Thesis

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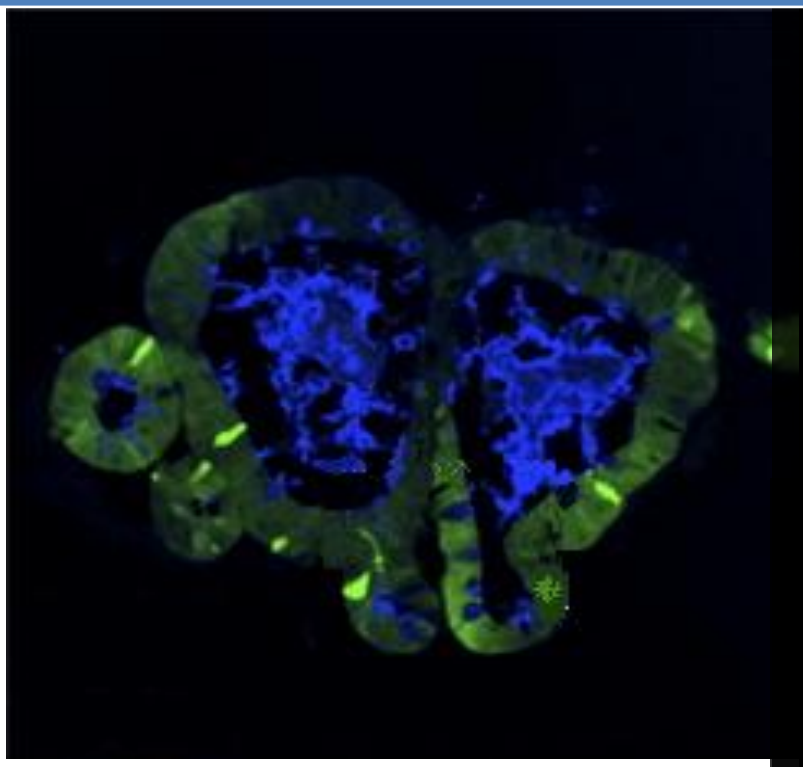
The Regeneration of Intestinal Tissue with Adult Stem cells

The transplantation of human intestinal stem cells (organoids) as an alternative to organ transplantation



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1- Introduction

1.1 *The Intestine*

The intestine (or bowel) is the section of the human gastrointestinal tract that begins at the pyloric sphincter of the stomach and ends at the anus. In humans and other animal species this organ is comprised of two segments; the small intestine (proximal) and the large intestine (distal). In vertebrates, the small intestine performs the primary function of the absorption of nutrients and minerals and is the region in which most digestion occurs [1]. This proximal section is subdivided into three regions: the duodenum, jejunum and ileum and is on average 700 cm in length and approximately 2.5-3 cm in diameter [2]. The large intestine, estimated to be 150 cm in length and divided into two segments (cecum and colon), functions mainly to absorb water from the residual indigestible food and remove waste material from the body [3]. The organ resembles a hollow tube and the lumen cavity is the zone in which the digested food travels and is the region from which nutrients are absorbed. Both sections of the intestine have the general structure of a luminal region surrounded by mucosa, which is in turn surrounded by submucosa, muscularis externa and finally the serosa (Fig 1A).

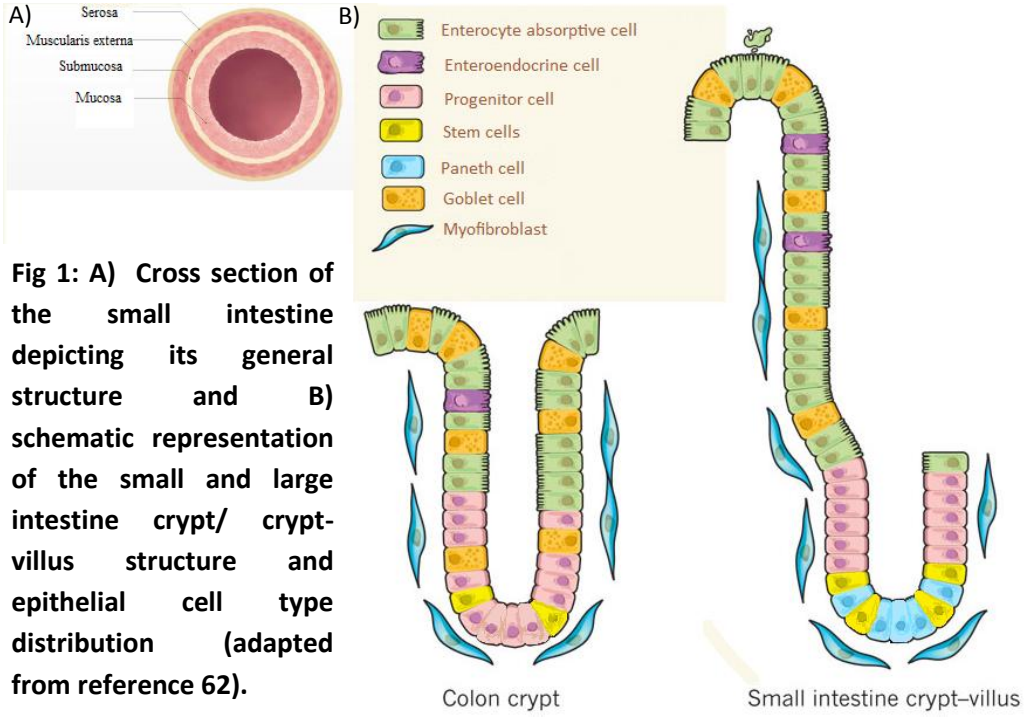


Fig 1: A) Cross section of the small intestine depicting its general structure and B) schematic representation of the small and large intestine crypt/ crypt-villus structure and epithelial cell type distribution (adapted from reference 62).

In the small intestine, the mucosa folds into structures known as villi (Fig 1B). Each villus contains capillaries and lymphatic vessels known as lacteals. These structures are used for the transport of amino acids and carbohydrates (capillaries) as well as lipids (lacteal). Due to these villus structures there is an increase in the intestinal surface area which is further amplified by the presence of microvilli on the epithelial cells of the villus. These structures work together in order to increase the surface area and optimize intestinal absorption. The large intestine does not have villus structures, but rather has invaginations (known as intestinal glands, Fig 1B). Glands found in the epithelial lining of the intestine are named crypts of Lieberkühn and constitute the region of the intestine from which various enzymes are secreted and where intestinal stem cells reside which produce new intestinal cells.

Beneath the epithelium lies the lamina propria, a region containing myofibroblasts, blood vessels, immune cells and nerves. The layer subsequent to this, the muscularis mucosa, is a layer of smooth muscle required for the movement of digested material along the gut and contains additional nerves and blood vessels. This layer is also involved in maintaining the shape of the intestine. Surrounding the first muscle layer is the muscularis externa, made up of longitudinal and circular smooth muscle, this stratum is involved in peristalsis and the movement of food material. The last layer of the intestine, the serosa, is comprised of loose connective tissue coated in mucus and acts to prevent damage to the intestine via rubbing from other tissues. The mesenteries hold all these structures together and suspend them in the abdominal cavity.

The epithelium of the intestine functions as both an immunological barrier and as the surface through which the absorption of nutrients takes place. It is comprised of various cell types, each with a specific structure and function that, on the whole, act together to facilitate the specific activity of the intestine. These major cell types are enterocytes, goblet cells, enteroendocrine cells, paneth cells and stem cells.

Enterocytes (absorptive cells) are simple columnar epithelial cells located in both the small intestine and colon. The microvilli on the apical surface of these cells increase their surface area

in order to achieve maximal absorption of molecules from the lumen of the intestine. These cells additionally contribute to digestion due to their possession of a glycocalyx surface coat containing digestive enzymes. They further play a secretory role by releasing immunoglobulin A, an antibody involved in mucosal immunity, into the lumen [4].

Goblet cells, although categorized as simple columnar epithelial cells, are glandular, secreting mucin that subsequently dissolves in water within the lumen to form mucus.

Enteroendocrine cells are the specific endocrine cells of the gut involved in the production of hormones required for motility, digestion and absorption and are located in the duodenum where they carry out their specific function.

Paneth cells, located at the bottom of the intestinal crypts, interspersing the stem cells (Fig 2), function to secrete immuno protective compounds (e.g: anti-microbial compounds) into the lumen [5]. The previously described major gut epithelial cell types migrate upward along the villi toward the lumen as they mature. Contrary to this, paneth cells migrate downward and are located in between stem cells where they carry out their vital function of defending the stem cells as well as assisting in the maintenance of the stem cell niche [5, 63].

Cells shed form the gut following senescence must be replaced in order to uphold the integrity of the intestine and its function. In order for this to occur a population of self-renewing cells that can differentiate into the four major epithelial cell types must exist [6]. These cells are aptly named intestinal stem cells. The number of stem cells per crypt is kept under strict control, indicating that the members of this population detect each other's presence [7]. Any changes in stem cells numbers subsequently influence not only the duration of the stem cell cycle but, additionally affect: the number of divisions prior to differentiation, the number of lineages the stem cell can generate and finally, the number of cells required for tissue regeneration following damage [7]. In current literature it is estimated that each crypt within the adult intestine contains fourteen to sixteen fully functional homogenous stem cells that are intermingled amongst paneth cells (Fig 2) [7]. Note, the daughter cells of these stem cells designated as transit amplifying (TA) cells are found mostly within the mid-crypt region from

which they differentiate. These cells are produced by the symmetric division of the parent stem cell following the cellular dynamics of a stochastic model [7]. In this model, the two cells formed as the result of one stem cell division do not display intrinsically different fates but rather, can become: two stem cells, one stem cell and one TA cell or two TA cells [7]. Thus, for stem cell numbers to be maintained, homeostatic mechanisms act on the stem cell population to cause the mean production of one stem cell and one TA cell. As a result of this type of division, the stem cells are said to follow neutral drift dynamics, causing crypts to drift to clonality within one to six months [7]. In cases of excess stem cell numbers, the surplus stem cells are removed via apoptosis while, in cases of extreme intestinal damage, new crypts can be generated from a single survivor [7].

