Generating and Utilising Murine Cas9-expressing Intestinal Organoids for Large Scale Knockout Genetic Screening

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Abstract

Organoid culture faithfully reproduces *in vitro* the *in vivo* characteristics of the intestinal/colon epithelium and elucidates molecular mechanisms underlying the regulation of stem cell compartment that, if altered, may lead tumourigenesis. CRISPR-Cas9 based editing technology has provided promising opportunities for targeted loss-of-function mutations at chosen sites in the genome of eukaryotes. Herein, we demonstrate a CRISPR/Cas9-mediated mutagenesis-based screening method using murine intestinal organoids by investigating the phenotypical morphology of cas9-expressing murine intestinal organoids. Murine intestinal crypts can be isolated and seeded into Matrigel and grown into stable organoid lines. Organoids subsequently transduced and selected to generate Cas9 expressing organoids. These organoids can be further transduced with the second lentiviruses expressing guide RNA (gRNA) (s) and screened for 8-10 days using bright-field and fluorescent microscopy to determine possible morphological or phenotypical abnormalities. Via phenotypical screening analysis, the candidate knockouts can be selected based on differential abnormal growth pattern vs their un-transduced or lenti-GFP transduced controls. Further assessment of these knockout organoids can be done via Phalloidin and Propidium iodide (PI) staining, proliferation assay and qRT-PCR and also Biochemical analysis. This CRISPR/Cas9 organoid mutagenesis-based screening method provides a reliable and rapid approach for investigating large numbers of genes with unknown/poorly identified biological functions. Knockout intestinal organoids can be associated with the key biological function of the gene(s) in development, homeostasis, disease progression, tumorigenesis and drug screening, thereby reducing, and potentially replacing animal models.

1. Introduction

Colorectal Cancer is the third most common cancer worldwide with approximately 1.1 million new diagnoses (6.1% of all cancers) in 2018 [1]. Regrettably, variations in treatment response and subsequent survival rates may be caused by significant heterogeneity of CRC tumours [2]. This variance makes the modelling of CRC in the laboratory challenging, with traditional 2D immortalised cell models limited by their inherent simplicity and genetic drift, reducing reproducibility and validity across functional genetics and drug response studies, while full *in vivo* models are vastly expensive and resource intensive, making them impractical for the majority of research centres. Therefore, studying of CRC and other cancers requires novel methods to be developed that facilitate more accurate investigations without the resource requirements of full *in vivo* modelling.

One of the most recent and promising modelling systems to be introduced is organoids: a 3D (3-dimentional), multicellular culture that mimics the phenotype of their original tissue and are compatible with many standard laboratory protocols. Stem cells from the organ in question are cultured with appropriate growth factors resulting in a small "organ in a dish" that can be maintained and expanded almost indefinitely. Organoids faithfully reproduce functional genetic or pharmacological changes visible in their originating organ, and have been cultured from the brain [3], retina [4], stomach [5], lung [6] and small intestine [7,8] among others from both human patients and mice, providing a crucial "middle ground" alternative between immortal cell culture and full *in vivo* animal models for functional generic analysis, high throughput drug testing [9], biobanking [10], tumour microenvironment [11], disease modelling [12,13] and targeted gene editing [14] via CRISPR/Cas9 technology, validating data between experimental models. When generated from both adjacent normal and matched tumour samples of an individual patient, epithelial organoids can provide an excellent tool for

researchers to investigate a wide range of cellular and molecular events directly related to individual patient care.

The CRISPR/Cas9 gene editing system provides a robust tool for generating genetically modified *in vitro* models. With recent systems capable of both "knock-in" and "knock-out" of chosen genes, CRISPR/Cas9 is used for generating *in vitro* cellular models for study. CRISPR editing has been successfully applied to cultured human organoids [15], demonstrating both an ideal platform and tool for assessing functioning of individual genes in multiple tissue types without the limitations and ethical implications of generating full *in vivo* models.

