Chapter 5

Gastric Cancer: A Stem Cell Disease?

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Additional information is available at the end of the chapter

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Abstract

Gastric stem cells have been recently identified and are not yet fully characterized. Each gastric gland or unit is composed of different specialized cells and a small number of discrete stem cells. These gastric stem cells play key roles. They have self-renewal and multipotent properties and are the origin of specialized gastric epithelial cells. These properties are the basis for the stem cells' role in tissue homeostasis, tissue repair, and cancer. In tumors, growing evidence indicates that a cell subpopulation with stem cell features, the so-called cancer stem cells (CSCs), represents the "fuel" for the tumor: they are at the origin of tumor initiation, growth, and dissemination, and they also display resistance to conventional chemotherapy treatments. The recent identification of CSCs in gastric carcinoma opens the door to the development of new therapeutic strategies targeting more specifically the CSCs at the origin of the disease, which is the third leading cause of cancer-related deaths worldwide.

Keywords: stem cells, gastric cancer stem cells, CD44, stomach cancer, *Helicobacter pylori*, chemoresistance, ALDH, markers

1. Introduction

Gastric cancer is the third leading cause of cancer-related deaths and the fifth most frequent cancer worldwide. The cancer in its nonmetastatic form is essentially treated by surgery associated with conventional chemotherapy or by chemotherapy alone when metastatic. Its poor prognosis, with less than 10% survival, is due to frequent relapses in metastatic forms even after multimodal therapy. This relapse is associated with the persistence of a cell subpopulation that has acquired or possesses intrinsic mechanisms to resist chemotherapeutic drugs. Indeed, gastric carcinoma, as other solid tumors, is heterogeneous and, a part of their cell population, the gastric cancer stem cells (GCSCs) are responsible for tumor initiation, progression,

recurrence, and metastasis. Herein, we first review the major markers of stem/progenitor cells in the stomach, then we describe the cells at the origin of gastric tumors, and finally, we focus on the characterization of the GCSC subpopulation.

2. Existence of stem cells in the stomach

In the stomach, the gastric epithelium is a physiologically self-renewing tissue with a cycle of 2–7 days. Anatomically, the stomach is divided into three main parts: the cardiac region (in humans) or the forestomach (in mice), the main body (corpus), and the distal part (antrum/pylorus). The mucosa of the stomach is composed of a glandular epithelium with millions of gastric units. Each gland is considered to be monoclonal [1] and is subdivided into the foveolus, isthmus, neck, and bottom regions (**Figure 1**). In the gastric corpus, the glands are long and, from the bottom to the top of the gland, contain zymogenic/chief cells implicated in digestion, parietal cells that are essential for acid production, enterochromaffin-like cells that control acid production, mucous neck cells, and superficial pit cells. In the antrum, the glands are shorter and are composed mainly of mucus-producing cells and enteroendocrine hormone-secreting cells that regulate acid and digestive enzyme production in the corpus. In both regions, some discrete gastric stem cells exist and are instrumental in stomach epithelium renewal under pathophysiological conditions.

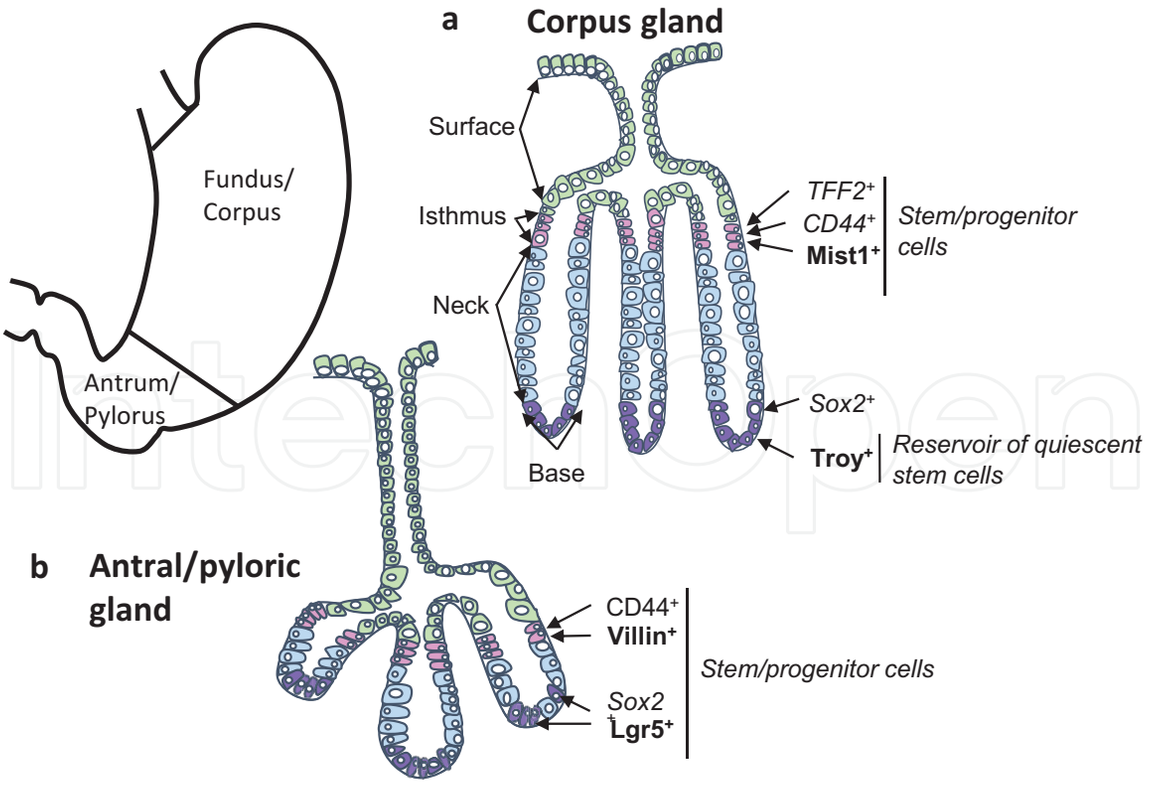

Figure 1. Architecture of the gastric glands and localization of stem/progenitor cells in the main parts of the stomach. (a) Fundic/corpus gastric gland. (b) Antral/pyloric gastric gland. Stem/progenitor markers identified by lineage tracing are indicated in bold.

