British Journal of Pharmacology

British Journal of Pharmacology (2017) **174** 4666–4683 4666

Themed Section: WNT Signalling: Mechanisms and Therapeutic Opportunities

REVIEW ARTICLE

Winding back Wnt signalling: potential therapeutic targets for treating gastric cancers

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Received 2 March 2017; **Revised** 17 May 2017; **Accepted** 22 May 2017

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Gastric cancer persists as a frequent and deadly disease that claims over 700 000 lives annually. Gastric cancer is a multifactorial disease that is genetically, cytologically and architecturally more heterogeneous than other gastrointestinal cancers, making it therapeutically challenging. As such, and largely attributed to late-stage diagnosis, gastric cancer patients show only partial response to standard chemo and targeted molecular therapies, highlighting an urgent need to develop new targeted therapies for this disease. Wnt signalling has a well-documented history in the genesis of many cancers and is, therefore, an attractive therapeutic target. As such, drug discovery has focused on developing inhibitors that target multiple nodes of the Wnt signalling cascade, some of which have progressed to clinical trials. The collective efforts of patient genomic profiling has uncovered genetic lesions to multiple components of the Wnt pathway in gastric cancer patients, which strongly suggest that Wnt-targeted therapies could offer therapeutic benefits for gastric cancer patients. These data have been supported by studies in mouse models of gastric cancer, which identify Wnt signalling as a driver of gastric tumourigenesis. Here, we review the current literature regarding Wnt signalling in gastric cancer and highlight the suitability of each class of Wnt inhibitor as a potential treatment for gastric cancer patients, in relation to the type of Wnt deregulation observed.

LINKED ARTICLES

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Abbreviations

M_3 receptor, ACh-muscarinic receptor-3; APC, adenomatous polyposis coli; BCL-9, B-cell lymphoma-9; CagA, cytotoxin-associated gene product; CBP, cAMP response element-binding protein; CK1, casein kinase 1; CRC, colorectal cancer; CRD, cysteine rich domain; CSC, cancer stem cell; CTNNB1, β-catenin; DKK, dickkopf; Dvl, dishevelled; ER, endoplasmic reticulum; FZD, frizzled; GAPPS, gastric adenocarcinoma and proximal polyposis of the stomach; GC, gastric cancer; GSK3B, glycogen synthase kinase-3β; *H. pylori*, *Helicobacter pylori*; HER2, human epidermal growth factor receptor 2; IWP-2, inhibitor of Wnt production-2; LEF1, lymphoid enhancer-binding factor-1; LGR5, leucine-rich repeat-containing G-protein-coupled receptor-5; LOF, loss of function; Mist1, muscle, intestine and stomach expression-1; MSI, microsatellite instability; PARsylation, poly(ADP-ribosyl)ation; PUMA, p53 up-regulated modulator of apoptosis; RNF43, ring finger 43; sFRP, secreted frizzled-related proteins; TNK1/2, tankyrase 1/2; TOP, T-cell factor optimal promoter; ZNRF3, zinc-ring finger 3

DOI:10.1111/bph.13890

Introduction

Gastric cancer (GC) is a frequent malignancy and is the third most common form of cancer-related death world-wide (Guggenheim and Shah, 2013). Approximately 1 000 000 new cases of GC are diagnosed annually, with a large proportion of cases reported within East Asia, South America and Eastern Europe (Rahman *et al.*, 2014). Wnt signalling regulates many cell functions, including proliferation, migration and cell death, and although it is essential for the development and homeostasis of several tissues, it is also deregulated in many cancers. The link between aberrant Wnt signalling and cancer is well characterized in intestinal, breast and liver cancers; however, its importance in the stomach is less well understood. This review will describe the current position of the field regarding the role of Wnt signalling in GC and how we might target the Wnt pathway to treat GC patients.

