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A unified model of the hierarchical and stochastic theories of gastric cancer

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Gastric cancer (GC) is a life-threatening disease worldwide. Despite remarkable advances in treatments for GC, it is still fatal to many patients due to cancer progression, recurrence and metastasis. Regarding the development of novel therapeutic techniques, many studies have focused on the biological mechanisms that initiate tumours and cause treatment resistance. Tumours have traditionally been considered to result from somatic mutations, either via clonal evolution or through a stochastic model. However, emerging evidence has characterised tumours using a hierarchical organisational structure, with cancer stem cells (CSCs) at the apex. Both stochastic and hierarchical models are reasonable systems that have been hypothesised to describe tumour heterogeneity. Although each model alone inadequately explains tumour diversity, the two models can be integrated to provide a more comprehensive explanation. In this review, we discuss existing evidence supporting a unified model of gastric CSCs, including the regulatory mechanisms of this unified model in addition to the current status of stemness-related targeted therapy in GC patients.

Gastric cancer (GC) is the third leading cause of death and the fifth most frequently diagnosed cancer in adults worldwide, with an estimated 0.72 million deaths and 0.95 million new cases in 2012 (Torre *et al*, 2015). Apparent tumour heterogeneity exists not only between sexes but also regarding the spatial-temporal distribution of the tumour. Overall, the incidence and mortality of GC are nearly twice as high in males as in females, and the disease usually occurs in individuals who are 45 years or older and predominantly from developing countries and regions, such as East Asia (Magee *et al*, 2012). Despite considerable advances in GC treatment and decreasing trends in incidence and mortality rates, many patients still die due to cancer progression, recurrence and metastasis. Due to a lack of effective screening methods, more than 50% of patients are in advanced stages at initial diagnosis, at which time most of these patients do not have the opportunity for radical surgery and are reluctant to undergo treatment with adjuvant therapy. The response rate (RR) of patients with advanced GC (AGC) to first-line chemotherapy is only 50%, and the median overall survival (OS) is less than 12 months (Cunningham *et al*, 2008; Kang *et al*, 2009). Furthermore, the OS of patients after second-line therapy is only ~6 months (Janowitz *et al*, 2016). In view of the failure of conventional therapy, substantial attention has been focused on the genetic and molecular mechanisms that initiate tumours and cause

drug resistance. More evidence is emerging to indicate that tumours are characterised by a hierarchical organisational structure, with cancer stem cells (CSCs) at the apex, and additional evidence suggests that successful eradication of CSCs may be a promising therapeutic approach for treating cancer.

Through improvements in cell sorting, gene labelling and lineage tracing, substantial evidence has emerged in support of the hierarchical model hypothesis. For example, CD34⁺CD38⁻ leukemia cells, which were the first CSCs identified using flow cytometry, demonstrated self-renewal ability and the capacity to differentiate into mature leukemic cells and initiate human acute myeloid leukemia after serial transplantation into immunocompromised mice (Bonnet and Dick, 1997). The lineage tracing of cells containing stemness markers has provided more direct, functional evidence for the presence of CSCs (Schepers *et al*, 2012; Hayakawa *et al*, 2015). Hence, it has been gradually accepted that the central features of CSCs are their unlimited self-renewal capacity and pluripotency and that this small number of cancer cells is responsible for tumour initiation, progression, recurrence and metastasis (Clarke *et al*, 2006). However, this model has been challenged based on results obtained using advances in high-throughput sequencing of multiple tumour genomes for detecting large variations in gene mutations within or between tumours

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(Gerlinger *et al*, 2012; Nik-Zainal *et al*, 2012) and using functional assays combined with genetic analyses. The diversity of driver mutations implies the presence of subclones and their role in tumour heterogeneity. In addition, the use of single-nucleus sequencing to investigate the genetic structure of single cancer cells by accurately quantifying the genomic copy number revealed the existence of distinct subclones among individual cells (Navin *et al*, 2011). Likewise, the single-cell DNA/RNA sequencing of circulating tumour cells (CTCs) has allowed the detection of heterogeneity in genome mutations and signalling pathways that may contribute to tumour progression and treatment failure (Yu *et al*, 2012; Heitzer *et al*, 2013; Miyamoto *et al*, 2015). Generally, the identification of different genetic subclones suggests that gene mutations and clonal evolution also have key roles in tumour initiation, progression and metastasis.

As discussed above, both the stochastic and hierarchical models are reasonable hypotheses that have been proposed to describe tumour heterogeneity, but alone, each model is insufficient to explain the diversity within tumours. In light of growing controversy, Kreso and Dick (2014) proposed that the two models could be integrated into a more comprehensive explanation. By combining genetic analyses with functional assays of tumour-initiating cells (T-ICs), it was found that T-ICs also contain genetic subclones, which influence their properties (Notta *et al*, 2011; Chow *et al*, 2014); that is, T-ICs can also evolve. Hence, it is reasonable to imagine that an early-stage tumour containing rare CSCs and other cells becomes more heterogeneous and invasive as advantageous mutations accumulate and that as the CSCs develop into several subclones, they achieve a higher capacity for self-renewal and thus form a greater proportion of the total cells (Figure 1). In addition, some studies have shown that sorted non-T-IC subpopulations can transform into T-ICs under specific conditions (Chaffer *et al*, 2013) via two possible mechanisms: by dedifferentiation of non-T-ICs (Schwitalla *et al*, 2013) or by occasional mutations in non-T-ICs that endow them with stemness. These studies highlight the plasticity of cancer cells and the dynamic process of tumour evolution, during which T-ICs and non-T-ICs can interconvert. Collectively, a unified model that is dynamically regulated by other determinants such as epigenetics (Baer *et al*, 2013; Klutstein *et al*, 2016), gene expression stochasticity (Elowitz *et al*, 2002; Kaern *et al*, 2005; Spencer *et al*, 2009), the CSC niche (Plaks *et al*, 2015) and the tumour

microenvironment (TME; Medema and Vermeulen, 2011) is proposed, and this model may provide an overall explanation for the biological properties of the tumour (Kreso and Dick, 2014).

In this review, we discuss the evidence for a unified model in describing gastric CSCs (GCSCs) and the regulatory mechanisms of this model in addition to the current status of stemness-related targeted therapy in GC patients.

ORIGIN OF GCSCS

On the basis of the Lauren classification, GC is primarily divided into two distinct categories: intestinal type and diffuse-type. The pathological process of intestinal type GC has been well studied; the gastric epithelium develops through sequential stages of chronic gastritis, atrophic gastritis, metaplasia, dysplasia and eventually cancer. However, it is unclear which determinants are involved and how those determinants result in the emergence of these morphologic changes, especially regarding the mechanism by which GCSCs originate. According to the unified model of carcinogenesis, tumour cells are composed of several subpopulations of CSCs and highly homogeneous non-CSCs, and these subclones could be produced by the mutation of a single CSC. Therefore, the following questions have been raised: what is the precursor of CSCs, and how does a normal cell obtain the ability to infinitely self-renew? Current evidence suggests that GCSCs likely originate from multiple cell types, including gastric stem cells (GSCs), glandular cells and bone marrow-derived cells (Figure 2A).

GCSCs originate from GSCs. In the human stomach, the epithelium of gastric units or glands is primarily composed of four types of functional cells: chief cells, parietal cells, mucous cells and enteroendocrine cells. The majority of these differentiated cells are short-lived and are rapidly replaced by new cells, and each gland is considered to be formed by polyclonal expansion of multiple stem cells (McDonald *et al*, 2008). Hence, it is widely accepted that the integrity of the fast-growing gastric epithelium is sustained by quiescent stem cells. Early evidence revealed by radiolabelling with ³H-thymidine and electron microscopy identified certain immature, granule-free cells with the highest labelling index in a region near the isthmus of units that were presumed to be GSCs (Karam and Leblond, 1993). The first specific

