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A Novel Culture System for Adult Porcine Intestinal Crypts

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Abstract

Background—Porcine models are useful for investigating therapeutic approaches to short bowel syndrome and potentially to intestinal stem cell (ISC) transplantation. While techniques for the culture and genetic manipulation of ISCs from mice and humans are well established, similar methods for porcine stem cells have not been reported.

Methods—Jejunal crypts were isolated from murine, human, and juvenile and adult porcine small intestine, suspended in Matrigel, and co-cultured with syngeneic intestinal subepithelial myofibroblasts (ISEMFs) or cultured without feeder cells in various culture media. Media containing epidermal growth factor, noggin, and R-spondin 1 (ENR medium) were supplemented with various combinations of Wnt3a- or ISEMF-conditioned medium (CM), and glycogen synthase kinase 3 inhibitor (GSK3i) and studied on cultured crypts. Cell lineage differentiation was assessed by immunohistochemistry (IHC) and quantitative PCR (qPCR). Cultured porcine cells were serially passaged and transduced with a lentiviral vector.

Results—Whereas ENR medium supported murine enteroid growth; it did not sustain porcine crypts beyond 5 days. Supplementation of Wnt3a-CM and GSK3i resulted in the formation of complex porcine enteroids with budding extensions. These enteroids contained a mixture of stem and differentiated cells and were successfully passaged in the presence of GSK3i. Crypts grown in media supplemented with porcine ISEMF-CM formed spheroids that were less well differentiated than enteroids. Enteroids and spheroids were transfected with a lentivirus with high efficiency.

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Discussion—We describe a method to maintain juvenile and adult porcine crypt cells long-term in culture. Porcine enteroids and spheroids can be successfully passaged and transduced using lentiviral vectors.

Keywords

porcine intestinal culture; enteroid; intestinal spheroid; intestinal subepithelial myofibroblast; transduction of intestinal epithelium

INTRODUCTION

Recent discoveries of mechanisms that regulate intestinal epithelial stem cell (ISC) growth and differentiation have made possible long-term *in vitro* culture and expansion of mouse and human intestinal epithelium (Sato et al. 2009; Sato et al. 2011a; Yin et al. 2014). Essential signaling pathways for the maintenance and differentiation of cultured intestinal epithelial stem cells have been described, and the importance of the stem cell niche is well recognized (Sato et al. 2011b). In particular, intestinal subepithelial myofibroblasts (ISEMFs) have been shown to be critical players in the dynamic interplay between epithelium and mesenchyme (Lahar et al. 2011; Yeung et al. 2011; Lei et al. 2014). However, while our *in vitro* armamentarium for interrogating ISC physiology is highly developed, preclinical utilization of intestinal tissue culture techniques in large animals have encountered many challenges, and most studies published to date remain centered around rodent models (Yui et al. 2012; Watson et al. 2014).

In order to improve the translational use of ISCs, data are needed from *in vivo* experiments performed in large animal models, which better predict the clinical response in human patients (Harding et al. 2013). This requires in-depth characterization of large animal models, including stem cell physiology, cell lineage identification, and development of a robust culture system, which may differ from the well-studied rodent systems. Pigs have been widely used for preclinical studies focused on intestinal physiology (Zhang et al.), and intestinal pathology (Stoltz et al. 2013; Linard et al. 2013). Porcine treatment models for short bowel syndrome have been established and these models may also prove useful as large animal systems to investigate and develop approaches to intestinal tissue engineering (Agopian et al. 2009; Sala et al. 2009). A recent study in neonatal piglets characterized the intestine of newborn animals and demonstrated successful intestinal culture (Gonzalez et al. 2013). However, studies in our laboratory showed that the culture conditions described for neonatal piglet intestinal crypts are not suitable to maintain juvenile or adult porcine crypts. Here we report investigations that make it possible to culture adult porcine crypt cells long-term and to transduce these cells successfully with lentiviral vectors.

MATERIALS AND METHODS

Porcine Crypt Isolation and Culture

Two-cm segments of mid-jejunum were isolated from 10 to 14 week old Yorkshire pigs (n=19) and 4–8 week old mini-Yucatan pigs (n=7). Each segment was placed in ice-cold PBS. The muscle layers were peeled away with forceps and the segment was cut open to

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expose the lumen. The mucosal surface was scraped with a glass coverslip to remove the villi. The segment was cut into 5 × 5 mm pieces and repeatedly washed in PBS until the supernatant was clear. Epithelial isolation was performed by incubating the tissue in 10 mM ethylenediaminetetraacetic acid (EDTA, Sigma, St. Louis, MO) and 1 mM dithiothreitol (DTT, Sigma) in PBS for 30 minutes at 4°C on a 50 rpm rocker. The supernatant containing villi and debris was decanted and discarded, fresh PBS was added, the tube was vortexed thrice for 10 seconds and the supernatant containing crypts was collected. This was repeated until six fractions were collected. The fractions were centrifuged at 200g for 2 min at 4°C then resuspended in PBS with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA). The fractions were combined and filtered through a 100 μm strainer, centrifuged at 200g for 2 min at 4°C and suspended in basic medium consisting of 1X antibiotic-antimycotic solution (ABAM), 2 mM glutaMAX, and 10 mM HEPES buffer in 5 mL advanced DMEM/F-12 (all from Invitrogen).