Matano et al. introduced CRISPR/Cas9-mediated sequential mutations in organoids derived from the normal human intestinal epithelium [16]. They demonstrated that engineered organoids harbouring mutations at driver genes including APC, SMAD4 and TP53 KRAS and/or PIK3CA grew independent of niche factors similar to the tumour counterparts and developed tumours upon transplantation kidney subcapsule in mice. These results highlighted the significance of morphological alterations in organoids. Inconsistent with the previous study, Drost and colleagues also utilized the CRISPR/Cas9 to engineer intestinal organoids mutated for APC, KRAS, TP53 and SMAD4. They revealed that simultaneous inactivation of APC and P53 drive the extensive aneuploidy, the main feature of tumour progression [17]. In another study, CRISPR/Cas9 mediated engineering of intestinal organoids in cystic fibrosis patients was successfully applied to repair the deletion at position 508 of cystic fibrosis transmembrane conductor receptor (CFTR) [18]. These studies have used CRISPR/Cas9 expression in organoids, but protein expression and success rate have varied considerably. Given the fragility of organoid cultures, electroporation and other invasive methods are unlikely to produce sufficient qualities for larger projects.

Herein, we present a method for isolating and culturing mouse intestinal crypts and culturing organoids, establishing Cas9 protein expression via lentiviral transduction, and subsequently using viral transduction of targeted Cas9 plasmids to generate genetic knockout organoid lines suitable for functional exploration via phenotypical or biochemical analysis. We propose that this CRISPR/Cas9-mediated mutagenesis-based screening method can provide a promising approach which facilitates the rapid and reliable identification and selection of new genes play role in development, and disease.

2. Materials

2.1 *Mouse intestinal organoid isolation*

1. HA-Rspo1-Fc cells: received from Prof. Hans Clevers laboratory.
2. Mice: C57BL/6J mice, approximately 4-6 weeks old were used. Mice were housed and bred in the transgenic animal facility of the Biomedical Service Unit at the University of Nottingham.
3. Fresh organoid medium: basal Advanced DMEM/F-12 medium, 2 mM L-glutamine, 100 U/ml Penicillin/Streptomycin and 10 mM HEPES was supplemented with N2 supplement (1x), B27 supplement (1x), 1 mM N-acetylcysteine to stimulate cell proliferation, 50 ng/mL murine recombinant epidermal growth factor (mEGF) to activate EGF signaling pathway, 1µg/mL R-spondin 1 (in-house) as Wnt agonist and 100 ng/mL Noggin (in-house) to inhibit BMP pathway.
4. EDTA (UltraPure, 0.5 M).
5. PBS(1X).
6. Matrigel.

2.2 *Lentivirus Production, transduction and generation of stable organoid lines, protein and RNA extraction and Phalloidin staining.*

1. Human HEK-293 (viral production cells).
2. Plasmids: pMD2.G and pCMVR8.74.
3. DMEM.
4. RPMI.
5. FBS.
6. L-Glutamine.

7. Penicilin/Streptomycin (P/S).
8. Opti-MEM.
9. Polyethylenimine (PEI).
10. Polybrene Reagent.
11. Fluorescent microscopy system.
12. Phalloidin.
13. DAPI mounting medium.
14. 8 µg/ml polybrene.
15. Paraformaldehyde.
16. Triton X-100.
17. Virus sucrose solution 50 mL in ultrapure water: 10% sucrose, 50 mM Tris HCL pH 7.4, 100 mM NaCL, 0.5 mM EDTA.
18. RIPA buffer [Radioimmunoprecipitation assay buffer (RIPA buffer; 150 mM sodium chloride, 1% NP-40 and 1% sodium dodecyl sulphate) containing 1x protease inhibitor cocktail].
19. TRIzol (TRI reagent).
20. RT-PCR, Immunofluorescence (IF) and western blots reagents, see published protocols [19,20].

3. Method

The brief step-by-step flowchart and a diagram of generating knockout intestinal organoids using the lentivirus-based system for Cas9/gRNA expression and subsequent analysis have summarised in Figures 1 and 2.

