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TITLE:

Establishment and Genetic Manipulation of Murine Hepatocyte Organoids

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SUMMARY:

A long-term *ex vitro* 3D organoid culture system was established from mouse hepatocytes. These organoids can be passaged and genetically manipulated by lentivirus infection of shRNA/ectopic construction, siRNA transfection and CRISPR-Cas9 engineering.

ABSTRACT:

The liver is the largest organ in mammals. It plays an important role in glucose storage, protein secretion, metabolism and detoxification. As the executor for most of the liver functions, primary hepatocytes have limited proliferating capacity. This requires the establishment of *ex vivo* hepatocyte expansion models for liver physiological and pathological research. Here, we isolated murine hepatocytes by two steps of collagenase perfusion and established a 3D organoid culture as the 'mini-liver' to recapitulate cell-cell interactions and physical functions. The organoids consist of heterogeneous cell populations including progenitors and mature hepatocytes. We introduce the process in detailed to isolate and culture the murine hepatocytes or fetal hepatocyte to form organoids within 2-3 weeks and show how to passage them by mechanically pipetting up and down. In addition, we will also introduce how to digest the organoids into single cells for lentivirus infection of shRNA/ectopic construction, siRNA transfection and CRISPR-Cas9 engineering. The organoids can be used for drug screens, disease modelling, and basic liver research by modeling liver biology and pathobiology.

INTRODUCTION:

Organoids are self-organized, three-dimensional (3D) *in vitro* clusters that include self-renewing stem cells and multi-lineage differentiated cells[1,2]. The organoids of many organs have been established either from pluripotent or adult stem cells by well-defined niche factors including the intestine, the brain, the colon, the kidney, the liver, the pancreas, the thyroid, the stomach, the skin, and the lung[3,4,5,6,7]. The organoids recapitulate physical cell functions by mimicking either

development (originated from embryonic or induced pluripotent stem cells, PSCs) or homeostasis/regeneration progression (originated from adult stem cells, ASCs), which opens up new avenues in disease research and therapy[8,9].

As the biggest organ in mammals, the liver is mainly responsible for storage, metabolism and detoxification. Two kinds of epithelial cell types, hepatocytes and cholangiocytes, construct the basic unit of a liver lobule. Hepatocytes are responsible for 70-80% of liver function[10]. Although the liver has remarkable regeneration capacity, rapid loss of hepatocyte features happens during traditional monolayer culturing by dysregulated cell polarization and dedifferentiation, which increases the need of researchers and clinicians to build 'gap-bridging' liver models in a dish. However, until recently the *ex vivo* expansion models from primary hepatocytes had not been well established[11,12,13,14,15]. Liver organoids can be established from embryonic/induced pluripotent stem cells, fibroblast conversion into hepatocyte-like cells, and tissue-derived cells. The development of liver organoids boosts the application of an in vitro model for drug screens and liver toxicity assays[16,17].

Here, we describe a detailed protocol for establishing liver organoids from murine primary hepatocytes. By using this protocol, we set up an *in vitro* culture system of hepatocyte organoids with two perfusions of collagenase. These organoids can be passaged for long-term expansion for months. Their physiological function is highly consistent with hepatocytes. Furthermore, we also provide a detailed description of how to perform genetical manipulation, such as lentivirus infection, siRNA transduction, and CRISPR-Cas9 engineering using organoids. The propagation of hepatocyte organoids shed light on the possibility of using organoids to understand liver biology and develop personalized and translational medicine approaches.

PROTOCOL:

All mice experiments were approved by the Animal Care and Use Committee of the School of Basic Medical Sciences at Shandong University and were carried out according to the License under Home Office guidelines and regulations (No. ECSBMSSDU2019-2-079).

NOTE: This protocol is mainly used to culture 3D organoids from isolated primary hepatocytes.

