ENTEROID CULTURE AND ITS IMPACT ON STEM CELL AND CANCER RESEARCH

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ABSTRACT OF THE THESIS

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The technique of enteroid cell culture has had an enormous effect on the study of intestinal stem cells as well as colorectal cancer, and will continue to aid scientists in making advances in these fields in the future. In this thesis, the intestinal epithelium is introduced and the cell types within it are explained, stating why it is so crucial to understand its properties. The brief history of enteroid culture is gone over, beginning with its inception in 2009 and the expansion and initial discoveries made with the technique up until and including 2012. Next, the main section of the thesis includes detailed protocols on how to extract intestinal crypts and grow enteroid cultures, as well as perform some modifying techniques such as lentiviral infection and the creation of enterospheres. My research and contribution to Dr. Verzi's lab was in the perfecting of these protocols. Finally, the thesis concludes with the most recent discoveries and advances made using the enteroid culture technique, and its potential for future innovation and discovery.

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Introduction

The small intestine is a fascinating organ in which cell types are diverse and cellular life processes are rapid (Peterson and Artis, 2014, Wells and Spence, 2014). Here, the primary goal of the cells which occupy the epithelium of the small intestine is the digestion and absorption of nutrients into the bloodstream. To accomplish this goal, several absorptive, secretory and maintenance cell types are necessary for the intestinal epithelium to function properly.

Goblet cells exist to secrete mucin into the small intestine, protecting it from shearing stress, chemical damage, and microorganisms which would otherwise harm the epithelium. Enterocytes, or absorptive cells, are the main cells which perform the primary function of the small intestine, the digestion and transport of amino acids, monosaccharaides, and lipids from within the intestinal lumen. In addition, these absorptive cells, on the villi of the intestinal epithelium, themselves have microvilli to further increase the absorptive surface area of the lumen. Finally, Enteroendocrine cells act as chemoreceptors in the small intestine, constantly monitoring the digestive tract and epithelium for hormones and harmful substances, initiating specific digestive actions when required based on signals sent throughout the body, and producing a specific hormonal response when needed. These cells are arranged on the epithelium of the small intestine into villus structures in order to increase the surface area for

absorption of nutrients, allowing the most absorptive potential in the smallest amount of physical space.

These digestive and absorptive processes are performed quickly and continuously, with the cells dying and being replaced every few days (Leblond and Walker, 1956), making the epithelium of the small intestine the most rapidly renewing tissue in the human body. New cells are created in the base of a structure called the intestinal crypt, a "valley" between the "hills" of the villi filled with undifferentiated cells that feed the villi with new absorptive and secretory cells constantly. For a visual representation of this, please see Figure 1. The intestinal epithelium*, both the crypts and the villi together as a single unit,* is constantly in motion, as old and overworked cells reach the top of the villus, they are eventually pushed off the tips of the villi and are digested in the intestinal lumen to make way for fresh cells generated at the base of the crypt.

The Intestinal crypt is a very different environment compared to the villus, where only three defined cell types exist, all performing very different functions. At the base of the crypt are small, thin cells known as Crypt Base Columnar (CBC) cells**.** Able to both renew themselves and produce every other type of cell in the intestinal epithelium, these cells are the point of origin for all of the cells which take part in the digestive and absorptive processes of the intestinal epithelium. These properties are why they are defined as the stem cells of the intestinal crypt. Flanking these CBC cells at the base of the crypt are the Paneth cells. Providing an essential niche for the Stem cells (Sato et al., 2011a), whose function is greatly reduced without them, they secrete protective antimicrobial agents,

lysozymes, and WNT signals to keep the stem cells healthy. Unlike all other differentiated cell types in the epithelium, these cells migrate downwards towards the base of the crypt after differentiation, and can survive for many months there before death. Finally, the most numerous of the cell types that inhabit the intestinal epithelium are the Transit Amplifying (TA) cells. These cells are born from the intestinal stem cells at the crypt base, and they themselves divide as they travel up the crypt wall on their way to the villus. As they do this, they differentiate into absorptive or secretory cells, fulfilling their function in aiding digestion (except for those who become Paneth cells). Figure 1 shows an image of the crypts and villi *in vitro*. Due to the combination of the variety of cell types present, the rapid cellular life cycle, and the importance of the function being performed, the intestinal epithelium is an ideal area of study for purposes both medical (including the treatment and study of cancers and IBD) and scientific (the study of the intestinal stem cells, rapidly making renewing and differentiating cells at the base of the crypt).

Colorectal Cancer is one of the most common types of cancer in the United States, with over 100,000 new cases each year and the third leading cause of cancer related death in the country (Cancer.org stats, 2014). Inflammatory Bowel disease, or IBD, which includes Crohn's Disease and Ulcerative Colitis, is a condition of the digestive tract that has the body's immune system attacking its own epithelial cells, causing chronic inflammation. This disease affects over 1.4 million Americans each year, and can currently only be treated but not cured. However, the new culture techniques discussed in this thesis provide a new opportunity to study this disease.

In this Thesis, I will examine the history, technique, findings, and potential of a technique known as Intestinal Enteroid Culture; which in recent years has allowed scientists to view the intestinal epithelium outside of a living organism, while the cells grow and divide. Residing in a Laminin-based matrix known as matrigel (Hughes et al., 2010), epithelial cells can be cultured and grown in structures called Intestinal Organoids, or Enteroids. These budded structures retain the basic crypt-villus morphology of the intestinal epithelium and can be passaged multiple times and exposed to reagents for experimental purposes. First, I will examine the origin of the technique and the first few discoveries made with it, laying the groundwork for future discovery and innovation. Then, I will go into protocols that I have adapted to culture and work with these enteroids. Finally, I will discuss recent advances that this technique has helped to discover in the fields of Stem Cell and Cancer research.

Origin of the Organoid Culture Technique

Rationale

One of the most efficient and versatile ways to study such diseases as IBD in order to learn more about their causes and effects on the body would be to observe the cells in culture. However, this has proven both extremely difficult and cumbersome over the years, in that when originally observed, intestinal epithelial cells die quickly when placed in culture after flow cytometry or differential centrifugation is used to separate them (Ferland et al., 1979). One study involved the harvesting of fetal intestinal epithelium,

which did manage to grow in culture for several days, but ultimately died and did not replicate the intestinal structure *in vivo* (Fukamichi et al., 1992). Another managed to obtain cell culture from adult rats, but it did not survive for more than two weeks and the crypt-villus morphology was not maintained (Evans et al. 1992). These methods, although obtaining a "pure" cell type sample, do not produce a culture that would replicate the cell growth conditions *in vivo*, in which the cells of the intestinal epithelium are organized into discrete crypt and villus structures which require the presence of other cell types to work as they are supposed to. In short, a cell culture system in which the basic epithelial structure is preserved (and functions in the same or similar way) had yet to be identified and used in study as of 2009. This posed a rather frustrating problem for researchers, with an area of study applicable to both medical and academic knowledge, there was no cheap and direct way of observing various effects on it in real time, and thusly making significant research contributions in this area proved to be quite difficult.

With current techniques, however, the intestinal epithelium is now able to be studied *ex vivo,* in which the epithelial structure remains biologically similar to its structure *in vivo*, but the cells are still able to be passaged, observed, and experimented on as they would in a cell culture environment. Before the advent of intestinal epithelial culture *ex vivo*, knowledge about the intestinal epithelium was a long and costly process, using and making transgenic mouse models in order to study the effects of several genes, with the most recent major discovery before the inception of organoid culture being the identification of LGR5 as an intestinal stem cell specific transmembrane protein (Hsu et

al., 1998). In the few short years since its discovery by Dr. Toshiro Sato and Dr. Hans Clevers, the technique has been diversified to work with several other types of cells including colon carcinoma (to study colorectal cancer) and even cells from human samples.

