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Long-term renewable human intestinal epithelial stem cells as monolayers: A potential for clinical use

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ABSTRACT

Purpose: Current culture schema for human intestinal stem cells (hISCs) frequently rely on a 3D culture system using Matrigel™, a laminin-rich matrix derived from murine sarcoma that is not suitable for clinical use. We have developed a novel 2D culture system for the *in vitro* expansion of hISCs as an intestinal epithelial monolayer without the use of Matrigel.
Methods: Cadaveric duodenal samples were processed to isolate intestinal crypts from the mucosa. Crypts were cultured on a thin coat of type I collagen or laminin. Intestinal epithelial monolayers were supported with growth factors to promote self-renewal or differentiation of the hISCs. Proliferating monolayers were sub-cultured every 4–5 days.
Results: Intestinal epithelial monolayers were capable of long-term cell renewal. Less differentiated monolayers expressed high levels of gene marker LGR5, while more differentiated monolayers had higher expressions of CDX2, MUC2, LYZ, DEF5, and CHGA. Furthermore, monolayers were capable of passaging into a 3D culture system to generate spheroids and enteroids.
Conclusion: This 2D system is an important step to expand hISCs for further experimental studies and for clinical cell transplantation.
Level of Evidence: 1 Experimental

In vitro intestinal epithelial cell culture can be used to increase our understanding of the basic biology of the intestinal epithelium and is an important tool for researching various intestinal disorders. In addition, this methodology holds the promise of expanding treatment options for various intestinal epithelial disorders [1,2]. Intestinal epithelial stem cells (ISCs) are crypt-based cells that continuously undergo self-renewal and differentiation to populate the various epithelial lineages of the gut [3]. Specifically, goblet cells, enteroendocrine cells, Paneth cells, enterocytes, tuft and M cells are six differentiated epithelial lineages that arise from ISCs [3]. Recent years have seen significant advancement in murine and human intestinal epithelial cell culture [2,4–18].

Spheroids and enteroids generated from dissociated crypts and ISCs are three-dimensional (3D) cell clusters [2,5,15,17]. Successful culture of dissociated crypts/ISCs requires several components, including appropriate matrices and Wnt agonists [11,12,19–28]. Most commonly, Matrigel™ is used as a 3D support matrix for *in vitro* ISCs cultures [2,15,18]. However, as this field advances toward clinical application, we must move beyond utilizing Matrigel as a matrix for *in vitro* cell expansion. Matrigel is a gelatinous protein mixture derived from Engelbreth-Holm-Swarm (EHS) mouse sarcoma [2,15] and is not FDA approved for clinical use. The protein mixture contains laminin-111, type IV collagen, perlecan, nidogen and small amounts of growth factors [29]. We sought to develop a system utilizing FDA approved matrices to rapidly generate and expand human intestinal stem cells (hISCs). Herein we describe a two dimensional (2D) *in vitro* culture system using thin coatings of type I collagen and recombinant human laminin to generate proliferating monolayers of hISCs. Furthermore, we demonstrate that

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such monolayers of cells can be induced to differentiate into mature intestinal epithelium.

1. Methods

1.1. Intestinal crypt isolation

Human cadaveric intestinal samples were obtained from Texas Organ Sharing Alliance (San Antonio, Texas) and were received en-bloc with the pancreas and spleen. The duodenum was carefully dissected from the head of the pancreas and then opened and washed with 1% chlorhexidine. A 4-cm segment of duodenum was used to isolate intestinal crypts as previously described [5,15–17]. In brief, the seromuscular layers were dissected and discarded, and the remaining mucosa was washed with ice-cold 10% phosphate buffered saline (PBS) (Fisher Scientific, Pittsburgh, PA). Diced mucosal segments were then treated incubated with 4 mM ethylenediaminetetraacetic acid (EDTA, Sigma, St. Louis, MO) and 1 mM dithiothreitol (DTT, Sigma, St. Louis, MO) at 4 °C for 30 min with gentle shaking to facilitate crypts dissociation. The remaining washing and centrifugation steps were identical to the previously published protocols [5,15–17]. Supernatant was discarded and the pellet containing isolated crypts was re-suspended in basic medium [Advanced Dulbecco's Modified Eagle Medium (ADMEM)/Ham's F12 (Invitrogen) with 2 mM GlutaMAX (Invitrogen), 10 mM HEPES (Invitrogen), and 1× Antibiotic-Antimycotic (Invitrogen)]. Crypt yield was determined by counting the number of crypts in 10 μl using an inverted light microscope.

1.2. Generating and passaging monolayers of epithelial cells

The culture methods are summarized in Fig. 1. Thin coats of bovine type I collagen (Purecol, Advance BioMatrix, Inc. San Diego, Ca) and recombinant human laminin isotypes 111, 211, 332, and 511 (Biolamina, Sundbyberg, Sweden) were used as support matrix for monolayer growth. Coats were prepared on 48-well Nunclon Delta-treated cell culture plates (Thermo Scientific, Waltham, MA). A 120 μl coating solution of type I collagen at a concentration of 100 μg/ml was pipetted into each well and incubated at 37 °C for one hour. After incubation, the coating solution was aspirated and crypts suspended in culture medium were added to each well. Laminin isotype coating solutions (111, 211, 332, and 511) were prepared as previously described [29]. After two hours incubation, the laminin coating solution was aspirated and crypts suspended in culture medium were added to each well.

