Cell Lineage Identification and Stem Cell Culture in a Porcine Model for the Study of Intestinal Epithelial Regeneration

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Abstract

Significant advances in intestinal stem cell biology have been made in murine models; however, anatomical and physiological differences between mice and humans limit mice as a translational model for stem cell based research. The pig has been an effective translational model, and represents a candidate species to study intestinal epithelial stem cell (IESC) driven regeneration. The lack of validated reagents and epithelial culture methods is an obstacle to investigating IESC driven regeneration in a pig model. In this study, antibodies against Epithelial Adhesion Molecule 1 (EpCAM) and Villin marked cells of epithelial origin. Antibodies against Proliferative Cell Nuclear Antigen (PCNA), Minichromosome Maintenance Complex 2 (MCM2), Bromodeoxyuridine (BrDU) and phosphorylated Histone H3 (pH3) distinguished proliferating cells at various stages of the cell cycle. SOX9, localized to the stem/progenitor cells zone, while HOPX was restricted to the +4/reserve stem cell zone. Immunostaining also identified major differentiated lineages. Goblet cells were identified by Mucin 2 (MUC2); enteroendocrine cells by Chromogranin A (CGA), Gastrin and Somatostatin; and absorptive enterocytes by carbonic anhydrase II (CAII) and sucrase isomaltase (SIM). Transmission electron microscopy demonstrated morphologic and subcellular characteristics of stem cell and differentiated intestinal epithelial cell types. Quantitative PCR gene expression analysis enabled identification of stem/progenitor cells, post mitotic cell lineages, and important growth and differentiation pathways. Additionally, a method for long-term culture of porcine crypts was developed. Biomarker characterization and development of IESC culture in the porcine model represents a foundation for translational studies of IESC-driven regeneration of the intestinal epithelium in physiology and disease.

Citation: Gonzalez LM, Williamson I, Piedrahita JA, Blikslager AT, Magness ST (2013) Cell Lineage Identification and Stem Cell Culture in a Porcine Model for the Study of Intestinal Epithelial Regeneration. PLoS ONE 8(6): e66465. doi:10.1371/journal.pone.0066465

Editor: Shree Ram Singh, National Cancer Institute, United States of America

Received April 13, 2013; Accepted May 5, 2013; Published June 28, 2013

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Funding: This work was supported by the National Center for Research Resources and the National Center for Advancing Translational Sciences, National Institutes of Health, through Grant Award Number UL1TR000083 (STM/JP). Additional support provided by R01DK091427 (STM), R03DK089126 (STM), NIH/NCSU Comparative Medicine and Translational Research Training Program (CMTRTP) T32RR024394. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Complete physiologic renewal of the intestinal epithelium occurs in approximately one week and is driven by a pool of IESCs at the crypt base [1]. This impressive rate of renewal is tightly controlled in homeostasis. Dysregulation of IESC renewal results in intestinal disorders such as small intestinal and colorectal cancer, which is the leading cause of digestive disease-related mortality [2,3]. Impaired epithelial renewal can lead to ulceration, chronic inflammatory responses and sepsis [4,5]. Since the description of IESCs in 1974 by Cheng and Lebond, investigators have attempted to understand the factors that control IESC-driven epithelial regeneration in physiology and disease [6].

In general, logistical and ethical issues minimize the use of humans or human- derived tissues for research and discovery pertaining to conditions of the intestinal epithelium. These obstacles highlight the need for a research model that closely mimics human intestinal anatomy, physiology, disease and injury processes. Currently, the vast majority of basic studies focused on intestinal epithelial diseases, injury and regeneration utilize rodent models. Rats and mice in particular represent an important, cost effective animal model for basic genetic, cellular and molecular biology of IESC-driven regeneration of the intestinal epithelium. Despite these advantages, significant differences between rodents and humans confound or prohibit translational studies [7].

Important anatomical, behavioral and environmental conditions that impact epithelial regeneration are more closely shared between pigs and humans than between mice and humans [8,9]. Pigs and humans share parallel mucosal barrier physiology, food intake, enteric microbiota composition, and pathogenicity of key...
disease causing microbes [7]. Pigs, like humans, are true omni-
vores and share similar metabolic and intestinal physiologic
processes [7,9]. A mucosal in vitro permeability study demonstrated
greater correlation between humans and pigs when compared to
rats [8]. Importantly, it has been demonstrated that pigs represents
a more physiologically relevant model of neonatal necrotizing
enterocolitis, intestinal ischemia-reperfusion injury, acute mesen-
teric ischemia, short bowel syndrome, AIDS-associated oppor-
tunistic Cryptosporidium infection, and stress-induced intestinal
dysfunction [10–22]. Additionally, a large animal model is likely
to serve as a more physiological relevant model to study segmental
assessment of radiation exposure, focally induced ischemia and
reperfusion as well as transplantation and cell-based therapies.

Severe intestinal disease necessitates approximately 200 intes-
tinal transplantations each year in the United States [2]. In a
prospective cross-sectional study of patients, 40% of visceral allo-
graft recipients died within 5 years of transplantation [23]. The
impact of digestive disease on rates of mortality and morbidity as
well as health care costs in the United States has created an urgent
need for advances in transplantation and tissue replacement
therapies [2]. A key factor to the success of many translational
studies is the gross size of the animal model. The small size of
the intestines of experimental rodent models often prohibits
probes or intervention as such tissue engraftment or transplantation. These
limitations further highlight the need for a large animal model to
advance cell or tissue based therapies.