Homeostasis of the stomach

The mammalian stomach is divided into two anatomically distinct regions; the corpus and the antrum. The corpus is responsible for the main digestive action of the stomach, releasing a cocktail of acids, enzymes and hormones, whereas the antrum produces large amounts of mucus and gastric hormones (Figure 1A). The stomach is lined by a simple columnar epithelium that is constantly renewed, a process driven by resident adult stem cells (Hoffmann, 2008; Barker *et al.*, 2010a). The gastric epithelium is organized into numerous mucosal invaginations called gastric units. Each gastric unit houses stem cells as well as the various differentiated cell types that perform distinct functions: mucus cells that secrete protective mucus, parietal cells responsible for secreting hydrochloric acid, chief cells that release active pepsin and several types of endocrine cells that secrete an array of hormones that aid and regulate digestion and absorption (Kim and Shivdasani, 2016). The precise architecture, cellular heterogeneity and turnover rate of the gastric units varies markedly between the antrum and corpus (Figure 1B, C) (Karam and Leblond, 1993; Barker *et al.*, 2010b). There are many facets that help regulate gastric epithelial homeostasis including key developmental pathways and interactions from underlying stromal and nerve cells (Hayakawa *et al.*, 2015; Kim and Shivdasani 2016). High Wnt signalling is observed transiently in the developing forestomach (Kim *et al.*, 2005), while in the adult stomach, expression is highest in the antrum where the Wnt target gene leucine-rich repeat-containing GPCR (**Lgr5**) marks a population of stem cells (Barker *et al.*, 2010a). Further functional experiments will be required to elucidate the full requirement for Wnt signalling during gastric homeostasis, as was recently shown for Notch signalling (Kim and Shivdasani, 2011; Demitrack *et al.*, 2015), but its critical inclusion in the culture medium of gastric organoids advocates its importance (Barker *et al.*, 2010a).

Histopathology of gastric cancer

Gastric cancer can be divided pathologically into broad classes: intestinal-type and diffuse-type as classified by Lauren (1965). Of note, each of these classes can be further broken down into sub-classes based on histopathological, anatomical and genomic characteristics. Proximal intestinal-type gastric, also referred to as proximal non-diffuse GC, is defined by tumours located in the gastric cardia, which may extend into the gastroesophageal junction (Figure 1A). Histological analysis of proximal intestinal-type GC reveals glandular dysplasia, which can be accompanied by chronic inflammation (Shah *et al.*, 2011). However, unlike the chronic inflammation associated with distal intestinal-type GC, which is often connected to *Helicobacter pylori* (*H. pylori*), carcinogenic