In adult organs, tissue stem cells are characterized by self-renewal and asymmetrical division properties, giving rise after mitosis to another stem cell and to a progenitor cell that will undergo expansion and then differentiation into mature cells. These stem cells reside in a physiologically limited and specialized microenvironment, called the niche, which is comprised of cells and extracellular matrices forming the surrounding stroma (including mesenchymal cells, vessels, nerves) and which plays a key role in the maintenance of the stem cell number and functions and in preventing tumorigenesis. The localization of the niche of stem/progenitor cells varies according to the part of the stomach considered: in the corpus, they are located in the isthmus just below the glandular narrowing, and in the antropyloric region, there are located at the bottom of the glands. Moreover, as is the case in other organs [2], the coexistence of two stem cell populations has been described in the stomach: (1) a population of dividing gastric stem cells recruited under "homeostatic conditions", expressing CD44 or Lgr5 markers and (2) a rare population of quiescent cells recruited mainly upon tissue damage, expressing Villin, Troy, and Mist1 markers (**Figure 1**).

2.1. Discovery of gastric stem cells and their markers

Using radiolabeling experiments and analyses of the cells by electron microscopy, Leblond et al. first identified a group of small undifferentiated and granule-free cells with the highest labeling index as the putative stem/progenitor cells. These cells are localized in the isthmus region from where they migrate toward both the pit and the bottom [3, 4]. However, the first evidence of the existence of multipotent stem cells in adult mouse gastric glands was found later using chemical mutagenesis of single cells and long-term gastric epithelial cell analyses where many clones spanned entire glands containing all specialized gastric cell lineages [5]. The use of inducible Cre recombinase activity to indelibly label putative stem/progenitor cells and their progeny in the stomach has been widely practiced and is considered as the gold standard method for lineage tracing studies. The first marker of gastric stem/progenitor cells revealed by lineage tracing in the gastric mucosa was **Villin**. *Villin-lacZ* transgenic mice revealed a rare population of quiescent β-galactosidase-positive cells located at the bottom of antropyloric glands or at the isthmus in the corpus [6]. These quiescent Villin$^+$ cells can be activated after stimulation by interferon-γ and moved from the isthmus toward the base of the gland to generate all of the specialized cells of the gastric glands. Villin$^+$ cells may act as a stem cell reservoir with a high proliferative potential to regenerate the gastric mucosa after injury. The presence of such a cell population that highly responds to inflammation is very interesting, especially in the context of gastric cancer which is initiated by a chronic inflammation of the gastric mucosa.

Leucine-rich G protein-coupled receptor 5 (**Lgr5**), a well-recognized stem cell maker in the intestine, is expressed at the bottom of prospective corpus and pyloric glands in the stomach of neonates, whereas its expression in adults is predominantly restricted to the bottom of pyloric glands in mice and in humans [7]. Lineage tracing experiments revealed that Lgr5$^+$ cells are multipotent stem cells responsible for the long-term renewal of the gastric epithelium. In vitro, single Lgr5$^+$ cells generated long-lived organoids resembling the pyloric epithelium in three-dimensional culture. Lgr5$^+$ cells divide symmetrically to generate clonal gland units via neutral competition and lateral expansion of stem cell clones via gland fission under non-damaging conditions [8].

These two markers identified stem cells in the gastric antrum/pyloric region, where most of distal gastric carcinoma arises in humans. In the corpus, some studies suggested that **Sox2**$^+$ cells may represent long-lived stem cells scattered throughout the isthmus and in the lower part of the gastric unit [9]. Trefoil factor family 2 (**Tff2**$^+$) cells were also described as short-lived progenitors in the isthmus region of the corpus [10]. More recently, lineage tracing experiments have shown that differentiated mature chief cells expressing the **Troy** marker at the base of the corpus gastric glands can generate entirely labeled gastric units over a period of several months in vivo and long-lived organoids in vitro [11]. This phenomenon is accelerated upon depletion of the proliferating isthmus compartment mediated by 5-fluorouracile treatment, suggesting that the gastric corpus also seems to contain two stem cell populations: (1) an actively dividing population located in the isthmus region and (2) a smaller reserve population of *Troy*$^+$ stem-like chief cells located at the base of the gland [11]. This property of a differentiated cell to reenter the cell cycle and to act as a multipotent stem cell highlights the plasticity of gastric epithelial cells. Surprisingly, Stange et al. detected Lgr5$^+$ cells at the base of the corpus glands using another Lgr5 reporter construction in transgenic mice, and transcriptomic analyses demonstrated that Troy$^+$ cells expresses several Wnt target genes including Lgr5 and CD44 [11].

Likewise, **Mist1** is a marker of stem-like quiescent chief cells located in the lower third of the glands and in rare single cells of the isthmus in the gastric corpus [12, 13]. The vast majority of Mist1$^+$ chief cells at the base of the glands are Lgr5$^+$, whereas Mist1$^+$ cells in the isthmus are Lgr5$^-$, and only 1.1% of them are proliferative. Ablation of Lgr5$^+$/Mist1$^+$ chief cells by expression of the diphtheria toxin in *Lgr5*-DTR-GFP transgenic mice results in an increase of Mist1$^+$ cells in the isthmus which reconstitute the entire glands, suggesting that Mist1$^+$ isthmus cells are multipotent stem cells [13]. Finally, Mist1$^+$ isthmus cells can form organoids in an Lgr5-independent manner in the corpus.