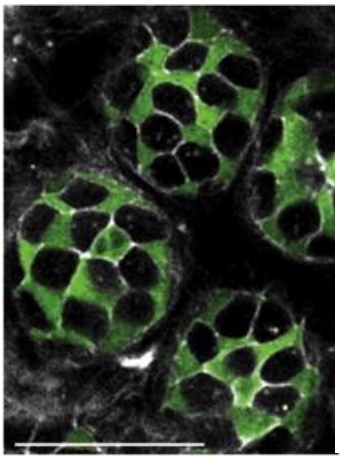


FIG 2: A confocal microscopy cross section of the small intestine that illustrates the distribution of paneth cells (black holes) in between *Lgr5-EGFP-Ires-CreERT2* stem cells (green) at the crypt base [7].

1.2 *Diseases of the Intestine*

Due to the importance of the intestine and the complexity of both its function and make up, it is of vital importance that the system works optimally on both the organ and cellular level. Any error, however slight, can cause disastrous effects. The following insert will illustrate the consequence of a malfunctioning intestine system by using three diseases. Beginning with a disease on the cellular level, Microvillous inclusion disease (MVID) is a congenital disorder affecting intestinal epithelial cells, more specifically enterocytes [8]. Upon a closer analysis of the intestinal enterocytes, it is revealed that there is partial or complete atrophy of the microvilli as well as inclusion bodies within the cell containing immature of fully differentiated

microvilli [8]. This disease causes persistent life threatening watery diarrhea, seen usually within the first days of life (early-onset) or in the first two months of life (late onset). Complications of the disease include intestinal failure [9], ischemia (as a result of hypovolemia) which causes neurological and psychological retardation [10], infections caused by catheter feeding and liver failure. Patients depend on parenteral nutrition however, this method of nourishment is not a permanent treatment with patients predominantly dying within the first 3 years of life. The only and final prospect for efficient treatment of these patients is small bowel transplantation in the hopes to achieve an increase in life expectancy and provide them with intestinal autonomy.

On the organ level, a second kind of intestinal disease can be described. This disease is known as Short Bowel syndrome (SBS), a disorder caused by the surgical removal of the small intestine, complete dysfunction of a section of the gut or in some cases a congenital anomaly [11]. The disease is only manifested when a minimum of two thirds of the intestine is removed with major complications such as malnourishment, pain, anemia and muscle spasms resulting from malabsorption. Due to the pathophysiology of the disease a process known as intestinal adaptation occurs in which physiological changes occur to the remaining portion of the small intestine in order to increase its absorptive power. Some of these changes include the enlargement of villi, increasing the remaining diameter of the small intestine and reduction in peristalsis [11]. Treatment includes anti-diarrheal medication, vitamin and mineral supplements, and total parenteral nutrition. However, even with intestinal adaptation and the administration of medication, in most cases children with congenital SBS have low survival rates [11] and adults show a 15-47% mortality rate [12]. A new frontier of treatment is thus being approached, that of intestinal transplantation. Research shows that this new therapy is proving to be more economical and successful than parentreal nutrition programs and work is currently being undertaken to further optimize the success of this treatment [12, 64].

On a systemic level we approach intestinal disease from an immunological point of view through a malady known as inflammatory bowel disease (IBD). Two major forms of IBD are Crohn's disease and ulcerative colitis [13]. Crohn's disease can affect any part of the GI tract,

with most cases starting in the terminal ileum while ulcerative colitis occurs mainly in the colon and rectum [14]. Although different in manifestation (Crohn's disease affecting the whole bowel and ulcerative colitis the gut epithelium), both disease show similar symptoms including: abdominal pain, vomiting, diarrhea, rectal bleeding, weight loss and an assortment of related complaints [13]. The exact cause of IBD is as of yet unknown, however, the disease-related inflammation is a result of a cell-mediated immune response within the gut mucosa that causes the release of cytokines, interleukins, tumor necrosis factor and other inflammatory factors [15]. Depending on the factors involved in the disease, treatment may involve immunosuppressive drugs, steroids, biologicals (e.g: TNF inhibitors) and in some cases surgery (removal of the affected area) [16]. Although fatality is rare, new treatments aimed at causing complete remission of the disease are currently in the pipeline.

Due to the fact intestinal diseases can be manifested on multiple levels, from the cellular to that of the organ, drug treatments currently in use vary in efficiency, success and quality. There are none to date that cause the complete remission or eradication of the disease state leaving patients with a grim future, one involving continual medication (some with severe side effects) and in most cases, a life expectancy below that of the population average. Even with intestinal transplantation as a final and best hope, complications due to donor matching such as graft-versus-host disease are still a reality. The future however, is not bleak. Current research in the field of gastroenterology is flourishing due to the discovery of a pioneering treatment method that in the future may provide patients with the ultimate cure to their disease. This treatment involves the use of intestinal stem cells in order to generate intestinal tissue that may eventually be transplanted into patients. This thesis thus aims to illustrate and provide a review of current research and literature surrounding the steps taken toward the generation of intestinal organoids for future transplantation and medical research. Providing both a clear overview, in-depth analysis and a look at future prospects in this field, this body of work will vividly and clearly analyze the past, present and future of intestinal stem cell research.

2-Identifying and Isolating Intestinal Cells

In order to begin with the process of understanding, creating and eventually culturing intestinal tissue, investigators had to learn to isolate and identify individual members of the intestinal epithelium cell population. Due to the abundance of the enterocyte, goblet, enteroendocrine and paneth cell populations, the processes developed and currently still used to isolate these cell groups are simple yet wonderfully concise and effective. In addition, as a result of the specificity of function of these cell populations, isolation techniques have focused on histology as well as specific secretory or protein factors of each individual population. These factors are considered to be characteristic to each cell group. Due to the efficiency of these isolation techniques and the advancement of scientific technique, research has progressed to permit the discovery of a multitude of factors expressed by these cells ranging, from cytokines and cluster of differentiation (CD) molecules to hormones and peptides. This section will however focus on the isolation techniques currently used in research involving intestinal epithelia.

2.1 *Histology*

Histology is used in order to determine the location of the specific cell population along the villus or within the crypt. Understanding the location of a cell population will not only contribute to an understanding of the cells specific function but, will also contribute to understanding the role of this cell population in the intestine as a whole. With this knowledge in hand, investigators can check if the intestinal structures they are working with (pre-existing or synthesized) have formed correctly and posses the correct morphology.

Histological analysis gives evidence toward the failure or success of an experiment as well as pathology (or lack thereof), by comparing the results of an experiment (structure, location and characteristics of the cell) to a given standard collected from healthy individuals. This method of analysis is one of the most basic and originating techniques yet still stands due to its simplicity and strength. Enterocytes, as the predominant epithelial cell type of the intestine are found in abundance upon histological review of the intestinal epithelium. Due to their function as absorptive cells, histological analysis reveals a high amount of small villi on the

apical surface that form what is called a brush border (Fig 3A) [17]. These microvillus structures are supported by microfilaments that make up part of a structure known as the terminal web, appearing as a thin line below the villus layer (Fig 3A). In addition to being tall, enterocytes also have a basally located nucleus that is both clear and oval containing several nucleoli [18]. A pale area around the nucleus signifies the location of the golgi apparatus which, is involved in the processing of some of the absorbed nutrients [17].

Interspersed amongst members of the enterocyte population are goblet and enteroendocrine cells. Goblet cells are specialized for mucous secretion, assisting in the lubrication and passage of food through the intestine. These cells exhibit a polarized morphology in which the apical portion of the cells is distended to form a cup shape (due to an abundance of mucinogen granules) and the basal portion is narrow and stem like (due to the absence of these granules) and contains the cell organelles. As a result of the many mucinogen granules, the nucleus is displaced to the basal end and neighboring cells are compressed. The nucleus appears as a compact darkly stained mass while the apical end containing the mucous droplet stains lighter and almost appears empty (Fig 3B) [17].

When looking at the enteroendocrine population, a sparse distribution of the cells is noted within the crypt or along the neck region of the villus (Fig 3C) [18]. Although at least 15 types of enteroendocrine cells exist (each producing a specific hormone) [18], these cells are histologically recognized due to their affinity to bind to specific metal stains (e.g: chromium or silver). Under an adequate resolution, the cells are further recognized for their pale cytoplasm, broad base and high basal density of secretory vesicles. However, this method of enteroendocrine cell recognition is not adequate to differentiate between the different types of enteroendocrine cells (hormone specific) thus, immunocytochemical methods of identification are preferred here [17].

Paneth cells, as previously mentioned, are located at the base of the intestinal crypts where they function to secrete antimicrobial proteins in order to protect intestinal stem cells [17]. These cells have the archetypal serous-secretory cell appearance, pyramidal in shape with a

basally located nucleus and a basophilic cytoplasm and an apical region containing zymogen granules (Fig 3D).

The final cell population to be isolated histologically is that of the stem cells. This group is particularly difficult to isolate due to the fact the cells remain indistinct when in the resting state. Thus, stem cells are only found histologically when they are undergoing mitosis. In this state, the cells exhibit dense and highly basophilic chromatin structures that are easily visible in the intestinal crypts, illustrated in Fig 3E.

Histology is however, analysis in a static system. Via this method of isolation and identification cells are no longer viable. In addition, this method although effective may in-fact be inaccurate due to factors such as: disease pathology, morphological overlap and most importantly, human error, where faults in methodology and analysis can create discrepancies. Thus, in order to isolate and analyze cells in a live system, investigators had to discover methods to viably identify and isolate individual cell populations. These new protocols allowed experiments to advance in both quality and accuracy, creating results that are considered comparable to *in vivo* conditions and recorded in real-time time amongst a myriad of other benefits, with each working towards more advanced experimentation. These methods are elaborated on further bellow.

