

Induction of different human enteroendocrine cells in intestinal organoids

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Method Article

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Abstract

Intestinal enteroendocrine cells (EECs) are key players in mammalian physiology as largest endocrine organ, and regulate metabolic processes such as insulin secretion and induction of satiety. EECs are rare cell types (less than 1% of the epithelium) and produce a large number of different hormones making these challenging to study. Model systems to assess the differentiation and function of these essential cell types have been lacking. In a novel approach, we describe the induction of human EECs in intestinal organoids. Moreover, in the manuscript linked to this protocol, we describe that manipulating the BMP signaling pathway allows to induce expression of specific sets of hormones that correspond to the EEC state in the crypt-villus axis. This differentiation platform can be used to study the function of EECs and could contribute to the development of drugs for diabetes and obesity.

Introduction

The protocol described here utilizes the previously developed human intestinal organoid system as a source of intestinal stem cells (1). Mouse EECs were successfully enriched in intestinal organoids before, containing up to 70% of the total organoid cells (2). In the protocol described here, we optimize and adapt the EEC differentiation protocol for human intestinal organoids to produce EECs in vitro. This protocol to produce human EECs is less efficient (max 10-20% EECs per organoid) than we have described for mouse, but is a first successful enrichment of human EECs other than neuroendocrine tumor cell lines. EECs survive as mature cells within these organoids for at least 3 days, a window long enough to assess their response to stimuli such as nutrients.

Reagents

• Collagenase type II (Gibco, cat. no. 17101015015) • Hyaluronidase from bovine testes (Sigma, cat. no. H4272) • Y-27632 dihydrochloride ROCK inhibitor (Abmole, cat. no. M1817) • DNase I (Roche, cat. no. 4536282001) • Advantec DMEM/F12 (Gibco, cat. no. 12634028) • HEPES (Life technologies, cat. no. 15630056) • Glutamax (Life technologies, cat. no. 35050038) • Penicillin/Streptomycin (Gibco, cat. no. 15070063) • Fetal bovine serum (FBS) (Sigma, cat. no. F7524) • B-27 Supplement (Gibco, cat. no. 17504001) • Basement membrane extract (BME) type 2 (Trevigen, cat. no. 3533-010-02) • Epidermal growth factor (Invitrogen, cat. no. E4127) • N-acetyl L-cysteine (Sigma, cat. no. A9165) • Nicotinamide (Sigma, cat. no. N-0636) • SB 202190 (Tocris, cat. no. 1264_ • A83-01 (R&D Systems, cat. no. 2939) • Prostaglandin E2 (R&D Systems, cat. no. 2296) • DAPT (Sigma, cat. no. D5942) • PD 0325901 (Sigma, cat. no. PZ0162) • Human BMP-4 (Peprotech, cat. no. 120-05ET)

Equipment

• Scalpel • Petri dish • 70 or 100 um strainer • 15 ml centrifuge tubes • 50 ml centrifuge tubes • P20 micropipette • P1000 micropipette • Centrifuge, cooled to 4C • Laminar flow hood • Water bath 37 deg •

Cell culture incubator (37C, 5% CO₂) • Greiner cell culture plate, suspension, 6-well, 12-well or 24-well • Glass pasteur pipette • Shaker at 37C