First Discoveries

To design a culture system where intestinal crypt proliferation maintained crypt-villus morphology as *in vivo*, several major factors had to be considered. The first thing that (Sato et al. 2009) did was a literature search for factors that enhanced crypt proliferation. Immediately, from studies by their own lab and several others, it was known that WNT singling was key to crypt proliferation, so a WNT agonist was one of the most crucial components. Kim et al. (2005) discovered that the WNT agonist R-Spondin 1, which was later found to bind to the intestinal stem cell marker LGR5 (Carmon et al., 2011), would introduce a large amount of proliferation in intestinal crypts. Due to this fact, R-Spondin 1 was chosen as the WNT agonist in the Culture media for intestinal crypts. Dignass et al. (2001) Showed that Epidermal Growth Factor (EGF) positively correlates with cell proliferation in the intestinal epithelium, and in transgenic mice who constitutively express Noggin, Haramis et al. (2004) discovered that the number of actual crypts in the intestine increased. These three components, plus the inclusion of N- acetylcysteine as a cell survival promoter, and N2 and B27 growth supplements, together make the core of ENR (EGF, Noggin, Rspondin1) Media, the

standard growth media used for intestinal crypts. One more issue still remained, in that these epithelial cells, when taken out of contact with the mesenchyme, would undergo anoikis. This issue is what prevented intestinal epithelial cell culture from replicating *in vivo* conditions prior, and (Sato et al. 2009) needed to overcome this before a culture system could be established.

(Sasaki et al. 2002) found out that Laminin a1 and a2 are enriched at the base of the crypt; a matrix rich in Laminin should possibly support standard crypt morphology without cell death. Due to BD Biosciences Matrigel being both a Laminin – rich media and it being used to support other types of epithelial cell growth while maintaining morphology, it was used to support the growth of epithelial cells *ex vivo*. The predictions made due to the literature search came true, as the main components of ENR media, when tested separately, could not support the growth of crypts or crypts would not passage properly without all main components. With all of them together, however, crypts actually grew in culture, into budded, spheroid structures called organoids. After the crypts were initially extracted from mouse tissue, they became tiny spheres within a few hours. After a few days, in a process similar to crypt fission, in which new crypts “bud” off of old crypts in order to fix damaged areas within the epithelium, the organoids began to have several buds off of the central sphere, which grew into structures reminiscent of crypts, and were in fact found to have paneth cells at the base of the crypt buds. Eventually, the original sphere began to fill with dead villus cells which *in vivo* would have gone into the lumen of the epithelium (See Figure 2 for an image of various types of organoids).

Of course, several factors needed to be considered before this could be dubbed an accurate model system. Before any experiments could be considered, it had to maintain its structure over several passages and not undergo anoikis or degenerate. In fact, the organoid culture was able to be cultured for over 8 months without any visible differences (Sato et al. 2009).

Mice with the transgene *Lgr5-EGFP-ires-CreERT2* have the ability to have GFP induced in LGR5-positive cells, which are generally accepted to be CBC Stem Cells in the intestinal crypt (Barker et al., 2007). These cells are intermingled between the Paneth cells that support them, and when cultured as enteroids, were seen to express GFP exactly as those *in vivo* would. When crossed with mice of the *Rosa26-LacZ* reporter line (which can be activated by the Cre Recombinase), lineage tracing of cells is available. When looked at under staining for LacZ, it was found that the organoids whose stem cells had glowed produced entirely blue crypts when looked at for LacZ, meaning that the organoids were being continuously grown and replenished via these stem cells, as the crypts behave *in vivo*.

Functionally similar to crypts taken from mice and grown in matrigel, the organoids were further examined for their similarity of structure to *in vivo* crypts. In the sorting analysis, non-epithelial cells were absent, and as in vivo, only a single layer of epithelial cells formed within the Matrigel Matrix. Just like the mature epithelium, older cells which were found to be enterocytes pinched off into the luminal central domain of each enteroid. Here, a system was not only established that could replicate the structure of

the intestinal epithelium in culture, but could in fact, with some difficulty, be grown from a single cell, showing more conclusively that these LGR5+ cells were in fact the stem cells of the intestinal crypt. This, as demonstrated by activities in (Sato et al. 2009) shown here, allowed scientists to study the epithelium in a manner far simpler than before, requiring multiple mice and only in vitro methods of observation. Growth of the enteroids has been modeled *in silico* (Buske et al. 2012), giving new insights into tissue formation, and have been used experimentally in new 3D Histopathology experiments (Chen et al. 2013).

Stomach Organoids

The technique was quickly put into practice that same year (Barker et al. 2009) where LGR5+ Stem Cells were being studied in the stomach. The Stomach also has a constantly renewing epithelium and its cells also arise in structures called pyloric crypts. Expressing behavior close to that of intestinal crypts, these, when harvested and grown in similar conditions (although WNT3A was found to be a required component of the growth media), were able to generate pyloric gastric units extremely similar to the intestinal organoids. However, they grew differently, and remained in a spherical shape without budding, even as they grew larger. Only with the addition of the growth factor FGF10 did the Pyloric Organoids grow into budded structures like the intestinal organoids. E-Cadherin staining was done for these cells to prove that they did indeed grow in a single-layer epithelium, just like the intestinal organoids, and lasted nine months as well

in culture and passage.(Barker et al. 2009). Here, this culture system helped greatly to establish not only the technique for working with stem cells from different parts of the digestive tract, but to establish the LGR5+ cells as stem cells in both the small Intestine and the Stomach.

Colon Organoids

The organoid culture system would be continued to be expanded with organoids derived from colonic crypts (Jung et al., 2011). Like the intestinal epithelium, the colon epithelium also renews itself regularly, with the stem cells at the base of the colon crypt the source Colorectal Cancer (van der Flier & Clevers, 2009). However, those Colonic stem cells had yet to still be properly identified.

Knowing EPHB2 is required to properly position cells along the crypt axis, and is most concentrated at the base of crypts, it was used as a marker to sort the colonic epithelial cells based on position in the crypt. By modifying the organoid culture system by supplementing Nicotinamide (Vitamin supplementation) and enhancing growth and survival with PGE2 (anoikis blocker), human colon crypts were able to grow into closed spheres, which proliferated rapidly and were able to be passaged. Confirming their hypothesis about EPHB2 being enriched in stem cells, when cells high in EPHB2 were sorted and grown in matrigel, they formed organoids at a much faster rate than cells low in EPHB2, supporting their findings.

When WNT3a and PGE2 were removed from the media to test the cells' ability to differentiate, the cells grew in a monolayer which contained all types of absorptive and secretory cells seen in the colon crypt *in vivo,* even after several passages in the standard Colonic crypt media which does not allow for differentiation. This shows that the cells remain multipotent even after being in an undifferentiated state for an extended period of time. This was the first major expansion of organoid culture technique into other tissues, showing its potential broad application in the study of epithelia in general.

New Discoveries (2011-2012)

In 2011, the first wave of new literature began to show the types of discoveries that can be made using Intestinal Crypt culture. In (Sato et al. 2011a), Paneth cells were confirmed to exist nestled between LGR5 expressing stem cells at the bottom of crypts both within the mouse via confocal microscopy, and in intestinal crypt culture. Since the organization of the cells was replicated so readily, the authors were now able to retest an old hypothesis that had been rejected (Paneth cells being critical to the intestinal crypt niche) in previous literature without the direct observation which organoid culture provides.

With a Crypt culture system had been preliminarily established, and that stem cells being the cells of origin for all the cells in the Organoids had been demonstrated, it followed that an entire organoid should be able to be formed from a single stem cell.

However, when the LGR5+ cells were sorted and grown in media, they died immediately without a Rho Kinase inhibitor and a Notch-Agonistic Peptide to maintain crypt proliferation, as Notch signaling is required for proliferation. Even with these additions, growing from a single LGR5+ stem cell was quite difficult as only 6% of the total sorted cells grew into organoids (Sato et al., 2009).