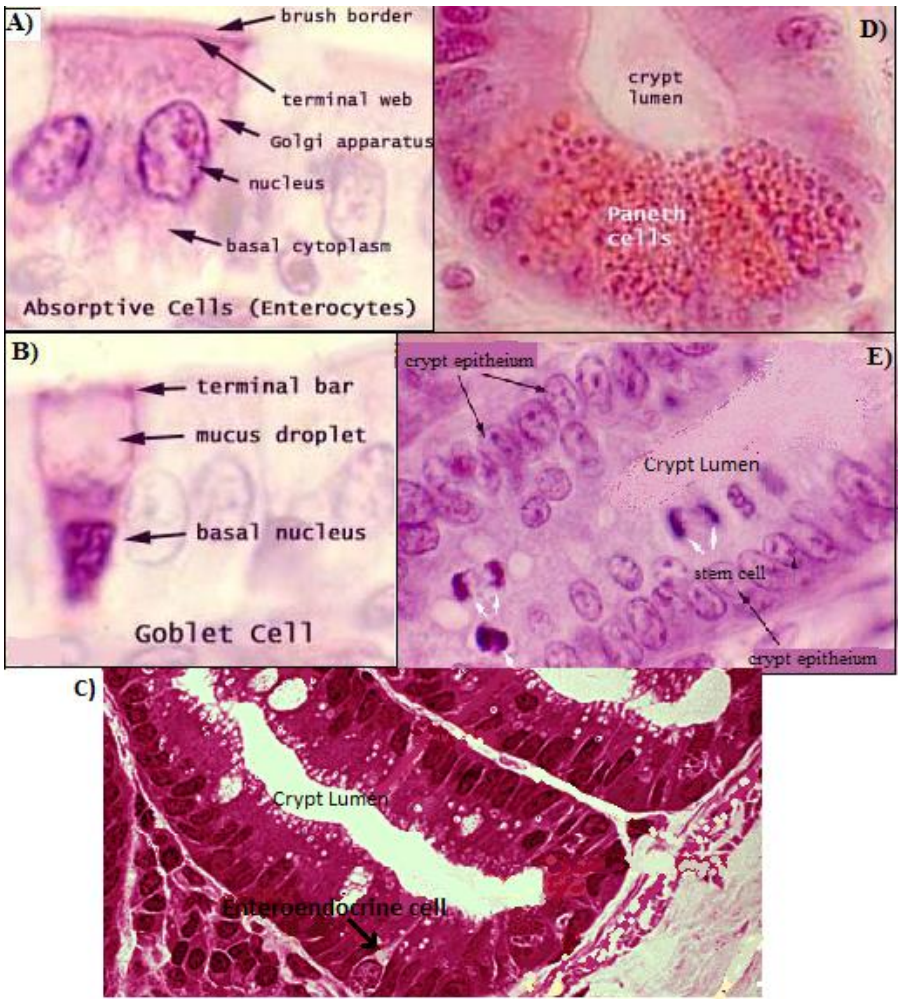


FIG 3: IIustrates the histological appearance of the 5 cell types found in the intestinal epithelium A) Enterocytes B) Goblet cells C) Enteroendocrince cells D) Paneth cells and E) stem cells [adapted from source 17].

2.2 *Confocal Microscopy and Flow Cytometry*

As previously stated, histological approaches although effective and used as a base line analyses for the identification and isolation of cells in the intestine, do not allow for the analysis or isolation of live cells or for real time cellular investigation. In order for investigators to achieve this higher level of experimentation, two methods were employed. Confocal microscopy, for real time (morphological) analysis of single cells as well as cell populations and flow cytometry (FACS) for cell identification and isolation.

2.21 Confocal Microscopy

Confocal microscopy is an imaging technique that uses point illumination and a spatial pinhole to increase optical resolution by the exclusion of out-of-focus light [19]. Through this method, light produced by fluorescence very close to the focal plane is detected creating excellent optical resolution. As a result of the technique used to increase resolution, signal intensity is weakened thus, long exposure times are required. Scanning occurs over regular rectangular or parallel lines in order to create two or three-dimensional images. In order to visualize different members of the epithelial cell population, the cells are incubated with fluorescent antibodies specific to particular proteins produced or expressed by that cell population. These proteins usually play a major role in one of the processes specific to the function of the cell type in question.

Enterocytes, are identified via the fluorescent labeling of the actin-binding protein villin (Fig 4A) [20,21]. This protein associates specifically with the actin core bundle of the microvilli of the brush border on enterocytes [22]. Even though the absolute function of the protein is not known, evidence supports its use as an enterocyte indicator in vertebrates due to its role in supporting the microfilaments of the brush border microvilli [21].

Goblet cells, on the other hand, are identified via their expression of the mucin 2 protein (MUC 2) (Fig 4B) [20]. Encoded by the MUC 2 gene, this protein is secreted from goblet cells in the intestinal epithelium where (along with other mucin like proteins) it polymerizes to form a gel that creates the vital insoluble mucous barrier required to protect the epithelium [23].

There are two markers/proteins commonly used to label enteroendocrine cells. These are chromogranin (Fig 4C) and substance P. Chromogranin is a member of the granin family, a group of closely regulated secretory proteins found in the cores of amine and peptide hormones [24].These acidic proteins are postulated to function as pro-hormones that form a variety of peptide fragments used for autocrine, paracrine and endocrine activities of most hormones [24]. Substance P is a member of the tachykinin neuropeptide family. These peptides excite neurons to cause vasodilation and the contraction of smooth muscles, including those

found in the gut [25,26]. Produced from a polyprotein precursor, substance P acts in unison with serotonin in the intestine in response to the presence of food in the lumen making the gut contract or, in cases of pathology, cause diarrhea and vomiting. Both substances are produced by an enteroendocrine cell sub-population known as enterochromaffin cells.

Paneth cells are identified by the presence of lysozyme. As previously mentioned, one of the main functions of paneth cells is to protect the villus stem cell population from pathogenic organisms. Lysozyme is an enzyme that catalyzes the hydrolysis of bacterial cell walls by attacking the peptidoglycan component of the wall specifically at the glycosidic bonds [27]. Paneth cells produce copious amounts of this enzyme as part of their protective activity thus, when fluorescent antibodies are used to label this particular enzyme, paneth cells are identified and located in the focal plane by the high degree of light/fluorescence emitted from these lysozyme containing vesicles [20].

The final intestinal epithelial population to be identified via confocal microscopy is the intestinal stem cells. Progress in the field of isolating and identifying intestinal stem cells has been slow over the past few decades due to the fact intestinal stem cells do not express specific biomarkers. Current research, however, uses a combinatorial technique to allow the identification and isolation of this population. By using flow cytometry some factors have been identified that are expressed specifically by the intestinal stem cell population. These factors include Sox9 (Fig 4E) and Lgr5 (Fig2). By labeling these molecules with enhanced green fluorescent protein (EGFP), stem cells were able to be visualized for the first time via confocal microscopy [28]. However, as previously stated, these stem cell markers were first isolated via flow cytometry thus, stem cell identification and isolation will be elaborated on in more detail in the following section that discusses the technique of flow cytometry.

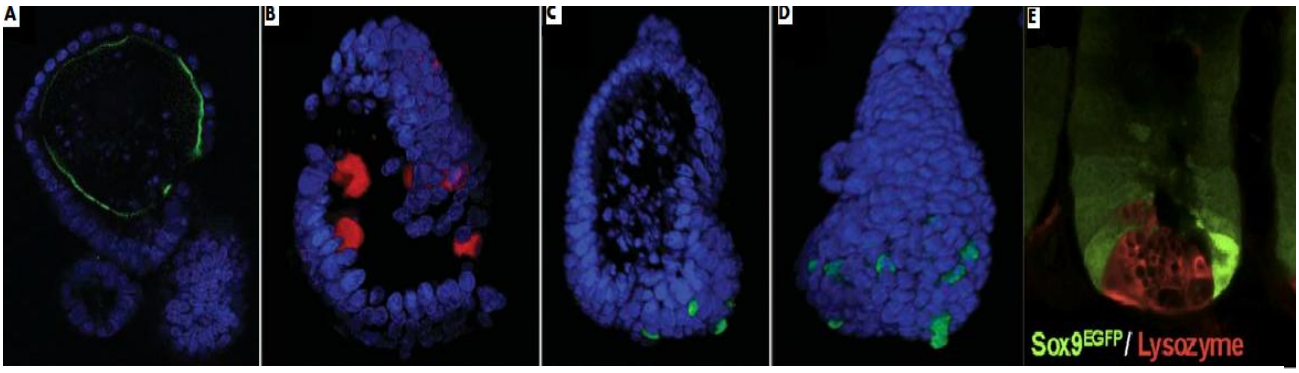


FIG 4: A confocal image illustrating the identification of the intestinal epithelium cell types via fluorescent antibody labeling of specific proteins produced by each cell type. A) Enterocyte identification via villin labeling. B) Goblet cell identification via Mucin 2 C) Enteroendocrine cell identification via Chromogranin and D) paneth cell identification via Lysozyme labeling [20]. E) Illustrates the presence of intestinal stem cells by the expression of EGFP-labeled Sox9 and paneth cells by labeling lysozyme red [28].

