The gastrointestinal tract is characterized by a self-renewing epithelium fueled by adult stem cells residing at the bottom of the intestinal crypt and gastric glands. Their activity and proliferation is strongly dependent on complex signaling pathways involving other crypt/gland cells as well as surrounding stromal cells. In recent years organoids are becoming increasingly popular as a new and powerful tool to study developmental or other biological processes. Organoids retain morphological and molecular patterns of the tissue they are derived from, are self-organizing, relatively simple to handle and accessible to genetic engineering. This review focuses on the developmental processes and signaling molecules involved in epithelial homeostasis and how a profound knowledge of these mechanisms allowed the establishment of a three dimensional organoid culture derived from adult gastrointestinal stem cells.
malignant transformation (Barker et al., 2007), making it a medical necessity to understand the processes regulating stem cell homeostasis. The impact of malignant transformation of gastrointestinal stem cells is evident in cancer statistics: stomach cancer and colorectal cancer are the 3rd and 4th most frequent reason for cancer associated death worldwide, accounting each for around 700,000 victims per year (Globocan, 2012).

Several decades of research and recent cutting edge findings have revealed the developmental processes and molecular mechanisms of tissue homeostasis taking place in the intestinal crypt and gastric gland. In this context it is noteworthy that numerous findings originated from comparisons with cancer development. Tumors as well as stem cells are influenced by their surrounding niche. The clarification of the fundamental mechanisms active in the intestinal stem cell niche allowed the reproduction of this niche in a 3D \textit{in vitro} culture system. Under selected growth conditions stem cells proliferate and form complex structures called organoids, which have been developed for the intestine (Sato et al., 2009) as well as for the stomach (Barker et al., 2010). Organoids represent an ideal tool to study developmental processes and tissue maintenance mechanisms due to their ease of genetic manipulation. The technique has spread widely within the community, mainly because of the potential of organoids to model \textit{in vitro} many complex \textit{in vivo} processes. In addition, although being somatic cells, they can be cultivated far beyond the Hayflick limit (Hayflick, 1965) while maintaining their genomic stability (Huch et al., 2015).

Here we present an overview of developmental processes and signaling pathways active in the mammalian gastrointestinal tract and how they translate into the establishment of organoid cultures. We discuss state of the art culture conditions as well as future challenges and opportunities in the field of gastrointestinal organoids.

2. Molecular mechanisms in the gastrointestinal tract

2.1. Intestine

The intestinal epithelium is composed of units formed by villi and crypts. In the embryonic intestinal tract proliferation is taking place in small invaginations developing into crypts (Patzelt, 1882; Pfitzner, 1880), which then give rise to villi and other crypts (Panneth, 1887). In the adult intestine cellular division is only occurring in the crypt but not in the villus (Bizzozero, 1893). The renewal of an entire unit is performed by a conveyor belt mechanism from the bottom of the crypt to the top of the villus driven by self-renewing, undifferentiated and multipotent stem cells (Friedman, 1945; Leblond and Messier, 1958; Leblond and Stevens, 1948; Quastler and Sherman, 1959; Stevens and Leblond, 1947; Walker and Leblond, 1958). Several potential stem cell populations have been proposed in the crypt (Behnke and Moe, 1964; Hampton, 1968; Kataoka, 1970; Troughton and Trier, 1969). One of them named crypt based columnar (CBC) cells is closely associated with Paneth cells at crypt bottoms (Cheng and Leblond, 1974). CBCs together with Paneth cells have long been proposed to form a restricted stem cell zone within the crypt (Bjerknes and Cheng, 1981a, 1981b, 1981c), a postulation that has been confirmed experimentally by lineage tracing experiments (Barker et al., 2007). Confined zones of proliferative and more differentiated regions can also be observed in tumors (Vermeulen and Snippert, 2014). Signaling pathways relevant in tumors such as e.g. the Wnt or Notch pathway often also play a decisive role in the stem cells of the respective tissue. In most colorectal cancers (CRCs) deregulation of genes associated with the Wnt pathway is an initiating event and results in enhanced proliferation (Groden et al., 1991; Kinzler et al., 1991; Korinek et al., 1997; Liu et al., 2000; Rubinfeld et al., 1993; Su et al., 1993). Interestingly, CRCs have overlapping gene expression patterns with intestinal crypts (van de Wetering et al., 2002), e.g. the surface protein Lgr5. Lineage tracing experiments revealed that single Lgr5$^+$ CBC cells are able to regenerate an entire crypt-villus axis. These cells are in a state of “stemness” and possess long-term self-renewal capabilities as well as multipotent differentiation abilities (Barker et al., 2007), thus fulfilling the definition of a stem cell (Lajtha, 1979). The size of the intestinal stem cell compartment remains constant over time. Cell population size is essential in this setting and involves self-renewing as well as differentiated cells. The cellular divisions follow a stochastic model with the stem cells dividing symmetrically upon the loss of a nearby cell, a concept known as neutral drift dynamics. In this stochastic model single intestinal crypts inevitably drift into monoclonality (Snippert et al., 2010). Several tissues contain Lgr5 positive stem cells e.g. the hair-follicles (Jaks et al., 2008) or the gastric antrum (Barker et al., 2010) making it a general marker of stemness in several Wnt-dependent tissues. Nevertheless, Lgr5 expression by itself does not convey stemness per se, as some differentiated cells can express Lgr5, hinting to the importance of additional factors such as the surrounding stromal niche in the transmission of stemness.