1. Establishment of Murine Hepatocyte Organoid Culture

NOTE: Extracellular matrix such as Matrigel or BME are used as the basement matrix to culture organoids. Store the extracellular matrix at -20 °C and pre-thaw it at 4 °C or on ice. Keep the extracellular matrix on ice during the experiments. The wash medium is Advanced DMEM/F12 supplemented with GlutaMAX, HEPES and penicillin/streptomycin. A standard incubator (37 °C, humidified atmosphere, 5% CO_2) is used for organoid culture.

1.1. Preparation of materials

1.1.1. Prepare the instruments required for the experiment: the mouse pillow, sterile surgical

instruments like tweezers, scissors, tubes, and a pump with a flow rate of 2-10 mL/min.

1.1.2. Prepare the reagents including the perfusion buffer, digestion buffer and make them sterile for hepatocyte isolation. Add fresh type IV collagenase (0.10 mg/mL) and $CaCl_2$ (0.50 M) and pre-warm the buffer at 37 °C in a water bath.

1.1.3. Prepare the conditional medium to produce R-spondin 1 and all chemical and growth factor stocks according to manufacturer's instructions.

NOTE: Avoid frequently freezing and thawing the conditional medium and stocks. Prepare the culture medium fresh and use it within one month. This is critical for organoid forming efficiency.

1.2. Murine hepatocyte isolation by liver perfusion and digestion

NOTE: Male or female mice between the ages of 12 weeks and 6 months were used for organoid culture. No obvious differences were found in organoid formation efficiency from the donor mouse age within this range.

1.2.1. Euthanize the mouse with an ethically approved method.

1.2.2. Fix the mouse on the mouse pillow and clean the abdominal fur and skin with 70% alcohol. Expose the abdomen and lift the liver above the rest of the body. Make a midline incision to expose the liver.

1.2.3. Fill the tube attached to the pump with perfusion buffer. Make an incision of the portal vein (about 1/3 of the diameter of the vein) and carefully place a catheter into it. Lift up the digestive organs to help insert the cannula. A tie with the surgical line is optional to avoid sliding out and rupturing.

1.2.4. Start the pump and perform perfusion at a flow rate of 5-7 mL/min with perfusion buffer. If the liver becomes pale immediately, cut the inferior vena cava to allow the perfusion buffer to flow through the whole liver and drive the blood out.

1.2.5. When the flow-out becomes clean, remove the cannula. It takes about 5-10 minutes.

1.2.6. Change the perfusion buffer with pre-warmed digestion buffer at a rate of 5 mL/min. Do not stop the pump until the liver becomes soft and swells. Remove the catheter when the liver stops swelling.

NOTE: It is very important to keep proper digestion time to avoid excessive digestion and get single liver cells.

1.2.7. Cut off the liver and place it in a 100 mm plate filled with 15 mL of cold wash medium. Cut the ligaments with small scissors. Tear the liver into pieces with pipette tips or forceps.

1.2.8. Pipette up and down gently to release the cells until the buffer is saturated with cells (12-15 times). Pour the cell suspension into a 50 mL tube through 70 μm filters.

1.2.9. Centrifuge for 1-3 minutes at 50 x *g* and 4 °C. Decant the supernatant. Optionally, resuspend the cells with wash medium or resuspend the cells with culture medium directly. Count the cells with a hemacytometer.

1.2.10. Distinguish live or dead cells by the cell counter machine after live cell dye staining.

NOTE: It is best to purify hepatocytes using cold 40% Percoll. After this step, viable hepatocytes will be at the bottom of the tubes.

1.3. Organoid culture and passage

1.3.1. Remove the supernatant and resuspend the hepatocytes with a mixture of extracellular matrix and culture medium in a ratio of 3:1.

NOTE: 10,000-20,000 cells in a 50-60 μL mixture per well for a 24-well plate are recommended. It is important to work quickly and keep the mixture on ice throughout the process. 200-500 organoids could be formed at this concentration.

1.3.2. Add the cell/mixture drop on the plate with small droplets to prevent the droplet from connecting together. Incubate the plate in the cell incubator at 37 °C, 5% CO_2 for 5-10 min and then take the plate out for 20-25 min until the extracellular matrix becomes solid.