Procedure

Establishment of organoid culture For large chunks of intestinal tissue, epithelial stem cells can be isolated as described previously and is based on mechanical dissection and chelation (1). Alternatively, for smaller pieces of the mucosa (<1cm), enzymatic dissociation can be performed using the following procedure: 1. Cut the tissue into small pieces using two scalpels. 2. Transfer the tissue fragments into a 15 ml falcon into 2 ml of Advanced DMEM/F12, supplemented with Y-27632 (10 μM), Collagenase type 2 (1mg /ml) and Hyaluronidase (10 μg/ml). Incubate for 30 minutes at 37C in a shaker. 3. Check the dissociated tissue for crypt isolation under a microscope. If no or few crypts are observed, extend the dissociation for another 30 minutes. 4. Add 8 ml of ice-cold Advanced DMEM/F12 and spin the fragments for 5 min at 4C. Aspirate the supernatant. 5. Resuspend the pellet in 10 ml of ice-cold Advanced DMEM/F12. If large fragments remain, filter the solution through a 70 or 100 μm strainer in a fresh 15 ml falcon. 6. At this stage, crypts can be quantified in 50 μl of medium to estimate the total number of fragments. 7. Spin the fragments for 5 min at 4C and aspirate the supernatant. 8. Resuspend the pellet in a mixture of Basement membrane extract (BME) and Advanced DMEM/F12. Especially larger pellets are easier to dissolve in Advanced DMEM/F12 first due to the viscosity of BME. BME can be diluted to 60% using Advanced DMEM/F12. Per BME droplet of 15 μl, plate approximately 150-300 fragments/crypts into a suspension plate (Greiner Bio-one). 9. Allow the BME to polymerize for at least 20 minutes at 37C. Add warmed human intestinal organoid as described previously.

Expansion of human intestinal organoids Human intestinal organoids can be expanded and passaged as described previously (1). Medium is refreshed every third day and organoids can be passaged 1:4-1:8 per week, depending on the regional identity of the tissue (ileum tends to grow slower).

Induction of EEC and other secretory cell differentiation 1. Differentiation of human intestinal organoids can be initiated 2 to 5 days post passaging, when organoids are between 100-200 μm in diameter. Ideally, 150-300 organoids are plated in 15 μl BME, but differentiation was achieved successfully with lower and higher concentrations as well. 2. Remove expansion medium and wash each well with 1-2 ml of Advanced DMEM/F12, depending on the size of the well. Try to remove as much as the wash solution as possible. 3. Add warmed differentiation medium to each well. Differentiation medium is prepared by withdrawing Wnt, Prostaglandin E₂, Nicotinamide, SB 202190 and A83-01 from the expansion medium. DAPT (10 μM) and PD0325901 (500 nM for duodenal, 1 μM for ileal organoids) are added to the differentiation medium. For induction of villus enriched hormones, BMP-4 (20 ng/ml) is added to the differentiation medium and Noggin removed. 4. Refresh the medium at day 1 and day 3 after differentiation initiation. 5. EECs are observed after 3-4 days of differentiation and organoids can be maintained up to 6 days. Typically, organoids were used for EEC experiments after 5 days of differentiation, at which the balance between cell survival and differentiation was most optimal. After that, apoptosis of the majority of differentiated cells will occur.

Timing

Starting from a patient isolate, differentiated organoids can be acquired within 3 weeks.

Troubleshooting

The inhibition of MEK signaling is optimal within a relatively narrow range of concentrations and the MEK inhibitor could be titrated (for PD0325901 between 100 nM and 5 μ M) when any of the following problems occur. Extensive cell death may occur with too high levels of MEK inhibition. Typically, faster proliferating organoids (such as duodenum) are more sensitive for MEK inhibition and require lower concentrations. Inefficient differentiation towards secretory cells but more towards enterocytes occurs when MEK inhibition is too low. Although the majority of organoids survive the differentiation protocol, 10% Wnt conditioned medium can be added to the differentiation medium to prevent most apoptosis. Additional Wnt does dampen BMP-induced upregulation of villus-hormones, and might therefore not be desired in all circumstances.

Anticipated Results

In the first 3 days of EEC differentiation, organoids typically stop growing, shrink in size and the epithelium becomes thicker (Figure 1). At day 5, cells with large vesicles can be observed (Figure 2), and are indicative of successful generation of secretory cells such as EECs. Differentiation medium without the Notch and MEK inhibitor generates organoids fully consisting of enterocytes. These can be recognized by extensive folding and/or a thick epithelial layer (Figure 3). Too low concentrations of the MEK inhibitor tend to generate enterocyte-enriched organoids with the same morphology as well. A comparison of RNA sequencing results showing Glucagon (crypt enriched), Neurotensin (villus enriched) and Chromogranin A (equal along crypt-villus) expression in control and EEC differentiation organoids in the absence or presence of BMP4 (Figure 4). Around 2% of the total number of cells will be EEC after the differentiation, but can reach as high as 10-20% in individual organoids (Figure 5).