In addition, the Runx1 enhancer element, **eR1**, is expressed in the isthmus and marks a small number of terminally differentiated chief cells at the base in the stomach corpus as well as near the bottom of the pyloric gland. eR1$^+$ cells generated entirely labeled gastric units after a year and formed organoids in vitro, suggesting that they are composed of gastric stem cells [14]. Nevertheless, it appears that some Runx1-expressing cells are stem cells, whereas others are differentiated cells, such as pit cells. Moreover, 80% of eR1$^+$ cells expressed Ki67, whereas only 1.1% of Mist1$^+$ cells in the isthmus expressed it, suggesting that Mist1$^+$ cells are quiescent cells and that eR1$^+$ cells are rapidly dividing cells [13, 14].

Additional markers have been proposed for gastric stem cells (e.g., DCKL1/DCAMKL1, CD133/PROM1, and CD44), but the multipotency of these cells has not yet been analyzed by lineage tracing [15, 16]. Khurana et al. found that **CD44** (cluster of differentiation 44) is mainly expressed at the base of antral/pyloric glands, in a region overlapping Lgr5, and in the isthmus region of the corpus glands [17, 18]. When parietal cell loss and atrophy were induced chemically or by *Helicobacter* infection, the CD44$^+$ cells expanded from the isthmus and replenished the base of the gastric units (**Figure 2**). CD44 expression is enriched in the Mist1$^+$ isthmus stem cell population in the corpus, suggesting again that they could represent stem/progenitor cells.

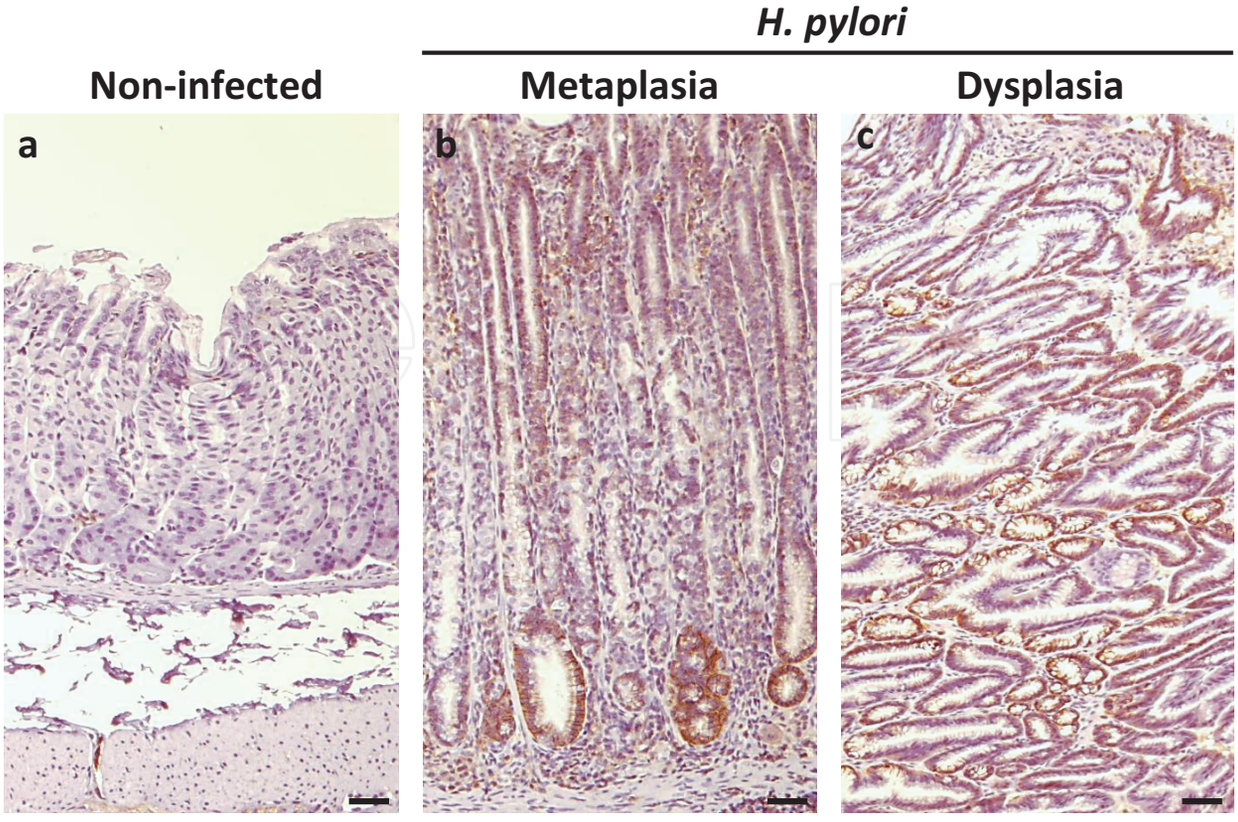

Figure 2. CD44 expression in *H. pylori*-induced metaplasia and dysplasia. Representative pictures of CD44 detection by immunohistochemistry in mouse stomachs: (a) normal gastric mucosa of a noninfected mouse; (b) metaplasia; and (c) intraepithelial dysplasia in *H. pylori SS1*-infected stomachs. Scale bars, 50 μm.

2.2. Factors sustaining gastric stem cell self-renewal and multipotency

Until very recently, suitable models to study gastric stem cells in vitro were lacking. Cell lines from cell banks are all derived from carcinomas, and primary culture of gastric epithelial cells from biopsies is not successful under conventional adherent culture conditions. Culture of antrum and fundus cells has been rendered possible very recently by the development of mouse and human protocols allowing the development of organoids, named gastroids, under three-dimensional culture conditions, in media containing epithelial growth factor (EGF) and Noggin, with either Wnt3A and R-spondin, a molecule binding Lgr4/5 and potentiating Wnt/β-catenin activity [19], or supplemented with the Notch ligand Jagged-1 [7, 13]. In vitro studies of organoid formation by gastric stem cells or gastric glands have allowed insight in the necessary growth factors and signaling molecules of the niche implicated in stem cell properties and gland formation and can offer new therapeutic applications in patient that suffer malignant diseases, for example, for ulcer treatment. Engevik et al. have shown that gastric stem cells/organoid isolated from young mice can be transplanted into sites of acetic acid-induced ulcer within the stomachs of older mice and that this results in accelerated repair injury [20].

