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# Exploring the human intestine in a dish:
# From physiology to disease

By

Ryan Ernest Forster

A dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy
in
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Professor Dirk Hockemeyer, Chair
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# ABSTRACT

Exploring the human intestine in a dish:
From physiology to disease
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Doctor of Philosophy in Molecular and Cell Biology
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The differentiation potential of pluripotent stem cells allows the possibility of making nearly any cell type given the right set of cues. As developmental biology continues to reveal how a cell is instructed to proceed to its fate, it is now possible to recreate *in vitro* methods to guide a cell to a specified fate. In addition to signaling cues in the form of growth factors or their analogs, some cells owe their identity to a specialized supportive environment that integrates a variety of cues. In the human intestine, the intestinal stem cell niche provides a physically supportive matrix with multiple overlapping gradients of growth factor signals. This niche can be reconstructed *in vitro* to maintain the progenitors that would maintain the epithelium for the life of a human. We call these *in vitro* tissues organoids. These cultures comprise progenitor stem cells that make the different cells of the intestine in a self-organizing tissue that has some of the functions of the intestine. While more complex than traditional cell cultures, an organoid is a reduced model of a tissue that allows for the observation of more stereotypical physiology and can be manipulated to model disease. This new mode of cell biology intersects with a time where genetic engineering technologies have expanded, become refined and now are easily accessible experimental systems.

Using gene editing in pluripotent stem cells we made a system that genetically identified a human intestinal stem cell that was derived from the spontaneous differentiation of embryonic stem cells into a benign tumor grown in a mouse. By creating a culture system to maintain these cells in a normal state long term *in vitro*, this has provided a model to biological questions that have not been experimentally achievable in human thus far.

# ACKNOWLEDGEMENTS

**DEDICATION**


To my Parents Russel and Marion Forster


Mom, you gave me a vision of simple moral clarity that guides me as faithfully now as it did as a child. All you have ever wanted for me is to be a good person with a happy and useful life and this has also been all I ever needed.


Dad, the short part of our lives we were able to share taught me to be curious and allowed science to shine a light on a fantastically rewarding inner life that I may never have discovered without those late nights watching PBS.


Thank you, Mom and Dad.

Fiat Lux!

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# Chapter 1

## Human intestinal physiology

## 1.1  Organization of the intestine

### 1.1.1  Introduction: intestine

The human intestine serves as the interface of nutrient and water exchange, secreting and reabsorbing nearly seven liters of fluid each day in the process of digesting food, maintaining a barrier from the microbiota and mobilizing waste (Kiela and Ghishan, 2016).  The focus of my thesis is the epithelial lining, the most highly proliferative tissue other than the blood. Somatic/adult stem cells of the intestine divide approximately once every day over the course of a human life. Groups of approximately six to twelve stem cells reside in isolation from the rest of the innumerable crypts, structures that are recessed along the basement membrane recessed into invaginations of the  intestinal stroma  (Finkbeiner and Spence, 2013a). How this tissue bears this extraordinary mitotic burden in the extreme environment of digestion, diverse microbial populations and a variety of changing xenobiotics with or without accumulation of genetic abnormalities is central to the motivation of this thesis. The tight regulation of genome integrity and proper cellular identity is essential for the prevention of cancer, but these systems can fail. This chapter does not attempt an exhaustive review of intestinal physiology or of intestinal cancer. Rather, my thesis work details my attempt to recapitulate a subset of normal features of intestinal proliferation and self-renewal to study the early genetic changes of colorectal cancer *in vitro* and to attempt to recapitulate findings from prior experiments in cells that have more of the features of human tissue than are seen in stable cell lines. Because of the advances in the fields of stem cell biology and genetic engineering, it is feasible to do genetic experiments in *in vitro* tissue culture with normal undifferentiated human cells. Years of experiments in mouse and other animal models, as well as clinical data from human patients, have informed the predictions I have used, and my aim in creating this system is to create a useful human model to study both cancer and intestinal physiology. My aim is to capture facets of human biology that may differ in other disease models and provide a means to do experimentation on human tissues with a greater ease and flexibility than can be done with human subjects. In order to provide a framework of understanding and to acknowledge aggregate years of work across several fields, I will first review a set of principles that were essential to my understanding of the intestinal epithelium, cancer, stem cells and genetic engineering. The scope of this introductory chapter

is to provide a framework for the rationale and the experiments described in Chapter two. In chapter three I will provide an outlook for the future potential for organotypic cultures particularly to disease modeling and the development and testing of therapeutics in addition to some of the more remarkable applications of these technologies since the inception of this thesis work.

### 1.1.2 Macroscopic architecture

The human intestine is separated into two main functional groups. The small intestine comprises the duodenum, jejunum and the ileum, which together provide for digestion and nutrient absorption. The large intestine comprises the cecum and the colon and is primarily responsible for the reabsorption of water and elimination of food waste. The intestine consists of multiple layers of diverse tissue from different lineages. Broadly these are suborganized (starting from the lumen) into the mucosa, submucosa, muscularis and the encasing serosa. The mucosa is the location of the epithelium, which is the focus of this thesis as it is the interface with the contents of the intestine and the tissue in which colorectal cancer arises in humans (Tomasetti et al., 2017).The epithelium is monolayer of columnar epithelium that lines the luminal surface, acts as: the interface of nutrient and water exchange, integration site of hormonal signals about feeding behavior and the physical barrier from the microbes that populate gastrointestinal (GI) tract (Turner, 2009). The intestinal mucosa comprises the epithelium and the underlying stroma called the lamina propria which is laminated with smooth muscles that gently agitate the epithelium against the exchange surface of the lumen. The stroma consists of fibroblasts, myofibroblasts, lymphocytes and capillaries which support the function of the epithelium and constitute its growth substrate by providing tissue architecture and growth factors that drive the maintenance of the stem cells and the constitutive regeneration of the epithelium (Wells and Spence, 2014). Additional layers contain both longitudinal and circular muscle to mobilize the food bolus through the digestive tract and a complex network of enteric innervations and arterial and veinous exchange to communicate feeding behaviors and signals and move nutrients to the liver respectively.

### 1.1.3 Intestinal epithelium

In the small intestine the epithelium is arranged in a monolayer of columnar cells that originate from invaginations in the stroma extending towards the lumen into an network of macroscopic villi. (Figure 1) Additionally, the apical surface of the terminally differentiated enterocytes develops microscopic villi that increase the surface area of the epithelial barrier. Together this increases the lumen-exposed surface area to nearly 32 m$^2$ a factor of 60-120 times in the small intestine where nutrient absorption takes place, and roughly 6.5 times in the colon where primarily water is reabsorbed (Helander and Fandriks, 2014). All of these cells are replaced approximately every five days by a system of differentiation starting from adult intestinal stem cells (ISCs) originally called crypt base columnar cells (CBCs). ISCs exist only in  a cellular niche of growth factor signals at the base of a structure originally named the crypts of Lieberkühn (Cheng and Leblond, 1974) or simply the crypt. At the base of the crypts higher levels of Wnt signaling and lower levels of bone morphogenic protein (BMP) signaling reinforce the adult stem cell identity and prevent differentiation. The niche facilitates the maintenance and proliferation of the pool of ISCs exists at the base of the crypt (Figure 1) (Barker et al., 2012; Potten et al., 1992). Radioactive labeling shows that intestinal proliferation is restricted to crypts, which also includes a column of highly mitotic progenitor cells just above the crypt base called the transient amplification compartment. Here cells physically mobilize inwards towards the lumen as they terminally differentiate into the functional exchange surface of the intestine and are eventually shed from the basement membrane in a process called anoikis: a programmed form of cell death that occurs in anchorage-dependent cells (Clevers, 2013). All the cells derive from the stem cells of the crypt (Barker et al., 2007) and of the progeny, two major differentiated epithelial lineages from: an absorptive enterocyte lineage that imports nutrients, and a secretory lineage. Paneth cells are secretory and provide niche support growth factors for CBC cells however they are predominantly a feature of the small intestine where they mobilize to the base of the crypt and live for 30-60 days (Farin et al., 2012). In addition to complimenting Wnt signaling from the stroma, these cells secrete antimicrobial cryptdins or defensins that protect the epithelium from microbes. Other cell types include Goblet cells that generate a microbial-protective and lubricating mucus layer; a variety of enteroendocrine cells that likely have myriad cryptic hormonal functions; and tuft cells that are

implicated in mechanosensation (Clevers, 2013) and chemosensation by mounting IL-25-associated immune responses during some pathogenic infections (Steele et al., 2016). All these cells are observed in different proportions in different regions of the intestine. For example in the small intestine there is a preponderance of absorptive enterocytes in the villus, a structure absent in the large intestine, and Paneth cells are very rarely observed in large intestinal crypts (Barker et al., 2008; Clevers and Bevins, 2013). The protective micro-environment of the crypt is the proliferative compartment of the epithelium where epithelial maintenance starts with the ISCs. As dividing cells crowd out of the crypt the cellular signals Wnt and BMP are arranged in countervailing cell fate signals established in opposing gradients along the axis of the crypt with Wnt highest at the base and BMP highest at the precipice of the crypt-villus (Farin et al., 2012). The growth factor cues transition across central portion of the crypt where cells undergo high levels of proliferation as they also differentiate into the post-mitotic cells of the villus. These cells defy the upward mobilization of other cells to move down to the crypt where they live for thirty to sixty days intercalated between stem cells at highest frequency in the proximal small intestine but are observed with decreasing frequency at more posterior regions (Clevers and Bevins, 2013; Farin et al., 2012; Sato et al., 2011b).
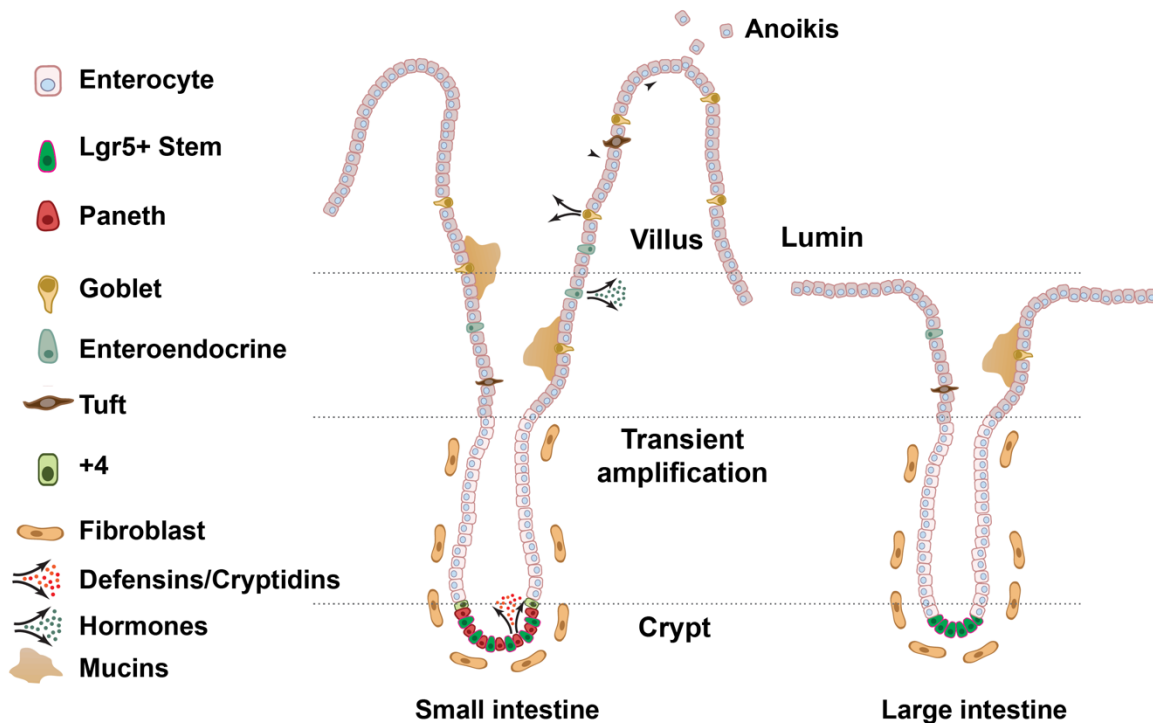
**Figure 1 Intestinal crypt organization**
The lumen of the intestinal tube is lined by a simple columnar epithelium with invaginations (crypts) in both the small and large intestine. The small intestinal crypt is accompanied by protrusions (villus) populated with enterocytes to aid in nutrient absorption whereas the large intestine lacks a villus and Paneth cells. Goblet cells are predominantly found in the ileum and the colon and secrete lubricating mucins. Other minor cell types include the hormone-secreting enteroendocrine cells, tuft/caveolated cells, cup cells, and M cells (latter two not shown). All of the cells of the epithelium are derived from the Lgr5$^+$ stem cells of the crypt except in cases of injury induction when cells from the +4 positions at the top of the crypt base can gain Lgr5 expression and stem cell capability.

### 1.1.4 Identifying the intestinal stem cell

In 1974 Cheng and Leblond discovered that small undifferentiated cells intercalated between the Paneth cells phagocytosed adjacent non-viable cells and conducted experiments exploiting this by treating mice with $^3$H-labled thymidine and harvesting intestine over a series of short times. They called these crypt base columnar cells (CBC cells), which have since been validated as the adult (or somatic) intestinal stem cells (ISCs) of the epithelium. These terms may be used interchangeably throughout. What the $^3$H (tritium) labeling experiments showed was that the remaining cells of the intestine retained this label, but that other cells processively in a "conveyer belt" mechanism became marked with this label  (Cheng and Leblond, 1974; Leblond and Messier, 1958). This indicated that the major cell types of the intestine all derived from these slender cells that intercalated the Paneth cells off the crypt and that they were the progenitors of the entire intestinal epithelium. This proposition turned out to be the case, curiously however, not entirely for the reasons that motivated them to believe this. At the time it was reasoned that stem cells must be mostly in a quiescent state as an evolved mechanism to maintain a pristine genome. They were believed at be responsible for maintaining a population of progenitor cells that would bear the burden of the many rounds of successive mitosis required to maintain and regenerate a tissue. Elements of these predictions still exist, but it is now known that not only are ISCs not quiescent, they divide nearly every day and that radiolabel retention was for an entirely different reason. More recently it has been shown that ISCs randomly segregate their chromosomes in a symmetric mitosis and that the reason the CBC cells retain a radiolabel in those first experiments is not because they are quiescent as was originally thought, it is because the

random segregation of chromosomes leads progressive loss of radiolabel over many divisions (Escobar et al., 2011). A group in the 1980's set forth the idea that there might be another population of intestinal stem cells that that were also capable of giving rise to all of the cells of the intestine located between two and eight cell diameters from the crypt and called this the +4 cell (Kaur and Potten, 1986; Potten, 1977, 1998). The proposition that these cells might be a second reserve, or facultative, stem cell population was in part based on the observations that these cells retained a radiolabel, a trait of quiescence once mistakenly generalized to stem cells under non-wound healing conditions. Additionally, these cells were more radiosensitive than other adjacent cells, which could indicate a geno-protective feature one might expect from a stem cell. Although these +4 cells retained a radiolabel, they were only semi-quiescent and, in this case, owed label retention to the fact that there were dividing asymmetrically and segregating and retaining radiolabeled DNA as proposed in the "immortal strand" phenomenon described by Cairns (Cairns, 1975; Potten et al., 2009). Most experimental evidence to date supports Leblond's original posit that the CBC cells are the maintenance stem cells of the intestine, which was demonstrated by rigorous lineage tracing experiments (Barker et al., 2007). It is now known from experiments in mice that either of these populations of cells can be ablated and reciprocally regenerate one another as daughter cells (Takeda et al., 2011; Tian et al., 2011a). It has further been shown that nearly all the intestinal epithelial cells have some potential for lineage plasticity or the ability to dedifferentiate back into adult stem cells. Cases of dedifferention upon ablation of the ISCs in mice have now been validated for +4 cells, enterocytes, secretory progenitors, Paneth cell precursors and enteroendocrine cells (Melo et al., 2017; Tetteh et al., 2016; Tian et al., 2011b; van Es et al., 2012).

## 1.1 Wnt signaling

### 1.2.1 Introduction: Wnt signaling

WNT signaling is mediated by secreted WNT ligands binding to receptors on the cell surface. Three Wnt signaling pathways have been characterized: the canonical Wnt pathway, the noncanonical planar cell polarity pathway, and the noncanonical Wnt/calcium pathway. The canonical Wnt pathway is the most well characterized and relies on β-catenin as its main effector protein. Planar cell polarity (PCP) and calcium

signaling have major roles in development and calcium release but are functionally divergent and will not be further described here. The following sections will cover the fundamental aspects of Wnt signaling that relate to the experiments in chapter two and the generation of an ISC reporter using the Wnt potentiator Lgr5.

## 1.2.2 Wnt in the Intestine

The intestine has become a model system for understanding WNT signaling and its role in stem cell dynamics. Normal epithelial homeostasis in the intestine is maintained by a population of highly proliferative intestinal stem cells that rely canonical Wnt signaling to maintain their normal stem cell identity, however Wnt also plays key roles in embryonic development of the intestine and in diseases like colorectal cancer. The major signaling pathways that facilitate the posterior patterning of the vertebrate embryo are Wnt, Fgf, retinoic acid (RA) and Bmp and gut tube morphogenesis can be partially recapitulated *in vitro* by stimulation with Fgf and Wnt and the maintenance of these cells is then also dependent on Wnt (Wells and Spence, 2014). This knowledge from developmental biology has been essential in the development of culture conditions for normal intestine, which are exquisitely reliant on Wnt. The current understanding of the role of canonical Wnt signaling in the intestine has been in the consolidation of observations in development, adult physiology and diseases of the gut like cancer. Experiments showing that inactivation of intestinal β-catenin in mice leads to a rapid loss of epithelial cells, starting at the crypt, blocks proliferation and increases terminal differentiation gave an indication that the role of Wnt may be in the maintenance of the ISCs (Fevr et al., 2007). Wnt signaling now known to be the master regulator of proliferation of intestinal in the normal intestinal crypt base, where ISCs reside (van de Wetering et al., 2002a). Furthermore, the establishment of organotypic cultures of mouse and human explants has shown that the long term maintenance of stem cells relies on Wnt signaling and a potent system of Wnt singling potentiation acting through a G- protein coupled receptor called Lgr5 and its cognate ligand R-spondin (Barker et al., 2007; Ootani et al., 2009b; Sato et al., 2011a; Sato et al., 2009).

Lgr5 is a transmembrane protein that when bound with its cognate ligand R-spondin (RSPO) potentiates Wnt signaling by stabilizing the Wnt receptor Frizzled (Ruffner et al., 2012). Later discovered to specifically

mark ISCs in mice, an set of Lgr5-reporter mice along with other made the Hans Clevers lab has been useful in both understanding Wnt signaling and intestinal biology as well as identifying and isolating the ISCs (Barker et al., 2007). Injection of Wnt agonist RSPO-1 into mice activation increases the numbers Lgr5$^+$ stem cell (Kim et al., 2005b). Converging findings show that intestine is dependent on canonical Wnt signaling not just for proper development, but for its ongoing maintenance, epithelial cell identities and normal function. The following passages will discuss some of the background of canonical Wnt signaling with respect to intestinal function, cancer and the identification of the intestinal stem cell.

### 1.2.3 Canonical Wnt Signaling

The Wnt pathway derives its name from a portmanteau of the Drosophila *Wingless* and the mouse *Int-1* genes. In 1982 researchers observed that the mouse *Wnt-1* gene (originally named *Int-1*) was aberrantly hyper-activated by integration of the Mouse Mammary Tumor Virus MMTV in virally induced mammary tumors (Nusse and Varmus, 1982). This secreted product of this gene was the first Wnt (*Wnt-1*) to be considered a proto-oncogene and was later discovered to be the *Wnt-1* counterpart, Wingless (*Wg*) in larval patterning in *Drosophila melanogaster* (fruit flies) (Rijsewijk et al., 1987). An entire field of research has developed from these early findings and now nineteen Wnt genes are known in mammals with twelve subfamilies showing conserved sequence similarity across many metazoans (Coudreuse and Korswagen, 2007). Wnts have a signal peptide sequence for secretion and are cysteine rich to promote stable and correct protein folding (Miller, 2002). As a long-rage secreted morphogen, Wnts are concentration dependent effecters exerting juxtacrine and paracrine effects on target cells within a spatial range of approximately 20-30 cell diameters making them excellent cues in spatially restricted micro-environments (Mikels and Nusse, 2006; Willert et al., 2003). Secreted Wnts bind to receptor complexes (including the transmembrane proteins Frizzled and Lrp5/6 Figure 2) on the cell surface initiating one of three disparate intracellular signaling pathways: canonical (T-cell factor [Tcf]/β-catenin) pathway, the non-canonical (planar cell polarity) or the Wnt/Ca 2+ pathway (Katoh, 2005; Kohn and Moon, 2005).

In a steady state absent Wnt stimulation a multiprotein complex adenomatous polyposis coli (APC) tumor suppressor protein, Axin

coordinate the kinase glycogen synthase kinase 3 (GSK3 β) to phosphorylate β-catenin, which then interacts with βTrCP leading to the constitutive degradation of β-catenin through a ubiquitin pathway (Cadigan and Nusse, 1997; Haraguchi et al., 2008).

When Wnt ligands are present, the Frizzled transmembrane protein interacts with Wnt and the LRP transmembrane protein, the intracellular portion of the LRP tail (phosphorylated) binds the scaffolding protein Axin and is thought to form a complex with Dvl, Axin and GSK3 thus impairing the kinase ability of GSK. Non-phosphorylated β-catenin is stabilized and quickly accumulates in the cytoplasm leading to flux into the nucleus where it interacts with TCF/LEF transcription factors to promote transcription of TCF/LEF target genes (Miller and Moon, 1996; Peifer and Polakis, 2000). Many Wnt dependent stem cells express members a transmembrane Wnt potentiator family named leucine-rich repeat-containing G protein-coupled receptor (LGRs). Discussed later, LGRs can be bound by R-spondins (RSPOs) to potentiate Wnt signaling. The Lgr5 when bound by R-Spondin-1acts by inhibiting the transmembrane E3 ubiquitin ligases RNF43 and ZNRF3 thereby stabilizing the Wnt receptor Frizzled from ubiquitylation that normally leads to endocytosis (Chen et al., 2013).
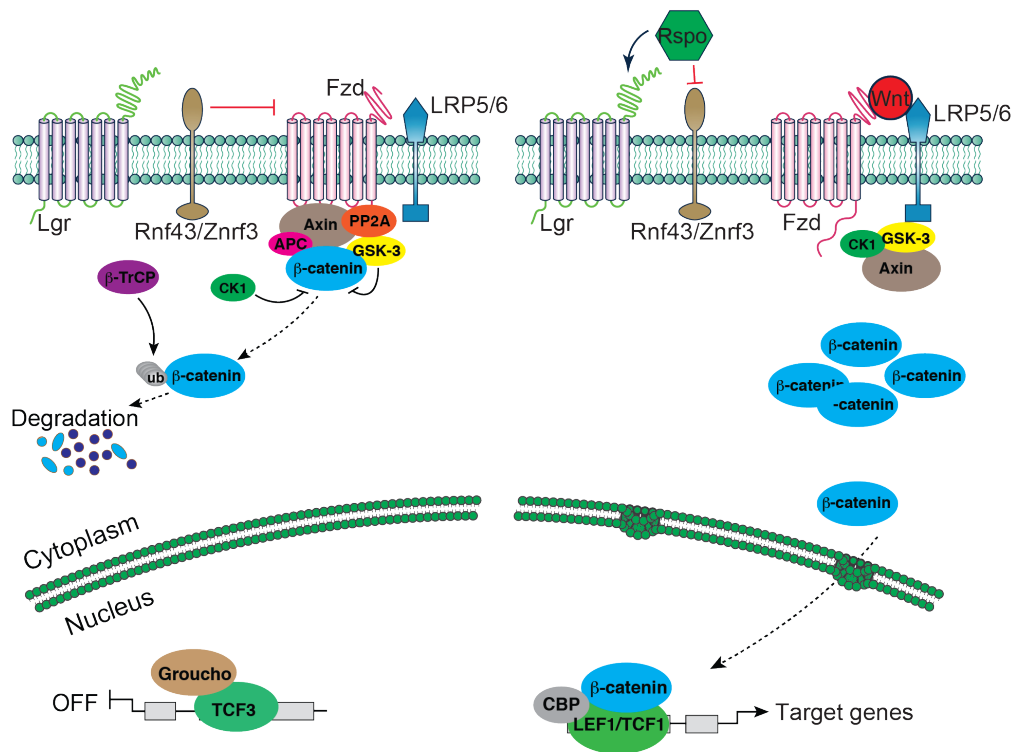
## Figure 2 Wnt signaling and R-Spondin potentiation

In the absence of Wnt ligand, glycogen synthase kinase 3 (GSK3 β) phosphorylates β-catenin, which triggers β-TrCP to initiate ubiquitin mediated degradation. However, in the presence of WNT ligand, the destruction complex (comprising GSK3, casein kinase (CK), Axin and adenomatous polyposis coli (APC)) is recruited to the Frizzled (Fzd)/LRP5/6 complex and inactivated. β-catenin accumulates and translocates to the nucleus, where it drives transcription of target genes. In cells expressing leucine-rich repeat-containing G protein-coupled receptor LGRs can be bound by RSPO ligands to inhibit the transmembrane ubiquitin ligases Rnf43 and Znrf3, stabilizing the Fzd protein by inhibiting ubiquitin mediated endocytosis.

