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Andrei I. Ivanov *Editor*

Gastrointestinal Physiology and Diseases

Methods and Protocols

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Gastrointestinal Physiology and Diseases

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Preface

The gastrointestinal tract is a unique, multifunctional organ in the human body. It is responsible for intake, digestion, and absorption of food, and excretion of bodily waste. It also houses a myriad of commensal and potentially pathogenic microorganisms that have profound effects on host development and homeostasis. Since these microorganisms have to be confined within the gut lumen, the gastrointestinal tract serves as the major impediment protecting internal tissues from invasion by harmful luminal microbes and exposure to their toxins. The gastrointestinal tract is also the largest organ of the immune system and is populated by specialized cells trained for border surveillance and recognition of external dangers.

Normal function of the gastrointestinal tract is frequently compromised by genetic factors, infections, stress, life habits, etc., that give rise to various diseases. Remarkably, impaired functions of this organ not only result in specific gastrointestinal disorders such as gastric ulcer, inflammatory bowel disease, or gastrointestinal tumors but also contribute to the development of other human pathologies including certain neurological and cardiovascular diseases, as well as diabetes. Collectively, these factors establish the study of the normal functions and disorders of the gastrointestinal system as one of the most important and exciting topics of modern biology and medicine.

The aim of *Gastrointestinal Physiology and Diseases: Methods and Protocols* is to provide an expert, step-by-step guide to a variety of techniques for examining the activity and regulation of the gastrointestinal system and for modeling the most common digestive diseases. This book is intended to target a large cohort of physiologists, cell and developmental biologists, immunologists, and physician-scientists working in the field of gastroenterology and beyond. This volume contains comprehensive and easy to follow protocols that are designed to be helpful to both seasoned researchers and newcomers to the field.

The protocols included in this volume are separated into five different parts. Part I (Chapters 1–9) describes in vitro and ex vivo techniques to study different aspects of the functions and differentiation of the gut mucosa, with particular emphasis on modern approaches to the growth, differentiation, and study of complex intestinal and gastric organoids. Part II (Chapters 10–15) outlines powerful in vivo imaging approaches to study biochemical alterations in epithelial cells, and to visualize leukocyte trafficking of in the gut during tissue inflammation and neoplasia. Part III (Chapters 16–20) presents protocols for the isolation, characterization, and therapeutic transfer of different types of intestinal immune cells. Part IV (Chapters 21–25) describes different animal models of gastrointestinal mucosal inflammation and injury. It describes classical models of chemically induced and infectious colitis in mice and also presents examples of the use of other model organisms in studying digestive disorders. Part V (Chapters 26–29) presents state-of-the-art animal models for studying tumor induction and development in the colon, stomach, and oral cavity.

I would like to thank all of the contributors for sharing their expertise and for carefully guiding readers through all the nuanced details of their respective techniques. I am very grateful to the series editor, Dr. John Walker, for his help during the editing process.

Richmond, VA, USA *Andrei I. Ivanov*

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Part I

In Vitro and Ex Vivo Systems to Study Gastrointestinal Functions and Diseases

Chapter 1

CRISPR/Cas9-Mediated Genome Editing of Mouse Small Intestinal Organoids

Gerald Schwank and Hans Clevers

Abstract

The CRISPR/Cas9 system is an RNA-guided genome-editing tool that has been recently developed based on the bacterial CRISPR-Cas immune defense system. Due to its versatility and simplicity, it rapidly became the method of choice for genome editing in various biological systems, including mammalian cells. Here we describe a protocol for CRISPR/Cas9-mediated genome editing in murine small intestinal organoids, a culture system in which somatic stem cells are maintained by self-renewal, while giving rise to all major cell types of the intestinal epithelium. This protocol allows the study of gene function in intestinal epithelial homeostasis and pathophysiology and can be extended to epithelial organoids derived from other internal mouse and human organs.

Key words Small intestinal organoids, Intestinal stem cells, CRISPR/Cas9, Genome editing

1 Introduction

1.1 The CRISPR/Cas9 Genome-Editing Tool

Clustered regularly interspaced short palindromic repeats (CRISPRs) are classes of repeated DNA sequences found in bacteria and archaea. Together with CRISPR-associated (Cas) genes they are part of an adaptive bacterial immune defense system, which confers resistance to foreign genetic elements such as phages [1]. The CRISPR/Cas immune defense process involves three steps. First, upon infection foreign DNA sequences are inserted as new spacers into the CRISPR locus. Second, the locus is transcribed into a single noncoding precursor CRISPR RNA (pre-crRNA) and is processed into short stretches of mature crRNA. Third, the mature crRNA forms a ribonucleoprotein complex with Cas proteins, which specifically recognizes and destroys the invading foreign DNA [2].

