molecule inhibitors of PORCN, such as IWP-2 [37] and C59. PORCN is a resident ER-protein with homology to o-acyl transferases and is likely involved in lipid modification of Wnt proteins [17, 38–40]. Since all Wnt proteins appear to require PORCN function for processing and subsequent secretion, blocking PORCN activity will produce an "all Wnt" mutant phenotype. Therefore, PORCN inhibition will likely block all Wnt signaling pathways, including noncanonical Wnt signaling.

More selective inhibition of Wnt/β-catenin signaling can be achieved with small molecules, such as IWR-1 and XAV939, that stabilize AXIN, a negative regulator of the pathway, thereby further destabilizing and lowering β-catenin levels [37, 41]. In addition, small molecules that block the interaction between β-catenin and TCF, such as iCRT3, are potent inhibitors of Wnt target gene activation [42].

Activation of WNT/β-catenin signaling can be monitored using a number of methods. Accumulation of β-catenin in the cytoplasm and nucleus is a reliable readout, however, due to high levels of β-catenin protein in complex with adherens junctions at the cell surface, its detection by immunoblotting or immunofluorescence is often technically challenging. In the case of hPSCs, activation of Wnt signaling, either with high doses of Wnt3a or GSK3β inhibitor, produces a clear change in cell morphology that is apparent within a few days after stimulation, as

described below. A more quantitative measure of Wnt signaling is the expression of Wnt target genes, which are detectable within hours of Wnt pathway stimulation. An extensive number of Wnt target genes have been described, however, their expression is often cell-context dependent. Among these, *Axin2* expression is reliably expressed in all cell types tested [43–45] and is often considered to be a universal Wnt target gene. In hPSCs, a large number of genes that includes *T brachyury transcription factor* (*T*) and *SP5* are potently activated by exogenous Wnt pathway activation. Expression of these target genes is observed during primitive streak formation and gastrulation, highlighting Wnt's role in these early developmental processes. The most convenient method to monitor Wnt pathway activation in cells is with reporter constructs comprising multimerized TCF binding sites, a minimal promoter and a reporter gene that encodes luciferase [46] or a fluorescent protein [47]. Stable transduction of these reporters provides a convenient and quantitative measure of Wnt signaling in hPSCs, as described in the Methods section below. In the following "Methods" section, we describe in detail how best to detect Wnt signaling activity in hPSCs.

2 Materials

2.1 Equipment

1. Biosafety cabinet, sterile, with vacuum line, suitable for tissue culture.
2. Desktop centrifuge capable of handling 15 and 50 mL conical polystyrene tubes.
3. Flow cytometer.
4. Hemocytometer.
5. Ice Bucket.
6. Inverted light microscope.
7. Micropipettes (2, 20, 200, 1000 μL capacity).
8. Motorized pipette aid for serological pipettes.
9. Real-time PCR Detection System.
10. Refrigerated desktop microcentrifuge capable of cooling to 4 °C.
11. Spray bottle (for 70% ethanol disinfectant).
12. Thermal cycler.
13. Timer.
14. Tissue culture incubator at 37 °C, humidified with 5% CO_2.
15. Tube rack for 15 and 50 mL conical tubes.
16. UV spectrophotometer.
17. Water bath, 37 °C for warming culture media.

2.2 Reagents, Stock Solutions and Consumables

1. 7TGP (Plasmid), Addgene, 24305. Lentivirus preparation with this vector requires 2nd generation packaging plasmids psPAX2, Addgene, 12260, and pCI-VSVG, Addgene, 1733.
2. Accutase®, Innovative Cell Technologies.
3. Bovine Serum Albumin (BSA).
4. CHIR 98014. Dissolve in DMSO to a final concentration of 25 mM, prepare aliquots and store at −20 °C protected from light.
5. Conical tubes, sterile, polystyrene (15 and 50 mL capacity).
6. Dimethylsulfoxide (DMSO),
7. Dulbecco's Modification of Eagle's Medium/Ham's F-12 50/50 Mix (with l-glutamine and HEPES).
8. Dulbecco's phosphate buffered saline, Ca^{2+} and Mg^{2+} Free (DPBS).
9. Ethanol (Absolute, Molecular Biology Grade).
10. Ethanol (For 70 % ethanol disinfectant).
11. Filter (0.22 μm, PES, syringe mounted).
12. Flow cytometry buffer: DPBS, EDTA 5 mM, BSA 1 %, NaN_3 0.1 %. This buffer is filtered through a 0.22 μm syringe filter to remove particulate matter.
13. Flow cytometry tubes.
14. Human pluripotent stem (hPS) cells, such as lines H1 (NIH Registration Number 0043), H9 (NIH Registration Number 0062), H9 *T-GFP* [48] HUES9 (NIH Registration Number 0022) or any induced pluripotent stem (iPS) cell line. For the methods described below (Subheading 3.1), 1 well of a 6-well plate of nearly confluent hPSCs will be sufficient. The morphology of normal hPSC colonies is shown in the top left panel of Fig. 2.
15. Ice.
16. Matrigel®. Thaw one vial of Matrigel® on ice overnight. Aliquot into prechilled sterile microcentrifuge tubes exactly according to the volume provided in the manufacturer's specifications. Each lot of Matrigel® requires a different aliquot volume. All Matrigel aliquots are later resuspended in 25 mL of ice cold DMEM/F12 regardless of lot.
17. MicroAmp Optical 96-well qPCR plate.
18. Microcentrifuge tubes (sterile, nuclease and protease free, 1.5 mL capacity).
19. Micropipette tips (2, 20, 200, 1000 μL capacity).
20. Nucleospin® RNA Kit.
21. Optical adhesive cover.

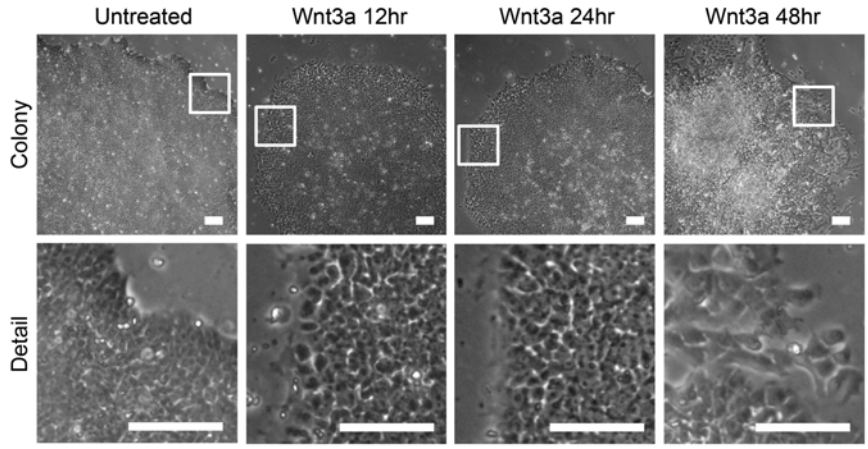

Fig. 2 Morphological changes of hESCs in response to Wnt3a**.** HESCs (H1) cells were either grown in standard culture conditions (Untreated) or treated with Wnt3a (1 nM) for 12, 24 and 24 h (hr). The images illustrate how hESC colonies treated with Wnt3a lose their characteristic compact colony with individual cells dissociating and adopting fibroblast-like morphologies. Scale bar = 100 μm

22. Pasteur pipettes (glass, autoclaved, for aspirating culture media and solutions in a sterile biosafety cabinet).
23. Penicillin–streptomycin (Pen/Strep) (*see* **Note 4**).
24. Primers for reverse transcription quantitative PCR (Table 2). Reconstitute lyophilized primers to 100 μM in molecular biology grade H_2O.
25. qScript cDNA SuperMix.
26. Rho-associated protein kinase inhibitor Y-27632 (ROCKi). Dissolve in DPBS at a final concentration of 5 mM to prepare a 1000× stock solution, prepare aliquots and store at −20 °C protected from light.

Table 2
List of primers for reverse transcription quantitative PCR (PCR)

Target	Forward primer	Reverse primer
AXIN2 (Wnt Target)	TATCCAGTGATGCGCTGACG	CGGTGGGTTCTCGGGAAATG
NANOG (Pluripotency)	TTTGTGGGCCTGAAGAAAACT	AGGGCTGTCCTGAATAAGCAG
POU5F1 (Pluripotency)	CTTGAATCCCGAATGGAAAGGG	GTGTATATCCCAGGGTGATCCTC
RPL37A (Internal Control)	ATTGAAATCAGCCAGCACGC	GATGGCGGACTTTACCGTGA
SP5 (Wnt Target)	TCGGACATAGGGACCCAGTT	CTGACGGTGGGAACGGTTTA
T (Wnt Target)	CTATTCTGACAACTCACCTGCAT	ACAGGCTGGGGTACTGACT

27. SensiFAST SYBR Hi-ROX Kit (2X qPCR Mix).
28. Serological pipettes (sterile; 5, 10, 25 mL capacity).
29. Syringe (Sterile, 50 mL, Luer Lock).
30. TeSR™-E8™ culture medium, or prepare as described [49].
31. Trypan blue solution (0.4%).
32. Water (Ultrapure™, DNase/RNase-free, distilled, molecular biology grade).
33. Wnt Buffer: DPBS, NaCl 1 M, CHAPS 1%, BSA 0.1%.
34. Wnt3a (Mouse, Recombinant Protein), reconstitute at 40 μg/mL (~1 μM) in sterile PBS+0.1% BSA. Or purify as described [18].

