Gastric Organoids: An Emerging Model System to Study *Helicobacter pylori* Pathogenesis

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Abstract *Helicobacter* research classically uses fixed human tissue, animal models or cancer cell lines. Each of these study objects has its advantages and has brought central insights into the infection process. Nevertheless, in model systems for basic and medical research, there is a gap between two-dimensional and most often transformed cell cultures and three-dimensional, highly organized tissues. In recent years, stem cell research has provided the means to fill this gap. The identification of the niche factors that support growth, expansion and differentiation of stem cells in vitro has allowed the development of three-dimensional culture systems called organoids. Gastric organoids are grown from gastric stem cells and are organized epithelial structures that comprise all the differentiated cell types of the stomach. They can be expanded without apparent limitation and are amenable to a wide range of standard laboratory techniques. Here, we review different stem cell-derived organoid model systems useful for *Helicobacter pylori* research and outline their advantages for infection studies.

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1 Introduction

1.1 Current Models for H. pylori *Research*

In 1983, Barry Marshall and Robin Warren described the staining, isolation and culture of curved bacilli from the stomach, now known as *Helicobacter pylori* (Warren and Marshall 1983). Their causative role in disease was highly questioned at the time. To demonstrate that this bacterium causes gastritis, they tried to infect healthy animals, such as pigs, rats and mice, but initially failed. So to fulfill Koch's postulates, Marshall himself drank a suspension of 10^9 colony-forming units of the bacteria, previously isolated from a gastritis patient (Marshall et al. 1985). He fell ill after about 1 week, with "feeling of fullness," vomiting and headache. Endoscopy confirmed the colonization of his stomach as well as mononuclear infiltrates (Marshall et al. 1985). This self-experiment did not only prove that *H. pylori* indeed causes acute gastritis, but also demonstrated the need for a study subject that reflects the natural host for infection research. Since then, several models have been established and we now have a variety of tools to analyze pathogenicity mechanisms.

Perhaps the most commonly used tools in *H. pylori* research are gastric epithelial cell lines that were originally derived from gastric tumors. They have advantages, since they are immortalized, cost-effective, accessible, easily maintained and a wide variety of experiments are well established. However, many molecular characteristics are modified in cancer cell lines, including genetic and epigenetic changes in tumor suppressor genes, oncogenes, cell cycle regulator genes, growth factors and their receptors. Additionally, cell lines have accumulated mutations during decades of culture in vitro and some are infected with viruses. The AGS cell line, for example, the workhorse of *Helicobacter* research, is commonly infected with parainfluenza virus type 5, influencing central pathways involved in immunity, proliferation and oncogenesis, such as signal transducer and activator of transcription (STAT) signaling as well as the interferon response (Young et al. 2007).

Primary cell culture models are generally a very good alternative to tumor cell lines, as they are not transformed. Culture models of human antral epithelial cells and normal gastric epithelial cells from biopsies have been developed (Boxberger et al. 1997; Richter-Dahlfors et al. 1998; Ootani et al. 2000), and some of these were already used to study bacterial infection (Richter-Dahlfors et al. 1998). While these studies laid important groundwork, the cells cannot be expanded and cultured long term as they lack the ability to renew themselves. Thus, a need to generate freshly isolated primary cells for each experiment makes this model less practical than others.

Conventional primary and tumor cell culture are two-dimensional (2D) and lack higher 3D organization. There have been efforts to grow cancer cell lines in gels composed of extracellular matrix proteins to support 3D organization. Indeed, in these cultures cancer cell lines can form 3D spheres and studies have shown that whether a cancer cell line was grown in 2 or 3D can influence the efficiency of applied drugs (Tung et al. 2011). This indicates that the 3D organization is an important feature of tissue reactions. The 3D tumor cell spheres, however, lack the combination of different cell types that are typical for tissue and organs. In the end, the stomach is—just as any other organ—much more complex than a monolayer of a single type of cells.

To analyze the impact of infection on any whole organ, such as the stomach, research currently has to turn to whole organisms. Many central descriptive studies, such as Warrens initial staining, are performed on human fixed tissue. This gives invaluable information allowing correlations to be made between human disease, marker expression and infection status. Experimental infection of humans has also been used for vaccine studies (Aebischer et al. 2008). Animal models include mice and gerbils, as well as pigs with a defined microbiota ("gnotobiotic" pigs) and even cats and dogs. All of them have their advantages and limitations. Because there are excellent recent reviews on animal models for *H. pylori* research (Lee 1998; Noto et al. 2016; Solnick et al. 2016), we will not discuss this here. Despite their usefulness, there is room between 2D monotypic cancer cell cultures and full organisms for new models that ideally combine the practicability, technical and ethical advantages of cancer cell lines and yet are closer to the human in vivo situation.

In the past decade, advances in stem cell research have allowed the development of new 3D stem cell-derived primary cell models called "organoids" for their striking resemblance to the in vivo counterpart tissue. In this review, we aim to provide an overview of gastric organoids for *H. pylori* infection. For this, we will introduce the development of organoids and their general advantages before discussing in detail the different types of gastric organoids, and their usefulness for *H. pylori* research.