Research in the past years has revealed a complex signaling network present in the intestinal crypt. CBC stem cell activity is maintained by continuous communication with the surrounding Paneth cells and surrounding stromal cells. Several signaling mechanisms are involved, among them Wnt, BMP/TGF-β, Notch and EGF with Wnt signaling being a key regulator of epithelial homeostasis and self-renewal activity (He et al., 2004; Schuijers et al., 2015). While the cells move along the crypt-villus axis they are exposed to a Wnt gradient. Stem cells become loaded with Wnt mediators that are produced by adjacent Paneth cells, which bind to their cognate Frizzled receptors (Yarin et al., 2016). Due to the local production and limited diffusion, Wnt molecules as well as their receptors are diminished through turnover by cellular division as the cells leave the stem cell zone and move away from Paneth cells. Besides Lgr5 the CBC stem cells express a whole set of further Wnt pathway associated genes like e.g. the transcription factor Ascl2 (van der Flier et al., 2009a, 2009b), which directly controls stemness in the intestinal crypts (Schuijers et al., 2015). The high Wnt activity in CBC stem cells is mediated by binding of secreted Rspondin family members to Lgr family members on the CBC membrane (Carmon et al., 2011; Glinka et al., 2011; de Lau et al., 2011). This binding potentiates the Frizzled mediated Wnt pathway activation. The molecular mechanism underlying this potentiation are two ubiquitin ligases, Rnf43 and Znrf3 (Koo et al., 2012), which mediate the endolysosomal degradation of Frizzled receptors by ubiquitination for fine regulation of Wnt activity in the intestinal stem cell. Upon binding of Rspondin to a Lgr receptor, Rnf43 and Znrf3 are recruited to a tripartite complex with Rspondin and Lgr5, sequestering them from Frizzled. As a result, robust activation of the Wnt pathway is achieved by blocking the inhibitory action of Rnf43 and Znrf3 (Hao et al., 2012). Another cell type at the crypt bottom relying on Wnt pathway activity is the Paneth cell. The downstream Wnt-target gene Sox9 is important in the development of Paneth cells (Bastide et al., 2007; Mori-Akiyama et al., 2007) as well as maturation of secretory precursor cells into Paneth cells (Andreu et al., 2005, 2008; van Es et al., 2005a). As already mentioned, Paneth cells also play an important role in stem cell niche formation at the crypt bottom (Fig. 1A). Besides this, Paneth cells are a major source of intestinal antimicrobial proteins in the form of lysozyme and defensins, protecting the crypts and thus the stem cells from microbial assaults. A defective defensin production followed by a break-down of the mucosal antibacterial defense is a critical pathogenetic factor in...
the development of inflammatory bowel disease (Wehkamp et al., 2005). Microbes induce signaling through toll-like receptors on intestinal stem cells, thereby inducing endoplasmatic reticulum stress and apoptosis (Afrazi et al., 2014). On the other hand, the immune system is actively involved in regenerating damaged intestinal epithelium though interleukin-22 (Lindemans et al., 2015). Commensal microbes also directly influence the epigenetic development of intestinal stem cells (Yu et al., 2015).

Besides Wnt signaling the intestinal stem cell state depends heavily on Notch pathway activity. This pathway controls the endodermal differentiation (Jensen et al., 2000) into the secretory lineage (Milano et al., 2004). Hes1/3/5 are the downstream target genes of the Notch pathway (Ueo et al., 2012) negatively regulating Atoh1 (aka Math1) (Yang et al., 2001). The cell fate is subsequently determined either by Hes expression, which induces an enterocyte fate (Jensen et al., 2000; Ueo et al., 2012) or by Atoh1, which promotes secretory lineage development (Shroyer et al., 2005; VanDussen and Samuelson, 2010; Yang et al., 2001). Inhibition of Notch gives rise to a vast number of goblet cells arising from converted stem cells under the action of Atoh1 (van Es et al., 2005b, 2010; Milano et al., 2004). Further developmental processes include bone morphogenic protein (BMP) signaling, which plays a role in differentiation and thus represents an antagonist to the Wnt pathway. It inhibits intestinal self-renewal by suppressing β-catenin activity (He et al., 2004). BMP antagonists, like Gremlin and Noggin, are an inherent part of the intestinal epithelial stem cell niche (Kosinski et al., 2007) and are important players for stem cell self-renewal (Davis et al., 2014; Haramis et al., 2004). They promote intestinal stem cell function by preventing BMP activity in the stem cell niche (Scoville et al., 2008). Epithelia commonly express the growth factor EGF, which induces cell proliferation and angiogenesis through PI3K/Akt, ErbB and MAPK/ERK signaling. Deregulation of these pathways (Davies et al., 2014; Feng et al., 2011) e.g. by Lrig1 knock-out (Powell et al., 2012; Wong et al., 2012) induces intestinal stem cell hyperplasia. Tight regulation of Egf signaling is therefore important for stem cell homeostasis and regeneration. After injury, Egf can be activated through the Hippo signaling pathway under the action of YAP which also transiently suppresses Wnt signaling and Paneth cell differentiation (Gorgoriff et al., 2015). Another relevant intestinal signaling pathway
is the Hedgehog pathway, which is involved in the patterning of the crypt-villus axis (Madison et al., 2005) and drives smooth muscle homeostasis (Zacharias et al., 2011). Some characteristics of intestinal tissue identity is influenced by the transcription factor Cdx2. Lgr5\(^+\) derived intestinal stem cells that loose functional Cdx2 start to express features of pyloric stem cells (Simmini et al., 2014).