3 Methods

3.1 Passaging hPSCs

To ensure reproducible experimental results, it is important that hPSCs be cultured and passaged in a consistent and controlled fashion. Here we lay out the critical details of hPSC passaging. All volumes and procedures are for 1 well of a 6-well plate. Scale volumes according to relative surface area when using different plate formats. All liquid handling must be performed within a sterile laminar flow biosafety cabinet. All nonsterile equipment, consumables and reagents (e.g., pipettes, sterile pipette tip boxes and bottles of medium) should be disinfected by spraying with 70% ethanol immediately prior to placement within the biosafety cabinet. Since FGF2 is labile at 37 °C, TeSR™-E8™ culture medium should not be warmed to 37 °C and should instead be brought to room temperature before use.

Coating plates with Matrigel® Matrix

1. Thaw an aliquot of Matrigel® on ice for 60 min. Meanwhile, prepare a 50 mL conical tube with 25 mL of DMEM/F12 medium, incubate on ice along with the thawing Matrigel® and remove TeSR™-E8™ culture medium from refrigeration to allow it to warm to room temperature, also 60 min.
2. Transfer the aliquot of Matrigel® and the tube of DMEM/F12 from the ice bucket to the biosafety cabinet. Using a micropipette (1000 μL capacity), quickly dilute the Matrigel® with 700 μL of ice cold DMEM/F12. Transfer the entire resuspended mixture to the conical tube of DMEM/F12 and mix by inverting gently ten times (*see* **Note 5**).
3. Coat 1 well of a 6-well plate with 1 mL of reconstituted Matrigel®. Incubate the plate for 30 min in a humidified incubator (37 °C, 5% CO_2). The plate may remain in the incubator until the cells are ready to be seeded (*see* below). Do not aspirate the reconstituted Matrigel® solution until immediately prior to adding the cells to the well.

Dissociating Cells with Accutase®

4. Aspirate the media from each well and wash with DPBS. Transfer 1 mL of Accutase® warmed to 37 °C for each well. Incubate at 37 °C for 3 min.
5. Remove the plate from the incubator and observe the level of dissociation using an inverted light microscope. Cells should be free floating, mostly single cells with some small clumps remaining. If the cells are not totally detached, return the plate to the incubator for 3 additional minutes and reassess (*see* **Note 6**).
6. Transfer the plate to the biosafety cabinet. Gently triturate the cell suspension with a micropipette to break up remaining clumps and transfer the resulting cell suspension to the 15 mL conical tube with TeSR™-E8™ culture medium.
7. Transfer the 15 mL conical tube with cells to a desktop cell centrifuge. Pellet the cells by centrifuging for 3 min at 200 × g-force (g). Ensure that the centrifuge is properly balanced. Meanwhile, prepare TeSR™-E8™ culture medium with ROCKi diluted 1:1000 (5 μM final concentration) for the resuspension of the cell pellet (1 mL) and plating in Matrigel® coated plates (2 mL).
8. Transfer the tube back to the biosafety cabinet. Aspirate the supernatant and use a micropipette to resuspend the cell pellet with 1000 μL TeSR™-E8™ + ROCKi. While the cells are still evenly suspended, use a micropipette to transfer 10 μL to a microcentrifuge tube and proceed with counting.

Seeding the Cells in a Matrigel® Coated Well

9. To count the cells, add 10 μL of trypan blue directly to the 10 μL cell suspension and mix gently and thoroughly by pipetting up and down. Transfer 10 μL of this suspension to a hemocytometer and count live (non-stained) cells using an inverted light microscope. Use the count to determine the density of live cells in each suspension. The twofold dilution with trypan blue must be factored in. Calculate the volume of cell suspension that contains 20,000 live cells. This is the volume of cells to be seeded in 1 well of a 6-well plate (*see* **Note 7**).
10. Transfer the Matrigel® coated plate from the incubator to the biosafety cabinet. Aspirate the Matrigel® matrix from the well and replace with 2 mL of TeSR™-E8™ + ROCKi (*see* **Note 8**).
11. Resuspend the cells (as they have likely partially settled at this point and are no longer uniformly suspended), transfer the volume containing 20,000 live cells to the appropriate well of the Matrigel® coated plate, and disperse the cells in the well by rocking the plate left to right three times, then forward and backward three times. Cells will begin to attach to Matrigel® coated wells very quickly, so it is important to evenly disperse the cells as quickly as possible to ensure even cell density across the well. When multiple wells are to be seeded, do not wait

until all wells have cells in them, but rather disperse the cells 1 well at a time as soon as they are transferred

12. Transfer the plate back to the incubator and let the cells rest overnight before feeding.
13. Feed the cells fresh TeSR™-E8™ culture medium (without ROCKi) daily until the next passaging (~80% confluency, 5–6 days) or until they are ready for experimentation (40–50% confluency; 4–5 days).

3.2 Manipulating WNT Signaling in hPSCs

All reagents for manipulating Wnt signaling in hPSCs should be resuspended in sterile tissue culture grade DPBS or DMSO according to manufacturer's recommendations. This protocol refers to recombinant Wnt3a protein, but any of the Wnt pathway modulators listed in Table 1 may be used in the same fashion as described below. All volumes are for 1 well of a 6-well plate. Scale volumes accordingly when using multiple wells or different plate formats.

1. In a sterile laminar flow biosafety cabinet, transfer the appropriate amount of TeSR™-E8™ culture medium (2 mL per well of a 6-well plate) and allow to warm to room temperature.
2. Thaw sufficient Wnt3a on ice and prepare a microcentrifuge tube of Wnt Buffer for control treatment. For a final Wnt3a concentration of 2.5 nM, 5 μL of protein (1 μM stock) is needed for every 2 mL of culture medium. For compounds prepared in DMSO, thaw at room temperature (DMSO is solid on ice and thaws slowly at room temperature).
3. Once the Wnt3a has thawed, disinfect with 70% ethanol and transfer to the hood.
4. Remove the plate of hPSCs from the tissue culture incubator and inspect them using an inverted phase contrast microscope to ensure that they are healthy and grown to the proper density, about 40–50% confluent. Move the cells to the biosafety cabinet.
5. Aspirate the culture medium from the well and replace with 2 mL of room temperature TeSR™-E8™ culture medium.
6. Using a micropipette, transfer the appropriate volume of Wnt3a directly to the well of freshly fed hPSCs (*see* **Note 9**). As a negative control, add an equal volume of Wnt Buffer to a separate well of hPSCs (*see* **Note 10**). Mix thoroughly by gently swirling. Return the cells to the incubator.
7. After 24 h of treatment, assess the level of Wnt pathway activation as discussed in the next sections. For longer periods of Wnt stimulation (2 or more days), hPSCs should be fed daily with TeSR™-E8™ culture medium + Wnt3a.

3.3 Detecting Wnt Activity

There are multiple methods for detecting Wnt pathway activation in hPSCs. In this section, we will describe how Wnt pathway activation can be evaluated by standard phase contrast microscopy, gene expression, and reporter gene expression.

3.3.1 Morphology Based Detection by Light Microscopy

Activation of the Wnt pathway in hPSCs leads to striking morphological changes reminiscent of gastrulation and the formation of embryonic germ layers. This morphological readout of Wnt pathway activation is reliable but not sufficiently quantitative for the fine-tuning of pathway activation that may be required for sensitive downstream applications including differentiation protocols.

1. Following treatment of hPSCs with Wnt3a (or other compound), image the well using an inverted phase contrast microscope. Using a camera, capture representative images of multiple colonies.
2. High Wnt pathway activation will result in heterogeneous cellular morphology often marked by epithelial-to-mesenchymal transition near the colony borders and mound formation near colony centers (Fig. 2).
3. Low or no Wnt pathway activation causes no significant changes to colony morphology relative to buffer treatment.

3.3.2 Gene Expression Based Detection by qPCR

Increased expression of known Wnt target genes (*AXIN2*, *SP5* and *T*) along with decreased expression of markers of pluripotency (*NANOG* and *POU5F1*) is a reliable and quantitative measure of Wnt pathway activation in hPSCs (Fig. 3). Cells should be harvested and processed quickly to ensure isolation of high quality RNA. Ensure that all reagents and materials, such as microcentrifuge tubes and pipette tips, are certified free of nucleases and proteases.

1. Following treatment of hPSCs with Wnt3a (or other compound), isolate total cellular RNA from the sample using the Nucleospin® RNA Kit (or comparable) according to the manufacturer's directions.
2. Quantify the RNA yield from the sample using a UV spectrophotometer. 1 well of a 6-well plate typically yields 10–30 μg of total RNA.
3. Utilize 1 μg of input RNA to perform first strand cDNA synthesis using the qScript™ cDNA Supermix kit (or comparable) according to the manufacturer's directions (*see* **Note 11**). Reaction mixture: 4 μL qScript™ cDNA Supermix; 1 μg RNA; H_2O to 20 μL final volume. Thermal cycler parameters: 25 °C, 5 min; 42 °C, 30 min; 85 °C, 5 min. Assume 1:1 copying of RNA to cDNA for downstream estimation of cDNA concentration (50 ng/μL) (*see* **Note 12**).