2 Understanding of Adult Stem Cells and Their Niche Led to the Development of Organoid Culture

2.1 The Gastric Epithelium

Anatomically, the stomach is divided into three different regions: the proximal cardia (human) or forestomach (mouse) followed by the corpus (also termed fundus or body), which is the largest part of the stomach. Distally, the antrum leads to the pyloric sphincter that controls the entrance into the intestinal duodenum. Only the murine forestomach is lined by a keratinized squamous epithelium—all other parts of murine and human stomach are lined by a simple columnar epithelium that is organized into many regular, flask-like invaginations called gastric pits. Each pit feeds into a gastric unit, which is divided into the regions isthmus, neck and base. Neck and base together are named gland (Fig. 1a). The epithelium constantly renews itself and parts of it turn over every three days. Thus, the stomach epithelium harbors a tremendous regenerative capacity and the stem cells that fuel this constant need for new cells have long been thought to reside in the tissue itself (Lee et al. 1982).

The isthmus is a site of massive proliferation from where the cells migrate toward either the pit or the gland. Pit cells are characterized by dense apical mucus and a short life span of about 3 days (Karam and Leblond 1993b). Cells migrating to the base of the gland differentiate into either mucus neck cells or pepsinogen-secreting chief cells, which can have life spans of up to 6 months

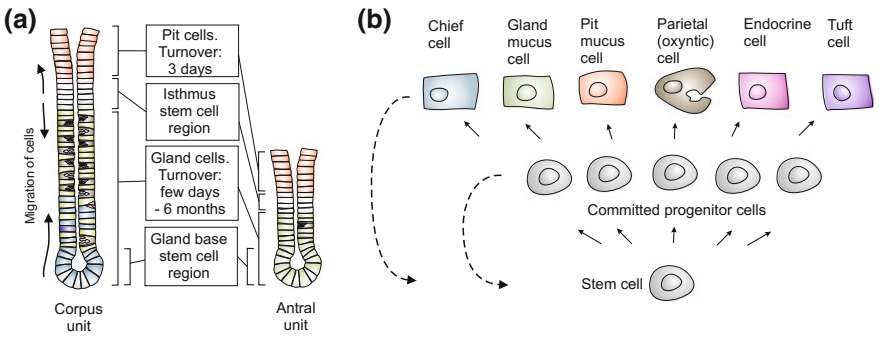

Fig. 1 Organization of gastric glands. *Left side*: Compared to antral units, corpus units are longer and characterized by high abundance of acid-producing parietal cells (*brown*). Each gastric unit is divided into the regions pit, isthmus and gland. The gland is further divided into the regions neck and base. Stem cells reside in the isthmus and at the base. The pit has very high turnover compared to the gland, where individual cells can have life spans of several months. The isthmus is the site of highest proliferation. *Right side*: Gastric glands harbor four main cell types: chief cells, gland mucus cells, pit mucus cells and parietal cells. Additional rare cell types are endocrine cells and tuft cells. Stem cells proliferate to give rise to generate committed progenitor cells, which can further proliferate and differentiate into the cell types of the stomach. Differentiated cells (i.e., chief cells) can gain stem cell capacity, indicating high levels of plasticity (*dashed arrow*)

(Karam and Leblond 1993a). Acid-secreting parietal cells migrate bidirectionally and are found in all regions of the gastric glands with highest numbers in the neck region (Karam 1993). The rare enteroendocrine cells, which account for <2% of the epithelium, are interspersed throughout the glands and secrete regulatory hormones such as gastrin, ghrelin, somatostatin and serotonin. The cellular composition of a gland defines its function, and glands in the corpus region are specialized to secrete acid and pepsinogen, while glands in the antrum are mainly mucus-producing. Mouse and human gastric glands have similar but not identical cellular compositions. Specifically, human antral glands are reported to contain higher numbers of chief and parietal cells than murine antral glands, which are virtually devoid of chief cells and contain only parietal cells when located at the intermediate zone toward the corpus (Lee et al. 1982; Lee and Leblond 1985a; Choi et al. 2014)

2.2 Gastric Stem Cells

It was long hypothesized that several tissues including stomach and intestine harbor resident adult tissue stem cells that constantly regenerate the organ during homeostasis (Fig. 1b). Early studies identified undifferentiated cells in the isthmus region of the glands (Lee and Leblond 1985b). The development of genetic lineage tracing empowered scientists to map the fate of the progeny from specific cells (reviewed in Kretzschmar and Watt 2012). Genetic marking of cells expressing the leucine-rich-repeat-containing G-protein-coupled receptor 5 (*Lgr5*) gene, which was originally described as a WNT response gene, showed that *Lgr5* identified very peculiar, slender cells wedged between the Paneth cells in the intestine, as well as cells at the base of gastric glands. Lineage tracing using *Lgr5-Egfp-IRES-CreERT2* mice, in which administration of tamoxifen induces irreversible genetic labeling in all *Lgr5*-expressing cells and their offspring, revealed that they can generate all cell lineages of the intestine as well as the stomach. Lineage tracing further showed that cells expressing the actin-binding protein villin (*Vil1*) (Qiao et al. 2007), sex-determining region Y (SRY)-box 2 (*Sox9*) (Arnold et al. 2011), gastrin receptor cholecystokinin B receptor (*Cckbr*) (Hayakawa et al. 2015b), transcription factor basic helix-loop-helix family member a15 (*Bhlha15* or *Mist1*) (Hayakawa et al. 2015a), or tumor necrosis factor receptor super family 19 (*Tnfrsf19*), encoding the protein TROY (Stange et al. 2013), have stem cell capacity in the stomach. Currently, the (possibly hierarchical) relations between the different stem cell populations are unclear (reviewed in Bartfeld and Koo 2016).