Sorting stem cells from Lgr5-EGFP-ires-CreERT2 mice and taking from the GFP-Hi population (enriched in stem cells), they were combined with Paneth cells sorted from CD24 expression. CD24 is expressed in cells in colon crypts which reside between LGR5+ cells, suggesting a strong relation to Paneth cells. When these cells were seeded in matrigel separately, Paneth cell clusters quickly died off, and only less than 10% of LGR5+ cells formed GFP-expressing Organoids that lasted long-term. However, when seeded together, these cells formed GFP-expressing organoids over 75% of the time. The cells were shown to associate naturally with each other using LGR5+ and Paneth cells sorted from RFP+ and YFP+ cultures (Sato et al. 2011a). With Organoid culture, a previously rejected hypothesis was able to be tested and verified, and a critical discovery about the properties of the cells in the crypt was made. Through a gene expression profile, even more evidence was found of the criticality of Paneth cells to the health of the Stem Cells, in that Notch ligands, WNT and EGF were all expressed by the Paneth cell, and all these are known to be essential signals for supporting the stem cell.

In the same study, it was also found that organoids, when overexposed to Wnt3A, had the normal crypt-villus domain became a large spherical cyst. That was believed due to

the fact that Wnt signaling is the main way by which cells in the intestinal epithelium generate a proliferative progenitor phenotype (van der Wetering et al., 2002). This was found though the examination of the effect of Rspondin1, a critical component of Intestinal Culture media (commonly referred to as ENR media). It was found to amplify local Wnt response to Wnt produced by Paneth cells. In short, the LGR5+ cells would get stronger signaling due to the effect of Rspondin1. Wnt3A in the ENR media was also able to overcome the 10% plating efficiency of single LGR5+ cells to form intestinal Organoids, allowing them to grow as if they were plated with Paneth cells. The experiment was repeated in a much more costly manner *in vivo* to confirm results, as Intestinal Organoid Culture was still very new. Using mice supposedly deleted of Paneth cells, they examined more closely and found that many crypts had at least one Paneth cell to support them, and there were also fewer stem cells as a result (Sato et al., 2011a).

In standard ENR media, colonic crypts did not survive in culture as well (1% survival of colonic crypts in ENR vs. 90% survival of intestinal crypts in ENR), so this called for a change of strategy (Sato et al. , 2011b). Putting their most recent discoveries about Paneth cells into practice, the Wnt signaling of colon crypts was monitored via crypts isolated from *Axin2-LacZ* mice. These colon crypts were found to lose all Wnt expression shortly after culture, while intestinal organoids continually express their own Wnt in culture. Since Wnt3A media had already been used in their previous experiment, it was simple enough to try and use the W-ENR (ENR + Wnt3A) media to culture the extracted colon crypts. This in fact increased survival tenfold and colonic crypts were able to

become organoids in matrigel culture. Due to the fact that the colon epithelium is made of only crypts and no villi, mature cells were found near the top of the crypts when extracted from the colon. When these mature cells were mechanically dissociated from the crypt base before culture, efficiency to form organoids was increased even more.

However, due to the effect of Wnt3A in intestinal organoids causing them to act more like undifferentiated progenitor cells, the same effect was similarly observed in the colon organoids, which had trouble differentiating in W-ENR. Once the culture was started, however, Wnt3A could be pulled after several days and the organoids would show signs of differentiation (Sato et al., 2011b).

Now attention could be turned to replicating models that could up until that point only be observed *in vitro*. Crossing Lgr5-GFP-ires-CreERT2 mice with APC flox/flox mice, one can use tamoxifen to activate Cre Recombinase and delete the APC gene, which cannot be knocked out from birth as the mouse will not be able to live without the gene at birth. Without APC, the WNT pathway is constitutively active. Here, crypts were harvested ten days after the induction of Tamoxifen, and grown according to normal culture protocols, with one notable exception. Since Rspondin1's purpose is to amplify WNT signaling, and here, WNT signaling is constitutively active, it was hypothesized that Rspondin1 was not needed to culture these APC- organoids, and this was shown to be correct in what the paper refers to as Adenoma Organoids. What was surprising, however, is that Noggin was not required to culture these adenoma organoids, which lost LGR5 a week after Noggin was withdrawn, but still lived (LGR5 loss also occurred in

normal intestinal organoids when Noggin was withdrawn). Thus, it was shown that Noggin was required for the maintenance of LGR5 expression in regular organoids but not Adenoma organoids (which grew as spherical cysts rather than budded structures). To show this even further, LGR5-GFP –Hi cells were sorted from LGR5-GFP-low cells in the same manner which had been used in previous papers to obtain LGR5+ cells for growing organoids. Here, however, when they were isolated from Adenoma Organoids, not only did the GFP-Hi cells have the ability to form organoids, but many of the GFP-low cells were able to as well.

Human Organoids

The enteroids culture technique was truly refined in (Sato et al. 2011b), in which not only mouse colonic organoid technique was perfected, but techniques to culture human intestinal and colonic organoids were perfected as well. This is especially important due to IBD directly affecting the colon and known to cause inflammatory phenotypes which would be invaluable to be observed in an *ex vivo* culture setting. Culturing human enteroids proved to be far more difficult than the simple addition of Wnt3A to ENR media, though. At first, human colonic organoids would only last around seven days before death. In an attempt to help the organoids survive, gastrin and Nicotinamide were added, Nicotinamide being especially important in that it was able to keep human colonic organoids alive in culture for one month in a budded state with distinct crypt domains. Afterward, they grew as spherical cysts similar to colonic and intestinal

organoids growing in Wnt3A supplemented media. Unfortunately, after three months, the cells stopped growing, a phenomenon observed in other human cultured cells (Shay et al. 2006). Eventually, after many trials, two small molecule inhibitors known as A83-01(AN Alk 4/5/7 inhibitor) and SB202190 (p38 inhibitor) proved to synergistically extend the life of the organoids to 6 months, and unlike previous attempts, these organoids developed into budded structures.

With these same conditions, human crypts from the small intestine were found to also be able to be cultured with similar results. This new culture, due to its sustainable effects on human intestinal and colonic organoids, was called HISC culture. Furthermore, their findings suggested links between Alk and p38 with maintenance of the human digestive tract epithelium, an effect which does not appear to be shared with mouse cells.

One important difference between human and mouse cultures was that in the standard HISC medium, enterocytes were the only differentiated cells found, with goblet and enteroendocrine cell differentiation blocked by Nicotinamide and SB202190. In these conditions, Lysozyme positive Paneth cells were only observed in human intestinal cultures and not in human colon cultures. When these two factors were removed from the media, goblet and enteroendocrine cells were once again observed.

Disease Models

Now that a (rather simple, compared to the human culture) system had been established to culture organoids representing a disease model, human colorectal cancer samples were attempted to be cultured. It was shown that due to the robustness of cancer cells, Noggin, RSpondin1, and even EGF were all unnecessary components in the media of human colorectal cancer organoids, which grew as irregularly shaped sphere as opposed to the neat, smooth spheres of Adenoma Organoids (Sato et al. 2011b).

One more human disease condition to be represented in the organoid culture system was Barret's Esophagus, a condition where intestinal goblet cells appear in the esophagus. Due to the condition having intestinal cells appear, extracted Barret's Esophagus cells were subjected to HISC conditions in a culture attempt, and could be maintained for a month as spherical cysts only. Only with the addition of Fibroblast Growth Factor 10 were the crypts able to form budded structures and be able to be cultured for a period of over three months. The Gamma Secretase inhibitor DBZ was actually able to block proliferation of the Barret's Epithelium cells, suggesting a possible avenue for treatment (Sato et al. 2011b).