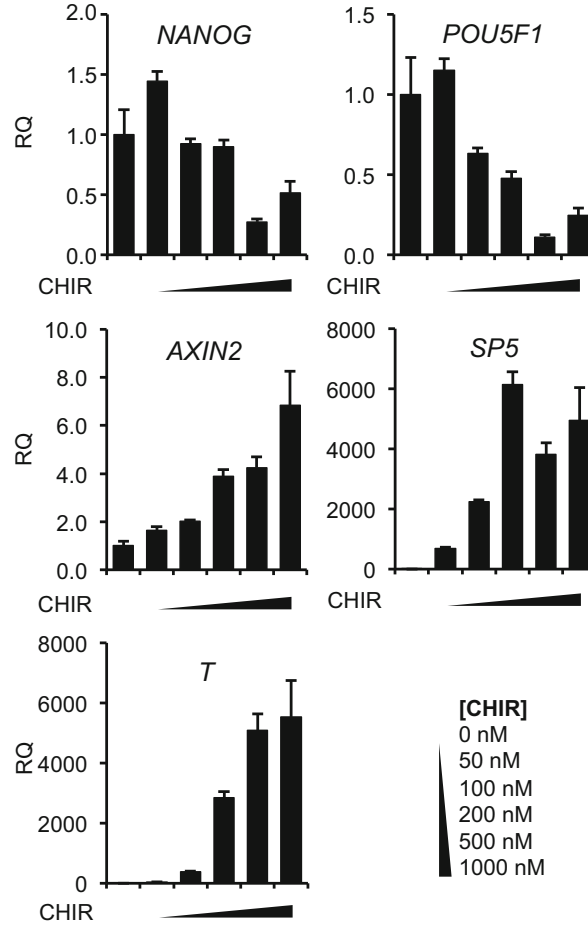

Fig. 3 Gene expression changes in response to Wnt pathway activation**.** HESCs (H1) were treated with the indicated doses of CHIR 98014 (CHIR) for 24 h. Gene expression was analyzed by reverse transcription quantitative PCR. Expression of the pluripotency markers *NANOG* and *POU5F1* (*OCT4*) steadily declines with increasing doses of CHIR, while expression of the Wnt target genes *AXIN2* and *SP5* steadily rises. Expression of the mesendodermal marker *T* (T brachyury transcription factor) also steadily rises

4. Prepare the Primer Mixes. For each gene target, mix the forward primer and reverse primer in molecular biology grade H_2O to a final concentration of 8 μM each. For example, if primer stocks have been reconstituted at 100 μM each, add 80 μL of forward primer, 80 μL of reverse primer, and 840 μL of H_2O. This primer mix is a 20× concentrate (the final concentration in qPCR reactions is 0.4 μM).
5. Prepare the primer master mixes (PMM) for qPCR. The volumes described below are for amplification in a 96-well plate format (10 μL per well). Adjust volumes accordingly for 384-well format (5 μL per well). For each test (one gene amplified in one sample),

three technical replicates of 10 μL final volume will be run on the plate. Each well will contain the following: 2× qPCR Mix, 5 μL; Primer Mix (8 μM Forward + 8 μM Reverse), 0.5 μL; H_2O, 3.5 μL; cDNA template, 1.0 μL. Therefore, to prepare PMM, ensure sufficient volume of 2× qPCR Mix, Primer Mix, and H_2O to fill 3.6 wells for each cDNA sample to be tested. The extra volume will account for pipetting error. For example, to analyze the expression of *AXIN2* in 4 samples, the PMM will contain 3.6 * 4 * (2× qPCR Mix, 5 μL; *AXIN2* Primer Mix, 0.5 μL; H_2O, 3.5 μL). The final volumes are then as follows: 2× qPCR Mix, 72 μL; *AXIN2* Primer Mix, 7.2 μL; H_2O, 50.4 μL. Make one PMM for each Primer Mix. Be sure that one PMM contains primers targeting an endogenous control gene whose expression changes little across treatment conditions.

6. Prepare the sample master mixes (SMM). For each cDNA sample to be analyzed, prepare labeled microcentrifuge tubes containing 31.5 μL of PMM from the previous step. There should be one tube for each individual PMM per cDNA sample to be tested. To each of these tubes, add 3.5 μL of the appropriate cDNA sample for a final volume of 35 μL. Mix thoroughly by pipetting up and down. Avoid air bubble introduction.
7. Open a tube of SMM. Using a micropipette, transfer 10 μL to three adjacent wells of a 96-well optical qPCR plate. Keep track of sample location. Repeat for the next tube of SMM in the next 3 wells and so on until all SMMs have been dispensed. Seal the plate using an optical adhesive cover. Ensure a complete seal over the top of each well. The plate may be centrifuged for 1 min at 1000 × *g* to collect all fluid to the bottom of each well.
8. Perform qPCR amplification on a Bio-Rad CFX96 or comparable instrument with the following parameters: 95 °C, 2 min; (95 °C, 5 s; 60 °C, 15 s) × 40 cycles.
9. Analyze the data using the software provided with the qPCR thermal cycler (Bio-Rad CFX™ Manager or comparable) (*see* **Note 13**).

3.3.3 Reporter Based Detection with Flow Cytometry

Fluorescent reporter constructs provide the most quantitative measurement of Wnt pathway activation and can be used to analyze or sort single cells grouped into sub-populations defined by responsiveness. Artificial reporters such as TOP-GFP (Fig. 4) can be introduced by lentiviral transgenesis followed by drug selection and single cell cloning (*see* **Note 14**). Reporters that express a gene encoding a fluorescent protein under the control of endogenous Wnt target gene promoters may more faithfully represent Wnt pathway activation. To that end, we have utilized the H9 *T-GFP* cell line [48], which expresses GFP under the control of the *T* promoter in response to Wnt pathway activation (Fig. 5). The following protocol describes the steps necessary for the analysis of one well of a 6-well plate.

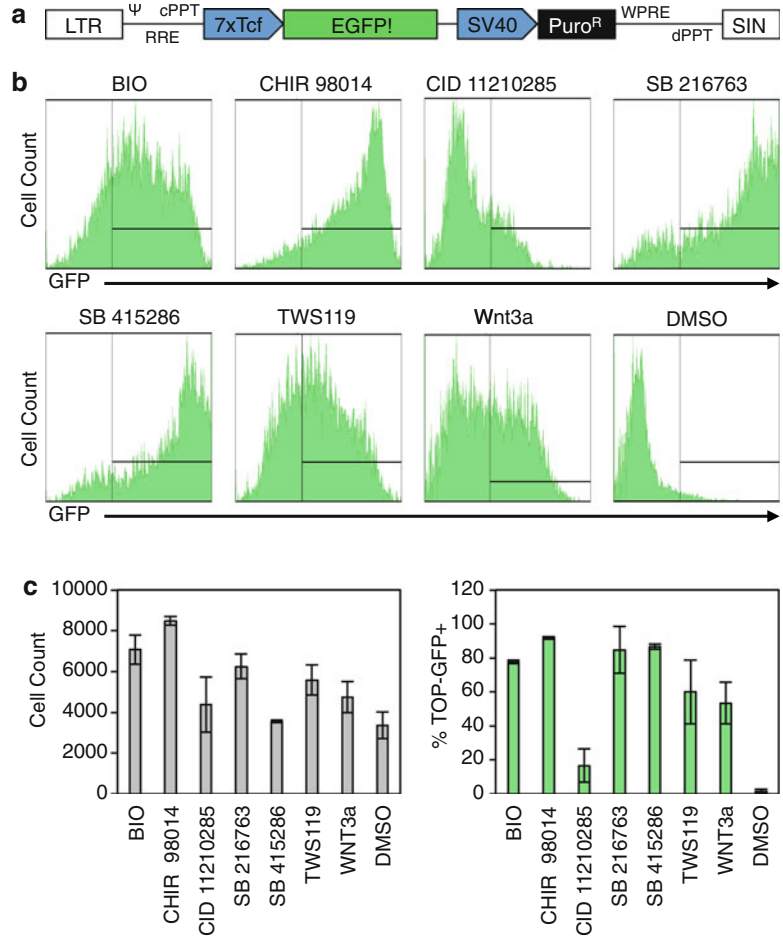

Fig. 4 Monitoring Wnt activity using a Wnt reporter assay**. (a)** Schematic of self-inactivating (SIN) lentiviral vector carrying a Wnt-inducible reporter. The Wnt-responsive promoter, designated by 7×Tcf, comprises seven multimerized Tcf binding sites (AGATCAAAGGGG) coupled to a minimal promoter. The vector also includes a drug selection cassette (SV40-Puro^R) [47]. Source: Addgene 7TGP, plasmid #24305. (**b)** Activation of Wnt reporter in hESCs. HUES9 cells were transduced with the lentiviral vector diagrammed in *panel A*, and a clonal, drug-selected line was isolated and screened for maximal Wnt responsiveness (described in ref. [61]). Here, this Wnt reporter cell line is treated with Wnt3a (2 nM) or with the indicated small molecule agonists of the Wnt pathway (each at 5 μM) for 48 h. GFP fluorescence was assayed by flow cytometry. With the exception of CID 11210285, these small molecules primary mode of action is through the inhibition of GSK3β. DMSO-treated cells served as a negative control. (**c**)**.** Quantitation of cell number (*left graph*) and GFP expression (*right graph*). The flow-cytometric data shown in *panel B* is illustrated in bar graph format. The cell count of cultures treated with certain GSK3β inhibitors (most notably BIO and CHIR 98014) is significantly elevated over DMSO- or Wnt3a-treated cultures, suggesting that GSK3β inhibition may promote proliferation independently of Wnt pathway activation