For crypt culture, crypts were pelleted with three quick spins and resuspended in growth-factor reduced Matrigel (BD Biosciences, San Jose, CA) and plated in 0.95 cm^2 culture plate wells (Thermo Scientific, Waltham, MA). After a 15-minute incubation period at 37°C, we added 250 μL culture medium, which consisted of basic medium (see above) with the addition of 1mM N-acetylcysteine (Sigma), 1X N2 supplement (Invitrogen), 1X B27 supplement (Invitrogen), with or without 50 ng/mL recombinant murine epidermal growth factor (EGF, Peprotech, Rocky Hill, NJ), 100 ng/mL recombinant murine noggin (Peprotech), and 500 ng/mL recombinant human R-spondin 1 (R&D Systems, Minneapolis, MN). This mixture was termed “ENR medium.” In addition, we tested effects of supplementation with ISEMF- or Wnt3a-conditioned medium (CM), 100 ng/mL recombinant human FGF10 (Peprotech), 1 mM valproic acid (VPA, Sigma), 10 mM nicotinamide (Sigma), 2.5 μM GSK3i (CHIR99021, Stemgent, Cambridge, MA), 10 μM p160ROCK inhibitor (Y27632, Stemgent), 10 μM p38 MAP kinase inhibitor (SB202190, Sigma), and 500 nM TGFβ receptor inhibitor (LY2157299, Selleck, Houston, TX). Colony forming efficiency (CFE) was determined by the number of live structures in each well on culture day 7 versus day 1.

ISEMF Culture and CM Collection

Pericryptal intestinal subepithelial myofibroblasts (ISEMFs) were isolated from porcine jejunum (n=10) as previously described for other mammalian species (Lahar et al. 2011). ISEMFs were cultured in low glucose DMEM with glutaMAX (Invitrogen), with 10% FBS, and 1X penicillin/streptomycin with 20 ng/mL EGF, 0.25 U/mL insulin, and 10 mg/mL transferrin in 6-well plates (Corning, Tewksbury MA). Once confluent, they were detached with 0.25% trypsin and 1mM EDTA, transferred and cultured in T25 or T75 tissue culture flasks (Corning). For co-culture experiments, ISEMFs were split and plated 2 days prior to co-culture at a seeding density of 37,500 myofibroblasts per 0.95 cm^2 well. For CM collection, ISEMF culture medium was collected after a 7-day incubation on confluent ISEMFs and mixed 1:1 with ENR medium, nicotinamide, Y2732, SB202190, and LY2157299. This was termed “spheroid medium.”

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Subculture technique

To passage enteroids, Matrigel was digested with 0.6 mg/mL dispase (Invitrogen) in PBS at 37°C for 10 minutes with gentle inversion every 2 minutes. After 3 quick spins, the supernatant was removed and the pellet was resuspended in PBS, passed thrice through a 25-gauge needle (BD Biosciences) and spun down. The pellet was then resuspended in Matrigel with or without 1 μM PGE_2 (Sigma) or 1 μM Jagged-1 (Jag1; AnaSpec, Fremont, CA) and plated. Culture medium after passage consisted of ENR medium with addition of 25% Wnt3a-CM, nicotinamide, CHIR99021, Y27632, SB202190, and LY2157299 ("enteroid medium") and was used with or without addition of VPA.

Murine and Human Cultures

In reference studies, murine intestinal crypt three-dimensional culture was carried out as described previously using 8–12 week old male and female C57BL/6J mice (n=9) (Jabaji et al. 2013; Lei et al. 2014). Human crypts were isolated from adult duodenal samples (n=9) as previously described (Lahar et al. 2011). Mouse and human crypts were either co-cultured on confluent mouse and human ISEMFs, respectively, or cultured without feeder cells. Co-cultures were supplemented with ENR medium. Without feeder cells, crypts were grown either in ENR medium alone or with addition of Wnt3a-CM (1:1) or 1 μM PGE_2.

Culture Temperatures

Murine, human and porcine crypts were cultured in a 5% CO_2 incubator at 37°C. To mimic the physiologic temperature of weaned pigs (39.3±0.3°C, (Dewey and Straw 2006)), cultures were also carried out at 39°C. Enteroids were grown in standard enteroid medium and assessed on culture day 6 for morphology and gene expression. The ability to subculture enteroids was assessed at both temperatures.

Cryo-preservation of Isolated Crypts

To assess freezing as an option for long-term storage, freshly isolated porcine jejunal crypts were suspended in 1 mL freeze preservation medium consisting of 40% FBS and 10% DMSO in 1X culture medium with no growth factors and snap-frozen. The cryovials were stored for two weeks at −80°C. For culture, crypts were rapidly thawed at 37°C (n=5), mixed with 10 mL cold PBS in a 15 mL conical tube, and spun down at 100g and 4°C for 10 min. The pellet was resuspended in Matrigel and plated as described above.

Transduction of Porcine Cells

Spheroids and enteroids were transduced with a non-concentrated pRRL-CMV-GFP lentivirus based on a modification of a previously described technique (Koo et al. 2012). In brief, spheroids and enteroids were subcultured as above and suspended in 50 μL culture medium with 8 μg/mL hexadimethrine bromide (Sigma). The lentivirus suspension was added to the dissociated structures in a 1:3 ratio by volume and spun down at 600 *g* and 34°C for 1 hour. Following a four hour incubation at 37°C, the cells were resuspended in Matrigel and plated. Twenty-four hours later, structures were split into single cells with dispase as above followed by TrypLE then pipetted 20 times. Transduction efficiency was

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quantified with a flow cytometer (LSR II, BD Biosciences) and compared to untransduced controls.