1.3.3. Add culture medium (500 μL per well for a 24-well plate) into the wells and return the plate to the incubator for organoid culture. Change the medium every 3-4 days. Observe the organoids until they are big enough to be passaged.

NOTE: A diameter with around 400-500 μm is suitable for passage of hepatocyte organoids.

1.3.4. Isolate organoids from the extracellular matrix with the detailed process below. Hold a glass Pasteur pipette in a flame to narrow its aperture (by ~1/2). Mechanically pipette up and down 5-10 times to dissociate the organoids into smaller organoids during passaging.

NOTE: During passaging, prepare pre-washed micropipette tips and tubes with tip-wash buffer to avoid the loss of organoids sticking to the tubes or tips.

2. Genetic manipulation of murine hepatocyte organoids

2.1. Single cells from hepatocyte organoids

2.1.1. Add 500 μL of cold wash medium/cell recovery solution to each well. Pipette up and down

to destroy the cell/extracellular matrix mixture with a micropipette and then transfer the organoid suspension to a 15 mL centrifuge tube on ice. Optionally, turn the tube up and down from time to time to thaw the mixture thoroughly.

2.1.2. Centrifuge for 5 min at 500-800 x *g* and 4 °C and discard the supernatant. Add 1 mL of trypsin solution to the pellet and pipette it to mix. Incubate at 37 °C for 5-10 min and then stop digestion by adding a 2x volume of wash medium.

2.1.3. Centrifuge for 5 min at 800 x g and 4 °C and discard the supernatant. Resuspend the pellet with culture medium and count the cells using a hemacytometer.

2.2. siRNA or plasmid transfection

NOTE: Smaller organoids with a diameter less than 50 µm or single cells from hepatocyte organoids are transfected with siRNA using Lipofectamine or plasmids using transfection reagent according to the manufacturer's instructions.

2.2.1. Mix siRNA or plasmids with transfection reagent. Add single cells from organoids and the Lipofectamine–RNA/Lipo-plasmid mixture (e.g., RNAiMAX) into 1-2 wells in the 24-well plate and centrifuge for 1 h at 500 x g and 32 °C. After centrifugation, incubate the plate in the incubator for 4-5 h at 37 °C.

2.2.2. Collect the cells and seed in extracellular matrix with culture medium at a concentration of 10,000 cells per well. Change the medium to the selection condition two days after transfection. Assess the organoid-formation efficiency after 10 days.

2.3. Lentiviral infection

NOTE: Small organoids with a diameter less than 50 µm or single cells from hepatocyte organoids are infected by lentivirus using infection reagents medium according to the manufacturer's instructions.

2.3.1. Mix 250 µL of the single-cell suspension from the organoid and 250 µL of viral particles in a 1.5 mL tube.

2.3.2. Plate the mixture and centrifuge for 1 h at 500 x g and 32 °C. Incubate for 4-5 h at 37 °C.

2.3.3. Collect the cells and seed in extracellular matrix with culture medium. Change the medium to the selection condition two days after transfection. Assess the organoid-formation efficiency after 10 days.

2.4. Genetic engineering by CRISPR/Cas9

NOTE: We provide video for this performance in the supplemental materials. Single cells from

organoids were infected with lentivirus or transfected by plasmids carrying sgRNA and Cas9.

2.4.1. Digest single cells from hepatocyte organoids cultured and passage according to the above method.

2.4.2. Infect single cells with sgRNA using Opti-MEM medium and infection reagents according to the manufacturer's instructions.

2.4.3. Mix 250 µL of a single-cell suspension and 250 µL of viral particles together in a 1.5 mL tube.

2.4.4. Centrifuge the 500 µL mixture for 1 h at 500 x g and 32 °C and then incubate for 4-5 h at 37 °C.

2.4.5. Collect the cell suspension and seed in extracellular matrix. Assess the organoid-formation efficiency after 10 days.