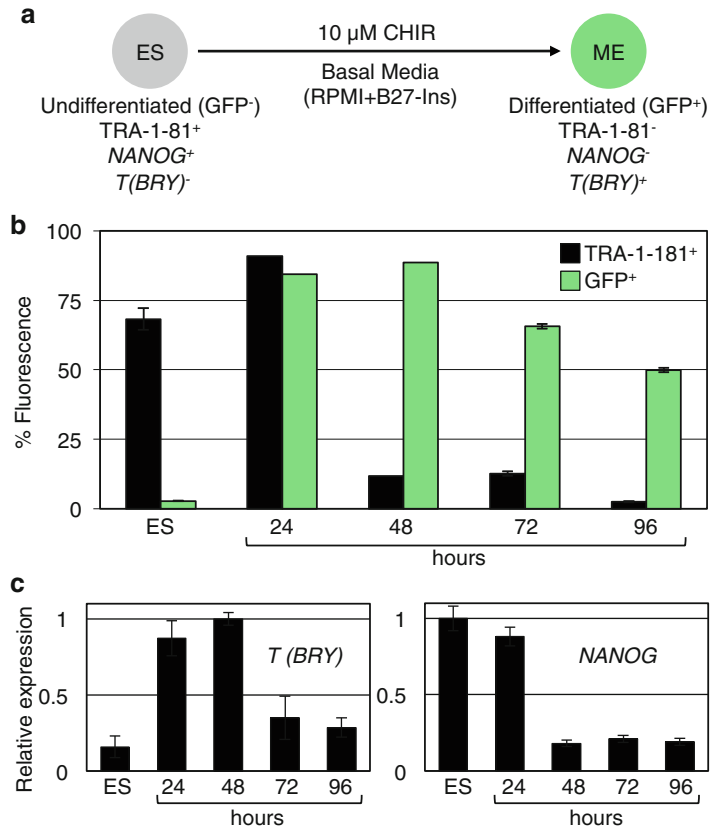

Fig. 5 Monitoring Wnt activity using a T-GFP hESC line**.** (**a**) Schematic of differentiation of hESCs (H9) carrying a T-GFP reporter construct (described in ref. [48]). Cells are treated with the GSK3β inhibitor CHIR 98014 (CHIR) in the indicated media. (**b)** Activation of the T-GFP reporter was quantified by flow cytometry at 24, 48, 72, and 96 h after treatment with CHIR. In addition, expression of the cell surface antigen TRA1-81 was monitored over the course of the treatment. (**c**)**.** Gene expression analysis of cells treated as described in *panel A* indicates a robust and transient activation of *T* and a steady decline of *NANOG* expression

1. Following treatment of hPSCs with Wnt3a (or other compound), dissociate the cells as described in Subheading 3.1, **steps 8–10**.
2. For each sample to be analyzed, prepare a 15 mL conical tube with 1 mL of flow cytometry buffer.
3. Remove the dissociating cells from the incubator and observe the level of dissociation using an inverted light microscope. Cells should be free floating, mostly single cells with small clumps. If the cells are not totally detached, return them to the incubator for 3 additional minutes and reassess (*see* **Note 6**).

4. Transfer the plate to the biosafety cabinet. Gently triturate the cell suspension with a micropipette (1000 μL capacity, set to 800 μL) to break up remaining clumps and transfer the resulting cell suspension to the 15 mL conical tube with flow cytometry buffer.
5. Transfer the 15 mL conical tube with cells to a desktop cell centrifuge. Pellet the cells by centrifuging for 3 min at 200 × *g*. Ensure that the centrifuge is properly balanced.
6. Transfer the tube back to the biosafety cabinet. Aspirate the supernatant and use a micropipette (1000 μL capacity) to resuspend the cell pellet with 1 mL flow cytometry buffer.
7. Transfer the cell suspension to a flow cytometry tube on ice.
8. Analyze GFP expression by flow cytometry using a BD FACSCanto™ II or similar instrument.

4 Notes

1. hPSCs are distinct from mouse embryonic stem cells (mESCs) in several critical ways: while mESCs are considered to be in a "naïve" state that most closely resembles the cells of the inner cell mass in the blastocyst, hPSCs are in a "primed" state that most closely resembles a slightly later developmental stage of the epiblast. The reader is directed to an extensively literature on this subject matter, including multiple reviews [50–52].
2. The literature is awash with the terms "canonical" and "non-canonical" Wnt signaling, however, the distinctions between these two pathways is not always clear. The canonical pathway, or Wnt/β-catenin pathway, is defined by the involvement of the downstream component β-catenin, which acts as a transcription factor to activate Wnt target genes. In contrast, non-canonical Wnt signaling encompasses a variety of pathways, including the most commonly referenced Wnt/calcium pathway and the planar cell polarity pathway [53–57]. Noncanonical Wnt signaling also potently antagonizes the Wnt/β-catenin signaling pathway [16, 58–60], highlighting the extent to which the various Wnt pathways are intertwined.
3. Other recombinant Wnt proteins, including Wnt2B, 4, 7A, 8A, 9A, 10B, 11, and 16B, are commercially available; however, their purification and activity(ies) have not been reported and detailed in the literature, and the reader should exercise caution in interpreting data obtained using these recombinant proteins.
4. Pen/Strep may be added to TeSR™-E8™ culture medium to prevent contamination of cell cultures but is not required. Pen/Strep is not detrimental to hPSC pluripotency, self-renewal, or differentiation.

5. Matrigel® begins to polymerize when removed from ice. It is important to work quickly and return the reconstituted Matrigel® to ice as soon as possible (e.g., immediately after dispensing it to wells). Reconstituted Matrigel® may be stored at 4–8 °C for up to 2 weeks.
6. Accutase® is gentler than other dissociation reagents, such as Trypsin. Cells can remain in Accutase® for up to 10 min if necessary without significant changes in viability or pluripotency. If the cells have not fully detached from the plate after 6 min, the Accutase® dissociation reagent may have lost activity. Make aliquots and avoid repeated warming of Accutase® to prevent diminished activity.
7. 20,000 live cells translates to roughly 2100 cells/cm^2, which for H1 cells in TeSR™-E8™ culture medium results in a culture that is ready to passage within 5 days. If other hPSC lines are used, it may be necessary to optimize cell seeding density. Generally speaking, hPSCs grown in TeSR™-E8™ culture medium proliferate rapidly. Tight control of initial cell seeding density is critical for the success of many hPSC protocols. Ensuring an accurate cell count is of the highest importance.
8. ROCKi is only used for 24 h after seeding and is withdrawn the following day.
9. Wnt3a protein activity diminishes rapidly in low protein growth media such as TeSR™-E8™. Do not premix Wnt3a protein with culture media. Wnt3a protein should be added directly to the wells following feeding and not prior to that time.
10. It is important to use Wnt Buffer as a negative control for Wnt treatment because these components are present in the lyophilized protein.
11. Accurate pipetting of RNA is crucial for the success of qPCR. If RNA is highly concentrated, make a dilution to avoid pipetting volumes under 2 μL.
12. Direct quantification of cDNA yield is unnecessary and will not be covered in this chapter. If you desire to quantify first strand cDNA directly, it is first necessary to remove RNA and free nucleotides via purification as these will inflate the observed concentration by UV spectrophotometry. Widely available PCR cleanup kits work well for this purpose.
13. Wnt pathway activation will lead to upregulation of the Wnt target genes *AXIN2*, *SP5* and *T*. The latter two genes are highly sensitive targets and display upregulation of several hundredfold 24 h post treatment with 2.5 nM Wnt3a. *AXIN2* upregulation following Wnt pathway activation is highly consistent and typically ranges between two and tenfold. In addition, the pluripotency markers *NANOG* and *POU5F1* exhibit

decreased expression following high levels of Wnt signaling for 24 h. A time course of treatment may also be of use to determine the kinetics of gene expression following Wnt pathway modulation.

14. The TOP-GFP viral vector, 7TGP, is a 2nd generation lentiviral vector. The virus may be produced and purified according to a protocol provided by The RNAi Consortium (TRC) (http://www.broadinstitute.org).