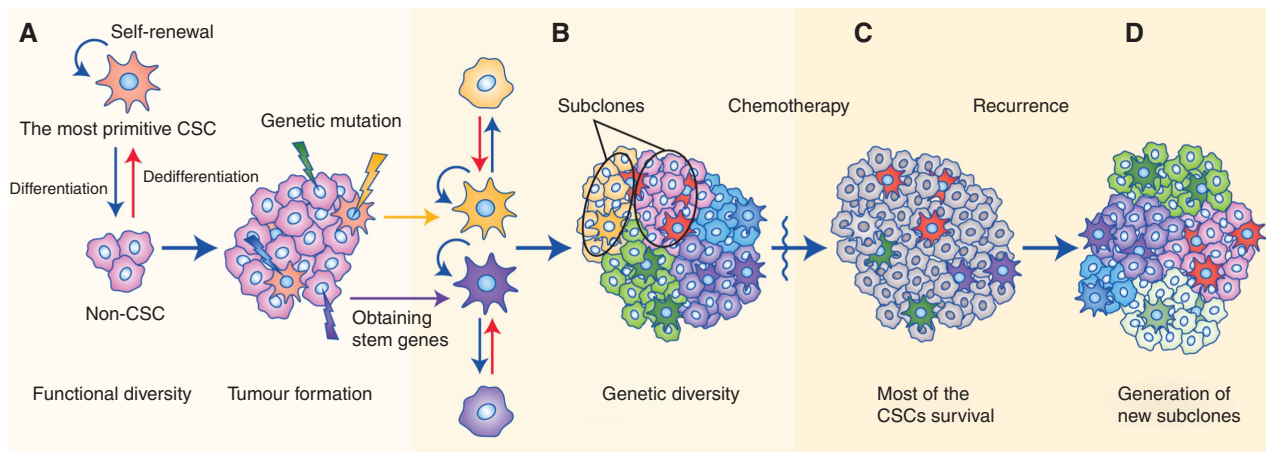


Figure 1. The unified model of cancer. (A) The most primitive CSCs continuously divide, self-renew, differentiate into non-CSCs and eventually form a tumour mass with functional diversity, during the course of which the cancer cells occasionally develop genetic mutations that can endow non-CSCs with stemness. (B) The tumour becomes more heterogeneous and invasive as advantageous mutations in the CSCs accumulate; in addition, several subclones develop, and the tumour cells confer a higher capacity for self-renewal and begin to make up a greater proportion of the total cells. (C) Most of the CSCs are drug-resistant and survive after chemotherapy or radiotherapy. (D) The residual CSCs cause tumour relapse, and more subclones are generated.

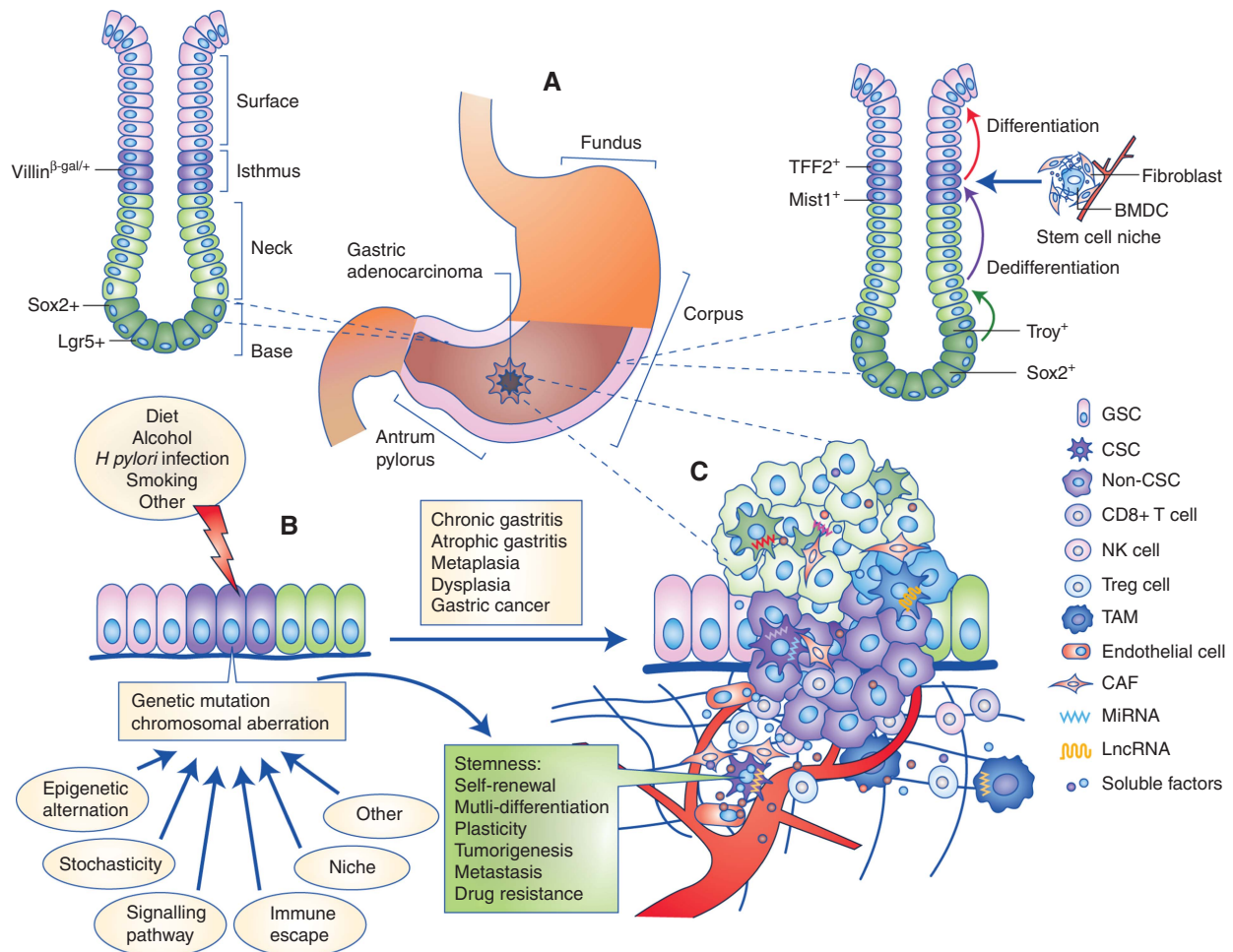


Figure 2. The combination of the hierarchical and stochastic theories of GC. **(A)** GC stem cells originate from gastric stem cells, dedifferentiated epithelial cells or bone marrow-derived cells. **(B)** The accumulation of cancer-associated mutations and chromosomal aberrations in the initiating cells, which are induced by multiple factors, such as diet, alcohol, *H. pylori* infection and smoking, promotes the pathological process of gastric adenocarcinoma, in which the gastric epithelium develops through sequential stages of chronic gastritis, atrophic gastritis, metaplasia (intestinal or spasmolytic polypeptide-expressing), dysplasia and eventually cancer. **(C)** The formation, invasion and metastasis of GC occur according to the unified model, which is dynamically regulated by other determinants such as epigenetic alternation, gene expression stochasticity, immune escape, niche, signalling pathways and networks of soluble factors. CAF = cancer-associated fibroblast.

identification of stem cells *in situ* by the Villin^{β-gal/+} marker demonstrated that this rare subpopulation is primarily located at or below the isthmus of pyloric glands (Qiao *et al*, 2007). Moreover, based on lineage tracing, cells in the isthmus that express trefoil factor family 2 (TFF2) messenger RNA have also been proposed as progenitor cells (Quante *et al*, 2010). Multiple specific markers have also been used to distinguish stem cells from mature epithelial cells in different sites within the stomach. Lgr5 is a well-established stem cell marker in the intestine and colon (Barker *et al*, 2007) that has been shown to be expressed strictly in the bottom of the adult human pyloric glands. Via stimulation of gastric units *in vitro* and lineage tracing *in vivo*, Lgr5⁺ cells have been shown to possess self-renewal capacity; therefore, Lgr5 can be considered an exclusive marker for GSCs (Barker *et al*, 2010). Above the bottom of the pyloric and fundic glands, Sox2⁺ stem cells without a marked overlap in Lgr5 expression were identified by using double-labelling methods and marker tracing (Arnold *et al*, 2011). Recently, some studies have found that a specific subpopulation of chief cells had the potential to function as reserve stem cells. A single Troy⁺ mature chief cell located at the base of the gastric corpus glands could generate an entire gland, based on lineage tracing, and could form long-lived organoids in a three-dimensional culture system (Stange *et al*, 2013). Likewise, Mist1-expressing cells

in the isthmus but not in the lower third of the gastric corpus units have also been observed to have self-renewal ability and to differentiate into multiple lineages of mature cells, such as mucous cells and chief cells. In addition, the use of transgenic mouse models also showed that Mist1⁺ isthmus cells could expand and evolve independently of Lgr5⁺ cells and are the origin of mature epithelial cells in the corpus units (Hayakawa *et al*, 2015). These results show the heterogeneity of stem cells in different regions of the stomach and even in different zones of an identical unit, which implies that GCSCs are heterogeneous.