This study focuses on eliminating many of the obstacles that
limit the pig as a translational model to study IESC-driven re-
generation of the intestinal epithelium. This work thoroughly cha-
racterizes the porcine intestinal mucosa by identifying, developing
and validating a comprehensive set of reagents to study porcine
stem/progenitor cells and their principal post-mitotic cell descen-
dants in situ and in culture.

**Materials and Methods**

**Ethics Statement**

All animal studies were approved by the Institutional Animal
Care and use Committee at North Carolina State University.

**Animals and sample collection**

Tissues were obtained from healthy 6–8 week-old wild type
Yorkshire cross pigs euthanized for reasons unrelated to this pro-
ject. Sections from the gastrointestinal tract including the duode-
umum, jejunum, ileum, proximal and distal colon were sharply
disected.

### Table 1. Functional/Cross Reactive Antibodies.

| Protein | Company   | Catalog #  | Host Species | Dilution 1° Ab | Antigen Retrieval | Functional for Western Blot/Band (KDa) |
|---------|-----------|------------|--------------|----------------|------------------|----------------------------------------|
| IESC    | SOX9      | Millipore  | ab5535       | rabbit         | 1:1000           | Yes                                    |
|         | HOPX      | Santa Cruz | sc-30216     | rabbit         | 1:500            | Yes                                    |
| Proliferative | PCNA    | Millipore  | MAB424R      | mouse          | 1:100            | Yes                                    |
| Goblet  | MUC2      | Santa Cruz | sc-15334     | rabbit         | 1:1000           | No                                     |
|         | UEA-1     | Sigma      | 19337 Atto 488|                | 1:500            | No                                     |
| Enteroendocrine | CgA  | Immunostar | 20086 (Porcine)| rabbit    | 1:1000           | Yes                                    |
|         | CgA       | Immunostar | 20085 (Bovine)| rabbit         | 1:1000           | No                                     |
|         | Gastrin   | Santa Cruz | sc-7783      | Goat           | 1:250            | Yes                                    |
|         | Glucagon  | Santa Cruz | sc-13091     | rabbit         | 1:250            | No                                     |
|         | Somatostatin | Santa Cruz | sc-7819     | Goat           | 1:500            | No                                     |
| Absorptive Endocrine | Sucrose Isomaltase | Santa Cruz | sc-27603 | Goat       | 1:500            | Yes                                    |
|         | CAII      | Santa Cruz | sc-17244     | Goat           | 1:250            | No                                     |
|         | CAII      | Santa Cruz | sc-17246     | Goat           | 1:500            | Yes                                    |
| Epithelial | EpCAM   | Biolegend  | 118212       | mouse          | 1:500            | No                                     |
|         | Villin    | Santa Cruz | sc-7672      | Goat           | 1:500            | Yes                                    |
| Apoptosis and Cell Cycle | Caspase 3 | Cell Signaling | 96615 | Rabbit       | 1:400            | Yes                                    |
|         | Phosph-histone H3 | Cell Signaling | 9701     | Rabbit         | 1:200           | Yes                                    |
|         | Phospho-histone H3 | Millipore | 6–570       | Rabbit         | 1:500           | Yes                                    |
|         | MCM2      | Santa Cruz | sc-9839      | Goat           | 1:200            | Yes                                    |
|         | Beta catenin | Cell Signaling | 2677 | mouse        | 1:200            | Yes                                    |

doi:10.1371/journal.pone.0066465.t001
Histological and Immunofluorescence Analyses

Tissues were rinsed with 1× phosphate-buffered saline (PBS) and opened longitudinally along the anti-mesenteric border. For immunohistochemical analysis, tissue was fixed in 10% formalin, embedded in paraffin and sectioned (~5–8 μm thickness). Slides were stained with hematoxylin and eosin to visualize crypt and villus morphology. For immunofluorescence, rinsed tissue was fixed in 4% paraformaldehyde (PFA) solution for 14–18 hours at 4°C. The tissue was transferred to 30% sucrose solution for at least 24 hours at 4°C, embedded in optimal cutting temperature (OCT) media, frozen and sectioned at 5–8 μm thickness using a cryotome and mounted on positively charged glass slides. Sections were washed three times with PBS to remove OCT. When necessary, heat induced epitope retrieval (HIER) was then performed by placing slides into reveal decloaker solution (Biocare Medical, Concord, CA) for 30 seconds at 120°C and then 90°C for 10 seconds, in a pressure cooker. The slides were allowed to cool at room temperature for 20 min prior to continuing. Tissue permeabilization was performed on all slides with PBS-0.3% Triton X-100 for 20 min, washed twice with PBS and incubated in blocking medium (Dako, Carpinteria, CA). Primary antibodies were applied to the tissue section in an antibody diluent (Dako) and incubated overnight at 4°C. Dilutions for functional antibodies were as follows: αSOX9 (rabbit, 1:1000, Chemicon/Millipore, Temecula, CA), αMucin2 (rabbit; 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), αLectin from Ulex europaeus-Atto 488 conjugate (1:500, Sigma-Aldrich, St. Louis, MO), αsucrase isomaltase (goat, 1:500, Santa Cruz Biotechnology), αCD326/EpCAM (rat, 1:500, BioLegend, San Diego, CA), αVillin (goat, 1:500, Santa Cruz Biotechnology), αCleaved Caspase 3 (rabbit, 1:400, Cell Signaling Technology, Inc., Danvers, MA), αGlucagon (rabbit, 1:250, Santa Cruz Biotechnology), αSomatostatin (goat, 1:500, Santa Cruz Biotechnology), αCarbonic Anhydrase (goat, 1:250, Santa Cruz Biotechnology), α...