2.22 Flow Cytometry

Flow cytometry is a general technique used to count and examine microscopic particles. However, for cell identification and isolation, a specific form of flow cytometry is used: Fluorescence-activated cell sorting (FACS). This is a specialized method via which a heterogeneous mixture of cells can be sorted into two or more containers according to the light scattering and fluorescent nature of each cell [29]. In this process, the desired cell suspension is passed through the centre of a thin, fast moving stream of liquid. A vibrating mechanism causes the stream of cells to dissociate into individual droplets (one cell per droplet). Just before the cells separate into droplets, the flow passes through a fluorescence measuring monitor that detects the fluorescent nature of each individual cell. Immediately after the cells have separated into droplets and been characterized, an electrically charged ring (charged according to the fluorescent intensity of the cell measured) gives the droplet an opposite charge. The charge droplet then travels through an electrostatic deflection system where it is directed into a specific container based on its charge. Note, even if the cells are not required to be separated, via this method each individual cell is characterized and information about their physical and chemical structure can be obtained [29]. Like confocal microscopy, fluorescently labeled antibodies are used to coat and isolate specific cell populations and the information gathered

can be plotted in one and two dimension. However, unlike confocal microscopy this method does not produce images but rather, allows a "high-throughput" quantification of given parameters [29]. Through FACS, individual cell populations can be viably isolated and used for further experimentation whilst, in confocal microscopy, information on the location of cells in the physiological context as well as the cellular location of proteins is obtained. The proteins and molecules labeled and used for confocal identification of cells can also be used for FACS identification and isolation of a desired cell population. In combination with the proteins used for enterocyte isolation in confocal microscopy, FACS uses additional surface markers in order to obtain pure samples of the enterocyte population. These markers are lectin [30], surface proteases (CD10, CD13 and CD26), antigen-presenting cell markers (CD13, CD14, CD35 and CD63), integrins (CD18 and CD61), specific epithelial/endothelial markers (CD21, CD31, CD47 and CD59) CD25, CD28 and lastly HLA-class II molecules [31]. Albers and Moore (1996) have shown that the lectin used for this method of isolation method named UEA-1, is a protein that binds specifically to the intestinal epithelial cell brush border and thus enterocytes [30].

As previously mentioned, scientist faced great difficulty when attempting to isolate and identify stem cell populations as a result of their lack of specific biomarker expression. This all changed however in 2009 when Sato *et al.* discovered that the gene Lgr5 was specifically expressed in cycling crypt-base columnar cells that were distributed amongst paneth cells. Using mice that contained a GFP/tamoxifen-inducible *Cre* recombinase cassette integrated into the Lgr5 locus, investigators could induce the expression GFP in Lgr5+ cells. Cells expressing high amounts of GFP were then isolated via FACS and cultured under specific conditions (further explained in section 4) and were shown to grow organoid structures (Fig 5). Of remarkable note was the fact that the organoids created from these cells contained polarized enterocytes, goblet cells, enteroendocrine cells and paneth cells [20], exhibiting a structure similar to organoids derived from whole crypts. The ability of these single cells to produce new differentiated cell populations while remaining undifferentiated themselves is a characteristic of stem cells.

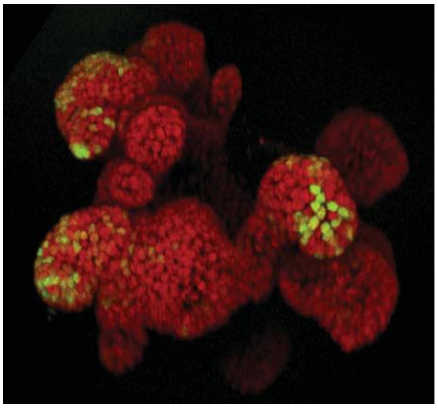


FIG 5: A 3D confocal image of an Lgr5–GFP organoid after 3 weeks of culture. Note the Lgr5-GFP cells are localized at the tip of crypt-like domains, the area that would be equivalent to the crypt base when extrapolated to *in vivo* conditions [20].

In 2010, Gracz *et al.* were able to highlight three additional markers for intestinal stem cells. The investigators discovered that Sox9-EGFP$_{low}$ expressing cells, similar to Lgr5+ cells, were able to produce organoid structures in culture. Sox9 is a known transcription factor that plays a role in maintaining the multipotent and proliferative capacity of stem cells in various tissues [32]. In addition, these Sox9-EGFP$_{low}$ expressing cells were enriched for Lgr5, expressed low/medium amounts of CD24, CD133 and Bmi1, a protein required for self-renewing cell divisions of adult hematopoietic stem cells [33]. The presence of multiple stem cell markers substantiated the conclusion that the cells isolated were in fact intestinal stem cells. As Gracz *et al.* illustrated, FACS is an effective method to viably isolate and identify specific cell populations. Furthermore, the investigators illustrated that labeling cells with multiple fluorescent antibodies is a more potent and efficient method by which to isolate pure cell populations. Enterocytes, paneth, goblet and enteroendocrine cells are all isolated with FACS by using antibodies specific to the hormone, cytokine or receptor expression profile of the desired cell population of interest.

Present day methods for isolating, identifying and analyzing intestinal cell populations combine histology, confocal and flow cytometry to isolate and analyze the cells used in the experiments. This methodological merger creates irrefutable results that are helping the field of cellular (stem cell) research flourish and move forward every day [see references 20 and 28 for examples of methodological fusion].

3-Control of Intestinal Growth and Cellular Differentiation

The development of the intestine and its epithelium during the embryonic stage till birth is well elucidated and understood by academics [34]. This chapter will however not focus on factors involved in the development of the intestine at the embryonic stage but rather on those required for the maintenance, differentiation and supply of intestinal epithelial cells in adults. A plethora of factors and pathways will subsequently be discussed in order to clearly explain and illustrate the background knowledge that is required when undertaking the task of eventually culturing intestinal organoids.

As previously stated, the intestinal epithelium is maintained by the asymmetric division of intestinal stem cells within the crypts of Lieberkühn. The progenitor cells produced as a result of intestinal stem cell division are undifferentiated. However, as these cells migrate to the crypt-villus junction morphological changes occur that result in the production of the four intestinal epithelial cell types [34]. This process of differentiation is closely regulated in order to prevent pathology. In addition, once differentiated, the cells produce additional factors that control not only their function, maintenance and differentiation but also that of neighboring cells. Of first and key importance to note is that the intestinal epithelium exists in a pattern known as the radial (RAD) axis [33]. This means that progenitor and proliferative cells reside at a deeper level than the lumenally located differentiated and functional cells. Thus, when pathways and factors control the development of this organ lining, this axis must always be maintained.

3.1 *Wnt/β-catenin/ Tcf4 pathway*

Wnt genes are known to encode proteins that are secreted [34]. The Wnt pathway is known to be of key importance in the maintenance of the crypt cell population in a proliferative state [35]. Certain members of the Wnt family, particularly WNT3, WNT6, and WNT9B, control canonical Wnt signaling in the intestinal epithelium with their expression restricted particularly to the crypts [35]. The crypt epithelial cells further express corresponding receptors which in turn activate a pathway that involves calcium and the nuclear translocation of β-catenin [36].

β-Catenin forms a complex with T cell factors (TCF), particularly Tcf4, mediating the transcriptional activation of Wnt target genes. The Wnt/β-catenin/Tcf4 pathway is of vital importance in maintaining the proliferating cells of the gut epithelium [37]. Further evidence for the pivotal functionality of this pathway comes from experiments in which the forced expression of the Wnt inhibitor dickkopfhomologue 1 (DKK1) in adult mice causes a loss of crypts and reduction in villus size and number [38]. Over activation of this pathway results in enlargened crypt structures, smaller villi and reduced migration of cells along the villi, suggesting that cells remain crypt-like and do not progress to their differentiated villus-like state [39, 40]. The Wnt pathway is thus strongly activated within the stem cell region and is important for maintaining the stem cell character [35]. This pathway is also involved in paneth cell differentiation thus, if a disruption in the signaling cascade occurs as a loss of Tcf4 signaling, the gut epithelial cells differentiate into enterocytes. However, if the signaling is blocked by DKK1, cells still differentiate into enterocytes but all classes of secretory cells are not formed [37,38]. In summary, the Wnt pathway has two effects. First, the maintenance of the stem cell compartment (proliferative and undifferentiated characteristics) and second, to provide cells with the potential but not the obligation to differentiate into secretory cells.

3.2 *Notch Pathway*

The Notch signaling pathway affects cell differentiation via lateral cell to cell interactions through its cell membrane located receptor Delta [34]. This pathway is active within the proliferative zone of the crypt. High levels of Notch induce the expression of transcription factors such as Hairy and enhancer of split 1 (Hes 1), a transcriptional repressor. By deleting Hes1 in mice or causing a mutation in the Delta1 receptor in zebrafish, the Notch pathway can be partially inactivated, which causes differentiation into goblet and enteroendocrine cells [35]. However, an overactive Notch pathway has the opposite effect, causing a drastic reduction in all three secretory cell types [35]. As previously stated, Hes1 is induced by Notch signaling and activates downstream targets such as mouse atonal homologue 1 (Math1), which is expressed on precursor secretory cells [34]. Math1 mutant mice are known to lack secretory cells but, maintain enterocyte cell generation.

Finally, the Notch and Wnt pathway are known to act together. Wnt signaling activates Notch activity and its pathway components which intern control lateral inhibition within the Wnt-activated population. This activity results in some cells being activated via Notch while others are not [35]. In addition, inhibition of the Notch pathways converts cells to a secretory phenotype and prevents proliferation, while over-activation of the pathway does not drive proliferation [41, 42]. Together these results indicate that the Notch and Wnt signaling pathways act together to maintain stem cells and all the proliferating cells in the crypt [35].

3.3 *Eph/Ephrin pathway*

The Eph/Ephrin pathway is involved in the regulation of epithelial cell migration and the position of the RAD axis [34]. Eph receptors and their ligands are controlled by the level of Wnt activation with Wnt signaling effecting the interaction between ephrin B1 and its receptors EPHB2 and EPHB3 [35]. EPHB2 and EPHB3 are expressed on the proliferative cells while ephrin-B1 is expressed on adjacent differentiated cells [34]. Wnt signaling is known to induce the expression of the EPH receptors while inhibiting the expression of the ephrin ligand. As a consequence, this pathway is crucial in the segregation of the proliferating cell compartment from the cells that have undergone post-mitotic differentiation. Evidence for this comes from experiments in which the deletion of the EphB2 and EphB3 genes causes proliferative cells to migrate across the crypt-villus axis and ephrin B1-expressing differentiated cells to spread throughout the crypt [43].