The mechanism of carcinogenesis is diverse in distinct phenotypes of GSCs, but the model of field cancerisation has been well documented. This model holds that a mutated GSC expands to the whole gland and forms a clonal patch by unit fission, which has been demonstrated previously by the detection of mitochondrial DNA mutations in normal and intestinal metaplastic mucosa adjacent to the tumour tissue of GC patients (McDonald *et al*, 2008). With the identification of GSC-specific markers, numerous driver mutations in the malignant progression of GSCs were verified by functional analysis in transgenic mouse models. A recent study showed that the inactivation of the tumour suppressor gene *Krüppel-like factor 4* (*Klf4*) in Villin⁺ gastric stem-like cells accelerated the malignant transformation of gastric

mucosa in the carcinogen-induced *Villin-Cre⁺;Klf4^{fl/fl}* mice (Li *et al*, 2012). The effect of aberrant Wnt pathway activity, such as the deletion of the *adenomatous polyposis coli* (*APC*) gene, on the carcinogenesis of *Lgr5⁺* stem cells was evaluated in the *Lgr5-CreERT2;APC^{fl/fl}* mouse model. The results showed that β -catenin^{hi} adenomas only in the pylorus were rapidly formed by the expansion of *Lgr5⁺* transformed stem cells after the mice were treated with tamoxifen to induce the loss of APC (Barker *et al*, 2010). A study regarding the effect of *Helicobacter pylori* (*H. pylori*) infection on *Lgr5⁺* stem cells also revealed that these bacteria could directly act on stem cells, promote proliferation and increase the expression of tumour-associated genes, such as the Wnt target gene *Axin2* and the anti-apoptosis gene *Olfactomedin4* (Sigal *et al*, 2015). Similarly, it has been demonstrated that mutations in distinct pathway components in isthmus *Mist1⁺* stem cells of the corpus gland were responsible for the differentiation of intestinal type and diffuse-type GC (IGC and DGC). Such effects have been observed for mutations in the *Kras* gene, facilitating the metaplastic/dysplastic transformation and expansion of *Mist1⁺* isthmus cells; aberrant *Notch* activation, resulting in the development of IGC; and mutations in *E-cadherin* and *transformation-related protein 53* (*TRP53*), inducing the formation and invasion of DGC containing numerous lineage-traced signet-ring cells in the context of chronic inflammation (Hayakawa *et al*, 2015). Collectively, it is presumed that GCSCs are likely derived from aberrant GSCs and that they possess various genetic and functional properties in disparate primary sites. This hypothesis would be more convincing if more in-depth genomic analyses and additional advanced bioinformatics techniques were applied to these stem cells.

GCSCs originate from dedifferentiated epithelial cells. Dedifferentiation is a cellular process by which mature, differentiated cells transform back to a primitive or fetal stage and lose certain characteristics, for example, their specific function or phenotype. Some researchers have suggested that the dedifferentiation of gastric epithelial cells (GECs) may be an aberrant change in the developmental cycle that gives rise to metaplasia, dysplasia and ultimately GC. A study showed that malignant changes in the gastric epithelium of Mongolian gerbils were associated with dedifferentiated mature cells defined by the expression of cytokeratin-7, which is mainly present in fetal epithelial cells (Kirchner *et al*, 2001). Recently, utilising lineage tracing to analyse the origin of the spasmodic polypeptide-expressing metaplasia (SPEM) cells induced by L-635 treatment in the *Mist1-CreERT2* mouse model, a study showed that the loss of acid-secreting cells under an induced inflammatory environment resulted in the rapid production and spread of SPEM that developed from transdifferentiated mature chief cells, which demonstrated the capacity of mature GECs to regain 'stemness' and to initiate a tumour in the presence of inflammation (Nam *et al*, 2010). To explore the mechanism by which gastric mucosa converts to intestinal type, research revealed that the homeobox protein CDX1 could directly bind to and activate the expression of genes encoding reprogramming proteins, such as *SALL4* and *KLF5*, which endow GECs with intestinal stem-like features. The long-term expression of CDX1 induced the generation of intestinal stem cell markers and the subsequent expression of intestinal mature cell markers, which indicated that mature GECs could dedifferentiate into stem-like cells and then transdifferentiate into intestinal type cells via the aberrant expression of stemness-related genes (Fujii *et al*, 2012). In addition, another four transcription factors, *Klf4*, *Oct 3/4*, *c-MYC* and *Sox2*, have also been demonstrated to induce the reprogramming of differentiated GECs into pluripotent stem cells (Aoi *et al*, 2008). Furthermore, the involvement of *H. pylori* in the generation of GCSCs via inducing the epithelial-mesenchymal transition (EMT) of GECs has been reported previously (Bessede *et al*, 2014;

Choi *et al*, 2015). In particular, the CagA protein of *H. pylori* was responsible for the transformation of GECs into a subset of cells with mesenchymal phenotypes and CSC features, including the activation of mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) signalling pathways (Bessede *et al*, 2014).

Although the above studies support the idea that mature GECs are likely to reacquire stemness properties and act as sources of GCSCs, the experimental techniques used in these studies were did not provide sufficient direct evidence of the capacity of mature GECs to dedifferentiate and initiate tumorigenesis. For example, the identification of cells by using specific markers could not definitively distinguish a bona fide stem cell from other cells expressing the identical marker; the observation of stemness characteristics may simply be due to the activation of stem cells among the studied cells. Hence, more rigorous and stronger evidence is needed to validate these data.

GCSCs originate from bone marrow-derived cells. Bone marrow-derived cells (BMDCs) are well known for their striking plasticity regarding their phenotype and function; they constantly migrate to peripheral tissues via chemotaxis towards chronic inflammation and injury, and they display the ability to differentiate into multiple lineages. To date, many biological effects on the gastric mucosa and stroma exerted by BMDCs have been demonstrated, including (1) contributing to the formation of a mesenchymal or perivascular stem cell niche (Quante *et al*, 2011; Hayakawa *et al*, 2015); (2) involvement in the repair of damage caused by chronic inflammation and the regeneration of glandular epithelium (Okamoto *et al*, 2002; Ferrand *et al*, 2011b; Bessede *et al*, 2015); (3) promotion of the proliferation and metastasis of GC cells (Quante *et al*, 2011; Hayakawa *et al*, 2015; Zhu *et al*, 2016a); and (4) acting as a potential source of epithelial cancer (Bessede *et al*, 2015). Despite massive investigative efforts, the mechanism underlying malignant changes in BMDCs is still not well-established. Early research using a mouse model revealed that chronic infection with *H. felis*, rather than acute injury or inflammation, resulted in the recruitment and repopulation of BMDCs into the gastric mucosa, and the progeny of the BMDCs developed subsequently through metaplasia, dysplasia and finally into epithelial cancer (Houghton *et al*, 2004). Recently, using a female mouse model treated with male mouse BMDCs expressing green fluorescent protein (GFP) and infected with different mouse-adapted *H. pylori* strains, metaplastic and dysplastic glands with GFP⁺ cells were detected in most of the mice after 1 year of infection with *H. pylori*, and nearly a quarter of the foci contained these BMDCs (Varon *et al*, 2012). In addition, fluorescence *in situ* hybridisation (FISH) for the Y chromosome was also performed on the stomach tissue sections of *Helicobacter*-infected mice in the above-mentioned two studies to further confirm that donor-derived engrafted BMDCs were the origin of the gastric mucosal cells expressing GFP and epithelial markers (Houghton *et al*, 2004; Varon *et al*, 2012). Thus, chronic inflammation and injury of the gastric mucosa may be a primary cause of BMDC homing. Moreover, the mechanism of BMDC migration in the epithelium is likely associated with the involvement of the nuclear factor-kappa B (NF- κ B) pathway via the secretion of chemokines (tumour necrosis factor- α , TNF- α ; Ferrand *et al*, 2011a). However, some results conflicting with the above evidence have also been presented. A study showed that syngeneic BMDCs labelled with GFP were only marginally scattered in stroma and not in glands of gastric neoplastic lesions or tumours induced by *N*-nitroso-*N*-methylurea (MNU) and *H. felis* infection in recipient mice (Yang *et al*, 2013). Likewise, opposite-sex donor BMDCs were only identified by FISH for sex chromosomes in perineoplastic stroma of secondary GC in an aplastic anaemia patient treated with allogeneic hematopoietic stem cell

transplantation (Worthley *et al*, 2009). The opposite conclusion is also possible because in the human case, chronic inflammation likely existed before transplantation, and in animal experiments, it is difficult to fully model the true conditions. However, the evidence for the properties of stem cells, which are presumed to originate from BMDCs, would be more reasonable if single-cell studies, lineage tracing and optimised animal models were performed.

IDENTIFICATION AND FUNCTIONAL VERIFICATION OF GCSCS

Although many experimental investigations have been performed on the identification and functional verification of GCSCs, the hierarchical model for stomach remains indefinite because of a lack of direct evidence. To date, there are several major methods that can be used to isolate GCSCs, including (1) identifying GCSCs by specific markers (Qiao and Gumucio, 2011); (2) separating GCSCs from side population (SP) cells (Fukuda *et al*, 2009; Nishii *et al*, 2009; Ehata *et al*, 2011; She *et al*, 2012; Hasegawa *et al*, 2014); (3) screening for GCSCs through drugs or radiation treatment (Xue *et al*, 2012; Xu *et al*, 2015b); and (4) capturing tumour cells from circulating blood, metastatic sites or peritoneal milky spots (Cao *et al*, 2009; Chen *et al*, 2012). For these selected CSC-rich subpopulations, two general approaches are used to validate the properties of stemness. One approach is the spheroid body formation assay *in vitro*; the other is the tumour-formation assay *in vivo*.

