Generation of gastric insulin-secreting organoids from human stomach sample

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Method Article

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Abstract

Stomach stem cells are accessible by biopsy and propagate robustly in culture, offering an invaluable resource for autologous cell therapies. Here we describe a detailed protocol to isolate, expand, engineer and differentiate human gastric stem cells (hGSCs) into pancreatic islet-like organoids containing abundant gastric insulin-secreting (GINS) cells that resemble beta-cells in molecular hallmarks and function. Sequential activation of the inducing factors NGN3 and PDX1-MAFA led hGSCs onto a novel differentiation path, including endocrine progenitor and GINS precursor, before adopting beta-cell identity, at efficiencies close to 70%. GINS organoids acquired glucose-stimulated insulin secretion in 10 days post differentiation.

Introduction

Gut stem cells are highly proliferative and power the weekly self-renewal of the gut mucosal lining\textsuperscript{1-3}. Harvested from biopsies, human gut stem cells can be propagated in culture as organoids or primary cell lines over many generations, providing abundant tissues for potential autologous transplantation therapies\textsuperscript{4-6}. Gut stem cells produce gut-specific tissues, including hormone-secreting enteroendocrine cells (EECs). Rare insulin expressing EECs have been reported in fetal human small intestine\textsuperscript{7}. Whether such cells secret insulin is unknown, but their presence suggests an intrinsic permissiveness for insulin production in the fetal if not postnatal intestine. Generating functional insulin-secreting cells has tremendous therapeutic value, offering treatments for insulin-dependent diabetes, including the autoimmune type 1 diabetes\textsuperscript{8-13}. An attractive feature of using gut stem cells to make beta-cell mimics is the ease of establishing autologous organoids from biopsies, which can enable mass production and personalized therapies.

Previously, we reported that co-expression of the endocrine regulator NEUROG3 (also known as NGN3) and pancreatic beta-cell regulators PDX1 and MAFA could induce insulin-secreting cells from murine intestine and stomach\textsuperscript{14}. Here, we provided a detailed step-by-step protocol to induce cultured hGSCs derived from human donors to differentiate into islet-like organoids at high efficiency, containing approximately 70% beta-like cells and other islet-like endocrine populations. The protocol contains 4 sections including (1) isolation of primary hGSCs from human stomach sample, (2) expansion and cryopreservation of hGSCs, (3) generation of Ngn3ER-hGSC line, and (4) generation of GINS organoids. GINS organoids generated from this protocol exhibited glucose responsiveness 10 days after induction.

Reagents

General reagents

Fibronectin (Sigma-Aldrich, F4759)

Matrigel (VWR, 47743-722)
DMSO (Sigma-Aldrich, 472301)

DPBS without calcium and magnesium (Thermo Fisher Scientific, 14190144)

BSA, fatty acid free (VWR, 10842-692)

4-Hydroxytamoxifen (4-OH-TAM, Sigma-Aldrich, H7904)

Polybrene (Millipore Sigma, TR-1003-G)

TrypLE (Thermo Fisher Scientific, 12604021)

Trypsin-EDTA (0.25%), phenol red (Thermo Fisher Scientific, 25200072)

Rabbit anti-MAFA (Bethyl; A700-067; 1:1000)

Opti-MEM I Reduced Serum Medium (Thermo Fisher Scientific, 31985062)

Sodium Pyruvate, 100 mM (Thermo Fisher Scientific, 11360070)

Lipofectamine 3000 Transfection Reagent (Thermo Fisher Scientific, L3000015)

Puromycin dihydrochloride (Sigma-Aldrich, P8833)

Lentivirus

Note: lentiviral plasmids are available from Addgene under a uniform biological material transfer agreement. Virus can be packaged as previously described\textsuperscript{15,16} or following the protocol on the website (https://www.thermofisher.com/us/en/home/life-science/cell-culture/cell-culture-learning-center/cell-culture-resource-library/cell-culture-transfection-application-notes/improve-lentiviral-production-using-lipofectamine-3000-reagent.html).