3.4 *Hedgehog Pathway*

In vertebrates the hedgehog family constitutes three important members: sonic hedgehog (Shh), Indian hedgehog (Ihh) and desert hedgehog (Dhh). These morphogens bind to two homologous receptors: patched 1 (Ptc-1) and patched 2 (Ptc-2). In embryonic development, these proteins signal along a pathway that results in the correct patterning of the tube axis and in its differentiation [35]. In adults however, the exact mechanism by which this pathway affects intestinal homeostasis is not known. In the adult small intestine Shh mRNA is detected at the base of villi near the stem cell region, positively regulating the proliferation of this cell

population [35]. Ihh, on the other hand, is expressed by colonic enterocytes and seems to play a role in their maturation while in the small intestine it is produced only in the proliferating stem cell region and thus at the base of crypts. Taken together these data suggest that Hedgehog pathway is important in the regulation of the stem cell compartment but, the exact mechanisms are still a matter of debate [34, 35].

3.5 *BMP pathway*

In the embryo, the BMP pathway is involved in regulating the number of crypt structures that are formed. However, in the adult, mutations in this pathway are known to manifest a pathology called juvenile polyposis syndrome (JPS). A mutation in SMAD4, a known component of the BMP pathway or, a truncating mutation in the bone morphogenic protein receptor (BMPR1A), results in an increase in the number of crypt structures [34, 35]. Research has also uncovered that Noggin, a secreted polypeptide that inhibits TGF-β signal transduction, functions to protect cells in the crypt region from BMP action, allowing these cells to continue to proliferate [35]. Additionally, the BMP pathway is positively regulated by the hedgehog pathway. Altogether, these data suggest that, in adults, the BMP pathway is vital in the mediation of hedgehog action and prevents ectopic crypt formation and thereby is involved in the maintenance of the stem cell compartment [44].

3.6 *Rho family of GTP binding proteins (Rho GTPase)*

Rho GTPase proteins are known to control the organization of the eukaryotic cytoskeleton [45]. By integrating signals from various pathways, the members of this protein family are key to the migration, proliferation and differentiation of cells [46]. Rac1, a member of this protein family, is known to activate the Jun N-terminal kinase (JNK) and p38 mitogen-activated protein (MAPK) pathway [34]. The expression of a dominant-negative form of the Rac1 protein in mice inhibits intestinal epithelial cell differentiation [34]. Furthermore, continuous Rac1 activation in adult mice causes increased cellular proliferation in the intestinal crypts causing the formation of wide villi [34]. Rac1 activation is known to particularly increase JNK phosphorylation in villous and intervillous cells. The activation of JNK as described above,

alters the actin cytoskeleton, affecting cellular migration and the position of cells along the RAD axis. Thus RhoGTPases are valuable in the process of cellular differentiation [47].

3.7 *Growth factors*

Continuously, new peptides are being discovered that are involved in the regulation of intestinal cell populations and function. This section will discuss seven of the most studied peptide growth factor families known to be involved in intestinal regulation. These are the: epidermal growth factor (EGF) family, the transforming growth factor beta (TGF-beta) family, the insulin-like growth factor (IGF) family, the fibroblast growth factor (FGF) family, the trefoil factor (TFF) family, the hepatocyte growth factor (HGF) family [48] and the R-spondin growth factor family [49]. These growth factors are known to act in a paracrine or juxtacrine way by binding to specific cell surface receptors located on desired cells [48]. Target cells produce and bind more than one type of growth factor. Therefore, these peptide populations and their activated pathways may show redundancies, overlaps and cross-overs. It is this network of action that makes it so difficult to determine the exact function of each peptide. Therefore, the descriptions that follow aim to provide a simple overview of the function that each growth factor family may have in the intestine and is summarized in Table 1.

The EGF family members bind to a variety of receptors of which the most common are the EGF receptors (EGFR). Research has shown that specific functions of EGF in the intestine include stimulation of proliferation (both epithelial and non-epithelial cells), promotion of growth and intestinal neoplasia, stimulation of angiogenesis, the enhancement of epithelial restitution and a variety of other functional changes, i.e. enzyme expression and nutrient transport [48].

TGF-β_1 is a prototypical member of the TGF family of peptides. Detected in all gastrointestinal tract tissues, this growth factor is known to affect intestinal epithelial cells by inhibiting proliferation and by enhancing epithelial restoration.

By acting through two cell surface receptor types (IGF type 1 and IGF type 2), IGF peptides are able to stimulate proliferation of both epithelial and nonepithelial cells as well as to promote intestinal wound healing [48].

FGFs act as autocrine growth factors and are known to stimulate intestinal epithelial proliferation as well as to cause epithelial restoration via a TGF-β dependent pathway.

TFF peptides are expressed in a region-specific pattern along the GI tract. TFF1 is expressed in goblet cells in the large intestine, whereas TFF2 is expressed on the distal ducts of the small intestine and TFF3 is expressed in goblet cells in both the small and large intestine. This family of peptides plays a pivotal role in the repair and healing of the gastrointestinal tract with TFF3 knock-out mice exhibiting low mucosal healing [48].

Although work on the function of HGF in the intestine is still pending, this peptide family has been implicated in the growth and morphogenesis of the intestine as well as in the remodeling of the tract following injury [48].

Lastly, the intestinal growth factor Rspo exerts its effect on the intestine through activation of the Wnt signaling pathway [49]. Each of the four known Rspo factors (1-4) binds to Lgr5 and its relatives Lgr4 and Lgr6. Binding of the Rspo proteins to Lgr5 and its homologue receptors triggers downstream Wnt signals through a family of G protein -coupled receptor proteins: frizzled. Signaling through frizzled-Lrp complexes allows Rspo proteins to enhance short-range Wnt signals released from paneth cells. This enhancement process explains the strong hyperplastic response of crypts to Rspo1, which is specifically known to enhance Wnt signals induced by Wnt3A [65]. By stimulating the expression of Wnt target genes, Rspo proteins are involved in the maintenance of the intestinal stem cell population.

As research continues, a larger number of specific pathways of activity will be uncovered, allowing for a deeper understanding of growth factor-directed intestinal epithelial growth and differentiation which will lead to greater progress in controlling and manipulating culture systems.

Table 1: Peptide growth factor families that specifically affect the mammalian intestine

Growth Factor Family	Members of relevance in mammalian intestine	Function in the Intestine
EGF/TGF-α	TGF-α, EGF, amphiregulin crypto, heparin-binding EGF, heregulin, betacellulin, pox virus growth factors	Stimulation of proliferation and growth Stimulation of angiogenesis Enhancement of epithelial restitution
TGF-β	TGF-β_{1-3}, inhibin A and B, follistatin, activin A and AB, Müllerian inhibiting substance, Vg1, vgr1, bone morphogenic proteins 1-7, decapentaplegic proteins	Inhibit epithelial proliferation Enhance epithelial restoration
IGF	Insulin, IGF I and II	Stimulate proliferation Promote intestinal wound healing
FGF	Acidic FGF (FGF-1), basic FGF (FGF-2) int-2 oncogene, K-FGF, FGF-5, FGF-6, KGF (FGF-7), FGF-18	Stimulate epithelial proliferation and restoration
TFF	TFF-1 (pS2), TFF-2 (spasmolytic polypeptide), TFF-3 (ITF)	Repair and healing
HGF	HGF proteins	Growth and morphogenesis Remodeling of the tract following injury
Rspo	$Rspo_{1-4}$	Stem cell maintenance

EGF (epidermal growth factor), FGF (fibroblast growth factor), IGF (insulin like growth factor), TFF (trefoil factor), TGF-α (transforming growth factor alpha), TGF-β (transforming growth factor beta). HGF (hepatocyte growth factor), Rspo (R-spondin). [Adapted from reference 48]

3.8 *Apoptosis pathway*

Apoptosis is a process of programmed cell death (PCD) that occurs as a result of biochemical changes that cause changes in cellular morphology and function [50]. This PCD is of vital importance in the maintenance of the integrity and homeostasis of the body, especially in the intestine where a high cellular turnover must be counterbalanced by an equally sustained loss of 'old' cells. For most cells in the body, the process of apoptosis follows a similar course triggered by both extracellular and intracellular factors. These factors can range from cell and

DNA damage to hormonal stimulation. However, all have the same outcome: cell death. The plethora of pathways that cause apoptosis are reviewed in reference [51]. However, we will discuss the discovery of a protein that is expressed specifically on intestinal epithelial cells undergoing apoptosis. This protein is known as liver kinase B1 (LKB1), a kinase encoded by the STK11 gene [52]. Cytoplasmic expression of LKB1 occurs in a gradient pattern along the villus with its expression being highest in older differentiated epithelial cells in comparison to those that are newly differentiated [34]. Of particular note, is the involvement of this protein kinase in the regulation of the p53-dependent apoptosis pathway of the intestinal epithelium [34]. Even though the process of PCD is understood in general, it is under continuous research in the field of intestinal epithelial maintenance as it is important in understanding new and inexplicable pathologies of intestinal cancer.

4-Culturing Organoids from Intestinal Stem Cells

Over the past decades, many researchers have tried to set up culturing methods for intestinal epithelial (stem) cells. Protocols to culture whole epithelial samples have been described, each with varying effectivity and each combating various difficulties faced in this system, ranging from controlling the differentiation status of the cells to increasing culture time. This section will only focus on the newly optimized and most efficient culturing protocols, with particular attention to the culturing of intestinal stem cells.

As is in most science, before experiments can be carried out with human tissue samples, experimental conditions and methodology are investigated in non-human species. Gastroenterological research has been carried out in a variety of species ranging from zebrafish to mice. We will focus on culturing procedures with murine intestinal tissue. Subsequently, extrapolating from these mouse data, we will also discuss experiments involving human tissue samples. Understanding these culture techniques could ultimately lead to their application in the clinical setting for the growth of human intestinal epithelial (stem) cells and the eventual production of therapeutic organoid treatments.