Figure 1. Markers to identify cells of epithelial origin. Identification of intestinal cells of epithelial origin within the porcine small intestine and colon are shown. Immunostaining for EpCAM, a pan-epithelial transmembrane protein, demonstrated expression in the basolateral membrane of all cells in the small intestine and colon arranged along the luminal monolayer of the epithelial mucosa. Immunostaining for Villin, a protein associated with the microvillar actin filaments, showed a gradient of expression localized to the apical border of small intestinal and colonic epithelial cells with increasing intensity in cells located closer to the lumen. All specific markers (red). Nuclei (blue). Scale bar 200 μm, inset scale bar 50 μm.
doi:10.1371/journal.pone.0066465.g001
Immunostaining for cleaved caspase 3, an indicator of apoptosis, marked a few expressing cells near the villus tip within small intestine and the crypt base. Immunostaining for BrdU, a marker of cells within the S stage of the cell cycle, was also localized to a subpopulation of cells within the crypt base. Immunostaining for pH3, a marker for cells between the G2-M stage of the cell cycle was similarly localized but to fewer cells. Immunostaining for PCNA, a general marker for cellular proliferation, demonstrated the greatest number of positive cells compared to the other markers of proliferation.

**Figure 2. Markers to assess proliferation and apoptosis.** Identification of proliferative cells in different stages of the cell cycle and those undergoing apoptosis in porcine small intestine and colon are shown. All proliferative markers localized to the nuclei of positive epithelial cells. Immunostaining for PCNA, a general marker for cellular proliferation, demonstrated the greatest number of positive cells compared to other markers of proliferation. Immunostaining for MCM2, a marker of cells at the G1 stage of the cell cycle, was localized to a subpopulation of cells within the crypt base. Immunostaining for BrdU, a marker of cells within the S stage of the cell cycle, was also localized to a subpopulation of cells within the crypt base. Immunostaining for pH3, a marker for cells between the G2-M stage of the cell cycle was similarly localized but to fewer cells. Immunostaining for cleaved caspase 3, an indicator of apoptosis, marked a few expressing cells near the villus tip within small intestine and the luminal surface of colon. All specific markers (red). Nuclei (blue). Scale bar 200 μm. doi:10.1371/journal.pone.0066465.g002

**qRT-PCR**

| Gene       | Primer 1 | Primer 2 | Sequence | Taqman assay
|------------|----------|----------|----------|--------------
| PCNA       |          |          |          |              |
| MCM2 - G1  |          |          |          |              |
| BrdU - S   |          |          |          |              |
| pH3 - G2-M |          |          |          |              |
| Casp 3     |          |          |          |              |

**Target validation by sequencing**

RT-PCR amplicons were analyzed on a 1.5% agarose TE gel to assess size. The amplicons were purified using the USB ExoSAP-IT PCR product cleanup reagent (Affymetrix, Santa Clara, CA). Samples were sequenced (GENEWIZ, Research.
PBS solution containing 30 mM EDTA, 10 mM DTT (Sigma-Aldrich), and 100 ng/mL penicillin/streptomycin at 4°C.

Transmission Electron Microscopy

Transmission Electron Microscopy (TEM) imaging was performed by the laboratory for advanced microscopy at North Carolina State University. The tissue fixation, preparation and image acquisition were performed as previously published [6,33,34].

Crypt isolation, Enteroid Culture and Analysis

Enteroids were cut into 5–8 μm sections and heat induced epitope retrieval was performed when necessary by heating in a decloaker solution (Biocare Medical, Concord, CA) to 120°C for 20 sec and then 90°C for 10 sec inside a pressure cooker. Slides were allowed to cool to room temperature for 20 min prior to staining. Enteroids were permeabilized in a 0.3% Triton X-100 PBS solution for 20 min and then blocked in protein block antibody diluent (Dako) and incubated for 2 hr at room temperature. Primary antibodies, αSOX9, αMucin2, αsuccrose isomaltase, and αPCNA, at the same dilutions previously described, were applied to the slides in antibody diluent (Dako) and incubated for 2 hr at room temperature. All secondary antibody incubations were performed with Cy3 conjugated antibodies (Jackson ImmunoResearch, West Grove, PA) diluted 1:500 in antibody diluent (Dako) incubated at room temperature for 45 min. Nuclei were marked with bisBenzimide H 33258 nuclear stain (Sigma/ Aldrich) diluted 1:1000 in PBS and applied for 5 min at room temperature. Confocal images were obtained using a Zeiss LSM 710 laser scanning microscope. The objective lenses used were X40 water and X63 oil with numerical apertures 1.1 and 1.4, respectively (G-Apochromat, Plan-Apochromat, Zeiss, Jena, Germany).

Results

Identification of cells of epithelial origin

To distinguish epithelial cells from those of mesenchymal and hematopoietic origin, we tested whether two candidate antibodies raised against mouse EpCAM (CD326) and human Villin would exclusively label pig epithelial cells. EpCAM is a pan-epithelial cell marker that targets the luminal surface of epithelial cells. HVAC is a hematopoietic marker. Both antibodies were validated against porcine tissue by immunohistochemistry on tissue sections from 2-14 day-old piglets. The antibodies stained cells of epithelial origin but not those of mesenchymal or hematopoietic origin (Table 2).

Transmission Electron Microscopy

TEM imaging was performed by the laboratory for advanced electron and light optic methods at North Carolina State University. The tissue fixation, preparation and image acquisition were performed as previously published [6,33,34].