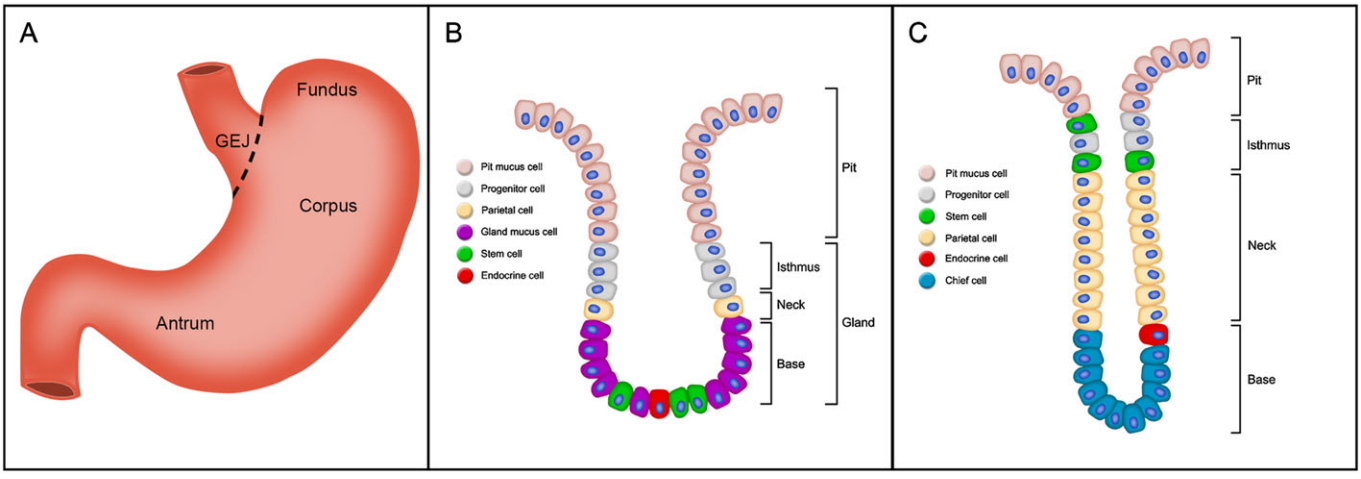

Figure 1

Anatomy of the mammalian stomach and mucosa. (A) Gross anatomy of the stomach, illustrating the gastroesophageal junction (GEJ), fundus, corpus and antrum. (B, C) Schematic of antral (B) and corpal (C) gastric units and their various epithelial cell types. Each unit is divided into a surface pit, isthmus, neck and base regions. Of note, the cellular composition and architecture varies between the antrum and corpus, which reflects functional specificity.

inflammation of proximal intestinal-type GC is often linked to gastric acid reflux (Crew and Neugut, 2006). Distal intestinal-type (non-diffuse) GCs are primarily located in the antrum but can occasionally be found in the body of the stomach (Wong and Yau, 2013) (Figure 1A). Distal intestinal-type GCs are well-differentiated and are composed of neoplastic gland-forming cells. Furthermore, this type of gastric tumour is often associated with *H. pylori* infection, the histopathology of which has been well characterized (Wong, 2016). Finally, diffuse GC, which is characterized by a diffuse pattern of cell infiltrate and poorly differentiated signet-ring cell clusters, is considered to arise *de novo* and is associated with loss of cell–cell contact *via* down-regulation/mutation to *CDH1* (E-cadherin) (Guilford *et al.*, 1998; 1999).

While surgical intervention is the common practice for GC treatment, most patients are often at an advanced stage of disease progression at the time of diagnosis, which limits the value of surgery. As such, chemotherapy is the next appropriate countermeasure for these patients. However, only 20–40% of patients respond to first-line chemotherapies that target DNA replication and repair mechanisms (folinic acid, fluorouracil and oxaliplatin, and folinic acid, fluorouracil and irinotecan), with a median overall survival of 6–11 months (Jemal *et al.*, 2011). The past decades of fervent research into gastric tumour biology has significantly illuminated our understanding of tumour genomics, heterogeneity, immunoediting and drug resistance, which have culminated in the development of molecular targeted therapies. Targeted therapy not only confers higher anticancer specificity and selectivity than chemotherapy but also reduces unwanted non-selective toxicity. While targeted therapies such as trastuzamab/pertuzamab [human epidermal growth factor receptor 2 (HER2)], certuximab (EGFR) and ramucirumab (vascular endothelial growth factor receptor-2) have been utilized in the clinic, often in combination with chemotherapy, they typically yield only a partial response and are prone to resistance (Blackham *et al.*, 2016). Of note, the proportion of Epstein–Barr virus positive GCs that display increased PD-L1 expression (Cancer Genome Atlas Research, 2014) has prompted several clinical trials testing 'breakthrough' immune checkpoint inhibitors (tremelimumab and nivolumab) in patients at various stages of the disease (Kang *et al.*, 2017) Despite the impressive antitumour effects of immune checkpoint inhibitors observed in other solid tumours, GC patients only show a modest increase in survival – 5.32 (nivolumab) versus 4.41 (placebo) months, highlighting the need for fully randomized trials to properly identify GC patients who will benefit from immunotherapies. The limited response and frequent resistance to first-line chemotherapy and adjuvant molecular therapies is partially attributed to an incomplete understanding of the biology of GC (Zhang and Fan, 2010). As such, the continual discovery of additional signalling pathways that drive gastric tumourigenesis and progression provide novel therapeutic avenues that are desperately needed in the clinic.

To date, there is a large quantity of data demonstrating that Wnt signalling plays a critical role in driving gastric tumourigenesis, invasion and metastasis (Radulescu *et al.*, 2012; Zhao *et al.*, 2013). Here, we review the relevant studies implicating aberrant Wnt signalling in GC and how Wnt-targeted therapies may offer therapeutic benefit to GC patients.