4.1 Whole Mouse Intestinal Tissue Culture

Mouse experiments reached new frontiers when Ootani *et al.* managed to utilize an air-liquid interface with 3D matrigel protocol to culture neonatal intestinal tissue samples [53]. Through their methodology, they were able to maintain intestinal proliferation and multilineage differentiation within a range of 30 to 350 days. The investigators initially created the long-term culture system by embedding minced intestinal tissue into a 3D type 1 collagen gel. This gel was then placed on a layer of collagen gel solution on an insert, which was placed in a larger dish containing medium, FCS and antibiotics. The culture medium was changed every 7 days in order to maintain nutrient supply and was kept in a humid 37°C atmosphere with 5% CO_2 [see further methodology reference: 53]. It was shown that cells grew well for a minimum of 30days and differentiated appropriately, forming crypt like structures within the monolayer. Finally these structures formed lumen-containing spheres that showed autonomous

contraction within outer surrounding muscle layers. Investigators noted however that, over time, proliferative zones and viable tissue became more sporadic [53]. This deterioration led investigators to postulate on the manipulation of stem cell-promoting pathways in order to create and maintain proliferative zones that could continuously supply fresh cells in order to create a more long-term expandable culture system. Therefore, investigators added a known Wnt signaling agonist, the recombinant Rspo1 to the culture medium. The addition of Rspo1 caused an increase in the number and size of intestinal spheres that were formed as well as the number of proliferative cells. The recombinant protein extended the culture time by 28days from the initial 7 that did not contain Rspo1 [53]. In summary, Ootani *et al.* concluded that the use of a 3D gel structure 1) facilitated stem cell behavior and tissue morphogenesis and 2) by mimicking normal tissue organization, the gel was able to induce suitable epithelial cell polarity and induce cell to cell signaling. Furthermore, the use of the air-liquid interface permitted long-term culture due to improved oxidation [53]. However, due to the use of minced cellular tissue samples, this method only allows mouse intestinal epithelium to be cultured in close proximity of myofibroblasts. Therefore, the growth of the intestinal epithelium was also dependent on the growth of these cells [53]. Moreover, researchers noted that even with the addition of Rspo1, cultures could only be extended for a limited number of days. This prompted the postulation that the limited culture time was due to a deterioration in myofibroblast condition or a lack of appropriate niche components. Ideally, effective long-term intestinal epithelial cell production and culture should a) not be dependent on the presence of other cells rather than intestinal epithelial cells b) be able to be cultured from stem cells rather than whole tissue samples and c) be able to be cultured ‘indefinitely’, as it is in *vivo*. Therefore, the work of Ootani *et al.* does not permit the culturing of intestinal tissue for patients suffering from cellular intestinal disease pathology, because whole tissue samples are cultured meaning that the pathological tissue itself will be cultured. In addition, the methodology is insufficient to produce enough viable, fully functioning cells.

4.2 Mouse Crypt and Intestinal Stem Cell Culture

Keeping in mind the pitfalls of the pioneering work performed by Ootani *et al.*, Sato *et al.* decided to create intestinal cell cultures based on intestinal epithelial stem cells. By isolating and appropriately culturing this cell population the investigators were be able to a) create cell cultures that could be expanded effectively for as long as desired, b) control the differentiation of the intestinal epithelial cells produced, and most importantly c) create a methodology that could be used for human tissues as well. First, a method for murine intestinal stem cells was developed. Sato *et al.* composed an effective protocol for the long-term culturing of single small intestinal crypts [20], which were mixed with matrigel and plated.

The reason for using matrigel was to allow the culturing of intestinal epithelial cells without the presence of myofibroblasts as it is known that isolated intestinal cells undergo a process known as anoikis (a type of PCD) when removed from the normal tissue context [54]. Matrigel is a gelatinous protein mixture that resembles the extracellular environment found in a multitude of tissues [55]. Matrigel is able to stimulate complex cell behavior due to the presence of structural proteins such as laminin, entactin and collagen [55]. Laminin is known to be enriched within the crypt base implicating that laminin-rich matrigel would be suitable to best simulate the crypt niche [20].

Next, Wnt signaling was provided through the addition of Rspo1 to stimulate crypt proliferation, EGF was added to induce intestinal proliferation and Noggin to induce further crypt expansion. The result of this culture protocol was the creation of organoids comprising of crypt domains encompassing a central lumen region containing apoptotic cells. The organoids themselves are made up of all four intestinal cell types including proliferative stem cell regions [20]. An even greater success of the protocol was that the organoids could be dissociated, replated and regrown (for more than 8 months) without the induction of stress or PCD-related genes (Fig 6) [20]. Investigators had thus created the optimal protocol for a long term intestinal cell culture. As a result of this establishment, stem cells could now be isolated (procedures described in section 2 and reference 20) and cultured using the new technique.

When single isolated stem cells were cultured under the previously mentioned conditions, crypts that were formed were entirely differentiated indicating that the stem cells did not retain their stem cell properties [20]. In order to combat this apparent loss in stem cell character, Rho Kinase inhibitor, Y-27632, was added to inhibit stem cell anoikis. In addition, it is known that cell-to-cell Notch signaling is required to maintain proliferating crypts thus, investigators altered the matrigel composition by adding Jagged-1, a known Notch agonistic peptide [20]. Under these new and improved conditions, the isolated stem cells were able to form large crypt organoids containing the various intestinal epithelial cells in their specific localization, either in the crypt or in the villus domains. These organoids were in fact identical to those produced from whole crypt structures, showed characteristic intestinal cell loss and could additionally be replated and regrown [20]. With this protocol, mouse intestinal stem cells could be isolated and expanded into small intestine epithelial structures similar to those of the normal gut.

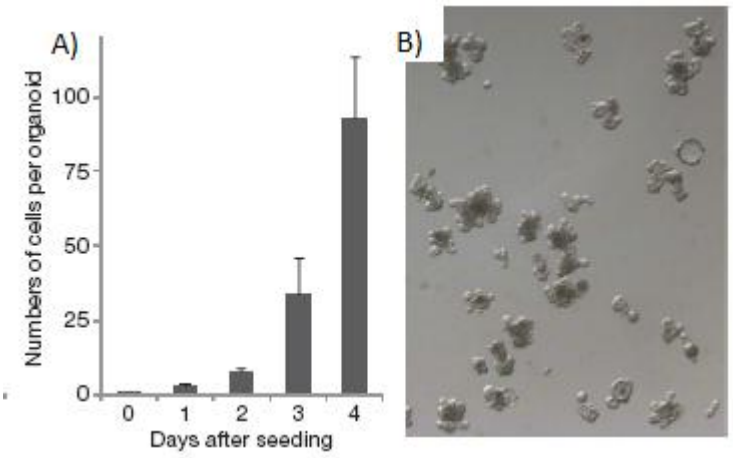


Fig 6: Illustrates the successful replating and regrowth of single-cells obtained from a single-cell-derived-organoid for 2 weeks. A) Clear exponential growth pattern following day one of seeding, the error bars indicate s.e.m. B) shows the well formed organoids formed following replating [20].

4.3 Human Intestinal Tissue Culture

After establishing the successful long-term culturing of mouse small intestine crypt and single stem cell-derived organoids, it was only a matter of time before the protocol was to be tested on mouse colonic cells and human intestinal tissue. Sato *et al.* thus adapted the new and improved culturing protocol to grow mouse colon and human small intestine and colon organoids [56]. Single cells or fragments of epithelium were embedded in matrigel and

stimulated with the growth factors ascertained from the previous protocol. It was noted that mouse colon epithelium, when cultured solely under these condition, had a low plating efficiency, could not be passaged and experiments were aborted within a short period of time [56]. As is known, intestinal stem cells require Wnt signaling to remain viable and active. Upon further analysis, the investigators noted that shortly after culture initiation, colonic crypts lost Wnt reporter expression, suggesting that colonic organoids did not produce enough Wnt signaling to maintain their stem cell population [56]. Subsequently, a Wnt ligand was added to the cultures (recombinant Wnt3A) in order to generate colonic organoids containing many stem cell-containing buds [56]. However, the addition of Wnt3A to small intestine cultures had an undesired side effect. Due to the continuous stimulation of the Wnt pathway, organoids consisted mostly of undifferentiated progenitor cells and failed to differentiate accordingly. Upon removal of Wnt3A, the organoids differentiated to produce all the appropriate epithelial lineages [56]. Notably, colonic stem cells were also successfully cultured using this method with the extra addition of Y-27632 (a selective kinase protein inhibitor of Rho-associated) for the first 2 days of culture in order to prevent stem cell anoikis. The investigators had now created a clear and well-balanced protocol for the long-term culture of single crypts as well as single sorted stem cells from the small intestine and colon of mice.

The next step would be to adapt these culture conditions for human intestinal tissue. Human colon crypts were cultured using the previously described conditions for mouse colon tissue. Under these conditions the crypts preliminarily survived, however, the cells disintegrated within 7 days [56]. In order to enhance growth efficiency and survival, a multitude of components (growth factors, hormones and vitamins) associated with intestinal activity and survival were screened. It was discovered that gastrin (which only had a marginal effect) and nicotinamide (which was indispensible for increasing culture time past 7 days), positively affected plating efficiency [56]. In addition, both factors did not affect cellular differentiation. The organoids could only be expanded for up to 1 month after which they became cystic structures and exhibited an abnormal morphology and eventually exhibited growth arrest after 3 months [56]. It has been observed in various primary cell culture conditions that cells undergo

two phases of growth arrest [57]. These phases are known as M1-senescence and M2-crisis. In order to further optimize culture conditions, the investigators screened mitogen activated protein kinases belonging to signaling molecules associated with colon cancer and of histone modifiers [56]. They found two small molecule inhibitors A83-01 and SB302190 that improved culturing efficiency. This may be due to the fact A83-01 is an Anaplastic lymphoma kinase (Alk) inhibitor and SB202190 is a P38 mitogen-activated protein kinase inhibitor, both of which are involved in cell stress and apoptosis. The inhibitors thus inhibited the two phases of growth arrest and allowed cells to be cultured for up to 6 months [56]. Combining all these factors, the human colonic organoids were shown to form budding structures containing the proliferating cells, comparable to intestinal crypt structures [56].