Transgenic Organoids

One more major advance in technique came from (Koo et al., 2011). Here, after so many advances in types of cells cultured, a method was invented that would finally allow researchers to study a transgenic model in the intestinal epithelium without relying on the cost and time it would take to make a full transgenic mouse model. The system designed by (Koo et al. 2011) favored a retroviral infection system based on the murine stem cell virus (MSCV). It was able to express GFP and was selectable by puromycin to ensure only that infected crypts were the ones which survived. This is necessary due to the fact that organoids are constantly regenerating, just like the *in vivo* system they are cultured from. Thusly, only organoids with stably infected stem cells (passing down the MSCV) will survive puromycin selection.

Using Wnt3A to expand the proliferative response of the cells and starting puromycin selection two days afterwards, they were able to infect the organoids with the MSCV system, allowing them to express GFP. Notably, the matrigel had to be mechanically separated from the organoids during infection, as they could not be infected if they were in matrigel at the time. After five weeks, 90% of the cells were able to show consistent expression of GFP.

A more complicated test followed, trying to replicate a system seen in an inducible knockout *in vivo*. The loss of function phenotype for the Notch pathway is shown by stem cells and TA cells quickly differentiating into goblet cells. Because such a switch would kill the organoids before they could grow if a mouse had the phenotype activated

and then its crypts harvested, the process needed to be done using the MSCV Retroviral system. Notch's function in the cell is to activate the TF (transcription factor) Hes1, which in turn activates Math1 (another TF) (Artavanis-Tsakonas et al., 1999). Math1 drives cells to differentiate into goblet cells. To accomplish this, the Retrovirus was modified to be able to induce knockdown or overexpression with Cre Recombinase (CreERT2), which is activateable by 4-OHT (4-hydroxytamoxifen). To induce overexpression, dsRed was inserted upstream of GFP, flanked by LoxP sites (floxed), so that before Cre is Activated by 4-OHT, dsRed will express, but when Cre is activated, dsRed is excised, and GFP is expressed.

To induce knockdown a miRNA system was introduced to follow GFP and express with it polycistronically. To combine this into the overexpression system, two types of LoxP sites were used in the virus. One type, when activated around the puromycin resistance gene and dsRed cassette, allowed them to flip with the GFP and miRNA cassette, effectively switching between them upon activation. With this system, the authors found a way to effectively switch between two phenotypes, all the while being able to observe the cells *ex vivo*. Of course, this system could not have been established without intestinal organoid culture.

Classification of Organoids

The rapid expansion in use of this new culture technique, and its proven versatility and usefulness in research led (Stelzner et al. 2012) and the NIH Intestinal Stem Cell Consortium to make firm guidelines on what each type of organoid culture should be called, so as to minimize nomenclature confusion in the future between literature from different labs.

The first are Epithelial Sheets, which as the name suggests are sheets of epithelial cells lifted off of the mesenchyme through EDTA chelation, and viewed essentially *in vitro*. This follows through with Isolated Crypts, which unlike Epithelial Sheets, are individual crypts that have been separated from the epithelium and mesenchyme. Then, the first true redefinition of terms comes through with the paper defining an Organoid as a truncation of an isolated crypt, with approximately twenty or so cells from the crypt bottom with some mesenchymal material intact. Finally, Mesenchymal Cells and Single Intestinal Epithelial Cells are self-explanatory categories. (Stelzner et al. 2012)

Enterospheres are defined as the “spherical cysts” which papers had referenced beforehand, growing under conditions such as Wnt3A ENR media and in some cases, HISC media. These grow as spheres of largely undifferentiated cells from the intestinal epithelium. The original budded structures formerly referred to as Intestinal Organoids or simply Organoids are now known as Enteroids, with their crypt and villus domains consisting of various differentiated and undifferentiated cell types.

Unfortunately adding a bit of confusion, the term Reconstituted Intestinal Organoid is now introduced. Very similar to enteroids, this is introduced as a catch-all term for a multicellular epithelial structure isolated from the colon or small intestine that has some mesenchymal cell elements. Induced Intestinal Organoids are multicellular structures coaxed into epithelial and mesenchymal differentiation form induced embryonic or pluripotent stem cells. Colonospheres and Colonoids are similar to Enterospheres and Enteroids, except derived from colon epithelium.

Detailed Protocols for Intestinal Enteroid and Enterosphere culture

Here, I will outline in detail the Enteroid Culture protocols that I have used and refined over the past several years in lab, and what components go into them. Perfecting these techniques and using them to aid several projects such as (Chahar et al., 2014) and (Perekatt et al., 2014) was the key experience of my research. These protocols cut down the time and effort in researching the precise workings of the mouse intestine, as well as providing a more accurate picture of the living intestinal epithelium than sections on a slide.

Preparation of Culture Media

Before crypts can be harvested, media must be prepared for the enteroids to culture. The components of this media are prepared as shown in Table 1.

Basal Culture Media (BCM) (1x)	Amount
Advanced DMEM/F-12 Life Technologies cat# 12364-10	500 ml
Penicillin/Streptomycin (5,000 U/ml) Life Technologies cat# 15070-063	5 ml
HEPES Buffer (1M) Life Technologies cat# 15630-080	5 ml
Glutamax (100x) Life Technologies cat# 35050-061	5 ml

The basal culture media (BCM) is Advanced DMEM/F-12 from Life Technologies (cat# 12364-10); Each 500 ml container supplemented with 5 ml Penicillin/Streptomycin, 5 ml HEPES Buffer (Life technologies cat# 15630-080, 100x), and 5 ml Glutamax (Life Technologies cat# 35050-061 100x).

From this, we take 11 ml BCM in a 15 ml conical tube to prepare a 4x mixture of ENR (EGF, Noggin, RSpondin1) media. This is supplemented with 1ml B27 Supplement (Life technologies cat# 17504-044) and 500 ul N2 Supplement (Life technologies cat# 17502-048). N-Acetyl-L-cysteine (Sigma-Aldrich Cat# A7250) is then added at concentration (100 ul of

500 mM). Epidermal Growth Factor (EGF) (Peprotech Cat# 315-09) is then added at concentration (5ul of 500 ug/ml), and finally, Noggin (Peprotech Cat# 250-38) is added at (50 ul of 100 ug/ml).

ENR Media	4x Amount	1x Amount
Basal Culture Media (BCM)	10.845 ml	48.345 ml
B27 Supplement Life Technologies cat# 17504-044	1 ml	1 ml
N2 Supplement Life Technologies cat# 17502-048	500 ul	500 ul
N-Acetyl-L-cysteine 500 mM Sigma-Aldrich Cat# A7250	100 ul	100 ul
Epidermal Growth Factor (EGF) 500 ug/ml Peprotech Cat# 315-09	5 ul	5 ul
Noggin 100 ug/ml Peprotech Cat# 250-38	50 ul	50 ul
Rspondin1 R&D Systems cat# 3474-RS-050	See Below	See Below
Totals:	12.655 ml	50.155 ml

Rspondin1 (R&D Systems cat# 3474-RS-050), perhaps the most critical component for normal Enteroid growth, is also the most expensive. A 50 ug vial can be resuspended in 100 ul BCM, with 50 ul being added to the ENR mix and the other 50 ul stored at -20°C for a second batch

used later. This 12.5 ML mix is then either diluted 4x into BCM to make 1x ENR media ready for use, or stored in 1 ml aliquots at -20°C until ready.

Our Lab uses a line of stably infected HEK293FT cells (Calvin Kuo Lab) which constitutively express Rspondin1. To maximize harvest, they are grown in standard 10% FBS DMEM for two to three days until nearly confluent. Then, they are switched to 10% FBS Supplemented BCM and let culture for 10 days. After this period, they are aliquoted into 1.5 ML tubes through a .2 u**m** filter, and stored at -20°C for future use in media. In our experience, supplementing 50 ul of RSpondin1 enriched media per 1ML of 1x EN (No RSpondin1) Media is enough to allow enteroid cultures to grow robustly.