Identification by specific markers. The antigen CD44 was the first putative marker used to identify GCSCs. The first study showed that CD44⁺ cells separated from three GC cell lines (GCCLs) through fluorescence-activated cell sorting (FACS) exhibited self-renewal capacity as indicated by spheroid formation *in vitro* and tumour formation in mice, while both the CD44⁻ cells and short-hairpin RNA (shRNA) CD44-knockdown cells exhibited a remarkable decline in tumorigenicity (Takaishi *et al*, 2009). Subsequently, CD44v8-10 was identified as the predominant CD44 variant and served as a GCSC marker through a series of experiments in primary tumour samples including the isolation and culture of primary GC cells (GCCs), limiting dilution assays and xenografts in NOD/SCID/IL2R γ ⁻ (NSG) mice (Lau *et al*, 2014). Strikingly, CD44⁺CD54⁺ CTCs captured in the peripheral blood of GC patients have also exhibited the ability to self-renew. Chen *et al* (2012), using an antibody combined with magnetic beads, separated up to 10³ CD44⁺ cells from the blood samples of seven chemotherapy patients, and cells from six of those patients successfully formed tumour spheres when passaged *in vitro*. Furthermore, after transplantation into SCID mice, CD44⁺CD54⁺ cells from these primary cultured cells quickly developed into tumours that were similar to the original lesions, and the remainder of the cells were negative for these markers. To improve specificity, the co-recognition of multiple markers with CD44 has been widely studied, and the relevant data are presented in Table 1. Recently, studies have focused on the mechanism of CD44⁺ cells functioning as GCSCs. Hedgehog (HH) signalling has been proven to have an important role in the maintenance of stemness of CD44⁺ CSCs, as HH signalling proteins are upregulated in

Table 1. Specific markers of gastric cancer stem cells

Markers	Source of specimen	Tumour spheroid formation	Tumour formation <i>in vivo</i>	Limiting dilution assay	Drug resistance	Differentiation capacity	Reference
CD44 ⁺	GCCLs	+	+	Not done	+	+	(Takaishi <i>et al</i> , 2009), (Yoon <i>et al</i> , 2014)
CD44 ⁺ /CD24 ⁺	Patient tissues	+	+	+	Not done	+	(Zhang <i>et al</i> , 2011)
CD44 ⁺ /CD26 ⁺	GCCLs	+	+	Not done	+	Not done	(Nishikawa <i>et al</i> , 2015)
CD44 ⁺ /CD47 ⁺	GCCLs	+	+	Not done	Not done	Not done	(Yoshida <i>et al</i> , 2015)
CD44 ⁺ /CD54 ⁺	Patient tissues	+	+	+	Not done	+	(Chen <i>et al</i> , 2012)
CD44 ⁺ /ABCG2 ⁺	GCCLs	+	+	Not done	Not done	Not done	(Liu <i>et al</i> , 2013a)
CD44 ⁺ /EpCAM ⁺	Patient tissues	+	+	+	+	+	(Han <i>et al</i> , 2011)
CD44v8-10 ⁺ /EpCAM ⁺	Patient tissues	+	+	+	Not done	Not done	(Lau <i>et al</i> , 2014)
CD44 ⁺ /Musashi-1 ⁺	GCCLs	Not done	+	Not done	+	Not done	(Xu <i>et al</i> , 2015a)
ALDH ⁺	GCCLs	+	+	+	+	Not done	(Nishikawa <i>et al</i> , 2013)
ALDH-3A1 ⁺	GCCLs	+	+	Not done	Not done	+	(Wu <i>et al</i> , 2016a)
ALDH ⁺ /REG4	GCCLs	Not done	+	Not done	Not done	+	(Katsuno <i>et al</i> , 2012)
CD49 ^{high}	Patient tissues	+	+	+	+	+	(Fukamachi <i>et al</i> , 2013)
CD71 ⁻	GCCLs	Not done	+	+	+	+	(Ohkuma <i>et al</i> , 2012)
CD90 ⁺	Patient tissues	+	+	+	+	Not done	(Jiang <i>et al</i> , 2012)
CD133 ⁺	GCCLs	+	+	Not done	+	Not done	(Zhang <i>et al</i> , 2016b)
CD133 ⁺ /SOX17 ⁺	GCCLs	Not done	+	Not done	Not done	+	(Fukamachi <i>et al</i> , 2011)
CXCR4 ⁺	GCCLs	Not done	+	+	+	+	(Fujita <i>et al</i> , 2015)
KIF1 ⁺	GCCLs	+	Not done	Not done	Not done	Not done	(Oue <i>et al</i> , 2016)
Lgr5 ⁺	Patient tissues	+	+	Not done	Not done	Not done	(Wang and Liu, 2015)
Oct4 ⁺ /Sox2 ⁺ /Nanog ⁺	GCCLs	+	+	Not done	+	Not done	(Liu <i>et al</i> , 2013b)
SALL4 ⁺	GCCLs	Not done	+	Not done	Not done	+	(Zhang <i>et al</i> , 2014)
Sca-1 ⁺	GCCLs	+	+	+	+	Not done	(Park <i>et al</i> , 2016)
TR3 ⁺	GCCLs	+	+	Not done	+	+	(Zhan <i>et al</i> , 2013)

Abbreviation: GCCLs=GC cell lines.

CD44⁺ GCCs, and the inhibition of HH signalling by Smoothed (Smo) shRNA or vismodegib results in a decrease in tumorigenesis and drug resistance of CD44⁺ cells and the full suppression of cancer cell proliferation, migration and invasion (Yoon *et al*, 2014). Another study also demonstrated the same functions of the SHH-GLI1 pathway and the effector protein ABCG2 in CD44⁺ Musashi-1⁺ GCSCs (Xu *et al*, 2015a).

CD90, another universal marker of multi-tissue stem cells and CSCs, has also been extensively investigated in GC. Jiang *et al* (2012) first observed that the CD90⁺ subpopulation in GC could be characterised as CSCs. In that study, the tumour hierarchy was successfully reconstructed using single cells that were selected from high-tumorigenicity mouse models established by using a different source of 10³ primary GCCs. The stemness properties of these single cells were also shown through the formation of *in vitro* spheroids and *in vivo* tumour masses. The comparison of RNA expression between spheroids and primary tumour cells revealed a marked upregulation of CD90. Then, after single CD90⁺ cells isolated via FACS were implanted into nude mice, nearly 90% developed into tumours. Recently, although an increasing number of specific GCSC markers have been found using the similar experimental techniques mentioned above (Table 1), studies utilising direct methods such as lineage tracing and single-cell analysis have not yet been reported. Furthermore, the expression of CSC markers is not always stable and reliable in different cells and at different times, perhaps due to the variability in mutations, origin of cells, frequency of tumorigenic cells, regulation of the TME or experimental techniques, resulting in the inability to purify true CSCs (Meacham and Morrison, 2013; Kreso and Dick, 2014). Hence, there are still many unknown variables in the hierarchical model of GC, and additional investigations in the heterogeneity and plasticity of CSC phenotypes are needed.

Separation from side population cells. Side population cells, the subpopulation of cells separated from the main population (MP) of cells by flow cytometric markers, have exhibited CSC-like features in many studies (Patrawala *et al*, 2005; Wang *et al*, 2007). With respect to GC, one study showed higher *in vivo* tumorigenicity of SP cells from GCCLs and from GC samples (Fukuda *et al*, 2009). Another study also supports this possibility through the use of spheroid- and tumour-formation assays with SP cells (She *et al*, 2012). However, our group (Zhang *et al*, 2013) previously found that only some of the SP cells possess stemness properties. Thus, the mechanism for distinguishing between SP cells and MP cells requires additional investigation.

Screening through the use of drugs or radiation treatment. Drug resistance, an inherent characteristic of quiescent cells, such as CSCs, may be a feasible approach for acquiring cancer stem-like cells. Confirming the CSC properties of drug-preconditioned GCCLs, recent research found that vincristine-treated cancer cells exhibited mesenchymal features, multi-drug resistance, high tumorigenicity and the abilities to self-renew and differentiate *in vitro* (Xue *et al*, 2012). Likewise, the stemness characteristics of residual cells from 5-fluorouracil (5-FU)-preconditioned GCCLs were also demonstrated by single-cell clonogenic assays and tumorigenicity assays (Xu *et al*, 2015b). Nevertheless, this method may be difficult to implement in primary tumour tissues due to a mix of various drug-resistant cells, such as normal tissue stem cells; therefore, it would be helpful to combine this technique with other identification methods.