### 2.2. Stomach

Anatomically the stomach can be divided into the fundus, corpus and the antrum, which opens into the duodenum. Each of these parts is lined by a glandular epithelium. Many mammalian animals instead of a glandular fundus possess a large forestomach lined with simple columnar epithelium. Glands begin as polyclonal structures in the embryo but become a monoclonal unit in the adult stomach (Nomura et al., 1998; Tatematsu et al., 1994). Since glands are individual units, a single monoclonal conversion in stem cells can affect the entire gland (McDonald et al., 2008; Tatematsu et al., 1994). The glands can be divided into the luminal pit region, the isthmus, the neck and the base. The isthmus shows a high cellular turnover and is the supposed location of a population of mostly quiescent stem cells (Karam and Leblond, 1993) activated upon IFN\(\alpha\) stimulation (Qiao et al., 2007). The isthmus stem cell, if it exists, might nevertheless also turn out to be vividly proliferating, similar to the Lgr5 stem cell of the intestine. Daughter cells of the isthmus stem cell population are thought to migrate bidirectionally to the top and the bottom of the gland (Bjerkses and Cheng, 2002). Their activity is under the control of Notch signaling (Kim and Shivdasani, 2011), as inhibition of Notch leads to differentiation of proliferating cells in the isthmus (Faure et al., 2015). The isthmus stem cells are suggested to reside in a specialized niche composed of Cxcl12(\(+\)) endothelium and Wnt5a-producing Cxcr4(\(+\)) innate lymphoid cells (Hayakawa et al., 2015). Although a specific marker of human isthmus stem cells is still missing, there is evidence that Aspm might be a marker in rats (Vange et al., 2015; Zhu et al., 2016), which nevertheless needs to be clarified by lineage tracing studies. Besides the isthmus region, a second region of stem cell activity has been identified in the stomach. The intestinal stem cell marker Lgr5 can be found at the bottom of stomach glands. Lineage tracing experiments proved that these cells can generate entire glands in the antrum/pyloric region (Barker et al., 2010). While in the antrum this cell population is frequently dividing, the corpus does not show prominent proliferative activity at gland bottoms. Nevertheless, a small population of chief cells expresses Troy (aka Tnfrs5f19) on their cell surface and lineage trace on a regular but infrequent basis into all differentiated cell types of the corpus gland (Stange et al., 2013). Upon epithelial injury the Troy\(^+\) stem cell population actively contributes to gland regeneration, thus acting as a reserve stem cell population. Activation of the Ras signaling pathway in these cells leads to metaplastic changes (Choi et al., 2016). It is yet to clarify by which mechanisms the isthmus and gland bottom stem cells compete in the homeostasis and regeneration of the glands.

Like in the intestinal epithelium, mesenchymal signaling is involved in the maintenance of gastric homeostasis. It is necessary for epithelial development, especially through Barx1 mediated inhibition of Wnt signaling (Kim et al., 2005a; Noguchi et al., 2015; Woo et al., 2011). Barx1 plays an important role in stomach patterning and is expressed in the gastric mesenchyme during development (Kim et al., 2007). Since Hedgehog signaling controls the mesenchymal development (Mao et al., 2010), it is indirectly also important for the patterning of the glandular epithelium. Shh-Wnt antagonistic interactions are required in several tissues for niche formation through expansion of stem cells (Ouspenskaya et al., 2016). Ihh and Shh are both present in the gastric epithelium and have a redundant function in gland differentiation (Kim et al., 2005b). Shh is mainly expressed in the corpus region and is lost in intestinal metaplasia of the stomach hinting to its importance in adult gland differentiation (van den Brink et al., 2002). Shh participates in parietal cell maturation during development (Stepan et al., 2005) and, importantly, parietal cells have an impact on gland maturation and morphology (van den Brink et al., 2001). They act as an endogenous source of Shh during gastric repair (Engevik et al., 2013; Xiao et al., 2013) and loss of parietal cell Shh expression induces hypergastrinemia and hyperproliferation of surface mucous cells (Xiao et al., 2010). Ihh is expressed in the pit region (Fukaya et al., 2006) and controls gastrin-induced proliferation of the adult stomach (Feng et al., 2014). Hedgehog signaling has yet been neglected in AdSC-derived organoid culture but has the potential to allow more effective differentiation. The use of the Wnt inhibitor Dkk1 together with Shh is used in murine embryonic stem cell derived cultures to form organoids comprising the mesenchyme and the epithelium (Noguchi et al., 2015). Stomach development is further dependent on FGF10 signaling (Nyeng et al., 2007; Spencer–Dene et al., 2006) especially in the glands (Shin et al., 2006) and on BMP signaling during embryonic development (Faure et al., 2013; Maloum et al., 2011).