So far, three types of CRISPR systems have been discovered. In contrast to the type I and type III CRISPR/Cas systems, the type II system relies on a single Cas protein for DNA interference, but—in addition to the crRNA—requires a tracrRNA bound to Cas9 [3]. The specificity of the CRISPR/Cas9 ribonucleoprotein

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complex to the invading DNA is mediated through Watson-Crick base pairing of a 20-nucleotide long stretch that is complementary between the crRNA and the invading DNA. The HNH and RuvC-like nuclease domains of the Cas9 protein then eliminate the foreign DNA by generating a double-strand break (DSB). In principle, any DNA sequence that is followed by a protospacer-associated motif (PAM), a conserved sequence of 2–5 nucleotides, can be recognized and cut by the CRISPR/Cas9 complex [3].

In 2012, the labs of Emanuelle Charpentier and Jennifer Doudna together adapted the type II CRISPR system for genome editing [4]. By combining the tracrRNA with the scRNA, a synthetic single guide RNA (sgRNA) was generated, which effectively targets Cas9 to a DNA sequence of interest and leads to the site-specific generation of a DSB [4]. Like in previously developed genome-editing tools, DSBs generated by CRISPR/Cas9 can modify the targeted DNA locus in two ways. First, in the absence of a homologous DNA template the DSBs can generate small insertions or deletions, as they are repaired by the error-prone non-homologous end-joining (NHEJ) pathway [5]. Second, in the presence of an exogenous homologous DNA template, the DSBs can be repaired by the homology directed repair pathway (HDRP), which allows to introduce specific DNA sequences and thus to precisely modify the genomic region [5]. In 2013, several research groups have demonstrated successful CRISPR/Cas9-mediated genome editing in a number of different organisms, ranging from plants to human cells [6, 7]. The easy design, high targeting efficiency, and low off-target mutation frequency of the CRISPR/Cas9 system rapidly made it the most commonly used genome-editing tool [3].

1.2 Mouse Small Intestinal Organoids

Mouse small intestinal organoids are in vitro-grown three-dimensional epithelial structures that closely resemble the in vivo gut epithelium. They can be established from single Lgr5+ stem cells, which are embedded in Matrigel and supplied with a cocktail of tissue-specific growth factors [8]. Like the in vivo gut epithelium, intestinal organoids contain a crypt-like compartment with self-renewing Lgr5+ stem cells, and a villus-like compartment with differentiated enterocytes, paneth cells, and enteroendocrine cells [8]. Minor changes in growth factor composition allow the growth of organoids from a range of human epithelial tissues [9–12]. Epithelial organoids are genetically and phenotypically stable [13] and can be genetically modified by CRISPR/Cas9-based genome editing [14–16].

In this protocol, we describe step by step how to edit the genomes of mouse small intestinal organoids using CRISPR/Cas9 in combination with DNA templates for homologous recombination (HR). In addition, we provide information on how to adapt the protocol for genome editing in organoids derived from different tissues.

2 Materials

2.1 Small Intestinal Organoid Culture Components

1. ECM matrix (*see* **Note 1**).
   (a) Corning® Matrigel® Growth Factor Reduced (GFR) Basement
   Membrane Matrix, cat no. 356231, or
   (b) Cultrex® BME2 RGF organoid matrix, cat no. 3533-005-02.
2. Intestinal organoid medium (*see* **Note 2**).
   (a) Advanced DMEM/F12 (Life Technologies).
   (b) GlutaMax (Life Technologies).
   (c) HEPES.
   (d) Penicillin-Streptomycin.
   (e) N2 supplement (Life Technologies, cat no. 17502–044).
   (f) B27 supplement (Life Technologies, cat no. 17504–044).
   (g) *N*–Acetylcysteine.
   (h) Murine recombinant EGF (Life Technologies, cat no. PMG8044).
   (i) Murine recombinant Noggin (PeproTech, cat no. 250–38).
   (j) Human recombinant R-spondin1 (PeproTech, cat no. 120–38).