Wnt5a, a noncanonical Wnt ligand, is highly expressed by $Cxcr4^+$ cells in the isthmus part of the corpus. Histological analyses show that Wnt5A is secreted by $Cxcr4^+$ resident hematopoietic

cells recruited to the isthmus and stimulated by Cxcl12 endothelial cell production. The efficiency of organoid formation is enhanced by Wnt5a or coculture with $Cxcr4^+$ intraepithelial gastric innate lymphoid cells [13], suggesting that cells in the niche regulate stem/progenitor proliferation.

The enteric nervous system also has the ability to regulate gastric homeostasis via direct innervation of the glands. In three independent mouse models of gastric cancer, Zhao et al. elegantly demonstrated that surgical or pharmacological denervation suppresses gastric tumorigenesis, even if performed at an early preneoplastic step [21]. Further analyses revealed that cholinergic nerves surround the base of glands and modulate epithelial stem cells through activation of the Wnt signaling pathway via the muscarinic acetylcholine receptor 3 (M_3R) expressed by $Lgr5^+$ cells. In stomach organoid models, coculture with neurons or treatment with pilocarpine, a cholinomimetic drug, increased organoid formation and the expression of *Lgr5* and *Cd44* stem cell markers, whereas the effects were reversed by botox treatment [21]. Another publication reported that $Dclk1^+$ tuft cells and nerves, the main sources of acetylcholine in the gastric mucosa, induced nerve growth factor (NGF) secretion from epithelial cells that expand enteric nerves and promote carcinogenesis [22]. Remarkably, *Tff2*-Cre;*R29*-NGF mice developed metaplasia and dysplasia by 8 months of age with $CD44^+$ dysplastic cell expansion and intramucosal adenocarcinomas by 18 months. Ablation of $Dclk1^+$ cells in this context led to the inhibition of epithelial proliferation and tumorigenesis in a M_3R-dependent manner [22].

The Notch signaling pathway is also inhibited in vagotomized mice [21]. The Notch inhibitor dibenzazepine (DBZ) reduced the proliferation in the isthmus region, decreased the Mist1-lineage tracing, and blocked the growth of corpus organoids in vitro, suggesting that Notch activity is important for corpus gastric stem cell maintenance and activity [13].

3. Is gastric cancer a stem cell disease?

Gastric carcinoma is a multifactorial disease, involving a chronic *Helicobacter pylori* infection as the main cause as well as the Epstein-Barr Virus to a small extent, diet (low vitamins, nitrosamines, chemicals, etc.), smoking, and genetic susceptibility of the host [23, 24]. At the histological level, the WHO described more than five different histological subtypes, divided into two main groups in the Lauren classification of gastric tumors, i.e., the intestinal type and the diffuse type [25]. At the molecular level, gastric carcinomas are classified in four main groups based on their mutational profile [26, 27]. These classifications are not currently used in clinical practice to orientate toward a specific targeted therapy.

More than 93% of distal gastric carcinoma cases are associated with a chronic *H. pylori* infection of the gastric mucosa [28]. Most of these cases represent the intestinal histological subtype. *H. pylori* infection induces a chronic inflammation of the gastric mucosa, i.e., gastritis. In 5–10% of cases, gastritis evolves into gastric or duodenal ulcer, and in 1% of cases, gastritis leads to stomach cancers. In this last case, the loss of specialized epithelial cells results in a chronic atrophic gastritis and to the compensatory cellular hyperproliferation and aberrant differentiation at the origin of the intestinal metaplasia (firstly appearing in the pylorus)

and/or spasmolytic polypeptide-expressing metaplasia (SPEM) (mainly in the corpus). These metaplastic lesions can further progress into dysplasia and ultimately into an intestinal-type carcinoma according to the Lauren classification [29, 30]. So, chronic atrophic gastritis is considered to be the first step of gastric adenocarcinomas. All of these lesions are well characterized histologically and can be reproduced experimentally in Mongolian gerbils [31] and in mice [32, 33] in response to *H. pylori* and *Helicobacter felis* infection. Gastric carcinoma of the diffuse type or signet ring cell carcinoma corresponds to poorly differentiated adenocarcinomas for which the glandular structure has disappeared. It is the second most frequent histological subtype of gastric tumors, which is frequently linked to sporadic or hereditary mutations of the *CDH1* gene encoding E-cadherin, and appears most of the time without precancerous lesions.

H. pylori is mainly present at the surface of gastric units but also as microcolonies deep in the stomach glands; the bacteria can interact directly with gastric stem/progenitor cells in the stomach of mice and humans. Regarding their long lifetime and high division ability, stem cells are more susceptible to accumulate genetic/epigenetic modifications than their progeny. Some current evidence suggests that, in the context of chronic *H. pylori* infection, gastric cancer stem cells originate from the transformation of stem cells of two different origins: local, for most of the cases, and to a lesser extent from bone marrow-derived stem cells that home into the gastric mucosa in response to the chronic injury mediated by *Helicobacter*, contributing to metaplasia and dysplasia [34–36]. It is important, however, to distinguish between the origin of cancer cells which initiate and drive the primary tumor development and those which accumulate and contribute to tumor growth once the process is initiated. These two main mechanisms leading to the emergence of gastric cancer cells will be discussed below.