Histology and Immunohistochemistry

Intestinal tissue was fixed overnight in 10% neutral buffered formalin and stored in 70% ethanol until further histologic processing. Intestinal cultures were fixed in 10% buffered formalin for 5 minutes then resuspended in 30 μL preheated Histogel (American MasterTech) and stored in ethanol as above. Specimens were embedded in paraffin and sectioned at 3 μm thickness. Hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) were performed per standard protocol (Lahar et al. 2011; Jabaji et al. 2013). Staining was done using antibodies against CD10 (56C6, Dako, Carpinteria, CA), lysozyme (ab74666, Abcam, Cambridge, MA), chromogranin A (20086, Immunostar, Hudson, WI) (Gonzalez et al. 2013), villin (1D2C3, Dako), E-cadherin (NCH-38, Dako), β-catenin (β-Catenin-1, Dako), p120-catenin (98, Ventana, Tucson, AZ), and cytokeratin 8 and 18 (EP17/EP30, Dako). Goblet cells were identified with the periodic acid-Schiff (PAS) stain (Brown et al. 1988). Porcine ISEMFs were plated in 0.95 cm^2 culture wells, fixed as above, and stained with antibodies against α-smooth muscle actin (α-SMA, M0851, Dako), desmin (M0760, Dako), and vimentin (ab92547, Abcam). For immunofluorescent stains, conjugated goat secondary antibody (Invitrogen) was added at 1:200 dilution and nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen).

Quantitative polymerase chain reaction (qPCR)

RNA was extracted with RNeasy mini-kit (Qiagen) and cDNA constructed with SuperScript III reverse transcriptase (Invitrogen). PCR was performed using SYBR green RT-PCR reagent kit (Invitrogen) and a real-time PCR thermal cycler (Applied Biosystems, Carlsbad, CA). PCR product quality was assessed with DNA electrophoresis. Primer sequences are shown in Table 1. Cycle numbers were normalized to beta-glucuronidase (Gusb) and to whole porcine jejunum.

Statistical Analysis

Statistical analysis was performed using the one-tailed Student's *t*-test assuming equal variance. $P<0.05$ was regarded as significant.

Ethics Statement

The animal research committee at UCLA approved use of murine and porcine tissue in this study (Protocol #2005-169 and 2013-007, respectively). The UCLA Institutional Review Board (IRB) approved procurement of de-identified human duodenal surgical specimens from the UCLA Translational Pathology Core Laboratory (Protocol #11-002778); the IRB waived the need for consent and approved the entire study.

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RESULTS

Comparison of Murine, Human and Porcine Intestinal Cultures

In co-culture with ISEMFs, growth of murine, human and juvenile or adult porcine crypts was supported by ENR medium; cultured crypts in all systems took on a combination of enteroidal, cystic, and flat morphologies (Fig. 1a–c). In the absence of ISEMFs, ENR medium was sufficient for budding enteroids to form from murine crypts (Sato et al. 2009), but not from human or juvenile and adult porcine crypts (Fig. 1d–f). The addition of Wnt3a-CM to ENR resulted in the formation of spheroids from murine but not human or porcine crypts (Fig. 1g–i). The addition of PGE_2 to ENR supported murine and human crypt cultures, producing a mixture of spheroids and small enteroids but was insufficient for the growth of porcine crypts (Fig. 1j–l). The addition to ENR medium of FGF10 or nicotinamide and the small molecule inhibitors of p160ROCK (Y27632), p38MAP kinase (SB202190), and TGFβ receptor (LY2157299; collectively “ENRnYSL medium”) resulted in colony-forming efficiency (CFE) <10% in the juvenile and adult porcine system (Fig. 1m). The addition of CHIR99021, a selective GSK3i to ENR medium, dramatically improved CFE to 62.5%±33.5% ($p=0.039$ vs. ENRnYSL medium). This improvement in CFE was more consistent when CHIR99021 was added to ENRnYSL medium with 1:1 addition of Wnt3a-CM (“enteroid medium”), resulting in 100% CFE. The addition of ISEMF-CM to ENRnYSL resulted in CFE of 65.8%±16.9%.

Characterization of Cultured Porcine Epithelium

Culturing porcine intestinal crypts in the enteroid medium resulted in a budding enteroid morphology (Fig. 2a) consisting of columnar epithelium (Fig. 2d) comparable to crypt cells *in vivo* (Fig. 2c). In contrast, addition of spheroid medium resulted in a spheroidal phenotype (Fig. 2b) with simple to cuboidal epithelium (Fig. 2e). Immunohistochemical analysis showed the presence of goblet, enteroendocrine, and rare Paneth cell lineages in enteroids consistent with the staining pattern of jejunal crypts, in contrast to spheroids, which lacked these differentiation markers (Fig. 2f–n) The enterocyte brush border marker CD10 was present in the core villus domain of enteroids and native intestinal villi but was absent in spheroids (Fig. 2o–q). The presence of Lgr5-positive stem cells, goblet, enteroendocrine, and Paneth cell lineages in enteroids and spheroids was also assessed by the RNA expression profiles of Lgr5, Muc2, Chga, and Lyz, respectively and compared to those of intact adult porcine crypts (Fig. 2r). Enteroids had reduced expression of Paneth, and goblet cell markers when compared to whole jejunum or intact crypts by RT-PCR. While the enteroendocrine marker chromogranin A was highly expressed in enteroids and spheroids, its expression was an order of magnitude lower in spheroids.

Using immunohistochemistry, we investigated the cell polarity of the cultured porcine structures. Both enteroids and spheroids showed evidence of cell surface polarity as evidenced by the basally located nuclei (Fig. 2d–e) and the cell membrane localization of E-cadherin, cytokeratin 8 and 18, villin, p120-catenin, and β-catenin (Fig. 3).