REPRESENTATIVE RESULTS:

The hepatocyte organoids from female Alb-Cre; Rosa26-GFP mouse (8 weeks) were observed five days after seeding (**Figure 1A**). The organoids are proliferating with Ki67 positive staining (**Figure 1B**). Interfering expression of representative genes in hepatocyte organoids either by siRNA transfection or lentivirus infection was examined by qRT-PCR and western blot. The results are shown in **Figure 2A** and **2B**. **Figure 2C** shows the selection results from liver organoids after 14 days of treatment with RSL-3 and the CRISPR/Cas9 genomic library screen. The results suggest these organoids could be expanded in vitro and genetically manipulated.

FIGURE AND TABLE LEGENDS:

Figure 1: Establishment and expansion and of murine hepatocyte organoids

(**A**) Representative image of hepatocyte organoids from Alb-Cre; Rosa26-GFP. The scale bar is 400 µm. (**B**) Representative image of whole mount staning of hepatocyte organoids with b-catenin and Ki67 antibodies.

Figure 2: Genetic manipulation of murine hepatocyte organoids

(**A**) The mRNA level of Hdac3 and Wee1 relative to GAPDH in hepatocyte organoids after transfected with siRNA. Error bars represent standard errors (n=3). (**B**) Western bolt analysis of organoids infected by lentivirus. (**C**) Representative image of liver organoids after CRISPR-Cas9 screen in culture medium with RSL-3 selection.

DISCUSSION:

The ability to culture mature hepatocytes over long periods is fundamental in studying liver basic science, drug toxicology and hepatotropic host-microbiology infections such as malaria and the hepatitis viruses. With a well-defined niche, the protocol here sets up a culture system for hepatocytes. This protocol drives mature hepatocytes to expand in 3D culture with a heterogeneity that recapitulates cell-cell interaction, most hepatocyte function and genetic

modifications, allowing the design of gene function studies and novel therapeutic avenues toward regenerative therapy.

High quality hepatocytes are very important for the establishment and genetic manipulation of hepatocyte organoids. The digestion time of collagenase treatment is quite critical. There remains some limitations in the method though. First, more niche factors are suggested in a trial to help the organoids expand longer. Second, the organoids do not like to be single cells consecutively and frequently. The hepatocyte-derived organoids appear to recapitulate the regenerative response of adult livers after partial hepatectomy, but they are still different from quiescent mature hepatocytes.

Of note, using hepatocyte organoids to expand hepatocytes open up new pathways for liver gene function studies. Co-culturing organoids with vessel cells, immune cells and hepatic viruses are expected to further study angiogenesis, microenvironments and infectious liver disease. A standard high-throughout screen system with these organoids is also recommended for an ex vivo detoxification study.

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DISCLOSURES:

The authors disclose no conflicts.

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Establishment of murine hepatocyte organoids Culture