References

1. Sato, T. et al. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology* 141, 1762–1772 (2011).
2. Basak, O. et al. Induced Quiescence of Lgr5+ Stem Cells in Intestinal Organoids Enables Differentiation of Hormone-Producing Enteroendocrine Cells. *Cell Stem Cell* 20, 177–190.e4 (2017).

Figures

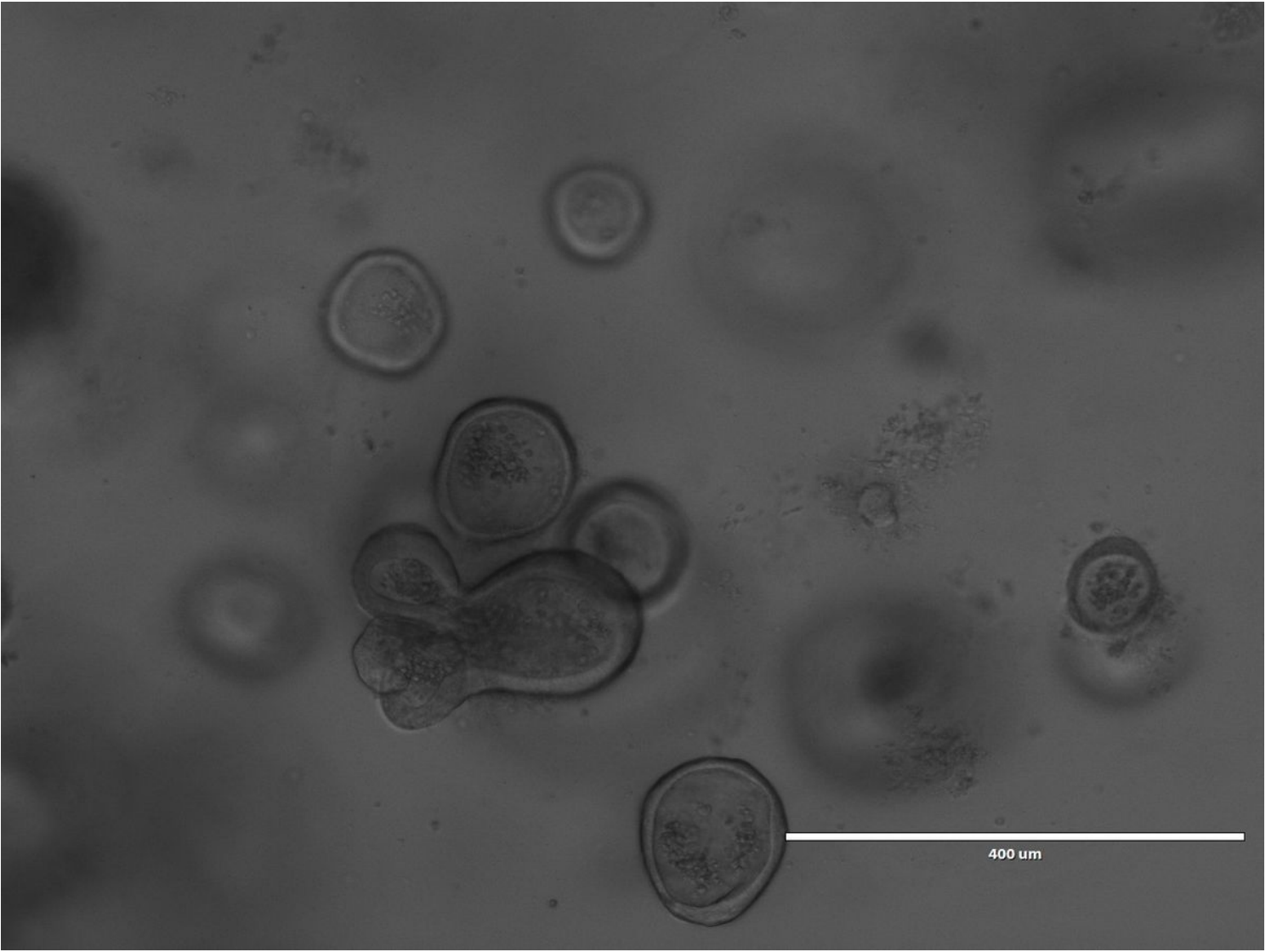


Figure 1

EEC differentiation day 3

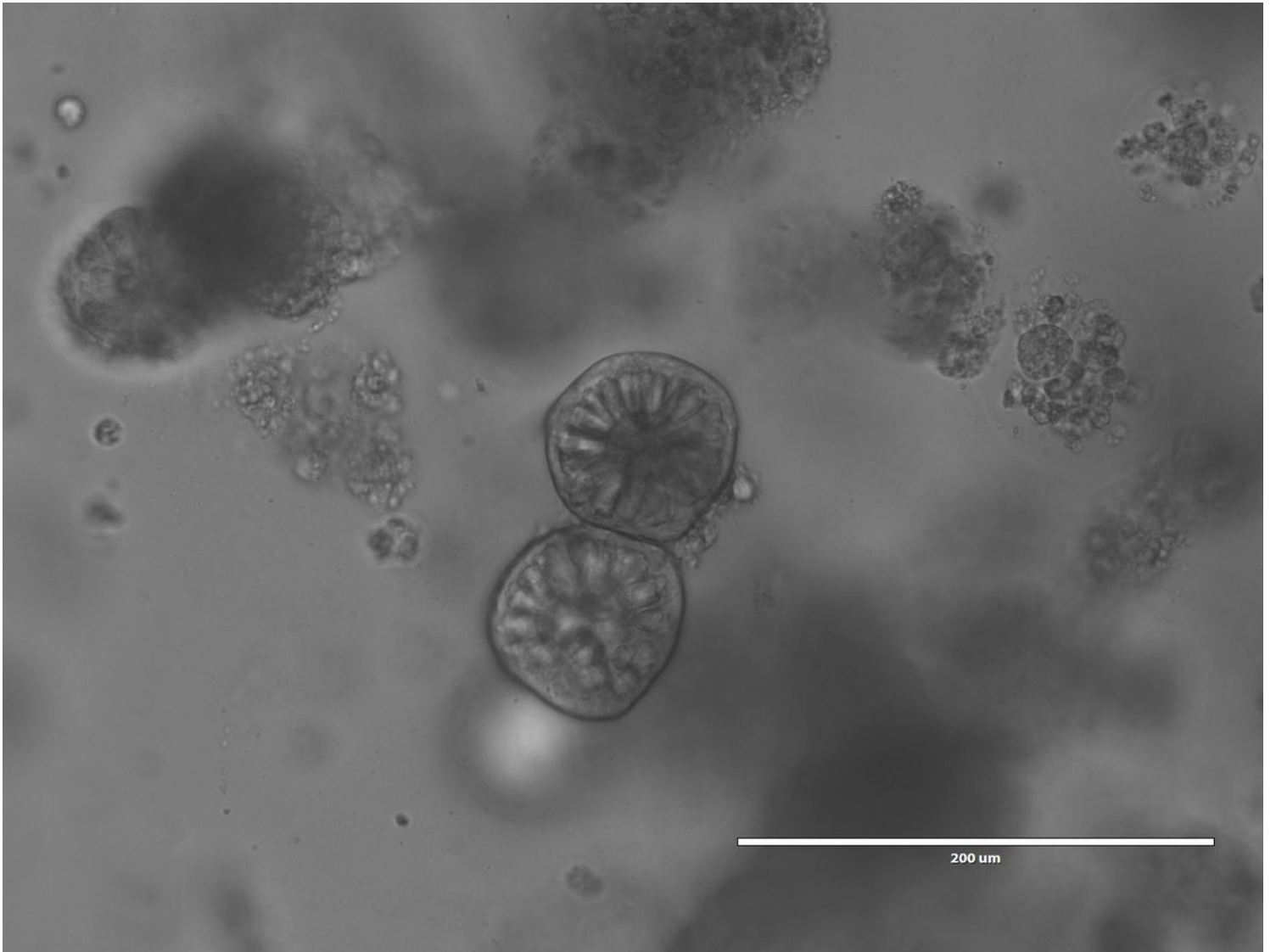


Figure 2

EEC differentiation day 5

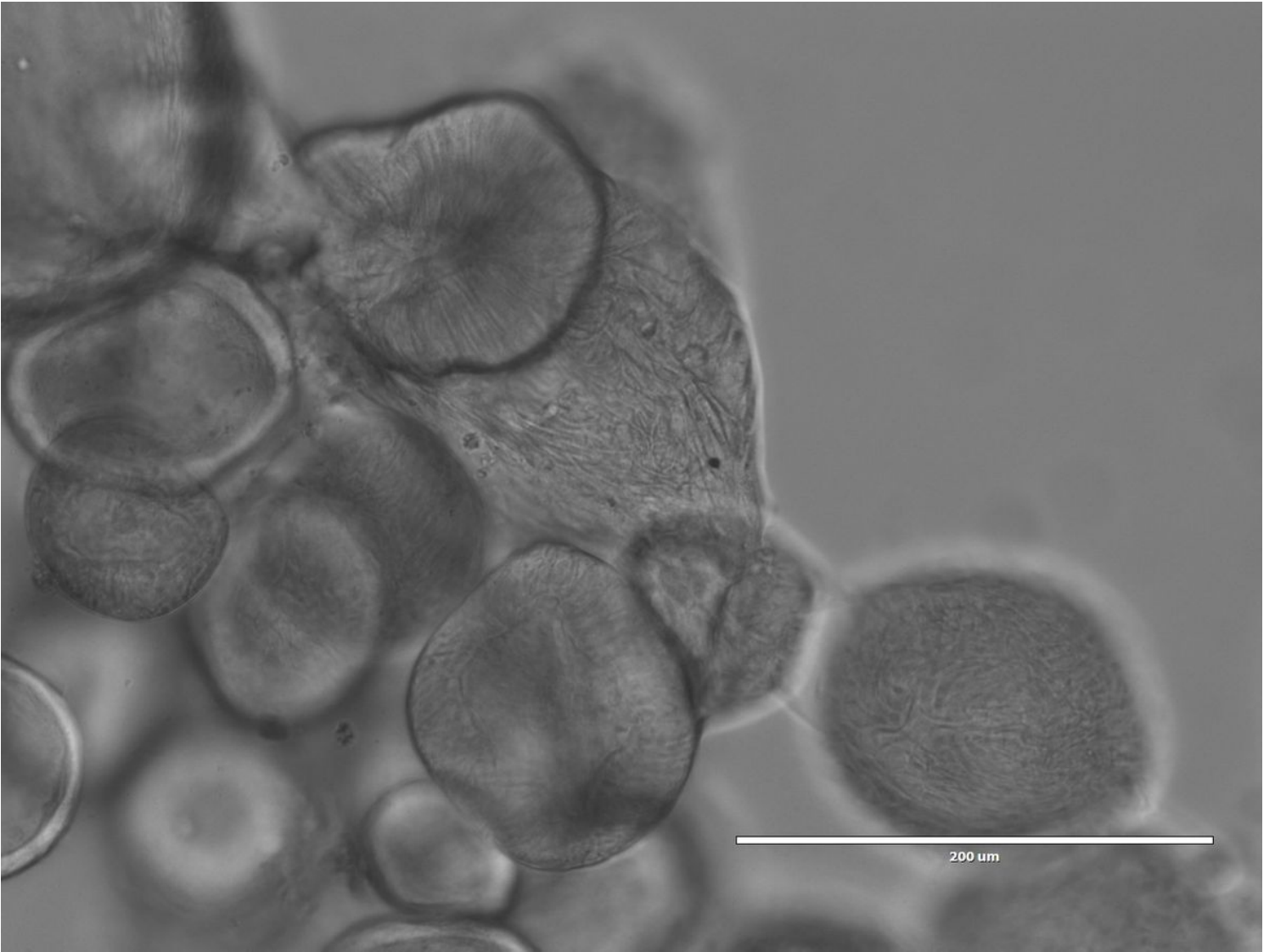