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Effect of Temperature on Porcine Crypt Culture

Enteroids grown at 37 or 39°C appeared to have similar budding structures on day 6 of primary culture (Fig. 4a–b). Enteroids from both temperature conditions were successfully subcultured, producing typical budding structures (Fig. 4c–d). The expression of Muc2, Chga, and Lyz in enteroids grown at the two temperatures was similar ($p>0.05$; Fig. 4e). The expression of Lgr5 was higher in enteroids grown at 37°C ($p=0.003$).

Cryo-preservation of Porcine Crypts

Porcine crypts were frozen at −80°C for two weeks then thawed and cultured as above in enteroid medium. Enterosphere formation was delayed after a freeze-thaw cycle compared to untreated control crypt cultures, but large budding structures were observed by culture day 7 (Fig. 4f).

ISEMF-CM Produces Intestinal Spheroids

Porcine ISEMFs showed markers analogous of ISEMFs isolated from murine and human intestines, namely αSMA and vimentin (Electronic Supplementary Material, Fig. S1a–b). In a few isolations we noticed an admixture of smooth muscle cells as demonstrated by positive desmin immunostaining (Electronic Supplementary Material, Fig. S1c). However, this did not result in differences in biological activity. ISEMF-CM mixed 1:1 with ENRnYSL medium produced spheroids (Fig. 2a–q, **right panels**).

Subculture

Long-term culture of porcine intestinal crypts was attempted with and without the addition of Wnt3a-CM, CHIR99021, PGE_2, Jag1, and ISEMF-CM. ENR medium supplemented with Wnt3a-CM alone did not support transfer and survival beyond four passages. Using enteroid medium, we were able to passage porcine crypts up to 10 times (mean 9.3±0.6 passages with an up to 640-fold expansion in cell mass (Fig. 5a–b). After 10 passages, there was arrest of growth and no further expansion. Jag1 had no discernible effect on enteroid passage, even when added in the presence of Wnt3a-CM and CHIR99021 or PGE_2 (data not shown). Addition of VPA to the enteroid medium resulted in the formation of budding enteroids in primary culture, but once subcultured small simple enteroids formed without buds (Fig. 5c), and these could only be passaged 2–3 times with limited expansion ability (up to 6-fold). This is in contrast to cultures maintained in enteroid medium, which maintain budding structures after subculture (Fig. 5d). The combination of Wnt3a-CM and PGE_2 (but not PGE_2 alone, Fig. 1l) produced spheroids and permitted up to 10 passages (9.5±0.7 passages; $p=0.39$ *vs.* enteroid medium, Fig. 5a) and up to 66-fold expansion. Spheroids cultured in ISEMF-CM were subcultured up to 8 times (6.3±1.5 passages, $p=0.02$ *vs.* enteroid medium; Fig. 5a); addition of PGE_2 did not improve the capacity of spheroids to undergo passage. The expansion capacity of spheroids in ISEMF-CM was limited to 10- to 12-fold over 6 weeks and 10 passages.

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Transduction

The transduction efficiency of enteroids and spheroids was 24.2%±0.21% and 65.3% ±4.95%, respectively (n=2; Fig. 6). Nearly all enteroids and spheroids contained GFP-positive cells but GFP expression within each structure was inhomogeneous (Fig. 6a–d).

DISCUSSION

Clinical utilization of intestinal stem cells requires preclinical assessment in animal models. In the area of intestinal mucosal stem cell transplantation, *in vivo* experience is thus far concentrated on rodents. Orthotopic transplantation of intestinal crypt organoid units has been previously demonstrated in mice and rats (Chen et al. 2006; Avansino et al. 2006). Furthermore, transplantation of mouse colonic stem cells was demonstrated in a murine colitis model (Yui et al. 2012). Long-term engraftment of murine and human ISCs co-cultured with ISEMFs has been shown in the subcutaneous position in mice (Lahar et al. 2011; Lei et al. 2014). These models demonstrate the feasibility of functional engraftment of cultured ISCs *in vivo*. However, these studies are limited by the inherent differences of rodents from humans in terms of intestinal physiology (Cao et al. 2006; Gibbons and Spencer 2011), although transplantation of intestinal crypts has been reported in one publication each for dogs and pigs, respectively (Agopian et al. 2009; Sala et al. 2009). Porcine models are well-established as large animal models for preclinical assays due to both anatomic and phylogenetic similarities of pigs and humans (Yandza et al. 2012; Swartz and Andreadis 2013). Thus, we pursued the establishment of culture methods for ISCs from juvenile and adult pigs by building on what has been learned from murine, human, and neonatal porcine ISC cultures (Sato et al. 2009; Jung et al. 2011; Gonzalez et al. 2013). Fetal porcine crypts were readily grown in our laboratory under the culture conditions established by Gonzalez et al. for neonatal pigs ((Gonzalez et al. 2013), data not shown). However, initial attempts to use this method for juvenile or adult porcine intestinal crypts were met with failure, leading us to consider that donor age may play an important role in intestinal culture of porcine cells, as has recently been shown in mice (Fordham et al. 2013).