### 3. Translating knowledge into the dish

#### 3.1. Intestinal Organoids

The establishment of an in vitro three dimensional gastrointestinal organ model has required a deep understanding of the mechanisms underlying cell homeostasis in this tissue. In contrast to cell lines derived from various colon cancers (Rutzky and Moyer, 1990), culturing AdSCs is far more difficult due to diverse growth factor requirements. Culture conditions for intestinal epithelium have been described, but these cultures are usually difficult to initiate and only allow culturing for a short period of time (Blay and Brown, 1984; Négre et al., 1983; Quaroni and May, 1980). This is species independent and has been performed with tissue from rats (Fukamachi, 1992), mice (Whitehead et al., 1999) and humans (Perreault and Beaulieu, 1996; Whitehead et al., 1999). Using collagen type I-coated dishes with a feeder layer of mesenchymal fibroblasts, it is possible to culture and characterize crypt epithelial cells for one to two weeks (Evans et al., 1992). Overcoming the limited time span as well as the unnatural two dimensional growth pattern were crucial steps to improve intestinal cultures. One approach is the use of an air-liquid interface with whole intestinal samples being embedded in a collagen-like gel (Ootani et al., 2009). The obstacle for a broad application of this system is the inability to passage and expand the culture. A characteristic of epithelial cells is their natural adhesion to the extracellular matrix. This matrix contains proteins like laminin \(\alpha1\) and \(\alpha2\), which are found to be enriched at the base of intestinal crypts (Sasaki et al., 2002). Intestinal cells without contact to extracellular matrix will undergo anoikis (Hofmann et al., 2007). Epithelial cells nevertheless successfully grow on Matrigel (Stingl et al., 2001), making it a perfect matrix for the growth of epithelium derived organoids. Matrigel is produced from Engelbreth-Holm-Swarm mouse sarcoma. It is rich in diverse ECM proteins such as collagen type IV, the above mentioned laminins, entactin, nidogen, heparin sulfate proteoglycans (Kleinman et al., 1986) and a variety of growth factors like EGF, bFGF NGF, PDGF, IGF-1 and TGF-\(\beta\). Matrigel is also suited as transportation medium for organoid xenografts and has a higher growth efficiency than collagen type I gel (Yui et al., 2012). These insights highlight the importance of a surrounding matrix with signaling activity for organoid growth.
As in the intestinal crypt, the key player in organoid architecture and growth is the Wnt pathway (Fig. 1A). Intestinal organoids grow out from Lgr5⁺ CBC cells (Sato et al., 2009). Organoid cultures can thus be started either by single sorted Lgr5⁺ CBC cells, or by plating whole crypts. In vivo the main actors are Wnt3 secreted by Paneth cells (Sato et al., 2011b), and Rspo2 being produced by the intestinal stroma (Kabiri et al., 2014; Storm et al., 2016). In vitro, R-spondin1 needs to be supplemented as a substitute for the missing stroma, whereas Wnt3a can be omitted from the medium once Paneth cells are present. The complex Wnt signaling network can be fine-tuned and adapted to the investigated tissue like in the murine colon, where organoids require the GSK-3 inhibitor CHIR99021 to grow (Yin et al., 2013). Wnt signaling independence can be achieved through depletion of the negative regulator APC (Schwitalla et al., 2013). The Wnt gradient along the crypt axis has it peak at the crypt bottom where the Lgr5⁺ stem cell/Paneth cell membranes form a pool for secreted Wnts (Farin et al., 2016) and diminishes towards the crypt-villus junction – it is very important for cellular differentiation along this axis (Fig. 1B). Nevertheless, against the general notion of differentiation being a one-way road, recent research has highlighted the plasticity of the intestinal crypt with committed progenitors being able to convert back to stem cells upon disturbance of stem cell homeostasis (Stange and Clevers, 2013). CBC depletion creates room next to Paneth cells, which is filled up by progenitors that “fall back” into the stem cell niche. Lineage tracing experiments have proven that several intestinal cell types are capable of lineage reversion into stem cells upon regaining contact to the stem cell niche. This phenomenon has been first shown for Dil1⁺ secretory progenitor cells, which adopt a stem cell state in organoid cultures upon exposure to Wnt3a, and in vivo upon depletion of CBC stem cells due to irradiation (van Es et al., 2012). Similarly, label retaining cells, which are normally committed to become Paneth or enteroendocrine cells, upon tissue damage can revert to stem cells (Buczacki et al., 2013). Other lineage tracing studies using a diverse set of markers of intestinal crypt cells like Hopx or Bmi1 have established their potential to regain stem cell potential after CBC depletion (Takeda et al., 2011; Tian et al., 2011). These markers are controversially discussed to be either +4 cell markers (Potten et al., 2009) or unspecific crypt cell markers (Muñoz et al., 2012). Even committed enterocyte precursors can dedifferentiate to stem cells upon acute CBC loss (Tetteh et al., 2016).

The complex BMP/TGF-β signaling pathway is responsible for cell differentiation (Fig. 1A). Intestinal BMP originates in the mesenchyme and particularly Bmp4 is responsible for differentiation and morphogenesis (Haramis et al., 2004; He et al., 2004; Zhang et al., 2009). Although organoids are exclusive epithelial entities, TGF-β signaling remains a crucial factor in these cultures, as it determines the long-term cultivation efficiency of the cells. Depletion of BMP antagonists like Noggin (Sato et al., 2009) or GREM1 (Davis et al., 2014) prevents cellular proliferation. Interestingly, inhibitors of the BMPs and TGF-β signaling pathway are necessary in adenomas and early colorectal cancers but become increasingly dispensable in advanced colorectal cancers (Fujii et al., 2016). Nevertheless normal human organoids are more sensitive to these pathways and require low doses of TGF-β kinase/activin receptor-like kinase (ALK5) inhibitors such as A83-01 for long term culture (Sato et al., 2011a). This prevents phosphorylation of Smad2/3 and the growth inhibition induced by TGF-β (Tojo et al., 2005). Organoids with CRISPR targeted SMAD4 grow regardless of TGF-β and BMP being present in the medium (Matano et al., 2015).

