Box 2: Activity testing of conditioned medium Timing 3 d

Additional material

- PEI transfection reagent (Polysciences, cat. no. 23966)
- HEK293T cells available from ATCC
- Plasmids for TOP/FOP assay: We use TOP 10 plasmid (WNT sensitive), FOP 10 plasmid (mutated TOP plasmid), Renilla luciferase (RL) and pcDNA 4 TO (all a kind gift by Hans Clevers, Hubrecht Institute). However there are other commercial solutions which work just as well.
- Dual-Luciferase® Reporter Assay System (Promega, cat. no. E1910)
- 96-well plate sterile, flat bottom, transparent (Sarstedt, cat. no. 83.3924)
- 96-well plate (Elisa reader compatible) sterile, flat bottom, white (Sarstedt, cat. no. 82.1581.110)
- Luminometer (e.g. Perkin Elmer, Victor™ X light)

Additional reagent setup

Preparation of PEI transfection reagent To make stock solution (10 mg/mL) add PEI into endotoxin-free dH_2O that has been heated to ~80 °C until powder is completely dissolved. Let it cool to RT. Neutralize to pH 7.0 with HCl, filter sterilize (0.22 µm), aliquot and store at -20 °C or preferably -80 °C. For PEI working solution (1 mg/mL), prepare with sterile ddH_2O dilution 1:10. Working stock can be kept at 4 °C (stable for about 2 months).

Procedure

Activity testing of conditioned medium: TOP/FOP assay

Critical TOP/FOP assay is one example to test the WNT3A/RSPO1 activity. There are other tests (e.g. GFP read-out) available. The TOP plasmid is a WNT-sensitive luciferase reporter plasmid. Increase in relative Firefly luciferase activity is observed once the substrate is added and WNT3A/RSPO1 had been produced successfully. FOP plasmid transfection is used as a

negative control (no Firefly luciferase activity should be measured). Renilla luciferase is detected to normalize each sample for transfection efficiency.

The example below is for WNT3A-CM, but the principle is the same for RSPO1-CM. However, RSPO1 does not activate alone, it only enhances WNT3A activation. Normally, in a first luciferase assay the activity of the WNT3A-CM produced is compared to the WNT3A-CM control and in a second assay the activity is measured again after adjusting the WNT3A-CM produced to the WNT3A-CM control (= WNT3A-CM diluted). The calculations for the second assay are presented below in the example.

Transfection in 96-well plates

1. Calculate the number of samples to prepare the master mix (**Supplementary Fig. 1a**).

   Example: Test of one harvest of produced WNT3A-CM. Each sample should be tested in triplicates. If measuring WNT3A-CM produced and WNT3A-CM diluted this results in 6 wells. As control, use 3 wells of WNT3A-CM control. Also include 3 wells each only L cell medium (no WNT3A activity). Everything should be tested with TOP plasmid ("TOP") and with the negative control FOP plasmid ("FOP"). Altogether 24 wells (12 wells TOP and 12 wells FOP).

   Critical step Without a control from a lab with running WNT3A-CM production there is no positive control. Insufficient WNT3A concentration can lead to slowly degrading cultures after months.

2. Pipette master mix (always calculate with some excess):

   Per well for TOP plasmid: Mix 135 ng pcDNA 4 TO DNA + 15 ng of reporter DNA (ratio TOP plasmid:Renilla is 9:1 resulting in 13.5 ng of TOP and 1.5 ng of RL) in 7.5 µL of DMEM (without FBS).

   Per well for FOP plasmid: Mix 135 ng pcDNA 4 TO DNA + 15 ng of mutated reporter DNA (ratio FOP plasmid:Renilla is 9:1 resulting in 13.5 ng of FOP and 1.5 ng of RL) in 7.5 µL of DMEM (without FBS).

Critical step pcDNA 4 TO is used to increase the amount of DNA which in turn increases transfection efficiency using PEI.

3. In another test tube, add 7.5 µL of FBS + 0.75 µL of PEI (per well).
4. Add 4 µL PEI solution (per well) to TOP and FOP plasmid DNA mix, respectively (total volume 11.5 µL each per well).
5. Incubate for 20 min at RT. During incubation trypsinize HEK293T cells.

   Critical step HEK293T cells must be passaged at least two times before transfection procedure.
6. Use a T75 culture flask with 70-80 % confluency.
7. Wash cells in PBS and then add 2 mL of TrypLE Express for 1-3 min.
8. Add 10 mL of growth medium (DMEM + 10 % FBS) to the culture flasks.
9. Count the cells using hemocytometer and calculate the number of cells per mL.
10. In a 96-well plate, you need 40,000 cells/well in a total volume of 100 µL. Prepare the cell suspension in growth medium based on the number of counted cells.
11. After 20 min add master mix to HEK293T cells:

    Master mix TOP plasmid already including PEI (11.5 µL per well) + cell suspension (per well 88.5 µL containing 40,000 cells).

    Master mix FOP plasmid already including PEI (11.5 µL per well) + cell suspension (per well 88.5 µL containing 40,000 cells).
12. Seed cells in 96-well plate (total volume 100 µL) and place in incubator for 48 h.
13. Replace medium (100 µL volume) with a mix of 50 % WNT3A-CM (according to pipetting scheme in **Supplementary Fig. 1a** either WNT3A-CM control, WNT3A-CM produced, WNT3A-CM diluted or L control cell medium) + 50 % DMEM containing 10 % FBS.