A brief overview of Wnt signalling

The Wnt signalling pathway is an ancient instructive genetic programme, which is conserved from humans through to *Hydra* (Phesse *et al.*, 2016). It plays a vital role for orchestrating complex cellular behaviours during development, tissue homeostasis and regeneration where it coordinates cell proliferation, cell fate decisions, cell motility and tissue polarity. Due to its biological pervasiveness, Wnt signalling is implicated in many human cancers and degenerative diseases following pathway deregulation (Clevers and Nusse, 2012). Historically, Wnt signalling is considered to operate in two distinct modalities based on downstream involvement – or lack thereof – of the cytoplasmic protein **β-catenin**. These are referred to from hereon in as β-catenin-dependent or β-catenin-independent Wnt signalling. Of the two Wnt pathway branches, the β-catenin-dependent pathway has received the most research interest and is thus better characterized, and as such will be the primary focus of this review.

Wnts are secreted lipid-modified glycoproteins that act as both short (Farin *et al.*, 2016) and long-range (Mulligan *et al.*, 2012) ligands to engage with cell surface receptors that can establish complex morphogen gradients, to promote subtle yet sophisticated biological outcomes, depending on cell and tissue context (Clevers, 2006; Clevers and Nusse, 2012; Clevers *et al.*, 2014). The hallmark of β-catenin-dependent Wnt signalling is the cytoplasmic accumulation of β-catenin. In the absence of Wnt activation, cytosolic levels of free β-catenin are kept to a minimum, despite the gene being continuously transcribed. This pool of free β-catenin is sequestered in a multi-protein 'destruction complex' that consists of adenomatous polyposis coli (APC) tumour suppressor protein, AXIN, **glycogen synthase kinase-3β (GSK3B)** and **casein kinase 1 (CK1)**. Formation of the β-catenin destruction complex induces phosphorylation of β-catenin by CK1 at Ser^{45}, which in turn primes GSK3B phosphorylation of β-catenin on Thr^{41}, Ser^{37} and Ser^{33} residues (Liu *et al.*, 2002). Phosphorylated β-catenin is ubiquitylated by the F-box-containing protein β-TrCP E3 ligase tagging it for proteasomal degradation (Aberle *et al.*, 1997; Kitagawa *et al.*, 1999; Li *et al.*, 2012). In the presence of Wnt, a heterodimeric receptor complex is formed, consisting of the seven-pass transmembrane protein frizzled (**FZD**) receptor and low-density lipoprotein receptor-related protein 5/6 (LRP5/6). Through an unresolved mechanism involving the adaptor protein Dishevelled (Dvl), both receptor components participate in separate intracellular interactions that trigger both the initiation and amplification of Wnt signalling *via* inhibition of the β-catenin destruction complex (Mao *et al.*, 2001; Zeng *et al.*, 2005; Zeng *et al.*, 2008; MacDonald *et al.*, 2009). This allows newly synthesized unphosphorylated β-catenin to accumulate, stabilize and translocate from the cytoplasm to the nucleus. β-catenin can then generate a transcriptionally active complex with T-cell factor/lymphoid enhancing factor

(TCF/LEF) family of transcription factors to induce Wnt target gene transcription (for detailed reviews on Wnt signal transduction, see Clevers and Nusse 2012; Niehrs, 2012; Acebron and Niehrs, 2016).

Recent experimental findings have revealed inherent pathway crosstalk and complexity that cannot be accounted for by current linear signal transduction models, with components at virtually every level of Wnt signal transduction being shown to affect both β-catenin-dependent and β-catenin-independent outputs (Topol *et al.*, 2003; Mikels and Nusse, 2006; Niehrs, 2012). As such, Wnt signalling is beginning to be viewed as a signalling network (van Amerongen and Nusse, 2009). An example of Wnt pathway complexity is the dazzling number of possible ligand–receptor interactions from the vast repertoire of mammalian Wnts (19), FZD receptors (10) and co-receptors (>6). These combinations influence just one facet of signal output following Wnt pathway activation and will yield different biological outcomes depending on cell/tissue context. This illustrates that despite major breakthroughs over the last several decades, there are still gaps in our understanding of how this pathway operates including precisely how Wnt-FZD receptor selectivity is achieved.