Human small intestinal crypts were also grown under these conditions and similarly flourished to produce organoids with the desired morphology [56]. However, as was noted in the mouse culture conditions, Wnt signaling also inhibited differentiation within the organoid, as was shown in the human colonic and small intestine organoid culture conditions as well. Therefore, withdrawal of Wnt signaling allowed enterocyte differentiation, but goblet cell and enteroendocrine cell differentiation was still inhibited. It was found that the removal of nicotinamide and SB202190 from the culture conditions allowed the cells to differentiate into goblet cells and enteroendocrine cells [56].

The culmination of the previously described investigations is the description of two new protocols that allow the long-term culture of both mouse and human small intestine and colon-derived crypts and stem cells (see Table 2 for summary of conditions). The organoids produced via this methodology are not only functionally similar to in vivo intestinal epithelial cells and morphology but also do not exhibit cellular mutations or transformations associated with long-term cultures. It is this final and most recent step in intestinal epithelial culturing experimentation that has brought science closer to the production of organoids containing functional cells that, in the future, may be transplanted into living organisms. For therapeutic purposes however, the small intestine will be the main focus of investigation as these patients rely on parenteral feeding while those that lack a colon are able to survive. What lies ahead is

an exciting journey of innovative science that aims to develop alternatives to organ transplantation.

Table2: A summary of the optimized culture conditions required to produce mouse and human small intestinal or colonic organoids.

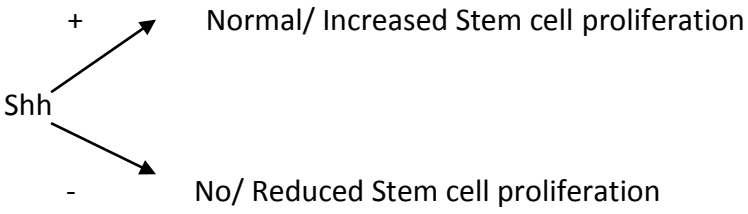
		Stem cell culture condition									**Differentiation condition**
Species organ		**Basal Culture Medium**	**EGF**	**Noggin**	**R-Spondin**	**Wnt3A**	**Nicotinamide**	**Gastrin**	**A-83-01**	**SB2021190**	**Withdraw (-) or add (+) indicating factors**
Mouse	Small Intestine	O	O	O	O	-	-	-	-	-	
	Colon	O	O	O	O	O	-	-	-	-	-Wnt3A
Human	Small Intestine	O +gastrin	O	O	O	O	O	O	O	O	-Wnt3A -SB202190 -nicotinamide
	Colon	O +gastrin	O	O	O	O	O	O		O	-Wnt3A -SB202190 -nicotinamide

Basal culture medium: advanced Dulbecco's modified eagle medium/F12 supplemented with penicillin/streptomycin, HEPES, Glutamax, 1xN2, 1xB27 and N-acetylcysteine. EGF (epidermal growth factor). [Adapted from reference 56].

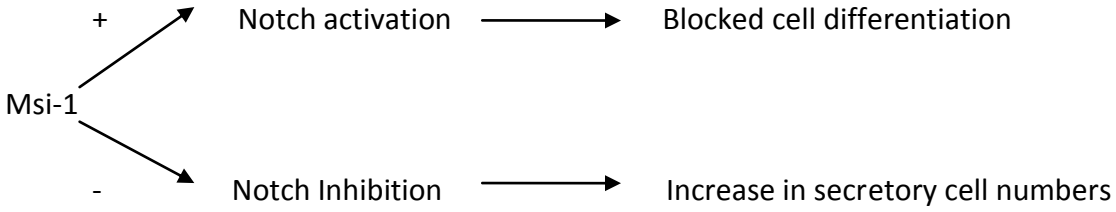
5- Discussion and Future Work

Thus far, science has attained the ability to successfully culture intestinal organoids. Using the techniques of Sato *et al.* (section 4.3) in combination with efficient and scientifically sound methods of analysis (section two), the goal of achieving intestinal tissue transplantation is on the horizon, however, the final synergy between laboratory and clinic remains out of reach. A few factors remain to be ascertained before this final fusion can occur. This section will thus discuss possible future work extending from the loop holes of the research that was described in the previous sections and aiming towards eventual clinical application.

As mentioned previously (section 3.4), Hedgehog proteins are involved in the regulation of the intestinal stem cell compartment [35]. The exact mechanism of this regulation is unknown. What is known is that the expression of these proteins is highest in the colon region with Sonic hedgehog (Shh) facilitating colonic crypt stem cell proliferation and Indian hedgehog (Ihh) positively affecting colonic enterocyte maturation [35]. The vital role that Shh seems to play in colonic stem cell maintenance must be analyzed further. Inhibiting this pathway or creating an inducible knockout of the protein during organoid growth can provide clear evidence toward the role of this protein in stem cell maintenance. If inhibition of Shh activity inhibits the proliferation of the stem cell compartment, a new method to increase colonic stem cell culturing could be discovered. Furthermore, if inhibition of stem cell compartment proliferation can be rescued by the addition of a Shh activator or stimulator, additional support will be provided for the addition of this factor to colonic culture systems, eventually optimizing colonic stem cell proliferation while simultaneously facilitating cellular differentiation. If Shh is determined to be of key importance then the simultaneous addition of both Wnt and Shh stimulation could synergistically increase culturing efficiency and stem cell proliferation, optimizing the culturing protocol even further.



The Notch pathway is known to be involved in maintaining the proliferative cells in crypts. A loss in Notch signaling leads to an increased number of secretory cells while a gain in signaling completely blocks cell differentiation [58]. Hes1 production occurs as a result of stimulating the Notch pathway [35]. The expression of the RNA-binding protein Musashi-1 (Msi-1) correlates with Hes1 expression [58]. In the embryo, it was shown that Msi-1 is capable of activating Notch signaling by repressing the translation of a Notch inhibitor (m-Numb) [58]. However, it is not known if Msi-1 can activate the Notch pathway in intestinal crypts. Thus, future work could uncover whether or not, like in the embryo, Msi-1 activates Notch signaling. In order to do this, researchers could over express Msi-1 protein or add a Msi-1 agonist to intestinal crypt culture in order to determine if this will cause a block in cell differentiation. Furthermore, complete inhibition Msi-1 activity could cause an increase in secretory cell numbers. If the afore mentioned hypotheses are true, a new mechanism to control intestinal epithelial cell differentiation will be discovered assisting researchers in creating the perfect niche to develop intestinal organoids.



As mentioned previously, BMP signaling prevents ectopic crypt formation and is positively regulated by the hedgehog pathway (section 3.5). However, the exact interaction between these two systems is unknown. The question of how BMP controls crypt formation and together with hedgehog regulates stem cell proliferation is a delicate balance of protein production and regulation. Mutations in the hedgehog pathway have been known to cause carcinomas [59], postulating that the BMP pathway controls ectopic crypt formation by manipulating specific hedgehog factors in order to promote proliferation, yet prevent over-stimulation and the formation of cancerous ectopic structures within the intestinal epithelium. Understanding this pathway could assist in creating a more exact culture niche and could also assist in uncovering new possible routes for cancer treatment.

Current approaches to use organoids in a therapeutic setting involve a simple yet effective technique. Via a controlled process, intestinal tissue is damaged. Following this damage, organoids are infused into the body where they make their way to the injured region. At the site of injury, the organoids attach and “patch up” the lesions (Fig 7 and Fig 8A). Through this process, the patches become healthy intestinal epithelium containing all differentiated cell types (Fig 8) ultimately restoring that specific region. In some disease cases however (e.g.: Crohn’s disease), the region to be reconstituted has been removed surgically in order to combat pathology. If, for example, the ileum has been surgically removed, patients lack bile salts and vitamin B12 uptake. It is yet unknown if ileum function can be restored in another part of the small intestine via this patchwork process. By using current knowledge on directing epithelial cell differentiation, future work can involve growing organoids constituting cells that have the missing specific function and testing whether or not these can be inserted into other regions of the intestine. Importantly, these patches should function sufficiently in order to provide the specific functionality of the missing region. However, currently, it remains unknown whether this method if intestinal reconstitution can be translated to humans in a clinical setting. Even though the greatest difficulty of producing a whole organ is avoided, this patchwork method could remain insufficient in providing sufficient amounts of nutrients to the body.

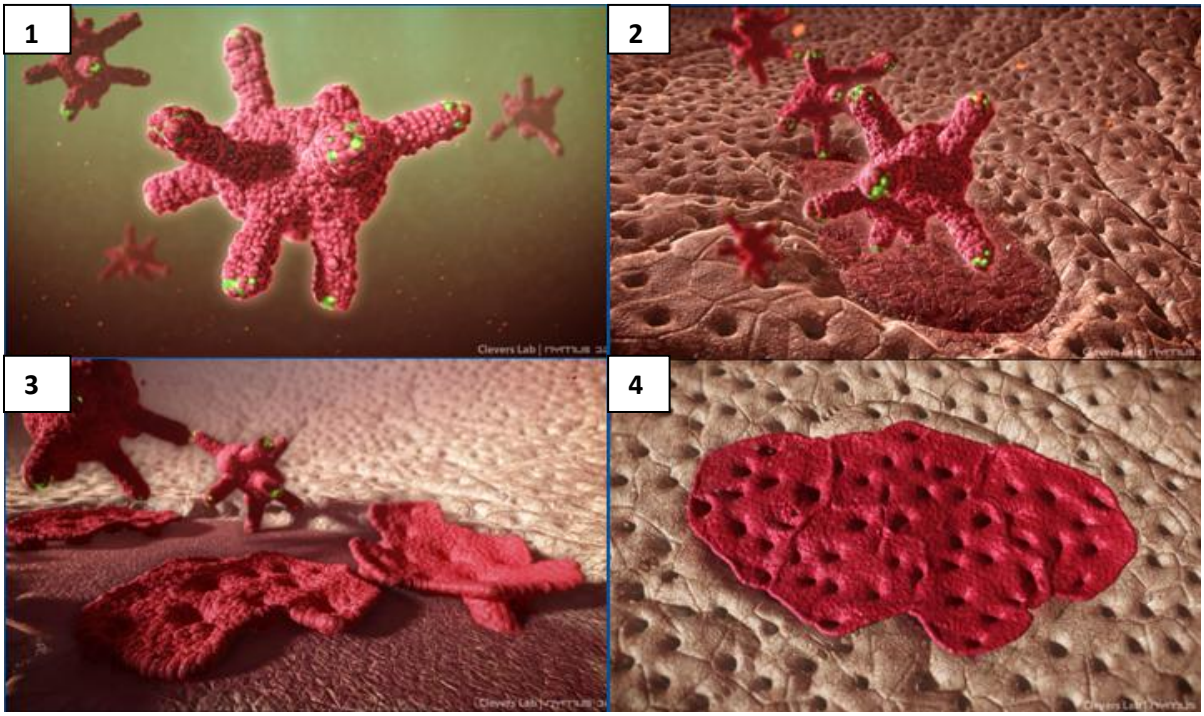


Fig 7: Illustrates intestinal epithelium reconstitution by organoids. Through the patchwork methodology, organoids are (1) infused into the body where they (2) travel to areas of damaged intestinal epithelium (3) attach and (4) repair lesions [copyright Clevers Lab].