Figure 3

Enterocyte differentiation day 5

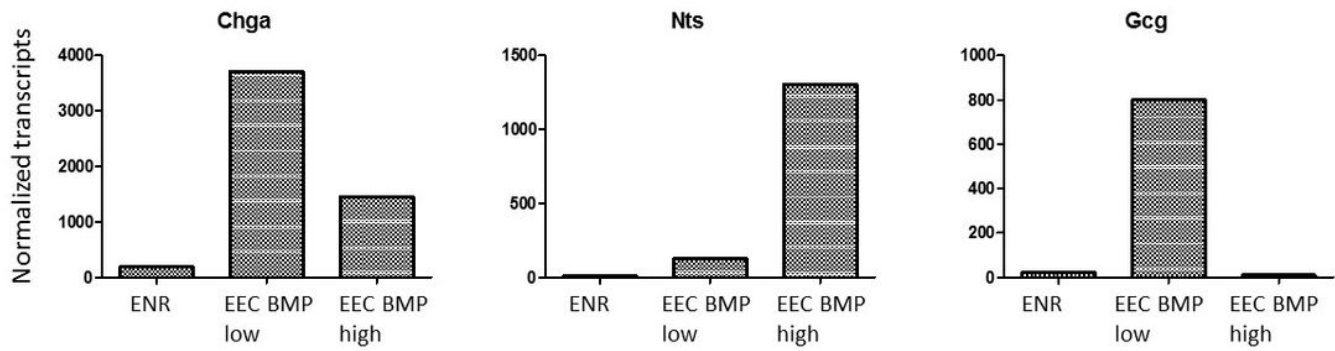


Figure 4

Hormones after differentiation protocol Organoids were differentiated either by withdrawing Wnt (ENR) or directed towards EECs using BMP low or high conditions. Normalized