    Critical Since you use a final concentration of 50 % WNT3A-CM (see **Table 1**) in the human gastric organoid medium for culturing, this percentage is to be tested in the TOP/FOP assay. For RSPO1 you use a final concentration of 10 % RSPO1-CM (see **Table 1**) in the human gastric organoid medium, so replace medium in step 13 accordingly: 50 % WNT3A-CM control + 10 % RSPO1-CM (either RSPO1-CM control,

RSPO1-CM produced, RSPO1-CM diluted or L control cell medium) + 40 % DMEM containing 10 % FBS.

14. **Critical step** Cells detach easily. Be very cautious and slow while changing medium.
15. After 24 h harvest cells for luciferase assay.

    Troubleshooting

Luciferase assay

16. Prepare required amount of 1 x lysis buffer (by diluting the 5 x lysis buffer included in the Dual-Luciferase Reporter Assay Kit in ddH_2O).
17. Harvest cells by replacing medium with 50 µL lysis buffer/well.
18. Incubate with lysis buffer by rocking the plate for 15 min at RT.

    Pause point Plate can be stored at -80 °C wrapped in parafilm. We analyze the plate within the same week. Warm plate to RT before continuing with the luciferase assay.
19. Using a multi-channel pipette, resuspend the content of each well and transfer 20 µL of each well in a 96-well plate (white and flat bottom).
20. Prepare the luminometer.
21. Add 20 µL of LARII to each well (prepared according to the Dual-Luciferase Reporter Assay Kit protocol and stored at -80 °C).
22. Read Firefly luciferase in luminometer.
23. Add 20 µL of Stop and Glo Reagent to each well (Reagents of the Dual-Luciferase Reporter Assay Kit, prepare fresh).
24. Read Renilla luciferase in luminometer.

    Critical step No absolute values are obtained, they are only relative. Numbers can vary each time the assay is performed. That is why the positive control is essential to compare to the produced conditioned medium (mentioned above) (**Supplementary Fig. 1b and 1c**).

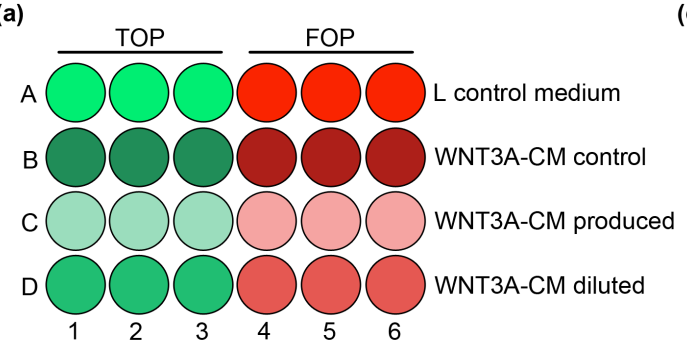
    Troubleshooting

Adjusting conditioned medium

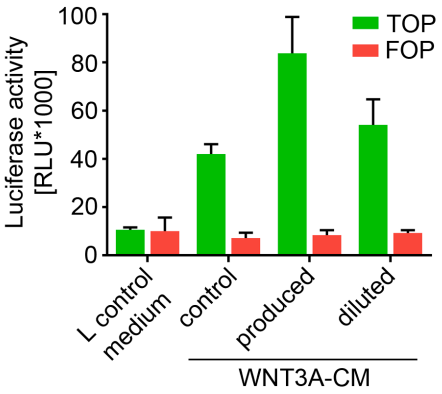
Critical Normally, the activity of the WNT3A- and RSPO1-CM produced is higher than that of the positive control (WNT3A- and RSPO1-CM control) in the luciferase assay and therefore needs to be diluted. Based on the assay results, using the formula $c_1v_1 = c_2v_2$ (c_1 is the activity level of the WNT3A- and RSPO1-CM produced obtained in the assay; v_1 is the volume of the WNT3A- and RSPO1-CM produced; c_2 is the activity level of the positive control), you can determine how much L control medium needs to be added to the WNT3A- and RSPO1-CM produced to get the same activity level as the positive control. To make sure that the WNT3A- and RSPO1-CM diluted has effectively the same activity level as the positive control, repeat the luciferase assay starting from the transfection of the HEK293T cells (**Box 2**, step 1).

SUPPLEMENTARY FIGURES

(a)



(c)



(b)

													Ratio (Firefly/Renilla)*1,000					
	TOP (Firefly)			TOP (Renilla)			FOP (Firefly)			FOP (Renilla)			TOP			FOP		
L control medium	7583	9358	7320	772702	802011	696375	8846	4359	2974	538279	538785	529196	9,8	11,7	10,5	16,4	8,1	5,6
WNT3A-CM control	26521	26143	24619	567065	657905	622578	4441	2528	3492	472032	500146	485033	46,8	39,7	39,5	9,4	5,1	7,2
WNT3A-CM produced	48430	38751	41906	478492	517720	555702	4379	2921	3229	407923	433753	415635	101,2	74,9	75,4	10,7	6,7	7,8
WNT3A-CM diluted	29043	25858	23428	453202	467026	545118	3740	4380	3472	403853	419809	428884	64,1	55,4	43,0	9,3	10,4	8,1

Supplementary Fig. 1: Example for TOP/FOP assay. (a) Layout of pipetting scheme for luciferase assay. Here shown for WNT3A-CM, but can be adopted for RSPO1-CM. (b) Table depicting example raw data from luminometer for Firefly and Renilla luciferase. Calculation of ratio (Firefly/Renilla)*1,000. (c) Graph showing average + SD for luciferase activity of tested samples.