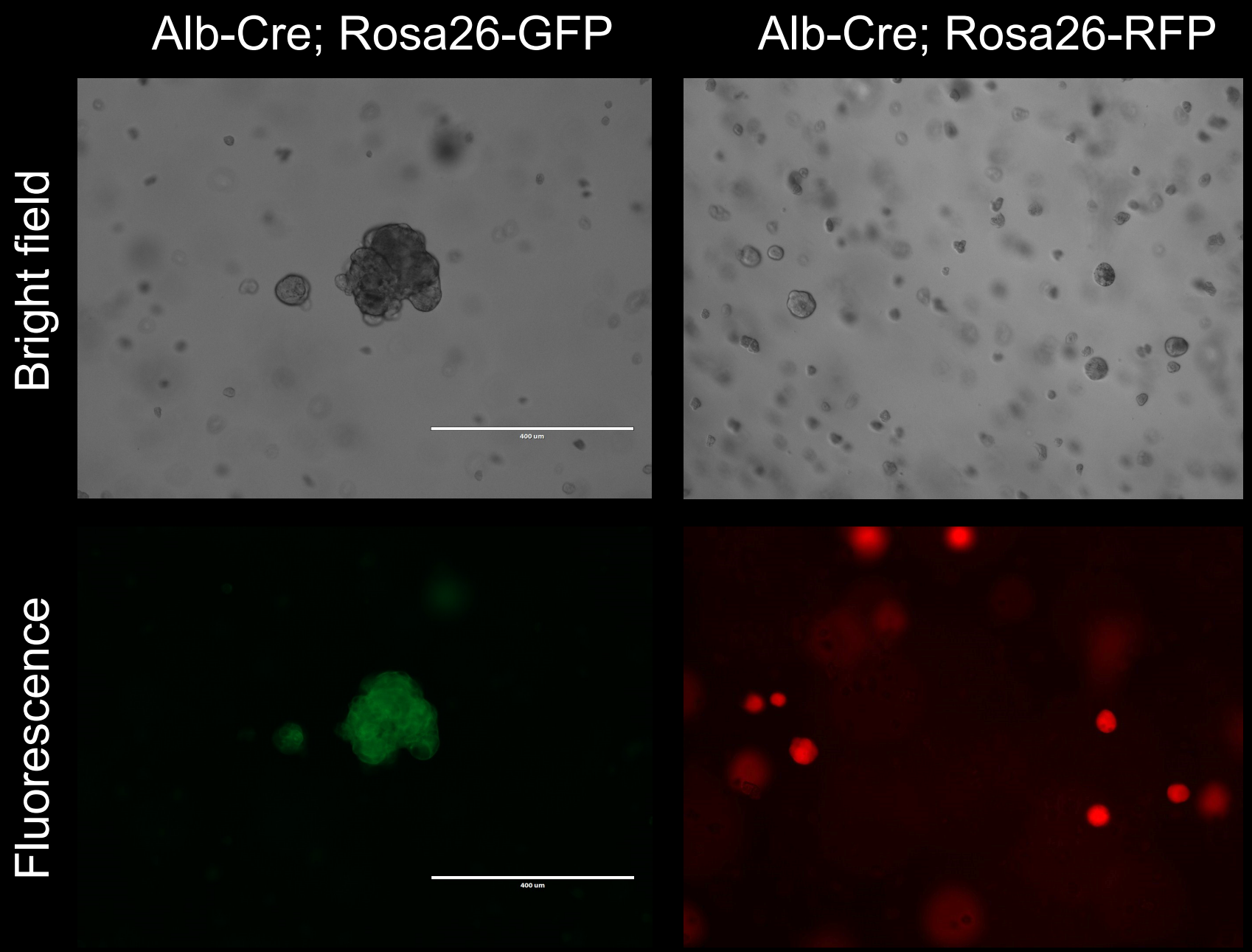

Figure 1A
(Refer to 1)