Homeostasis and proliferation of tissue-resident stem cells is maintained by the stem cell niche. Niche factors for gastric stem cells include Wnt family members (WNT), Notch, epidermal growth factor (EGF), fibroblast growth factor 10 (FGF-10) and gastrin (GAST) as well as an absence of bone morphogenetic protein (BMP) (Goodlad et al. 1987; Wang et al. 1996; Nyeng et al. 2007; Bredemeyer et al. 2009; Barker et al. 2010; Radulescu et al. 2013; Demitrack et al. 2015; reviewed in Bartfeld and Koo, 2016).

2.3 Organoids: Self-organizing Three-Dimensional Systems of Stem Cells and Differentiated Cells

The identification of the *Lgr5*$^+$ stem cells and their niche factors laid the groundwork for their maintenance in culture. In 2009, Toshiro Sato and colleagues in the laboratory of Hans Clevers published a landmark study, in which single sorted murine intestinal *Lgr5*$^+$ stem cells were seeded into an extracellular matrix and embedded in a medium containing the essential niche factors R-spondin1 (RSPO, a WNT agonist), EGF and noggin (NOG, a BMP antagonist). These four components (matrix, RSPO, EGF and NOG) allowed the stem cells to proliferate and expand. Initially, the cells form small cysts, but after few days, buds appear and grow into the matrix. The cells become polarized, with the apical side facing the lumen of the cystic structure. Individual cells differentiate into the specific cell types of the intestine, and a single organoid contains stem cells, progenitor cells, Paneth cells, enterocytes, enteroendocrine cells and goblet cells. Moreover, the cells even self-organize into domains that resemble the intestinal crypts, harboring mostly stem cells interspersed between Paneth cells and progenitor cells, and villus structures harboring enterocytes, goblet cells and enteroendocrine cells (Sato et al. 2009). Probably, the most important feature that makes these organoids so attractive for the laboratory is that they are long-lived: They can be split and re-seeded, frozen and thawed, and appear to have unlimited expansion capacity. Further, they are amenable to a wide range of standard laboratory techniques, including microscopy, RNA analysis, protein analysis, genetic manipulation using lentiviruses, bacterial artificial chromosomes (BACs) or genome editing based on clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) (CRISPR/Cas9) (Koo et al. 2012; Li et al. 2012; Schwank et al. 2013a; Andersson-Rolf et al. 2014; Bartfeld et al. 2015; Drost et al. 2015). Organoids can be grown from different vertebrates and different tissues, such as small intestine, colon, stomach, liver, pancreas, prostate, fallopian tube, gallbladder and others (Sato et al. 2009; Barker et al. 2010; Huch et al. 2013; Karthaus et al. 2014; Boj et al. 2015; Kessler et al. 2015; Scanu et al. 2015). Since the development of conditions for the growth of human organoids (Sato et al. 2011), it became clear that organoids can be grown from apparently every patient, from biopsies as well as from resection material (VanDussen et al. 2015; Fujii et al. 2016). Established living biobanks of intestinal organoids have also enabled drug testing (Van de Wetering et al. 2015). After the establishment of an organoid line, it can be used in a similar way to standard cell lines, with the important difference that organoids are untransformed, primary cells that faithfully mimic the epithelial organization in 3D. For further excellent general overviews about organoid cultures, we would like to direct the reader to other reviews (Sato and Clevers 2013; Lancaster and Knoblich 2014; Huch and Koo 2015; Fatehullah et al. 2016; Werner et al. 2016; Dedhia et al. 2016). Here, we will focus on gastric organoids and their use as an infection model for *H. pylori*.

3 Gastric Organoids

3.1 Organoids Grown from Adult or Pluripotent Stem Cells

Gastric organoids have been generated from tissue-resident adult stem cells (ASCs) (Barker et al. 2010) as well as from two types of pluripotent stem cells (PSCs): induced PSC (iPSC) and embryonic stem cells (ESCs) (McCracken et al. 2014) (Fig. 2). A distinctive difference between ASC- and PSC-derived organoids is the presence of mesenchymal cells. Initial seeding of ASC-derived organoids as well as PSC-derived organoids contains epithelial as well as mesenchymal cells. During further expansion by splitting and re-seeding, ASC-derived organoids become purely epithelial. Thus, this model allows the initial observation of mesenchymal–epithelial interactions and long-term study of pure epithelium. PSC-derived organoids are usually limited in their expansion and remain a combination of epithelium and mesenchyme.