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Chapter 17

Directed Endothelial Progenitor Differentiation from Human Pluripotent Stem Cells Via Wnt Activation Under Defined Conditions

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Abstract

Efficient derivation of endothelial cells and their progenitors from human pluripotent stem cells (hPSCs) can facilitate studies of human vascular development, disease modeling, drug discovery, and cell-based therapy. Here we provide a detailed protocol for directing hPSCs to functional endothelial cells and their progenitors in a completely defined, growth factor- and serum-free system by temporal modulation of Wnt/β-catenin signaling via small molecules. We demonstrate a 10-day, two-stage process that recapitulates endothelial cell development, in which hPSCs first differentiate to endothelial progenitors that then generate functional endothelial cells and smooth muscle cells. Methods to characterize endothelial cell identity and function are also described.

Key words Human pluripotent stem cells, Endothelial progenitors, Endothelial cells, Wnt signaling, Chemically defined, Growth factor-free, Serum-free, Small molecules

1 Introduction

Human pluripotent stem cells (hPSCs) possess great potential for the study and treatment of vascular diseases due to their capacity for unlimited self-renewal and ability to form any somatic cell type [1–4]. Functional endothelial cells and their progenitors differentiated from hPSCs could be beneficial for many potential applications, including disease modeling, drug discovery, and cellular therapies [5–8]. To realize this potential, it is necessary to be able to control hPSC differentiation to endothelial lineages with high efficiency and reproducibility in a scalable and cost-effective manner.

Over the past decades, three major approaches: (1) coculture of hPSCs with mouse stromal cell lines [9, 10], (2) embryoid body (EB) formation [11–13], and (3) 2D directed differentiation techniques [14–17], have been developed to induce endothelial differentiation from hPSCs. These protocols utilized small molecules, growth

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factors, and extracellular matrix proteins to specify hPSCs to EC fates. Some of these protocols have reported generating endothelial cells with a purity of 20–50% in different hPSC lines. However, the efficiency of these distinct differentiation protocols is highly variable between cell lines and experimental repeats within the same line. In addition, the inclusion of growth factors and xenogeneic components increases the complexity and cost of these approaches, limiting their application as a model to study vascular development.

In this protocol, we provide a simple and robust differentiation platform for the generation of endothelial progenitors and functional endothelial cells from hPSCs in a completely defined, growth factor-free and serum-free system by temporal modulation of Wnt/β-catenin signaling via small molecules. This protocol is based on our earlier reports that Wnt signaling can direct hPSCs through mesoderm progenitors to cardiovascular cell types [18, 19] and is composed of three major steps: (1) induction of endothelial progenitors from hPSCs by temporal modulation of Wnt signaling, (2) a single magnetic-activated cell sorting (MACS) step to obtain pure endothelial progenitors, and (3) directed differentiation of endothelial progenitors into endothelial cells. We also provide procedures for performing flow cytometry and immunostaining analysis, and cryostorage and thawing of these hPSC-derived endothelial cell progenitors. The culture system described here can generate homogenous cultures of pure endothelial cells and their progenitors within 10 days.

2 Materials

Prepare all reagents and solutions in a sterile environment, or filter the unsterile solution using Stericup or Steriflip filtration systems after preparation. Prepare and store all reagents in a refrigerator (4 °C) for up to 3 months unless indicated otherwise. Diligently follow all waste disposal regulations and biological safety protocols when disposing waste materials.

2.1 Cell Culture and Differentiation Reagents

1. Stem cell culture media: mTeSR1 (STEMCELL Technologies, Vancouver, BC, Canada) or Essential 8™ (E8) (Life Technologies, Grand Island, NY, USA).
2. l-Ascorbic acid stock solution (100 mg/ml): dissolve 5 g l-ascorbic acid (Sigma, St. Louis, MO, USA) into 50 ml sterile MilliQ water. Aliquot into 1 ml samples and store at −20 °C for up to 1 year.
3. DMEM/Vc medium (500 ml): add 500 μl of 100 mg/ml l-ascorbic acid solution to 500 ml DMEM basal medium.
4. LaSR basal medium (500 ml): add 6.25 ml GlutaMAX (Life Technologies, Grand Island, NY, USA) and 305 μl 100 mg/ml l-ascorbic acid into 500 ml advanced DMEM/F12 medium.

5. Y27632 (5 mM): dissolve 10 mg Y27632 (Tocris, Minneapolis, MN, USA) in 6.24 ml sterile phosphate-buffered saline (PBS), aliquot 100 μl samples into sterile 1.5 ml tubes and store at −20 °C for up to 1 year.
6. CHIR99021 (36 mM): dissolve 25 mg CHIR99021 (Selleckchem, Houston, TX, USA) in 1.49 ml DMSO, aliquot and store at −20 °C for up to 1 year.
7. Matrigel-coated plates: add 1 ml of cold (4 °C) DMEM/F12 into one Matrigel aliquot (2.5 mg), and use a P1000 tip to thaw and dissolve the Matrigel. Immediately transfer the solution to a 50 ml conical tube on ice that contains 23 ml cold DMEM/F12. Mix well and add 1 ml/well Matrigel in DMEM/F12 for 6-well plates, 0.5 ml/well for 12-well plates. Allow the Matrigel to sit for at least 30 min at room temperature before use.
8. Synthemax II-SC stock solution (1 mg/ml): dissolve 10 mg Synthemax II-SC substrate (Corning, New York, NY, USA) in 10 ml of sterile water, mix well and store at 4 °C for up to 6 months.
9. Synthemax-coated plates: add 150 μl of 1 mg/ml Synthemax II-SC stock solution into 6 ml of sterile water and mix well by gently pipette. Add 1 ml/well Synthemax in water for 6-well plates, 0.5 ml/well for 12-well plates, and allow the Synthemax to sit at room temperature for at least 2 h. Aspirate the remaining solution and the Synthemax-coated plates are ready to use.
10. Accutase (Innovative Cell Technology, San Diego, CA, USA).

2.2 Magnetic-Activated Cell Sorting (MACS)

1. EasySep™ Magnet (STEMCELL Technologies, Vancouver, BC, Canada).
2. EasySep™ FITC Positive Selection Kit (STEMCELL Technologies, Vancouver, BC, Canada).
3. CD34-FITC conjugated antibodies (Miltenyi Biotec, San Diego, CA, USA).
4. EGM-2 BulletKit (Lonza, Basel, Switzerland).
5. Fetal bovine serum (FBS, Life Technologies, Grand Island, NY, USA).
6. Stericup and Steriflip filtration systems (Millipore, Billerica, MA, USA).
7. FlowBuffer-1 (500 ml): dissolve 2.5 g BSA in 500 ml PBS and filter using a 500 ml Stericup filtration system. The solution can be stored at 4 °C for up to 6 months.
8. 40 μm Falcon cell strainer (Corning, New York, NY, USA).
9. Collagen IV (1 mg/ml): dissolve 5 mg collagen IV in 5 ml sterile water, aliquot and store at −20 °C for up to 1 year.

10. Collagen IV-coated plates or coverslips: add 60 μl of 1 mg/ml collagen IV into 6 ml sterile water and gently pipette the solution to mix well. Immediately add 1 ml/well collagen IV in water for 6-well plates, 0.5 ml/well for 12-well plates with or without sterile coverslips. Allow the collagen IV to sit at 37 °C for at least 30 min before use (*see* **Note 1**).

2.3 Characterization of hPSC-derived Endothelial Cells

1. DMEM10: add 50 ml FBS to 450 ml DMEM, and filter with a 500 ml Stericup filtration system.
2. 1 and 4% formaldehyde: add 187.5 μl or 1 ml of 16% formaldehyde into 3 ml PBS. We do not recommend storing the solution.
3. 1% Triton X-100 solution (500 ml): add 5 ml Triton X-100 into 495 ml PBS and shake the bottle to dissolve the Triton.
4. 5% nonfat dry milk, 0.4% Triton X-100 solution: add 0.5 g nonfat dry milk powder and 4 ml of 1% Triton X-100 solution into 6 ml PBS. We don't recommend storing this solution.
5. Human endothelial-SFM medium (Life Technologies, Grand Island, NY, USA).
6. Antibodies used in this chapter: *see* Table 1.

2.4 Cryostorage and Thawing of Cells

1. Freezing container.
2. EC freezing medium (50 ml): add 15 ml FBS, 5 ml DMSO, and 50 μl of 5 mM Y27632 into 30 ml EGM-2 medium, and filter with a 50 ml Steriflip filtration system.
3. Cryogenic tubes (2 ml).

3 Methods

3.1 Endothelial Progenitor Differentiation with Gsk3 Inhibitor

A summary of this protocol is shown in Fig. 1. The endothelial cell progenitors can be generated in albumin-containing LaSR basal medium with 6 μM CHIR99021 or albumin-free DMEM/Vc medium with 5 μM CHIR99021 within 5 days.