Approximately 200–250 crypts per well were seeded to generate monolayers. In order to promote growth of undifferentiated monolayers, cultures were supported with 50% complete medium [basic medium, 1 mM N-acetylcysteine (Sigma), 1× N2 supplement (Invitrogen), 1× B27 supplement (Invitrogen)], 100 ng/ml recombinant murine Noggin (PeproTech), 50 ng/ml recombinant murine EGF (PeproTech), 1 μg/mL recombinant human (rh) R-spondin 1 (R&D Systems, Minneapolis, MN), 10 μM ROCK-inhibitor (Sigma)] and 50% intestinal subepithelial myofibroblast (ISEMF) conditioned medium (CM), which we denote as ENRY-ISEMF-CM. ISEMF-CM was prepared using pediatric human ISEMFs. ISEMFs were cultured for 5–7 days in Dulbecco's Modified Eagle Medium (DMEM)/Low Glucose/GlutaMAX (Invitrogen), 10% FBS (Invitrogen), 1 × Antibiotic-Antimycotic (Invitrogen), 0.25 U/mL insulin (Sigma), 20 ng/mL recombinant murine EGF (PeproTech, Rocky Hill, NJ), and 10 μg/mL transferrin (Sigma).

After 3 to 5 days of growth, monolayers reached 90–100% confluency and were sub-cultured as follows. TrypLE (Life Technologies) was pre-warmed at 37 °C and 150 μl of TrypLE containing 10 μM ROCK-inhibitor was added to each well containing a confluent monolayer. Monolayers were incubated for 5 to 7 min at 37 °C to facilitate detachment of monolayers into single cells. TrypLE was then quenched using 10% FBS in ADMEM/F12. Cells were split 1:2 or 1:3 and re-seeded on a new thin coat of type I collagen, laminin-111, laminin-211, laminin-332, or laminin-511. Doubling-time was measured at time of sub-culture by using a hemocytometer.

1.3. Generating and passaging more differentiated monolayers

Thin coats of type I collagen and laminin isotypes (111, 211, 332, and 511) were prepared and crypts were seeded to generate monolayers as described above. In order to promote growth of differentiated monolayers, cultures were supported with 50% complete medium, 5 μM GSK-inhibitor (Stemgent, Cambridge, MA), 10 μM SB 431542 (Tocris Bioscience, R&D Systems), and 50% L-Wnt3A conditioned medium (CM), which we denote as ENRYG-50Wnt3A. The 50% L-Wnt3A

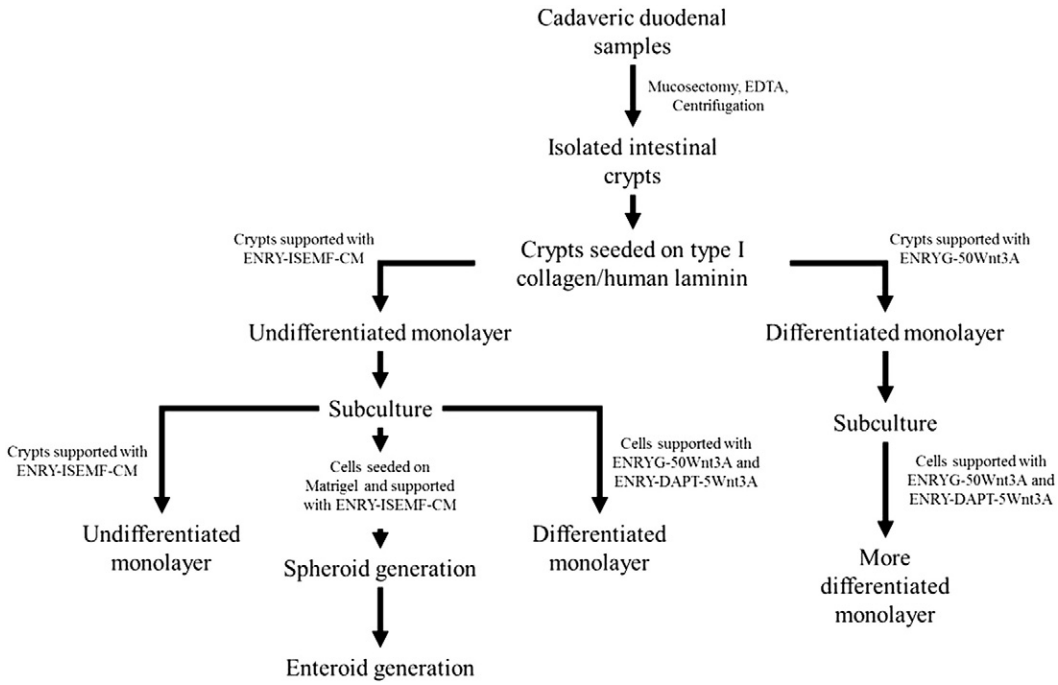

Fig. 1. Flow chart describing culturing methodology.