Capturing from circulating blood, metastasis sites or peritoneal milky spots. Metastatic cells from original tumours are well known for their capacity to initiate tumours. Milky spots, namely lymphoid tissues in the abdominal cavity, have been indicated as early infiltration sites of the peritoneal dissemination of GCCs (Tsujiyama *et al*, 1996). A milky spot has been assumed to be a

'natural filter' for capturing GCSCs, as a location in which abundant macrophages are cytotoxic against non-CSCs and contribute to a niche for CSC survival (Cao *et al*, 2009). A recent study reported many macrophages and metastatic lesions observed by electron microscopy and DiI-labelled MFCs (a murine GCCL) in omental milky spots (OMSs) in a mouse model, and the researchers verified that OMS-derived cancer cells exhibited remarkable CSC properties, such as higher tumorigenicity through a limiting dilution assay *in vivo* and higher expression of CSC markers (Cao *et al*, 2011). Furthermore, milky spots were also considered to provide a hypoxic microenvironment for GCSCs through the overexpression of hypoxia inducible factor-1 α (HIF-1 α) and to enhance the survival and self-renewal capacity of the CSCs in them (Miao *et al*, 2014). These reports offer an alternative route for investigating the mechanism of cancer cell metastasis and the heterogeneity of CSCs between primary and metastatic sites.

EVIDENCE OF THE CLONAL EVOLUTION MODEL OF GASTRIC CANCER

The heterogeneity, the phenotypic characteristics and the biological function of stomach tumours have been described in many studies over the past several decades; however, genetic variation was not investigated based on a comprehensive molecular evaluation until the emergence of next-generation high-throughput sequencing technology. The first study using whole-exome sequencing found massive somatic mutations including 20 candidate driver genes in 22 matched pairs of GC samples and healthy controls. They reported that mutations of a top putative driver gene, *ARID1A*, were more frequent in the microsatellite instability (MSI) and Epstein-Barr virus (EBV)-infected types of GC and showed a link with a trend of better prognosis (Wang *et al*, 2011). Similarly, another exome sequencing study showed recurrent gene mutations including in the proto-oncogene *PIK3CA* and the tumour suppressor genes *TP53* and *ARID1A* in 15 GC samples, as well as frequent genetic abnormalities including in the E-cadherin family gene *FAT4* and the chromatin remodelling genes (*ARID1A*, *MLL3* and *MLL*), in a prevalence screening among 110 GC samples. The functional assays suggested that inactivation of the *FAT4* and *ARID1A* genes was correlated with malignant behaviour of GC such as cellular proliferation, invasion and migration (Zang *et al*, 2012). The first whole-genome analysis using massively parallel signature sequencing and DNA paired-end tag sequencing in 2 gastric adenocarcinomas (GACs) provided the first detailed reconstruction of a mutant genome and revealed the integrative characteristics of mutational processes in GC compared with exome sequencing (Nagarajan *et al*, 2012). Subsequently, many mutations and epigenetic alterations were identified using similar techniques in different subtypes of GC, and the corresponding functional verification implied their core roles in initiation and progression of GC (Jaiswal *et al*, 2013; Kakiuchi *et al*, 2014; Liang *et al*, 2014; Shimizu *et al*, 2014; Wang *et al*, 2014; Wong *et al*, 2014; Zhou *et al*, 2014; Hu *et al*, 2016; Li *et al*, 2016a, b; Lim *et al*, 2016b). Recently, based on the molecular sequencing and analysis platforms, a robust and comprehensive classification was presented, dividing GC into four molecular subtypes and characterising somatic genomic mutations, alterations in gene expression and signalling pathways by analysing 295 human GACs (Cancer Genome Atlas Research Network, 2014). The following subtypes were defined: (1) EBV-infected tumours, which are mainly characterised by recurrent *PIK3CA* mutations, amplification of the genes for the non-receptor tyrosine kinase *JAK2* and the immune suppressive proteins PD-L1/2, and extensive DNA hypermethylation; (2) MSI tumours, which exhibit increased mutation frequency and hypermethylation, including the epigenetic silencing of the mismatch repair gene *hMLH1*;

(3) genomically stable tumours, which are rich in the diffuse histological subtype, *E-cadherin* and *RHOA* (a member of Ras superfamily) gene mutations and structural rearrangements, especially in the fusions between the *CLDN18* (a member of the claudin family) gene and the *ARHGAP26/6* (Rho GTPase-activating proteins) gene; and (4) chromosomal instability (CIN) tumours, which are distinguished by frequent aneuploidy, the overexpression of p53 (*TP53* mutation) and recurrent genomic amplifications of receptor tyrosine kinases (RTKs), such as EGFR and VEGFR, and downstream effector RAS proteins. Later, the Asian Cancer Research Group proposed a different type of molecular classification (Cristescu *et al*, 2015) to establish an association of the diverse patterns of molecular alterations with tumour progression and prognosis. This proposal defined four subtypes: (1) the mesenchymal-like type, with the worst prognosis and highest recurrence rates; (2) the MSI type, with the best OS and the lowest recurrence rates; (3) the TP53-active; and (4) TP53-inactive types with intermediate clinical outcomes. Furthermore, distinct somatic mutations in peritoneal metastatic tumours *vs* primary tumours were identified (Lim *et al*, 2016a; Liu *et al*, 2016). In general, numerous indisputable pieces of evidence have proven the genetic heterogeneity among different subtypes and even within the same subtype of GC, and functional tests have supported that various driver mutations contributed to the clonal expansion of cancer cells endowed with advantageous functions and characteristics toward a favourable direction for survival. Consequently, recurrent genetic alterations constantly change the phenotypic and functional properties of GC for adapting to hostile environments, implying an important role of clonal evolution in GC heterogeneity and development.

THE COMBINATION OF THE HIERARCHICAL AND STOCHASTIC THEORIES OF GASTRIC CANCER

In light of the studies on the origination and identification of GCSCs, the existence of a functional hierarchy in GCCs may be confirmed. However, the GCSCs identified based on specific markers also possess distinct functions and phenotypes. Despite the lack of an exclusive marker, the deficiency of cell separation and purification technologies and the disparity among cell sources, the combination of the hierarchical and stochastic theories is likely to provide a reasonable explanation for how primitive GCSCs continually optimise their own genome, improve the pattern of gene expression and generate various subclones to adapt to the ever-changing TME (Figure 2B). According to the evidence on the origin of GCSCs, the accumulation of diverse cancer-related mutations induced by carcinogenic factors in stem cells that are derived from the epithelium, mesenchyme or dedifferentiated mature cells results in the formation of the most primitive CSCs, which show distinct genotypes in a variety of histopathological and anatomic sites, even within a single gastric unit, due to the presence of different types of stem cells and genetic alterations. Primitive CSCs acquire the properties of unlimited self-renewal and multilineage differentiation potential and gradually create a hierarchical structure in the tumour lesion. Concurrently, these CSCs evolve into different subclones to survive and to evade the immune system, and the dominant clones generate their respective hierarchical organisations, while the remaining cells are eliminated. Although this hypothesis has been supported by single-cell analyses of other cancers (Navin *et al*, 2011; Lawson *et al*, 2015), genetic and functional studies on GCSCs at the single-cell level are lacking. Hence, using identification, isolation, dynamic observation and deep sequencing techniques with single GCSCs is expected to contribute to existing knowledge and to allow for the study of different subclones of GCSCs via the combination of genomic analyses with functional assays.

REGULATORY MECHANISMS OF THE UNIFIED MODEL OF GC

CSC niche. It is generally accepted that dormant stem cells in a given tissue reside in a specific anatomic location, namely the stem cell niche, where a specialised microenvironment is provided to regulate stem cell fate (Voog and Jones, 2010). Likewise, according to previous studies, there is also a niche around CSCs in tumours (Plaks *et al*, 2015). The CSC niche, containing distinct stromal cells such as fibroblasts, endothelial cells, extracellular matrix and networks of soluble factors and blood vessels (Figure 2C), has an important role in the maintenance of CSC properties and plasticity (Borovski *et al*, 2011). However, the components and the principally regulated pathways of niches in GC are largely unknown. Some studies have suggested that bone marrow-derived myofibroblasts (BMFs), which are involved in the formation of the niche, promote the development and progression of GC (Worthley *et al*, 2009; Quante *et al*, 2011; Shibata *et al*, 2013; Zhu *et al*, 2016a). Using an inflammation-induced GC model, Quante *et al* (2011) found the expansion of MFs in the BM niche at the earliest stage of GC development and subsequent migration into injury sites. The BMFs created a niche that promoted tumorigenesis in TGF- β - and Cxcr4/Cxcl12-dependent pathways. Similarly, another study revealed that the activation of the IL-6/HGF/STAT3 pathway in BMFs-stimulated cancer cells to upregulate TGF- β 1, and vice versa. This interaction between BMFs and GCCs is responsible for the regulation of stemness and tumorigenesis (Zhu *et al*, 2016a). Recent research confirmed that BM-derived Cxcr4⁺ intraepithelial gastric innate lymphoid cells (ILCs) adjacent to Mist1⁺ stem cells in the isthmus area functioned as niche cells to maintain the properties of GSCs (Hayakawa *et al*, 2015). The other essential aspects of the CSC niche, such as hypoxia (Lu *et al*, 2015; Zhang *et al*, 2016a), tumour-associated macrophages (Wan *et al*, 2014; Zhou *et al*, 2015; Jia *et al*, 2016; Raggi *et al*, 2016), blood vessels (Borovski *et al*, 2011) and vasculogenic mimicry (Sun *et al*, 2016), have also been studied for their effects on the stemness of CSCs in various types of tumours. Although the relevant research on GC is insufficient, it can nevertheless be speculated that the niche is important for the survival and evolution of GCSCs.