3.1 Mouse intestinal organoid isolation

1. Sacrifice the mouse and excise the whole intestine from the abdominal cavity.
2. Clean the specimen carefully with ice-cold PBS to eliminate external connective tissues and internal faeces (*see* **Note 1**).
3. Cut a section from duodenum, jejunum and ileum and open longitudinally.
4. To detach the villi, subdivide every piece in small fragments of approximately 1mm and wash with cold PBS several times, into a 50 mL falcon tube.
5. Once the PBS become clear, incubate the intestinal pieces with 3 mM EDTA in PBS for 45 min at 4 °C to separate the crypts by chelation.
6. Transfer the fragments to a falcon containing 10% FBS/PBS and agitate frequently and subsequently filter through a 70 μm strainer.
7. Following the filtration, collect the crypt fragments on top of the strainer and transfer to a new falcon containing 10% FBS/PBS.
8. Agitate and pass through the filter (this process repeated 3-4 times).
9. Evaluate the filtered medium by using an inverted microscope in order to choose the best fraction in term of purity and crypt concentration [8,21].
10. Centrifuge crypts at 300 *xg* for 5 min. Remove the supernatant carefully after the centrifugation step and re-suspend crypts in ice-cold Matrigel.

11. Add 25 µL of crypts-containing Matrigel in pre-warmed 48-well plate. Next, transfer the plate to a 37 °C, 5% CO2 incubator for approximately 10 minutes to allow Matrigel to solidify.
12. After 5-10 min in 37 °C incubator, distribute 300 µL of fresh organoid medium into each well to cover the Matrigel drop. Culture the crypts into 37 °C, 5% CO2 incubator and replace the organoid medium every 48 h. Monitor regularly organoid growth by inverted BF microscopy (*see* **Note 1**).

3.2 Lentivirus Production

1. Seed HEK293-T cells in a total of 10 mL DMEM per 75 cm^2 flask. Keep the density of cells around 70-80% at the day of transfection (no greater than 90%) (*see* **Note 2**).
2. Agitate plate crossways to spread cells evenly. Incubate overnight at 37 °C.

Day 1

3. Calculate volumes required for plasmid transfection and PEI (final volume per plate 15 µg LV construct, 5 µg pMDG2 packaging, 10 µg pCMV R8.74 packaging plasmid, 1.5 mL Opti-MEM per 75 mL flask) (*see* **Note 3**).
4. Add 1.5 mL Opti-MEM to a 15 mL tube per each flask to be transfected.
5. Add the required amount of packaging and transgene plasmids (no4) to the tube per each flask to be transfected.
6. Prepare the PEI concentration (3-4 µL/1 µg plasmid DNA).
7. Incubate for 5 min at room temperature.
8. Add the PEI to each tube containing the plasmid DNA (*see* **Note 4**).
9. Incubate the mixture for 20 min at room temperature.
10. Add the Opti-MEM/plasmid (packaging plasmid and PEI) gently to flask, leave the flasks for 6-9 h. Gently agitate every 2 h for best results.

11. Check cells after 6 h, and then change with complete DMEM medium. Incubate overnight 37 °C.

Day 2-3

12. If gRNA has fluorescent reporter gene, check transduction efficiency. Transfer supernatant from flasks into 50 mL falcon tube and filter through 45 µm filter unit (*see* **Notes 5** and **6**).
13. Replace the medium with complete DMEM medium (3 flasks at a time) and incubate for a further 24 h.
14. Store virus-containing medium at 4 degrees. (cover tubes with parafilm. Please note that these samples are highly dangerous and should be securely stored).

Day 4

15. Repeat virus harvest to have 3 days of collection [aprox 60 mL of virus containing medium per plasmid]. Mark samples with day of collection. The titre usually declines with each day. Some detachment of cells may also occur.
16. Pipette into ultracentrifuge tubes containing 1 mL of virus sucrose solution (20%), ~7-8 mL viral supernatant per tube, ensure equal volumes to balance.
17. Ultracentrifuge at 10,000 rpm for 4 h at 4 °C.
18. Discard supernatant by careful pipetting into beaker of Virkon. Carefully aspirate walls of tube to dry, without touching bottom/pellet (pellet may be invisible, and care must be taken not to disturb).
19. Optional: allow tube to air dry under hood for 10-15 min to maximise supernatant removed.
20. Add 500 uL of organoid medium or complete ADMEM to each tube. Leave the tubes overnight at 4 °C.
21. Take dry ice. Transfer all virus into one tube, pipette several times before transfer to disaggregate the virus particles. Try to avoid bubbles as they reduce useful volume.

22. Aliquot 50-60 uL into Eppendorf's. Transport on dry ice and store at -80 °C. Try not to freeze/thaw virus aliquots several times as transduction efficiency is reduced with each cycle (*see* **Notes 3**).