The main technical differences between the systems regard the source and the handling: On the one hand, ASC-derived organoids are generated directly from tissue and mature within 1–2 weeks (depending on the tissue type). Once established, it appears that they can be expanded for an unlimited period of time. Lines can also be shared between laboratories, so for research on organoids expertise in their establishment is not required. On the other hand, PSC-derived organoids are generated from ESCs or iPSCs and each experiment requires their differentiation

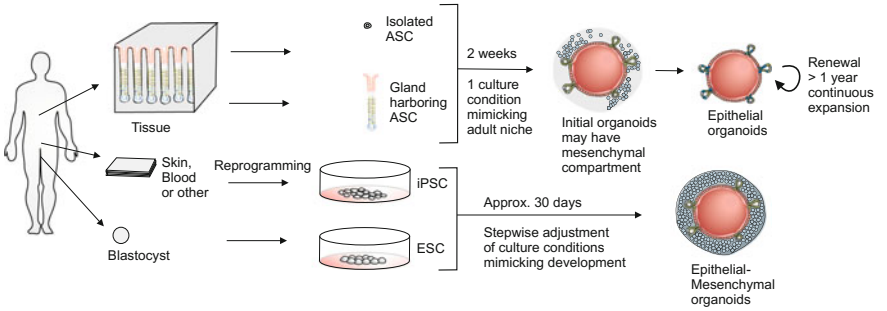

Fig. 2 Generation of organoids. Organoids can be generated either from tissue-resident adult stem cells (ASCs) or from pluripotent stem cells (PSCs). PSCs can be obtained either from blastocysts (embryonic stem cells, ESCs) or by reprogramming of differentiated cells from skin, blood or other tissues (induced PSCs, iPSCs). To generate organoids from ASCs, single ASCs can be isolated, for example based on marker gene expression. Alternatively, whole glands can be isolated from the tissue. Placed in optimal culture conditions, isolated glands round up, stem cells proliferate, and daughter cells differentiate to generate 3D organoids that harbor stem cells next to differentiated cells. After seeding, the culture may contain a mesenchymal compartment. By expansion (simple splitting and re-seeding), pure epithelial cultures can be maintained long term. To generate organoids from PSCs, lines of iPSCs or ESCs are cultured under stepwise changes of conditions to mimic the exposure to different developmental cues during organ development. Duration and steps vary between protocols. Resulting organoids have epithelial and mesenchymal compartments

from PSCs toward the (adult) epithelium, which usually takes about 30–60 days and a fine-tuned sequence of growth factors and inhibitors to lead the cells through the different stages of embryonic development and tissue-specific patterning. PSC-derived organoids have a limited life span; thus, each experiment starts with pluripotent cells and the purity of gastric cells (and the presence of non-wanted phenotypes) in the final culture needs to be monitored (Fig. 2) (reviewed in Wells and Spence 2014).

3.2 *Long-Term Self-renewing Purely Epithelial Organoids*

The first gastric organoid system to be developed was a direct advancement of the above-mentioned original intestinal culture system from the Clevers group (Sato et al. 2009). Meritxell Huch isolated murine antral gastric glands, embedded them into extracellular matrix and identified WNT, FGF-10 and GAST as stomach-specific niche factors that had to be added to the culture in addition to the intestinal factors RSPO, EGF and NOG to allow growth of the glands into organoids (Barker et al. 2010). Murine antral organoids grow into more cystic structures than their intestinal counterparts, but also have small buds that harbor the *Lgr5*$^{+}$ stem cells. Cells in the organoids differentiate into mucin 6 (MUC6)+ neck cells, MUC5AC+ pit cells, pepsinogen C (PGC)+ chief cells and enteroendocrine cells. After seeding, they expand within about 1 week to full size and can be split at a ratio of 1:5 every week, and cultures can be expanded long term (9 months at initial publication) (Barker et al. 2010). The same conditions also allow culture of organoids from the murine corpus (Stange et al. 2013; Schumacher et al. 2015a) and from isolated single *Tnfrsf19*/TROY+ chief cells (Stange et al. 2013). Corpus organoids also harbor neck, pit, chief and enteroendocrine cells (Stange et al. 2013). In addition, parietal cells are present in the initial seeding, but numbers decrease over time (Schumacher et al. 2015a) (Fig. 2).

To enable research on human stem cells, the culture system was then adapted for human intestine, which in addition to WNT, EGF, NOG, and RSPO requires inhibition of transforming growth factor beta (TGF-β) signaling, inhibition of p38 signaling as well as addition of nicotinamide to inhibit sirtuin activity. A Rho-kinase (ROCK) inhibitor is also added after seeding to inhibit anoikis (Sato et al. 2011). The conditions used for growing mouse gastric organoids plus the addition of the nicotinamide allow initial growth of human organoids (Bertaux-Skeirik et al. 2015) but not the long-term expansion (Bartfeld et al. 2015). The culture conditions used for growing human intestinal organoids also support growth of gastric glands into spheres (Schlaermann et al. 2016). The minimal and optimized culture for human gastric stem cells (including only EGF, NOG, RSPO, WNT, FGF10, GAST and TGF-β inhibition) allows the growth of gastric glands or single cells into complex organoids with a cystic body comprised mostly of pit cells and glandular buddings harboring neck cells, chief cells and rare enteroendocrine cells (Bartfeld et al. 2015). By manipulating the culture conditions, researchers

were able to differentiate organoids into three different types distinguished by differences in the mucus-producing cells. The first one consists of all the four gastric lineages, termed "complete type," the second has only the gland domain and is termed "gland type," and the third contains mainly pit cells and hence is termed "pit type." Although these cultures lack parietal cells, they contain the other four different cell types and can be maintained for more than a year without loss of expansion or differentiation capacity (Bartfeld et al. 2015).