Wnt3A is a common supplement used in Enterosphere and Enteroid cultures, and we derive it from a line of stably transfected WNT3A L Cells (From ATCC), grown in 10% FBS DMEM supplemented with G418 (500 ug/ml). This media is used to grow and passage the cells. When the cells get near confluence, replace the media with 10% FBS BCM. After four days, harvest the media and run it through a .2 u**m** filter, label it Batch#1, and store it at 4°C, replacing the 10% FBS BCM afterwards. After another four days, harvest the media and run it through a .2 u**m** filter into the first batch of Wnt3A – Supplemented BCM, combining the two batches. This can be stored long term at 4°C. To make W-ENR Media, when resuspending 1 ml of 4x ENR media into BCM, simply replace 2 ml of the BCM with 2 ml of the Wnt3A-BCM. In other words, to go from 1 ml 4x EN media to 4 ml 1x W-ENR media, simply add 200ul Rspondin1-Supplmented BCM, 800 ul BCM, and 2 ml Wnt3A-BCM.

Harvesting of Crypts from Mouse Intestine and Primary Culture Preparation

Before beginning harvest, it is important to have an aliquot of Matrigel ready, as well as what type of matrigel to have. To properly visualize Enteroids and Enterospheres, Growth Factor Reduced, Phenol Red Free Matrigel is required (Fisher Scientific Cat# CB-40230C). It arrived frozen and must be thawed overnight at 4°C in ice. Then, it can be aliquoted and kept frozen at -20°C for most practical use. Too Much freeze-thaw harms the quality of the matrigel. I recommend 500 ul or 1 ml aliquots depending on frequency of use. Either way, begin to thaw the aliquot (always at 4°C on ice) 1-1.5 hours before beginning crypt harvest.

Our experience has shown that the age of the mouse matters little in crypt quality, although an older mouse will have a larger intestine and be easier for someone inexperienced with dissection to harvest. Begin by euthanizing the mouse via $C0_2$ and cervical dislocation. Expose the mouse's ventral side and spray it with 70% ethanol to clean the area, then make a vertical incision close to the genital and continue to cut towards the head, stopping at the ribcage. Take care not to pierce any organs and cause bleeding. Cut along the sides of the mouse near your first cut to expose the intestine. With forceps, carefully remove or put aside any fat deposits blocking your view and work area, and begin to carefully brush aside the majority of the intestine until you can see the part of the colon leading to the rectum near the back of the lower edge of the work area. Carefully cut the colon at this point and begin to pull the entire intestine out of the mouse from this end. If you are worried the intestine will break due to pressure,

try to cut some of the connecting tissues as you see them, with care not to cut the intestine itself. When you have fully extended the intestine from the mouse, cut it as close to the stomach s possible, and place it in a petri dish filled with cold 1X PBS, on ice. Next, take a syringe and fill it with cold 1X PBS, and attach a piece of rubber tubing securely to the dispensing end.

We will be using the proximal half of the small intestine as it contains the most LGR5+ cells Cut the Intestine at what point you desire and discard the rest along with the mouse body (unless needed for other purposes unrelated to enteroid/Enterosphere culture). Attach the intestine's open end to the piece of rubber tubing at the end of the syringe and flush out any undigested food remaining in the intestine. Then, with a single blunt edged scissor, carefully cut open the intestine so that it goes from being a tube to a sheet. Cut this into three approximately equally sized pieces to minimize overlapping of the intestine onto itself and allow for more crypts to come free later.

To prepare for washing the crypts, get three 50 ml and two 15 ml conical tubes ready. Fill each 50 ml tube with approximately 25 ml of cold 1X PBS, and each 15 ml tube with 2mM EDTA diluted in 1x PBS. Now, add your three pieces of intestine to one of the 50 ml tubes and close the cap. Invert several times to wash the intestine. Do not shake the tube as you may lose crypts in the wash; only invert. Shaking will lessen your total crypt yield at this step; the purpose of the washes is to remove excess villi and debris. Repeat this two more times with the other two 50 ml tubes to ensure a clean wash, then transfer the intestine pieces to the 1st 15 ml tube. Put this tube at 4°C in a rotator for 5

minutes, being careful to close the cap tightly. Then, transfer the intestine pieces to the second 15 ml tube, and put it back in the rotator at 4°C, this time for 40 minutes. This will loosen the crypts from the mesenchyme of the intestine.

Once the 40 minutes is up, take the tube out and shake vigorously until the PBS is mostly opaque with dislodged crypt and villus particles. This PBS/EDTA/Crypt/villus mix should then be put through a 70 uM filter into 40 ml cold 1X PBS. Once all the liquid has run through, discard the filter and centrifuge the new 50 ml of liquid containing the crypts at 200g for 3 minutes at 4°C. When this is done, pour off the supernatant and resuspend the pellet in 50 ml 1X cold PBS. Repeat the centrifugation step, only this time; resuspend the pellet in 1 ml cold 1X PBS. Keep the crypts cold at all times to minimize cell death.

At this point, the crypts should be ready to plate, but it will be likely that there will be several times more crypts than than needed for standard culture. Now, 10 ul of the 1 ml crypt suspension should be taken and observed under a microscope. Count how many crypts you can see in the droplet. 50 crypts is a standard amount for a single well in a 24 well plate. Less and it will be too sparse, more and the crypts will not grow properly.. Usually, 10 ul contains approximately 40-60 crypts in a fairly opaque crypt-PBS mix, but it should always be adjusted based on what type of experiment is to be done with the culture. Remember to keep the crypt-PBS suspension well mixed to obtain a representative number of crypts per well. Collect your desired amount of crypts in a 1.5

ml tube and centrifuge it at 200 g for 2 minutes. Carefully pipet out the PBS supernatant.

Immediately put your crypt tube on ice, along with an aliquot of matrigel. All following steps should be done in an HEPA-filtered chemical hood to avoid bacterial contamination. Resuspend your crypts in Matrigel, with 50 ul of Matrigel for every well desired to seed in a 24 well plate. The Matrigel should be very liquid now, but it hardens quickly once put at room temperature, so keep it on ice as much as possible.

Here, careful pipetting is a necessity. Ideally, a bubble of matrigel containing crypts as close to the center of the well as possible is what is most optimal for observation and culture, in each 50 ul aliquot. If the Matrigel touches the side of the well, the crypts will still grow, but they will be harder to visualize. It is also critical to keep bubbles out of the matrigel, as they will make viewing the crypts extremely difficult. Rubbing the 1.5 ml tube between your fingers for a few seconds to solidify the matrigel extremely slightly (so it does not spread out on the bottom of the well to the edge), then pipet two to four wells (50 ul matrigel/crypt mix each) in succession. The best technique I have found is to pipet slowly, starting with a sphere of matrigel forming at the tip of the pipet, then lowering it onto the plate. This will minimize spread and help prevent the matrigel bubbles from touching the side of each well.

Once the seeding of each well is finished, place the 24 (or other size) well plate into a 37C incubator for 10 minutes to allow the matrigel to harden. After this point, carefully

fill each well with 500 ul of ENR media (for growing Enteroids) or W-ENR Media (for growing Enterospheres).

Care and Passaging of Enteroids

From here on, the growth media should be changed every two days (though the first feeding can last 3 days if necessary). After approximately seven to ten days, the enteroids or enterospheres should be gaining confluence and a large number of dead cells building up in the center of each one. Passaging is necessary to maintain a healthy culture and repeatable experimental data.

To passage enteroids or enterospheres, and get rid of dead cell debris, begin by breaking up the matrigel, while still in the well, with a P1000 pipet tip. When it is sufficiently broken, pipet it up and down several times against the bottom of the cell plate in order to break up the crypts. When it is sufficiently broken, the option exists at this point of suspending the enteroids in Trypsin for 1-2 minutes to break them up even more. However, it is critical that they are in Trypsin for only this short amount of time, and then immediately diluted to 10 ml with BCM. Usually, this trypsin step is not necessary for passage and is not recommended (due to risk of culture loss) unless there is significant difficulty in breaking up the enteroids. If they are kept in much longer, then they will likely be close to broken up into individual cells and the culture may be lost. After the cells are in 10 ml of BCM (with or without trypsin), centrifuge them at 200 G for 5 minutes in order to pellet, decant the BCM, and resuspend the cells in another 10

ml BCM for a second wash at 200 G for 5 minutes. Afterwards, decant the BCM once more, and resuspend the cells in matrigel, plating them at whatever ratio desired, with 50 ul of Matrigel (for a 24 well plate) per 50 crypts as the standard.