## 1.2.4 Wnt signaling and colorectal cancer

Among the suppressors of Wnt signaling the tumor supressor *adenomatous polyposis coli* (*APC*) is the most frequently mutated gene in human cancers. Truncating mutations in APC are the cause of familial adenomatous polyposis (FAP), a precondition where *APC* germline mutations at the 5' end of the gene leads to hundreds of polyps in the large

intestine (De Queiroz Rossanese et al., 2013). The incidence of FAP in the population is approximately 1 in 8000 (Bisgaard et al., 1994) and as described with Knudson's two-hit hypothesis (Knudson, 1971), colorectal tumors from FAP patients carry additional somatic *APC* mutations or loss of heterozygosity (LOH) at this locus in addition to the original germline mutation (Ichii et al., 1993; Miyoshi et al., 1992; Solomon et al., 1987). The idea is that a predisposing mutation poises a cell for a cancer that is set into motion upon the acquisition of a second mutation in another gene, or in the case of APC, the other allele. Somatic mutations in *APC* occur in ~80% of sporadic colorectal tumors and appear to have a selective constraint to the region called the mutation cluster region (MCR) between codons 1,284 and 1,580 of the *APC* gene and nearly always lead to a truncated *APC* fragment (Dundar et al., 2007) rather than the RNA quality control mechanism nonsense mediated decay (NMD). Activation of the WNT pathway, usually through inactivating mutations in APC, leads to the formation of benign polyps. The "adenoma–carcinoma sequence" proposes acquisition of sequential genetic alterations facilitates the progression of colorectal cancer (CRC) (Fearon and Vogelstein, 1990). Wnt signaling mutations, typically APC, lead to benign polyps while subsequent mutations in EGF receptor (EGFR), Kirsten rat sarcoma viral oncogene homolog (*KRAS*), P53 and transforming growth factor (TGF)- β pathways promote CRC with a metastatic phenotype (Fearon, 2011; Vogelstein et al., 2013a). Mutations that confer selective growth advantages by deregulating driver pathways involved in differentiation, cellular proliferation and apoptosis are thought to follow mutations in APC because of both the observed prevalence of APC mutation and that when looking at genetic changes in benign adenomas versus blood from the same subjects, mutations in APC are the most prevalent of early genetic change (Lin et al., 2017). The complex genetic differences across somatic tissues, and between human subjects, coupled with knowledge that mouse models of CRC fail to fully recapitulate some of the major phenotypes of human CRC (McCart et al., 2008) was a prime motivating factor in the development of my thesis work. By creating a human organoid system to grow normal intestinal epithelium, I could introduce mutations observed at the early steps of cancer to test whether they happened in a dependent sequence and model how these pathway deregulations interact influence the phenotypic changes seen from the progression through benign adenoma to invasive metastatic carcinoma.

## 1.2.5 Canonical Wnt Signaling (Tcf/β-catenin) and adult intestinal stem cells

The understanding of the essential role of Wnt signaling came from the convergence of three areas of studies in Wnt signaling: the intestinal developmental defects seen in neonatal rodents with impairments in Wnt signaling, the catastrophic intestinal defects seen in adult mice where Wnt signaling was interrupted, and the links between activating mutations in the Wnt signaling pathway to human intestinal neoplasms and cancers. The transcription factor Tcf4, when bound to its effector β-catenin in the nucleus, activates Wnt target gene expression. The first indication that β-catenin played an essential role for ISCs came from a double knockout of Tcf4 and another contemporaneously discovered transcription factor Tcf3 (Tcf3 later shown to have a role in Notch signaling) were genetically deleted in mice and resulted in a phenotype where intestinal crypts failed to form (Ikawa et al., 2006; Korinek et al., 1998b). This intestinal failure phenotype was recapitulated with the ectopic application of the secreted Wnt antagonist Dickkopf-1 (Dkk1) and also β-catenin knockout mice (Ireland et al., 2004; Kuhnert et al., 2004; Pinto et al., 2003). It was evident that significantly impairing Wnt signaling at nearly any point in its respective signaling cascade had pathological effects on both intestinal development and maintenance. Separately, in an attempt to understand the genetic contributions to colorectal cancer in humans, it was noted that intestinal neoplasms from adenomas to carcinomas most frequently have activating mutations in β-catenin (Morin et al., 1997) or the protein binding partner and negative regulator of β-catenin APC (85% of sporadic colorectal cancers). (Munemitsu et al., 1995; Rubinfeld et al., 1993; Su et al., 1993) In 90% of these cases the mutation results in a protein fragment of APC that occurs in surrounding the β-catenin binding motif (Fodde et al., 2001) and these mutations are present in the earliest of these growths (adenomas) and in more advanced cancers, but not in the blood of the same subjects giving the field some indication that activating mutations in Wnt signaling might be driving the earliest stages of cancer (De Benedetti et al., 1994; Miyoshi et al., 1992). Nearly 15% of CRCs are in familial (i.e. inherited) context such as familial adenomatous polyposis (FAP) where individuals develop hundreds to thousands of adenomatous polyps in the large intestine at an early age and invariably progresses to malignant colorectal cancers if not surgically removed (Lynch and de la Chapelle, 2003). Mutation analysis indicated that adenomas in FAP subjects that developed

cancer had often experienced mutations in the other APC allele or lost APC expression by chromosome missegregation (Baker and van Deursen, 2010; Fodde et al., 2001; Hao et al., 1998; Miyaki et al., 1992). Because benign adenomas were the earliest stage of human colorectal cancer and the genetic evidence was that Wnt played a major role in both intestinal cancer and normal intestinal stem cell maintenance, some proposed a model in which tumors arose from neoplastically transformed stem cells at the base of the crypt, progress to adenomas which next expand by crypt fission towards the intestinal lumen in a 'bottom-up' fashion (Preston et al., 2003). Others predicted that dysplastic cells located on the surface epithelium, next laterally migrate, and then grow down crypts through a 'top-down' mechanism (Shih et al., 2001). A model of colorectal cancer formation that started with early observations of clinical and histopathological suggested that genetic alterations drove cells through successive waves of clonal expansion from adenoma to carcinoma to metastatic cancer (Nowell, 1976; Sugarbaker, 1985).This model still persists and has been refined as reviewed by (Vogelstein et al., 2013b) and that the first mutation (called the Gatekeeper) occur most often in APC (Kinzler and Vogelstein, 1997). This linked the idea that a required pioneering or *gatekeeper* mutation in colon cancer happened in a gene that increased Wnt signaling and that adult intestinal stem cells relied on Wnt to maintain the adult stem cell identity. Implicit in this was the notion that the cell of origin for most CRC was an adult stem cell that had mutation or aberration that increased Wnt signaling. These cells would clonally expand until other driver mutations genetic alterations occurred and allow the adenoma to progress to other stages of cancer and that the accumulation of these mutations is what lead to metastatic cancer(Vogelstein et al., 2013b).

From the study of tissues of subjects with Wnt related mutations and experiments manipulating Wnt in rodents it became clear that aberrantly high Wnt signals upregulate a battery of target genes. However most of these genes were expressed throughout the crypt and transient amplification compartment leading up to the villus so it was difficult to determine if there was any kind of hierarchical role in Wnt signaling that would indicate a specific Wnt expression signature specific to adult intestinal stem cells (van de Wetering et al., 2002b). Entering the 2000s it was evident that intestinal maintenance was heavily regulated by Wnt based on the fact that disrupting Wnt signaling in caused epithelial failure,

but in order to determine if the failure was a result of intestinal stem cell loss there was a desire to somehow specifically lable the ISC. The link to intestinal maintenance and Wnt led some to look at the expression of Wnt target genes expressed at higher levels in the crypt cells than in the more differentiated villus. In order for any these target genes to be used as a specific marker of stem cell identity, the expression would need to be restricted only to the master progenitor cells which most thought were the crypt base columnar cells at the base of the crypt first described by Cheng and Leblond in the 1970s. In colorectal cancer cell (CRC) lines, a subset of these Wnt upregulated genes had expression restricted to the crypt and one of them, the leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5 also called Gpr49) had expression restricted exclusively to the CBC cells (van de Wetering et al., 2002b; Van der Flier et al., 2007). This lead to the generation of a series of reporters that lead to the first genetic labeling an adult stem in mice cell to prove it was responsible for the generation of all the cells of the intestinal epithelium (Barker et al., 2007).

**1.2.6 LGR5- stem cells and Wnt**

Years of debate about which intestinal cell was the stem cell somewhat settled when Lgr5 was bona fide as an intestinal stem cell marker. Lineage tracing experiments showed that mice with an inducible Cre knock in allele crossed with the *Rosa26-lacZ* reporter background generated all of the epithelial lineages, and that mice with either *Lgr5-LacZ* or *Lgr5-EGFP* specifically marked the crypt base columnar cells and had the same morphology as noted by Cheng and Leblond, and further that they did *not* mark the +4 cells described by Potten (Barker et al., 2007). In the intervening time, several other Lgr5^{+} stem cell populations have since been identified in other tissues including ectoderm (Yu et al., 2017) mesoderm (Lee et al., 2017) and even in dedifferentiated cancer cells of some tumors (de Sousa e Melo et al., 2017; Shimokawa et al., 2017) leading has led to the use of Lgr5 as a prognostic marker in cancer (Han et al., 2015).

The Lgr5 protein is a seven-pass transmembrane protein from the rhodopsin G-coupled receptor family that, like other LGR proteins, bind secreted Rspo ligands. In the ligand bound state, Lgr5 inhibits transmembrane E3 ubiquitin ligases Rnf43/Znfr3, thereby stabilizing the Wnt receptor Frizzled and agonizing the Wnt signal (de Lau et al., 2014). Lgr5 acts as a potent agonist of Wnt signaling and within intestinal

epithelium is only expressed by a small pool of 12–15 ISCs. In mice, the Lgr5^{+} CBC cells are long-lived stem cells that maintain an isolated stem cell pool in each crypt and give rise to the major cell types of the intestinal epithelium including the secretory (Paneth, goblet, and enteroendocrine cells) and the absorptive enterocytes (Barker et al., 2007). The ability of these genetically labeled mice to show both self-renewal and multipotency, the central features of adult/somatic stem cells, was further demonstrated in organotypic mouse explants called organoids in an experiment where a single genetically labeled Lgr5^{+} was shown competent to give rise to all the same cell types *in vitro* (Sato et al., 2009).

## 1.2.7 LGR5 and Wnt-independent determinants of ISCs

The validation of Lgr5 as both a marker of adult stem cells and a critical modulator of their activity via its role as an effector of Wnt/R-spondin (Rspo) signaling proved technically useful in studying the maintenance of the intestinal epithelium and in many cases the potentiation of Wnt signaling, but the details of the scope of its cellular functions are still just becoming understood. In cell-cell adhesion studies Lgr5 was shown to phosphorylate GTPase-activating protein 1 (IQGAP1), a scaffolding binder of Rac1 and Cdc42 affecting activities of the Wnt pathway, F-actin, MAP kinases, and Rho GTPases and the actin cytoskeleton. (Carmon et al., 2017). The Paneth cells found predominantly in the proximal small intestine provide a supportive role in Wnt signaling, however they also appear to serve a metabolic support role that is specific to intestinal stem cell biology. Intestinal stem cells have higher respiration and a pyruvate versus lactate ratio than Paneth cells, which actually produce some of the lactate that the ISCs use as energy, thereby activating the differentiating p38MAPkinase signal via reactive oxidative species ROS (Rodriguez-Colman et al., 2017) Mitochondrial pyruvate carrier (MPC) is upregulated as intestinal epithelium differentiates and when pyruvate genes are inhibited in both mice or fruit flies it causes a significant expansion of ISCs both *in vivo* and *ex vivo* (Martinez-Outschoorn et al., 2017; Rodriguez-Colman et al., 2017).Taken together these observations imply integration between cell adhesion, cell signaling and metabolism in the control of stem cell maintenance and differentiation.

## 1.3 Pluripotency and multipotency – *in vivo* and *ex vivo*

### 1.3.1 Introduction: Stem cells

The detailed understanding of the biology of a somatic tissue maintenance and regeneration became more tractable with the advent of genetic editing discussed later. Developmental biology as coined by Paul Weiss and N. J. Berrill in the 1950s to include embryology and the study the differentiation of cells from the stem cell stage and onwards, how cell lineages differentiate, and the processes dictate the formation of tissues and organs (Gilbert, 2017). The intersection of genetic manipulation and stem cells has been a boon across many areas in biology, but the accessibility of embryonic stem cells and the public funds to conduct research went through an unstable period because of ethical concerns about research using human embryonic tissues. In 2001 the United States limited federal funding of research on human embryonic stem cells (hESCs) but allowed research to continue on lines established before the ban. Then in 2006 Shinya Yamanaka of Kyoto University in Japan revealed a means to reprogram somatic cells into a pluripotent state that appeared to have many of the same capacities as stem cells derived from embryos. Then in 2009, the restrictions for publicly funding embryonic stem cell research were lifted, and a new set of standards addressing ethical concerns were established for the derivation of embryonic stem cells. The effect of this was that many of the lines in use could still be used and shared among scientists, although some lines lacking the documentation needed to meet the standards for approval could no longer be supported by public funds (Tanne, 2010; Waite and Nindl, 2003). Cellular reprogramming (discussed more later) led to expanded use of *in vitro* culture and differentiation methods for both embryonic and somatic stem cells in the intervening time, but it is worth appreciating that progress in the fields related to this technology was seriously limited by the embryonic ban for nearly a decade. As the biology of stem cells gained in understanding, the field of developmental biology contributed a huge body of knowledge concerning the processes by which cells differentiate into somatic tissues. From this intersection came methods for differentiating embryonic and the newer reprogrammed cells IPSCs called induced pluripotent stem cells. Pluripotency is the capacity for a cell to generate all the different cell types of the body, a feature shared by iPSCs and ES cells. Many of the first cell

types made were terminally differentiated cells, meaning they did not give rise to other cell types other than daughter cells of the same cellular identity. But it was known that many animal tissues were maintained by long lived cells that gave rise to a variety of different types of cells in that tissue that are called somatic or adult stem cells. One key difference between adult and embryonic stem cells is their differing potential in the number and type of somatic cell types they can give rise to. ESCs are pluripotent, meaning they can become all cell types of the body, while *somatic* or *adult* stem cells are limited to differentiating into the respective cell types of their tissue of origin when in the context of cues they receive in their natural cellular niche (Wagers and Weissman, 2004). Understanding how the differentiation potential of stem cells is established and maintained has led to the key understanding that often in addition to nutrient and growth factor dependencies many of these cells have mechanical dependencies that are now being decoded in an emerging field of *in vitro* and *ex vivo* organotypic cultures called organoids. It will be useful here to discuss some of the historic contours of stem cell biology.

## 1.3.2  Embryonic stem cells

The first isolation of mouse embryonic stem cells (ESCs) was in 1981 (Barker et al., 2008; Evans and Kaufman, 1981; Martin, 1981)  and human ESCs (hESCs) we isolated nearly a decade later from a human blastocyst (Thomson et al., 1998). It was subsequently determined that totipotency could be conferred to the nucleus of a terminally differentiated cell by transferring it into the nuclear compartment of an oocyte (Gurdon et al., 1958). This discovery made it clear that the necessary factors for reprogramming a cell into its earliest sate of stemness could be ectopically applied to a differentiated nucleus to reprogram it into a cell that could make an entire sexually fertile animal. The defining factors for the induction of pluripotency, transcription factors (i.e. *Oct4* and *Sox2*) and two proto-oncogenes (*Klf4* and c-*Myc*), were identified is screening experiments that revealed the ectopic expression of just these four factors was all that was required to revert a somatic cell into a stem cell (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). This knowledge made it possible to induce or derive embryonic stem cells capable of producing representing all three germ layers of the body (pluripotent). The validation of pluripotency for mouse ESCs was typically evidence of all three germ layers in a

chimera or by tetraploid complementation (Okita et al., 2007; Zhao et al., 2009). For human cells, the pluripotency assay was a teratoma formation assay. For this, presumed pluripotent cells are injected subcutaneously into immune compromised mice to form benign tumors and if the cells are pluripotent they will spontaneously differentiate into tissues from each of the three germ layers consisting of mature and immature cells with normal karyotypes (Oosterhuis and Looijenga, 2005; Pierce et al., 1960). By withdrawing the pluripotent stem cell niche factors (for human cells: high FGF2 with either a mouse fibroblast monolayer substrate *or* a combination of insulin, selenium, transferrin, L-ascorbic acid, and TGFβ/NODAL) (Chen et al., 2011) ES cells also spontaneously differentiate into poorly organized aggreges of cells and tissue called embryonic bodies that could also generate all three germ layers.

### 1.3.3  Advent of three-dimensional tissue culture

Historically, three-dimensional (3D) cultures were derived from fragments of organs (usually epithelium) (Barcellos-Hoff et al., 1989; Petersen et al., 1992) and mechanically and enzymatically dissociated into different types of 3D gels in an attempt to recapitulate tissue development that in 2D was not only lacking proper cellular organization, but also failed to recapitulate cellular functions (Barcellos-Hoff et al., 1989). Possibly the first 3D culture was done by a zoologist that dissociated sponge adhere to glass plates, but if the layer was washed off the cells would aggregate and start to differentiate into distinct structures with architecture reminiscent of the original sponge (Wilson, 1907). Much later studies reported  dissociated chick embryos self-assembled into 3D structures seen in embryos (Moscona and Moscona, 1952) and formation of complete chick organs from single cells (Weiss and Taylor, 1960). This advance was followed by an *ex vivo* reconstitution of mouse mammary glands function that produced milk, even after enzymatic disaggregation and reformation of the tissue (Lasfargues, 1957). In 2008 apico-basally polarized cortical tissues with spectacularly similar patterning as seen *in vivo* were generated from ESCs (Eiraku et al., 2008) and in 2009 mini-guts that produced all the major cell types of the mouse intestinal epithelium were formed from a single cell (Sato et al., 2009) and an *in vitro* method of directly inducing hESCs was discovered (McCracken et al., 2011; Spence et al., 2011). Since then human optic cups and mini-brains with multi-layered cortex (Lancaster and Knoblich, 2014) have been reported and the field of 3D or organoid cultures

has led to a number of useful disease models. Confusion about the clear definition on an "organoid" from the mid-twentieth century when organelles in eukaryotic cells were called organoids to the present when some simple tissue aggregates have also been so named. The prevailing consensus comes from three criteria proposed by Lancaster and Knoblich who proposed that an organoid should have the following three qualities: (1) multiple organ-specific cell types (2) capacity to recapitulate some specific function of the organ (eg. excretion, filtration, neural activity, contraction) (3) spatial organization similar to an organ (Lancaster and Knoblich, 2014).

### 1.3.4  Intestinal organoids

Long term culture of mouse organoids from dissociated intestine was reported by (Ootani et al., 2009a) when at the same time Sato and collogues generated organized crypt-villus organoids from single cells derived from mice genetically labeled with a fluorescence marker at the Lgr5 gene. Grown in a laminin rich 3D matrix, the medium contained three of the essential growth factors for the maintenance of the adult stem cells of the intestinal crypt: R-spondin-1, Noggin and epidermal growth factor EGF.  These cultures had organization similar to that seen in mice and contained Lgr5$^+$, Paneth, goblet, enteroendocrine, and enterocyte cells, however these cultures live only about one month as opposed to prior mouse explants that grew for many months (Sato et al., 2009) . Biopsies directly from human small intestine and large intestine were also derived from the same group with the addition of a few more small molecule inhibitors, but these lacked the major crypt protrusions seen in mouse and constituted mostly a metaplasia of almost entirely adult stem cells except with withdrawal of growth factors (Sato et al., 2011a). These experiments overlapped at a time when the more facile CRISPR-Cas9 gene editing methods were emerging (Jinek et al., 2012; Jinek et al., 2013) (discussed in greater detail below) and within three years two related groups had recapitulated (in organoids) the early stages of human cancer from benign adenoma through cultures that had acted as metastatic carcinoma in kidney capsule xenograft experiments  (Drost et al., 2015; Matano et al., 2015). The direct differentiation of human intestinal organoids was done by induction of definitive endoderm that then budded with epithelial structures that would make organoids that did not show mature phenotypes for 100 days, but this group followed up with experiments that transplanted this

tissue in the kidney capsule of a mouse where it later developed into fully mature tissue (Finkbeiner et al., 2015). The following chapter describes in detail a method for deriving human intestinal organoids that make all the major cell types of the intestine in sustained long-term cultures (Forster et al., 2014). Later protocols describe methods to direct organoids specifically into colonic tissues with the identification of more specifically hindgut markers (Munera et al., 2017). As potential disease models, one of the chief ambitions for organoid cultures was to study the effect of gene mutations in a more tractable system. The following will cover fundamental mechanisms that relate to the genetic engineering of tissue cultures.