transcript numbers of hormones determined by RNA sequencing are shown.

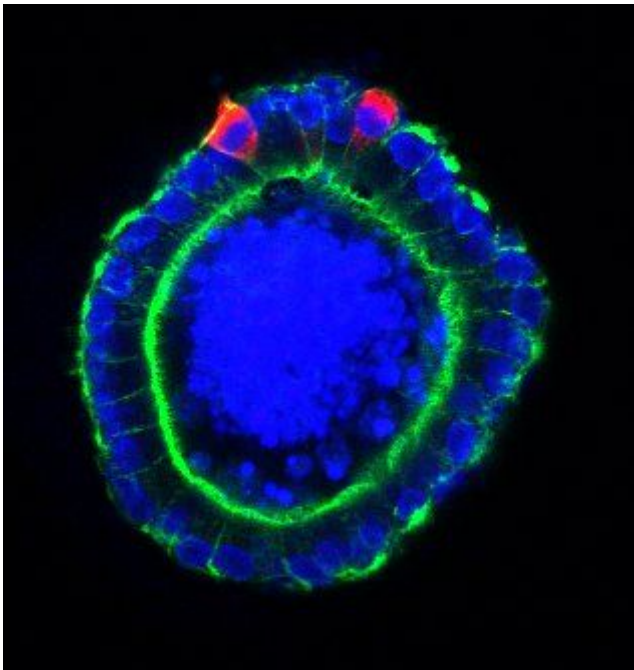


Figure 5

Enteroendocrine cells in organoids Staining for phalloidin (green) and Chromogranin A (red) of EEC differentiated organoid.