Box 1: Production of WNT3A-, RSPO1- or NOG-conditioned medium Timing 2-5 weeks

Additional material

- L cells WNT3A, control L cells (kindly provided by Hans Clevers, Hubrecht Institute) and RSPO1 cells (kindly provided by Calvin Kuo, Stanford University). Note: ATCC also has WNT3A and RSPO1 cell lines, but we have no experience with them.
- mNOG cells (kindly provided by Hans Clevers, Hubrecht Institute)

  Caution All cell lines used should be regularly checked for mycoplasma infection.
- DMEM, high glucose, GlutaMAX™, pyruvate (Thermo Fisher Scientific, cat. no. 31966021)
- FBS (Thermo Fisher Scientific, cat. no. 10270106)
- Penicillin-Streptomycin (Thermo Fisher Scientific, cat. no. 15140122)
- Zeocin™ (Thermo Fisher Scientific, cat. no. R25001), stock concentration 100 mg/mL; end concentration 125 µg/mL
- Geneticin (G418; Santa Cruz, cat. no. sc-29065A), stock concentration 400 mg/mL; end concentration 500 µg/mL
- Human recombinant Noggin (NOG; PeproTech, cat. no. 120-10C)
- Filter bottle UltraCruz® Filter Flask, 500 mL, 0.22 µm (Santa Cruz, cat. no. sc-200255)
- T25 tissue culture flask (Sarstedt, cat. no. 83.3910.002)
- T75 tissue culture flask (Sarstedt, cat. no. 83.3911.002)
- T175 tissue culture flask (Sarstedt, cat. no. 83.3912.002)
- Cell culture dish 152 cm^2 (Sarstedt, cat. no. 83.3903)

Procedure

1. Follow option A to produce WNT3A- or RSPO1-CM or option B to produce NOG-CM.

   (A) WNT3A- and RSPO1-conditioned medium

Critical Functional WNT3A (and RSPO1) is the most critical factor for organoid growth. Every batch needs to be tested for activity. It is most helpful to request a sample of WNT3A- and RSPO1-CM from a lab that has organoid cultures running. Once you receive this, make your own WNT3A- and RSPO1-CM (WNT3A- and RSPO1-CM produced) and test it in comparison to the received WNT3A- and RSPO1-CM (WNT3A- and RSPO1-CM control) using the TOP/FOP assay (**Box 2**). This will help you to quantify the activity of your own produced WNT3A-CM (and RSPO1-CM). Dilute your own WNT3A- and RSPO1-CM (WNT3A- and RSPO1-CM diluted) down to the level of the control WNT3A- and RSPO1-CM using supernatant from control L cells. Then store aliquots of this for future references. This way you keep your WNT3A- and RSPO1 at the right activity level over the coming years.

Conditioned medium production

i. Take control L, WNT3A and/or RSPO1 cells from liquid nitrogen stocks and briefly thaw at 37 °C in a waterbath.
ii. Pipette cells into 15 mL Falcon tubes with 10 mL growth medium (DMEM + 12 % FBS) and spin for 5 min at 300 g. Discard supernatant, resuspend cells in 12 mL growth medium and plate in T75 culture flask (cultured at 37 °C with 5 % CO_2).
iii. When cells are 50 % confluent, add Zeocin.
iv. Once cells are ~70-80 % confluent, split the culture flask in 5 x T175 culture flasks in growth medium and add Zeocin to only one of the culture flasks.
v. When the cells in the culture flasks containing the growth medium without Zeocin are confluent (after 3-4 d), trypsinize for 2-3 min, resuspend the cells of each culture flask in 10 mL growth medium, pool cell suspension of all culture flasks together and plate in 25 x 152 cm^2 dishes (2 mL/dish) before adding 19 mL growth medium/dish.
vi. After 7 d in the incubator, the medium can be harvested. Spin down the medium for 5 min at 400 g to remove floating cells and filter through 0.22 µM filter bottle.

Troubleshooting

vii. Store aliquots (e.g. in 50 mL tubes) at 4 °C. Test the activity of the medium in the TOP/FOP assay (**Box 2**).

Pause point Medium can be stored long-term at -20 °C. To ensure functionality of produced factors, we advise to use medium within 6 months. Nevertheless, this will depend on individual storage and we also used media after several years without experiencing loss of function.

Critical step Avoid freeze/thaw cycles. This may affect WNT3A and RSPO1 activity.

viii. Use the remaining confluent T175 culture flask in growing medium with Zeocin to repeat the procedure. Cells can be used for up to 10 passages to harvest more batches of conditioned medium.

Troubleshooting

Critical Every batch needs to be tested. However, to reduce workload, we prefer to produce a large batch of several liters, test the WNT3A and RSPO1 activity in the TOP/FOP assay (**Box 2**), adjust the WNT3A and RSPO1 activity by dilution, then aliquot and store at -20 °C until usage. For a lab starting WNT3A- and RSPO1-CM production, it may be helpful to first keep the volume smaller and test a range of harvests, e.g. just a few plates and collect 10 separate harvests from them, and test every harvest in the TOP/FOP assay. This will help to evaluate how the production works in your hands. With experience, you can then pool harvests and only measure the activity of the pool.

(B) NOG-conditioned medium

i. Take mNOG cells from liquid nitrogen stocks and briefly thaw cells in 37 °C waterbath.

ii. Pipette cells into 15 mL Falcon tubes with 10 mL growth medium (DMEM + 10 % FBS) and spin for 5 min at 300 g. Discard the supernatant, resuspend cells in 12 mL growth medium in T25 culture flask (cultured at 37 °C with 5 % CO_2).

iii. When cells are 100 % confluent, transfer to a T75 culture flask.

iv. When cells are 50 % confluent, add G418 antibiotic.

v. Split the confluent culture flask in 5 x T175 culture flasks in growth medium and add G418 to only one of the culture flasks.

Critical step NOG cells have different antibiotic than WNT3A and RSPO1 cells (G418 vs. Zeocin).

vi. When the culture flasks in growth medium without G418 are confluent (after 3-4 d) trypsinize the cells, pool cell suspension of all culture flasks together and plate in 25 x 152 cm^2 dishes (2 mL/dish) before adding 19 mL growth medium/dish.

vii. After 7 d in the incubator the medium can be harvested. Spin down the medium for 5 min at 400 g to remove floating cells and filter through 0.22 µM filter bottle.

viii. Store the medium at -20 °C.

ix. If producing large amounts, pool all the batches and test activity.

x. Use the remaining confluent T175 culture flask in growth medium with G418 to repeat the procedure. Cells can be used for up to 6-8 passages to harvest more batches of conditioned medium.

Pause point Medium can be stored long-term at -20 °C. To ensure functionality of produced factors, we advise to use medium within 6 months.

Critical step Avoid freeze/thaw cycles as this can affect activity.

xi. To check activity use 2 %, 5 % and 10 % of NOG-CM with other growth factors and inhibitors. Compare organoid growth with the current batch.

Critical step There is no commercial quantification assay available. You can however compare the activity of the in house-produced NOG-CM to commercial NOG. The produced NOG-CM is directly tested on running organoid cultures in a 48-well plate under the following conditions: Without NOG-CM, commercial NOG, NOG-CM in use (positive control), 2 % produced NOG-CM, 5 % produced NOG-CM and 10 % produced NOG-CM. The different media are changed every alternate day, after 14 days in culture, the organoids are split and seeded in a new 48-well plate under the same conditions as above. The first differences in growth can be observed after the first passage.