In addition to Wnt and BMP/TGF-β signaling several other pathways play an important role in the creation of the stem cell niche in vitro (Fig. 1A). As in the in vivo situation, Notch signaling plays a major role in intestinal organoids. Organoids have the capability to self-organize, with stem cells and Paneth cells grouping around each other and creating crypt-like structures in vitro (Sato et al., 2011b). This implies the importance of direct cell-to-cell signaling between Paneth cells and intestinal stem cells. The intestinal stem cells express Notch on their surface, which is in constant contact with the ligand Dl4 expressed by Paneth cells (Sato et al., 2011b). When contact to Paneth cells is lost, most undifferentiated cells turn into goblet cells (Pellegrin et al., 2011). In contrast cells deficient for Atoh1 (Durand et al., 2012) or under p38 (Sato et al., 2011a) or histone deacetylase (Yin et al., 2013) inhibition can grow without adjacent ligands and form organoids lacking the secretory lineages. Another factor responsible for cell growth and proliferation is EGF. EGF signaling is required for crypt growth and consequently for long-term expansion of organoid cultures (Sato et al., 2009). Within the stem cell niche it is produced by Paneth cells (Sato et al., 2011b). EGF is very important in organoid proliferation because inhibition of EGF results in a slow proliferation rate in human intestinal organoids (Matano et al., 2015). The action of EGF is regulated through negative feedback loops and organoids with activating KRAS or PI3KCA mutations (Matano et al., 2015) as well as inactivated Lrig1 (Wong et al., 2012) or TRPV1 (de Jong et al., 2014) are able to grow without EGF. Other alternative growth factors are the mesenchymal produced IGF1 (Lund et al., 1988; Reynolds et al., 2014; Simmons et al., 2007) and TGFβ produced by Paneth cells (Sato et al., 2011b) or HB-EGF (Chen et al., 2012), all of which can be used in organoid culture to replace EGF. Akt/mTOR signaling is another important mechanism maintaining crypt homeostasis by coupling nutrient availability and stem cell function (Yilmaz et al., 2012).

A number of supplements further increase the efficiency of organoid culture initiation and propagation. Antibiotics and fungicides are used in organoid cultures, especially from human origin, to prevent infections from contaminations. The supplement B27 increases sphere-forming efficiency and sustains the propagation of tumor spheres (Chen, 2011). Nicotinamide (niacin) is the amide of nicotinic acid (Vitamin B3) and is required in the synthesis of the coenzymes NAD⁺ and NADP⁺. N2 is a 100 times concentrate of Bottenstein’s N-2 formulation (Bottenstein and Sato, 1979) lacking thryoxine and triiodothyronine. It is used in FCS free media for growth of post-mitotic cells. N-acetylcysteine is an antioxidant directly scavenging ROS and partially via ERK1/2 activation (Zhang et al., 2011). It is also a source for cysteine in the generation of the antioxidat glutathione. Additionally it has mucolytic properties. Primary cells that are taken into cell culture experience severe levels of stress. Y-27632, an inhibitor of the Rho-associated protein kinase, can be added to the medium and prevents anoikis induced cell death. SB202190 is an effective and selective inhibitor of p38 (Manthey et al., 1998) thus influencing differentiation, proliferation and apoptosis. It is mainly needed in the culture of human colon and colorectal cancer organoids (Sato et al., 2011a). Colon organoids generally require several additional components to form long-term organoid cultures. The brain derived neurotrophic factor BDNF is a growth factor, playing a role in cell survival and increasing culture efficiency of mouse colon organoids (Sato et al., 2011a). PGE2 is required for normal human colonic and tumor organoids (Sato et al., 2011a). It is an activator of the Wnt pathway (Goessling et al., 2009) and maintains primary Lgr5 colorectal organoid culture viability and rates of cell proliferation (Fan et al., 2014). Valproic acid (VPA) is a histone deacetylase inhibitor that can activate Notch signaling (Greenblatt et al., 2007; Stockhausen et al., 2005) and is needed for mouse colon organoids, maintaining the self-renewal of Lgr5⁺ cells (Yin et al., 2013). Thus, culture conditions vary depending on the tissue of origin (Tables 1,2). Media compositions can vary between protocols, exemplary compositions are given in Table 2. Apart from the medium composition, organoid handling is straight forward and can be
Paneth cells turn differentiating progenitors into CBCs mediated by Wnt and Notch signaling as discussed above. Interestingly Paneth cells themselves form without Notch signals but under strong Wnt conditions (van Es et al., 2005a; Farin et al., 2012). In Wnt free medium organoids start to self-organize and to form more complex structures. Local production of Wnt by Paneth cells, in conjunction with Rspo provided in the medium, induces organoid budding which protrude from the central cyst (Farin et al., 2012; Sato et al., 2011b)(Fig. 1B). The migration of progenitors and differentiated cells is orchestrated by the Wnt pathway and repulsive EphB/EphrinB interactions (Batlle et al., 2002). As EphB2 is highly expressed at human colon crypt bottoms, it can be used to isolate and culture human colonic stem cells (Jung et al., 2011). EphB3 is expressed by Paneth cells whereas differentiated cells express the membrane bound EphrinB ligands. As TA cells travel along the Wnt-gradient they gradually lose their EphB expression and show an exogenous source of Wnt is still required for organoid growth.