## 1.4   DNA repair


### 1.4.1 Introduction: Gene editing

In order to specifically edit mammalian genomes, efficient DSB formation is the first step in a two-step process. Ectopically introducing one of a variety of increasingly programable site specific nucleases creates a double stranded DNA break that will be recognized by cellular machinery from either of the two endogenous DSB repair pathways. From there changes can be made through an error-prone process resulting in ligation of the chromosome ends called non-homologous end joining NHEJ, or by co-opting a homology directed repair (HDR) pathway that normally uses a sister chromatid for a synthesis dependent repair. By providing a DNA repair template with intentional changes made to the sequence, a cell can template the repair of a damaged chromosome with intentional changes to the sequence. Both of these processes are now widely exploited in genetic engineering. NHEJ frequently results in indels that can be positioned to knock out gene function, and HDR can be coopted to introduce mutations or even entirely new sequence to specific sites in the genome. Applications of gene editing are ever expanding in cells animals and now humans from simple gene knockouts induced by randomly changing exomic coding frames, to recapitulating disease related mutations in cells, to adding larger stretches of ectopic DNA coding fluorophore or luciferase reporters recombineering cassettes and nearly any desired DNA expression system. Practically speaking, this technology can be used to carefully reconstruct cellular or whole animal models of biological importance to study disease, physiology, and tissue repair and

regeneration. The following will put into context this important technology that was essential to nearly every aspect of this thesis work.
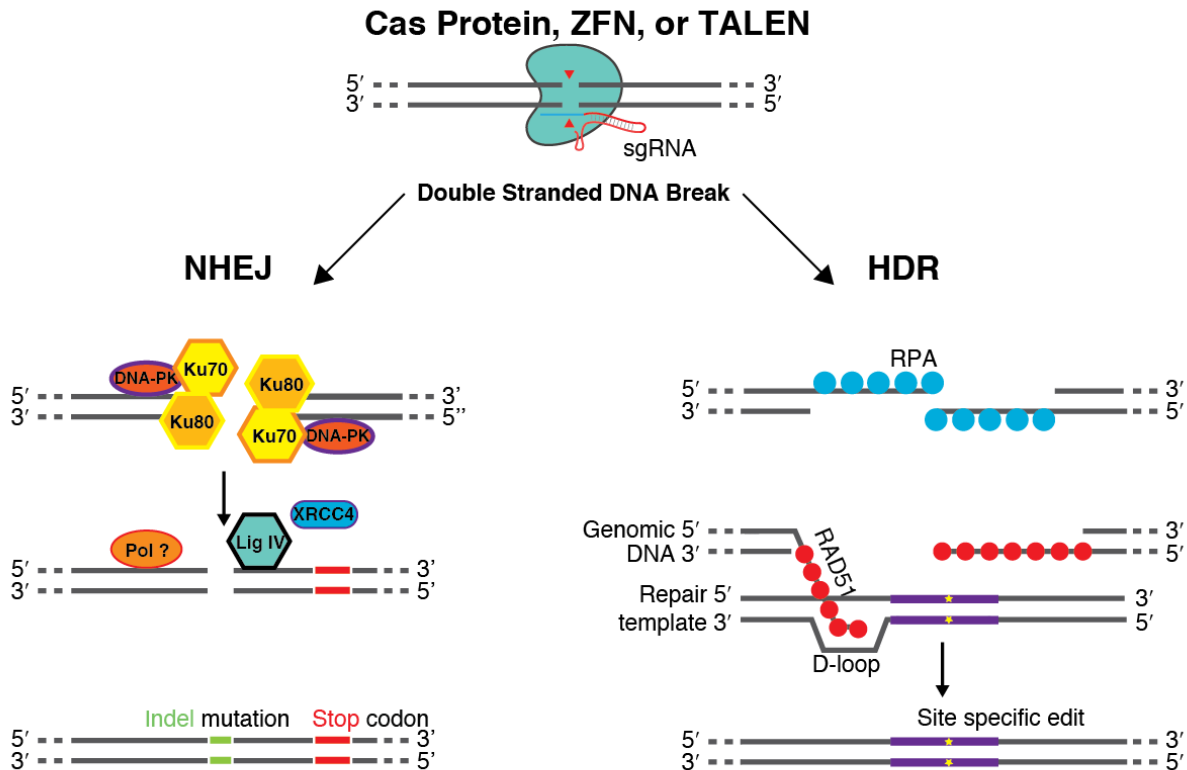


## Figure 3 Gene editing with site specific nucleases: NJEJ and HDR

After a site-specific nuclease induces a double stranded DNA break on a chromosome, the break can be repaired by one of two major DNA repair pathways in vertebrates. Left: Double-strand breaks are recognized primarily by the Ku protein, which consists of two subunits, Ku70 and Ku80. Ku then recruits the DNA-dependent protein kinase, DNA-PKcs which leads to the activation the Artemis nuclease, which trims back overhangs to prepare for the ligase complex XRCC4 or XLF (XRCC4-like factor; also called Cernunnos), and DNA ligase IV. The POL X family of DNA polymerases can participate in filling in the gaps of lesions. Right: To generate the substrate for binding of Rad51, DNA ends are processed to yield 3′ single-stranded DNA (5′–3′resection). Replication protein A (RPA) binds the single-stranded DNA and is later displaced by Rad51 aided by the mediator proteins Rad52 or BRCA2.The RAD51 filaments that result search for a homologous sequence (here the ectopic DNA repair donor template) in double-stranded DNA and the single-stranded DNA invades donor double-stranded DNA to form a joint

molecule with a displaced strand (D loop). DNA polymerase δ extends the 3′end from the broken chromosome using the donor strand as a template. To resolve the intermediate by synthesis-dependent strand annealing, the newly synthesized DNA that is made from the donor repair template is displaced (strand displacement) and anneals to complementary sequences exposed by 5′–3′resection of the other side of the broken chromosome.

## 1.4.2 DNA Repair: non-homologous end joining

In Vertebrates, double stranded breaks (DSBs) in DNA arise during V(D)J recombination and immunoglobulin heavy chain class switch recombination, DNA replication errors, ionizing radiation, reactive oxygen species, and cleavage by nuclear enzymes. Often the DNA ends are modified or have mismatching overhangs that preclude direct ligation and undergo resection by nucleases to directly produce microhomology, or polymerases can add nucleotides. Some DSBs can be directly joined by ligases complexed with repair machinery. The three general steps of NHEJ are DNA end-binding and bridging, terminal end processing, and ligation. In DNA End-binding and Bridging DSB are recognized by the Ku70–Ku80 heterodimer (Ku) , which complexes with the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) (Figure 3) (Meek et al., 2008). DNA-PKcs can phosphorylate several nuclear proteins *in vitro*, including DNA ligase IV and XRCC4. Because NHEJ requires two blunt DNA ends in order to rejoin the chromosome, terminal end processing of modified terminal nucleotides or resection/fill in of overhangs may be required. In cases of DNA resection, DNA-PKcs and complexed with Artemis (Gu et al., 2010) which autophosphorylates and becomes competent to cut boundaries of single/double stranded DNA (Chang et al., 2015). In NHEJ in humans, DNA polymerase μ (Pol μ) and Pol λ can interact with Ku to incorporate nucleotides with or without template DNA (Bebenek et al., 2014; Bertocci et al., 2006; Moon et al., 2014). Once blunt DNA ends are produced DNA ligase IV and X-ray repair cross-complementing protein 4 (XRCC4) can bind Ku (Costantini et al., 2007), and ligate DNA. NHEJ is often called an error-prone pathway because of the variety of insertion or deletion possibilities for the resulting sequence. NHEJ can resolve DNA breaks without sequence changes, but often because of the need for resection and fill in to create microhomology or unmodified blunt DNA ends, nucleotides at the DNA ligation boundary are added or removed. These changes can have significant impact in coding and non-coding regions of

the genome and the application of site nucleases can exploit this property in gene regulation covered later in this chapter.

### 1.4.3  DNA Repair: homology directed repair

Homology directed repair (HDR) is sometimes called "error free" repair because a homologous DNA is used as a template to repair a broken chromosome. Active in S and G2 phases of the cell cycle, DNA at a double stranded break Mre11-Rad50-NSB1 (MRN) and CtIP resect 5' -3' to produce a 3' single stranded stretch of DNA that is bound by the high-affinity single-stranded binding protein RPA. BRCA2 is the principal mediator for the nucleation of RAD51 deposition where RPA is subsequently replaced with RAD51 forming a synaptic filament that searches for ssDNA–dsDNA homology (Galletto et al., 2006) and initiates homology search for matching dsDNA sequence (Benson et al., 1994). Invasion of homologous DNA creates a D-loop that is stabilized by RPA and the annealed ssDNA acts to prime polymerase δ or polymerase ε (Wilson et al., 2013)and DNA is polymerized to the extent needed for the newly synthesized of stretch of DNA to re-pair with the other end of its broken chromosome and the and is filled in by synthesis.

### 1.4.4  Site- specific nucleases

Introducing deliberate and site-specific changes in the genomes of living cells (gene editing) currently relies on the introduction of double stranded DNA breaks to create insertion/deletion of random nucleotides through the NHEJ DNA repair pathway or creating sequence changes by providing an ectopic DNA repair template that is coopted into the HDR pathway. Currently this is done by introducing one of a variety of engineered "site-specific-nucleases" into cells to home in on a specific sequence and produce a double stranded break. In 1994 Maria Jason accomplished the first site specific mutation of a mammalian chromosome using the I-Sce-1, a mitochondrial intron-encoded endonuclease from *S. cerevisiae* (Rouet et al., 1994). Many years of technological development and discovery is overlooked here to summarize that there are now three main nucleases used for gene targeting: zinc-finger nuclease (ZFN), Transcription activator-like effector nuclease (TALEN) or CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats) CRISPR-associated (*Cas*) genes.

The zinc finger domain is among the most common type of DNA-binding motifs found in eukaryotes and zinc-finger domains have been commercially produced to recognize nearly all of the 64 possible nucleotide triplets. Using computational prediction modules of zinc fingers can be assembled based on prediction to target DNA sequences with a chimerically fused nuclease to produce a double stranded break. Pre-selected zinc-finger modules can be linked together in tandem to target DNA sequences that contain a series of these DNA triplets (Beerli et al., 2000; Gonzalez et al., 2010; Kim et al., 2011; Liu et al., 1997). Transcription activator like effector (TALE) proteins from the plant pathogenic bacteria genus *Xanthomonas*, similar to zinc fingers, are 33–35 amino acid repeat domains that recognize single nucleotides, however these proteins can be assembled modularly with far less constraint or prediction than the previous ZFN technology (Christian et al., 2010; Miller et al., 2011; Mussolino et al., 2011). CRISPRs (clustered regularly interspaced palindromic repeats) were first bioinformatically discovered in *Escherichia coli (Ishino et al., 1987)* and many other bacterial genomes eventually to be determined to be an adaptive defense system that uses antisense RNAs in conjunction with nucleases to defend against viral infection (Tang et al., 2002). From this body of findings the CRISPR Cas9 system for the purpose of gene editing was developed showing that trans-activating crRNA (tracrRNA), a small RNA that is trans-encoded upstream of the type II CRISPR-Cas locus in *Streptococcus pyogenes*, could be synthetically adapted to recognize most specific sequences in a genome with relatively little constraint when co-expressed with the Cas9 nuclease (Jinek et al., 2012; Jinek et al., 2013). In practical terms, this meant that almost anyone that could transfect commonly used cells could simply clone a short oligonucleotide to clone into a plasmid and they could start gene editing. The simplest form of gene editing with a site-specific nuclease is to introduce a double stranded break in a gene that results in an out-of-from indel mutation that creates a gene knock out. For more sophisticated applications of creating point mutations, reporters and gene fusions the codelivery of a DNA repair template would be required.

### 1.4.5 Gene editing with repair templates

Since Maria Jasin's groundbreaking accomplishments is site-specific gene editing  (Rouet et al., 1994) the proposition of editing cells to study gene function has been for many equal parts delight at the possibility of

discovery and frustration at the ruinously low efficiency of template mediated repair. Early work like that of Rudnicki, Jaenisch, Carrol (Carroll, 2004; Rudnicki et al., 1992) and others soldiered on, but the programable nucleases (ZFN, TALEN, Cas9) began the democratization of gene editing. Hockemeyer and collogues used ZFNs to label the pluripotency factor OCT4 (POU5F1) and in the same study targeted the *AAVS1* locus located on chromosome 19, encoding the *PPP1R12C* thereby demonstrating that reporters and overexpression can be targeted in ES cells without any apparent biological defects and that these cells could also be differentiated into other mature cell types. Of note, these strategies relied on antibiotic selectable markers that dramatically increase targeting efficiency. In 2011 Soldner and collogues made a model of Parkinson's disease by editing single base substitutions recapitulating the monogenic disorder in otherwise isogenic ESCs for the first time, but with an experimental targeting rate of only about one percent in a very expensive and labor intensive set of experiments that for some other gene-edits simply does not work at all (Soldner et al., 2011). Few reports have convincingly claimed greater consistently higher efficiencies than this to date. Most strategies rely on using antibiotic selection or accepting the DNA lesions left behind after excision of recombineering technologies such as Flp/FRT and *loxP* (Yang et al., 2017). Nonetheless, the potential for modeling diseases in engineered cells, particularly monogenic diseases that can be made in cells that are in matched isogenic backgrounds overcomes so many of the confounding features of previous disease models this area has continued to gain a tide of interest.

## 1.4.6  Disease modeling

Animal modeling, which is overwhelmingly done in mice, has provided a wealth of information and expedited the path to drug discovery. However, differences in absorption, distribution, metabolism, and excretion (ADME) of xenobiotics biological differences such as the types of cancers, lifespan and the extensively inbreed nature of lab mice presents an obstacle to making direct conclusions from these experiments (Rangarajan and Weinberg, 2003; Shanks et al., 2009). Human cellular models share the same genetic origin but stable cells lines are often transformed into immortality using perturbations to the normal quality controls such as cell cycle checkpoints and have further been shown to have not only abnormal karyotype, but ongoing karyotypic instability over ongoing passages

(Stepanenko and Dmitrenko, 2015). Genetic engineering methods such as reprogramming with integrating viruses and even simple genetic modifications at specific loci can have the unintended consequences of incomplete epigenetic remodeling and disruption of neighboring genes or RNA's expressed from the corresponding antisense locus (Carey et al., 2011; Lister et al., 2011) and in female cell lines, mosaicism and unstable X-inactivation can perturb expression patters of genes and confound the interpretation of phenotypes. (Mekhoubad et al., 2012). Because humans typically vary from the annotated reference genome at several thousand protein coding sites, comparing iPSCs derived from different donors there is a hazard in drawing strong conclusions from disease versus normal models (McCarthy et al., 2013; Tennessen et al., 2012). For these and other reasons the central aims of my thesis were to refine methods for genetically engineering both pluripotent stem cells and human derived tissues, and to create a robust differentiation protocol for differentiating stem cells with the end goal of having isogenically matched cultures that differed only at the mutation of interest.

# Chapter 2

# Human intestinal tissue with adult stem cell properties derived from pluripotent stem cells

## 2.1 Summary

We developed a derivation method for human intestinal organoid cultures from hPSCs. Isolating intestinal cells from hPSC-derived teratomas yields intestinal cultures similar to organoids isolated form primary tissue. Molecular and histological analysis demonstrates that hPSC-derived organoids comprise the major cell types of the intestinal epithelium including adult stem cell-like cells. Transcriptional profiling reveals that hESC-derived organoids are highly similar to organoids isolated from primary tissue.

## 2.2 Abstract

Genetically engineered human pluripotent stem cells (hPSCs) have been proposed as a source for transplantation therapies and are rapidly becoming valuable tools for human disease modeling. However, many applications are limited due to the lack of robust differentiation paradigms that allow for the isolation of defined functional tissues. Here we derive adult stem cells from hPSCs using an endogenous LGR5-GFP reporter that give rise to functional human intestinal tissue comprising all major cell types of the intestine. Histological and functional analysis revealed that such human organoid cultures could be derived with high purity, and a composition and morphology similar to cultures obtained from human biopsies. Importantly, hPSC-derived organoids responded to the canonical signaling pathways that control self-renewal and differentiation in the adult human intestinal stem cell compartment. This adult stem cell system provides a platform to study human intestinal disease in vitro using genetically engineered hPSCs.

## 2.3 Introduction

Human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs)(Takahashi et al., 2007), collectively referred to as pluripotent stem cells (hPSCs), are currently used in disease modeling to address questions specific to humans and to complement our insight gained from other model organisms (Soldner and Jaenisch, 2012; Soldner et al.). Genetic engineering using site specific nucleases has recently been established in hPSCs (Dekelver et al., 2010; Hockemeyer et al., 2009; Hockemeyer et al., 2011; Yusa et al., 2011; Zou et al.), allowing a level of genetic control previously limited to model systems. We can now target gene knockouts, generate tissue-specific cell lineage reporters,

overexpress genes from a defined locus, and introduce or repair single point mutations in hPSCs. Realizing the full potential of hPSCs will require robust differentiation protocols. Most current protocols isolate individual cell types rather than establish functional tissues. While the former can identify cell autonomous phenotypes, the study of cell non-autonomous disease mechanisms necessitates a defined tissue context where individual cell types are represented with the same stoichiometry and architecture as occurs *in vivo*. The recent establishment of human intestinal tissue as *in vitro* organoid cultures from human hPSCs and primary tissue presents a major advance towards such model system for human tissue (Jung et al., 2011; McCracken et al., 2011; Ootani et al., 2009a; Sato et al., 2011b; Sato et al., 2009; Spence et al., 2011). Intestinal organoid cultures comprise tissue-specific differentiated cell types and adult stem-like progenitor cells that self-renew and differentiate, by growth factor induction, into the respective cell types of the intestinal epithelium. Here, we establish a protocol that can enrich for intestinal cells with adult stem character. We first generated an hESC line using gene editing that specifically labeled intestinal adult stem cells using a fluorescent reporter placed into an endogenous gene and then used this cell line to identify and isolate adult stem cells from a pool of heterogeneous cell types during the differentiation of hPSCs.

We focused on LGR5 (McDonald et al., 1998), which belongs to the leucine-rich repeat-containing G protein-coupled receptor protein class. LGR5 functions within the WNT (Wingless-related integration site)-signaling cascade, which maintains the adult intestinal stem cell compartment (de Lau et al., 2011). LGR5 is activated by its ligand, R-Spondin  (RSPO1) (Carmon et al., 2011; de Lau et al., 2011; Kim et al., 2005a; Ruffner et al., 2012), and has been shown by genetic lineage tracing experiments to mark intestinal stem cells (Barker et al., 2007). LGR5-expressing cells at the base of the intestinal crypt exhibit WNT-dependent self-renewal and can differentiate into all cell types of the adult intestine (Snippert et al., 2010). Together, LGR5-expressing cells and Paneth cells form the adult stem cell niche and are sufficient to establish *in vitro* organoid cultures from mice (Sato et al., 2011b). Such murine *in vitro* organoids can be maintained over time in 3D Matrigel cultures under defined conditions that either support WNT- dependent self-renewal of the adult stem cells or differentiation by the withdrawal of WNT and Notch signaling (Korinek et al., 1998a; Pellegrinet et al., 2011; van Es et al., 2005). Similarly, human organoid

cultures lacking stromal components can be derived from primary tissue biopsies when supplemented with additional small-molecule signals (Jung et al., 2011; Sato et al., 2011a; Sato et al., 2009) and *in vitro* hPSC-derived organoids can be maintained under a variety of conditions (Jung et al., 2011; McCracken et al., 2011; Sato et al., 2011a; Spence et al., 2011; Wang et al., 2013) and used in human disease modeling (Dekkers et al., 2013). Importantly, LGR5-positive mouse colon cells can form organoids that can be expanded *ex vivo* and allogenically transplanted into colitis models (Fordham et al., 2013a; Yui et al., 2012), suggesting that human intestinal tissue might be amenable to transplantation therapies.

Here we report tools that allow for the isolation of adult intestinal stem cells and intestinal organoid cultures from direct differentiation of hPSCs using standard teratoma differentiation assays. Cultures with posterior gut qualities and expression profiles closely resembling human intestinal tissue can be derived and progressively enriched. Our strategy is based on directly isolating LGR5-positive intestinal cells, a cell type that is only acquired over extended periods by other protocols (Cao et al., 2011; McCracken et al., 2011; Ogaki et al., 2013; Spence et al., 2011; Wang et al., 2011). Functional analysis and mRNA expression profiling of these organoid cultures confirmed the presence of a subset of adult stem cells. We expect that genetically engineering the parent hPSCs of an *in vitro* human intestinal tissue will provide a model to investigate human intestinal pathophysiology.

## 2.4 Results

### 2.4.1 Genome editing the endogenous human LGR5 locus in human pluripotent stem cells to isolate adult intestinal stem cells

Based on previous findings in mice that used LGR5 expression to identify (Barker et al., 2007) and isolate (Sato et al., 2011b) adult intestinal stem cells, we tested whether a similar approach could be used to isolate and characterize gastrointestinal tissue derived from hPSCs. We developed two Zinc Finger Nuclease (ZFN) pairs that target the LGR5 gene either in the first (LGR5-GFP [N-term]) or last coding exon (LGR5-GFP [C-term]) (Figures 4A, S5A). We co-electroporated these ZFNs and their corresponding donor plasmids into WIBR3 hESCs (Lengner et al., 2010) to integrate a GFP cassette together with a PGK-Puromycin into the LGR5 locus (Figure 4A). Southern Blot analysis using external and internal

probes (Figures 5A) and sequence analysis in subsequent RNAseq, thus confirming the correct junction of the LGR5 gene with the GFP reading frame at either position. The targeting efficiencies for the generation of LGR5-GFP $^{N-term}$ and LGR5-GFP $^{C-term}$ cell lines were 18.75% and 6.25%, respectively.
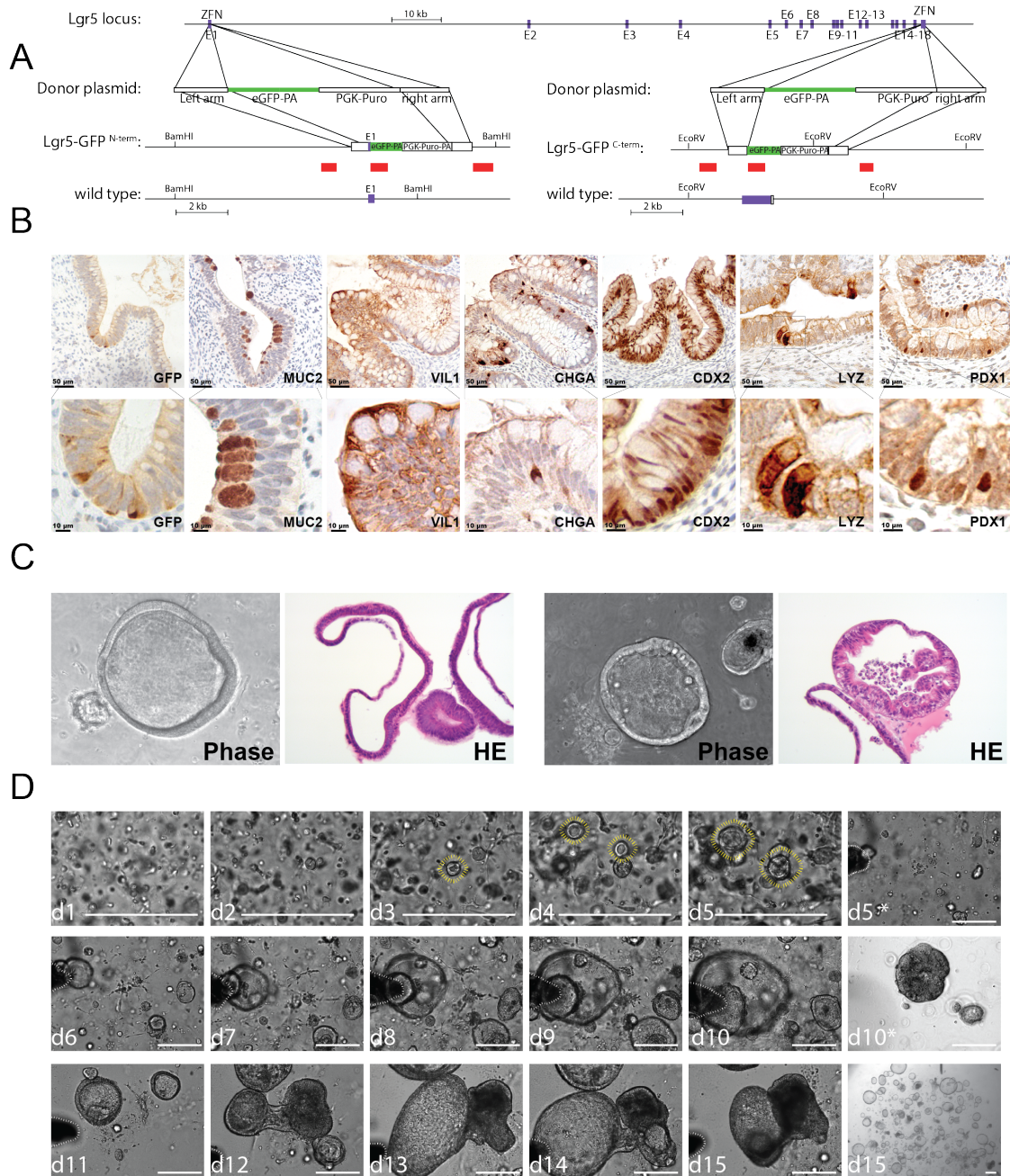
## Figure 4: Generation of LGR5-GFP reporter hESCs using ZFNs

(A) Schematic overview depicting the gene editing strategy for the LGR5 locus using a ZFN targeted to either the first or last coding exon of LGR5. Southern blot probes are shown as red boxes, exons are shown as blue boxes, and the vertical arrow indicates respective LGR5 ZFN cut sites. Below are the donor plasmids used to target the LGR5 locus to generate either an N-terminal (LGR5-GFP [N-term]) or C-terminal (LGR5-GFP [C-term]) LGR5-GFP reporter: pA, polyadenylation sequence; PGK, phosphoglycerate kinase promoter; Puro, puromycin resistance gene; eGFP, enhanced green fluorescent protein. Shown below the donor plasmids is the LGR5 locus after targeting with the respective donor plasmids.