3.1. Tumors can originate from epithelial stem cell transformation

Interestingly, the parietal cell atrophy induced after *H. pylori* infection causes an increase in the proliferation of stem/progenitor cells in the isthmus [37] that is associated with an induction of CD44 expression in this region, which then expands toward the bottom of the gastric unit [17, 35, 36] (**Figure 2**). Sigal et al. also reported an increase of the number of antral $Lgr5^+$ cells in *H. pylori*-associated gastritis and carcinoma in humans. As these cells are susceptible to DNA damage, it suggests that $Lgr5^+$ stem cells could be at the origin of cancer [38, 39]. An $Lgr5^+$ gene signature in pyloric gastric units identified Wnt target genes, including *Sox9* and *Cd44*, suggesting canonical Wnt signaling activity at the base of the pyloric glands. Gastric cancer patients exhibit a dysregulation of Wnt signaling [21]. Spontaneous Wnt activation in the mouse model APC^{min} leads to the development of gastric adenomas in the pyloric region; *Apc* depletion specifically in $Lgr5^+$ via a single tamoxifen injection in $Lgr5\text{-}EGFP\text{-}CreERT2;APC^{flox/flox}$ mice leads to adenoma formation in the gastric antrum, but not in the corpus [7].

KRAS is one of the most commonly mutated oncogenes in gastric cancer. The *Kras* mutation in the $Mist1^+$ isthmus cells, and not in the $Mist1^+$ chief cells, results in the formation of metaplastic/dysplastic foci from the isthmus to the bottom of glands. $Mist1^+$ stem cells give rise to intramucosal intestinal-type gastric cancer induced by *Apc* loss of function but only in the context of KRAS-induced metaplasia [13].

E-cadherin expression is lost in most diffuse-type gastric carcinomas, but E-cadherin loss alone is not sufficient to initiate diffuse-type gastric cancer in mice [40]. Loss of the tight junction protein IQGAP1 is also insufficient to induce diffuse-type gastric carcinoma in transgenic mice after challenge with *Helicobacter* infection, as it indeed promotes intestinal-type carcinogenesis [41]. To try to reproduce the diffuse-type gastric cancer, some authors infected *Mist1*;CreERT2;*Cdh1*$^{flox/flox}$ mice with *H. felis* to induce chronic inflammation. In this context, E-cadherin loss in Mist1^{+} cells resulted in the development of diffuse-type gastric carcinomas [13]. The double inactivation of E-cadherin and p53 in a conditional mouse model also successfully led to metastatic diffuse-type gastric cancer [42].

3.2. Tumors originating from bone marrow-derived cells

Some data have shown that bone marrow-derived cells (BMDCs) can migrate to peripheral tissues in case of injury or inflammation where they are engrafted and participate in tissue repair, giving rise to all cell lineages. Houghton et al. showed that BMDCs are recruited into the gastric mucosa of C57BL/6 mice chronically infected by *H. felis* and contribute overtime to metaplasia, dysplasia, and finally cancer [34]. In fact, to the contrary, they found that it was a rare event in the context of a normal homeostasis, without injury. Results from this study did not exclude the possibility that BMDCs participate in the development of lesions via fusion with epithelial cells. Our group confirmed these observations in the same mouse genetic background but with different strains of the human pathogen *H. pylori* and found that nearly a quarter of high-grade dysplastic lesions are composed of BMDCs [35]. BMDC epithelial gland repopulation was significantly associated with pseudointestinal metaplasia, suggesting that BMDC recruitment may play a role in preneoplastic lesion progression. These BMDCs are recruited only in response to chronic *H. felis* and *H. pylori* infection but not in response to acute injury [34]. BMDC recruitment occurs in response to the secretion of several chemokines such as SDF1 and TNFα by infected epithelial cells in a NF-κB-dependent manner [34, 43]. Once recruited, the BMDCs can differentiate into local gastric epithelial cells via transdifferentiation or cell/cell fusion with local gastric epithelial cells [35, 44]. Interestingly, BMDC recruitment into the gastric mucosa was a late event in the cascade of gastric carcinogenesis, occurring only in infected animals of more than 1 year of age. In these chimera mice, metaplastic lesions were comprised, inside the same gland, of a mosaic of tagged-BMDCs and native gastric epithelial cells, revealing a multiclonal composition [35]. These metaplastic lesions are now considered as a "point of no return," after which, most of the time, eradication of *H. pylori* cannot lead to a regression of the metaplastic and associated dysplastic lesions, because mutations deregulating stem cell properties and proliferation are already present. A monoclonal conversion will occur during the evolution of intestinal metaplasia toward dysplasia and finally carcinoma. As the mice never develop real metastatic gastric adenocarcinoma in contrast to the human situation, there is no evidence in the literature to date of the role of BMDCs composing metaplastic/dysplastic lesions as the tumor-initiating cells in invasive gastric adenocarcinoma. However, it is very interesting to note that BMDCs were also detected in gastric carcinoma of the esophagus in mice models and in humans, which also develop on a background of chronic inflammation and intestinal metaplasia cascade [45, 46]. Very few studies have been described in humans to strengthen the results obtained in animal models, because there is a limited possibility to trace BMDCs in an individual developing a carcinoma of the GI tract. The only technical approach

tested was to detect the Y chromosome of BM cells of a male donor in female transplanted patients by fluorescence in situ hybridization (FISH). In such transplanted cases having developed carcinoma of the GI tract, BMDCs were detected in some rare cases of carcinoma and dysplasia of the esophagus [45, 46]. Concerning the stomach, the study of Whortley et al., performed on only four cases of sex-mismatch transplanted cases having developed gastric carcinoma, failed to report a carcinoma composed of cells of BM origin. However, one of the cases showed aneuploidy, so a contribution of the BMDCs cannot be totally excluded [47].