Lentiviral Infection Protocol

Infecting Enteroids with gene expression cassettes in plasmids is perhaps one of the most straightforward ways to take advantage of *ex vivo* nature of enteroid culture. With it, you can observe epithelial phenotypes and cell behaviors without the costly and time consuming process of making a transgenic mouse. Before I begin elaborating on the technique, it is important to note that most institutions and companies require a separate lentiviral training course for anyone who is going to be performing experiments with it.

In our lab, a Lentiviral infection system based upon (Koo et al. 2011) was used to infect enteroids grown in W-ENR media (to maximize proliferative cells to be infected, otherwise infected cells will die quickly). We used Lipofectamine 2000 (Life Technologies cat#11668-027) and its standard protocol to prepare our lentiviral plasmid for transfection into 293FT cells grown to 80% confluence. The media is changed to collection media the next morning, and two days later, the collection media is itself collected into an ultracentrifuge tube. Our lab found that the most successful infections came from two 15cm cell plates which were transfected with the same plasmid and combined. Ultracentrifugation was done at 18,000 G for 1.5 hours in Beckman coulter

Thinwall Ultra-clear tubes (Fisher Cat# NC9146666). In the original protocol, centrifugation is done overnight, but we found that this worked as well for our lentiviral infections.

After ultracentrifugation, media was decanted into 10% bleach waste and viral pellet was left to resuspend in 500 ul W-ENR media overnight. On the next day (day of infection), the virus/W-ENR mix was removed and spun down at 15,000 G in 1.5 ml tubes to remove cell debris, and subsequently placed in new tubes with 0.5 ul Polybrene for better viral infection (Millipore cat # TR-1003-G) and 0.4 ul Rho Kinase Y-27632 to prevent cell death (Millipore cat# SCM075) (Final Concentration, 10 uM).

To prepare enterospheres for infection, passage approximately five days before your estimated day of infection, with the Enteroids growing in W-ENR media. On the day of infection, passage the enterospheres as you would normally until you are at the step before matrigel resuspension. For a robust infection, it is highly recommended that you use Trypsin for 1-2 minutes during passage. Here instead, resuspend the Enterosphere pellet in your virus/W-ENR media mix, and place it in a centrifuge spinning at 200G for 6 hours at 37C. This process was termed Spinoculation by (Koo et al. 2011). If you do not have a temperature controlled centrifuge available, our lab has found that spinning for 30 minutes and then placing the tubes in 37C incubator for 6 hours also suffices for a suitable infection. After the 6 hours, remove the virus media from the crypt pellet (centrifuge to separate if necessary). Add matrigel and continue as with normal passaging.

Enteroid Selection Treatments

Many protocols require the supplementation of media with additives to ensure that only the organoids needed in the experiment are the ones that live (or die). In my experience I have used supplementation with Puromycin for Lentiviral treatments, as well as the addition of Tamoxifen for treatments of enteroids taken from transgenic mice whose gene of interest is excised by Cre Recombinase upon induction of Tamoxifen.

Here, all that it needed is to add your Tamoxifen at Final Concentration (1 uM) into each desired well, and let it sit in the incubator for the standard feeding period of two days. In my experience, Tamoxifen needs to be added only once for the effects to become permanent in that particular well, even after passaging. This is likely due to the fact that Cre Recombinase has permanently excised the gene. For Puromycin, the final concentration in each well so as not to kill the cells is (1 ug/ml) in 500 ul of ENR media in a 24 well plate. After selection and/or infections, Enteroids were imaged on a Zeiss Axiovert 200M microscope, with Retiga-SRV CCD (Q-Imaging). You can see some examples of images taken with this microscope in figure 3.

Recent Advances –

Discoveries in Stem Cell Function enabled by Enteroid Culture

In 2013, with the enteroid culture technique now being used by a multitude of labs, a plethora of new discoveries were made relating to both cancer and the functions of the LGR5+ CBC stem cells. Yin et al. (2013) used the technique to make headway in the understanding of what makes these stem cells both live and die on their own, and why they do not proliferate when put into culture individually (without paneth cells). Realizing that these ISCs (Intestinal Stem Cells) and their fates are controlled by the Wnt, Notch, and BMP pathways, they set out to find a way to replicate their effects without the use of Paneth cells to support the ISCs.

Their work began by using the familiar Lgr5-EGFP-IRES-CreERT2 mouse line to isolate crypts and grow them in ENR media, the ISCs fluorescing due to LGR5-Dependant GFP expression. As observed previously, when isolated, **L**GR5+ cells grow very poorly, and mostly die in ENR media. Additives such as CHIR 99021 and Valproic acid (notch inhibitor) were added to ENR media in an effort to change dependence on the notch pathway and increase proliferation. ENR-C media also increased GFP+ cells and GFP intensity, while ENR-V decreased differentiation. Combining these two in what was then called ENR-CV media significantly increased the proliferation and expression of GFP+ cells, promoting self-renewal and decreasing differentiation.

Cultures in ENR-CV were found to lack a population known as GFP-low in cell sorting experiments. This population normally represented cells that had just begun to

differentiate from stem cells to TA cells. After cell sorting experiments, GFP+ cell growth and ability to form enteroids was tested in both ENR-C and ENR-V media. Separately, ENR-C increased the ability of single GFP+ cells to form enteroids by 50-fold, but did not maintain GFP expression well, while ENR-V did the exact opposite. When cultured singly in the combined CV media, they could be passaged multiple times and form enterospheres with high efficiency.

From ENR treated crypts, GFP-low cells were obtained and exposed to CV media. They were actually found to revert to a GFP-Hi state in culture. CV media also allowed epithelial stem cells from colon and stomach to be cultured on their own as well. A gene expression analysis of Colonoids confirmed that LGR5 expression was up and differentiation marker expression was down. Altogether, this showed that ENR-CV media promotes maintenance of the stem cell state. QPCR and microarray analysis revealed that other cell markers, such as goblet and Paneth cells, were downregulated, while stem cell markers were upregulated. When returned from CV to ENR media, the Enterospheres grew into the standard budded enteroid structure.

The next experiment was trying to induce differentiation in Enterospheres grown in CV media via additives now that the incredible step of successfully maintaining the stem cell state was obtained. In a manner similar to their previous attempts with inhibitors, a WNT inhibitor (IWP-2) and a Notch Inhibitor (DAPT) were used. In replacing CV with DAPT, LGR5 expression decreased and differentiation markers increased. With IWP-2 differentiation markers increased, but not all at the same levels, with enterocyte

markers showing the most increase. When examining the expression of cells grown in ENR-V secretory cell markers were either greatly reduced or abolished, but not enterocytes. Combining ENR-V and IWP-2, they were in fact able to induce cells specifically to differentiate into enterocytes. On the other side, the combination of ENR-C and DAPT induced cells to differentiate into Paneth cells, and IWP-2 and DAPT together promoted Goblet cell differentiation. This was incredibly important in that it showed, for the first time, targeted differentiation *ex vivo*.

(Heijmans et al., 2013) used enteroid culture to more closely examine the poorly understood process by which the stem cells differentiate into TA cells as the epithelium grows. Generally, in cell differentiation, many proteins (mostly transmembrane) are processed in the endoplasmic reticulum. Grp78 is a chaperone which binds to specific receptors on the ER during a stress response called UPR (unfolded protein response). In UPR, the PRK-like ER Kinase (Perk) phosphorylates the translation initiation factor eIF2a. This eventually leads to an increase in ER capacity, resolving the stress, which kills the cell if it is not managed in this manner.