3.3 Transduction and generation of stable organoid lines

1. Passage the organoids into sufficient number of wells prior LV transduction [typically 10 confluent organoids per well, 3-4 wells per transduction]. Culture the organoids in Wnt3a supplement to form hyper proliferative structures.
2. Break the basement matrix containing the mature organoids with 1 mL pipette in chilled PBS.
3. Transfer the fragments into a 15 mL tube.
4. Centrifuge, 3 min for 300 *xg*.
5. Aspirate the PBS and the basement matrix residue as much as it is possible and keep the pellet. Pellet may be completely invisible.
6. Repeat step 2-6 twice more.
7. Re-suspend the pellet with PBS (300 µL) and break down the fragments with 200 mL pipette (20-30X).
8. Centrifuge, 3 min for 300 *xg*.
9. Make up medium containing polybrene to working concentration 16 µg/mL (final 8 µg/mL).
10. Re-suspend the pellet with the high titre LV and polybrene and leave the mixture for 4 h in 37-degree incubator (*see* **Note 5**).
11. Agitate the virus containing the organoid fragments every 30 min, using the 200 µL pipette to maximize the transduction efficacy.
12. After 4 h, centrifuge the transduced organoids for 3 min for 300 *xg*.

13. Discard the supernatant and add required amount of basement matrix and mix the pellet gently using a 200 µL pipette.
14. Seed the organoids (25 µL) in each well of 48 well plate.
15. Incubate the organoids in incubator for 5 min and add the complete organoid medium as described.
16. Start the selection process for the generation of the stable organoid lines from 48 h post transduction. (G418: 200 µg/uL, puromycin: 1 µg/uL).
17. Continue the selection process until the negative control (un-transduced organoids are completely dead).
18. Passage the resistant (survived), stable (transduced) organoids and continue antibiotic selection.
19. After 2nd round of the selection, expand the organoids in free-antibiotic medium (*see* **Notes 7-11**).
20. Freeze organoids. Transport on dry ice and store at -80 °C. The cryopreservation of organoids enables the freezing of knockout organoid lines post-screening (*see* **Note 12**).
21. Validation and exploration the selected organoid line via RT-PCR, WB and Phalloidin staining (see protocols 3.4, 3.5 and 3.6).

3.4 Organoid protein extraction

1. Western blot validation requires sufficient number of organoids (at least 6 wells of 20-30 mature organoids/well).
2. When the organoids are grown after 6-9 days, follow the steps 1-8 of transduction protocol.
3. Re-suspend and incubate the organoids pellet in 70-80 µl of RIPA buffer for 20 min in cold room, while rocking.

4. Lyse and homogenise the organoids using a syringe (microlance) occasionally within the incubation time.
5. The lysate can be used directly for the western blot analysis.

3.5 Organoid RNA extraction

1. Extract total RNA from organoids using TRI reagent according to the manufacturer's instructions.
2. To remove the Matrigel, incubate organoids with Matrigel cell recovery solution for 3 h at 4 °C.
3. Wash with PBS twice.
4. Centrifuge into microcentrifuge tubes.
5. Homogenise organoid pellet manually with 500 µL of TRI reagent and incubate for 5 min at RT. To maximize the homogenising, the organoids can be agitated using a 200 µL pipette every 1 min.
6. Add 100 µL of chloroform per 500 µL of TRI reagent to the samples and incubate for 3 min at RT. Mix vigorously; then, centrifuge the samples at 12,000 *xg* at 4 °C for 15 min to separate RNA (aqueous phase).
7. Transfer the upper phase containing RNA to a fresh tube and mix with 250 µL of isopropanol per 500 µL of Trizol. After 10 min at RT, precipitate RNA at 12,000 *xg* for 10 min at 4 °C (*see* **Note 13**).
8. Wash the pellet two times with cold 75% ethanol by centrifugation at 7,400 *xg* for 8 min at 4 °C, air dried and re-suspend with 20 µL of DNase/RNase free water.
9. Measure RNA concentration by Nanodrop and store the samples at -80 °C.