Epigenetic alterations. Epigenetics refers to heritable alterations in gene expression without the involvement of changes in nucleotide sequences. Similar to genetic mutations, epigenetic alterations also have an irreplaceably important function in the malignant transformation of somatic cells and in the development and progression of cancer. In GAC, many mechanisms of variation have been studied, including DNA hypermethylation, histone modification and epigenetic silencing of noncoding RNAs. Using combined transcriptome and epigenome sequence analyses in EBV-infected GCCLs, it was revealed that the transcription of 216 genes was downregulated due to EBV-related hypermethylation and that tumour-associated proteins, such as Indian hedgehog and TraB domain-containing, were overexpressed with a remarkable increase in gene methylation (Liang *et al*, 2014). In addition, the epigenetic silencing of mismatch repair genes and tumour suppressor genes, including *MLH1*, *CDH1* and *CDKN2A*, was also identified in distinct subtypes of GC (Ushijima and Hattori, 2012; Cancer Genome Atlas Research Network, 2014). Furthermore, many studies (Peterson *et al*, 2010; Tomita *et al*, 2011; Haam *et al*, 2014; Chen *et al*, 2015; Guo *et al*, 2015; Park *et al*, 2015; Kim *et al*, 2016; Yang *et al*, 2016; Yoshida *et al*, 2017) have demonstrated the effects of aberrant epigenetic activation and of the inactivation of diverse genes, including *Tff1*, *Tff2*, *BACH2*, *Sema3E*, *OLFM4*, *TET1*, *TLR4*, *GDF1*, *FAT4* and so on, on GC carcinogenesis and progression through *in vivo* and *in vitro* functional experiments. For instance, Tomita *et al* (2011) observed an extensive

suppression of *Tff1*, a tumour suppressor gene, which is associated with DNA methylation and histone modification at the *Tff1* promoter in epithelial cells of MNU-treated wild-type mice, and subsequent GC initiation. Additionally, there was an enhanced tumorigenic capacity in gastrin- and *Tff1*-deficient mice and, conversely, a repression in hypergastrinemic mice. Hence, it was suggested that the progressive epigenetic silencing of *Tff1* by promoter methylation, which could be reversed by gastrin, contributed to GC initiation (Tomita *et al*, 2011).

The ability of long noncoding RNA (lncRNA; Endo *et al*, 2013; Hu *et al*, 2014; Okugawa *et al*, 2014; Fan *et al*, 2016; Guo *et al*, 2016; Lu *et al*, 2016) and microRNA (miRNA) to regulate GC development and progression is also being elucidated (Table 2). In CD44⁺ GC stem-like cells, it was found that the marked upregulation of miR-106b and the related downregulation of its target protein Smad7 resulted in activation of the TGF- β /Smad pathway, thereby enhancing stemness characteristics such as self-renewal, EMT and invasion (Yu *et al*, 2014). Likewise, another study showed that the overexpression of miR-19b/20a/92a promoted sustained stem-like properties through activating the Wnt- β -catenin pathway in EpCAM⁺CD44⁺ GCCs (Wu *et al*, 2013). Furthermore, our group and other researchers revealed an important role of lncRNA and miRNA in regulating CD44 expression of GCCs (Hashimoto *et al*, 2010; Hu *et al*, 2014;

Wei *et al*, 2016). Recently, Bintu *et al* (2016) investigated the regulatory mechanism of epigenetics at the single-cell level and proposed a three-state dynamic model of epigenetic silencing, in which the state of gene expression was stochastically converted among activation, reversible silencing and irreversible silencing in an all-or-none manner, namely, by regulating the fraction of repressed cells but not the level of gene expression. Hence, epigenetic aberrations contributed to the distinct functional types and phenotypes of individual GCCs, particularly the initiation, persistence and progression of CSCs; however, additional functional assays are necessary to validate the emergence of epigenetic alterations in identifying potential therapeutic targets.

Other regulatory mechanisms. Almost all of the genetic and epigenetic functions of cells are performed and regulated in the context of various types of signalling pathways, and the dysregulation of such pathways contributes to tumorigenesis. For example, the Wnt pathway significantly influences the development of various tumours (Takahashi-Yanaga and Kahn, 2010). Many studies have shown that the canonical Wnt/ β -catenin pathway is involved in the acquisition and maintenance of the stem-like features of GCCs (Wang *et al*, 2013; Mao *et al*, 2014; Yong *et al*, 2016; Zhou *et al*, 2016), and non-canonical Wnt pathways, especially those activated by Wnt5a, are important in

Table 2. Effects of microRNAs on the stemness properties of gastric cancer cells

MiRNA Expression	Source of specimen	Targets	Functional validation <i>in vitro</i> and <i>in vivo</i>	Reference
MiR-19b/20a/92a \uparrow	EpCAM ⁺ CD44 ⁺ GCCs	E2F1 \downarrow HIPK1 \downarrow Wnt/ β -catenin pathway \uparrow	The capacity of self-renewal and proliferation \uparrow	(Wu <i>et al</i> , 2013)
MiR-23b \downarrow	GCCLs	Notch 2 receptor \uparrow Ets1 \uparrow E2F1 \uparrow	Tumorigenesis, growth, progression and tumorsphere formation \uparrow	(Huang <i>et al</i> , 2015)
MiR-29c \downarrow	GCCLs	ITGB1 \uparrow	Tumorigenesis, proliferation, adhesion, invasion and migration \uparrow	(Han <i>et al</i> , 2015)
MiR-34 \downarrow	P53-deficient GCCLs	Bcl-2 \uparrow Notch \uparrow HMGA2 \uparrow	Tumorigenesis and tumorsphere formation \uparrow	(Ji <i>et al</i> , 2008)
MiR-106b \uparrow	CD44 ⁺ GCCLs	Smad7 \downarrow TGF- β /Smad pathway \uparrow	The capacity of self-renewal, EMT and invasiveness \uparrow	(Yu <i>et al</i> , 2014)
MiR-124 \downarrow	GCCLs	SMOX \uparrow H ₂ O ₂ production \uparrow	Not done	(Murray-Stewart <i>et al</i> , 2016)
MiR-133b \downarrow	GCCLs	FSCN1 \uparrow	Proliferation, invasion and migration \uparrow	(Guo <i>et al</i> , 2014)
MiR-141 \downarrow	GCCLs	TAZ \uparrow	Proliferation, invasion and migration \uparrow	(Zuo <i>et al</i> , 2015)
MiR-145 \downarrow	GCCLs	N-cadherin (CDH2) \uparrow	Invasion and migration \uparrow	(Gao <i>et al</i> , 2013)
MiR-148a \downarrow	GCCLs	ROCK1 \uparrow	Invasion and migration \uparrow	(Zheng <i>et al</i> , 2011)
MiR-149 \downarrow	GCCLs and human tissues	IL-6 \uparrow PTGER2/EP2 \uparrow	The effect of CAFs on EMT, invasion and stemness of GC \uparrow	(Li <i>et al</i> , 2015)
MiR-155-5p \downarrow	GCCLs and human tissues	NF- κ B p65 \uparrow	Transition of BM-MSc to GC-MSc \uparrow	(Zhu <i>et al</i> , 2016b)
MiR-181c \downarrow	GCCLs	NOTCH4 \uparrow KRAS \uparrow	Tumorigenesis \uparrow	(Hashimoto <i>et al</i> , 2010)
MiR-200b \downarrow	GCCLs	ZEB1/2 \downarrow	Invasion and migration \uparrow	(Kurashige <i>et al</i> , 2015)
MiR-210 \downarrow	GCCLs	STMN1 \uparrow DIMT1 \uparrow	Chronic gastric diseases and tumorigenesis \uparrow	(Kiga <i>et al</i> , 2014)
MiR-328 \downarrow	GCCLs	CD44 \uparrow	Tumorigenesis, resistance to drugs and ROS \uparrow	(Ishimoto <i>et al</i> , 2014)
MiR-373 \downarrow	GCCLs	CD44 \uparrow	Stemness \uparrow	(Wei <i>et al</i> , 2016)
MiR-448 \uparrow	GCCLs	KDM2B \downarrow Myc \uparrow	Glycolytic metabolism \uparrow	(Hong <i>et al</i> , 2016)
MiR-483-5p \uparrow	Spheroid from GCCLs	Wnt/ β -catenin pathway \uparrow	Growth, invasion and self-renewal of GCSCs \uparrow	(Wu <i>et al</i> , 2016b)
MiR-490-3p \downarrow	GCCLs	SMARCD1 \uparrow	Tumorigenesis, growth, migration and invasiveness \uparrow	(Shen <i>et al</i> , 2015)
MiR-508-5p \uparrow	GCCLs	ABCB1 \uparrow ZNRD1 \uparrow	Multi-drug resistance \uparrow	(Shang <i>et al</i> , 2014)
MiR-1290 \uparrow	GCCLs	FOXA1 \downarrow	Proliferation and migration \uparrow	(Lin <i>et al</i> , 2016)