(B) IHC staining for indicated proteins in teratoma sections derived from LGR5-GFP [N-term] hESC reporter cells, MUC2= mucin2, VIL1= villin1, CHGA= Chromogranin A, CDX2= caudal type homeobox 2, LYZ= lysozyme, PDX1= pancreatic and duodenal homeobox 1).

(C) Phase contrast and hematoxylin and eosin staining (HE) of epithelial organoid cultures arising from LGR5-GFP sorting experiments. See quantification in table 1.

(D) Bright-field images of developing organoids (outlined by yellow lines) derived from nonsorted single-cell suspensions after matrigel embedding. The demarcation outlined by white lines in bright-field images taken on days 5*-15 was used as a reference point for tracking organoids clustered near this region. Cells were passaged every five days– here, an image before and after (*) passaging is shown. All bars 200µm.
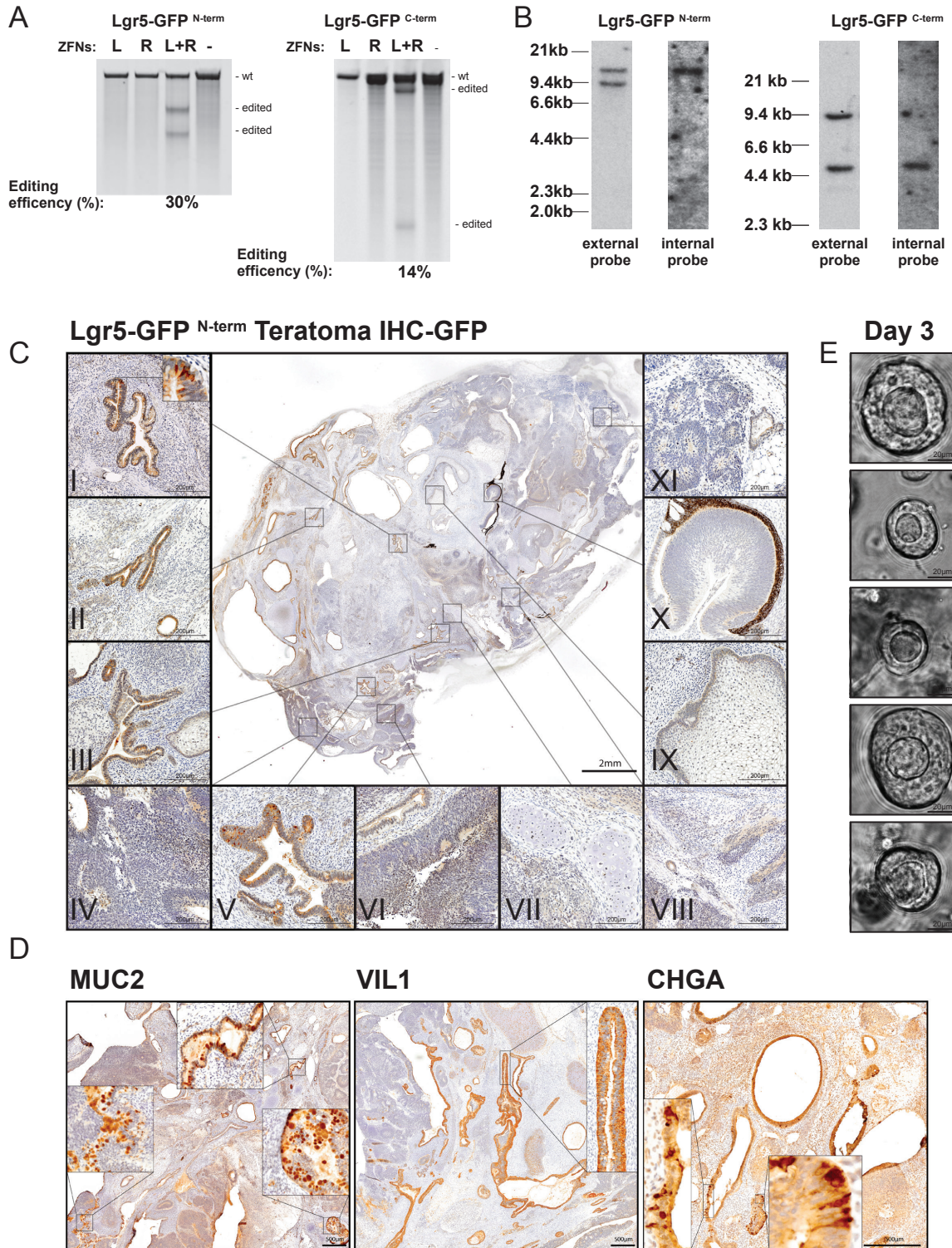
**Figure 5: Genome editing of LGR5 locus in WIBR3 hESCs, Teratoma histology and organoid formation**

(A) Validating genome-editing activity of engineered ZFNs at the first exon of LGR5 (LGR5 N-term) in K562 cells. Following transient transfection of the ZFN expression construct, the stretch of the LGR5 locus surrounding the respective ZFN cut sites was PCR-amplified, and genome editing efficiency was measured using the Surveyor nuclease assay. The degree of target locus disruption was quantified and is shown below each lane.

(B) Southern blot analysis of WIBR3 hESCs targeted in the LGR5 locus with the respective donor plasmid shown in Figure 4A. Genomic DNA was digested with HindIII for LGR5-GFP N-term targeted cells and with EcoRV for LGR5-GFP C-term targeted cells. DNA was hybridized with the $^{32}$P-labeled external 3′-probe or with the internal eGFP probe. Expected fragment sizes for LGR5-GFP N-term are: WT = 4.2 kb, targeted = 6.8 kb for both probes. Expected fragment sizes for LGR5-GFP C-term are: WT = 4.2 kb, targeted = 6.8 kb for both probes.

(C) Immunohistochemical staining for indicated proteins in teratoma sections generated from LGR5-GFP N-term hESC reporter cells (GFP= enhanced green fluorescent protein, large panel shows whole teratoma section with regions of interest showing GFP positive cells in intestinal regions (I-III and V) or other cell types where interpretable [IV: not specified (NS), VI: NS, VII: cartilage, VIII: NS, IX: NS, X: ectoderm/neuroepithelium, XI: neural rosettes].

(D) Immunohistochemical staining for MUC2= mucin2, VIL1= villin1, and CHGA= Chromogranin A in the context of lower magnification images of teratoma slices. Higher magnification images of independently derived organoids (n=5) on the third day after embedding single-cell dissociated teratoma samples into matrigel. Image shows representative pictures of the sub fraction of organoids that form a central lumen at day three.

Initially we characterized this new LGR5 reporter system using a previously established protocol for the direct differentiation of hPSCs to intestinal cells (McCracken et al., 2011; Spence et al., 2011) (data not shown). In order to enrich more of the later steps of differentiation with increased LGR5 expression, we capitalized on our previous observation that in teratoma formation assays, some regions of the teratoma differentiate into intestinal-like tissues (Figures 4B, S5SC-D). When we performed these teratoma formation assays with the LGR5-GFP reporter cells, we were able to specifically detect GFP expression by IHC staining in regions forming a polarized and specialized epithelium (Figure 4B). GFP expression was restricted to intestinal tissues stained positive for CDX2 and VIL1 (Figures 4B, S5C-D) where neighboring cells stained positive for intestinal markers mucin 2 (MUC2), Lysozyme (LYZ), chromogranin A (CHGA) and at a low frequency for PDX1 (Figure 4B,S1C, S1D). No significant GFP-positive staining in any other tissue type present in the

teratoma, such as neuronal rosettes, cartilage or smooth muscle was detected (Figure 5C). Further, we could only detect GFP positive cells in teratomas derived from LGR5-GFP [N-term] (n=3) by IHC, but not in those derived from wild-type cells (n=3) or from LGR5-GFP [C-term] cells (n=3) although these did functionally sort by fluorescence-activated cell sorting (FACS). These observations could suggest a functional difference between the two reporter systems and could be due to differential stability, processing, or localization of the LGR5-GFP fusion protein.

## 2.4.2 Isolation of intestinal organoids from LGR5-GFP hPSCs

The detection of LGR5-GFP positive cells in teratomas prompted us to isolate these cells and to test their ability to generate intestinal organoid cultures. We dissociated teratomas derived from LGR5-GFP hPSCs to a single cell suspension and used FACS to isolate individual cells based on their GFP expression (Table 1 upper and S2B). LGR5-GFP-positive cells were embedded in Matrigel and cultured using conditions previously described for human intestinal organoid cultures (Jung et al., 2011; Sato et al., 2011a; Sato et al., 2011b; Spence et al., 2011; Wang et al., 2013). Under these conditions we observed multicellular epithelial structures, with a central lumen after three days, that proliferated into uniform, long-lived organoids with a morphology previously reported for human intestinal organoids isolated from primary tissue (Fordham et al., 2013a; Jung et al., 2011; Sato et al., 2011a) (Figures 4C-D, S5E, S11A-D).

It is important to note that in independent experiments, these organoids were generated with high frequency from GFP-positive cells (Table1 top), although they could also be robustly generated from non-sorted teratoma cells. Undifferentiated hESCs (Figure 11A) as well as the sorted GFP-negative fraction failed to give rise to organoids (Table1 top) although this does not exclude that LGR5-negative cells could give rise to organoids under alternative conditions. The most prominent non-organoid cells found to grow in the 3D matrix –regardless of whether cells were GFP-sorted or not– were single cells with neuronal precursor morphology. These contaminating cells were removed at each passage, enriching for nearly homogenous long-term (>150 days) organoid cultures that could be maintained regardless of whether they were derived from GFP-positive cells or from non-sorted teratomas (Figures 4C,4D, S5A-D).

WIBR3-hESC Derived

Teratoma-derived organoids[a]

| Teratomas | GFP Negative | | | GFP Positive | | | Total | | |
|---|---|---|---|---|---|---|---|---|---|
| | No. of organoids formed | No. of cells sorted | Cells forming organoids (%) | No. of organoids formed | No. of cells sorted | Cells forming organoids (%) | No. of organoids formed | No. of cells sorted | Cells forming organoids (%) |
| Lgr5-GFP N-term | 0 | 1,000 | 0 | 23 | 1,000 | 2.3 | ~50 | >50,000 | <0.1% |
| Lgr5-GFP N-term | 0 | 1,710 | 0 | 101 | 1,710 | 5.91 | ~50 | >50,000 | <0.1% |
| Lgr5-GFP N-term | 0 | 1,675 | 0 | 21 | 1,675 | 1.25 | ~50 | >50,000 | <0.1% |
| Lgr5-GFP C-term | 0 | 4,203 | 0 | 38 | 4,203 | 0.9 | ~50 | >50,000 | <0.1% |
| Lgr5-GFP C-term | 0 | 6,613 | 0 | 81 | 6,613 | 1.22 | ~50 | >50,000 | <0.1% |
| Lgr5-GFP N-term | 0 | 6,046 | 0 | 3 | 6,046 | 0.05 | ~50 | >50,000 | <0.1% |

Organoid-rederived organoids[b]

| Organoids | GFP Negative | | | GFP Positive | | | Total | |
|---|---|---|---|---|---|---|---|---|
| | No. of organoids formed | No. of cells sorted | Cells forming organoids (%) | No. of organoids formed | No. of cells sorted | Cells forming organoids (%) | No. of organoids formed | No. of cells sorted | Cells forming organoids (%) |
| Lgr5-GFP N-term | 0 | 1,500 | 0 | 7 | 1,500 | 0.47 | 7 | 15,000 | 0.05 |
| Lgr5-GFP N-term | 24 | 6,700 | 0.36 | 140 | 6,700 | 2.09 | 65 | 13,000 | 0.5 |
| Lgr5-GFP N-term | 3 | 7,960 | 0.04 | 20 | 7,960 | 0.26 | 15 | 38,450 | 0.04 |

[a]Six independent teratomas FAC-sorted and assayed for organoid formation.
[b]Three organoids derived from three independent unsorted teratomas (dissociated, FAC-sorted, and assayed for organoid formation).

## Table 1. Organoid formation assay

| | | | | |
|---|---|---|---|---|
| duodenum #1 | primary organoid | wt human biopsy | NA | WENR+ inhibitors |
| duodenum #2 | primary organoid | wt human biopsy | NA | WENR+ inhibitors |
| ileum #1 | primary organoid | wt human biopsy | NA | WENR+ inhibitors |
| ileum #2 | primary organoid | wt human biopsy | NA | WENR+ inhibitors |
| rectum #1 | primary organoid | wt human biopsy | NA | WENR+ inhibitors |
| rectum #2 | primary organoid | wt human biopsy | NA | WENR+ inhibitors |
| intestinal organoid#1 | hESC-Organoid | WIBR3- N-term | 1 | GF-Media |
| Diff. intestinal organoid#1 | hESC-Organoid | WIBR3- N-term | 1 | *GF -Media+DAPT |
| hESC#1  LGR5-GFP N-term | hES | WIBR3- N-term | 1 | hES MEDIA |
| intestinal organoid# 2 | hESC-Organoid | WIBR3- C-term | 2 | GF-Media |
| Diff. intestinal organoid# 2 | hESC-Organoid | WIBR3- C-term | 2 | *GF -Media+DAPT |
| hESC#1  LGR5-GFP  C-term | hES | WIBR3- C-term | 2 | hES MEDIA |
| hESC#1 (WIBR#3) | hES | WIBR3 | 3 | hES MEDIA |
| intestinal organoid#3a | hESC-Organoid | WIBR3 | 3a | GF-Media |
| teratoma #4 | Teratoma | WIBR3-AI9 | 4 | Mouse |
| intestinal organoid#4a | hESC-Organoid | WIBR3-AI9 | 4a | GF-Media |
| teratoma #5 | Teratoma | WIBR3-AI9 | 5 | Mouse |

| | | | | |
|---|---|---|---|---|
| intestinal organoid#5a | hESC-Organoid | WIBR3-AI9 | 5a | GF-Media |
| teratoma #1 | Teratoma | WIBR3 | 1 | Mouse |
| teratoma #2 | Teratoma | WIBR3 | 2 | Mouse |
| intestinal organoid#3b | hESC-Organoid | WIBR3 | 3b | GF-Media |
| intestinal organoid#4b | hESC-Organoid | WIBR3-AI9 | 4b | GF-Media |
| intestinal organoid#5b | hESC-Organoid | WIBR3-AI9 | 5b | GF-Media |
| intestinal organoid# 6 | hESC-Organoid | BGO1 | 6 | GF-Media |
| Diff. intestinal organoid#6 | hESC-Organoid | BGO1 | 6 | *GF -Media+DAPT |
| intestinal organoid# 7 | hESC-Organoid | BGO1 | 7 | GF-Media |
| Diff. intestinal organoid#7 | hESC-Organoid | BGO1 | 7 | *GF -Media+DAPT |
| background#4 | Non-Organoid | WIBR3-AI9 | 4 | GF-Media |
| background#5 | Non-Organoid | WIBR3-AI9 | 5 | GF-Media |
| hESC#6-7 (BGO1) | hES | BGO1 | 6,7 | hES MEDIA |

## Table 2: Genetic background and culture conditions for RNAseq

RNA samples were analyzed by RNAseq (column 1). The second column (tissue) indicates the tissue type of each RNA sample. RNA from primary organoid samples was isolated from organoid lines that were both cultured for 1-6 months and derived from duodenum, ileum, or rectum biopsies of human subjects as described previously (Sato et al., 2011a) media called WENR+inhibitors elsewhere. hESC-derived organoids, hESC and teratoma indicates RNA samples that were isolated from these steps in the differentiation protocol. Non-organoid material indicates single-cell contaminants that were removed by the enrichment protocol described as "single cell background" in the text. The third column indicates the "*Genetic Background*" of the samples respecting the parent tissue. hESC-derived organoids were isolated from 5 genetically distinct hESC

lines: WIBR3, WIBR3 Lgr5-GFP [N-term], WIBR3 Lgr5-GFP [C-term], WIBR3-AI9, and BGO1. Column 4 shows the correspondence of independently derived teratomas (1-7) to the parental hESCs and derived organoid samples and shows biological replicates (indicated a or b). The last column shows the specific growth conditions as described in the methods section with GF equivalent to the stem cell growth factors except when differentiation required WNT3a withdrawal (*GF-Media+DAPT).

## 2.4.3 hPSC-derived intestinal organoids comprise all major cell types of the intestine

To characterize hPSC-derived intestinal organoids, we performed qRT-PCR analysis of cultures derived from GFP-FACS experiments, as well as from non-sorted teratoma cells, followed by RNAseq analysis of samples of tissues from different stages of our differentiation paradigm and primary intestinal biopsy derived organoids (Supplemental Table 1). These analyses revealed the specific upregulation of intestinal stem cell markers such as LGR5, KLF5, and SOX9, and intestine specific genes such as VIL1, MUC2, and TFF3 (Figure 13E-F). Marker gene expression for non-intestinal cell types such as FGF8 and Nestin and Sox1 (neural) or MIXL1 (mesoderm) were significantly decreased in hPSC-derived intestinal organoids. Genes not-associated with high intestinal stem cell expression showed low and variable expression levels in the GFP-sorted organoids, and pluripotency genes such as Nanog were absent. These data indicate that hESC-derived organoids were no longer pluripotent, had an intestinal expression pattern, and could be isolated without GFP-sorting (Figure 6 and 7A, 13F). Organoid cultures failed to form tumors when injected subcutaneously into immunocompromised mice (data not shown).

**Figure 6: Isolation of intestinal organoids independent of the LGR5-GFP reporter system**

Expression profiling of organoids from the GFP positive and nonsorted LGR5-GFP teratoma cells compared to hESCs and fibroblast-like cells. qRT-PCR for the indicated genes in hESCs, organoids derived from eGFP positive cells (n=3), and nonsorted (n=3) cells of LGR5-GFP [N-term], hESCs and fibroblast-like cells (derived from hESCs, as described previously (Hockemeyer et al., 2008), expressing telomerase from the AAVS1 locus) Relative expression levels were normalized to baseline expression of these genes in hESCs. Data are biological replicates of independent experiments, bars represent the SEM.
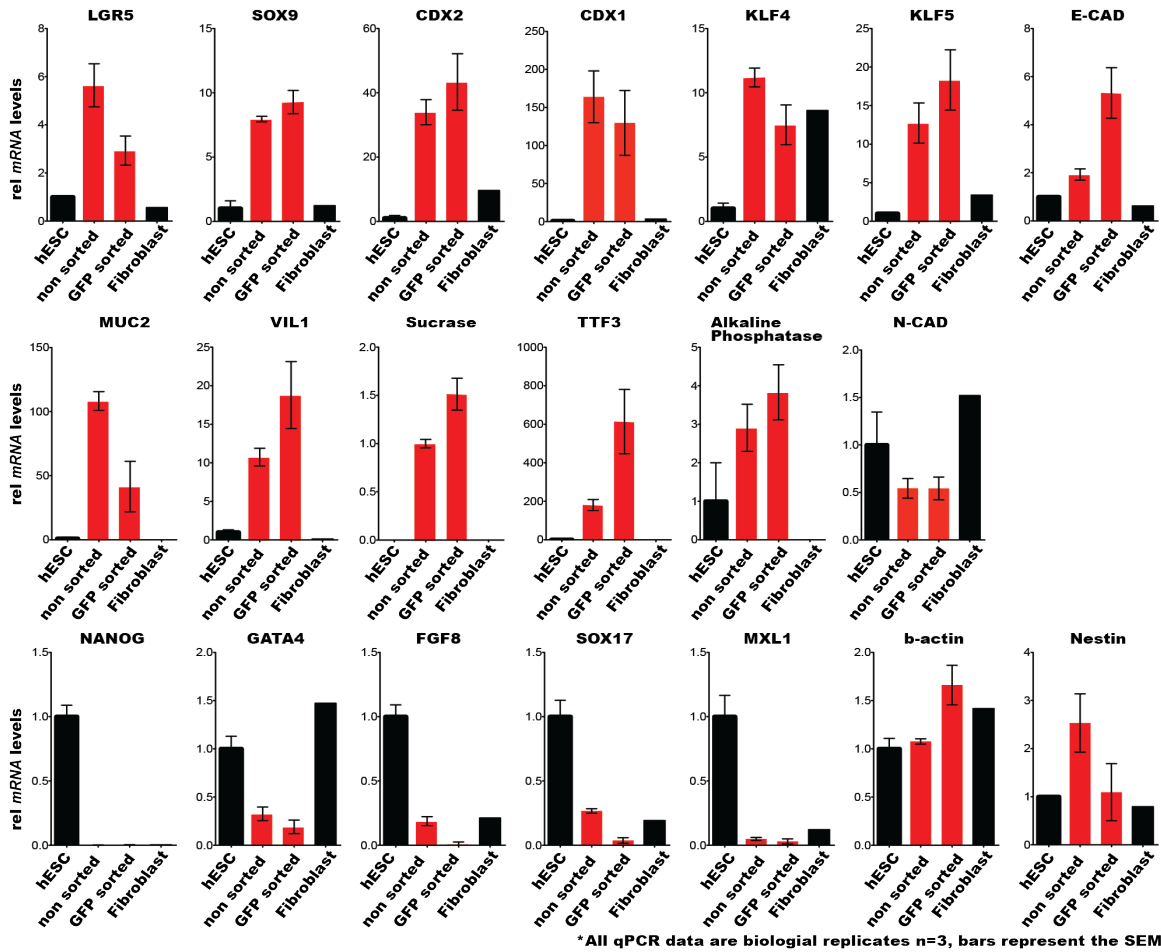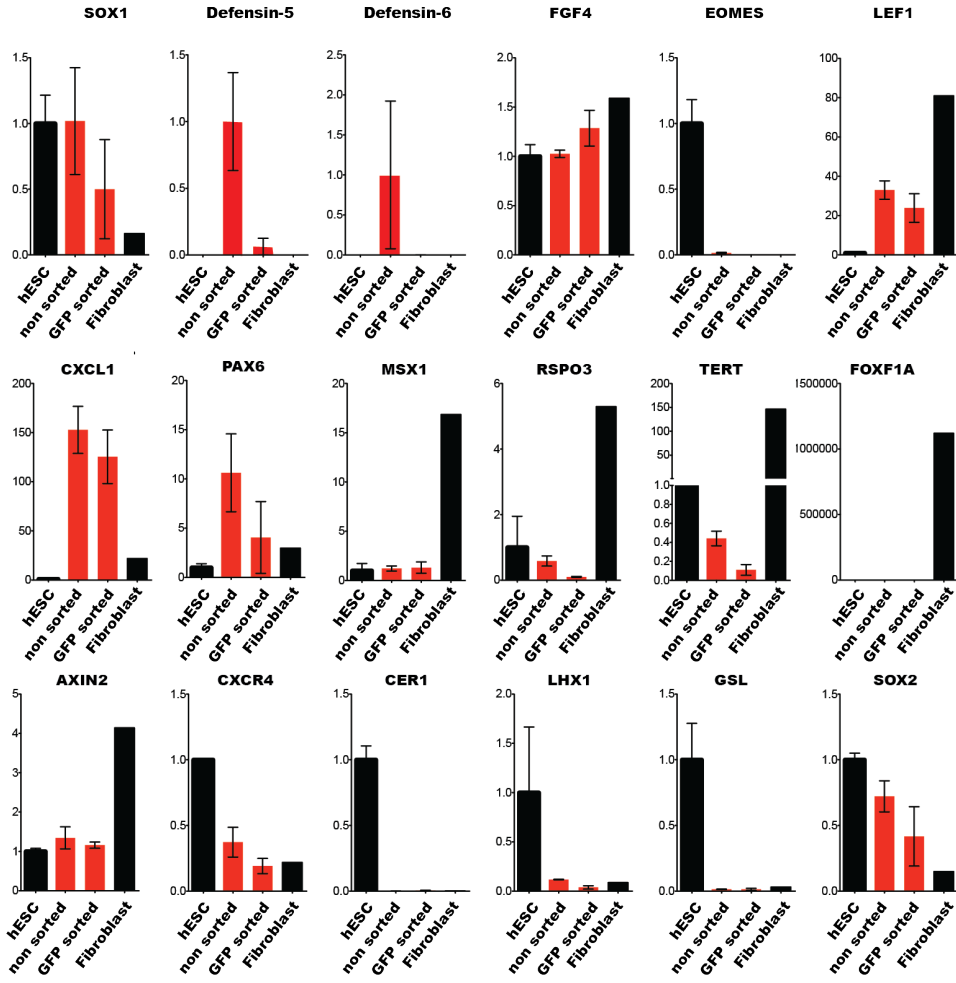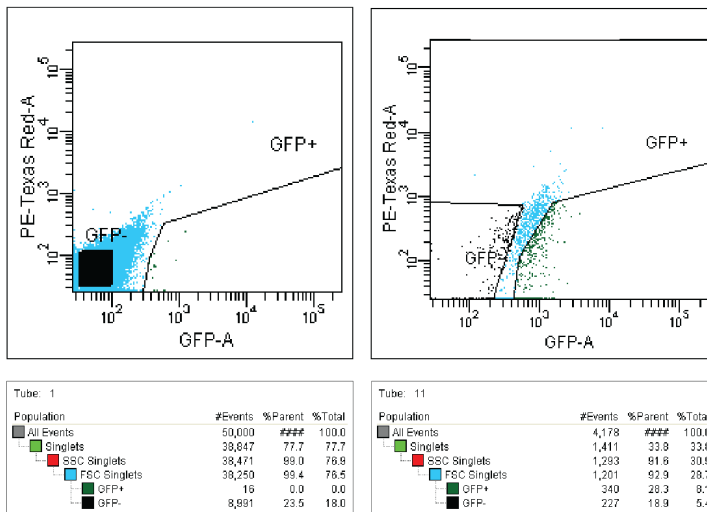
**Figure 7: Isolation of intestinal organoids: the LGR5-GFP reporter system**