Table 2. Non Functional Antibodies.

| Protein      | Company   | Catalog # | Host Species |
|--------------|-----------|-----------|--------------|
| IESC         | LGR5      | sc-68580  | Goat         |
|              | LGR5      | TA301323  | Rabbit       |
|              | SOX4      | sc-17326  | Goat         |
|              | SOX17     | sc-17355  | Goat         |
|              | DCAMKL1   | AP7219b   | Rabbit       |
|              | DCAMKL1   | 37994     | Rabbit       |
|              | MS11      | AB5977    | Rabbit       |
|              | OLFM4     | AB85046   | Rabbit       |
|              | OLFM4/GC-1| Sc-84274  | Rabbit       |
|              | CD24      | 557436    | Rat          |
|              | CD24      | 1279      | Mouse        |
| Progenitor   | NEURO D   | sc-1084   | Goat         |
|              | HES1      | sc-13844  | Goat         |
|              | HES1      | D153-3    | Rabbit       |
|              | NSUN1     | sc-83439  | Goat         |
|              | NOTCH1    | sc-6014   | Rabbit       |
| Proliferative| Ki 67     | sc-7846   | Goat         |
|              | cMYC      | sc-764    | Rabbit       |
|              | Cyclin D1 | RMAB003   | Rabbit       |
| Paneth       | Lysozyme  | RP028     | Rabbit       |
|              | Lysozyme  | sc-27598  | Goat         |
|              | cKIT (CD117)| MBL | 566 | Rabbit |
| Enteroendocrine| Synaptophysin | ab52636 | Rabbit |

doi:10.1371/journal.pone.0066465.t002
transmembrane protein that functions as a homotypic calcium-independent cell adhesion molecule [35]. EpCAM expression was observed in the basolateral membrane of all cells arranged along the luminal monolayer of the epithelial mucosa (Figure 1) [35]. In the small and large intestine, Villin expression is localized to the apical border of the intestinal epithelial cells due to its association with the microvillar actin filaments [36]. Villin expression demonstrated a gradient of increased staining intensity from the crypt toward the lumen as has been described in normal mammalian tissue (Figure 1) [36].

Markers of proliferation and apoptosis identify cells within each stage of the cell cycle

Assessing the proliferative capacity of IESCs and their progenitors is essential for monitoring regenerative responses in the small intestine and colon. Proliferating Cell Nuclear Antigen (PCNA) is accepted as a general proliferation marker and localized to the nucleus of the majority of cells constituting the crypt base in porcine tissue (Figure 2). Minichromosome Maintenance Complex 2 (MCM2) serves as a biomarker for cells that are peaking at G1-S phase [28]. The thymidine analogs BrdU or EdU are well established markers for cells in S-phase [29]. Both MCM2 and BrdU were localized within the nuclei of a subset of cells within the proliferative zone (Figure 2). Histone H3 is phosphorylated (pH3) at the end of prophase and represents a suitable marker for cells in G2-M-phase of the cell cycle. pH3 positive cells marked a minority of cells within all crypt bases consistent with the limited number of cells at this point in the cell cycle [30]. Immunostaining jejunal and colonic tissues for each of these proliferation markers demonstrates robust cross-reactivity with cells located in the proliferative zone of the crypt as has been demonstrated in mice (Figure 2) [28,37,38].

These validated antibodies for porcine gut tissue represent a comprehensive set of reagents for detailed study of the proliferative response in physiology, disease and injury induced regeneration.

Interrogating apoptotic dynamics is equally important to understanding mechanisms underlying a regenerative response [39]. Caspase3 cleavage represents the execution phase of apoptosis [39]. The antibody against cleaved human caspase 3 (CASP3) marked few cells at the villus tip in porcine small intestine and colon where an apoptotic event known as ‘anoikis’ typically occurs (Figure 2) [26]. Rare cells at the base of the crypts were observed which is consistent with rare apoptotic incidences in physiologic renewal.

Identification of stem and progenitor cell populations

Next, we aimed to distinguish stem/progenitor cells from fully differentiated lineages. Recent evidence supports the presence of IESCs that exist in different states of proliferative capacity [40,41]. Crypt-based columnar ‘active’ stem cells (CBCs) are located intercalated between Paneth cells in mice, are constantly dividing, and primarily responsible for the burden of homeostatic epithelial regeneration. Unfortunately, the commercially available antibodies used to detect the CBC population, LGR5, OLFM4, and CD24 did not demonstrate cross reactivity with active CBC stem cells in porcine intestinal tissue (data not shown; Table 2). However, antibodies raised against SOX9, a member of the SRY-family of transcription factors primarily expressed in CBCs and transit-amplifying progenitor cells, demonstrated the ability to detect proliferating cells in the base of the small intestine and colonic crypts with distinct localization to the nucleus of positive cells (Figure 3) [31,42,43].
Identification of absorptive cell lineage

Enterocytes function to absorb nutrients, electrolytes and water and are the predominant cell type within the intestinal mucosa [33]. The histologic identification of this cell lineage is used to assess whether appropriate cellular differentiation is occurring during a regenerative response [45,46]. Immunostaining for the digestive enzymes, sucrase isomaltase (SIM) and carbonic anhydrase II (CAII) clearly demonstrates localization to the apical brush-border of the absorptive enterocytes in the small and large intestine, respectively. [47,48]. There was no positive immunostaining of SIM in the colon or CAII in the small intestine indicating these antibodies are suitable to differentially distinguish between these two absorptive cell types (Figure 4).