As Sato et al. have shown previously, the addition of various growth factors (EGF, Noggin, and Rspondin1) is necessary and sufficient for the long-term culture of mouse intestinal crypts in the absence of mesenchymal cells (Sato et al. 2009). Building on this knowledge and our experiences with murine, human and neonatal porcine cultures, we developed a culture protocol for juvenile and adult porcine small bowel crypts (Jung et al. 2011; Sato et al. 2011a). We show that ENR medium, which is sufficient for murine crypt culture, does not support porcine crypt growth. While addition of PGE_2 allowed us to culture human crypts, this culture condition was non-supportive of juvenile and adult porcine cultures. In the case of the pig, we found that addition of CHIR99021, a specific GSK3 inhibitor, dramatically improved short-term survival of primary cultures. This is likely a result of expansion of the stem cell population (Yin et al. 2014). These differences highlight the importance of optimizing culture conditions for intestinal epithelium from different species.

Similarly, while the neonatal culture medium (Gonzalez et al. 2013) supports the growth of fetal and neonatal porcine intestinal crypts in primary culture, in our experience, this medium does not permit culture of juvenile or adult porcine crypts for more than 4–5 days.

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Our data support the notion that, in comparison to their fetal and neonatal counterparts, adult pig intestinal crypts in culture are more Wnt-dependent as evidenced by the robust effects of GSK3 inhibition on enteroid survival in primary culture and following subculture. Canonical Wnt signaling is needed for the adult porcine jejunal crypts to survive in the absence of ISEMFs. Comparable differences have also been observed between fetal and adult intestinal crypts from mice (Mustata et al. 2013).

Passage of juvenile and adult porcine intestinal cultures poses another special challenge. In murine cultures, simple mechanical passage of enteroids in ENR medium, with or without chemical detachment of cells, is feasible (Sato et al. 2009; Fuller et al. 2012). In contrast, human colonic enteroids require the addition of recombinant human Wnt3a, nicotinamide, the TGFβ inhibitor A83-01, and the p38 MAP kinase inhibitor SB202190 to ENR medium for successful transfer and subculture (Jung et al. 2011). We found these procedures unsuited for splitting and passaging of adult porcine intestinal enteroid cultures and developed a novel enteroid subculture technique. Once budding enteroids were produced in primary culture, these were split mechanically to break off the buds. We then evaluated the effect of various additives on subculture survival. Jag1, a notch activator important for stem cell regeneration (VanDussen et al. 2012), and VPA, a histone deacetylase inhibitor (Yin et al. 2014), had no discernible effect when added after passage. In contrast, addition of CHIR99021 (a GSK3i), which we had found to be essential for short-term enteroid survival, made all the difference, allowing up to 10 successful enteroid passages. In subsequent studies, we assessed the effects of PGE_2 because of its role in inhibition of anoikis, mitogenic signaling (Jung et al. 2011), and its effect on upregulating or stabilizing Lgr5 (Al-Kharusi et al. 2013). While addition of PGE_2 alone to ENR medium did not support growth after subculture, simultaneous addition of ENRnYSL medium, Wnt3a-CM and PGE_2 resulted in a spheroidal morphology and lack of budding extensions and this mixture permitted up to 10 passages in culture.

Unlike mice and humans, pigs have a relatively high resting physiologic body temperature (39.3±0.3°C) (Dewey and Straw 2006). However, porcine tissue culture has traditionally been performed at 37°C (Mueller et al.; Wang et al. 2011; Gonzalez et al. 2013) despite the paucity of literature to document the physiologic basis of this culture temperature. To assess the effect of culture temperature on porcine intestinal cultures, we isolated porcine jejunal crypts and cultured them in enteroid medium at either 37°C or 39°C. There were no differences in expression of differentiation markers at either temperature; the exception was a higher expression of Lgr5 expression at 37°C, though enteroids at both temperatures were subcultured equally successfully. Normal growth of porcine intestinal cultures at 37°C obviates the need for maintaining incubators at different temperatures – a practical consideration for laboratories where cells from various species are maintained in the same incubator.

We demonstrated successful cryo-preservation of adult porcine intestinal crypts with ability of the thawed crypts to form enteroids. Similar data has been demonstrated using murine enteroids (Yui et al. 2012; Fuller et al. 2012). Ability to cryo-preserve isolated intestinal crypts has important potential implications both for research and therapeutic purposes and provides the ability to share intestinal tissue among collaborating institutions.

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While we have now shown that porcine crypts can be successfully cultured and transduced in enteroid and spheroid media, a method for long-term culture of juvenile and adult porcine crypts beyond several months remains elusive. The practical utility of long-term culture revolves around the use of porcine models in tissue engineering experiments – long-term culture is required for ISC expansion in intestinal tissue regeneration (Bitar and Raghavan 2012; Orlando et al. 2013). To this end, a more formal mechanistic approach will likely be needed to uncover important pathways in adult porcine intestinal cultures. Comparison of intestinal epithelial culture in the adult porcine model with those in the murine and human systems, as we have done here, is likely to be the most expedient route to pursue this mechanistic approach thanks to the large volume of preexisting data on murine and human intestinal cultures.

In summary, we describe a novel method to maintain juvenile and adult porcine intestinal crypts in culture over several weeks and show improved short-term plating efficiency in the setting of continuous canonical Wnt stimulation by inhibition of GSK3. We demonstrate that porcine crypts can be induced by specific supplements to form spheroids or budding enteroids that can be transduced with lentiviruses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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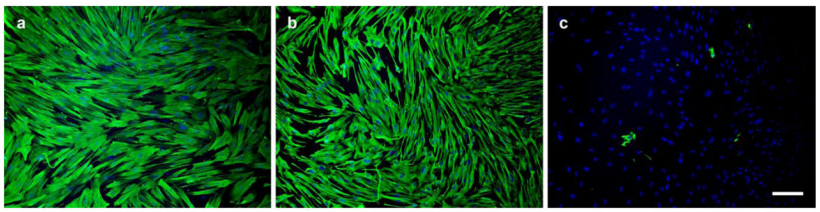