ASC-derived organoids are probably the most practical, because they have the ability to self-renew and thus can be cultured long term (Fig. 3, right column). After establishment, they have a short maturation time of only one (for mouse) or two (for human) weeks. An interesting feature of this system is further that it allows the expansion of healthy tissue stem cells, as well as cells from metaplasia, such as Barrett's esophagus (Sato et al. 2011), or gastric cancer cells (Bartfeld et al. 2015). Thus, both cancer and healthy organoids can be established from the same patient, rendering this an ideal model for drug testing (Bartfeld et al. 2015; Van de Wetering et al. 2015). While establishment of cell lines or patient-derived xenograft models is only successful from a comparatively small number of patients, organoids can be grown from apparently every patient. Thus, uniquely, organoids allow the establishment of living biobanks and the genotypes in the biobanks cover the range known from large-scale genetic screening of patients, indicating that the generation

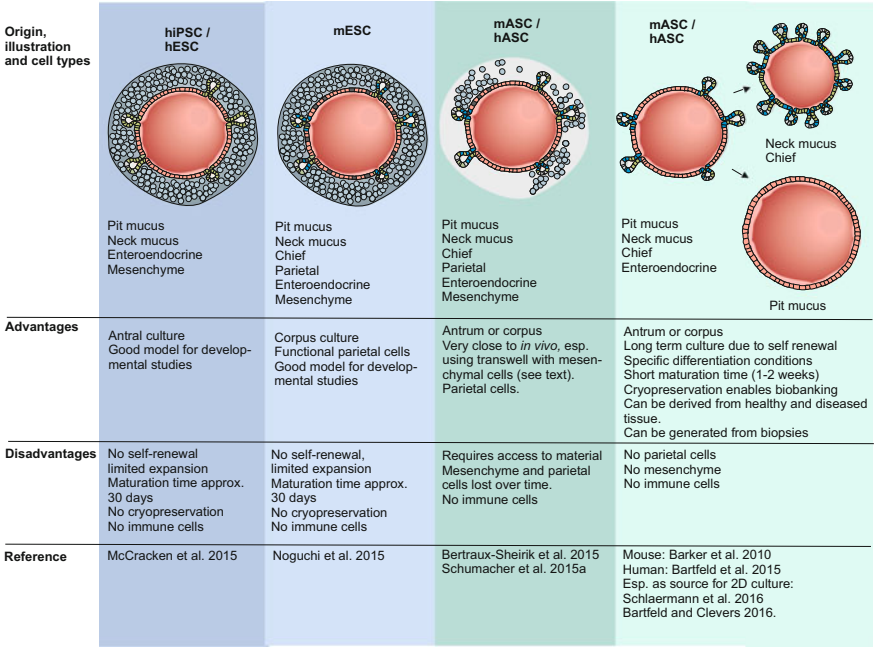

Fig. 3 Overview of different gastric organoid systems, their cellular composition, advantages and disadvantages. See text for more details on each system

of organoids is non-selective and thus represents the entire spectrum of patient genotypes (Van de Wetering et al. 2015; VanDussen et al. 2015; Fujii et al. 2016). This is a unique feature of organoids and enables research approaches targeting patient specificity: (i) experimental analysis of a specific parameter in a wide range of patients to identify patient-specific risk factors for disease development and (ii) the use of a specific subset of organoids, for example based on the genotype, to determine a response, for example to test the efficiency of a drug for a subset of patients. While all other methods (discussed below) also allow establishment of organoids from individual patients, this specific system is the only one that can be expanded long term and allows the generation of frozen stocks that can be stored in a living biobank to be thawed and expanded again for later studies.

Because of their purely epithelial nature, ASC-derived organoids allow the study of isolated epithelium without potentially confounding factors from mesenchyme or the immune system. Because the growth conditions maintain stem cells and progenitors, these organoids also allow the study of a possible direct effect of infection on stem cells. This may also have high impact for the clinic, as cancer can originate from stem cells (reviewed in Mills and Sansom 2015).

3.3 Epithelial/Mesenchymal Organoids Derived from Adult Stem Cells

In the initial seeding, ASC-derived gastric organoids cultured by the above-described or similar, adapted methods (Schumacher et al. 2015a; Bertaux-Skeirik et al. 2015) may also contain a mesenchymal layer (Feng et al. 2014). Interestingly, this coincides with the presence of parietal cells in initial culture of corpus organoids (Schumacher et al. 2015a), indicating that the development of parietal cells may depend on interactions with the mesenchyme, a hypothesis that is supported by ESC-derived organoids (Noguchi et al. 2015). In ASC-derived organoids, markers of mesenchymal cells and parietal cells decline with time and especially after passage (Feng et al. 2014; Schumacher et al. 2015a) (Fig. 2). Addition of immortalized stomach mesenchymal cells in the lower compartment of a transwell, with the matrix-supported organoids in the upper compartment, increased expression of differentiation markers including expression of the H^+/K^+ATPase of parietal cells (Schumacher et al. 2015a). Thus, short-term culture of mouse or human ASC-derived gastric organoids allows the study of mesenchymal–epithelial interactions and also contains parietal cells. These features are, however, not supported by long-term expansion of the cultures.