Identification of secretory cell lineage

In mice and humans three primary secretory lineages exist, enteroendocrine cells, goblet cells and paneth cells. Enteroendocrine cells represent a minor population of cells that secrete various hormones that regulate gut physiology and appetite control [49]. Chromogranin A (CgA) is an acidic glycoprotein that localizes within secretory granules of nearly all enteroendocrine cells and is considered a general marker for all enteroendocrine cell subtypes [50]. Immunostaining for CgA localized to the cytoplasm of a minority of cells throughout the length of the small intestine and colon, consistent in morphology with enteroendocrine cells found in other animal species [31,51] (Figure 5).

Subtypes of enteroendocrine cells could also be identified in pig intestinal epithelium. Gastrin (GAST) is a hormone secreted by G cells in the stomach and duodenum, and it functions as both a mucosal growth factor and stimulator of mast and parietal cells [49]. An antibody against human Gastrin (GAST) identified a minority of cells in the duodenum while no immunostaining was observed along other segments of the small or large intestine, an observation consistent with the expression pattern of GAST observed in humans [49] (Figure 5). Somatostatin (SST) is a hormone secreted by delta cells throughout the length of the intestine and functions to block the release of many gut hormones ultimately affecting epithelial transport and intestinal motility [49,52]. SST-positive cells were observed intermittently in all segments of the porcine intestine (Figure 5). The main role of glucagon-like peptide 1 (GLP-1) is to delay gastric emptying and signal post prandial satiety and that of glucagon-like peptide 2 (GLP-2) is to stimulate mucosal enterocyte proliferation [53]. Reactivity to the glucagon antibody, with cytoplasmic localization, was observed within the enteroendocrine cells along the proximal distal axis of the small intestine (Figure 5) consistent with that observed in mice and humans [53,54].

Secretory goblet cells produce mucus that are integral to intestinal physiology by providing protection of the epithelial surface as well as aiding in absorption [55]. Immunostaining for Mucin 2 (MUC2) exclusively marked mucous-producing cells along the entire length of small and large intestine as well as the crypt-villus axis (Figure 6). Positive staining was localized to the cytoplasm of positive cells and within the luminal surface which is the expected location of mucinous secretions. Ulex europaeus agglutinin-1 (UEA-1) is a lectin that specifically binds to alpha-linked fructose receptors located on cell surface glycoproteins and glycolipids and is used to detect both goblet and Paneth cells in mice [56]. UEA-1 bound to the mucinous secretions of goblet cells in fixed tissue sections, UAE-1 is likely suitable for fluorescence activated cell sorting of live cells [37].

The existence of the Paneth cell in the pig remains disputed [57,58]. No cosin or toluidine blue staining, which typically marks...
apically located granules in Paneth cells, was identified in the crypt base of porcine small intestine (data not shown). In mice lysozyme expression is a biomarker for Paneth cells [59,60]. Lysozyme staining was not observed in the epithelium of porcine small intestine despite the use of multiple anti-lysozyme antibodies (data not shown; Table 2).

TEM characterization of porcine crypt-based cells

Transmission electron microscopy allows for the morphological identification of cell lineage by the presence or absence of sub-cellular features [6,33,61–63]. Comparative analysis of crypt-based cells from various organisms has enabled the characterization of cell types that are consistent with particular lineages [6,33]. A complete description of the pig small intestine and colon, to the best of our knowledge, has not been previously described. At least two distinct cell types were distinguishable in the crypt base of the pig small intestine and colon (Figure 7). Multiple irregularly shaped, small, columnar cells with basally located nuclei and scarce cytoplasm, consistent in appearance with CBC cells of mice and humans [33,64], were interspersed between large pyramidal shaped cells with large supranuclear clear mucoid vesicles and small electron dense bodies (Figure 7). The appearance of these large mucoid filled cells was not entirely consistent with the accepted morphological features of Paneth cells in other mammalian species [63,65]. The goblet cell within the small intestine and colon possessed small basally located nuclei that were notably distended apically with mucinous globules consistent with the accepted ultrastructural appearance in mammalian intestinal tissue (Figure 7) [61,66]. The enteroendocrine cells of the pig intestine demonstrated a narrow apex and wide base with many small, spheroidal, electron dense granules in the infranuclear region as is classically described in mammals (Figure 7) [33,66]. Other key features of these cells include multiple organelles and a lack of secretory granules within the cytoplasm and a position closer to the gut lumen in both the small intestine and colon, as has been described in other mammals [Figure 7] [33,66]. These electron micrographs of the porcine crypt base represents a foundation for morphologic description of cells in normal, injury and disease states.

Assessment of gene expression in stem/progenitor and differentiated cell lineages

Measuring gene expression is essential to understanding mechanisms of injury, disease and the stem cell-driven regeneration. Because qPCR primer sets to detect target gene expression in pig are limited, we designed and validated primers to genetic biomarkers of stem/progenitor cells, differentiated cell lineages, and important signal transduction pathways involved in regeneration [38,44,68,69]. Twenty one candidate PCR primers were designed and six were previously described [27,70,71] (Table 3). Amplification of cDNA generated from total intestinal RNA demonstrated expression of Wnt3a+ and Lgr5+, which are important regulators of IESC maintenance [59,59,72]. Genes important for Notch pathway regulation, Atoh1+, Dll4+, and Hes1+, were amplified from epithelial-derived cDNA. These genes are critical for appropriate cell differentiation and fate and important to interrogate in regeneration [38]. ‘Active’ stem cell markers, Olfm4+, Ascl2+, Sox9+, CD24+, were used to monitor stem cell renewal and maintenance. Hallmark genetic biomarkers for differentiated lineages were detected by amplification of Muc2+ and Itf+ (goblet cells), CgA+ and Cck+ (enteroendocrine cells), and Sglt1+ and L-Fabp+ (absorptive enterocytes) [43,73–75]. Single amplicons of the appropriate size were verified by gel electrophoresis and DNA sequencing of all amplicons validated target gene
amplification. Primers efficiencies were calculated using the equation, Efficiency = 10^{(\frac{-1}{\text{slope})} - 1} and demonstrated >92% efficiency (Table 3). Under the conditions described, no primer-dimers were observed.