Abbreviations: CAF = cancer-associated fibroblast; EMT = epithelial-mesenchymal transition; GCCLs = GC cell lines; GCSC = gastric cancer stem cell; GC-MSc = gastric cancer tissue-derived mesenchymal stem cell; ROS = reactive oxygen species.

regulating EMT and initiating GCSCs (Kanzawa *et al*, 2013; Hayakawa *et al*, 2015). The HH family, as another key regulator during embryonic development, has also been implicated in the formation of diverse tumours (McMillan and Matsui, 2012). In human gastric glands, the SHH pathway has important roles in self-renewal (Song *et al*, 2011), chemotherapy resistance of CSCs (Yoon *et al*, 2014; Xu *et al*, 2015a) and tumour angiogenesis (Chen *et al*, 2011). In addition, multiple signalling pathways, including those associated with Notch-1 (Yeh *et al*, 2009; Hong *et al*, 2014)/Notch2 (Tseng *et al*, 2012; Huang *et al*, 2015), STAT3 (Jenkins *et al*, 2005; Ernst *et al*, 2008; Tye *et al*, 2012), TGF- β /Smad (Yoo *et al*, 2008; Yu *et al*, 2014; Yang *et al*, 2016), TNF- α /TNFR1 (Oshima *et al*, 2014), PI3K/AKT/mTOR (Yun *et al*, 2014), c-Met (Yashiro *et al*, 2013) and Ras/Raf/MEK/ERK (Bessedes *et al*, 2014), are likely to be associated with the malignant behaviour of GCSCs. In total, all of these pathways form a complex signalling network inside and outside cancer cells via crosstalk between different axes and via mutual regulation by their products, such as cytokines, chemokines, transcription factors and other regulatory proteins. In addition, there are many unknown or unclear mechanisms, such as the stochasticity of gene expression and the plasticity of cancer cells that promote CSC development and evolution. Therefore, it is impossible for a single theory to elucidate this complicated biological system, whereas a unified model is better able to present a comprehensive and reasonable explanation.

CLINICAL TARGETED THERAPY

The primary methods of targeted therapy in GC involve small molecules, tumour vaccines, monoclonal antibodies (mAbs) and adaptive cellular immunotherapies, including the chimeric antigen receptor T-cell (CAR-T) strategy. Regarding GCSCs, the major drug treatment targets are specific antigens and stemness-related signalling pathways.

Targeting specific antigens. CD44 is a surface marker that is expressed in GC stem-like cells. A study found that CD44v promoted glutathione synthesis and inhibited the activity of reactive oxygen species, thereby resulting in tumour growth via interaction with xCT (a glutamate-cystine transporter; Ishimoto *et al*, 2011). Accordingly, a dose-escalation clinical study for targeting CD44v⁺ CSCs by the oral administration of sulfasalazine (SSZ, an inhibitor of xCT) in patients with AGC was conducted. The results showed that SSZ was safe and efficacious in patients, some of whom exhibited decreased levels of CD44v⁺ cells and glutathione in their biopsy samples (Shitara *et al*, 2016). Moreover, several types of mAbs, such as bivatuzumab mertansine (BIMI1) and RO5429083, have been designed to fight CD44⁺ cells. However, multiple phase I clinical trials in different tumours showed that a few patients treated with CD44v6-targeting BIMI1 had fatal drug-associated adverse events, and the overall assessment of the benefits and risks was negative (Tijink *et al*, 2006; Rupp *et al*, 2007; Sauter *et al*, 2007; Riechelmann *et al*, 2008). Thus, the further clinical development of BIMI1 was terminated. RO5429083, also known as RG7356, is a humanised IgG1 mAb that is specific to CD44. In a phase I study, among 44 patients with refractory or relapsed acute myeloid leukemia, two patients treated with RG7356 had complete responses, and one had stable disease, and the side effects were generally mild or moderate (Vey *et al*, 2016). The study of RG7356 in patients with CD44⁺ advanced solid tumours, including GC, was sponsored by Roche (NCT01358903), and it is expected to reach a favourable outcome.

Epithelial cell adhesion molecule (EpCAM) is a transmembrane glycoprotein in normal epithelia and is upregulated in epithelial neoplasms. In addition, it is a marker widely expressed in CSCs and is involved in the activation of a stemness-related pathway

(Gires *et al*, 2009; Munz *et al*, 2009). EpCAM has long been considered a potential target for antitumour therapy, and vaccination with EpCAM protein has been proposed (Mosolits *et al*, 2004). Furthermore, clinical trials of mAbs have ranged from testing the first murine IgG2a antibody, termed edrecolomab (Mosolits *et al*, 2004), to the EpCAM/CD3 bi-specific antibody MT110 (NCT00635596) and catumaxomab (Heiss *et al*, 2010; Goere *et al*, 2014; Bokemeyer *et al*, 2015), and ultimately to human engineered or fully human antibodies including ING-1 (Mosolits *et al*, 2004) and adecatumumab (MT201; Schmidt *et al*, 2012). Heiss *et al* (2010) found that catumaxomab provided a significant clinical benefit to GC patients with malignant ascites in a phase II/III trial. Another study has also demonstrated the safety and efficacy of catumaxomab in patients with resectable GC when serving as a part of a combined modality therapy (Bokemeyer *et al*, 2015). Moreover, a phase II trial of the intraperitoneal infusion of catumaxomab to treat postoperative GC patients with resectable primary lesions and peritoneal carcinomatosis is currently underway (Goere *et al*, 2014). In addition, a clinical study (NCT02725125) aiming to assess the safety and efficacy of EpCAM-targeted CAR-T for GC is currently recruiting patients.

CD133⁺ cells represent a subpopulation of CSCs in many solid tumours (Grosse-Gehling *et al*, 2013). Although several mAbs (Swaminathan *et al*, 2010; Huang *et al*, 2013) against CD133 have been developed, retrospective clinical trials are lacking. Recently, our research team successfully generated anti-CD133 CAR vector-transduced T cells, and a phase I clinical study (NCT02541370) on the safety and efficacy of our CART133 formulation for treating relapsed or chemotherapy-refractory advanced malignancies including GC is currently recruiting patients. In addition, the University of Minnesota has sponsored a clinical trial to test CD133KDEL toxin (an anti-CD133 ligand-directed, non-immunogenic pseudomonas toxin) in solid tumour patients (NCT02845414).

Programmed cell death-ligand 1 (PD-L1) is now considered a critical component of the immunosuppressive microenvironment in GAC (Thompson *et al*, 2016). Multiple inhibitors of immune checkpoints have been applied to clinical therapy, especially the anti-PD-1 mAb pembrolizumab, which has shown manageable side effects and a 22% (8/36) overall response rate in a phase I study of PD-L1⁺ AGC patients (Muro *et al*, 2016). However, another multicenter study on the anti-PD-L1 mAb nivolumab showed no benefit in 7 GC patients (Brahmer *et al*, 2012). Many promising studies of combination treatments of pembrolizumab or nivolumab with different therapies and new drugs including atezolizumab (a human engineered anti-PD-L1 mAb) and CA-170 (a PD-L1/2 and VISTA checkpoint inhibitor) are underway in GC patients.

Lgr5 and Sox2 are thought to be specific markers of GCSCs and normal stem cells (NSCs). A Sox2-targeted plasmid DNA vaccine has been developed and used to treat patients with advanced breast cancer (NCT02157051). In addition, an anti-Lgr5 humanised mAb, BNC101, is currently being tested in a phase I clinical study of patients with metastatic colorectal cancer (NCT02726334). The expected results may promote the application of these drugs in GC patients.

Targeting stemness-related signalling pathways. HH pathway inhibitors have been designed to target four major sites, specifically SMO, Hh ligand, Hh acyltransferase (HHAT) and GLI. To date, the drugs applied in clinical trials only include SMO inhibitors, such as vismodegib (GDC-0449), sonidegib (LDE225), saridegib (IPI-926), taladegib (LY2940680), glasdegib (PF-04449913), TAK-441, BMS-833923 (XL139) and LEQ506. Although some have exerted targeted efficacy against tumours (Justilien and Fields, 2015; Migden *et al*, 2015; Robinson *et al*, 2015), the clinical results have not shown clear improvements in terms of therapeutic effects

in GC patients. A randomised, controlled, multicenter phase II clinical trial conducted by the NCI to evaluate the efficacy of chemotherapy (FOLFOX) with or without vismodegib in advanced gastric and gastroesophageal junction (GEJ) cancer patients has been completed, and it revealed no significant differences in the outcome measures, including progression-free survival (PFS), relative risk (RR) and OS (NCT00982592). Likewise, the GC patients in multiple phase I clinical trials rarely experienced a confirmed complete or partial response to other SMO inhibitors, such as the objective response being 0/1 for sonidegib by Rodon *et al* (2014) and 0/1 for TAK-441 by Goldman *et al* (2015). Nevertheless, some studies have been conducted, including a phase 1b/2 clinical trial of taladegib in combination with chemotherapy and radiotherapy to treat patients with oesophageal or GEJ adenocarcinoma (NCT02530437) and some GC-related clinical studies.