Unfortunately, in those models, full proof of the concept that gastric stem cells or other populations of differentiated cells or BMDCs are the cells of origin of cancer has not been found, because the tumorigenic effect mediated, for instance, by *Apc* and *Cdh1* inactivation or by *Kras* oncogenic activation in non-stem cell populations, i.e., in progenitor or differentiated cells, has not been followed. Moreover, in contrast to squamous skin tumors [48] or intestinal adenomas [49], the in vivo contribution of GCSCs to tumorigenesis has not yet been fully elucidated. Nevertheless, regardless of their origin, dysplastic lesions and gastric adenocarcinomas are composed of $CD44^+$ cells (**Figure 2**) [17, 36] that have been recently described to possess cancer stem cell properties [50, 51].

3.3. Tumor originates from dedifferentiation of epithelial cells

In an inflammatory setting, differentiated cells can reacquire the ability to divide and to give rise to all cell lineages. In vitro, *H. pylori* infection induces a destabilization of cell/cell junctions and an elongated phenotype associated with motility [52]. We reported that *H. pylori* infection leads to the generation of a $CD44^+$ cancer stem-like cell population with mesenchymal phenotype and tumorigenic properties, through complex signaling pathways involving activation of the mitogen-activated protein kinase ERK, c-Jun N-terminal kinase, miR200, and NF-κB signaling pathways, leading to the activation of ZEB1 and Snail1 transcription factors, the main drivers of the epithelial-mesenchymal transition (EMT) [36]. We showed that this effect was associated with the bacterial oncoprotein CagA produced by *H. pylori* and with secreted factors such as hepatocyte growth factor (HGF) as described by others [36, 53]. In vivo, the expansion of the compartment of $CD44^+$ stem cells at the isthmus in the corpus and at the base of the glands in the antro-pyloric region is associated with the expression of mesenchymal markers in the context of *H. pylori*-associated gastritis, metaplasia, and dysplasia, in humans and in wild-type mice [23, 36, 41]. We recently showed that invalidation of *iqgap1*, a partner of E-cadherin at the cell/cell junctions, increased EMT both in vitro and in vivo, promoted *H. felis*- and *H. pylori*-induced regenerative hyperplasia expressing CD44 and mesenchymal markers, and accelerated and worsened metaplasia and dysplasia development, reinforcing the causal link between EMT and emergence of CSC-like cells [41].

4. Properties of gastric cancer stem cells

Tumor cells are heterogeneous in terms of mutations carried, susceptibility to drugs, markers expressed or morphology, and not all are tumorigenic. This genetic heterogeneity would come not only from intrinsic factors such as genetic mutations acquired progressively and amplified

within new clones but also from extrinsic factors related to the variation of the tumor microenvironment [54, 55]. To explain these observations, two concepts have been proposed: the cancer stem cell (CSC) theory, also named the hierarchical model, and the stochastic model. In the stochastic model, all cancer cells have similar tumorigenic properties, with cancer arising after a series of genetic and epigenetic events leading to successive waves of clonal selections depending on the proliferative and survival benefits acquired. In the hierarchical model, there is a cellular hierarchy between cancer cells inside the tumor, with CSCs being at the origin of the more or less differentiated cells, not all proliferative and tumorigenic, composing the tumor mass. CSCs represent a small percentage of tumor cells and possess particular properties compared to non-CSCs (**Figure 3**): (1) the first and most important is their capacity to self-renew and divide asymmetrically and to generate a new CSC and a non-CSC progenitor cell, a property that maintains a constant CSC pool; (2) CSCs are able to initiate tumor growth when injected in low cell numbers in immunocompromised mice; (3) CSCs display differentiation properties giving rise to the more or less differentiated cells composing the tumor mass, reconstructing the tumor heterogeneity observed within the primary tumor; (4) CSCs have increased resistance to current chemo- and radiotherapies; and (5) CSCs express