   Preparation of the intestinal organoid medium: First supplement 500 ml of Advanced DMEM/F12 with 5 ml 100× Glutamax, 5 ml 1 M HEPES, and 5 ml 100× Penicillin-Streptomycin. This Advanced DMEM/F12+++ medium is stable at 4 C° for at least one month. To prepare 20 ml of intestinal organoid medium, supplement the Advanced DMEM/F12+++ medium with 400 µl of 50× B27, 200 µl of 100× N2, 50 µl of 500 µg/ml *N*-acetylcysteine, 2 µl of 500 µg/ml mouse EGF, 20 µl of 100 µg/ml mouse recombinant Noggin, and 20 µl of 1 mg/ml human recombinant R-spondin1. Intestinal organoid medium is stable for at least two weeks at 4 C°.
3. Recovery™ Cell Culture Freezing Medium (Life Technologies).
4. Cryopreservation cell freezing containers.
5. 24-well and 48-well cell culture plates.

2.2 Transfection and Clonal Selection of Small Intestinal Organoids

1. Lipofectamine 2000 (Life Technologies).
2. Opti-MEM (Life Technologies).
3. Y-27632 dihydrochloride (Sigma-Aldrich).
4. CRISPR/Cas9 plasmids: pSpCas9(BB)-2A-GFP(PX458), pSpCas9(BB)-2A-Puro (PX459) V2.0 (available via Addgene).

5. Trypsin replacement solution (TrypLE) (Life Technologies).
6. Murine recombinant Wnt-3a (Millipore, cat no. GF154).
7. 4-Hydroxytamoxifen (Sigma-Aldrich).
8. Purelink Genomic DNA Extraction kit.
9. Puromycin.
10. Nicotinamide.
11. Parafilm.
12. Refrigerated centrifuge with microtiter plate carrier.
13. Thermal cycler.

3 Methods

3.1 Design and Generation of CRISPR/Cas9 Genome-Editing Vectors

For the design and the cloning of CRISPR/Cas9 vectors, we advise to follow the Nature Protocol from the Zhang lab [5]. We generally use the pSpCas9(BB)-2A-GFP CRISPR/Cas9 plasmid, and insert the specific target sequence as described in their protocol.

The design of the template DNA for homologous recombination depends on the application. We generally synthesize plasmids with a 500 bp homology region up- and downstream of the desired nucleotide change. Close to the modified nucleotide (<50 bp), we insert a puromycin resistance cassette flanked with loxP sites. This setup allows efficient screening for homologous recombination events using antibiotics and subsequent cassette excision (*see* **Note 3**).

3.2 Establishment of Small Intestinal Crypt Cultures

The establishment of organoid cultures from freshly isolated murine small intestinal crypts is described in Sato and Clevers, 2013 [17]. This protocol explains all experimental steps in detail and lists all required reagents. Please follow this protocol to establish and passage murine small intestinal organoids.

3.3 Cryopreservation of Organoids

After establishing a new organoid line, we recommend cryopreserving the line, and always starting from an early passage when gene-editing experiments are repeated.

1. After establishing a new organoid line, passage the culture once or twice prior to cryopreservation (*see* **Note 4**).
2. Approximately 7 days after seeding, replace the mouse small intestinal organoid medium with 1–2 ml of cold basal culture medium, and disrupt Matrigel by gently pipetting with a p1000 pipette.
3. Transfer organoids from one well into a 15 ml falcon tube, and disrupt them by gently pipetting 10–15 times with a fire-polished Pasteur pipette.

4. Centrifuge organoids at 150 × *g* for 5 min at 4 C°, remove the supernatant, and resuspend the cell pellet in 0.5 ml of ice-cold Recovery™ Cell Culture Freezing Medium.
5. Transfer the cell suspension into 1.5 ml cryogenic storage tube and put the tubes in a CoolCell® cryopreservation container. Immediately transfer the container to the −80 C° freezer, and keep it at −80 C° for 24 h. Afterwards tubes can be moved to the liquid nitrogen container for long-term storage.