Fig. 1. Comparison of murine, human, and porcine intestinal cultures

(a–c) Intestinal crypts from all 3 species produce enteroids, spheroids, and flat structures when co-cultured with ISEMFs and grown in ENR medium. (d–f) Intestinal crypt culture without ISEMFs. (d–f) Only murine crypts survived in ENR medium. (g–i) Addition of Wnt3a-CM produced spheroids in the murine culture system and short-lived enteroids in the human system, but did not support adult porcine crypt growth. (j–l) Addition of PGE_2 to ENR medium resulted in spheroid formation in murine and human culture systems but was not supportive of adult porcine crypt culture. (m) Comparison of day 7 CFE among various culture media combinations demonstrating a notable survival benefit through addition of either CHIR99021 or ISEMF-CM. Scale bar, 200 μm. NS, non-supportive.

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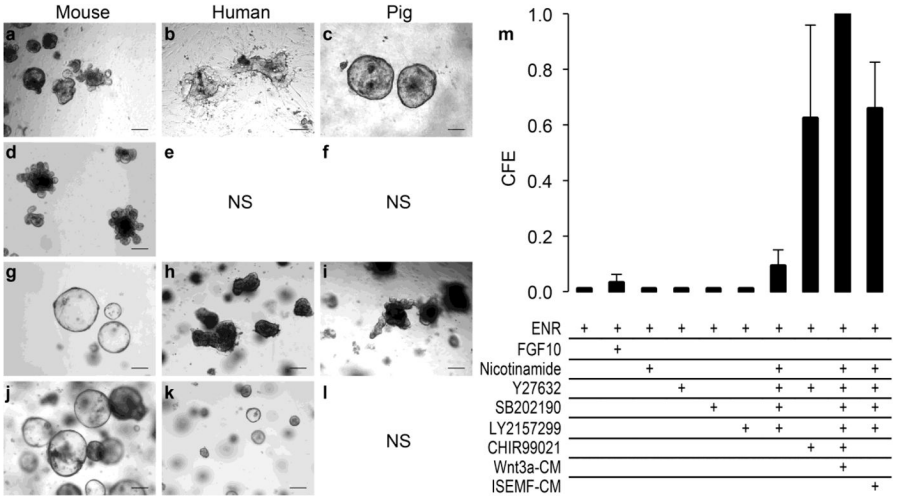

Fig. 2.

Characterization of cultured porcine enteroids (a–q, middle panels) **and spheroids** (a–q, right panels) **compared to porcine jejunum** (c–q, left panels): (a–b) Brightfield, (c–e) H&E, (f–h) PAS, (i–k) chromogranin A, (l–n) lysozyme, (o–q) CD10. (r) Gene expression of porcine crypts, enteroids and spheroids normalized by housekeeping gene Gusb and whole porcine jejunum gene expression. In (l–q), nuclei are counter-stained with DAPI. Scale bar, 200 μm (a–b) and 50 μm (c–q).

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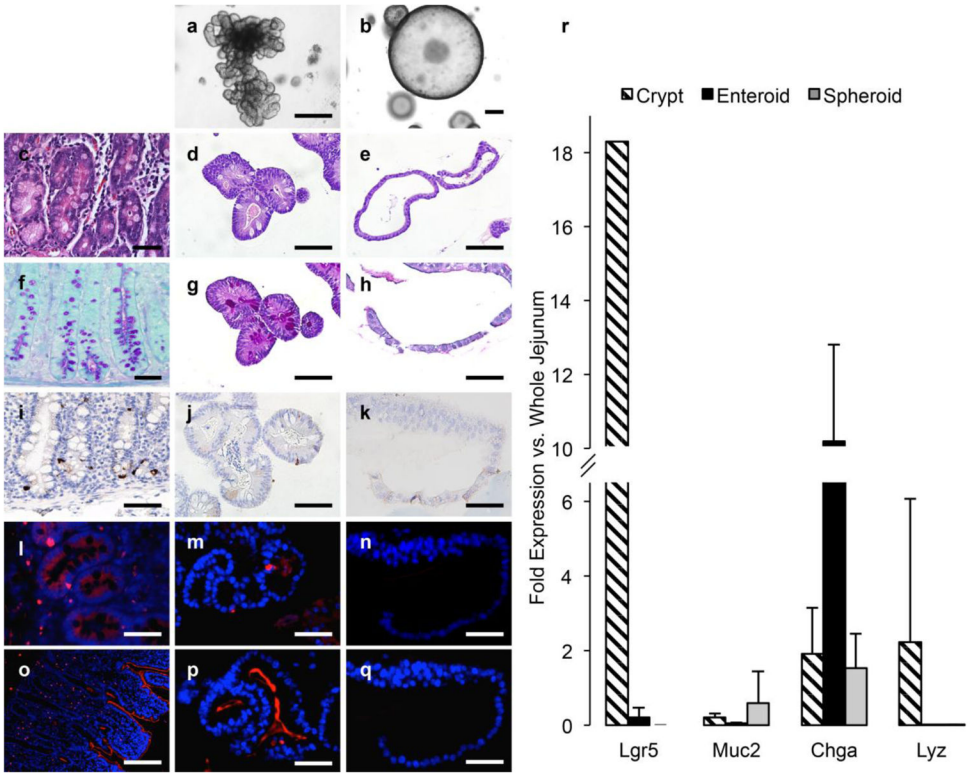

Fig. 3.