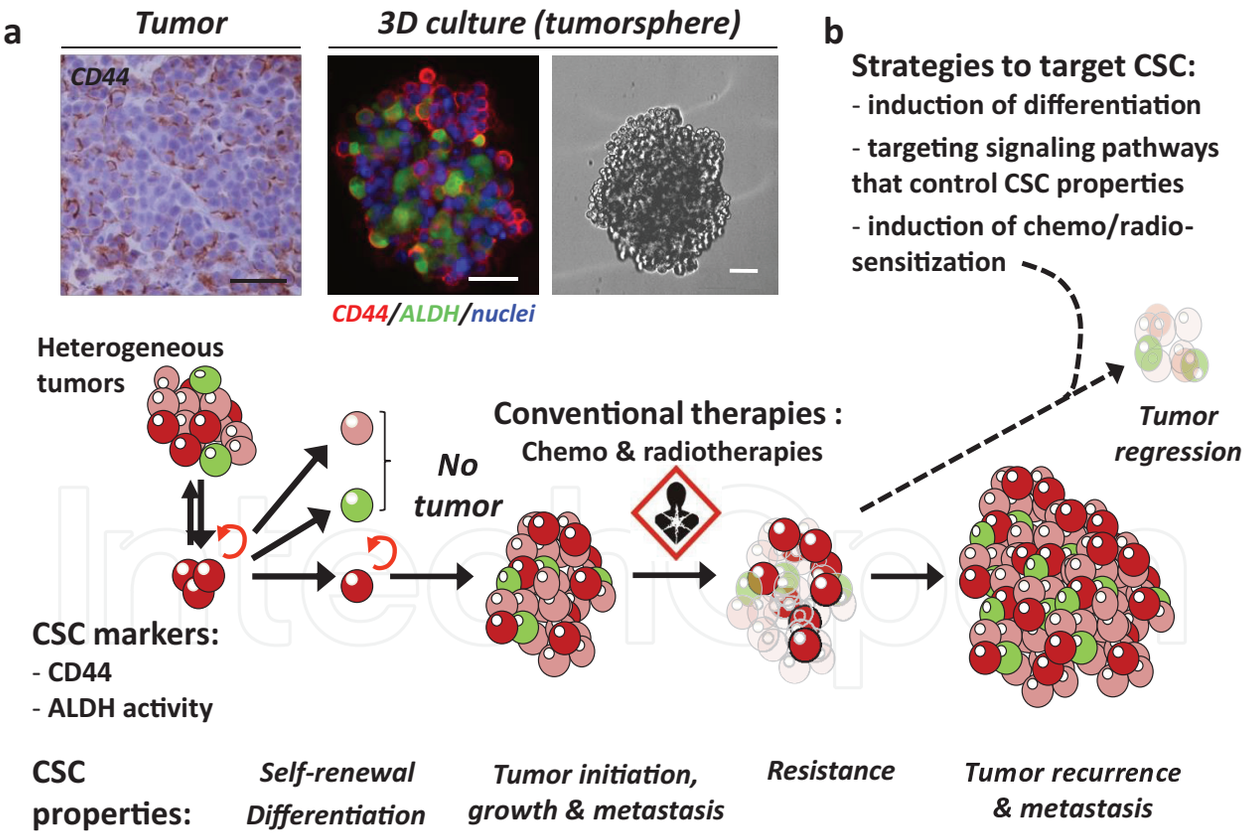

Figure 3. Hierarchical model illustrating the heterogeneity of the tumors. (a) Representative images of CD44 detection by immunohistochemistry on tumor tissue section (left panel) and of the detection of CD44 by immunofluorescence and ALDH activity (Aldefluor™ reagent) with Hoescht 33342 dye (middle panel) on tumor spheres of MKN45 gastric cancer cells (right panel, phase contrast microscopy). Scale bars, 50 μm. (b) Hypothetical strategies to target CSC to cure gastric cancer. The main gastric CSC markers are CD44 and ALDH activity. Cancer stem cells represent a subpopulation of cells implicated in tumor initiation, growth, metastasis, and chemo-/radio-resistance.

specific markers [55]. This hierarchical model is not exclusive but is now the most accepted model with the recent identification of CSCs in most cancers since their first discovery in acute myeloid leukemia in 1995, then in solid tumors in 2003, and more recently in gastric carcinoma. However, we must keep in mind that this hierarchical model is also subjected to clonal evolution even if it has not been clearly demonstrated for gastric carcinoma [56].

4.1. Functional characterization of gastric cancer stem cells

By taking advantage of the capacity of stem cells to self-renew, differentiate, and initiate tumors, functional assays have been developed to evaluate the amount of GCSCs or to isolate them from a global population of tumor cells. In vitro, under conditions in which cells are seeded at low-density in low-adherent plates, without serum and in the presence of some growth factors such as EGF, bFGF, and insulin, only GCSCs can survive, self-renew, and form tumorspheres [36, 50, 51, 57]. Long self-renewal ability is evaluated after tumorsphere dissociation into single cells and several passages; indeed only GCSCs can generate tumorspheres after several passages [51, 58]. In vivo, frequency of GCSCs in a given population is determined after subcutaneous xenograft in immunocompromised mice using different cell doses and an analysis of their ability to initiate a new heterogenous tumor after several weeks. Immunocompromised mouse phenotypes, nonobese diabetic/severe combined immunodeficiency (NOD/SCID) and NOD/SCID/IL2Rg− (NSG), can influence the CSC frequency as reported in the case of melanoma by Morrison's group [59]. In both methods, cells were seeded for an extreme limiting dilution assay, and a mathematical method was applied to calculate the CSC frequency in a given cell population [51, 60].

4.2. Phenotypic characterization of gastric cancer stem cells

Several phenotypic characteristics have been proposed to isolate GCSCs using fluorescence-activated cell sorting (FACS), including (1) the expression of cell membrane markers (or combinations of markers), and among them CD44; (2) the exclusion of Hoechst 33342 dye by the "side population" of cells (SP cells); and (3) the enzymatic activity of aldehyde dehydrogenases (ALDH).

CD44 was among the first markers of CSCs described in solid tumors and initially in breast carcinoma [61]. CD44 is a type I transmembrane glycoprotein expressed in many normal and tumoral cells. It plays a role in adhesion/homing, supporting cell migration and transmitting survival signals, thereby being pro-oncogenic by nature. The principal ligand of the CD44 receptor coordinating signalization is hyaluronic acid, but it can also interact with additional molecules of the extracellular matrix, such as collagen, fibronectin, fibrinogen, laminin, or osteopontin [62]. Cytoplasmic partner molecules of CD44 are the cytoskeletal proteins Ezrin, Radixin, Moesin, and Ankyrin, which influence the signaling pathway. Loss of CD44 in mice models results in a decrease in gastric mucosa proliferation in the isthmus region. The critical role of CD44 in proliferation involves its interaction with hyaluronic acid and the downstream activation of the STAT3 signaling pathway [17], RhoGTPases, the PI3K/AKT pathway, and the MAPK signaling pathway [63]. CD44 is encoded by the 20-exon *CD44* gene, in which exons 1–5 and 16–20 are spliced together and translated into CD44s, the standard or small isoform.

In addition, the variant exons 6–15 can be alternatively spliced and assembled in different combinations with the standard exons to generate other variant (CD44v) protein isoforms [62, 64]. We and others reported that CD44 is expressed following *H. pylori* infection in patients and in mouse models in the case of regenerative hyperplasia, intestinal metaplasia, dysplasia, and gastric carcinoma (**Figure 2**) [35, 36, 50, 51, 65]. Histological and molecular analyses of tumor collections have shown that CD44 is positively and significantly associated with tumor recurrence and mortality in gastric cancer, and the expression of CD44 and CD44v has also been associated with metastasis formation [57, 65–70].