Establishment of murine hepatocyte organoids Culture. Refer to 1

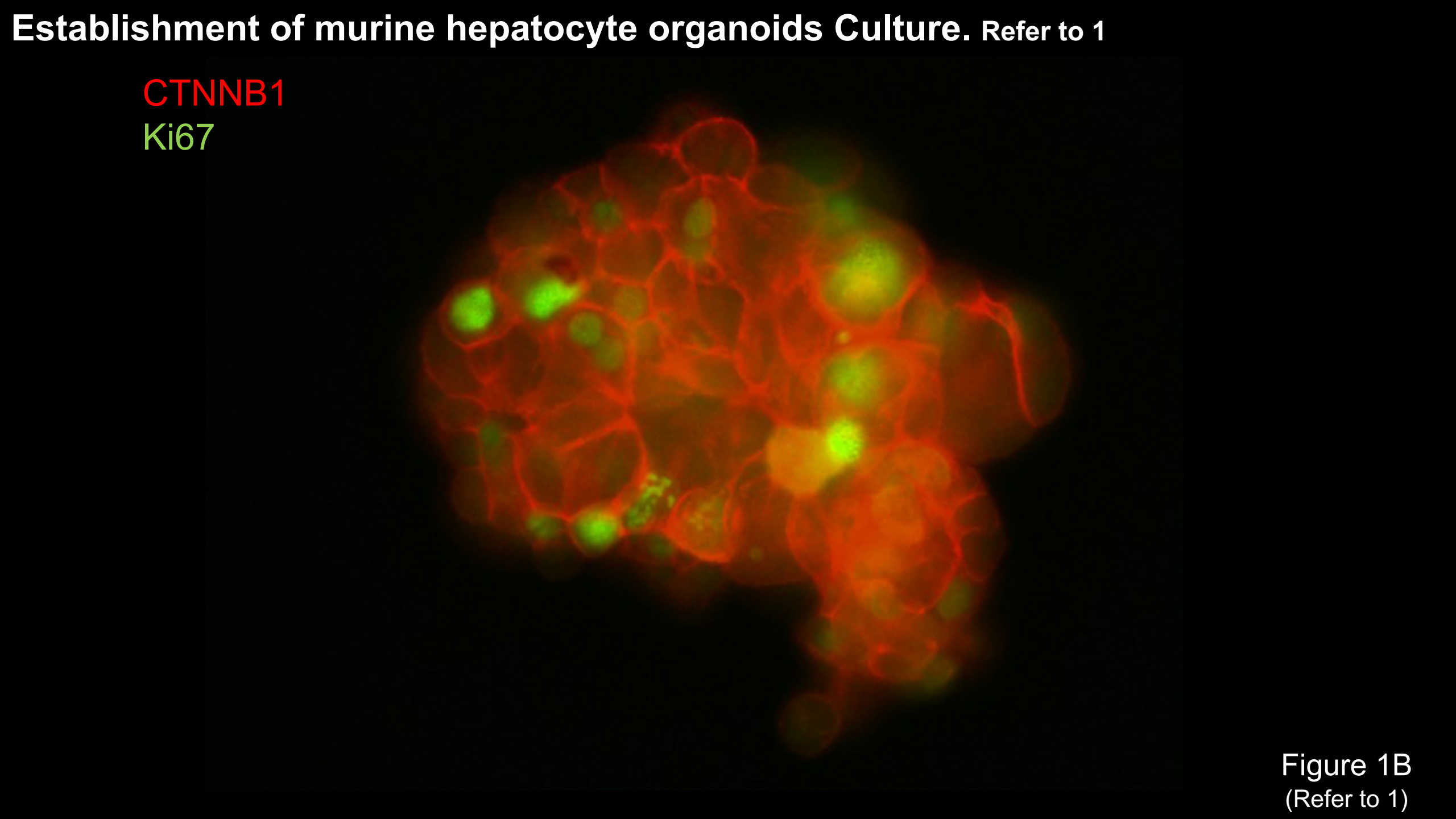

Figure 1B
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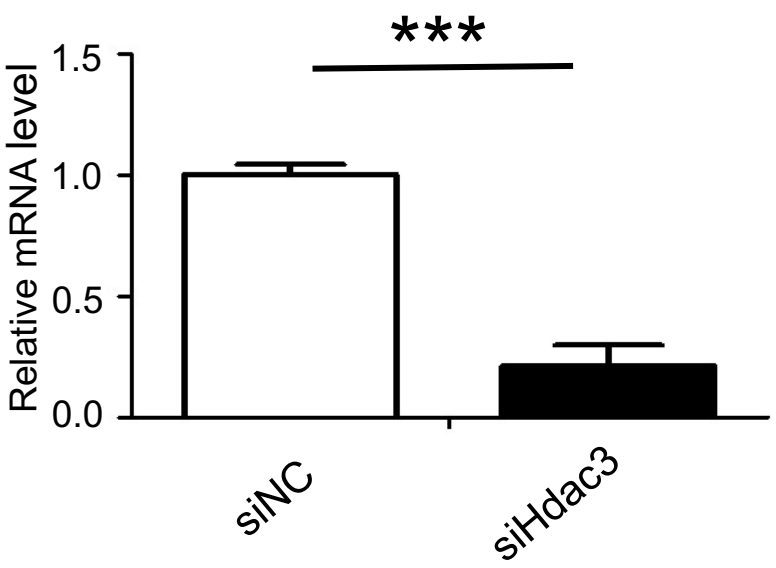
Genetic manipulation of murine hepatocyte organoids