Table 1

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Table 2

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Fig. 2. Architecture and differentiation conditions for intestinal and gastric organoids. The structure of the intestinal and gastric units are shown. Organoids are represented at initiation, during expansion and in their established form with the composition of the respective growth media.

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Thus, cKit+ cells only partially take over the function of Paneth cells in colonic organoids. Their in vivo function remains to be elucidated. Stem cells within the organoids retain their self-renewal and multilineage differentiation properties even after long periods of culture, which has been convincingly demonstrated by their ability to functionally engraft into wounded colon (Yui et al., 2012). This raises hope for future applications in regenerative medicine and disease treatment.

3.2. Stomach organoids

The development of stomach organoids was facilitated by the fact that many components of the intestinal system could be transferred (Fig. 2). This was helpful, as the gastric stem cell niche and its underlying mechanisms are much less well defined as compared to the intestine. Using a variation of the intestinal culture protocol, it is possible to create organoids from pylorus glands and stem cells marked by Lgr5 (Barker et al., 2010) as well as from corpus glands and stem cells marked by Troy (Stange et al., 2013). These stomach-derived organoids consist mainly of chief cells and mucus neck cells, and can differentiate into pit cells and enteroendocrine cells when certain stem cell growth promoting medium components are omitted. Of note, parietal cells are not formed in these cultures, indicating that certain differentiation inducing components are missing in the culture medium. Nevertheless, when these organoids are co-cultured together with immortalized stomach mesenchymal cells, a differentiation into all lineages is possible, hinting to the importance of the mesenchyme to direct stem cell vs. differentiation fates in the stomach epithelium (Schumacher et al., 2015) (Fig. 2). Additional medium components compared to the intestinal organoid system are the growth factor FGF10 and the peptide hormone gastrin. FGF10 is required for cell proliferation and gland formation in the gastric epithelium (Shin et al., 2006) and controls stomach morphogenesis (Nyeng et al., 2007). FGF signaling has also been shown to play a crucial role in gastric cancer (Hattori et al., 1990). Gastrin is involved in gastric epithelial cell proliferation and maturation (Jain and Samuelson, 2006). It is responsible for gastric epithelial cell homeostasis (Todisco et al., 2015) under the control of Ilh (Feng et al., 2014). Stomach organoids have been shown to be useful to study stomach biology, e.g. they have been used for H. pylori infections (Bartfeld et al., 2015; Schlaermann et al., 2016) and physiological studies on intraluminal pH variations (Schumacher et al., 2015). Disease modeling using organoids and genetic engineering has been elegantly reviewed elsewhere (Werner et al., 2016). In short, organoids can be modified using a variety of genetic tools making it possible to study gene function and model various diseases. For instance it is possible to overexpress or knock down certain genes in organoids (Koo et al., 2011) or repair genetic deficiencies using the CRISPR/Cas9 system (Schwank et al., 2013).

3.3. Gastrointestinal cancer organoids

Adult gastrointestinal stem cells are believed to constitute the cells originating cancer (Huels and Sansom, 2015). Therefore, stem cell based organoid cultures represent an attractive in vitro system to model these diseases. Tumor progression is associated with the accumulation of defined driver or gatekeeper mutations. In colorectal cancer, APC is the first hit in cancer initiation, followed by KRAS, TP53 and SMAD (Vogelstein et al., 1988). To study their effects and importance in combination, artificial cancer organoids can be created by sequentially mutating them in organoid cultures using various techniques of genetic engineering (Drost et al., 2015; Fujii et al., 2015; Matano et al., 2015). In addition, novel cancer candidate genes identified e.g. by genome sequencing can be validated (Li et al., 2014; Nadauld et al., 2014). In addition, the effect of restoration of the function of oncogenes can be tested (Dow et al., 2015). Besides modeling cancer starting from normal organoids, it is also possible to take patient derived colorectal tumors directly into organoid culture (Sato et al., 2011a) (Table 2). Colorectal cancer organoids recapitulate the genetic diversity of the original tumors (Fujii et al., 2016; van de Wetering et al., 2015). A potential application of patient derived tumor organoids is the possibility to perform drug screenings (van de Wetering et al., 2015). However, the possibilities of organoids go even far beyond this. Personalized medicine is in great need of a robust system that allows response prediction of a certain targeted therapy. Due to the high complexity of genetic alterations in tumors a prediction solely based on bioinformatics is often impossible. Altogether organoids present a promising approach opening a broad range of new possibilities to study gastrointestinal cancers and their therapy.