Takaishi et al. were the first to propose CD44 as a marker of GCSCs in a study performed on several gastric cancer cell lines [50]. Their $CD44^+$ cells were able to form tumorspheres and initiate tumors after subcutaneous and orthotopic engraftment in mice, and they were resistant to anticancer drugs, whereas $CD44^-$ sorted cells were not. Moreover, it seems that CD44 is not only a GCSC marker, but it also plays an oncogenic role, assessed by a decrease in tumor growth using siRNA targeting CD44. More recently, further relevant results from patient-derived xenograft models (PDXs) of gastric carcinoma have confirmed that CD44 is also a marker of GCSCs in primary gastric carcinoma. The FACS-sorted $CD44^+$ cells, but not their $CD44^-$ counterpart, displayed CSC properties such as growing as tumorspheres in vitro and lead to tumor formation in vivo that reconstitute the heterogeneity of the primary tumor of the patients and are more chemoresistant [51, 71, 72]. ESA, CD24, CD133, and CD166 are also expressed by $CD44^+$ cells, but they do not allow a better enrichment of GCSCs in combination with CD44 compared to CD44 alone [51, 73]. Although CD44 marks GCSCs, not all $CD44^+$ cells are tumorigenic [51]. CD44v8-10, also named CD44E, has been identified as the predominant CD44 variant expressed in gastric cancer cells, and its expression is low in normal tissues [57]. It plays a functional role in tumor initiation, most likely by increasing CSC resilience to adverse conditions such as hypoxia or oxidative stress. Indeed, there is evidence that CD44v8-10 stabilizes the cystine-glutamate transporter subunit xCT and promotes the synthesis of glutathione, thereby protecting cancer cells from reactive oxygen species [70]. Depletion of the expression of CD44 leads to a decrease in the tumorigenicity of cancer cell lines [50], and Yoon et al. demonstrated implication of the Hedgehog signaling in the maintenance, chemoresistance, and migration capacity of the GCSC $CD44^+$ cells [74].

ALDH activity has also been described as a GCSC marker [51, 75]. In an extensive screening of the expression of 10 putative CSC surface markers, as well as in eight PDX models, we found that CSCs expressed both CD44 and ALDH activity and that ALDH activity revealed a subpopulation within the $CD44^+$ cells that possessed CSC properties, i.e., the ability to generate a new heterogeneous tumor in vivo and a tumorsphere in vitro. Xenograft experiments using the ELDA mathematical model showed that the frequency of GCSCs expressing CD44 and ALDH was 0.1–3.5% of the cancer cells [51]. These $CD44^+/ALDH^+$ cells did not incorporate the vital DNA dye Hoechst 33342, whereas the $ALDH^-$ cells incorporated it, suggesting that $CD44^+/ALDH^+$ cells may correspond to **SP cells** with CSC properties as previously described in gastric carcinoma cell lines by others [76–78]. The ability of $CD44^+/ALDH^+$ cells to efflux the Hoechst 33342 dye and to resist conventional chemotherapy was reversed by treatment with efflux pump inhibitors [51]. Nevertheless, Takaishi et al. found that both gastric SP and

non-SP cells possess a tumorigenic ability in vitro and in vivo [50]. Therefore, the detection of the SP cells does not seem to be a good marker for GCSCs; rather the best markers to detect them are CD44 and ALDH.

4.3. Missing data: implication of gastric cancer stem cells in metastasis?

Another important property of CSCs is their ability to initiate metastasis. Metastasis is a rare event [79] requiring the acquisition of invasive properties through epithelial-mesenchymal transition (1) to escape from the niche of the primary tumor in order to disseminate to distant organs after extravasation as circulating tumor cells (CTCs) and (2) to initiate secondary tumors [80]. We reported that the $CD44^+$ cells with CSC-like properties induced by *H. pylori* infection but not the $CD44^-$ cells overexpressed mesenchymal markers such as Vimentin and Zeb1 and downregulated epithelial markers and tumorigenic, migratory, and invasive properties [36, 81]. Chen et al. identified CTCs characterized by $CD44^+CD54^+$ expression in the peripheral blood from patients with gastric cancer which were able to form tumorspheres and generate heterogeneous tumors when injected into immunodeficient mice; these CTCs had a self-renewal capability both in cell culture and in mouse models [82]. This study suggested that $CD44^+CD54^+$ CTCs could represent metastatic GCSCs. Nevertheless, the characterization of the CSC subpopulation capable of initiating metastases needs to be determined.

5. Conclusion

Since 2007, researchers have increased efforts to identify real gastric stem cells, the cell population capable of replenishing an entire gastric gland containing of all cell lineages. Many of the markers involved have been reviewed here, and their stemness properties have been clearly demonstrated in mouse models. It will be of interest to understand why there are different localizations of stem cells, one in the isthmus and one at the bottom of the gland. These two stem/progenitor cell niches could play different roles, one being more proliferative than the other one which seems to behave like a reservoir, but they could also play distinct roles in response to different stimuli and damage to the gastric mucosa.

Regarding to the gastric tumor, an in-depth analysis of putative CSC markers identified CD44 as well as ALDH activity as the "gold" gastric CSC markers in cancer cell lines and in PDX models [51]. Determination of the signaling pathways controlling their properties is now instrumental to find new targeted therapies for gastric cancer, for which there is a crucial unmet need to find new efficient therapy. In this aim, we have shown by different strategies that the targeting of gastric CSCs expressing CD44 by blocking specific microRNAs or by inducing their differentiation by all-trans retinoic acid allows inhibition of tumor growth in vivo [58, 83].

Nevertheless, the characterization of gastric CSCs was limited in some publications by the cellular model used. To date, the best models to study the efficiency of new therapeutic strategies on primary gastric CSCs remain PDX models, with the restriction that almost all of those described are subcutaneous engraftments which never give rise to metastasis. Moreover,

mouse models of gastric carcinogenesis induced by *Helicobacter* infection and/or carcinogens do not reproduce invasion of the deeper layers of the stomach, peritoneal carcinomatosis, and distant metastases as in humans. Consequently, there is an urgent need to develop mouse models of metastatic gastric carcinoma, in order to study the efficiency of new therapeutic strategies targeting CSCs not only on tumor initiation but also on metastasis formation.

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