In vitro culture of porcine crypts
Long-term culture of intestinal epithelial stem cells in mice and humans has only recently been accomplished and has revolutionized the ability to conduct detailed mechanistic studies in a highly controlled manner [38,76–78]. Pig crypts isolated from jejunal tissue were introduced into a modified 3-dimensional (3-D) culture environment similar to the culture conditions that support growth of mouse and human enteroids [42,76–79]. Within 24 hours of plating whole pig crypts in matrigel with defined medium, enterosheres formed (Figure 8, day 2) [80]. These structures persisted until day 4 and then began to convert into enteroids that possessed columnar epithelial cells and primitive crypt buds (Figure 8, day 4). Mature crypt buds developed by day 14 and by day 21 enteroids were fully formed (Figure 8, day 14 and 21). Enteroid cross sections demonstrated the presence of SOX9+ stem/progenitor cell populations, PCNA+ zones of proliferation, MUC2+ goblet cells, CgA+ enteroendocrine cells, and SIM+ absorptive enterocytes (Figure 9). Fully developed enteroids were allowed to persist in culture for two weeks at which point they were passaged. To date, enteroids have been passaged 8 times representing a total of 4.5 months in culture. There has been no apparent decrease in enteroid formation over this time.

Discussion
A recent NIH symposium entitled “Improving Animal Models for Regenerative Medicine” focused on the development of large animal models for the study of human disease [81]. The motivation for the symposium was the persistent failure in translating murine models to clinical treatments [81]. The utility of the pig as a large animal model has been well-documented for many body systems [15–21,82–88]; and the similarities between the pig and human gastrointestinal system position the pig as a promising species for animal models of gastrointestinal disease. Seminal advances made in murine intestinal stem cell biology now position investigators to answer clinically relevant problems from the perspective of stem cell-driven epithelial regeneration. Data presented in this study lay the foundation for developing the pig as a large and physiologically relevant animal model for these
studies. This study identifies, develops and validates a range of genetic biomarkers and crypt culture strategies that will enable investigators to assess stem cell maintenance and potency in both the small and large intestine of pig models of physiology, injury and disease.

Few studies use commercially available antibodies on porcine intestinal tissue to assess cell lineage allocation, thus limiting the ability to effectively monitor and analyze epithelial regenerative responses [10,25–27,89,90]. To address this problem, we identified a comprehensive set of commercially available antibodies that would cross-react with target pig proteins to enable detection of stem/progenitor and post-mitotic lineages. The ability to specifically observe epithelial dynamics during physiology and disease is critical to understanding and quantifying regenerative processes [10]. Appropriate designation of EpCAM expressing cells, for example, can offer insight into epithelial cell-cell adhesion, migration, signaling, differentiation and proliferation since it plays a key role in these cellular functions [35]. Additionally, assessing the proliferative capacity of IESCs and their progenitors is essential for monitoring regenerative processes and therapeutic interventions [10]. Appropriate designation of EpCAM expressing cells, for example, can offer insight into epithelial cell-cell adhesion, migration, signaling, differentiation and proliferation since it plays a key role in these cellular functions [35].

Identification of stem and progenitor cell populations has proven critical to deciphering important cellular pathways as well as the impact and response of these cells to injury. In mice, ‘active’ IESCs are marked by Lgr5, Olfm4, Ascl2, Sox9 [31,43,59,60, 73,74,91,92]. Unfortunately, of the commercially available antibodies for CBCs tested, only SOX9 demonstrated positive staining. Sox9 is a member of the SRY-family of transcription factors that is primarily expressed in crypt-based columnar stem cells and transit-amplifying progenitor cells [31,42,43]. Enteroendocrine and Tuft cells also express very high levels of Sox9 and recent evidence suggests that the Sox9 high population has ‘reserve’ IESC capacity [31,42–44,75]. Slower dividing ‘reserve’ or facultative stem cells primarily reside above the Paneth cell compartment in mice and humans [41,44]. These have been historically termed ‘the +4 stem cells’ which denotes the cell position in the crypt base where they most likely exist [93]. As the name suggests, facultative IESCs appear to respond to injury stimulus to re-enter an active state to regenerate the epithelium [44,69,94]. To some extent, the reserve IESC population is marked by Bmi1, Tert, Hopx, and Lrig1 in mice [37,95–101]. Immunostaining for HOPX will enable future studies utilizing porcine models to evaluate the role of these putative ‘reserve’ IECs during and following intestinal injury.