4. Concluding remarks and future perspectives

Understanding the mechanisms of epithelial homeostasis is the key to a wide range of new discoveries. A deep knowledge of developmental processes and molecular mechanisms in the adult tissue allows the creation of more and more complex cell culture models such as organoids. There should be a clear definition of the terms and concepts used in the young field of organoid research, such as the difference between spheroids and organoids. As we define it, organoids are three dimensionally growing entities consisting of normal, non-transformed cells exhibiting the morphological as well as the molecular patterns of the tissue they are derived from. Of note, they comprise differentiated cells pursuing the physiological functions of the tissues they are derived from. Furthermore, they are accessible to genetic manipulation, which allows the modification of genes of interest and the documentation of the effect on the molecular and phenotypic level. Owing to these characteristics they are suited for analyzes on different levels: from simple morphological studies to advanced molecular investigations. They can be used e.g. for stem cell research, disease treatment or translational cancer research. Due to their high degree of similarity to the in vivo situation, they can also be used as an animal-saving model. Although the culture methods are already well developed, especially for intestinal cultures, several aspects require further improvements. One aspect is the culture components, for example Matrigel is derived from a mouse sarcoma cell line which prevents its use in clinical applications. This is also true for the other culture components, which need to be adapted to fulfill strict clinical requirements. Another aspect is related to the state of organoid (stem) cells. A population of intestinal progenitor cells labeled retaining cells (LRcs) do not contribute to the maintenance of the stem cell pool in vivo in homeostasis, but are key players after intestinal injury (Buczacki et al., 2013). In vitro, they form organoids with the same efficiency as Lgr5+ cells. Troy+ reserve stem cells of the stomach are mostly quiescent during in vivo homeostasis, can be induced to divide upon tissue damage, and proliferate vividly in organoid cultures (Stange et al., 2013). These two examples show that the state of cells in homeostasis and regeneration can vary and that organoids represent more the regenerative state. In summary, organoids remain for the moment a tool for basic research focusing on regenerative studies, but will certainly be driven towards clinical applications in the near future.

Many questions are yet unanswered regarding molecular processes taking place from early human embryonic development to adult tissue homeostasis. Organoids have already helped to reveal new insights in those tissues for which these cultures could be developed. Expanding the organoid library to other tissues holds
promise for new discoveries also in these organs. AdSC organoids have the potential to clarify how entire organ systems grow and (self-) organize. Furthermore, the knowledge gained through such studies has the potential to open new ways for regenerative therapies by expanding individual cell types or growing even more complex tissues for transplantation. Future research using gastrointestinal organoids may deepen our knowledge on intrinsic and extrinsic factors involved in the morphogenesis of the gut, define key players in the regulatory networks and look for further markers of the different cell types. These findings can be used as a basis for broader studies on the molecular mechanisms along the crypt/villus axis or in gastric glands. Especially the latter needs to be further investigated, since the mechanisms defining the two stem cell populations at the bottom and in the isthmus as well as their complicated interplay are only poorly understood. Varying stomach organoid culture conditions might be a useful tool to discover the still elusive isthmus stem cell. The interactions of the stem cells with the surrounding mesenchyme are also of importance, especially in the gastric epithelium. In this context, AdSC organoids can allow spatio-temporal studies through co-culturing with mesenchyme, potentially allowing the identification of the endogenous factors playing a role in this very intimate relationship. This should ultimately lead to a better understanding of the processes involved in tissue development, maintenance, differentiation, repair and, but also in tumor growth and progression.

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References


Faure, S., Georges, M., McKey, J., Sagnol, S., de Santa Barbara, P., 2013. Expression

Huch, M., Gehart, H., van Boxtel, R., Hamer, K., Blokzijl, F., Verstegen, M.M.A., Ellis, J., Ydbio.2016.08.010i

Nakanishi, Y., Taniguchi, H., Sakamoto, H., et al., 2006. Hedgehog signal acti-
thelial cells in primary serum-free culture. J. Cell Sci. 103, 511