1. Culture the hPSCs on Matrigel or Synthemax-coated 6-well plates in mTeSR1 or E8 medium to 80–90% confluence using instructions provided in our previous protocol [20]. Aspirate the medium and add 1 ml of room temperature Accutase to each well. Incubate the plate in a 37 °C, 5% CO_2 incubator for 8 min.
2. Add 0.5 ml of mTeSR1 or E8 into each well of the 6-well plate and pool all of the cells into a 15 ml conical. Mix well and count the total cell number with a hemocytometer. Centrifuge the cells at 200 × *g* for 5 min.
3. Aspirate the supernatant, resuspend the cells in mTeSR1 or E8 + 5 μM Y27632 at a cell density of two million cells/ml, and plate 0.1–0.5 million cells/well in each well of a 12-well plate. Add mTeSR1 or E8 + 5 μM Y27632 medium to each

Table 1
Antibodies used in this study

Antibody	Source/isotype/clone/cat. no.	Dilution
CD31-APC	Miltenyi Biotec, mouse IgG1, Clone: AC128	1:50
CD34-FITC	Miltenyi Biotec, mouse IgG2a, Clone: AC136	1:50
CD31-FITC	Miltenyi Biotec, mouse IgG1, Clone: AC390	1:50
VE-cadherin	Santa Cruz, mouse IgG1, Clone: F-8 sc9989	1:100
KDR	Santa Cruz, mouse IgG1, Clone: A-3 sc6251	1:200
vWF	Dako, rabbit IgG, Cat. No: A008202-5	1:500
Brachyury	R&D Systems, Goat IgG, Clone: AF2085	1:100
Oct4	Santa Cruz, Mouse IgG2b, sc5279	1:100
Secondary antibody	Alexa 488 Chicken anti-Gt IgG/A-21467	1:1000
Secondary antibody	Alexa 488 Goat anti-Ms IgG1/A-21121	1:1000
Secondary antibody	Alexa 488 Goat anti-Rb IgG/A-11008	1:1000
Secondary antibody	Alexa 594 Goat anti-Ms IgG2b/A-21145	1:1000
Secondary antibody	Alexa 594 Goat anti-Rb IgG/A-11012	1:1000
Secondary antibody	Alexa 647 Goat anti-Ms IgG2b/A-21242	1:1000
Secondary antibody	Alexa 647 Goat anti-Rb IgG/A-21244	1:1000

well to make a final volume of 1 ml in each well of the 12-well plate. This time point corresponds to day -3 (*see* **Note 2**).

4. Day –2 and day –1, aspirate the medium and replace with 2 ml room temperature mTeSR1 or E8 per well of the 12-well plate.
5. Day 0, prepare 6 μM CHIR99021 LaSR basal medium or 5 μM CHIR99021 DMEM/Vc medium. Add 4 μl of 36 mM CHIR99021 into 24 ml LaSR basal medium to make 6 μM CHIR99021 LaSR basal medium, or add 3.33 μl of 36 mM CHIR99021 into 24 ml DMEM/Vc medium to make 5 μM CHIR99021 DMEM/Vc medium. Aspirate the old medium and then add 2 ml LaSR basal medium or DMEM/Vc with CHIR99021 per well of 12-well plate (*see* **Note 3**).
6. Day 1, aspirate the medium from each well of the 12-well plate and replace with 2 ml room temperature 6 μM CHIR99021 LaSR basal medium or 5 μM CHIR99021 DMEM/Vc medium. Put the plate back into the 37 °C, 5 % CO_2 incubator (*see* **Note 4**).
7. Day 2, day 3, and day 4, aspirate the medium and replace with 2 ml room temperature LaSR basal medium or DMEM/Vc medium per well of the 12-well plate (*see* **Note 5**).

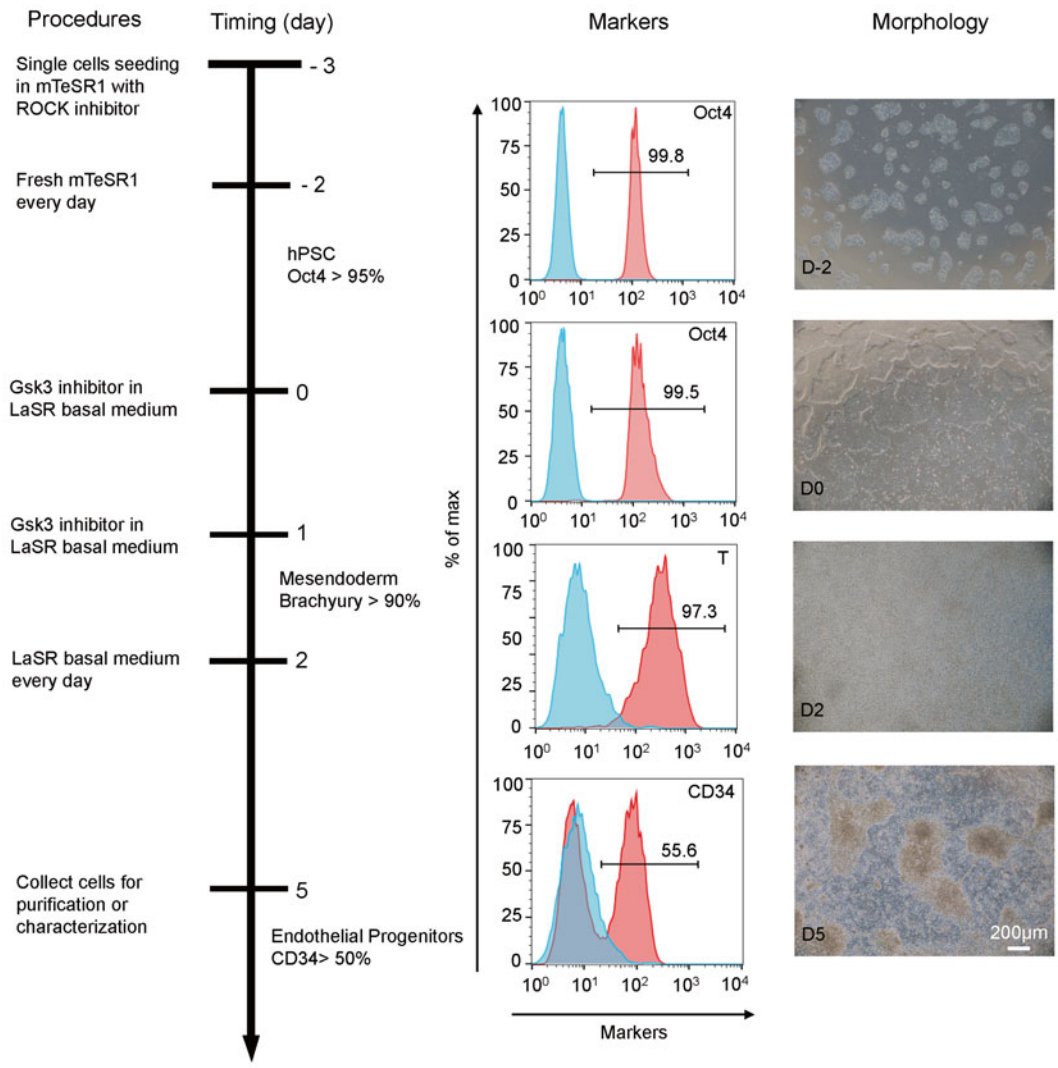

Fig. 1 Schematic of the protocol for the differentiation of endothelial progenitors from hPSCs with small-molecule modulators of canonical Wnt signaling. Bright-field images of the typical morphology of day −2, day 0, day 2, and day 5 cells from 19-9-11 are shown at × 4 magnifications along with flow cytometry analysis of indicated markers. Scale bar, 200 μm

3.2 Purification of Bipotent Endothelial Progenitors

A schematic summary of this purification step is shown in Fig. 2.

1. At day 5 (or other days determined optimal for generating CD34+/CD31+ cells), aspirate the old medium, add 1 ml room temperature Accutase to each well, and incubate in a 37 °C, 5% CO_2 incubator for 6–10 mins.
2. Pipette 5–10 times with a P1000 tip to singularize the cells and then filter the cell suspensions one by one through a 40 μm Falcon cell strainer into a 50 ml conical tube containing 12 ml DMEM10 medium (*see* **Note 6**).