(A) Expression profiling of organoids from GFP positive and unsorted LGR5-GFP teratoma cells compared to hESCs and fibroblast-like cells. Quantitative RT-PCR for the indicated genes in hESCs, organoids derived from eGFP-positive cells (n=3) or nonsorted (n=3) cells of LGR5-GFP [N-term], hESCs and fibroblast-like cells [derived from hESCs, as described previously (Hockemeyer et al., 2008), expressing telomerase from the AAVS1 locus]. Relative expression levels were normalized to expression of these genes in hESCs. Error bars indicate the SEM. Genes such as PAX6, SOX2 and Defensins 5 and 6 are depreciated in GFP sorted cells.

(B) Left: Representative FACS analysis (top) and sorting statistics of LGR5-GFP [N-term] hESCs isolated from teratoma explants. Gates were chosen based on undifferentiated hESC cells.
Right: Representative FACS analysis (top) and sorting statistics of LGR5-GFP [N-term] hESCs isolated from teratoma explants not sorted for GFP but cultured for 5 days prior to this analysis in organoid culture conditions. Gates and settings of FACS were identical in both plots.

We found a nearly identical expression profile between organoid cultures that had been generated using either the LGR5-GFP marker and unsorted teratoma cells, suggesting that our culture method was selective for intestinal stem/progenitor cells and that though functional, the LGR5-GFP reporter was dispensable for the isolation of intestinal organoid cultures by this method. These observations indicate that our protocol may be generalized to other genetically engineered hPSCs without the LGR5 reporter.

The major differentiated cell types of the intestinal epithelium include Paneth cells, enterocytes, goblet cells and enteroendocrine cells. We asked whether these cell types could also be detected in our hPSC-derived intestinal organoids. Immunohistochemical (IHC) analysis showed that the hPSC-derived organoids comprise a mostly polarized epithelium including differentiated cell types with distinct morphologies and specific marker gene expression (Figure8). We identified cells with robust expression of CHGA, a marker for enteroendocrine cells, MUC2, a marker for goblet cells, as well as VIL1, marking the villi of enterocytes (Figure 8). Furthermore, we observed a subset of organoids contained cells that were enriched for lysozyme staining (Figure 9).
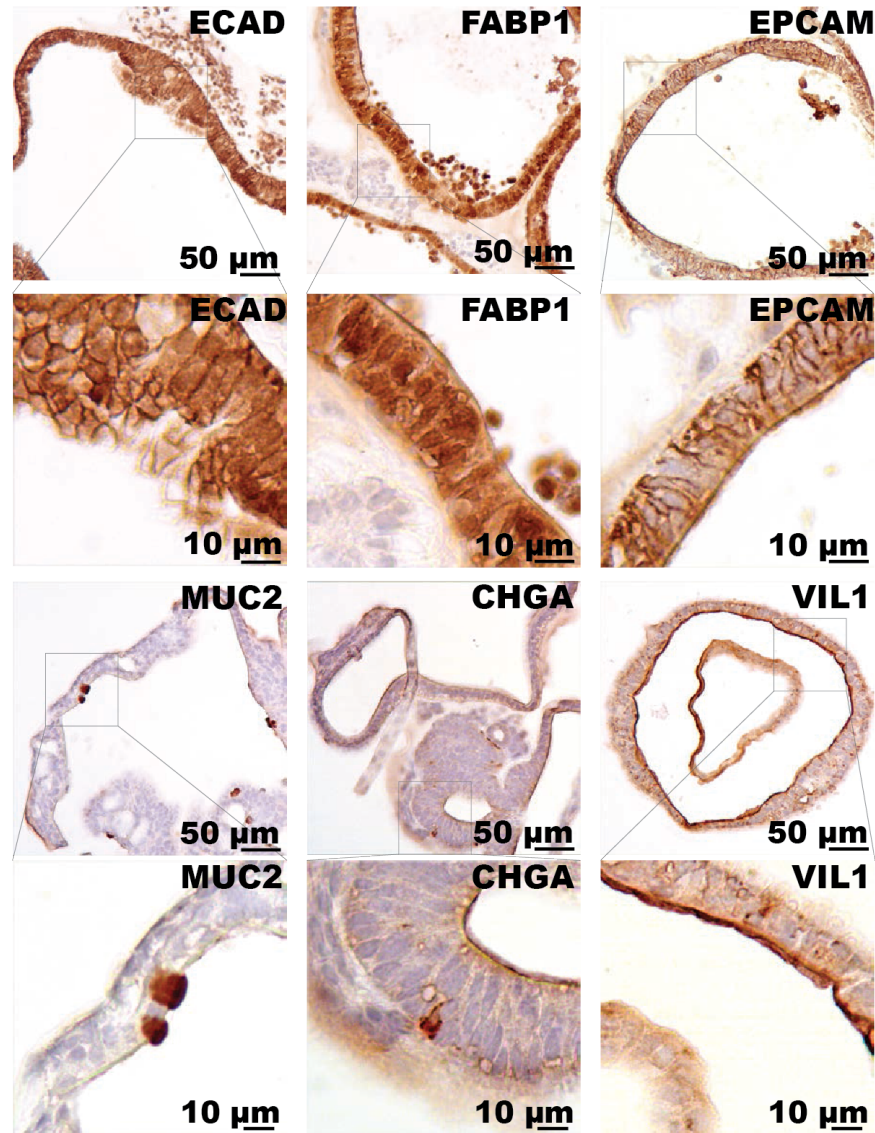
**Figure 8: hESC-Derived Organoids Comprise Specific Cell Types Characteristic of the Human Intestinal Epithelium**

(A) IHC staining for intestinal marker proteins in sections of hESC-derived organoids generated from wild-type hESC reporter cells. ECAD1, E-cadherin; FABP1, fatty acid-binding protein 1; EPCAM, epithelial cell adhesion molecule; MUC2, mucin2; CHGA, chromogranin A; VIL1, villin1. The expression of adult intestinal marker proteins for the major cell types of the adult intestinal tissue prompted us to further analyze the composition and subcellular organization of hESC-derived organoids by electron microscopy (EM). Micrographs showed that organoids were organized in a polarized epithelium, with nuclei positioned proximal to the basolateral membrane (Figure 10A). The apical cellular membranes were characteristic of enterocytes with highly organized microvilli (Figure 10B-II and DIII) and tight junctions connecting neighboring enterocyte-

like cells (Figure 10B-I and DIII). Secretory cells (Figure 10C) with a goblet shape that contained large vesicles coming from a well-defined rough endoplasmic reticulum (Figure 10D I-II) were embedded in the enterocytes. Vesicles of these cells were localized at the apical side of the cell, suggesting secretion toward the lumen of the organoid (Figure 10).
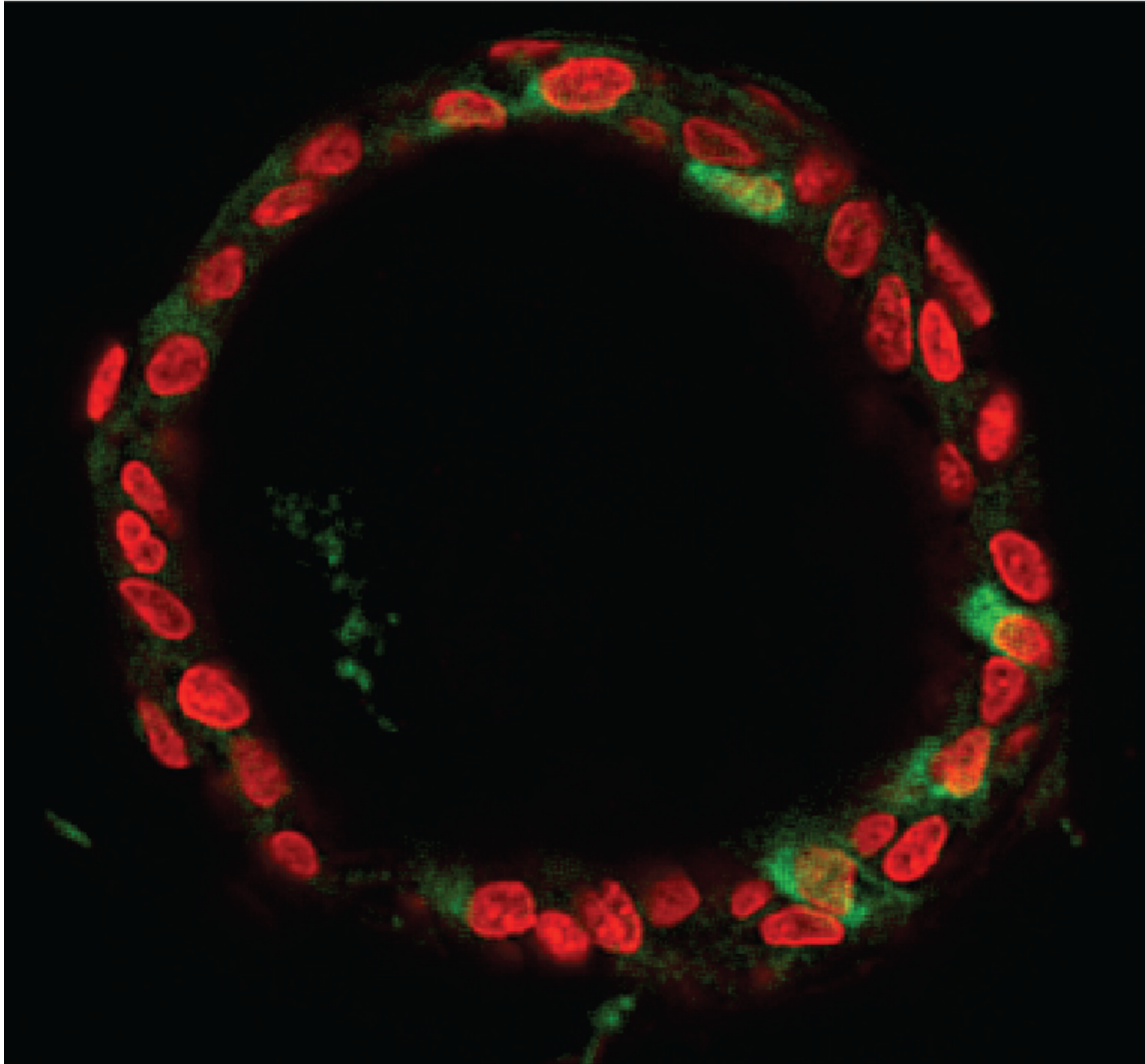


**Figure 9: hESC-derived organoids contain Lysozyme positive cells indicating the presence of Paneth cells.**

Immunofluorescence staining of a hESC-derived human intestinal organoid stained for Lysozyme (green) and DAPI (red) by confocal as in Supplemental movie 1.
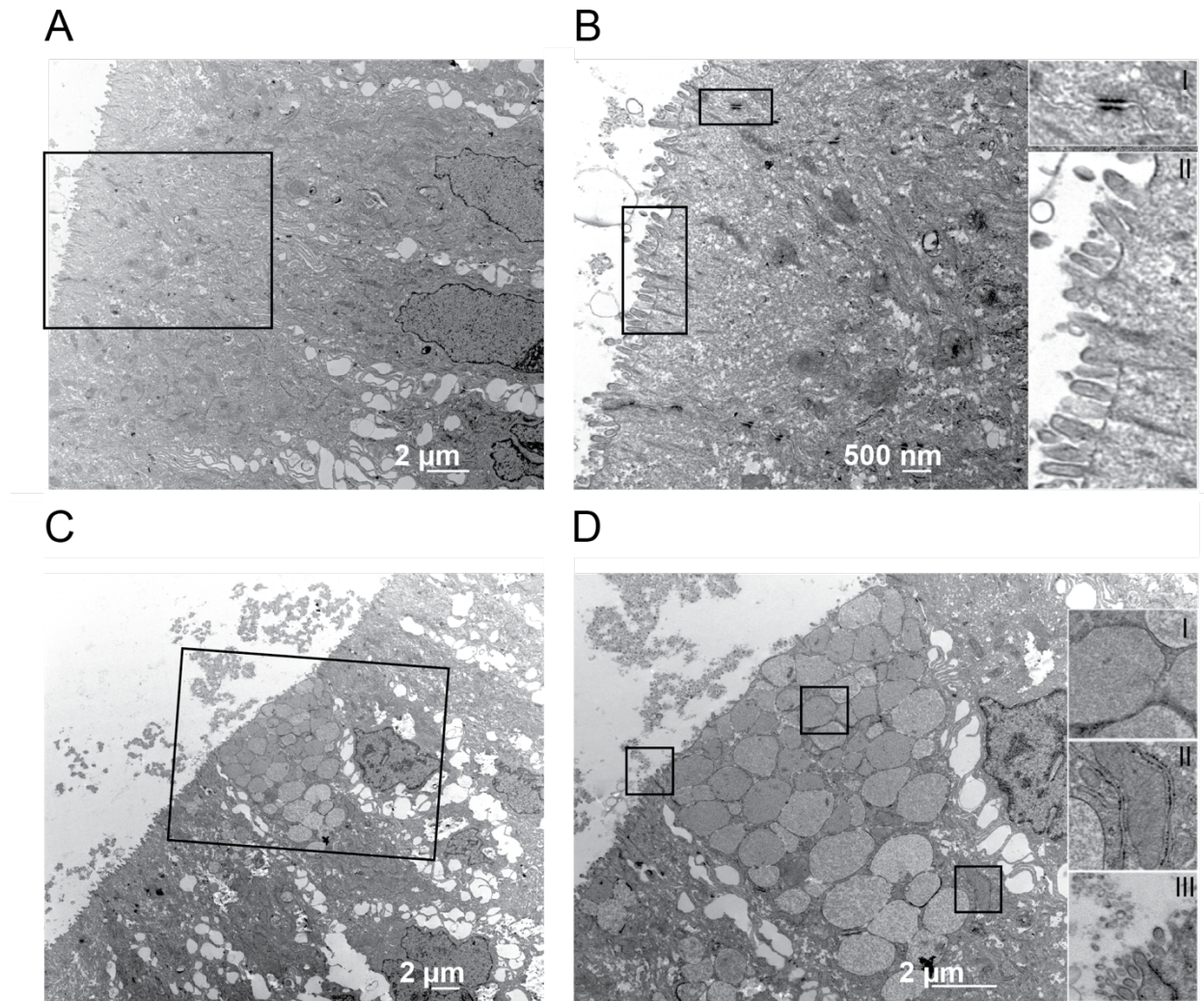
**Figure 10: Subcellular Organization of Organoids Resembles the Structure of the Human Intestinal Epithelium**

(A) Electron micrograph of hESC-derived organoids. Shown is a representative image with enterocyte-like cells forming a polarized epithelium. The lumen of the organoid is oriented to the left (apical), while the nuclei are aligned along the right (basal).

(B) Higher-magnification images of the micrograph shown in (A). Insets indicate areas of increased magnification. Images show the microvilli lining the luminal cell surface, vesicles in the luminal space (II), and the tight junctions connecting adjacent enterocyte-like cells (I).

(C) Electron micrograph of hESC-derived organoids. Shown is a representative image of a goblet-like secretory cell that is embedded into a sheet of enterocyte-like cells. Orientation of the image as in (A).

(D) Higher-magnification images of the micrograph shown in (C). Insets indicate areas of increased magnification. Images show magnification of vesicles (I), rough endoplasmic reticulum at the base of the vesicles (II), and tight junctions and cellular debris shed into the organoid's lumen (III).
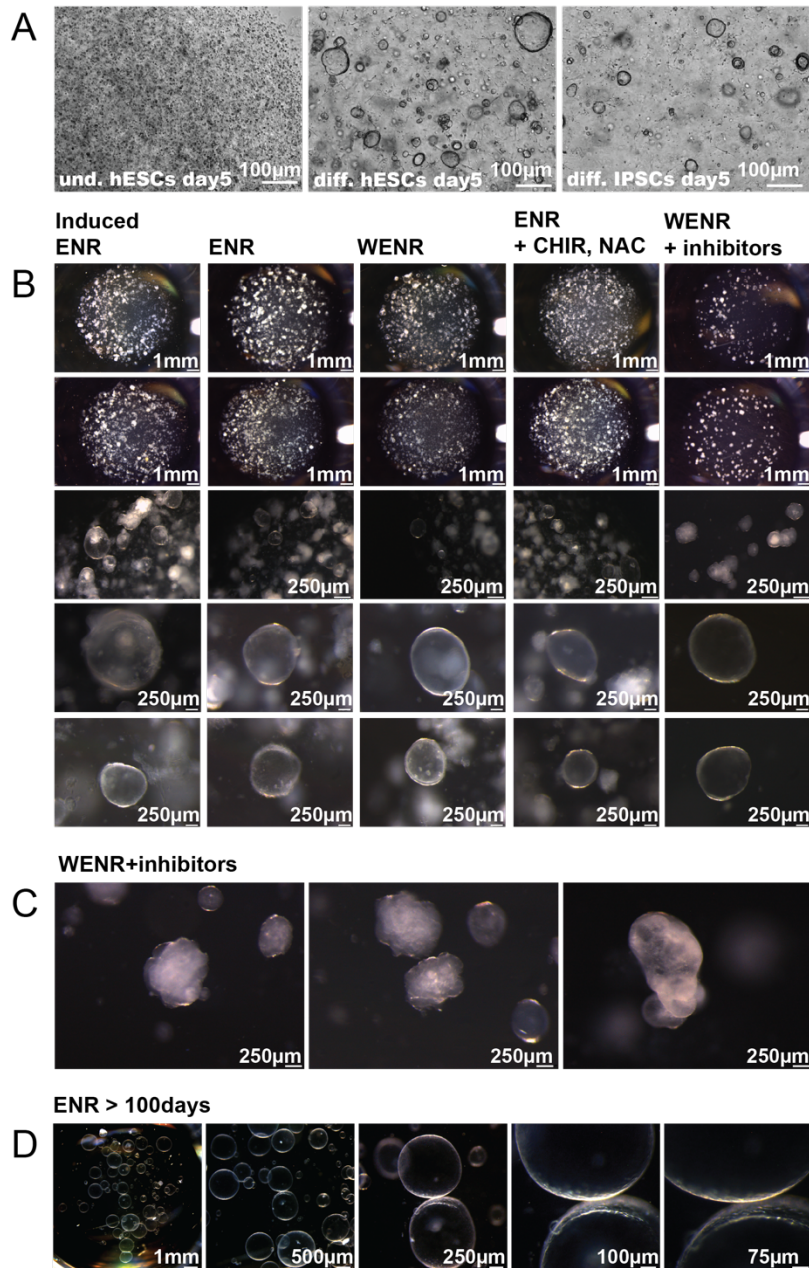


**Figure 11: hESCs and iPSCs can form intestinal organoid cultures when differentiated into a teratoma but not when directly embedded into Matrigel.**

(A) Left- Undifferentiated hESCs after 5 days grown under intestinal stem cell conditions and embedded into matrigel. Middle panel and right panel show teratoma cells isolated from hESCs and iPSCs after 5 days– grown in parallel under identical conditions.

(B) Increasing magnification top to bottom shows LGR5-GFP N- and C- term derived teratoma cells assayed for the ability to form organoids. Conditions were adapted as described by (Spence et al., 2011). Cells labeled "Induced" ENR were cultured for 4 days in 500ng/ml FGF4 and 500ng/ml WNT3a (as described in (Spence et al., 2011) and then continuously cultured in ENR. Cells labeled ENR and WENR were cultures as described in methods; ENR/CHIR/NAC as described by (Wang et al., 2013); or WENR + inhibitors as described by (Sato et al., 2011).

(C) A fraction of organoids in "WENR + inhibitors" media showed morphological changes not observed in other conditions but did not explicitly bud into crypt-like structures after >30 days in culture.

(D) Long-term cultures (>100 days shown) of organoids grown in WENR (not shown) or ENR persist and exhibit increasingly uniform morphology over time.