Immunohistochemical characterization of cell polarity in porcine jejunum (left) **and cultured porcine enteroids** (middle) **and spheroids** (right): (a–c) E-cadherin, (d–f) cytokeratin 8 and 18, (g–i) villin, (j–l) p120-catenin, and (m–o) β-catenin. Scale bar, 50 μm.

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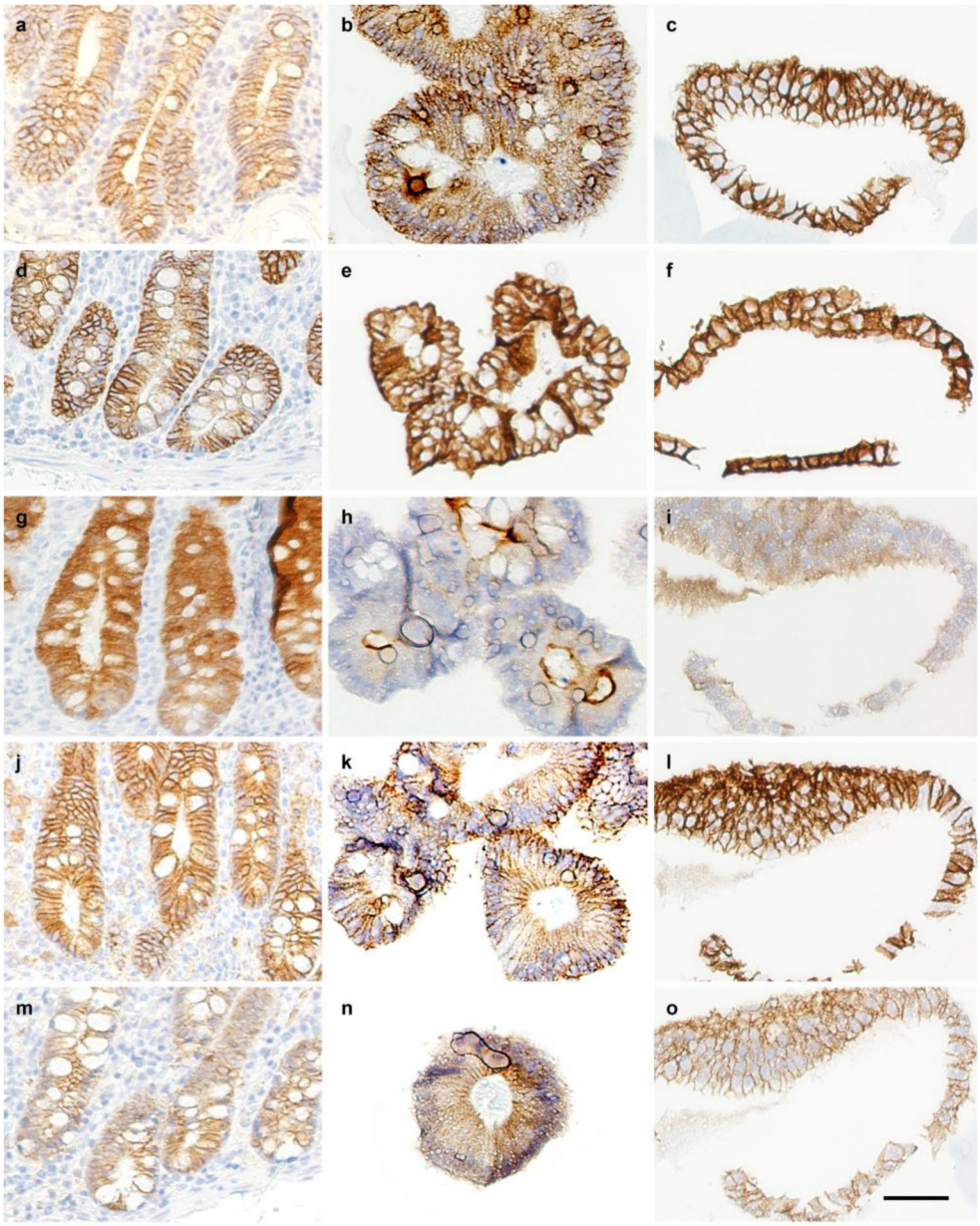

Fig. 4. Effect of culture temperature on enteroid growth

Enteroids on culture day 6 cultured at 39 °C (a) and 37 °C (c). Subcultured enteroids 3 days after initial subculture at 39 (b) and 37 °C (d). (e) Gene expression of enteroids grown at the two culture temperatures for 7 days. (f) Thawed crypts produced enteroids; culture day 7. Scale bar, 500 μm (a–b) and 200 μm (c–d, f).

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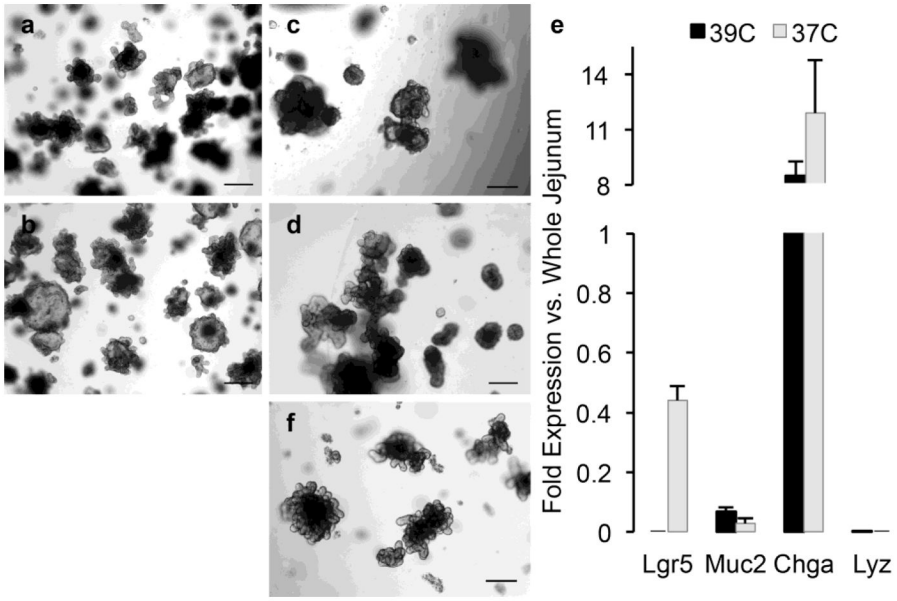