There are three major types of Notch pathway-targeted drugs: (1) Notch receptor inhibitors: brontictuzumab (OMP-52M51, anti-Notch1), tarextumab (OMP-59R5, anti-Notch2/3) and BMS906024 (pan-Notch inhibitor); (2) inhibitors of the Notch ligand DLL4: MED10639, demcizumab (OMP-21M18), OMP-305B83 and enoticumab (REGN421); and (3) γ -secretase inhibitors: MK0752, RO4929097, PF03084014, BMS986115, LY3039478 and LY900009. However, only a few solid tumour patients have experienced

complete responses to selected drugs, including MK0752 (Krop *et al*, 2012; Piha-Paul *et al*, 2015) and PF03084014 (Messersmith *et al*, 2015). Moreover, many drugs induce a variety of severe adverse events such as intractable diarrhoea, vomiting, skin disorders and hypophosphatemia (Takebe *et al*, 2014), perhaps due to the wide distribution of the Notch pathway in normal tissues, especially NSCs. Hence, the antitumour activity of Notch pathway inhibitors must be confirmed based on the results of ongoing clinical trials.

Drugs targeting the Wnt pathway have been developed and have emerged for clinical use within the last 5 years. The mechanisms associated with targeting the Wnt pathway have been categorised as follows: (1) targeting the Wnt receptor: Foxy-5 (mimicking the effects of Wnt-5a), OTSA101 (anti-Fzd10), ipafriccept (Fzd8-Fc, OMP-54F28); (2) β -catenin antagonists: PRI-724, CWP232291 (CWP291); (3) PORCN protein inhibitors: WNT974 (LGK974), ETC-1922159 (ETC-159), CGX1321; and (4) other inhibitors: DKN01 (LY2812176, anti-DKK1), LY2090314 (anti-GSK3 β). A phase I clinical trial that assessed the combination treatment of LY2090314 with pemetrexed and carboplatin in patients with solid tumours reported an overall response rate of 5/41, but two GC cases had no objective response (Gray *et al*, 2015). Most of the clinical studies on targeting the Wnt pathway are in progress, although a few have been terminated or suspended due to severe adverse events.

Table 3. Clinical trials of targeted therapy against CSC-related targets in AGC patients

Target	Drug	Mechanism	Registration	Phase	Treatment	Results
CD44v	Sulfasalazine	xCT inhibitor	EPOC1205	I	Sulfasalazine	Positive
C-MET	Rilotumumab/AMG-102	Anti-HGF mAb	RILOMET-1/ NCT01697072	III	ECX with or without Rilotumumab	Terminated duo to increased death risk
C-MET	Rilotumumab/AMG-102	Anti-HGF mAb	RILOMET-2/ NCT02137343	III	CX with or without Rilotumumab	Terminated duo to increased death risk
C-MET	Onartuzumab/MetMab	Anti-MET mAb	NCT01590719	II	FOLFOX6 with or without Onartuzumab	MET ⁺ PFS: 5.95 mo vs 6.80 mo ($P=0.45$), OS: 8.51 mo vs 8.48 mo ($P=0.80$); Shah <i>et al</i> , 2016)
C-MET	Onartuzumab/MetMab	Anti-MET mAb	NCT01662869	III	FOLFOX6 with or without Onartuzumab	MET 2+/3+ PFS: 6.9 mo vs 5.7 mo ($P=0.223$), OS: 11.0 mo vs 9.7 mo ($P=0.062$); Shah <i>et al</i> , 2015)
C-MET	Tivantinib/ ARQ-197	c-Met receptor tyrosine kinase inhibitor	NCT01611857	II	Tivantinib + mFOLFOX	ORR: 10/34 with 1CR, 9PRs, PFS: 6.1 mo, OS: 9.6 mo (Pant <i>et al</i> , 2015)
C-MET	Savolitinib/AZD6094/ Volitinib	c-Met receptor tyrosine kinase inhibitor	NCT02447380 and NCT02447406	II	Volitinib + docetaxel	Ongoing
C-MET	AMG 337	c-Met receptor tyrosine kinase inhibitor	NCT02344810	I/II	FOLFOX6 with or without AMG 337	Ongoing
EpCAM	Catumaxomab	Anti-CD3 and Anti-EpCAM mAb	NCT00836654	II/III	paracentesis with or without catumaxomab	Positive
EpCAM	Catumaxomab	Anti-CD3 and Anti-EpCAM mAb	NCT01784900	II	Surgical resection + catumaxomab	Terminated
EpCAM	Catumaxomab	Anti-CD3 and Anti-EpCAM mAb	No	II	Chemotherapy + Surgery + catumaxomab	4-Year DFS and OS rates: 38 and 50%
EpCAM	CAR-T	Anti-EpCAM	NCT02725125	I/II	CAR-T cells	Ongoing
mTOR	Everolimus/RAD001	mTOR inhibitor	GRANITE-1/ NCT00879333	III	BSC with or without Everolimus	PFS: 1.7 mo vs 1.4 mo ($P=0.78$), OS: 5.4 mo vs 4.3 mo ($P=0.124$); Ohtsu <i>et al</i> , 2013)
PD-L1	Pembrolizumab	Anti-PD-L1 mAb	KEYNOTE-012/ NCT01848834	Ib	Pembrolizumab	Positive RR: 8/36 with 8PRs
PD-L1	Nivolumab/MDX1105	Anti-PD-L1 mAb	NCT00729664	I	Nivolumab	Negative RR: 0/7
Raf	Sorafenib/ BAY 43-906	Tyrosine kinase and Raf kinase inhibitor	ECOG5203/ NCT00253370	II	Sorafenib + docetaxel + cisplatin	ORR: 18/44 with 18 PRs, PFS: 5.8 mo, OS: 13.6 mo (Sun <i>et al</i> , 2010)
SHH	Vismodegib/GDC0449	SMO inhibitor	NCT00982592	II	FOLFOX with or without Vismodegib	PFS: 8.4 mo vs 8.8 mo ($P=0.87$), OS: 12.1 mo vs 15.4 mo ($P=0.253$)
SHH	Taladegib/ LY2940680	SMO inhibitor	NCT02530437	Ib/2	Taladegib pre- or with Chemo radiation	Ongoing

Abbreviations: AGC = advanced gastric cancer; BSC = best supportive care; CR = complete remission; CSC, cancer stem cell; CX = Cisplatin and Capecitabine; DFS = disease-free survival; ECX = Epirubicin, Cisplatin and Capecitabine; FOLFOX = Fluorouracil, Leucovorin and Oxaliptin; mo = months; ORR = overall response rate; OS = overall survival; PFS = progression-free survival; PR = partial remission; RR = response rate.

The clinical trials of other agents aimed at targeting CSC-related pathways in GC patients are shown in Table 3. Although the majority of existing results have demonstrated negative or limited efficacy, we believe that targeted therapy for CSCs is a feasible and promising approach for developing cures for cancer in light of successful analogous research in multiple types of tumours.

FURTHER PERSPECTIVES

Currently, the primary problems with regard to the safety and efficacy of targeted therapies against solid tumours are off-target effects and drug resistance, a situation that largely reflects the complexity and dynamic variation of cancer cells. Technological advances in molecular biology have revolutionised our understanding of tumour development. In particular, sequencing techniques have confirmed multiple subclones with distinct genomes in tumours, and functional experiments have revealed rare subpopulations with stemness properties, indicating that tumour growth can be considered as an evolutionary tree, in which the most primitive CSCs, representing the trunk, continuously divide, self-renew and differentiate. Subsequently, new mutations emerge as branches, and the main subtypes are gradually formed due to selection by the TME. However, most of the mechanisms underlying the unified model remain unclear. Even within a single genotype, each individual cancer cell possesses different gene expression levels, phenotypes and functional characteristics, and this diversity is constantly regulated. Hence, many problems remain to be resolved, such as how heterogeneity is generated among different CSCs with the same genome, whether cancer could be cured by killing CSCs just as one may cut down the trunk of a tree, and so forth. Additional promising studies are currently focused on investigating the mechanisms of heterogeneity in CSCs at the single-cell level, which will likely aid in addressing the current challenge of developing clinical CSC-targeted treatments.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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