Lenti-EF1\textsubscript{α}-Ngn3ER-2A-PuroR-2A-mCherry (EF1\textsubscript{α}, EF-1\textsubscript{α} promoter; 2A, 2A peptide for polycistronic gene expression; \textit{PuroR}, puromycin resistant gene)

Lenti-CMV-Pdx1-2A-Mafa (CMV, CMV promoter; 2A, 2A peptide for polycistronic gene expression)

Cell lines

DR4 MEF, irradiated (Thermo Fisher Scientific, A34966)

293FT (Thermo Fisher Scientific, R70007)
**DMEM complete medium**

DMEM, high Glucose, GlutaMAX, pyruvate (Thermo Fisher Scientific, 11-965-118)

10% FBS (R&D systems, S11150)

100 U/mL Penicillin-Streptomycin (Thermo Fisher Scientific, 15-140-122)

**hGSC isolation medium**

F12K (Thermo Fisher Scientific, 21127030)

2 mg/mL Collagenase IV (Worthington Biochemical, LS004188)

(optional) 2 U/mL DNase I (Worthington Biochemical, LS002007)

**hGSC medium**

66.7% DMEM, high glucose (Thermo Fisher Scientific, 11-965-118)

33.3% F12K (Thermo Fisher Scientific, 21127030)

18% FBS (R&D systems, S11150)

10% R-Spondin-2 conditioned medium (RS2 cell line is a gift from Dr. Xi He from Children's Hospital Boston; may be substituted by Recombinant Human R-Spondin 2 (PeproTech, 120-43))

10 mM Nicotinamide (Sigma-Aldrich, N5535)

25 μM Primocin (Invivogen, ant-pm-1)

1 μM A8301 (Cayman, 9001799)

5 μg/mL Insulin (Sigma-Aldrich, I0516-5ML)

10 μM Y-27632 (LC Laboratories, Y-5301)

1 μM DMH1 (Cayman, 16679)

50 ng/mL EGF (R&D Systems, 236-EG-01M)

2 μM T3 (Sigma-Aldrich, T6397)
**GINS medium**

Advanced DMEM/F12 (Thermo Fisher Scientific, 12634010)

10 mM HEPES (Thermo Fisher Scientific, 15-630-080)

1X GlutaMAX (Thermo Fisher Scientific, 35050061)

1X B-27 (Thermo Fisher Scientific, 17504044)

1X N-2 (Thermo Fisher Scientific, 17502048)

500 μM N-Acetyl-L-Cysteine (NAC) (Sigma-Aldrich, A9165)

25 μM Primocin (Invivogen, ant-pm-1)

10 mM Nicotinamide (Sigma-Aldrich, N5535)

1 μM A8301 (Cayman, 9001799)

10 μM Y-27632 (LC Laboratories, Y-5301)

**Equipment**

Tissue culture humidified CO2 incubator

Biosafety cabinet

Water bath

P10 micropipette and sterile tips

P20 micropipette and sterile tips

P200 micropipette and sterile tips

P1000 micropipette and sterile tips

Pipet-Aid

5 mL sterile serological pipets (VWR, 89130-896)

10 mL sterile serological pipets (VWR, 89130-898)
25 mL sterile serological pipets (VWR, 89130-900)
15 mL centrifuge tube (VWR, 10026-076)
50 mL centrifuge tube (VWR, 10026-078)
Plate and tube centrifuge (Eppendorf, 5810R)
Aggrewell™400, 24 wells, Starter Kit (STEMCELL Technologies, 34450)
TC treated 48-well cell culture plate (VWR, 10062-898)
TC treated 6-well cell culture plate (VWR, 10062-892)
TC treated Dishes 100 × 20 mm (USA Scientific, CC7682-3394)

Procedure

Isolation of primary hGSCs from human stomach sample

1. Prepare 10 mL of hGSC isolation medium per stomach tissue sample (~2 cm³).
2. Warm the isolation medium at 37°C in water bath.
3. (CRITICAL) Cut tissue into tiny pieces that can go through 10-mL pipet.
4. Coat inner surface of a 10-mL pipet and a P1000 tip with BSA (1% in PBS).
5. Wash tissue with 10 mL of cold DPBS with pipetting using the coated pipet.
6. Let the tissue sink to the bottom.
7. Discard supernatant.
8. Repeat step 5-7 until supernatant looks clean.
9. Spin down at 100 g for 3 min.
10. Discard supernatant.
11. Resuspend pellet in the warmed isolation medium using the coated pipet.

12. (CRITICAL) Incubate tissue at 37°C in the water bath for 15-30 min with pipetting rigorously using the coated 10 mL pipet (when the size of tissue allows, use the coated P1000 tip instead) until clusters of crypt cells released. NOTE: duration of this step should be adjusted case by case.

13. (Optional) Let stand for 1 min and collect the tissue that sink to the bottom and repeat step 11-12 in a separate tube.

14. Neutralize cells with F12K supplemented with 10% FBS.

15. Spin down at 500 g for 5 min.


17. Resuspend pellet in hGSC medium and seed cells on 1 well of 6-well plate that coated with confluent inactivated MEF feeder cells.