Toshimitsu, K., Nakazato, Y., Kawasaki, K., et al., 2016. A colorectal tumor or-

Yoshida, T., Sugimura, T., Terada, M., 1990. K-sam, an ampli-

A., Clevers, H., 2004. De novo crypt formation and juvenile polyposis on BMP

D., Ruffner, H., et al., 2012. ZNRF3 promotes Wnt receptor turnover in an

Stem Cell Pool. Gastroenterology 141, pp. 1003

Differentiation in the Colon Epithelium but Does Not Expand the Presumptive


A., Clevers, H., 2004. De novo crypt formation and juvenile polyposis on BMP

Nature 526, 715

Nature 303, 1684

Nature 530, 340

Nature 530, 340

Nature 530, 340

Nature 530, 340

Nature 132, 587

Nature 132, 587

Nature 132, 587

Nature 132, 587

Nature 132, 587

Nature 132, 587

Nature 132, 587

Nature 132, 587

Nature 132, 587

Nature 132, 587

Nature 132, 587

Nature 132, 587

Nature 132, 587

Nature 132, 587

Nature 132, 587

Nature 132, 587

Nature 132, 587

Nature 132, 587

Nature 132, 587

Nature 132, 587

Nature 132, 587

Nature 132, 587

Nature 132, 587
Rubinfeld, B., Souza, B., Albert, 0., Müller, O., Chamberlain, S.H., Masiarz, F.R., Mu-
memitsu, S., Polakis, P., 1993. Association of the APC gene product with beta-
Moyer, Mary Pat, Poste, George H. (Eds.), Colon Cancer Cells. Academic Press,
GTH-1 and 2 in the rat, human and adult mouse tissues: an immunochemical approach.
Exp Cell Res. 275, 185–199.
Sato, T., Vries, R.G., Snippert, H.J., de Wetering, M., Barker, N., Stange, D.E., van
epithelial cell cultures from adult human colon, adenoma, adenocarcinoma, and Barrett's epithelium. Gastroenterology 141, 1762–1772.
Sato, T., van Es, J.H., Snippert, H.J., D'Eugenio, N., Vg, R.G., van de Wetering, M., Barker,
N., Shroyer, N.F., van de Wetering, M., Clevers, H., 2011b. Paneth cells constitute the
Schlaermann, P., Toelle, B., Berger, H., Schmidt, S.C., Klaman, N., Ordemann, J.,
parietal cell culture system for modelling Helicobacter pylori infection in vitro. Gut 65,
202–213.
Schuwirth, J., Juncker, J.P., Mokry, M., Hatzis, P., Koo, B.-K., Sessili, V., van der Flier,
Schumacher, M.A., Ahara, E., Feng, R., Engebri, A., Shroyer, N.O., Otten, K.M.,
Worrell, R.T., Montrose, M.H., Shroyer, N.O., Zavros, Y., 2015. The use of
murine-derived fundic organoids in studies of gastric physiology: the use of
Schwan, G., Koo, B.-K., Sessili, V., Dekkers, J.F., Heo, L., Tempe, T., Sasaki, N.,
Schwall, S., Fingerfe A. beim, Cammerare, P., Nebelsiek, T., Götstra, S., Ziegler, P.,
Canli, O., Heimann, H., Huel, D., Moreaux, G., et al., 2013. Intestinal tumour
igenesis initiated by differentiation and acquisition of stem-cell-like properties.
Cell 152, 25–38.
Shin, M., Noji, S., Neubig, D., van de Wetering, M., Barker, N., Clevers, H., 2009. GFRα1 is required for cell pro-
liferation and gland formation in the stomach epithelium of the chicken embryo.
downstream of Math1 to control intestinal secretory cell subtype allocation and
Simmini, S., Bialecka, M., Huch, M., Kester, L., van de Wetering, M., Sato, T., Beck, F.,
van Oudenaarden, A., Clevers, H., Desamps, J., 2014. Transformation of
intestinal stem cells into gastric stem cells on loss of transcription factor Gata2.
Nat. Commun. 5, 5728.
Simmons, J.G., Ling, Y., Wilkins, H., Fuller, C.R., D’Ercole, A.J., Fagem, J., Lund, P.K.,
2007. Cell signaling effects of insulin receptor-substrate-1 deficiency on normal and
Stange, D.E., Koo, B.-K., Huch, M., Kester, L., de Wetering, M., Sato, T., Beck, F.,
van Oudenaarden, A., Clevers, H., Desamps, J., 2014. Transformation of
intestinal stem cells into gastric stem cells on loss of transcription factor Gata2.
Nat. Commun. 5, 5728.
Simmons, J.G., Ling, Y., Wilkins, H., Fuller, C.R., D’Ercole, A.J., Fagem, J., Lund, P.K.,
2007. Cell signaling effects of insulin receptor-substrate-1 deficiency on normal and
Stange, D.E., Koo, B.-K., Huch, M., Kester, L., de Wetering, M., Sato, T., Beck, F.,
van Oudenaarden, A., Clevers, H., Desamps, J., 2014. Transformation of
intestinal stem cells into gastric stem cells on loss of transcription factor Gata2.
Nat. Commun. 5, 5728.
Simmons, J.G., Ling, Y., Wilkins, H., Fuller, C.R., D’Ercole, A.J., Fagem, J., Lund, P.K.,
2007. Cell signaling effects of insulin receptor-substrate-1 deficiency on normal and
Stange, D.E., Koo, B.-K., Huch, M., Kester, L., de Wetering, M., Sato, T., Beck, F.,
van Oudenaarden, A., Clevers, H., Desamps, J., 2014. Transformation of
intestinal stem cells into gastric stem cells on loss of transcription factor Gata2.
Nat. Commun. 5, 5728.
Simmons, J.G., Ling, Y., Wilkins, H., Fuller, C.R., D’Ercole, A.J., Fagem, J., Lund, P.K.,
2007. Cell signaling effects of insulin receptor-substrate-1 deficiency on normal and
Stange, D.E., Koo, B.-K., Huch, M., Kester, L., de Wetering, M., Sato, T., Beck, F.,
van Oudenaarden, A., Clevers, H., Desamps, J., 2014. Transformation of
intestinal stem cells into gastric stem cells on loss of transcription factor Gata2.
Nat. Commun. 5, 5728.
Simmons, J.G., Ling, Y., Wilkins, H., Fuller, C.R., D’Ercole, A.J., Fagem, J., Lund, P.K.,
2007. Cell signaling effects of insulin receptor-substrate-1 deficiency on normal and


esophageal septation and epithelial differentiation. PLOS One 6, e22493.


dependent high-purity cultures of Lgr5+ intestinal stem cells and their progeny. Nat. Methods 11, 106–112.


Zhang, F., Lau, S.S., Monks, T.J., 2011. The cytoprotective effect of N-acetyl-L-cysteine against ROS-induced cytotoxicity is independent of its ability to enhance glut
tathione synthesis. Toxicol. Sci. 120, 87–97.


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