In parallel developments, Ootani and colleagues established a system for primary cell cultures that contain epithelial as well as mesenchymal cells. Their early work had shown that gastric primary cells build short-lived but well-differentiated monolayers of columnar MUC5AC+ pit cells when embedded in a collagen matrix

and exposed to an air–liquid interface (Ootani et al. 2003). In 2009, they published an intestinal culture system, in which neonatal tissue from murine intestine was seeded into collagen matrix. The tissue pieces built spheres that consisted of an inner polarized layer of epithelial cells surrounded by mesenchymal myofibroblasts. Supported by additional RSPO, the intestinal air–liquid interface cultures survived for one year with high levels of proliferation in the first month, which later decreased. Adult tissue-derived cultures clearly had shorter in vitro viability (Ootani et al. 2009). Murine neonatal gastric tissue also formed 3D epithelial/mesenchymal structures in the collagen-based air–liquid interface system. They could be maintained for an average of one month with a maximum of 80 days and mostly differentiated into MUC5AC+ pit cells and to a lesser extent into MUC6-positive neck cells and few parietal cells (Katano et al. 2013; Li et al. 2014). When these cultures were generated from mice harboring mutations predisposing for cancer development [such as in the genes Kirsten rat sarcoma viral oncogene homolog (*Kras*) or tumor protein p53 (*p53*)], gastric cultures could be passaged and expanded at least six passages (Li et al. 2014).

This organoid system is extremely close to the in vivo situation (Fig. 3, third column). Because it maintains parietal cells, the acidic environment of the stomach can be mimicked and the effect of the infection on parietal cells can also be studied. This has high clinical impact, because loss of parietal cells and accompanied antralization of the tissue is a pathologic feature of chronic *H. pylori* infection in patients. This system, however, requires access to human material, as it has a limited life span.

3.4 Epithelial/Mesenchymal Organoids Derived from PSC

Understanding and in vitro recapitulation of the adult stem cell niche provided the last building block for the generation of human gastrointestinal cells from PSCs. Contrary to ASCs, which are programmed to generate all cells of one particular tissue (such as the stomach), PSCs can become all cells of any tissue (such as stomach, nervous system or heart muscle) (Fig. 2). In two studies, McCracken and colleagues described the precise choreography of stepwise differentiation, mimicking the stages of embryonic development necessary to differentiate human PSCs into intestinal (McCracken et al. 2011) or gastric cells (McCracken et al. 2014). Generally, in this type of experiment, the stepwise differentiation is indicated by markers characteristic of specific developmental stages. For gastric development, human ESCs or iPSCs were first differentiated into definitive endoderm by adding activin A and BMP4. Next, the addition of WNT3a and FGF4 induced caudal-type homeobox 2 (*Cdx2*) expression, a marker for mid-hindgut. Simultaneous inhibition of BMP signaling by NOG resulted in the generation of foregut, as indicated by expression of the foregut endoderm marker sex-determining region Y (SRY)-box 2 (*Sox2*). Transferring these cultures to 3D extracellular matrix and treating them with EGF and retinoic acid (RA) induced antral development, characterized by

expression of pancreatic and duodenal homeobox 1 (*Pdx1*) and *Sox2*. After 34 days of stepwise differentiation, human gastric organoids expressed markers *Muc5AC*, *Muc6* of mucus pit and neck lineages as well as markers for several types of enteroendocrine lineages. Numbers of enteroendocrine cells were increased at low levels of EGF, indicating a previously unanticipated role for EGF in enteroendocrine cell development (McCracken et al. 2014).

Using a similar stepwise path through the developmental stages of the stomach, Noguchi and colleagues generated corpus organoids from mouse ESCs (Noguchi et al. 2015). In the first step, they cultured ESCs in suspension. In this condition, they grow into 3D aggregates called embryoid bodies, which consist of cells from all three germ layers. Transferred to adherent culture conditions and exposed to sonic hedgehog (SHH) and the Wnt antagonist Dickkopf 1 (DKK1), the embryoid bodies formed primordium spheroids expressing *Sox2* and the mesoderm marker BarH-like homeobox 1 (*Barx1*). The spheroids were embedded in extracellular matrix and supplemented with FGF10, WNT, NOG and RSPO. After a total of 60 days of differentiation, this led to formation of corpus organoids containing not only pit cells, neck cells and enteroendocrine cells, but also fully mature chief cells and functional parietal cells. Chief cells were able to secrete pepsinogen and parietal cells secreted acid upon stimulation with histamine (Noguchi et al. 2015).

Organoids derived from PSCs have been extremely useful for the study of developmental processes (Fig. 3, two left columns). They also provide the possibility to study the impact of infections on functional parietal cells, with high medical importance as outlined above. Demonstration of parietal cell development from human PSCs will be an important development in the future.