18. Maintain cells in hGSC medium at 37 °C in a 5-7.5% CO₂ humidified incubator.

19. Change medium every other day.

**Expansion and cryopreservation of hGSCs**

**Note:** hGSC colonies should be passaged when the colonies begin to make contact to each other (~70% confluency).

1. Wash cells twice with DPBS.

2. Incubate cells in TrypLE for 10-12 min.

3. Detach cells by pipetting.

4. Transfer cells into a centrifuge tube that contains DMEM complete medium.

5. Centrifuge cells at 300 x g for 5 min.

6. Resuspend pellet in hGSC medium and then seed cells on an inactivated-MEF-coated dish. **Note:** Split hGSCs every 4-6 days at a ratio between 1:3 and 1:5.

7. Maintain hGSCs in hGSC medium at 37 °C in a 5-7.5% CO₂ humidified incubator.

8. Change medium every other day.
9. For hGSC cryopreservation, pellet from step 6 should be resuspended in freezing solution (10% DMSO in FBS) and frozen using standard mammalian cell cryopreservation protocol.

**Generation of Ngn3ER-hGSC line**

**Note:** Ngn3ER-hGSCs were labeled with mCherry constitutively by incorporation of a polycistronic cassette EF1a-\textit{Ngn3ER-PuroR-mCherry} (\textit{PuroR}, puromycin resistant gene).

1. Seed $10^5$ hGSCs in 1 well of 6-well plate coated with confluent inactivated MEF 24 hours prior to lentiviral transduction.

2. Wash cells with DPBS once.

3. Overlay cells with 2 mL of hGSC medium containing 10 $\mu$g/mL polybrene and $\sim 10^6$ TU of Lenti-EF1a-\textit{Ngn3ER-PuroR-mCherry}. **Note:** Lentivirus can be prepared in bulk and titrated in 293FT by mCherry$^+$ cells quantification.

4. Spin the cell culture with lentivirus in plate at 1000 g for 30 min at 37°C.

5. Culture the infected hGSCs at 37°C in a 5-7.5% CO$_2$ incubator for 48 hours.

6. Change culture medium to hGSC medium containing 1 $\mu$g/mL puromycin bidaily for 2 weeks.

7. The line can be expanded or cryopreserved.

**Generation of GINS organoids**

1. \textit{Ngn3ER} activation (Differentiation to endocrine progenitors, day 0-2)

   (1) Seed \textit{Ngn3ER}-hGSCs 4-5 days prior to differentiation.

   (2) Wash cells once with DPBS.

   (3) Overlay cells with hGSC medium containing 1 $\mu$M 4-OH-TAM.

2. Pdx1-Mafa transduction (Differentiation to GINS precursors, day 2-6)

   **Note:** coat the inner surface of pipet tips and centrifuge tubes with 1% BSA before experiment.

   (1) Coat dishes in DMEM containing Fibronectin (1:50) and Matrigel (1:50) for 2 hours or overnight.

   (2) Gently wash cells with DPBS twice.
(3) Incubate cells in DPBS for 10 min.*

(4) Detach cells by pipetting vigorously using BSA-coated pipet tips.*

(5) Transfer the cells in a BSA-coated centrifuge tube.*

(6) Centrifuge at 100 g for 3 min.*

(7) Remove supernatant carefully. *Note: cell debris should be removed if they retain in supernatant.

(8) Dissociate cells in TrypLE at 37°C for 10-15 min with pipetting every 3-5 min.

(9) **(CRITICAL)** Neutralize cells in complete DMEM when most of the cells are single cells, doublets or small clusters that contain 3-5 cells.

(10) Spin cells at 100 g for 5 min.

(11) Resuspend cells in medium composed of 50% of hGSC medium, 50% of GINS medium, and 10 μg/mL polybrene.

(12) Infect cells with Lenti-CMV-Pdx1-2A-Mafa at a multiplicity of infection (MOI) of 10. **Note:** Lentivirus should be prepared in bulk and titrated in 293FT by immunostaining for Mafa and quantification.

(13) Spin the cell culture with lentivirus in plate at 1000 x g for 30 min at 37°C.

(14) Transfer infected cells to Fibronectin/Matrigel coated dishes (~10^7 cells per 10-cm dish).

(15) Two days post infection, change medium to 75% GINS medium and 25% hGSC medium.

*Note: for extremely large-scale production, consider skipping step 3-7, which was designed to remove sticky cell debris and undifferentiated hGSCs.

3. **GINS organoid formation (day 6-21)**

(1) Dissociate cells in TrypLE for 5 min.

(2) Neutralize cells in DMEM complete medium.

(3) Spin cells at 300 g for 5 min.

(4) Resuspend pellet in GINS medium and count cells.

(5) Aggregate cells in AggreWell400 (typically 2.0-2.4 million cells/well) using the manufacturer's recommended protocol. **Note:** Aggregates normally form within 24 hours.