3.4 Preparation of Organoids for Lipofection

1. Start with organoids from an early passage. If cryopreserved organoids are used, thaw a vial in a 37 C° water bath and immediately suspend in 10 ml of basal culture medium containing 10 % FBS. Centrifuge at 150 × *g* for 5 min at 4 C°, remove supernatant, repeat the washing step with basal culture medium, and resuspend the pellet in 150 μl of ice-cold Matrigel.
2. Divide the Matrigel cell suspension as hemispheric droplets into three wells of a 24-well plate, and incubate in a 37 C° incubator for 10 min for Matrigel polymerization (*see* **Note 5**).
3. Add mouse intestinal organoid medium supplemented with Wnt-3a (100 ng/ml) and nicotinamide (10 mM) to the wells, and change medium every 2–3 days (*see* **Notes 6** and **7**). Approximately once a week organoids can be passaged.
4. After two passages organoids should be cystic, and are ready for transfection.
5. Replace the intestinal organoid medium with 1 ml of cold basal culture medium, and disrupt Matrigel by gently pipetting with a p1000 pipette. Transfer organoids from four wells (~100 organoids per well) into one 15 ml falcon tube, and break them by gently pipetting with a fire-polished Pasteur pipette.
6. Centrifuge at 150 × *g* for 5 min, and wash the pellet with 5 ml of ice-cold basal culture medium to fully remove Matrigel (*see* **Note 8**).
7. Resuspend the pellet in 4 ml of pre-warmed TrypLE and incubate at 37 C° for 5 min in a water bath (*see* **Note 9**).
8. Centrifuge at 150 × *g* for 5 min at 4 C°, and resuspend the pellet in 450 μl mouse small intestinal organoid medium supplemented with Wnt-3a (100 ng/ml), nicotinamide (10 mM), and the Rho kinase inhibitor Y-27632 (10 nM).
9. Transfer the cell suspension into one well of a 48-well plate, let the cells sink to the bottom, and analyze cell density under the microscope. Cells should be 70–90 % confluent.

3.5 Lipofection of Organoids with CRISPR/Cas9 Plasmids

We recommend also reading the Lipofectamine® 2000 reagent protocol on the Life Technologies webpage: http://tools.lifetechnologies.com/content/sfs/manuals/Lipofectamine_2000_Reag_protocol.pdf

1. Dilute 2 μl of Lipofectamine reagent in 25 μl of Opti-MEM® medium.
2. Dilute plasmids (0.5 μg CRISPR/Cas9 vector and 0.5 μg HDR plasmid) in 25 μl of Opti-MEM® medium.
3. Mix diluted DNA and diluted Lipofectamine reagent, and incubate for 5 min.
4. Add the 50 μl Lipofectamine-DNA complex gently to one well of dissociated organoids.
5. Seal the plate with parafilm, and centrifuge 60 min at 600 × *g* at 32 °C.
6. Discard the parafilm, and incubate the plate for another 2–4 h in a tissue culture incubator.
7. Collect the transfected cells in a 15 ml falcon tube, spin at 150 × *g* for 5 min, and resuspend the pellet in 100 μl of ice-cold Matrigel.
8. Divide the Matrigel cell suspension into two wells of a 24-well plate, and incubate in the 37 C° incubator for 10 min for Matrigel polymerization.
9. Add mouse small intestinal organoid medium supplemented with Wnt-3a (100 ng/ml), nicotinamide (10 mM), and Y-27632 (10 nM), and place the plate into the tissue culture incubator.

3.6 Selection of Genome-Modified Organoids

1. 3 days after transfection start with the antibiotics selection (500 ng/ml puromycin) (*see* **Note 10**).
2. When drug-sensitive organoids start to grow out, pick individual organoids from the Matrigel under a binocular microscope using a p200 pipette, transfer them individually into 1.5 ml tubes, and split them by pipetting with the p200 pipette.
3. Centrifuge for 5 min at 900 × *g*, resuspend in 100 μl ice-cold Matrigel, and plate cells in a 24-well plate. After Matrigel polymerization add normal small intestinal organoid medium (*see* **Note 11**).
4. After expansion of clonal organoids, use 1–2 wells for genomic DNA isolation. For the isolation you can use the PureLink® Genomic DNA extraction kit from Life Technologies or other standard genomic DNA isolation procedures.
5. Analyze the genome by PCR and Sanger sequencing to identify clones with correct HR events. Use primer pairs that bind within the puromycin resistance cassette and up- or downstream of the