3.5 2D Monolayers Derived from Epithelial Organoids

While 3D cultures have their clear advantages, long-term organoid cultures from adult stem cells can also be used as a source for primary cells seeded in conventional 2D settings, either adherent on plastic, grown on a layer of extracellular matrix, or supported by a transwell (VanDussen et al. 2015; Bartfeld and Clevers 2015; Schlaermann et al. 2016). The ease and simplicity by which cells of various origins can be cultured long term makes it a practical alternative for primary cultures directly from tissue, freeing the researcher from the dependence on surgical specimens.

In summary, gastric organoids represent a useful bridge between traditional cell culture and in vivo models. Given the diversity of organoids grown from human and mouse tissue in vitro, it will be important to consider the experimental strengths and limitations of each system when designing experiments. For example, ASC-derived stomach organoids can be generated in less than two weeks, whereas PSC-derived stomach organoids require more than five weeks. The fine-tuned steps undertaken for the development of PSCs into gastric organoids may pose an initial barrier for inexperienced researchers. On the other hand, PSC-derived organoids

will be the best source to answer developmental questions. Interactions with the mesenchyme or parietal cells can best be studied in unpassaged ASC- or PSC-derived cultures.

4 The Use of Stem Cell-Derived Organoids for *H. pylori* Research

Organoids hold great promise as new tools for infection research. Future studies will show whether they can recapitulate phenotypes that are only present in 3D, deliver relevant results for infection-induced carcinogenesis that were impossible to obtain in mutated cancer cell lines or identify patient-specific responses and risk factors for disease development. However, one of the many exciting possibilities of organoids that have already been published is to study cell types that could previously not be cultured, for example mucus gland cells or parietal cells.

When Marshall infected himself with *H. pylori*, he developed acute gastritis characterized by polymorphonuclear neutrophil leukocyte infiltrates (Marshall et al. 1985). Today, it is known that inflammation is driven by the potent neutrophil attractor interleukin 8 (IL-8), which is a target gene of the central pathway of innate immunity, nuclear factor kappa B (NF-κB) (reviewed in Shimoyama and Crabtree 1998). Despite its importance, it is still not well understood how *H. pylori* activates NF-κB. In human gastric cancer cell lines, NF-κB activation depends on the cytotoxicity-associated gene pathogenicity island (*cag*PAI) of the bacterium, but not on the gene *cagA* (Bartfeld et al. 2010; Sokolova et al. 2013). In human gastric organoids, *H. pylori* also strongly induces NF-κB and expression of its target gene *IL-8*. However, contrary to what has been shown in cancer cell lines, *IL-8* expression does not depend on the presence of a functional *cag*PAI in *H. pylori* (Bartfeld et al. 2015). While the bacterial factor inducing NF-κB remains elusive (Backert and Naumann 2010), the study indicated an influence of the differentiation of the host cells: Organoids that contain mainly cell lineages of the gland produce higher levels of IL-8 than organoids that contain mainly cell lineages of the pit, leaving room for speculation whether this may be due to expression of a putative receptor or a possible barrier function of the pit mucus (Bartfeld et al. 2015). This demonstrates the unique potential of organoids: No in vitro system before has allowed the parallel infection of pit or gland cells. Similarly, another study used the unique possibility of organoids to study the response of previously non-culturable parietal cells (Schumacher et al. 2015b). A central pathologic feature of *H. pylori*-induced chronic gastritis is the loss of parietal cells, which leads to normal chief cell differentiation and antralization of corpus tissue (reviewed in Mills and Sansom 2015). Parietal cells are the main providers of SHH (Van Den Brink et al. 2001), which induces BMP in the mesenchyme and is necessary for differentiation (Shyer et al. 2015). Schumacher and colleagues examined *Shh* expression in gastric organoids in response to *H. pylori* infection. Importantly, these ASC-derived

organoids were used without passaging; thus, they contained parietal cells. Two days after infection, *H. pylori* induced *Shh* in organoids and the induction was abrogated by pretreatment with NF-κB inhibitor IV, a form of resveratrol (Schumacher et al. 2015b). It will be most interesting to follow up on the interplay between *H. pylori*, NF-κB, *Shh* induction and parietal cell survival in this unique model.

While the new possibilities of organoids are promising, it is also necessary to sound out which previously known hallmarks of infection are recapitulated in the new model. So how well can *H. pylori* infect gastric organoids? Several studies laid the groundwork and addressed colonization, adherence to epithelial cells, bacterial replication, CagA translocation and induction of host cell responses such as apoptosis and proliferative response (Posselt et al. 2013; Backert et al. 2015).