(6) Change medium every 2-3 days.
(7) Maintain organoids in GINS medium at 37 °C in a 5-7.5% CO₂ humidified incubator.

**Troubleshooting**

**Isolation of primary hGSCs from human stomach sample**

**Step:** 3.

**Problem:** Tissue cannot go through 10-mL pipet.

**Possible reason:** Tissue is not cut thoroughly.

**Solution:** Put the tissue at the bottom of a sterile Eppendorf tube and use fine scissors to cut vigorously.

**Step:** 5.

**Problem:** Tissue stuck in pipets or tips.

**Possible reason:** The pipets/tips are not coated with BSA.

**Solution:** Make sure the pipets/tips are coated by pipetting the 1% BSA solution a few times.

**Step:** 12.

**Problem:** No cell clusters release.

**Possible reason:** Did not use the right collagenase or did not pipet vigorously.

**Solution:** Make sure you use the right type of collagenase at the recommended concentration. After a few times of pipetting using 10-mL pipet, use BSA-coated P1000 tip to pipet.

**Expansion and cryopreservation of hGSCs**

**Step:** 3.

**Problem:** Cells cannot detach.
**Possible reason:** (1) Cells have been neutralized before pipetting. (2) Colonies are too big and hard to detach.

**Solution:** (1) Detach the cells in TrypLE before neutralization. (2) Incubate cells in TrypLE for additional 2-3 min.

**Problem:** Colonies do not form in 3-4 days after passage.

**Possible reason:** Over-digestion.

**Solution:** Cells should remain in small clusters during the procedure. Single cells grow slowly.

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**Generation of Ngn3ER-hGSC line**

**Step:** 7.

**Problem:** No cells survive after selection.

**Possible reason:** The infection fails

**Solution:** Make sure the virus prep works.

**Problem:** Few cells are mCherry* after selection.

**Possible reason:** Selection fails

**Solution:** Make sure the antibiotics can kill negative cells in 3-5 days. The concentration of the antibiotics may need optimization.

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**Generation of GINS organoids**

1. Ngn3ER activation (Differentiation to endocrine progenitors, day 0-2)

**Problem:** Morphology does not change on day 2
**Possible reason**: The morphological change on day 2 is subtle.

**Solution**: The morphology changes is cell line dependent. Some lines show more dramatic changes than the others. Clearer boundaries between cells in colonies should be observed.

2. Pdx1-Mafa transduction (Differentiation to GINS precursors, day 2-6)

**Step**: 4

**Problem**: Cells do not detach.

**Possible reason**: Cells are not differentiating.

**Solution**: Make sure the selection of *Ngn3ER*-hGSC works and the cells can differentiate. Without Pdx1-Mafa transduction, cells should differentiate into gastric endocrine cells.

3. GINS organoid formation (day 6-21)

**Problem**: Endocrine progenitors do not differentiate into GINS cells

**Possible reason**: Lentivirus transduction fails

**Solution**: Make sure the virus prep works; MOI may need optimization; dissociate the endocrine progenitors into single cells or doublets to increase infectivity; polybrene should be added during infection; use immunostaining to confirm the expression of Pdx1/Mafa.

**Time Taken**

**Isolation of primary hGSCs from human stomach sample**

2-3 hours

**Expansion and cryopreservation of hGSCs**

1 hour based on one 10-cm dish scale.

**Generation of *Ngn3ER*-hGSC line**

1.5 hours excluding antibiotics selection
**Generation of GINS organoids**

1. Ngn3ER activation (Differentiation to endocrine progenitors, day 0-2)
   5 min

2. Pdx1-Mafa transduction (Differentiation to GINS precursors, day 2-6)
   2 hours

3. GINS organoid formation (day 6-21)
   1 hour

**Anticipated Results**

**Isolation of primary hGSCs from human stomach sample**

30-40 primary hGSC colonies would be visible in 1-2 weeks.

**Expansion and cryopreservation of hGSCs**

Cell doubling time is approximately 48 hours.

Mucus may be secreted by spontaneously differentiated cells.

**Generation of Ngn3ER-hGSC line**

Approximately 99% of the cells should be mCherry$^+$ after the procedure.

**Generation of GINS organoids**

1. Ngn3ER activation (Differentiation to endocrine progenitors, day 0-2):

Colonies begin to fall apart. Cell morphology changes. Cells stop growing. Some cells die.
2. Pdx1-Mafa transduction (Differentiation to GINS precursors, day 2-6):

Cells spontaneously cluster on day 5-6. Some cells die.

3. GINS organoid formation (day 6-21):

Cells aggregates into organoid within 24 hours. Organoids secret insulin in response to glucose as early as 4 days post aggregation.

References


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