## 2.4.4 hPSC-derived intestinal organoids contain cells with characteristics of adult intestinal stem cells

Adult stem cells have the ability to self-renew and the restricted capacity to differentiate into defined cell types.  The human intestinal epithelium is a highly proliferative tissue with a turnover rate of ~5 days in differentiated cells outside the crypt. Single-cell-derived intestinal organoids could be maintained in long-term cultures (>6 months), suggesting the presence of a self-renewing cell population. To further test for the presence of such adult stem cell-like cells, we assayed the minimum growth factors required to establish and maintain the organoid cultures. As expected for a system that originates from adult intestinal stem cells, we found phenotypic growth dependence on EGF, Noggin, and RSPO1 (Figure 12A and B), factors also found to be essential in the mouse organoid system and human primary cultures. (Barker et al., 2007; Sato et al., 2009; Spence et al., 2011) Alternative conditions that were previously reported to support human organoid cultures (Jung et al., 2011; McCracken et al., 2011; Spence et al., 2011; Wang et al., 2013) also allowed the derivation of organoids from teratoma samples with a similar morphology (Figure 11B-C) described by (Finkbeiner and Spence, 2013b; Sato et al., 2009; Stelzner et al., 2012).
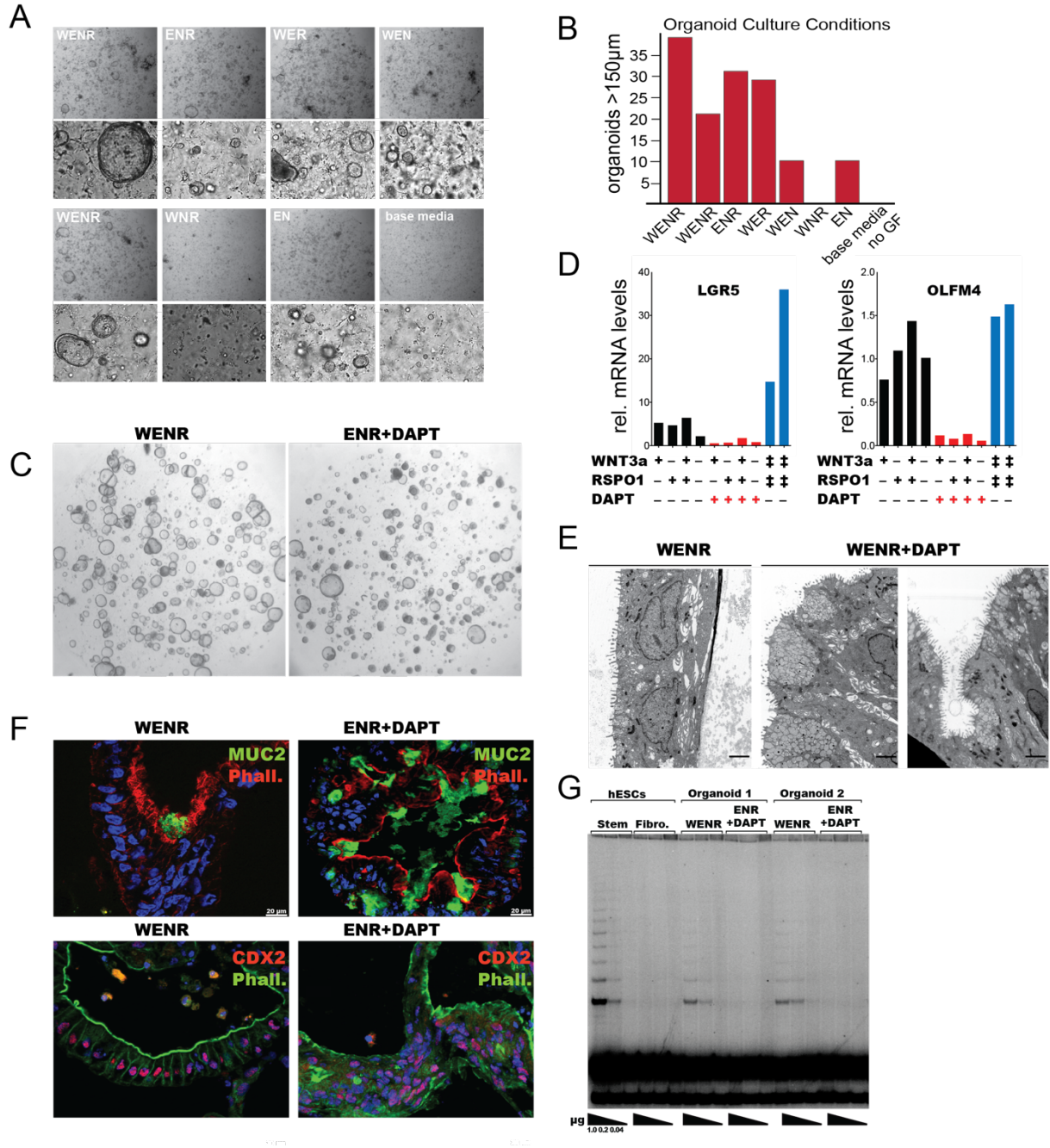
**Figure 12: WNT and Notch Signaling Is Required to Establish and Maintain Cells with Adult Stem Cell Properties in hESC-Derived Intestinal Organoids**

(A) Bright-field image of organoids at day 5 of their derivation under the indicated culture conditions at two different magnifications. Growth factors (GFs): WENR [W, Wnt3a; E, EGF; N, Noggin; R, R-spondin-1] were supplemented in the combinations indicated. No GFs were added to "base media."

(B) Quantification of images shown in (A). The graph shows the number of organoids larger than 150 mm from a single teratoma isolation cultured in parallel under the conditions described in (A), where organoids were grown in 50 ml solidified Matrigel in 500 ml media/well in a 24-well plate.

(C) Bright-field image of organoids at day 15. The image to the left shows a culture at day 15 when grown in WENR, and the right image shows the same cells when switched at day 10 to differentiation media.

(D) qRT-PCR for the intestinal stem cell markers LGR5 and OLFM4 in a single organoid culture treated in parallel with different culture conditions. The z symbol indicates 1,000 ng/ml and 200 ng/ml of RSPO1 and WNT3a, respectively, and + indicates 200 mg/ml and 50 ng/ml of RSPO1 and WNT3a, respectively. Where indicated, DAPT was added at 10 mM.

(E) Electron micrographs of the cells shown in (C). Size bars indicate 2 mm.

(F) IF staining of cryosectioned organoids cultured in either stem cell media (WENR) or differentiation media (10 mM ENR+DAPT added for 4 consecutive days). Top: MUC2 (green), phalloidin (red), and DAPI (blue). Bottom: CDX2 (red), phalloidin (green), and DAPI (blue).

(G) TRAP assay (Kim and Wu, 1997) of hESCs, hESCs differentiated into fibroblast-like cells (Hockemeyer et al., 2008), intestinal organoids grown in stem cell media (WENR, n = 2) or differentiation media (10 mm ENR+ DAPT, n = 2). Shown is a 32P autoradiogram of TRAP activity described for decreasing amounts of protein extracts (1.0–0.4 mg) for the indicated cell types (hESCs [WIBR3]; Fibro., fibroblast-like cells derived from WIBR3).
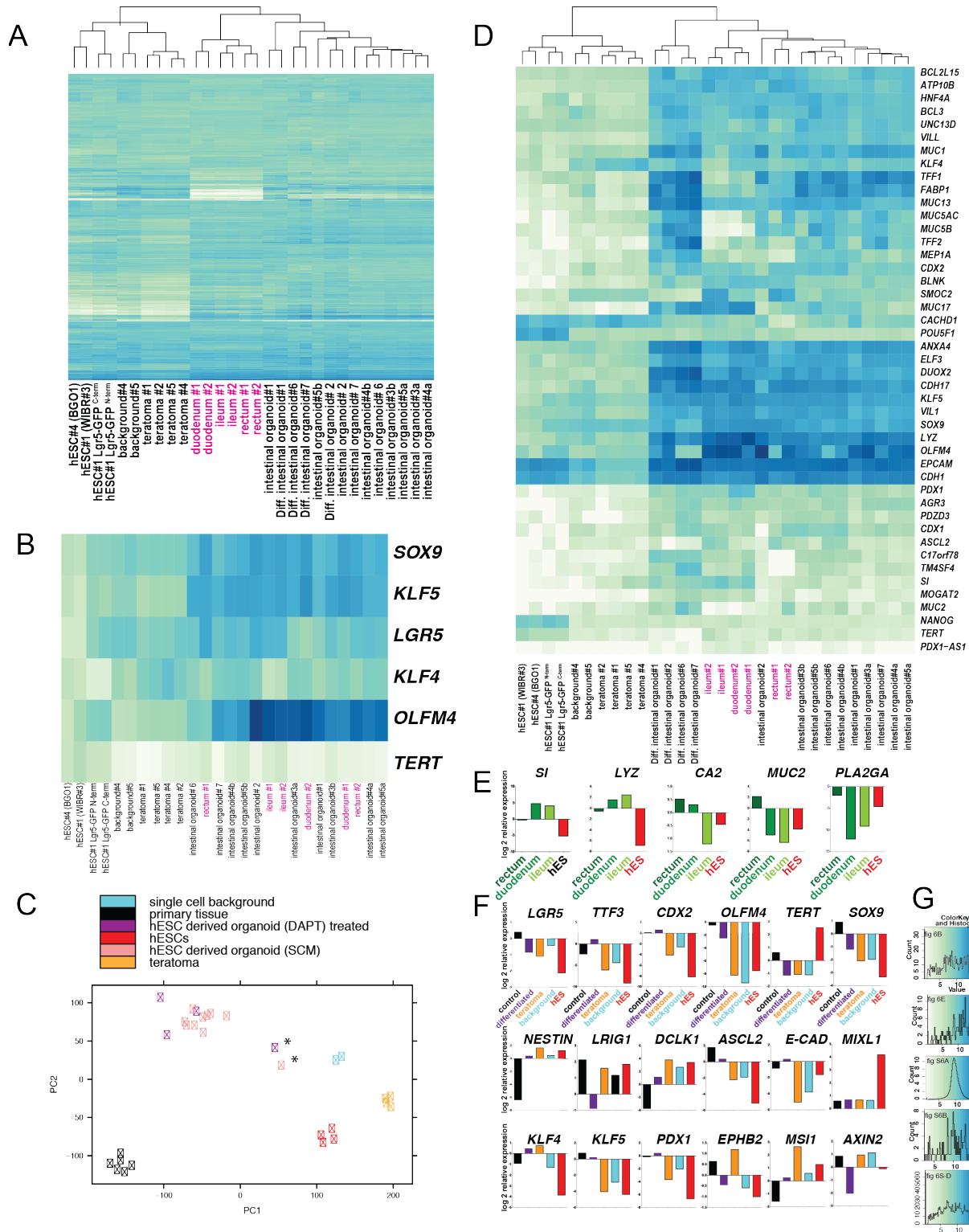
**Figure 13: hESC-derived organoids share a transcriptional profile with primary intestinal tissue derived organoids and display the characteristic responses to differentiation stimuli**

(A) Heat map of hESCs (n=4), teratoma samples (n=4), bulk hESC-derived organoid cultures grown in WENR (n=10), bulk cultures of hESC-derived organoids grown in differentiation medium (n=4), and primary- duodenum (n=2), rectum (n=2) or ileum (n=2) derived organoids. Shown are the Euclidian distances calculated from the expression of the top 5000 differentially expressed transcripts.

(B) Heat map as in 13A but here intestinal stem cell marker genes.

(C) First two components of principal component analysis of expression data from all samples, labeled by sample type. Samples indicated with * were contaminated with non-organoid teratoma cells "single cell background cells" (organoids #1 and organoids #1-differentiated). Early analysis of sample #1 showed unexpected expression; however, subsequent organoids were cultured more stringently as described in methods. "Single cell background" is the RNA isolated from the non-organoid teratoma cells that are removed but might represent some residual contamination.

(D) Heat map displaying unbiased cluster analysis of the samples analyzed in Figure 14B, but for an extended subset of genes with intestine specific expression and function. Shown are the Euclidian distances calculated from the relative expression of the genes as determined by RNASeq.

(E) Intermediate DESeq calculation of RNAseq results. Shown is the log2 fold change of three types of human-derived intestinal samples and parental hESCs relative to organoids cultured in WENR media (methods).

(F) Intermediate DESeq calculation of RNAseq results. Shown is the log2 fold change of intestinal stem cell marker genes shown in Figures 6 and S7 for control (all human primary derived samples), differentiated (ENR+DAPT), parental hES, teratoma, and the background (non-organoid cells removed from the cultures) relative to organoids cultured in WENR.

(G) Histograms for heat maps generated for Figures 14B, 14E, S13A, S13B, and S14D.

To further validate the presence of cells with adult stem cell-like characteristics in hPSC-derived intestinal organoids, we took advantage of the LGR5-GFP reporter system. First, we isolated organoid cultures from LGR5-GFP derived teratomas without FAC-sorting for GFP. After maintaining these cells for 5 days in 3D cultures we investigated whether LGR5-GFP positive cells were still present and able to form organoids after single cell dissociation. We found that sorting for GFP fluorescence enriched for cells competent to form organoids when compared to the GFP negative cell fraction (Table 1). Since LGR5 in mice marks intestinal stem-, but not the differentiated, cell types, this finding suggested that our culture conditions maintained cells with adult stem cell properties sufficient to form new organoids from single cells (Table 1 and Figures 4D, S5).

To further substantiate the presence of functional adult stem-cell-like cells in the hPSC-derived organoid system, we then cultured hPSC-derived organoids under conditions known to either promote self-renewal (high WNT signaling) or induce differentiation (Notch inhibition) of intestinal stem cells. We found that reduced WNT signaling by withdrawal of either WNT or RSPO1 reduced proliferation and decreased expression of the stem cell markers LGR5 and OLFM4 (Figure 12C and D), and induced differentiation into secretory cells as indicated by a shift in cellular morphology detected by EM and immunofluorescence (IF) staining (Figure 12E-F). These cells showed an increased number of vesicles at the apical/luminal boarder (Figure 12E), maintained CDX2, increased MUC2 and showed a density of filamentous actin at the apical border by IF (Figures 10, 12E-F).

Long-term maintenance of adult stem cells requires telomerase expression and enzymatic activity (Günes and Rudolph, 2013). Telomerase activity in human intestine is restricted to the stem cell compartment and is silenced upon differentiation of these cells (Schepers et al., 2011). We find that hPSC-derived organoid cultures can be maintained for several months, suggesting the presence of a self-renewing cell type. To functionally test whether hPSC-derived organoids contained telomerase-positive cells, we measured telomerase *in vitro*. As a positive control, we confirmed that the high level of telomerase activity in hESCs was significantly downregulated upon differentiation into fibroblast-like cells (Figure 12G). Notably, we could detect robust telomerase activity in 3 out of 4 independently derived intestinal organoid cultures when grown under adult stem cell conditions by telomeric repeat amplification protocol (TRAP) assay (Kim and Wu, 1997) (Figure 12G). However, this activity was abrogated upon terminal differentiation of organoids by WNT-signaling withdrawal and inhibition of gamma -secretase by DAPT. These functional data suggest that cells with adult stem cell properties, such as self-renewal and multipotency, represent a subpopulation of organoid cells that can respond appropriately to differentiation cues.

### 2.4.5 hPSC-derived intestinal tissue organoids are closely related to human primary intestinal tissue-derived organoids

To determine the degree to which hPSC-derived organoids could recapitulate primary cultures established from adult intestinal tissue, we evaluated the expression profile using next-generation RNA sequencing (RNAseq) of hPSC-derived intestinal organoids cultured in media that

either supported stem cell maintenance (n=10) or induces terminal differentiation (n=4). We compared these to the parental hESC lines (n=4) and teratoma samples (n=4); and to organoids generated from adult human tissue samples biopsied from duodenum (n=2), ileum (n=2), or rectum (n=2). Unbiased complete linkage clustering for the top 5000 most differentially expressed transcripts across these samples revealed that hESC-derived organoids closely resembled organoids isolated from the intestines of human subjects (Figures 14A, S13A). In contrast, teratoma samples were most similar to human ES cells (Figures 14A, S13A, S13C). Transcriptional profiling of organoids derived from an independent hESC line (BG01) confirmed that organoids could be derived irrespective of the genetic background of the hPSC cell line with high similarity to primary tissue-derived organoids (Figures 14A-B, S13A-D). hESC-derived intestinal organoids grown in WENR-media expressed analogous levels of intestinal adult stem cell marker genes (LGR5, OLFM4, KLF4, KLF5, SOX9 and TERT) and differentiated/secretory linage genes (e.g. KRT20, VIL1, MUC2, LYZ, CA2, SI, PLA2GA) as found in organoids derived from primary tissue, with the greatest similarity to rectum-derived samples (Figures 14B, 14E, S13D-G).

In contrast to previously establish protocols, our intestinal organoids did not result in a significant budding of crypt like structures, characteristic of mouse small intestine cultures. The cystic morphology of our organoids was previously described for developmental immature stages of enterosphere differentiation as well as for adult colon-derived organoids (Fordham et al., 2013a; Jung et al., 2011; Sato et al., 2009; Stelzner et al., 2012). While expression levels of several marker proteins for intestinal function, such as PLAG2A, TFF1 and TFF2 (Figure 13SD-F) offer evidence of cell maturation of hESC-derived organoids, the full complexity of intestinal maturation is not recapitulated by this *in vitro* culture. The complex maturation signals required from the neural and vascular plexus and the mesenchyme are yet to be defined in an *in vitro* culture (Wells and Spence, 2014). Nevertheless, our protocol results in organoids that express adult stem cell markers to the same extent as primary tissue-derived organoids, suggesting that they are beyond a developmental stage that was recently described for human embryonic-like intestinal progenitor cells (Fordham et al., 2013a) (Figures S13B, S13D-F).
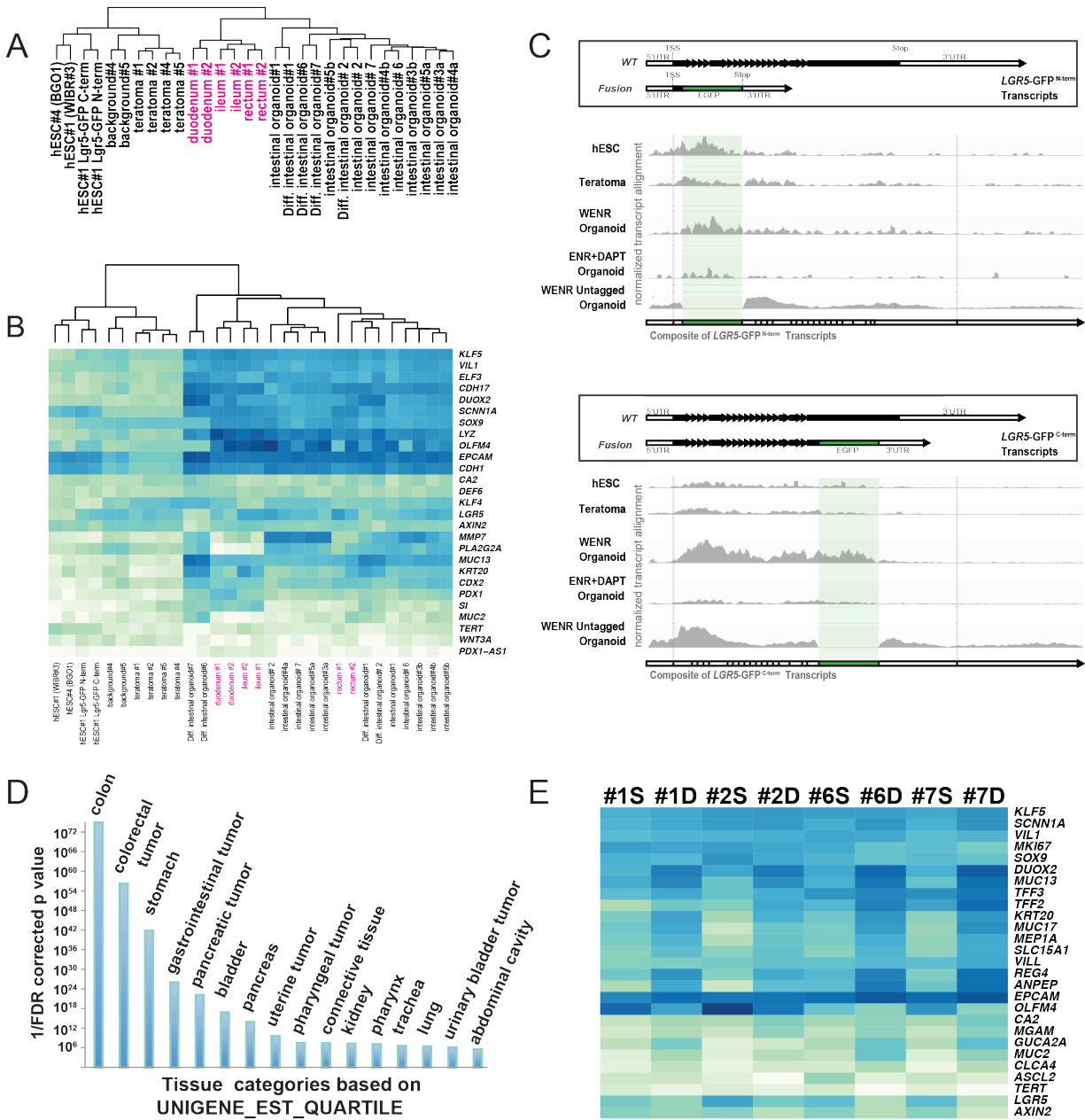
**Figure 14: hESC-Derived Organoids Share a Transcriptional Profile with Primary Intestinal Tissue-Derived Organoids and Display the Characteristic Responses to Differentiation Stimuli**

(A) Cluster analysis of hESCs (n = 4), teratoma samples (n = 4), bulk hESC-derived organoid cultures grown in WENR (n = 10), bulk cultures of hESC-derived organoids grown in differentiation medium (ENR+DAPT, n = 4), and primary duodenum- (n = 2), rectum- (n = 2) and ileum- (n = 2)

derived organoids. Euclidian distances calculated from the abundance levels of the top 5,000 differentially expressed transcripts are also shown in Figure S5A.

(B) Heatmap displaying an unbiased cluster analysis of samples analyzed in (A) for a limited selection of genes relevant to intestinal expression and, in most cases, reported functions. Shown are the Euclidian distances calculated from the relative expression of the genes as determined by next-generation RNA-seq expression analysis.

(C) Density maps of RNA-seq reads mapped to the genetically engineered LGR5-GFPN-term and LGR5-GFPC-term locus. The top boxes show the predicted transcripts for each allele based on validated gene editing described in Figure 4A. Shaded in green is the region mapping to the coding sequence for the eGPF fusion reporter. Shown across each histogram are the alignments to Lgr5-eGFP fusions predicted for each cell line. RNA was collected from the parent hESC cell lines, the intermediate teratoma samples, intestinal organoids, and intestinal organoids differentiated by the withdrawal of WNT3a and the addition of DAPT.

(D) Gene Ontology analysis for tissue-specific expression using the Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/home.jsp) and the "UNIGENE_EST_QUARTILE" expression profile database. Analyzed were genes significantly (FDR corrected p value < 0.05) unregulated (>22.5-fold) in hESC-derived intestinal organoids (n = 10) compared with hESCs (n = 4).

(E) Heatmap displaying an unbiased cluster analysis of organoid samples (from groups 1, 2, 6, and 7 as described in Table S1) analyzed in (A) for selected genes that are associated with the differentiation of adult intestinal stem cells. Shown are pairwise hESC-derived intestinal organoid cultures (n = 4) grown in either WENR stem cell media (S) or differentiated in ENR+DAPT (D).

Next, we attempted to address the positional identity of these organoids with respect to anterior/posterior gut development. We found expression of the caudal type homeobox genes CDX1 and hindgut markers CDX2 (Sherwood et al., 2009) PLA2G2A, CA2 and MUC2 in hPSC-derived organoids and in rectum-derived tissues (Figures 14, S13D-E). The midgut

gene PDX1 was enriched in duodenal-derived samples only, while organoids expressed levels more comparable to distal gut (ileum/rectum) (Figure 14B). This analysis suggests that our differentiation protocol may bias for relatively mature hindgut/large intestinal-like tissue.