Fig. 5. Subculture of porcine intestinal epithelium

(a) Maximum number of passages for various culture conditions. (b) Fold-expansion of porcine enteroids grown in enteroid medium. (c) Addition of VPA resulted in small enteroids without buds and limited expansion ability. (d) Budding was preserved in enteroids subcultured in enteroid medium without VPA. Scale bar, 500 μm.

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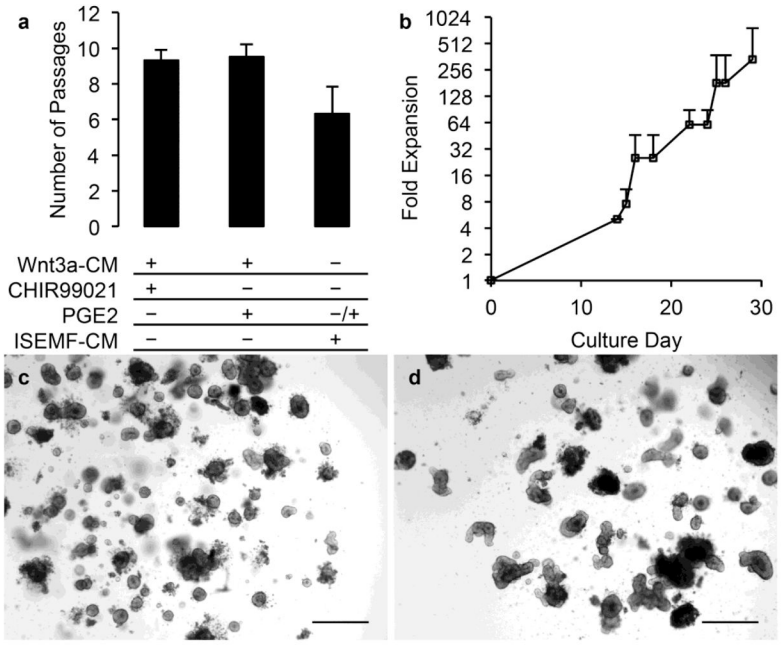

Fig. 6. Transduction of porcine intestinal enteroids and spheroids

Brightfield and FM of transduced enteroids (a, b) and spheroids (c, d), respectively. (e) Transduction efficiency of enteroids and spheroids compared to non-transduced controls. (f) Scatter plot of cultured porcine cells. (g–j) Flow cytometric quantification of GFP-positive cells among (g) untransduced enteroids, (h) untransduced spheroids, (i) transduced enteroids, and (j) transduced spheroids. Scale bar 100 μm (a–b) and 200 μm (c–d).

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Table 1

Primer sequences (5′ to 3′) used for qPCR; bp, base pair. Sequences marked with an asterisk (*) were obtained from Gonzalez et al. 2013.

Gene	Forward Primer	Reverse Primer	Product Size (bp)
Lgr5	CCTTGGCCCTGAACAAAATA	ATTTCTTTCCCAGGGAGTGG	110*
Olfm4	GTCAGCAAACCGGCTATTGT	TGCCTTGGCCATAGGAAATA	226*
Bmi1	TCATTGATGCCACAACCATT	TGAAAAGCCCCGGAACTAAT	189*
Lyz	GGTCTATGATCGGTGCGAGT	AACTGCTTTGGGTGTCTTGC	220
Muc2	CTCAGCCGGGATCCAATCTC	GAAAGCCCCGGTGTAACCAT	249*
Chga	TGAAGTGCATCGTCGAGGTC	GAGGATCCGTTCATCTCCTCG	104
Sglt1	TCACCAAGCCCATTCCAGATG	GCTTCTTGAATGTCCTCCTCCT	109
Vil1	GTTGTGGGAGTCACCCTCTG	GTGCTGACTTGGCCATACCT	109
Gapdh	GTGCTGAGTATGTCGTGGAG	TGATCTTGAGGCTGTTGTCATAC	168
Gusb	TAACAAGCACGAGGATGCAG	TCCTCTGCGTAGGGGTAGTG	128*
18S	TGGAGCGATTTGTCTGGTTA	ACGCTGAGCCAGTCAGTGTA	197
Cdx2	CGAAAGACAAATACCGAGTCGTG	CGGCCTTTCTCCGAATGGT	99
Cdh1	TGGGCCGAGTGAGTTTTGAA	TGACTGTAACCACACCGTCG	102
Acta2	TGTGACAATGGTTCTGGGCT	CACCATCACCCCCTGATGTC	108
Des	TGACCCAGGCAGCCAATAAG	CGATCTCGCAGGTGTAGGAC	99
Vim	TCCAAGTTTGCCGACCTCTC	GACTCGTTGGTCCCCTTGAG	140