Finding Grp78 and its components low in stem cells and high in TA cells the closer they were to the villus, testing in Paneth cells showed that only a subset of them expressed high levels. Once again using the Lgr5-GFP mouse line to sort for “Stemness”, in this experiment the level of GFP fluorescence was graded on a more specific scale, with 5 as the highest and 1 as the lowest. Analysis of Stem Cell markers confirmed they were the highest in the 5 population, and UPR components showed an inverse correlation with

these stem cell markers, meaning that their ERs are not stressed nearly as much as the differentiating TA cells.

Now that the difference in UPR activity was confirmed, the next thing that was attempted was to induce stress into intestinal epithelial stem cells. Some *in vitro* experiments were done, but to truly show the effects in the setting most similar to the organism biologically, enteroid culture was required. Activating the UPR response with SubAB, stem cell markers were nearly completely lost, and crypt domains of the organoids began to disappear after 48 hours. This was when Villin and P21 became upregulated, which suggested that the enteroids were differentiating into enterocytes. ER overload response pathways were among the most significantly upregulated, strongly suggesting that an ER stress response was key in reducing stemness.

Enteroids were then collected from mice that held Grp78 as a conditional allele that could be cut out via CreERT2 activation via tamoxifen. It was quickly found that these enteroids, once exposed to Tamoxifen, differentiated and rounded up. When attempted to be passaged, no new cells were observed, showing that without proper mediation of ER stress, differentiation was quick and complete, as well as non-reversible. RNA expression analysis confirmed these results with significant downregulation of stem cell genes, and an *in vivo* experiment confirmed this as well.

Next, the authors used (Koo et al., 2011)'s technique of MSCV viral infection to find out if Perk signaling is sufficient for stem cell and intestinal differentiation in general. The virus was encoded with an shRNA against Perk, and during infection, the organoids were

grown in ENR-C media to produce an enterosphere phenotype. When ENR-C was withdrawn, control organoids quickly began budding, but when exposed to the shRNA, they remained as enterospheres until about three weeks. This showed that without Perk, differentiation is greatly lessened. Stem cell markers were also upregulated when three distinct shRNAs were used against Perk, showing that ER stress plays a key role in Stem Cell differentiation in homeostatic conditions.

Discoveries in Intestinal Tumorigenesis

In (Onuma et al., 2013) the technique of enteroid culture was taken even further in the attempt to reproduce inactivation of the gene APC in an *ex vivo* culture. The transgenic mouse model of APC- has been used many times in previous studies, and combined with gene expression profiles, a large amount of genes related to colorectal cancer have been revealed. However, up until recent times, each gene has had to be tested for its effect in a modified mouse, greatly slowing the progress of knowledge in this area. The authors also note that even when the genes are analyzed in traditional cell culture lines, they might not always be able to be extrapolated to tumorigenesis *in vivo.*

It is for this reason why (Onuma et al., 2013) used a lentiviral system (in that it is able to infect both dividing and nondividing cells) to try to suppress APC function *ex vivo* without using crypt samples taken from a transgenic mouse. Infecting by first dissociating the matrigel, a very high infection efficiency of 75% after 20 days and several passages (Onuma et al. 2013) was achieved. Using this technique, similar to our

own unpublished technique, five shRNA clones targeted to APC were introduced into the enteroids, but all of them failed to produce enteroids that grew. This was not due to the shRNA system as shRNA against other proteins produced viable enteroids. Finally, by infecting with all five APC shRNA together, viable enteroids were produced that grew into enterospheres quickly. Puromycin resistance (Which as shown from (Sato et al. 2011), is required for infections to remain in the enteroids, as it was in our own experiences) was confirmed, as well as loss of APC expression and β-Catenin accumulation. A system that reproduced the effects of APC-deficient transgenic mice was created.

Next, enterospheres infected with the APC shRNA were dissociated and injected into nude mice to see if they could form tumors within the mice. In fact, high proliferation was seen via ki67 labeling and β-Catenin accumulation. Overall, the rate of tumor formation via injection of APC shRNA enterospheres into nude mice was 63% (Onuma et al., 2013). (Onuma et al., 2103) then tried to replicate the act of crossing transgenic mice to see the effects of two modifications together. P53 and PTEN shRNA were cointroduced along with the APC shRNA and the rate of tumor development and size increased rapidly.

Turning away from APC briefly, the next gene co-tested was Kras, which is commonly mutated in human colorectal cancer. The APC deficient enterospheres were introduce into mice with a Cre-Lox system attached to expression of Kras. When both were activated together, tumors arose so rapidly that the nude mice died within two weeks of

injection (Onuma et al. 2013). When Kras was activated on its own, tumors formed, but they were gone 6 weeks after injection, showing that Kras on its own is not strong enough to create lasting tumors.

Finally, when analyzing the tumors and enteroids for stem cell markers, common Intestinal stem cell markers such as LGR5 and BMI1 were not found, but Cancer Stem Cell Markers such as CD44 and CD133 were, suggesting that the population of cells underwent a transformation from intestinal stem cells to cancer stem cells. This study was able to use the enteroid culture system to actually observe a critical moment in cell structure change in an ex vivo setting, the change from stem cells to cancer stem cells, and thusly provided an incredible starting point for future studies on how these changes occur and what can be done to prevent or halt them in vivo.

(Fatehullah et al. 2013) used enteroid culture in their experiments to observe for the first time, changes in tissue polarity that take place accompanying cancer developments. The study, as said by the authors, could only be done because of the advent of enteroid culture. Using the APC fl/fl (A gene which is mutated extremely commonly in Colorectal Cancers) mouse model to culture enterospheres from, they immediately noticed that these APC deficient crypts form enterospheres in ENR media, with the lack of any Wnt3A or other additives. Paneth cells, which normally cluster at the bottom of crypts alongside the stem cells, are here randomly distributed throughout the enterosphere, as they found using the Lysozyme marker to detect Paneth cell locations. Phosphohistone 3 Antibodies were used to detect mitotic cells. In normal enteroids, they are limited to

the crypt domain above the Paneth cells. In these APC- Enterospheres, they are randomly distributed throughout. Ki67 staining for Proliferation revealed likewise, that they were randomly distributed throughout the enterosphere, and even Paneth cells, which do not normally divide, held this marker. This led the authors to conclude that the crypt-villus axis has been lost and that the APC- Enterospheres are reminiscent to cysts which form in Adenomas, elevating them to the status of a model system which can be used to study tumor progression.

Normally, in tumors, when APC is lost, so is tissue organization. Beta-Catenin (which is regulated by APC, and localizes to the cell membrane) was stained for in these enterospheres, as was ZO1, a protein involved in maintaining epithelial integrity and tight junctions. Interestingly, these expression patterns were maintained in the APC- enterospheres as well. F-actin, Ezrin, Integrin, and E-cadherin distribution was also normal regardless of APC deletion. Na+/K+ ATPase was also, for the most part, normally distributed by its restriction to lateral domains. Altogether, this showed that despite the loss of APC, Cell Polarity is more or less maintained in spite of a complete loss of Tissue Polarity. Without the enteroid culture technique, only assumptions and experiments in less representative tissues could be made.

CONCLUSION

Recently, the use of the enteroid culture technique has been used to show that LGR5+ Liver cells can regenerate and expand into enteroids as well (Hutch et al. 2013), and samples of intestinal stem cells taken from fetal mice behave in an entirely different manner than those taken from adults (Mustata et al. 2013). In (Schwank et al., 2013), another breakthrough was made in infecting enteroids with a BAC, allowing them to express transgenes and markers in a method that costs only a fraction of the cost and time of making a transgenic mouse.