3.6 Phalloidin staining of organoid culture

1. Select 5-6 wells of confluent organoids and add 500 µL of 4% PFA for fixation. Incubate the organoids in 4 ℃ for 1 h.
2. Discard the PFA gently, collect the organoids in a tube (15 mL falcon) and wash the organoids with PBS. Then centrifuge for 3 min, 300 *xg*.
3. Repeat this step twice.
4. Discard the PBS and permeabilize the organoids with 500 µL of 0.5% Trtiton X 100 for 30 min at RT.
5. Wash twice with PBS, 5 min.
6. Add 800 µl in each tube with Phalloidin 1:500 diluted, keep in darkness and incubate for 40 min at RT.
7. Wash twice with PBS, 10 min.
8. Discard the PBS and lay out the organoids in the microscopy slide.
9. Mounting with DAPI.

4. Notes

1. Intestinal organoids provide an excellent *in vitro* model to investigate the molecular mechanisms of CRC. Our experience indicates that initial isolation of can produce between 8-12 wells of 10-20 organoids per well. Initial isolation will also produce a significant amount of extraneous debris that can be cleared during the first and second post—isolation subcultures. Not all organoids will successfully proliferate/differentiate and will die during this initial stage. Each well of 10-12 organoids can generally be subcultured 1:3 with minimal loss. Over-seeding organoids will cause starvation and loss during the growth period, while under-seeding can lead to reduced subculture efficiency.
2. The HEK293T cells should be healthy, of low passage number and in the exponential phase of growth. Make sure they are passaged regularly and do not allow to reach confluence.

3. Do not add antibiotics to virus harvesting medium and try not to freeze/thaw virus aliquots several times as transduction efficiency is reduced with each cycle.
4. For the transfection, polyfectamine and other related reagents can be used instead.
5. We found viral transduction efficiency was very poor when initial plasmid transfection was lower than 50%. Reducing viral dilution post-titration may improve efficiency if gRNA has low transfection rate.
6. Please ensure that your Cas9 plasmid selection marker is different to that in your prospective plasmids.
7. Organoid morphology is not exact and large number of images is necessary to quantify any potential changes based on knockout.
8. In the present study, monitoring the phenotypical alterations of knockout organoids vs controls enabled the analysis and selection of candidate genes mediated by the abnormal morphology. Of note, not all the proteins of interest are expressed in intestinal organoids and some may not show a significant phenotypical change due to non-essential functions or functional compensation by their paralogues.
9. As far as we are aware, this is the first study to utilise organoids for large scale screening of a targeted group of genes using stable and abundant Cas9 expressing organoids. While several studies have been published on the uses of organoids in cancer studies [22,23], drug resistance [24], the use of engineered organoids has been limited up to this point.
10. Organoids represent a new opportunity and hope for both biomedical research and personalised, regenerative medicine, particularly for the generation and transplant of patient-derived tissue that will prevent rejection and immune responses. However, current organoid methodology is still limited in both size and practicality, with the lack of vasculature limiting the maximum size of organoid models before necrosis occurs in the central cells due to lack of growth factors, and extracellular matrix materials such as

Matrigel will break down if samples expand greatly. One recent paper by Grebenyuk & Ranga [25] discussed combining multiple organoid types in the same dish to potentially produce hybrid tissue samples that could grow too much greater sizes due to vascularisation, possibly removing that limitation. While organoids have been demonstrated to self-assemble into *ad-hoc* structures roughly analogous to their source anatomy [26], they are currently unsuitable to such delicate physical engineering.

11. With CRISPR/Cas9 producing accurate mutations and organoids faithfully representing their origin tissues, it is now possible to model how individual genes may affect multiple tissue types or explore how a specific tissue type is affected by knockout without the cost and ethical considerations of *in vivo* alternatives. This method demonstrates generating CRISPR/Cas9 organoids and subsequent knockout organoid lines, representing a significant step forward in reducing and replacing the use of animal models in research. While organoids will not completely replace animal models in research, they provide both a middle ground option and triage method for future research.
12. This provides the opportunity to rapidly expand organoid use across research groups, collaborations and bioscience by allowing collaborative researchers to share engineered organoids, and the production of an "organoid bank" in a similar way to standard cell lines.
13. Incubation of the organoid lysate in isopropanol step overnight can be done to increase higher yield of RNA.

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