The ability to observe and quantify fully differentiated cells is critical to understanding the dynamics of epithelial regeneration in normal homeostasis, injury, and repair. CgA was used as a general marker of enteroendocrine cells but multiple sub-types were also characterized. The hormones produced by all of the enteroendocrine cells are integral to crypt cell physiology. Proglucagon, for example, is produced and cleaved within the L-type enteroendocrine cells into glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2).
peptide-2 (GLP-2) [54]. Interestingly, glucagon-like peptides demonstrate immunoreactivity to antibodies against glucagon, the product of cleaved proglucagon in the pancreas [54]. The potential therapeutic benefits of both GLP-1 and GLP-2 are of interest for multiple important human diseases. GLP-1 is integral to both signaling satiety and in glucose homeostasis. GLP-1 based treatments are now well established in the management of type 2 diabetes and have been proposed for the treatment of obesity [102]. Research into the therapeutic benefits of GLP-2 administration to hasten epithelial proliferation with direct stimulatory effects on the stem cell population following intestinal resection have also been studied [12,103–105]. The ability to clearly identify GLP-1 and GLP-2 producing cells may then prove integral in pig models that study weight management, diabetes and short bowel syndrome [12,102,103,106].

Electron microscopy supported the immunohistochemical findings on porcine small and large bowel showing clear ultrastructural characteristics of lineage states along the crypt villus axis. Electron microscopy provided morphological evidence suitable for identification of cells consistent with CBC stem cells, goblet cells, enteroendocrine cells and absorptive enterocytes. TEM studies have and will continue to contribute to understanding the ultrastructural cellular changes that occur during disease and repair processes as well as following signaling pathway manipulation that may not be possible with immunohistochemical studies [95,107,108]. TEM has been utilized, for example, to visualize invasion of intestinal epithelium by viral particles

| Target | Sequences of primers (5’ to 3’) | Annealing Temp(°C) | Product Size | Reference |
|--------|---------------------------------|-------------------|--------------|-----------|
| House Keeping Genes | | | | |
| Gapdh | ATCCTGGGCCTACCTGAGGAC AAGTGCTGCTGAGGCAATG | 60 | | |
| Gusb | TAACGACAGAAGGGATGCTG TCCCTCTGAGGGTATGG | 60 | 129 | Author |
| 18S | TGGAGGCATTGTGCTGTTA ACCTGAGCCAGCTGTA | 60 | 200 | [71] |
| Lgr5 | CCTTCGCCCTGACGAAATA ATTCCTTTCCAGGAGTGG | 60 | 110 | Author |
| Olfr4 | GTACAAGAACGCCCTGATGG TGCATTGCTGAAAGAATA | 60 | 226 | Author |
| Sox9 | CGGTTGCAGACAGAATAAGC GTAATCGGGGCTGCTTCTC | 60 | 229 | Author |
| Ascl2 | GAGCTCTCGACTTCCTCAG TCCCACTAGGCCCTGTC | 60 | 204 | Author |
| CD24 | TAAGAGCAGCGTGCTCCTC ACGGAGAGGGGAGGAGGAC | 62 | 283 | Author |
| +4 Stem Cells/Quiescent | | | | |
| Bm1 | TCATTGATGCCCAACACTT TGAAGGCGCCCGAATAT | 60 | 189 | Author |
| Proliferative Cells | | | | |
| PcnA | TACCTGAAAGGGGCGAAGATAGTTGCTGACACTGGCATACGGT | 60 | 192 | [27] |
| Atog | CACGGGCTAACGCGCCTGGTGCTCGT | 60 | 234 | Author |
| Rex1 | ATTCCTTGCTCCGGCTGCCGCTGGT | 62 | 279 | Author |
| Goblet Cells | | | | |
| Muc2 | GGGCTACTGTTGAGGAGGAT ATGGTCCTGGAAGA | 60 | 249 | Author |
| Ift | TGGTTTGCAGAAACCCTGCC GGAGGATGCTGCAGGAGG | 60 | 220 | Author |
| Enteroendocrine Cells | | | | |
| CgA | GACCTCGCTCCTCAAAGGGACCA TGGTGCGCGGGGGTTCTT | 60 | 332 | Author |
| Cck | CAAAGGTTAGACCGGCGAGTC CGCGGGTCTCTAGGAATTGA | 60 | 217 | Author |
| Enteroocytes | | | | |
| Sglt1 | GCAGCTGGTGCTCTCACTCATG CAAACTCTGGAATGACTACGG | 60 | 113 | Author |
| L-Fabp | CCAGGAATACAACTGAGGAGCCCCTCTCCGGTCCATCGAGG | 60 | 225 | Author |
| Growth Factors and Signaling Molecules | | | | |
| Tgfβ | CAGCTGGTGCTCCTCCTTCTTGTG | 62 | 189 | Author |
| Egl | TGGCGAGATGCGGGAATATCA AAGGTTATCTAAGAACAG | 60 | 262 | Author |
| Dll4 | TCATCATGGAACGCGCCGCCGCTTCGCTATAGACCTG | 60 | 224 | Author |
| Wnt3a | GCAGCGCTCCTCAGGAGAAGAAG TGCAGTGACAGAGG | 60 | 201 | Author |
| Wnt11 | CGGGTGACTAGTGCATACGAG TCTCTGAAGATCCGGGAGG | 60 | 259 | Author |
| Lyz | GGTGTGATGCTGCTGGGTA AACTCTTTGTCCTGTCG | 60 | 220 | Author |
| pBD1 | ACCGGTCTGCTTCGCTGCTGCTGCAATCCTCTAGG | 60 | 233 | Author |
| pBD2 | ATGAGGCCCTCCTGCTGCTGACTACTCTCCTGCTGGT | 60 | 259 | [70] |
| Apoptosis | | | | |
| Casp3 | ACCCAAACTTTTCAATATCA ACCAGGTGCTGAGAATATGC | 60 | 143 | [27] |

Table 3. Primers designed for gene expression in porcine intestine.
detailed evidence of the impaired structural integrity of epithelial tight junctions in disease [108–110].