In this thesis, I have outlined to the best of my ability why the technique of enteroid culture has been a fundamental and revolutionary discovery in the fields of stem cell and cancer research pertaining to the intestine and colon. My hope is that this technique will continue to expand its applications even further as it had in 2013 and be of even more use in the research of functioning cell cultures in their proper biological arrangements. In the future, I can see enteroid being used for the purpose of creating varied and accurate types of disease models for both mouse and human, and using them to test possible therapies, as well as continuing to harness their similarity to in vivo to study cell differentiation and behaviors as they would in a living organism.

In addition, I hope that the protocols I have provided here are of use to anyone wanting to begin the technique for themselves in a detailed, precise manner. I'd like to thank you for reading my thesis and I hope that it has educated you and excited you about the future discoveries that can be and are being made with this incredible technique.

Figures

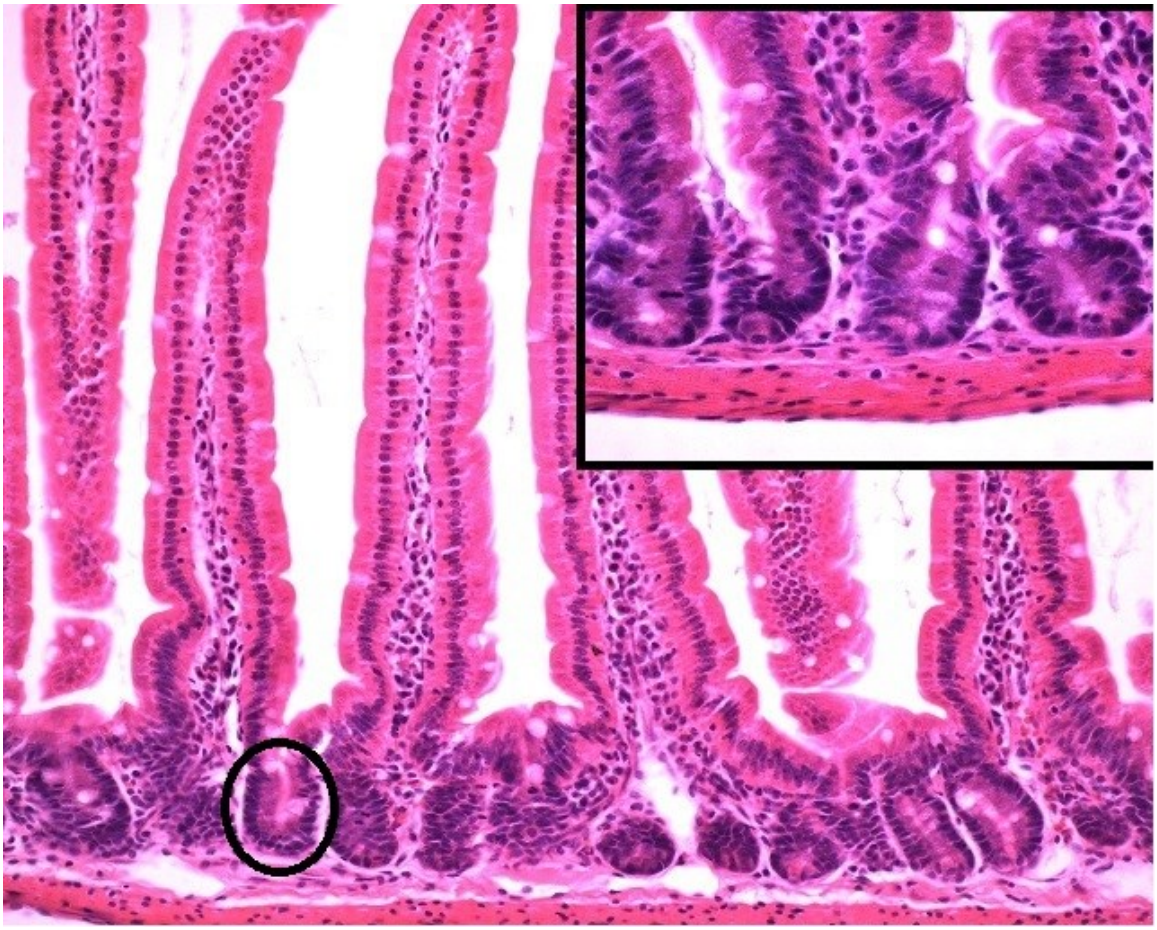

Figure 1. A histology image of a section of small intestine taken from a mouse. In the larger image, the size differential between the villi and the crypts (one encircled and zoomed in in inset) can be seen. This image was taken at 20X magnification with Hematoxylin and Eosin staining.

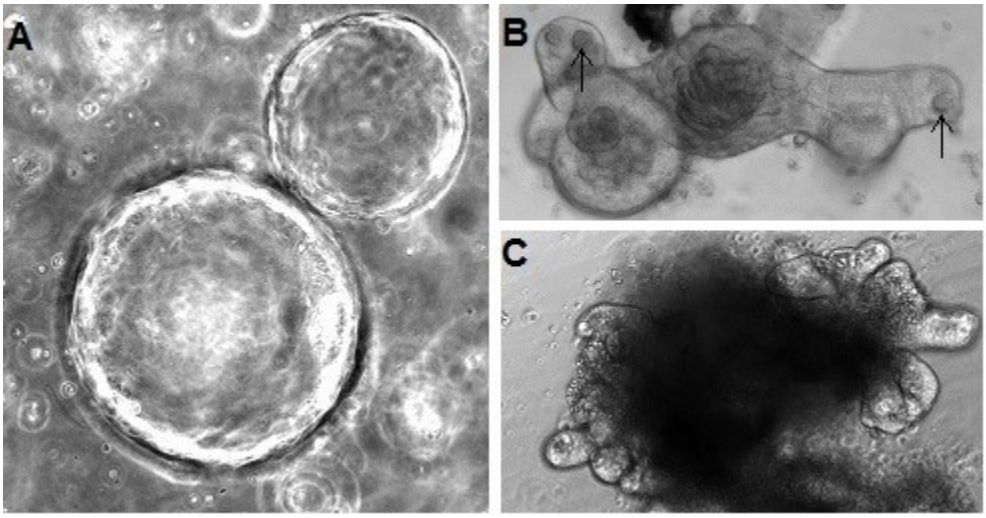

Figure 2. Types of intestinal organoids seen in culture. A. Enterospheres grown from isolated crypts using W-ENR media. 3 days old, 20x magnification. B. A young enteroid grown in ENR media. Paneth cells are pointed out at the end of the buds. 3 days old, 20x magnification. C.A mature Enteroid, ready for passage. Here, the lumen has grown dark and has become filled with dead cells. 8 days old, 10x magnification.

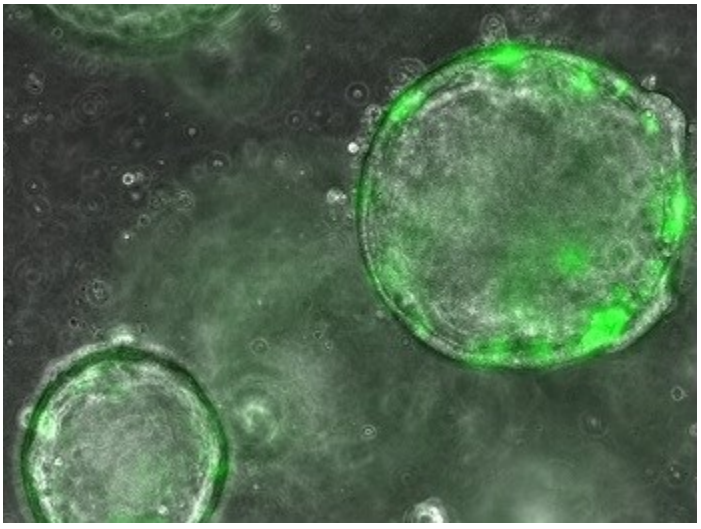

Figure 3. Enterospheres infected with a GFP-Expressing Lentiviral plasmid. 20x Magnification. Grown in W-ENR media.

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