To further substantiate that hESC-derived organoids specifically express intestinal adult stem cell markers, we analyzed the expression profile of the LGR5-GFP reporter gene in hESC-derived organoids by RNAseq. Here we found that both the LGR5-GFP [N-term] and LGR5-GFP [C-term] reporter recapitulated the expression of the endogenous LGR5 gene and contained sequence that aligned across the expected fusion junction by RNAseq (Figures 4A, 14C). While the LGR5 reporter was expressed in hESCs and slightly reduced in tetratoma cells, it was higher in the bulk analysis of organoids that were maintained under stem cell conditions. Importantly, LGR5-GFP expression was significantly repressed in organoid cultures under differentiation conditions (Figure 14C). These data not only validate our LGR5-GFP reporter systems, despite showing minimal fluorescence by microscopy, but also support the hypothesis that a defined organoid system containing adult stem cells can be successfully generated from hPSCs by this method. Furthermore, Gene ontology analysis for genes that were differentially expressed between hESCs (n=4) and hESC-derived intestinal organoids (n=10), revealed a significant enrichment of genes associated with the gastrointestinal system (Figures 14D). Categories enriched include the *Epithelial Differentiation* and *Digestion* for biological processes and various *Peptidase activities* for biological function. We also analyzed the genes that were differentially expressed between the human primary-derived samples and the hESC-derived organoids (see Supplemental material for further discussion of the RNAseq data).

Finally, we analyzed independently derived organoid cultures that were grown in the absence of WNT3a and in the presence of DAPT. Transcriptional changes showed that this specific change in WNT and Notch signaling resulted in the induction of intestinal-specific differentiation markers and a reduction of adult intestinal stem cell markers (Figures 14E S13D).

In summary, genome wide expression analysis confirmed the following hypotheses:  1) organoids are similar to primary intestinal tissue– specifically large intestine; 2) organoids express adult intestinal stem cell markers such as SOX9, OLFM4, KLF4, KLF5, LGR5 and TERT; 3) The

genes expressed indicate that organoid cultures comprise a relatively mature hindgut-like tissue; 4) LGR5 and the LGR5-GFP reporter expression are upregulated in mature organoids; and 5) organoids respond to the canonical cues that also determine differentiation and self-renewal in the intestine of adult humans.

## 2.5 Discussion

### 2.5.1 A human LGR5-GFP reporter system allows the isolation of intestinal tissue organoids

Here we report on the development of a method that allows robust derivation of human intestinal adult stem cells and organoid tissue from hPSCs. We used ZFN-mediated genome editing of the endogenous LGR5 locus to generate a reporter system based on the study in mice that established LGR5 as a bona fide marker of adult intestinal stem cells (Barker et al., 2007). Analogous to previous mouse experiments, we demonstrate here that generating either an N- or C-terminal reporter of human LGR5 allows the specific isolation and enrichment of cells with the ability to form organoid cultures from hPSCs despite showing minimal fluorescence by microscopy. Our results provide proof of concept for using site-specific gene editing technology to isolate novel human cell types from hPSCs.

The intestinal organoids that we generated are similar to tissue isolated directly from humans, as they express gastrointestinal-specific genes and contain highly organized and differentiated cell types such as goblet-, enterocyte- and enteroendocrine-like cells. In addition to the tissue organization, the cellular and subcellular morphology of hESC-derived organoids is reminiscent of primary organoid cultures. When grown under conditions that promote differentiation, organoids consist of an epithelial sheet of polarized cells with a subcellular organization characteristic of specific cell subtypes such as secretory cells. Expression profiling suggests that our differentiation protocol may bias for relatively mature hindgut/large intestinal-like tissue. We do not know if this is an intrinsic feature of the teratoma or a function of our culture conditions. However, we find that a subset of organoids contain cells that stain positive for the small intestinal marker gene lysozyme, suggesting that our protocol does not strictly discriminate against more anterior regions of the intestine. As we can detect lysozyme- and PDX1-positive cells in the parent teratoma samples,

it seems possible that our method could also be refined to specifically isolate and enrich for small intestinal cellular subtypes.

## 2.5.2 Modeling intestinal function and disease using hESC-derived Intestinal tissue

For many research applications including nutrient uptake, drug delivery, metabolic regulation and human disease modeling such as intestinal cancer, rodent models do not fully recapitulate human intestinal physiology. However, organoids derived from hPSCs may provide a novel platform for *in vitro* disease modeling and drug screening to complement experiments in conventional animal models.

The organoid-system established here will advance disease modeling because the differentiation protocol does not rely on the LGR5 reporter and because the committed intestinal stem cells can be derived from hiPSCs and hESCs. We demonstrate that hESCs that are genetically engineered by TALEN and ZFN-mediated genome editing can subsequently be used to generate intestinal organoids, suggesting that genome editing approaches such as ZFN, TALEN and CRISPR-mediated gene knockout and transgenesis will facilitate the study of intestinal biology. Gene editing will allow the generation of isogenic disease-specific cell lines, circumventing the problem of heterogeneity in human samples, eliminating phenotypic heterogeneity of *in vitro* phenotypes (Soldner and Jaenisch, 2012; Soldner et al.) and thereby complementing current approaches that directly genetically modify intestinal organoid cultures (Miyoshi and Stappenbeck, 2013; Schwank et al., 2013).

## 2.5.3 hESC-derived organoids represent a novel *in vitro* adult stem cell system

Adult intestinal stem cells are a highly specialized and differentiated cell type that is formed through a well-timed and coordinated developmental process. Our isolation procedure for hPSC-derived intestinal adult stem cells suggests that these developmental cues can be sufficiently recapitulated when hPSCs form a teratoma. Key features of the differentiation process during teratoma formation that allow for the establishment of adult stem cells include the extended time that the tissue is allowed to form without perturbation, the optimal nutrient/hormonal conditions, and the unconstrained 3D growth in the subcutaneous

compartment. In the future, these particular characteristics will have to be recapitulated in a xeno-free differentiation paradigm. Considering that LGR5 has recently also been implicated in the adult stem cell function and maintenance of other tissue types, it will be interesting to investigate if this strategy of genetically engineering hPSCs to carry reporter genes can be applied to the culture of other adult stem cell types that previously could not be isolated.

## 2.6 Material and Methods

### 2.6.1 Teratoma formation and analysis
hESCs were collected by collagenase treatment (1.5mg/ml) and separated from feeder cells by sedimentation. Cells were resuspended in 250µl of phosphate buffered saline (PBS) and injected subcutaneously into NOD-SCID mice (Taconic). Tumors (<1.5cm) formed within 4-8 weeks when teratomas were isolated and disaggregated to single cells or fixed in formalin for analysis of hematoxylin and eosin staining. Immunostaining of paraffin section was performed with standard techniques using a rabbit polyclonal anti-GFP antibody (Abcam 290).

### 2.6.2 Culture of Intestinal Epithelial Organoids from hESCs
Single cells isolated from teratoma were embedded at 4°C in 50µl Matrigel containing 1µM JAG-1 (AnaSpec; no. 61298) and incubated at 37°C for 10 min.  Media (500µl/well) comprised 1:1 DMEM F-12 and Neurobasic with N2, B27, L-glutamine, NEAA, PS, and with growth factors 200ngml$^{-1}$WNT3A,1µgml$^{-1}$RSPO1,50ngml$^{-1}$ EGF,100ngml$^{-1}$ Noggin, and 10µM ROCK inhibitor changed each 2 days. Organoids were passaged using 5 min dispase digestion, subcultured and gravity separated from single cells using PBS 0.5%BSA wash. After five minutes PBS 0.5%BSA 2mM EDTA incubation and hES media inactivation, tissue was peleted resuspended in 4°C matrigel as described above. When indicated, WNT was withdrawn and 10µM DAPT was added to the cultures for four days to induce differentiation.
Human subject tissues:
The ethics committee of the University Medical Centre Utrecht obtained approval for this study and written informed consent was obtained. Organoids were generated from biopsies obtained for diagnostic purposes and maintained in culture (for 1 to 6 months) as described (Sato et al., 2011a).

### 2.6.3 Immunofluorescence staining

Organoids were fixed in 3% paraformaldehyde with 5% sucrose in PBS (pH 7.4) for 30 min. Samples were embedded for cryo-sectioning, sectioned at 10µM and mounted on poly-L-lysine coated slides. Samples were permeabilized with TBST (TRIS-buffer saline with 0.5% Triton X-100) and incubated at 4°C 1:5000 dilution with shaking overnight. After washing the slides with TBST at 4°C three times, slides were incubated with the appropriate Alexa Fluor secondary antibodies (1:1000) and of a phalloidin conjugate (1:1000). Afterwards, slides were washed as previously, stained for DAPI and mounted.

### 2.6.4 Transcriptional profiling

A detailed protocol can be found in the Supplemental material. Briefly, RNAseq library was following the standard Illumina library preparation protocol. Ribosomal RNAs were depleted using a oligo(dT) 25 magnetic bead kit (Life Technologies), and the libraries were generated using the PrepX™ SPIA® RNA-Seq and PrepX Library kit (IntegenX) according to the manufacturer's protocol. Reads were mapped to the Ensembl cDNA release 72 (Flicek et al., 2013) with Bowtie2 version 2.1.0 (Langmead and Salzberg, 2012) using the parameters –rdg 6,5, –rfg 6,5, and –score-min L,-.6,-.4. Transcript abundances were calculated with eXpress version 1.4.0 (Roberts and Pachter, 2013) and the resulting effective counts for each transcript were used to calculate fold changes. Effective counts were also processed with DESeq version 1.12.0 (Anders and Huber, 2010) to identify statistically significant differentially expressed genes and transcripts. FPKM available in supplement and raw data for RNAseq is available at http://www.ncbi.nlm.nih.gov/geo/ (GSE56930).

### 2.6.5 ZFN-driven targeted genetic engineering of the LGR5 locus in hESCs and hiPSCs using ZFN-mediated homologous recombination

hESCs and hiPSCs were cultured in Rho Kinase (ROCK)-inhibitor (Calbiochem; Y-27632) 24 hours prior to electroporation. Cells were harvested using 0.25% trypsin/EDTA solution (Invitrogen), and $1 \times 10^7$ cells were resuspended in phosphate buffered saline (PBS) and electroporated with 40 µg of donor plasmids (previously described in (Hockemeyer et al., 2009) or designed and assembled by D.H.) and 5 µg of each ZFN or TALEN encoding plasmid (Gene Pulser Xcell System, Bio-Rad: 250 V, 500µ F, 0.4 cm cuvettes). Cells were subsequently plated on MEF feeder

layers (DR4 MEFs for puromycin selection) in hESC medium supplemented with ROCK-inhibitor for the first 24 hours. Individual colonies were picked and expanded after puromycin selection (0.5 µg/ml) 10 to 14 days after electroporation. hESC-derived fibroblast-like cells used as negative controls for the expression analysis shown in Figure 6 and Figure 13 were generated from hESCs overexpressing hTERT from the AAVS1 locus (unpublished data) as previously described (Hockemeyer et al., 2009).

### 2.6.7 ZFN design and ZFN expression plasmids

ZFNs against the human LGR5 locus were designed using an archive of validated two- finger modules (Perez et al., 2008; Urnov et al., 2005); complete sequences of the ZFNs, which carried obligate heterodimer forms of the FokI endonuclease (Miller et al., 2007).

### 2.6.8 ZFN target sites

ZFN Target site and corresponding recognition alpha helices (NH2 to COOH) engineered against the LGR5 locus were as follows: N-term L ZFN target– AATGACAGTGTGTGGGGC, DRSHLTR, RSDHLTT, RSDSLLR, LQHHLTD, DRSNLSR, LRQNLIM; N-term R ZFN target– CCGACGGCAGGAtGTTGCT, QSSDLSR, YKWTLRN, QSGHLAR, QSGDLTR, RSDTLSQ, RSDDRKK; C-term L ZFN target – ACAGTTTAATGGGGG, RSAHLSR, RSDHLST, QSANRTK, TSGSLSR, QSSVRNS; C-term R ZFN target– GGGGTCATCGCAGCAGTG, RSDSLSV, QSGDLTR, QSGDLTR, DTGARLK, DRSALSR, RSDHLSR. The ZFNs were tested by transient transfection into K562 cells followed by Surveyor (Cel-1) endonuclease-based measurement of NHEJ at the target locus exactly as described (Miller et al., 2007; Perez et al., 2008).

### 2.6.9 hESC culture

Cell culture was carried out as described previously (Soldner et all 2009). All hESC lines were maintained on a layer inactivated mouse embryonic fibroblasts (MEFs) in hESC medium (DMEM/F12 [Invitrogen] supplemented with 15% fetal bovine serum [Hyclone], 5% KnockOutTM Serum Replacement [Invitrogen], 1mM glutamine [Invitrogen], 1% nonessential amino acids [Invitrogen], 0.1 mM β-mercaptoethanol [Sigma], penicillin/streptomycin [Gibco], and 4 ng/ml FGF2 [R&D systems]). Cultures were passaged every 5-7 days either manually or enzymatically with collagenase typeIV (Invitrogen; 1.5 mg/ml).

### 2.7.0 Immunocytochemistry

Immunostaining of paraffin section was performed with standard techniques using the following antibodies: anti-GFP (Abcam 290), Sox9 (AB5535 Millipore), Mucin2 (H-300 Santa Cruz), Villin (C-19, sc-7672 Santa Cruz), chromogranin A (SP-1 ImmunoStar), EphB2 (AF467 R&D System), Epcam AF960 R&D systems, FABP1 (HPA028275 Sigma), E-cadherin 610181 BD), Lysozyme (Dako), CDX2 (CDX2-88 BioGenex), PDX1 (abcam® ab47267) and appropriate Molecular Probes Alexa Fluor® dye conjugated secondary antibodies (Invitrogen). Phalloidin was Alexa Fluor® 488 Phalloidin or Rhodamine Phalloidin (Molecular Probes – life Technologies).

## 2.7.1 Electron microscopy analysis
The material was fixed in 2.5% gluteraldehyde, 3% paraformaldehyde with 5% sucrose in 0.1M sodium cacodylate buffer (pH 7.4). When possible, the organoids were pelleted. Larger organoids were carried through processing in mesh bottom baskets.  Material was post-fixed in 1% OsO4 in veronal-acetate buffer, stained en bloc overnight with 0.5% uranyl acetate in veronal-acetate buffer, dehydrated and embedded in Embed-812 resin. Ultra-thin sections were cut on a Reichert Ultracut E microtome with a Diatome diamond knife, stained with uranyl acetate, and lead citrate. The sections were examined using a FEI Tecnai spirit at 80KV and photographed with an AMT ccd camera.

## 2.7.2 RT-PCR analysis
RNA was isolated using either the RNeasy Mini Kit (Qiagen) or Trizol extraction followed by ethanol precipitation. Reverse transcription was performed on 150ng of total RNA using oligo dT priming and Thermoscript reverse transcriptase at 50°C (Invitrogen). qRT-PCR was performed in an ABI Prism 7000 (Applied Biosystems) with Platinum SYBR green pPCR SuperMIX-UDG with ROX (Invitrogen) using primers that were in part previously described (Soldner et al., 2009).

**qRT-PCR Primer sequences**
SOX9F CTGAGCAGCGACGTCATCTC
SOX9R GTTGGGCGGCAGGTACTG
KLF4F ACCAGGCACTACCGTAAACACA
KLF4R GGTCCGACCTGGAAAATGCT
KLF5F CCCTTGCACATACACAATGC

KLF5R GGATGGAGGTGGGGTTAAAT
EOMESF AAGGCATGGGAGGGTATTAT
EOMESR AAACACCACCAAGTCCATCT
VillinF ACACAGGTGGAGGTGCAGAAT
VillinR GGTTGGTCGCTGTCCACTTC
TTF3F CTTGCTGTCCTCCAGCTCT
TTF3R CCGGTTGTTGCACTCCTT
FOXF1F CACTCCCTGGAGCAGCCGTAT C
FOXF1R AAG GCTTGATGTCTTGGTAGGTGA
CDX1F AGCCGTTACATCACAATC
CDX1R GAGACTCGGACCAGACCT
CDX2 B F GAGCTGGAGAAGGAGTTT
CDX2 B R GGTGACGGTGGGGTTTAG
MUC2F TGGGTGTCCTCGTCTCCTACA
MUC2R TGTTGCCAAACCGGTGGTA
ALPF GCAACCCTGCAACCCACCCAAGGAG
ALPR CCAGCATCCAGATGTCCCGGGAG
Sucrase (SI) F TGGCAAGAAAGAAATTTAGTGGA
Sucrase (SI) R TTATTCTCACATTGACAGGATC
Defensin-5 F GACAACCAGGACCTTGCTATCT
Defensin-5 R ACGGGTAGCACAACGGC
Defensin-6F GACAACCAGGACCTTGCTATCT
Defensin-6R ACGGGTAGCACAACGGC

## 2.7.3 Telomeric repeat amplification protocol (TRAP)

Organoids were isolated from matrigel using dispase, washed with PBS and lysed with HLB buffer (20mM HEPES, 2mM MgCl2, 0.2mM EGTA) and 10% glycerol supplemented with 0.5% CHAPS, 1mM DTT and 0.1mM PMSF. The lysate was rocked at 4° C for 30min and cellular debris was removed by centrifugation. Dilution of the supernatant (0.04 to 1μg of total protein) was incubated with 0.1μg of the TS primer (AATCCGTCGAGCAGAGTT) for primer elongation in TRAP PCR buffer (20mM Tris-HCl pH8.0, 2.5mM $MgCl_2$, 68mM KCl, 0.05% Tween20, 1mM EGTA) including 1mM dNTPs at 30 C° for 1hr. The following PCR reaction was performed supplied with PCR master mix including 0.04μg of the reverse ACX primer (GCGCGGCTTACCCTTACCCTAACC), [α-^{32}P]dGTP (3000 Ci/mmol; PerkinElmer), 1.25 unit of Taq DNA polymerase and 0.04μg of semi-competitive primer sets [TSNT forward primer; ATTCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT, NT reverse

primer; ATCGCTTCTCGGCCTTTT) as previously reported (Kim and Wu, 1997)]. The PCR products were analyzed by non-denatured polyacrylamide gel electrophoresis.

## 2.7.4 Transcriptional profiling

RNAseq library was prepared at the FGL at UC Berkeley following the standard Illumina library preparation protocol. Total RNA isolation was performed on a pooled culture of organoids from a single 50μl matrigel matrix in the respective condition, colonies of hES cells, or a triturated pellet of minced teratoma. cDNA libraries for high throughput sequencing were prepared from 50~100ng of total RNA from each sample. Ribosomal RNAs were depleted using a oligo(dT) 25 magnetic bead kit (Life Technologies), and the libraries were generated using the PrepX™ SPIA® RNA-Seq and PrepX Library kit(IntegenX) according to the manufacturer's protocol. Then, samples were quantified using the Qubit and PCR amplified for 18 cycles to incorporate indexes and flow cell-binding regions. Final libraries were quantified using the Qubit, Bioanalzyer and qPCR before being sequenced on the HiSeq2000 for 50bps Single-End reads with multiplexing using V3 SBS chemistry reagents.

Reads were mapped to the Ensembl cDNA release 72 (Flicek et al., 2013) with Bowtie2 version 2.1.0 (Langmead and Salzberg, 2012) using the parameters –rdg 6,5, –rfg 6,5, and –score-min L,-.6,-.4. Transcript abundances were calculated with eXpress version 1.4.0 (Roberts and Pachter, 2013) and the resulting effective counts for each transcript were used to calculate fold changes. Effective counts were also processed with DESeq version 1.12.0 (Anders and Huber, 2010) to identify statistically significant differentially expressed genes and transcripts. For bar graphs of gene expression, transcripts were grouped by their originating gene, based on their Ensembl annotation. The expression level of a given gene was calculated as the sum of the estimated abundances of all of its transcripts. The resulting intermediate log2 fold analysis shown as relative expression bar graphs used DESeq normalized expression results to generate pair wise analysis. Experiments were clustered using complete linkage clustering applied to abundance estimates and projection of the transcript abundance matrix onto the first two principal components was used to visualize similarity between experiments. IGV version 2.3.13 (Thorvaldsdóttir et al., 2013) was used to create read coverage tracks. FPKM available in supplement and raw data for RNAseq is available at http://www.ncbi.nlm.nih.gov/geo/  (GSE56930).

## 2.8 Additional discussion of the transcriptional profiling data

### 2.8.1 Single cell contaminants can account largely for the expression differences between the hESC derived organoids and primary tissues samples

We also analyzed the genes that were differentially expressed between the human primary-derived samples and the hESC-derived organoids (Figure 15). Unexpectedly we found a significant enrichment of genes overexpressed in hESC-derived organoid samples, particularly organoid sample #1. We hypothesized that this difference in gene expression was the result of small amounts of differentiated teratoma cells contaminating the hESC-derived organoids. To test this, we isolated the residual contaminating single cells embedded in the matrigel and performed transcriptional profiling of both hESC-derived organoids and these single-cell contaminants that are normally discarded. Indeed, transcriptional profiling of these contaminating cells showed elevated expression of genes with non-intestinal ontology that can largely explain the transcriptional differences between hPSC-derived organoids and primary tissue cultures. Cultures subsequent to the derivation organoid sample #1 were passaged more stringently against the non-enteric teratoma cells as described in the methods and aligned much closer to the profiles of human-derived samples.
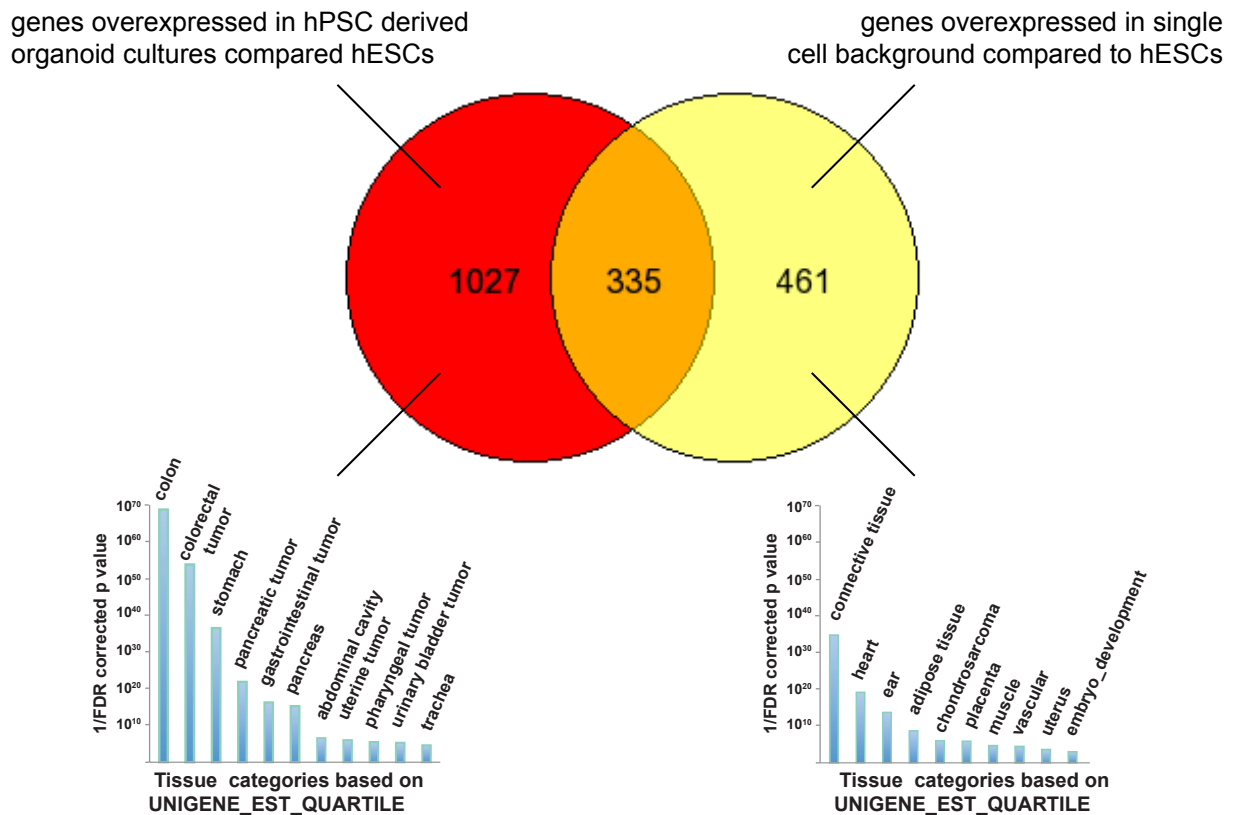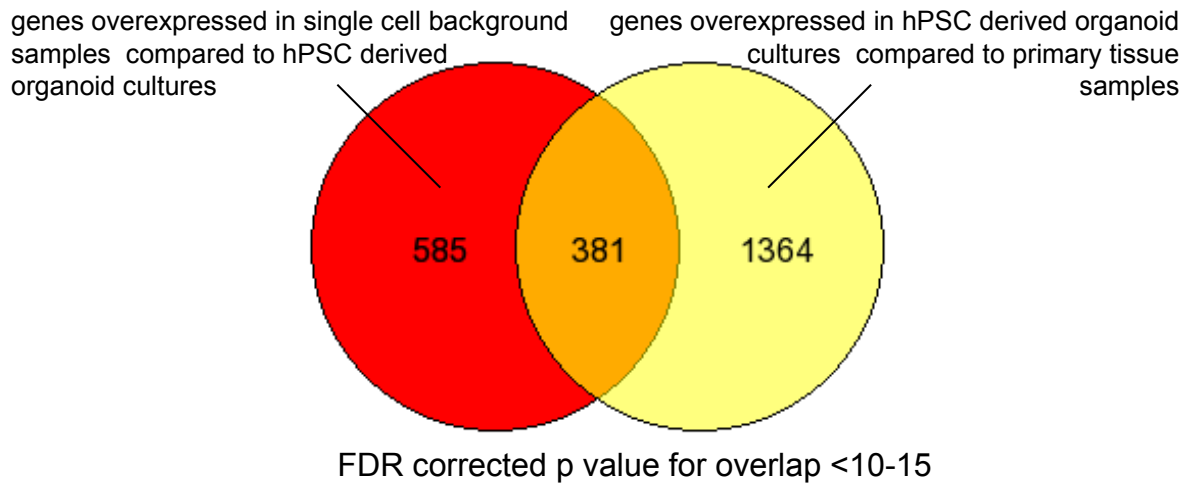
Figure 15: Gene ontology