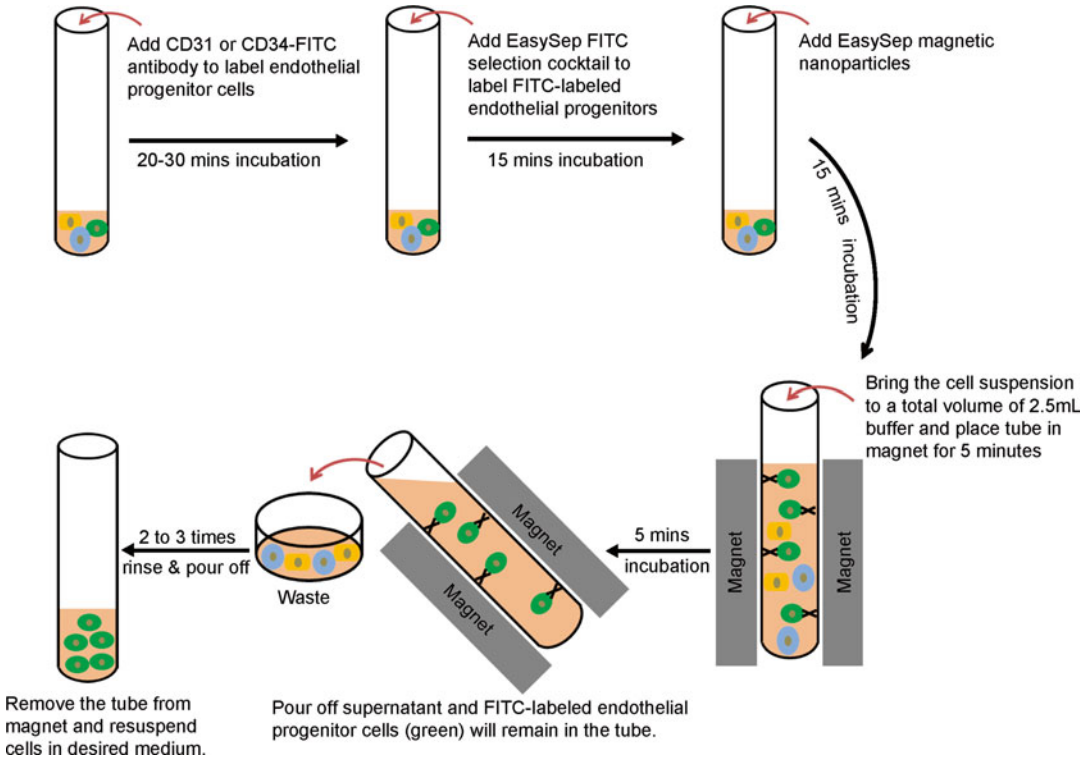

Fig. 2 Schematic of the protocol for the purification of hPSC-derived endothelial progenitors

3. Count the cells with a hemocytometer, split the filtered cell suspension evenly into two 15 ml conical tubes, centrifuge the cells at 200 × *g* for 5 min, and aspirate the supernatants.
4. Add 10 ml FlowBuffer-1 to resuspend the two cell pellets into one single 15 ml conical tube, centrifuge the cells at 200 × *g* for 5 min, aspirate the supernatant, and resuspend the cell pellet at a concentration of 1×10^7 cells per 100 µl FlowBuffer-1 with a 1:50 CD31 or CD34-FITC antibody. For samples containing 1×10^7 cells or fewer, resuspend in 100 µl FlowBuffer-1.
5. Incubate the mixture in dark at room temperature for 20–30 min, add 2 ml FlowBuffer-1, take 100 µl samples to perform flow cytometry analysis and centrifuge the remaining cells at 200 × *g* for 5 min (*see* **Note** 7).
6. Aspirate the supernatant, resuspend the cell pellet at a concentration of 1×10^7 cells per 100 µl FlowBuffer-1, and add EasySep FITC Selection Cocktail at 10 µl per 100 µl cell mixture. Mix well and incubate at room temperature for 15 min.
7. Add the well-mixed Magnetic Nanoparticles at 5 µl per 100 µl cell mixture, mix well and incubate at room temperature for 10 min.

8. Bring the cell suspension to a total volume of 2.5 ml FlowBuffer-1, mix the cells, and then transfer into a flow tube. Next, place the flow tube (without cap) into the magnet and set aside for 5 min.
9. Pick up the magnet, and in one continuous motion invert the magnet and flow tube, pouring off the supernatant fraction. Leave the magnet and tube inverted for 2–3 s, then return to an upright position (*see* **Note 8**).
10. Remove the flow tube from the magnet, add 2.5 ml FlowBuffer-1, and mix the cell suspension by gently pipetting up and down for 2–3 times. Place the flow tube (without cap) back in the magnet and set aside for 5 min.
11. Repeat **steps 9** and **10** two to three times, and then **step 9** once more. Remove the flow tube from the magnet and resuspend cells in an appropriate amount of desired medium for further use (*see* **Note 9**).

3.3 Extended Culture of hPSC-derived Endothelial Progenitors to Endothelial Cells

1. Take a collagen IV-coated 6-well plate from 4 °C and place it at room temperature for 15 min to warm up.
2. Add 1×10^5 purified CD34+ cells in 2.5 ml EGM-2 medium with 5 μM Y27632 per well of a 6-well plate (*see* **Note 10**).
3. The next day, aspirate the medium in each well and replace with 2 ml fresh room temperature EGM-2 or Endothelial-SFM medium.
4. On day 3 of extended culture and every 3 days thereafter, aspirate the medium from each well and add room temperature EGM-2 or endothelial-SFM medium at a volume of 2 ml per well. On days 6–10, endothelial cells are ready for characterization.

3.4 Characterization of hPSC-derived Endothelial Cells and Their Progenitors

Cells from Subheading 3.1, **step 7** and Subheading 3.3, **step 4** should express markers of endothelial progenitors and endothelial cells, respectively. To characterize these cells, perform immunostaining (option A) or flow cytometry analysis (option B). We recommend flow cytometry analysis for quantitative analysis of the purity of hPSC-derived endothelial cells and their progenitors. Antibody combinations of CD31/CD34 and VE-cadherin/von Willebrand factor (vWF) are recommended for double staining.

(A) Immunostaining analysis of hPSC-derived endothelial cells and their progenitors

1. Day 6–10 post-addition of EGM-2 medium in Step 3.3.4, wash the differentiated cells with 1 ml PBS per well in a 6-well plate. Aspirate the PBS, add 1 ml Accutase per well, and incubate in a 37 °C, 5 % CO_2 incubator for 10 min.

2. Pipette 5–10 times with a P1000 tip to singularize the cells and then transfer the 1 ml cell suspension into a 15 ml conical containing 2 ml DMEM10 medium.
3. Count the cells with a hemocytometer, centrifuge the cells at 200 × *g* for 5 min, and aspirate the supernatant.
4. Resuspend the cell pellet in EGM-2 medium + 5 μM Y27632 at a concentration of 100,000 cells/ml. Plate 1 ml of the resuspended cell solution in each well of a 12-well plate containing a collagen IV-coated coverslip. Incubate the plate at 37 °C, 5% CO_2 for 2 days without medium change to allow cell attachment (*see* **Note 11**).
5. After 2 days, aspirate the medium and add 1 ml of PBS per well to wash the cells. Aspirate the 1 ml PBS supernatant.
6. Add 1 ml of 4% formaldehyde per well and incubate for 15 min at room temperature to fix the cells. Aspirate the formaldehyde solution and then add 1 ml of PBS per well and aspirate to rinse the cells. Repeat the PBS rinse step twice.
7. Add 300 μl 5% nonfat dry milk, 0.4% Triton X-100 in PBS per well and then add primary antibodies into individual wells according to Table 1. Incubate at room temperature for 1 h or at 4 °C overnight. Antibodies for cell characterization include, but are not restricted to, CD34, CD31, VE-cadherin, von Willebrand factor (vWF), and ICAM-1.
8. Aspirate the antibody solution. Add 1 ml of PBS to each well and then aspirate the PBS. Repeat this wash three times.
9. Dilute the secondary antibodies specific to the primary IgG subtype at 1:1000 in 5% milk, 0.4% Triton X-100. Add 300 μl of secondary antibody solution to each well and then incubate for 30 min at room temperature in dark.
10. In dark, aspirate the secondary antibody solution. Add 1 ml of PBS to each well and then aspirate the PBS. Repeat this wash three times.
11. Seal the coverslips with Gold Anti-fade reagent with DAPI to glass slides. Examine the slides with an epifluorescence microscope. Typical CD34, CD31, VE-cadherin and vWF patterns of hPSC-derived endothelial progenitors (Fig. 3a, b) and endothelial cells (Fig. 3e, f) are shown in Fig. 3.

(B) Flow cytometry analysis of hPSC-derived endothelial cells and their progenitors

1. Wash the differentiated cells with 1 ml PBS per well in a 12-well plate (from Subheading 3.1, **step 7**) or 6-well plate (from Subheading 3.3, **step 4**), aspirate the PBS, add 1 ml Accutase per well, and incubate in a 37 °C, 5% CO_2 incubator for 10 min.