In the human stomach, the majority of *H. pylori* is found motile in the mucus layer and only a part of the bacterial population (between 0 and 41%) is found adherent to the epithelial cells (Hessey et al. 1990). Adherence of *H. pylori* to epithelial cells contributes to transient or persistent colonization of the host gastric mucosa and to efficient delivery of virulence factors such as CagA (Blaser et al. 1995; Blaser and Kirschner 1999). Naturally, the bacterium first interacts with the apical side of the epithelium. As the apical side normally faces the lumen of the organoids [although exceptions have been reported (Huang et al. 2015)], bacteria are commonly microinjected into the luminal space. Here, bacteria face a thick mucus barrier shielding the epithelial cells. Cancer cell lines that were classically used for *H. pylori* research, such as AGS cells, lack this mucus expression—thus, this is one of the factors that render organoids much closer to the in vivo situation, especially because the bacteria establish their replicative niche in this mucus layer in vivo (Schreiber et al. 2004). Indeed, studies in organoids demonstrated localization of bacteria close to or within the mucus layer after injection (Wroblewski et al. 2014; Bartfeld et al. 2015; Schumacher et al. 2015b; Bertaux-Skeirik et al. 2015). Bacteria are viable inside the organoids, as shown through re-culturing of previously injected bacteria (Bartfeld et al. 2015; Bertaux-Skeirik et al. 2015), and can also replicate there (Bertaux-Skeirik et al. 2015). In vivo, *H. pylori* uses taxis toward urea and away from gastric acid to reach the mucus (Schreiber et al. 2004; Huang et al. 2015) and this chemotaxis may in part also be functional in organoids, as they secrete urea, and this indeed attracts *H. pylori* (Huang et al. 2015). The contribution of a possible pH gradient to chemotaxis in organoids is currently unclear: There are some systems that have parietal cells and therefore should generally be able to establish a pH gradient (see above), but this has not been demonstrated. It will be interesting in the future to study a possible effect of a pH gradient on *H. pylori* localization and replication.

In contrast to classical cancer cell culture studies, in which often 100 bacteria are added per host cell, many of which also attach due to the lack of a physical barrier, in organoids attachment to epithelial cells is a comparatively rare event, but it can be observed with electron microscopy (Bartfeld et al. 2015). This opens up the possibility to study attachment, its prerequisites and local consequences under more physiological conditions, which is of medical interest because attachment rates of

bacteria in human gastritis patients vary with disease state: High attachment rates coincide with high pathology (Hessey et al. 1990).

After attachment, *H. pylori* translocates at least one bacterial protein into the host cell, CagA (Covacci et al. 1993; Backert et al. 2000). There, CagA is phosphorylated by Src (Selbach et al. 2002) and Abl family kinases (Mueller et al. 2012). While CagA phosphorylation in organoids remains to be shown, it can be detected in monolayers of cells derived from human organoids (Schlaermann et al. 2016), indicating that *H. pylori* is generally able to translocate CagA into cells derived from organoids. Bacterial CagA interacts with the tyrosine kinase receptor c-MET, leading to increased cell proliferation and morphological changes such as cell elongation. The interaction depends on the type IV secretion system (T4SS), encoded by the *cag*PAI, suggesting that c-MET interacts only with translocated CagA (Churin et al. 2003). In human gastric organoids, this interaction can also be observed, giving an indication that CagA may also be efficiently translocated into primary cells (McCracken et al. 2014; Bertaux-Skeirik et al. 2015). Also, CagA-dependent doubling in proliferation has been observed, based on quantification of newly incorporated labeled nucleoside analogs (5-ethynyl-2'-deoxyuridine, EdU) in organoids (Wroblewski et al. 2014; McCracken et al. 2014; Bertaux-Skeirik et al. 2015). This may be due to c-MET activation and not compensation for *H. pylori*-induced cell loss in the organoids, as levels of apoptosis were unchanged (Wroblewski et al. 2014). In infected organoids, c-MET co-immunoprecipitated with cell surface antigen cluster of differentiation 44 (CD44) and hepatocyte growth factor (HGF) and this interaction depended on CagA. Proliferation was dependent on this interaction, as organoids derived from a CD44-deficient knockout mouse model showed neither complex formation nor proliferation (Bertaux-Skeirik et al. 2015).

In summary, many characteristic hallmarks of *H. pylori* infection such as attachment, colonization, NF-κB activation, CagA-c-MET interaction and increased host cell proliferation have been recapitulated in organoids, indicating that they are a useful new model for *H. pylori* research. Further, several studies highlighted previously unknown aspects of host response to infection, using the unique features of organoids, for example the effects of host cell differentiation on the innate immune response.

5 Concluding Remarks

In addition to their invaluable utility for better understanding development, organoids open up new avenues for a wide range of applications. They have been used to model cancer development using stepwise addition of cancer mutations using CRISPR/Cas9 (Matano et al. 2015; Drost et al. 2015) or knockout mice (Li et al. 2014). CRISPR/Cas9 has also been used to repair a genetic mutation in the CFTR gene in organoids generated from cystic fibrosis patients (Schwank et al. 2013b). Organoids have been transplanted into the mouse colon (Yui et al. 2012). Biobanks

of colon cancer organoids (Van de Wetering et al. 2015; Fujii et al. 2016) represent the range of mutations known from previous genetic analysis and can be used for drug screening (Van de Wetering et al. 2015). Human gastric organoids can also be grown from tumors (Bartfeld et al. 2015), and it will be interesting to see whether this technique can be expanded to tissue from gastric precancerous lesions in the future.

For infection biology, and especially for *H. pylori* research, organoids promise not only a unique resource for primary cells, but also the potential to study the different cell lineages in the stomach in 3D organization. As the presence of parietal cells seems to coincide with the presence of mesenchymal cells, the interplay between infection, mesenchyme and parietal cells may unravel functional insights into the early steps of metaplastic changes in gastric tissue that are characterized by depletion of parietal cells. Biobanks of organoids may identify interindividual factors that play a role in infection and could have a high clinical relevance as putative risk factors for cancer development.

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