**Top:** Venn diagram depicting the overlap of genes significantly overexpressed ($p<0.05$ and $2^{2.5}$-fold upregulation) in single cell background samples compared to hPSC-derived organoid cultures and
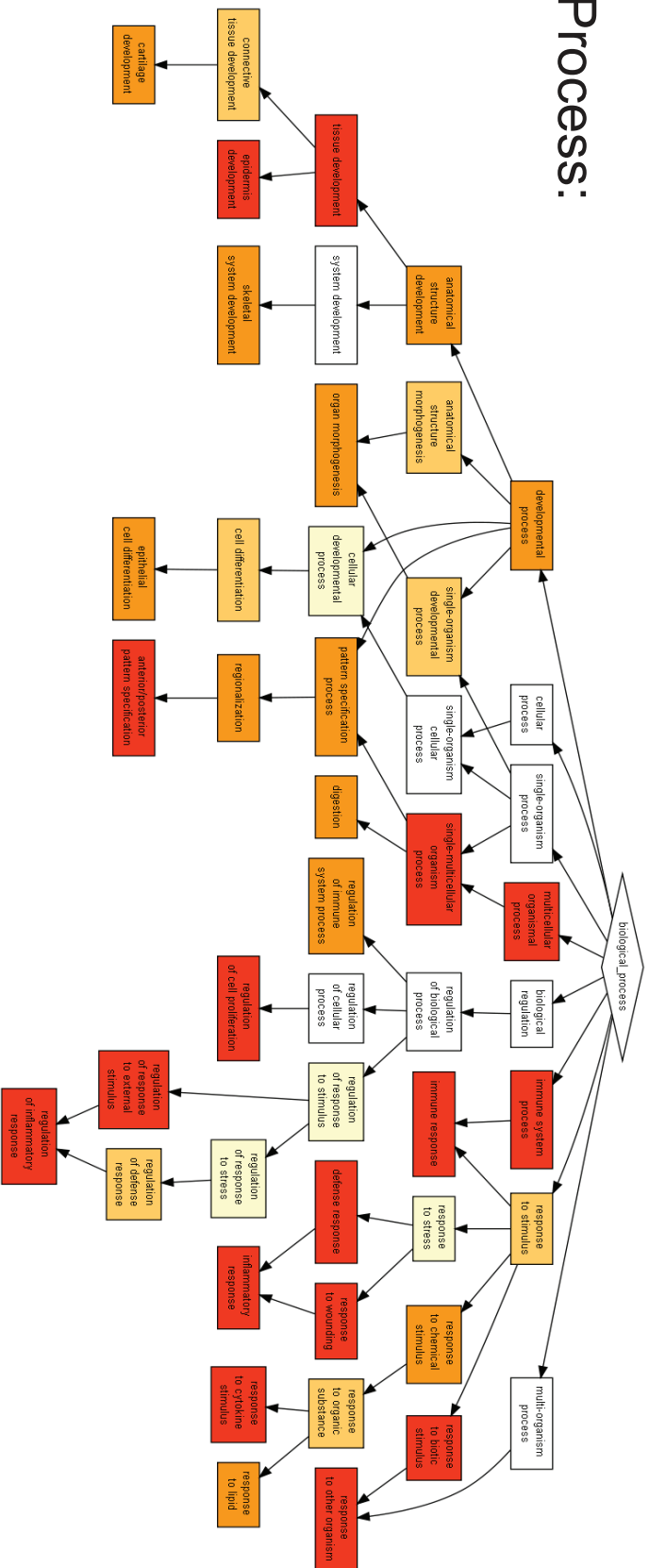
genes overexpressed in hPSC-derived organoid cultures compared to primary tissue samples. Red circle indicates total number of genes overexpressed in background samples (non-organoid outgrowth of teratoma cells) compared to hPSC-derived organoid cultures. Yellow circle indicates total number of genes overexpressed in hPSC-derived organoid cultures compared to primary-derived tissue samples. The high amount of overlap between both gene lists suggests that a significant fraction of the difference between hPSC-derived organoid cultures and primary-derived intestine tissue comes from contamination by background material. P-value for overlap was calculated using a hypergeometric distribution and found to be less than $10^{-15}$.

**Bottom:** Venn diagram depicting the overlap of genes significantly overexpressed (p<0.05 and $2^{2.5}$-fold upregulation) in hPSC-derived organoid samples compared to hPSCs as well as genes overexpressed in single-cell background samples compared to hPSC samples. Red circle indicates total number of genes overexpressed hPSC-derived organoid samples compared to hPSCs. Yellow circle indicates total number of genes overexpressed in single-cell background samples compared to hPSC samples. Shown below is the tissue specific expression gene ontology analysis for these genes using the Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/home.jsp) and the "UNIGENE_EST_QUARTILE" expression profile database. Analyzed were genes significantly (FDR corrected p value <0.05) unregulated (>$2^{2.5}$fold) in hESC-derived intestinal organoids (n=10) compared to hESCs (n=4).


## 2.8.2 Functional Gene ontology analysis of hPSC-derived organoids

Diagrams show the gene ontology using the Gene Ontology enRIchment anaLysis and visuaLizAtion tool (http://cbl-gorilla.cs.technion.ac.il). (Figure 16) Genes were ranked by differential expression between hPSC-derived organoids and hESCs. Depicted in the following three panels are labeled (biological) **Processes**, **Components**, and **Functions** that are significantly enriched in hPSC-derived organoids.

# Process:

# Component:
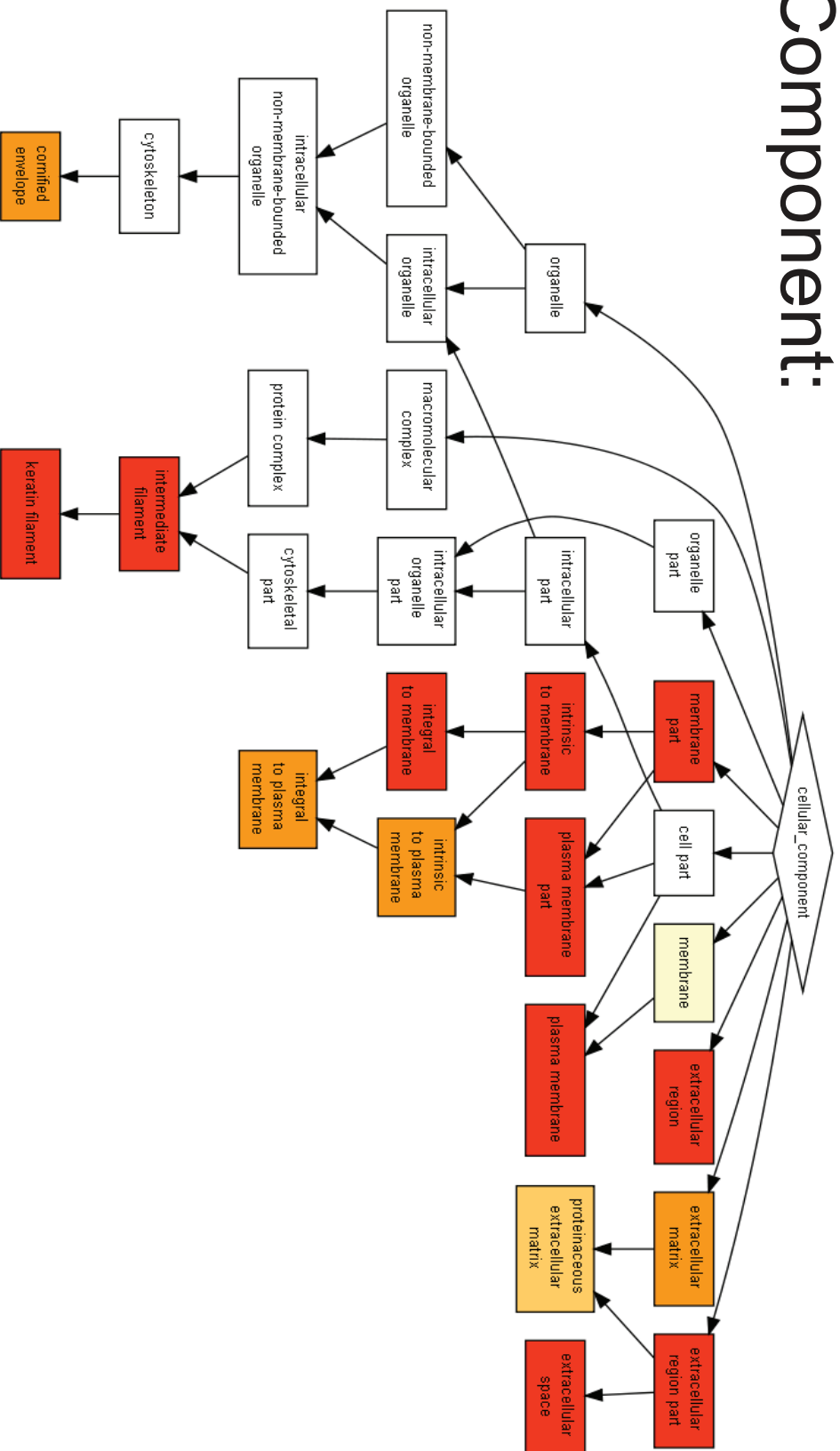
# Function:



**Figure 16: Functional Gene ontology**

## 2.9 Acknowledgments

# Chapter 3: Perspectives

## 3.1 Perspective: organoid disease modeling

Common cancer models include patient-derived tumor xenografts, genetically altered mice, and stable cancer cell lines. Cell lines are derived from human subjects but are then adapted to two-dimensional tissue culture conditions. This inherently selects for outgrowth of rare clones with considerable genetic changes resulting in a loss of the heterogeneity inherent in tumors. Animal models continue to recreate the most complex features of physiology, but species differences are not always evident and sometimes are only unmasked in drug development when human trials begin. Although organoids certainly cannot fully replace the current methods for modeling disease, they can cast light onto aspects of disease that that are unknown or difficult to recreate in these systems.

To date, intestinal organoid culture systems has overcome several significant technical limitations inherent it previous *in vitro* lines of study. The culture of long term biopsy derived organoids have now been established for colon (Sato et al., 2011a), liver (Huch et al., 2015), pancreas (Boj et al., 2015), prostate (Karthaus et al., 2014), stomach (Bartfeld et al., 2015), fallopian tube (Kessler et al., 2015), taste buds (Ren et al., 2014), salivary glands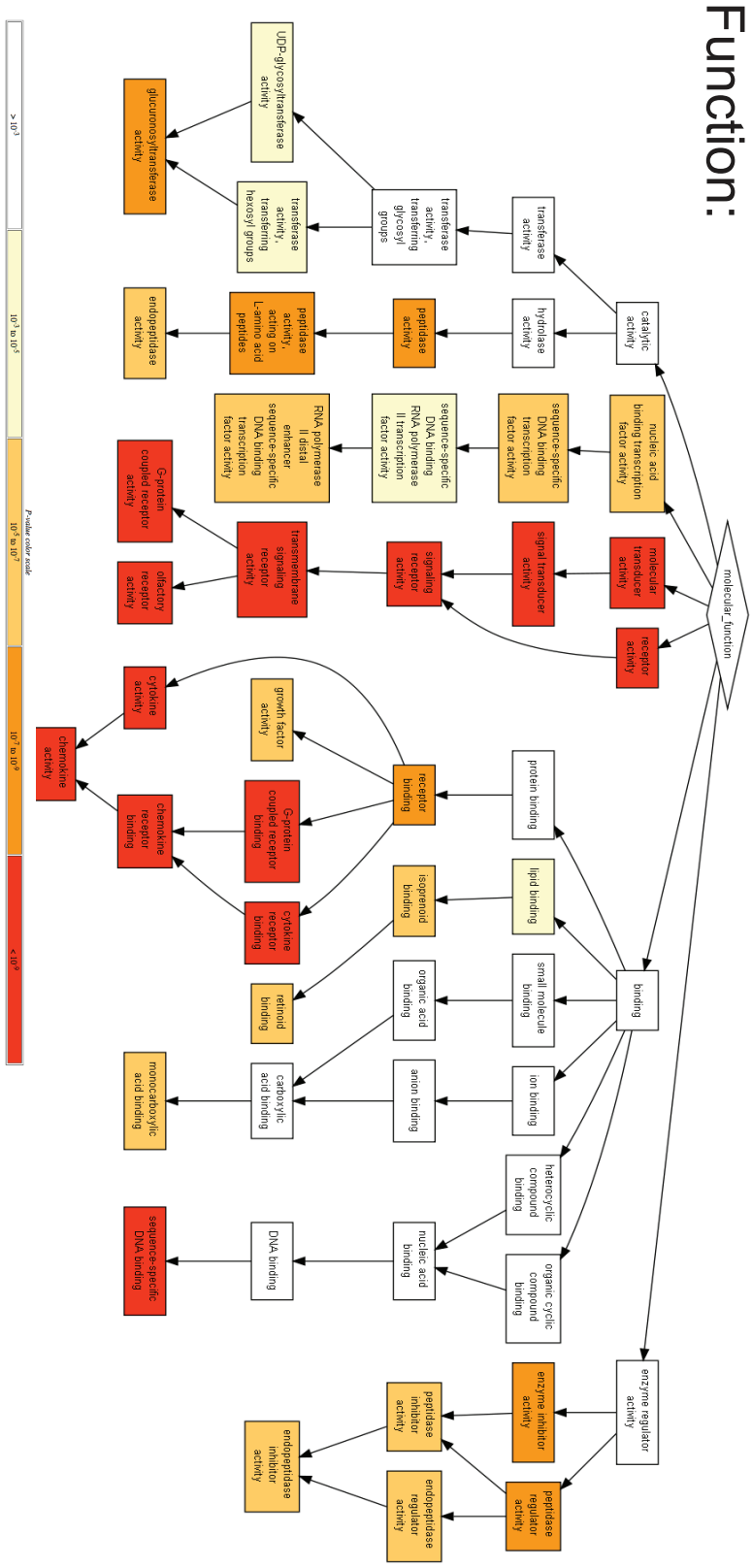 (Maimets et al., 2016), esophagus (DeWard et al., 2014), lung (Rock et al., 2009), endometrium (Turco et al., 2017) and breast (Sachs et al., 2018) and many of these and other tissues such a cerebral cortex have also been created by directed-differentiation protocols. One of the outstanding difficulties in organoids made from pluripotent stem cells is the ability to produce fully mature tissues. Some organoid systems produced from pluripotent stem cells have signatures of immature tissue. For example, human intestinal organoids make the cell types of human adult intestine, self-organize and have some of the functional capacities of mature tissue, however they still bear some immature expression of fetal tissue. Fetal derived intestinal organoids from humans and mouse have shown that cultures can be derived in the immature state and subsequently induced to partial maturity with the addition of growth factors (Fordham et al., 2013b). In mammalian neonates, rapid intestinal maturation happens at first exposures of the intestinal epithelium to milk and endogenous food, which also well as the colonization of the gut by commensal and pathogenic microorganisms, which produce a plethora of metabolites (Koenig et al., 2011). One group had made immature organoids from

pluripotent stem cells that were later implanted into an immune system-privileged compartment in a mouse kidney and show increased expression of maturity by maturity markers as well and some of the expected histological changes (Watson et al., 2014) so there may be unknown factors in the mouse that confer developmental changes. The development of refined protocols as well as validation of *in vitro* stem cell derived cultures by comparison with tissue-similar biopsy-derived cultures will aid in the ability to understand and recapitulate more and more functional aspects of mature tissue. Setting doubts about maturity aside, there might be utility in capturing and exploiting immature developmental states. In a study of human norovirus infection in intestinal organoids, it was discovered that the virus multiplied in biopsy-derived organoids but not in hPSC-derived organoids. Rather than a failure, this creates an opportunity to explore the slight biological differences in these similar systems understand disease mechanisms like viral replication (Hill and Spence, 2017). Because organoids represent a reductionist system, it will always take careful consideration when considering to what degree the readouts of an organoid system can provide useful insight into physiology and pathologies.

Some of successful applications of organoid cultures were by groups looking at host pathogen interactions. Short-chain fatty acids serve as a nutrient and signaling molecules in mammals and are produced by microbial fermentation of dietary fiber (Park et al., 2016). Even the constituent molecules of some bacterial cell wells have been found protect the cells of organoid cultures from oxidative induced cell death. These facet of pathology that would be difficult to detect or deconvolute in either transformed cell lines or the complex microbiome of whole animals (Nigro et al., 2014), but an organoid system simplifies the experimental background while still exhibiting normal cellular physiology. An landmark study designed a proliferation-reporter assay to screen a panel of 92 microbial metabolites for the effects of proliferation in  organoids and found that butyrate suppresses proliferation of stem cell enriched organoids (Kaiko et al., 2016), a potential lead on diseases of unknown origin like irritable bowel. These and numerous other studies are using organoids to produce viable infections with Helicobacter pylori (H. pylori), Zika virus (ZIKV), and Norovirus (NoV) particularly to model the cell intrinsic and innate immune responses to the early stages of infection and ongoing viral replication mechanisms.

With respect to cancer, two studies highlight best the features of organoids that give these complex cultures some of the experimental flexibility absent in some other systems. As illustrated earlier, organoids can be maintained long term in defined culture systems that recapitulate supportive features of the niche. Because colon cancer typically arises over a long-time frame and acquires a confounding number of passenger mutations during the acquisition of the essential driver mutations, understanding the adenoma carcinoma sequence reviewed by (Vogelstein et al., 2013b) is a particularly interesting application of intestinal organoid disease modeling. It was observed that mutations in signaling pathways could affect the growth factor requirements in intestinal cultures. In epithelial organoids from APC-deficient subjects, R-spondin was no longer required to stimulate Wnt signaling (Sato et al., 2011a). The rationale for the generation of the organoids described in chapters one and two was that a human intestinal organoid culture could reduce the complexity of diseases like cancer that have important driver mutations that are difficult to deconvolute from the large number of passenger mutations. Two studies published close together did this in an elegant set of experiments mutating the tumor suppressors and oncogenes, in colon organoids grown from normal human colon tissues (Drost et al., 2015; Matano et al., 2015). Because of the difficulty in genetically engineering primary organoids, both groups used CRISPR/Cas9 gene editing to induce mutations and then withdrew growth factors that acted in corresponding pathways as a means of positively selecting clones. This produced an invasive metastatic carcinoma with a minimal set of required driver mutations. The mutations that had been inferred to be the drivers of cancer by so many prior models did in fact recapitulate the features of the adenoma carcinoma sequence and lead to cancer.

A major hurdle in development of new therapeutic regimens is the translation of knowledge from bench to bedside. Already some of the gaps in the ability of current models to fully recapitulate patients' disease have been filled by the new capabilities of organoids. As a reduced experimental system there are context dependent considerations for organoids, as in any model that is only a mimic of actual disease. One important finding in the area of hepatic organoids was that they express cytochrome 450 enzymes at physiologic levels when induced to differentiation (Huch et al., 2015; Katsuda et al., 2017). These enzymes are responsible for the metabolism of nearly all the small molecule drugs and small differences can have a

huge impact on the efficacy, dosage and safety in humans. Notably mice have experienced significant expansion of the CYP gene superfamily as compared to humans (102 vs. 57) (Guengerich, 1997) and it is not always clear whether mice will metabolize drugs the same way humans would. As this is often the last stage before human trials, organoids could play a key role much earlier in the development process for drugs and potentially avert harm and years of wasted resources.

Progress on the culture of existing systems as well as new derivation protocols continue to move the field forward. Already evidence is emerging that indicates patient organoids might be a means to tailor therapies in personalized medicine. Biobanks of organoids from patients that had matched samples of normal and pathological tissue have been established for intestine and in one assay looking at 20 cancer organoids an assay determined that only one of the samples was susceptible to inhibition by a Wnt secretion inhibiting drug (Chen et al., 2009). It would be useful to test organoid cultures with uniform genetic backgrounds to see if mutagenic assays could test what kinds of genetic changes lead to chemotherapeutic drug resistance. By using CRISPR/Cas9 screening libraries or radiation-induced mutagenesis, one can envision survival-based assays that could test genome wide arrays of mutations for the ability to confer resistance. By further applying advances in bioengineering, some of the remaining undefined features of three-dimensional culture and variations in mechanical properties such as stiffness can be addressed. Artificial matrices abound but still need improvement to provide all of the unknown essential qualities of Matrigel. Control of mechanical signals are available in the form of photolabile PEG hydrogel that can be induced to locally degrade with light and other photo-initiators that can similarly cause stiffening (Guvendiren and Burdick, 2012). In addition to mimicking essential signals from native extracellular matrices ECMs and providing a better mimic of the stroma, microfluidics could bring organ-on-a-chip closer to fruition by mimicking circulation and respiration. The idea of organ on a chip is that a patient could have iPSCs made from their own cell and have a tissue, or several tissues made at a micro scale to directly test which treatments would work best. Microfluidics provide the essential functions of vasculature for delivering oxygen and nutrients, while removing waste. These systems could also flow in timed releases of developmental growth factors to make better organoid cultures or even screen therapeutic drugs. Outstanding questions remain in larger questions such as the direct use of

organoids in regenerative medicine. We cannot rule out the possibility that genetic or epigenetic aberrations may be caused by adapting explants to organoid culture.  Current organoids are mainly comprising a subcomponent of a tissue from one germ layer in some cases poorly defined stromal cells while most tissues consist of specified stromal cells, muscle, blood vessels, and immune cells which support the normal development and maturation as well as the functional capacity of these organs. By cautiously studying the development and maintenance of these tissues and the requirements met by their tissue compartments, new methods may continue to advance the ability to generate mini-organs from the reprogrammed cells or explants of patients to directly determine which therapies would be most appropriate for a specific patient.

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