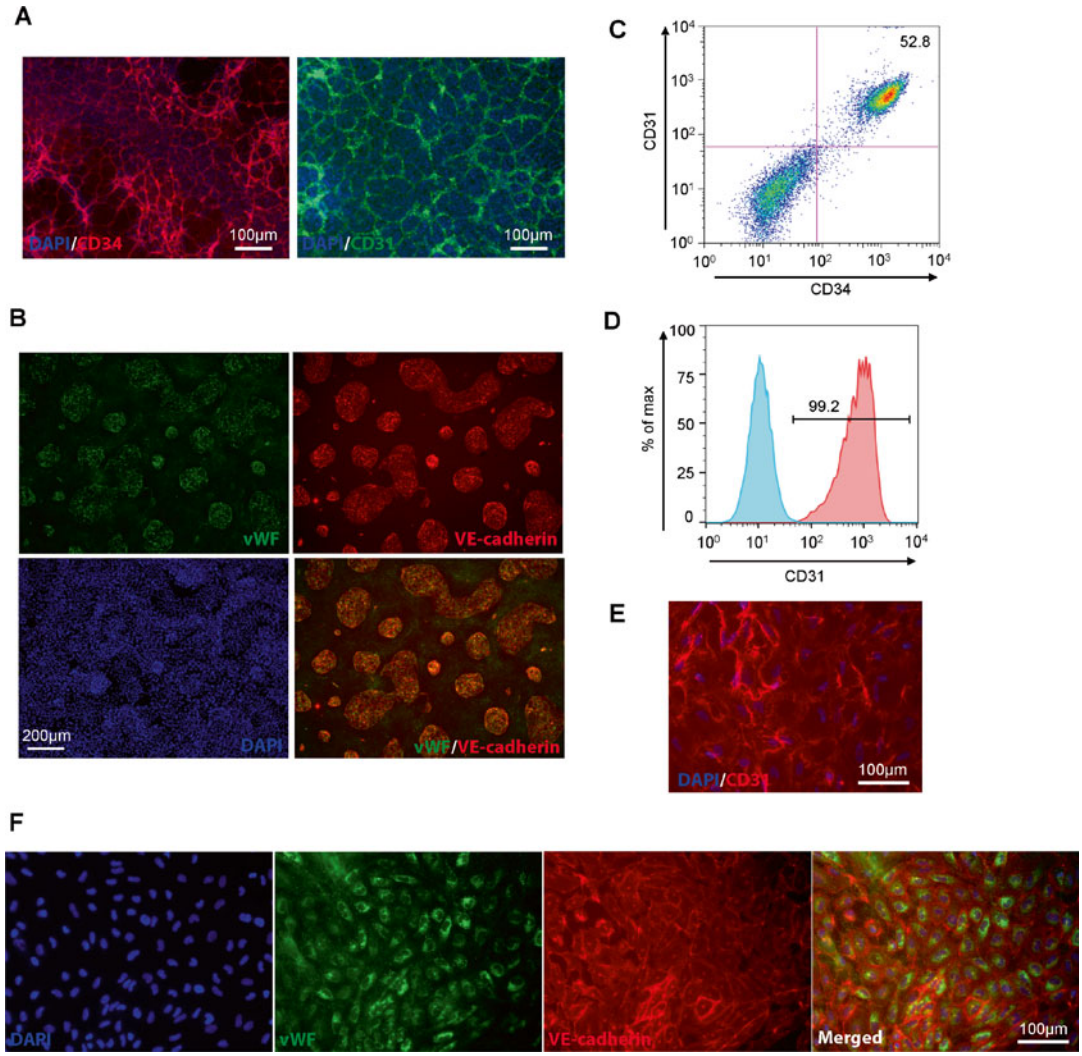

Fig. 3 Characterization of endothelial cells and their progenitors derived from hPSCs. (**a–c**) 19-9-11 iPSCs were differentiated as illustrated in Fig. 1. At day 5, presort CD34+ cells were immunostained for CD31/CD34 (**a**) and VE-cadherin/vWF (**b**) and quantitatively analyzed for CD31/CD34 (**c**). (**d–f**) the post-sort CD34+ endothelial progenitors were quantitatively analyzed for CD31 (**d**) and immunostained for CD31 (**e**) and VE-cadherin/vWF (**f**)

2. Pipette 5–10 times with a P1000 tip to singularize the cells and then transfer the 1 ml cell mixture into a 15 ml conical containing 2 ml DMEM10 medium.
3. Count the cells with a hemocytometer, centrifuge the cells at 200 × *g* for 5 min, and aspirate the supernatant.
4. Add 1 ml of 1 % formaldehyde to resuspend the cell pellet and then incubate at room temperature for 20 min. Next, centrifuge the cells at 200 × *g* for 5 min, aspirate the supernatant and

then resuspend the fixed cells in 1 ml of FlowBuffer-1 per tube. Calculate the cell density based on the cell count obtained from previous step (*see* **Note 12**).

5. Add one million cells into a 15-ml tube containing 2 ml of FlowBuffer-1, centrifuge the cells 200 × g for 5 min and then aspirate the supernatant. Repeat this wash two times to remove the formaldehyde.
6. Resuspend the cell pellet in 100 μl of FlowBuffer-1 with the appropriate dilution of primary antibody. Antibody combinations of CD31/CD34 and VE-cadherin/vWF are recommended for double staining. Incubate for 1 h at room temperature or at 4 °C overnight.
7. Wash the cells with 2 ml of FlowBuffer-1 and resuspend the cell pellet in 100 μl of FlowBuffer-1 containing 1:1000 dilution of secondary antibody. Incubate for 30 min at room temperature in dark.
8. Wash the cells with 2 ml FlowBuffer-1 twice, resuspend the cell pellet in 300 μl FlowBuffer-1, and transfer into flow round-bottom tubes. Place the flow tubes on ice and perform the flow cytometric analysis with a FACSCaliber. A representative result of CD31/CD34 double staining of presort endothelial progenitors is shown in Fig. 3c, and a CD31 staining result of post-sort endothelial cells is shown Fig. 3d.

3.5 Cryostorage and Thawing of Endothelial Cells and Their Progenitors

1. Following purification of CD34+ endothelial progenitors in Subheading 3.2, **step 11** or dissociation of the culture with Accutase as described in Subheading 3.4 A, **steps 1–3**, resuspend endothelial progenitors (from Subheading 3.2, **step 11**) or endothelial cells (from Subheading 3.4 A, **step 3**) at a density of 2×10^6 cells per ml of EC freezing medium.
2. Aliquot 1 ml of the cell suspension into each cryovial.
3. Freeze in a Mr. Frosty™ freezing container at −80 °C overnight.
4. Transfer the cryovials to liquid nitrogen for long-term storage. These cells can be stored in liquid nitrogen for at least 1 year.

Thawing cells

5. Partially thaw the cell vials in a 37 °C water bath.
6. Transfer the partially thawed cells to a 15-ml conical tube containing 5 ml DMEM10 medium.
7. Centrifuge the cells at 200 × *g* for 5 min, and aspirate the supernatant.
8. Gently resuspend the cells in 1 ml of EGM-2 medium and plate into collagen-coated 6-well plate at 0.5 million cells per cm^2 with 5 μM Y27632. Addition of 10% FBS or albumin may help to increase cell attachment and viability.

9. The next day, aspirate the medium in each well and replace with 2 ml fresh room temperature EGM-2 or endothelial-SFM medium.
10. Resume culturing of cells at Subheading 3.3, **step 4**.

4 Notes

1. Pre-coated Corning BioCoat™ plates (laminin, collagen I, collagen IV, etc.) can also be used to culture endothelial cells and their progenitors.
2. Optimizing cell density is very critical for efficient endothelial cell differentiation. The initial plating density and/or the time of expansion prior to initiation of differentiation may require optimization for different cell lines or expansion conditions. We recommend plating 0.1–0.5 million cells per well of 12-well plate and expanding the cells for 2 or 3 days prior to initiation of differentiation.
3. Though we identified 6 μM or 5 μM CHIR99021 as the optimal concentrations in LaSR basal medium and DMEM/Vc medium, respectively, for the cell lines that we tested, other lines may respond to CHIR99021 treatment differently. Therefore, optimization of CHIR99021 concentration may be required. We recommend testing 4–10 μM CHIR99021.
4. If many cells are dead or detached from the surface, decrease the concentration of CHIR99021 or reoptimize the initial cell seeding density.
5. Extended culture in LaSR basal medium or DMEM/Vc medium after CHIR99021 treatment is important to achieve a high yield and purity of endothelial progenitors. Though we identified a 3-day culture after CHIR99021 treatment as the optimal condition for the cell lines that we tested, other lines may need more time to reach maximum purity. We recommend testing 3–5 days for the extended culture. If cells ball up during the culture, decrease initial cell seeding density.
6. Filtering the cell mixture is critical for the subsequent purification step since cell clusters will negatively affect the purification performance.
7. Flow cytometry analysis is necessary for the subsequent purification step since the purity of CD31+CD34+ cells will directly affect the performance of magnetic-activated cell sorting. It is recommended that the presort cell mixture contains at least 15% of CD34+CD31+ cells before proceeding to the next step.
8. Do not shake or blot off any drops that may remain hanging from the mouth of the flow tube.

9. Additional rounds of MACS are recommended when the CD34+ or CD31+ cell purity is not sufficient. Purified cells can be also frozen for future use.
10. Including a ROCK inhibitor is very important for high hPSC-derived endothelial progenitor recovery after purification. Addition of 10% FBS or albumin will also increase cell attachment and viability.
11. For endothelial progenitors, resuspend CD34+ cells (from Subheading 3.2, **step 11**) in EGM-2 medium with 5 μM Y27632 at a concentration of 100,000 cells/ml.
12. Do not use cold methanol or Triton X-100 to permeabilize the cells since methanol and Triton X-100 treatment will destroy surface proteins for subsequent flow cytometry analysis. For flow cytometry analysis of intracellular proteins such as brachyury, please follow instructions provided in our previous protocol [20].

Acknowledgments

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Delivery of the Porcupine Inhibitor WNT974 in Mice

Li-shu Zhang and Lawrence Lum

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The percentage in Error: In *2.1 Drug Preparation*, Point 6 was incorrectly noted as 5 % Carboxymethylcellulose in chapter 12, page no. 112. The correct percentage is 0.5 % Carboxymethylcellulose.

The online version of the updated original chapter can be found under
http://dx.doi.org/10.1007/978-1-4939-6393-5_12

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