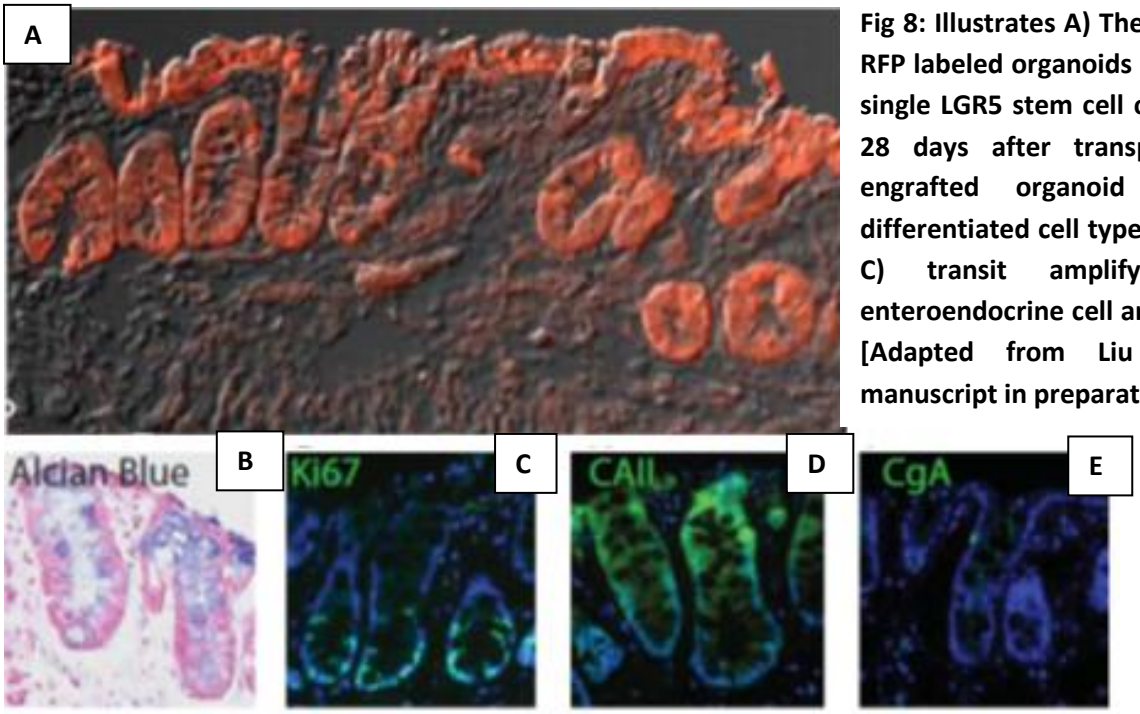


Fig 8: Illustrates A) The engraftment of RFP labeled organoids originating from single LGR5 stem cell organoid culture 28 days after transplantation. The engrafted organoid produces all differentiated cell types: B) goblet cell, C) transit amplifying cell, D) enteroendocrine cell and E) colonocyte [Adapted from Liu and Clevers, manuscript in preparation]

If in fact the “patchwork” method is unsuccessful, a new hurdle arises for the use of organoids in transplantation. The hurdle being that the organoids have to be upscaled to allow sufficient amounts of tissue for transplantation. Organoids that are currently produced are small enough to rely on diffusion to feed the cells. However, once larger organoids need to be grown or, organoids need to be connected together to form a sufficient transplantable structure, diffusion will no longer suffice. Investigators could begin to attempt to combat this problem by introducing factors that stimulate angiogenesis in the hopes that alongside intestinal tissue growth and enlargement vascular structures can develop simultaneously. If this approach were to work, it could be postulated that the organoids could be grown to a larger size due to a sufficient nutrient supply. Another mechanism by which to assist in the process of upscaling could be the use of polyester scaffolds. Chen *et al.* [60] have shown that growing neointestinal tissue on biodegradable polyester scaffolds allowed the cells to attach and grow in a 3D space with sufficient nutrient flow through the matrix. The investigators demonstrated that intestinal organoids could be engrafted onto biodegradable polyester scaffolding with reformation of an intestinal mucosal layer [60]. In addition, it was noted that scaffolding consisting of a larger pore size promoted vascularization and tissue proliferation. Taking these factors into consideration it can be postulated that once a sufficient number of organoids has been cultured according to the previously mentioned successful protocols, they can be engrafted onto a biodegradable scaffolding that resembles a part of the intestinal tube. Once the scaffolding is covered with a sufficient layer of intestinal tissue it may be inserted into the living organism and attached to the pre-existing intestinal tissue. Here the tissue will continue to grow in an environment with sufficient nutrient supply, while the biodegradable polyester structure will slowly dissolve, eventually leaving an intestinal structure that will develop and function accordingly. Due to a lack of scientific technology and the difficulties faced in growing whole organs, this approach can only be undertaken in the future when advancements in the field of organoid development have occurred. Research will thus be focused on optimalizing the more manageable patchwork method.

Regardless of the method used for organoid transplantation, patchwork or bigger structures, two factors will remain uncertain and may hinder human application. The first is that the organoids are grown in matrigel that is derived from a non-human species. It is not known whether or not organoids produced in this environment exhibit or display these non-human proteins. If this is the case, tissue grown under these conditions could elicit a form of graft versus host disease in which the immune system rejects the transplanted tissue. The second would be the functionality of the reconstituted organ. Even if the tissue appears functional in a synthetic environment, it is unknown whether the same activity will be seen in the *in vivo* situation. Here the cultured tissue may be unable to tolerate immune modulating molecules like cytokines and it is not known whether the organ will have the immunological capacity to be able to withstand the intestinal flora. All these seemingly trivial questions are pivotal in determining the success of the transplanted tissue.

As a final pivotal requirement, all products to be used or tested in humans must conform to Good Manufacturing Practice (GMP) protocols. These protocols regulate all the aspects of the manufacturing of a product to make sure that the final processing outcome is of a certain quality and safety standard. GMP protocols specific to the products being produced and tested as well as their target species. This section will discuss GMPs specific for the manufacturing of products to be used in humans. The basic principles of GMP protocols apply to the manufacturing and quality control, the facility, the equipment and to personnel [61]. At each step of work, quality control assays are taken beginning with the facility design (e.g: room spacing, hygiene and air systems), this is thoroughly checked in order to assure that no cross-contamination will occur. Next, starting materials (e.g: containers and media), ancillary components and sera are checked and recorded from batch to batch [61]. In addition, all donor material, whether autologous or allogeneic, must be tested for viral or bacterial infections. This step is carried out for the safety of both the donor as well as for the personnel in order to lower biohazards to a minimum and prevent cross contamination upon storage [61]. All personnel must be highly qualified, regularly trained and undergo routine medical checks to assure ultimate safety and quality. Personnel are additionally provided with standard operating

procedures (SOPs) at all times to continuously cross reference, ensuring that they are performing their tasks according to specific steps and standards. Additionally, consecutive steps should not be taken simultaneously within the same environment and all equipment, reagents and containers must be clearly labeled and stored under appropriate conditions. This process of checking is carried out at each manufacturing step and all data is documented in detail, supported with evidence and stored for a minimum of 15 years [61]. As a final step to the GMPs, all components of the process and the product itself can be traced back to their original supplier and all complaints are thoroughly investigated with appropriate measures being taken where they are needed. If on the whole the organoid production process follows these rules and regulations then, transplantation into human subjects will be permitted.

As a whole, research in the field of intestinal epithelial cell culturing and organoid formation has grown leaps and bounds. With the use of effective isolation techniques, tweaked highly effective protocols and sound methodologies to isolate, grow and identify intestinal epithelial cells, organoid production and implementation in the human setting and at the clinical level is feasible in the near future. By successfully creating upscaling methods to form tissues that are compatible both on the immunological and functional level, patients suffering from debilitating intestinal diseases will be offered the hope of a disease-free future. In growing organs from stem cells autologous to the patient, factors such as rejection are circumvented and patient intestines can regain normal functional and nutrient absorbing capabilities. Diseases such as microvillus inclusion disease could be successfully treated through the patchwork, infusion and reconstitution process, providing patients with the opportunity of a parenteral feeding-free future. However, although promising, there remain pros and cons to the situation at hand. When all future work is achieved, it remains unknown how each individual patient will react to the treatment. Some patients may, for example, have full repair of all induced intestinal damage while some may not. Additionally, varying disease types or states may affect the success or failure of treatment, exemplified by patients suffering from Crohn's disease (disease at the immune level) in which neither the patchwork method or whole organoid-produced intestinal tissue will be a sufficient cure for the disease. In all, the future

clinical application of this treatment method will remain uncertain until safe clinical trials are performed. The laboratory science can only be tweaked to create a full and effective treatment if preliminary clinical evidence leads it in the right direction. For the benefit of all patients from present to future, this synergy between laboratory science and clinical practice is crucial.

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