Quantitative gene expression analysis is highly sensitive and contributes to a more complete characterization of intestinal epithelium in the pig. In this study, the PCR primers were specifically designed to amplify target genes currently used as molecular signatures for each cell type and important signaling molecules known to regulate intestinal homeostasis. Interpreting gene expression dynamics during and following intestinal injury can give insight into the cell populations, CBCs (Lgr5\textsuperscript{+}, Olfm4\textsuperscript{+}, Sox9\textsuperscript{+}, Ascl2\textsuperscript{+}, CD24\textsuperscript{+}) versus ‘reserve’ stem cells (Bmi1\textsuperscript{+}), that are compromised or stimulated in the process [44,69,95]. Additionally, the impact on stem cell homeostasis (Wnt3a\textsuperscript{+}), proliferative capacity (PCNA\textsuperscript{+}), and cell fate determination (Atoh\textsuperscript{+}, Hes1\textsuperscript{+}) can be evaluated to interpret epithelial regenerative capacity [44,69,111]. Ultimately, by evaluating the specific post-mitotic cell populations, absorptive enterocyte (Sglt1\textsuperscript{+}, L-Fabp\textsuperscript{+}) or specific secretory cells (Muc2\textsuperscript{+}, Itf\textsuperscript{+}, CgA\textsuperscript{+}, Cck\textsuperscript{+}) that are sensitive to or upregulated in response to injury, potential therapeutics aimed at signal pathway manipulation can be pursued [38,75,112,113]. Having defined qPCR primers allows for thorough and reliable interpretation of cellular pathway dynamics, and therefore, insight into mechanistic processes controlling epithelial regeneration.

An intriguing observation in cross species comparison of the small intestine epithelium is the presence of the Paneth cell lineage in some species and the absence in others. Paneth cells are long-lived post-mitotic cells that reside in the base of the small intestinal crypts in some species. In mice, Paneth cells have been implicated in serving as a ‘nurse’ cell for the CBC stem cells by secreting WNTs and presenting NOTCH-ligands [38,59,60]. In the colon, evidence indicates that a c-KIT\textsuperscript{+} MUC2\textsuperscript{+} cell may serve as an analogous counterpart to the Paneth cell in the colon [114]. The existence of Paneth cells in pigs is still debated [57,58]. Morphologically, Paneth cells can be identified by the ultrastructural presence of an elongated flattened nucleus, large cytoplasm and secretory granules [63,65]. In our porcine studies, TEM and immunostaining do not support the presence of a bona fide Paneth cell; however, our study presents data that is consistent with the interpretation that a Paneth cell equivalent, similar to that of the mouse colon, may be present in pig small intestinal crypts.

Besides enabling detailed mechanistic studies in vitro, long-term crypt culture from the pig small intestine represents a significant advancement toward developing tissue-engineering strategies and stem cell-based therapies. Organoid units from primary small intestine have been placed on biodegradable scaffold tubes and
then implanted into the omentum of an autologous host [25]. The engineered tissue demonstrated morphological characteristics of small bowel 7-weeks post implantation. While these studies used biopsied samples derived from resected tissue, it is likely that a therapeutic mass of tissue would be required to be clinically relevant. The culture method developed in this study will enable a small number of crypts, perhaps from a biopsy, to be expanded ex vivo to increase the mass of epithelium required to test therapeutic strategies for tissue replacement. Detection of successful and functional tissue replacement is fundamental to monitoring outcomes of these new approaches to treat disease and injury to the intestinal epithelium. This new long-term crypt culture model also represents a significant advancement toward development of pharmaceutical screening modalities, stem cell therapy models, and tissue replacement strategies that can all be tested in a translationally relevant context. The comprehensive set of reagents identified, developed and validated will serve as a foundation for using the pig as a translational model to study stem cell-driven regeneration of the intestinal epithelium.

Figure 9. Markers to identify cell lineages within in vitro cultures. The identification of specific cell lineages within in vitro cultures of porcine crypts is shown. The existence of stem/progenitor and differentiated lineages were confirmed in enteroids utilizing the established genetic biomarkers for cell lineage identification: anti-SOX9 (stem/progenitor), anti-PCNA (proliferation), anti-CgA (enteroendocrine), anti-MUC2 (goblet) and anti-sucrase isomaltase (absorptive enterocyte) antibodies. All specific markers (red). Nuclei, blue. Scale Bar 50 μm, inset scale bar 10 μm.

doi:10.1371/journal.pone.0066465.g009

Acknowledgments

The authors gratefully acknowledge Dr. Jody Gookin from the Department of Clinical Sciences at NCSU-CVM for her assistance with the development of the in vitro culture of porcine crypts as well as donation of tissue for BrdU staining. We thank Odessa Marks, Seywon Koh, Jaewook Chung and Ling Guo for their assistance with RTqPCR and Dr. Victoria Newton for her assistance with immunofluorescence. We also thank Wendy Savage of the Education Media & Design department and Dr. Jeanette Shipley-Phillips of the Laboratory for Advanced Electron and Light Optical Methods (LAELOM) at NCSU.

Author Contributions

Conceived and designed the experiments: LMG STM JAP ATB. Performed the experiments: LMG IW. Analyzed the data: LMG JAP ATB STM. Contributed reagents/materials/analysis tools: JAP ATB STM. Wrote the paper: LMG IW JAP ATB STM.
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