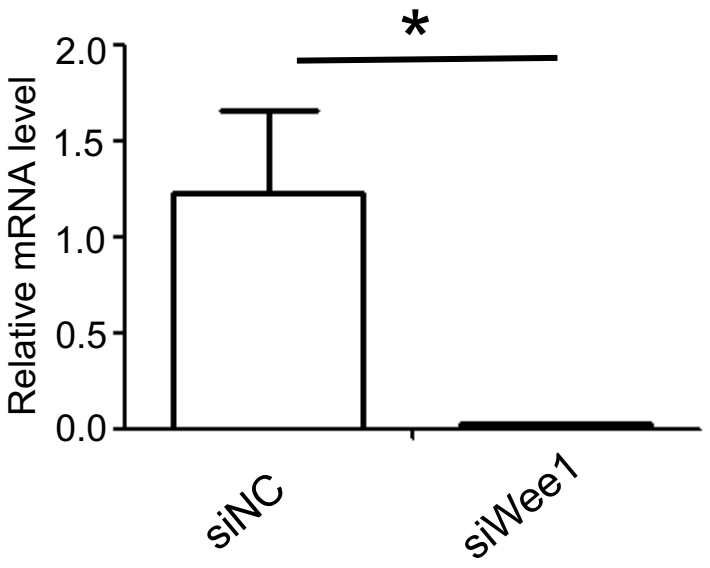


Figure 2A
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Figure 2B
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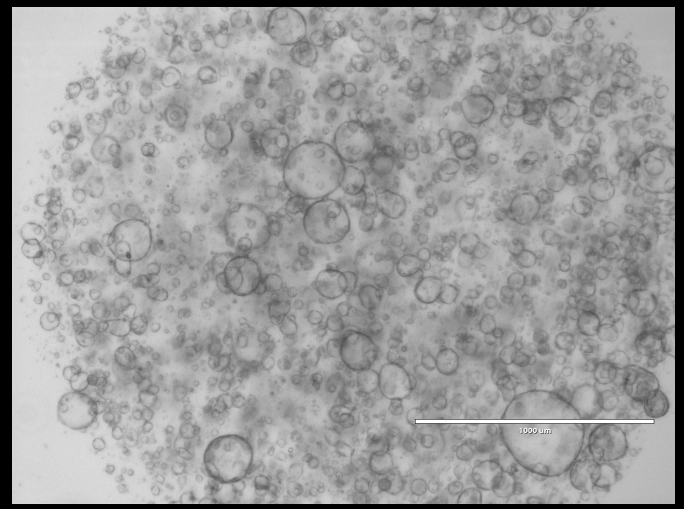
Genetic manipulation of murine organoids

Control



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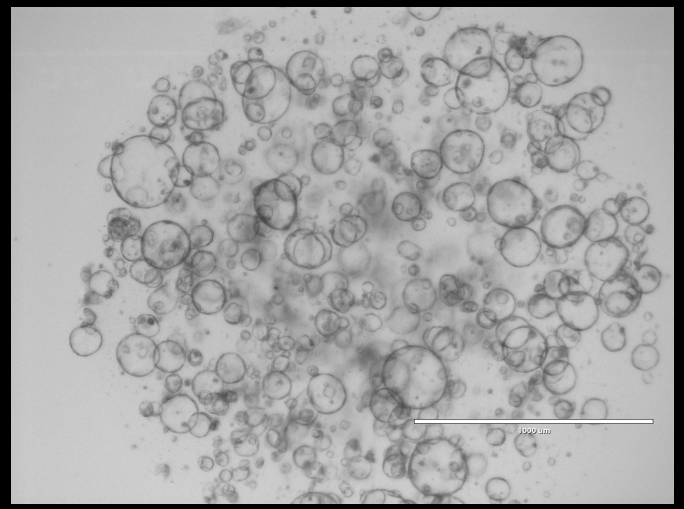

Figure 2C
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