Duplicitous Wnt signalling – regulator of both normal and cancer stem cell biology

Many adult tissues, such as the skin and gut, undergo constant renewal, meaning, a balance between cell extrusion and replacement by newly born cells. It is now understood that cellular attrition, either through natural exhaustion or injury, within diverse tissues is fuelled by stem cells. Stem cell activity is often controlled by the micro-environment (niche) so that stem cell output is matched to the homeostatic needs or regenerative demands of the tissue (Clevers *et al.*, 2014). Wnt signalling controls stem cell activity in a variety of tissues such as the intestines, stomach, skin, bone and haematopoietic system (Clevers and Nusse, 2012; Visvader and Clevers, 2016). For example, intestinal stem cells sustain the constant turnover of the intestinal epithelium and express the cell surface receptor LGR5 (Barker *et al.*, 2007). *Lgr5* is a Wnt target gene (Barker *et al.*, 2007), and *Lgr5*$^+$ intestinal stem cells require the Wnt receptor **FZD$_7$** to maintain homeostasis in the intestinal epithelium (Flanagan *et al.*, 2015). Indeed, LGR5 is a co-receptor for Wnts, which are expressed by neighbouring Paneth cells in the small intestine (Sato *et al.*, 2011) and c-kit$^+$ goblet cells and Reg4$^+$ deep secretory cells in the colon (Rothenberg *et al.*, 2012; Sasaki *et al.*, 2016). *Lgr5*$^+$ stem cells are also located in the gastric epithelium suggesting that Wnt signalling also regulates gastric homeostasis. This is supported by the observation that deletion of *Fzd7* in the gastric epithelium is deleterious and triggers rapid repopulation (Flanagan *et al.*, 2017). The TNF-family receptor *TNFRSF19* (*Troy*) was recently identified as a marker of a subset of chief cells which act as 'reserve' stem cells in the stomach following injury, which are characterized by an elevated expression of Wnt target genes (Stange *et al.*, 2013). Together, these data suggest Wnt signalling regulates gastric stem cell activity; however, the full extent of Wnt-regulated homeostasis in the gastric epithelium is relatively poorly understood in comparison with that in the intestine.

The cancer stem cell model suggests that tumour growth is driven by a small sub-population of cells [cancer stem cells (CSCs)] rather than the bulk of the tumour cells (Visvader, 2011). Over several years, this model has been refined as researchers have discovered significant plasticity between CSCs and non-CSCs within a tumour depending on the exposure of cells to growth factors and cytokines expressed by tumour cells or surrounding cells. Importantly, despite the progress that has led to an improved standard of care, resistance to chemotherapy, whether intrinsic or acquired, is a complex and multifactorial phenomenon and remains the main cause of treatment failure and death in GC patients (Brungs *et al.*, 2016). Gastric CSCs (gCSCs) have shown to be resistant to GC therapy and subsequently responsible for tumour recurrence and metastasis (Mayer *et al.*, 1993; Brungs *et al.*, 2016). Consequently, identifying the mechanisms of CSC regulation and maintenance is crucial to clarifying how these mechanisms influence the development of chemoresistant tumour cells in GC patients. Indeed, the similarities between normal adult somatic stem cells and CSCs suggest that the same signalling pathways that are involved in regulating somatic stem cell maintenance are also involved in the regulation of CSCs. As such, deregulated Wnt signalling has shown to increase 'stemness', trigger transformation and influence the development of chemoresistant CSCs (Sansom *et al.*, 2007; Barker *et al.*, 2009; Melo *et al.*, 2017). For instance, an elevated expression of the transcription factor and Wnt target gene *SOX9* (Blache *et al.*, 2004) is observed in human GC patients and correlates with decreased patient survival (Santos *et al.*, 2016). Targeted knockdown of *SOX9* is sufficient to reduce CSCs viability/formation with concomitant suppression of Wnt signalling and target gene expression, which was phenocopied following β-catenin knockdown (Santos *et al.*, 2016). Importantly, the increase in SOX9$^+$ resistant cells following exposure to cisplatin was largely prevented in SOX9 knockdown GC cells, demonstrating that agents targeting SOX9-β-catenin signalling can overcome chemoresistant cells (Santos *et al.*, 2016). Similarly, **Wnt6** expression is positively associated with tumour stage and inversely correlates with response to the anthracycline chemotherapeutics epirubicin and doxorubicin (Yuan *et al.*, 2013). Treatment with epirubicin or doxorubicin increases Wnt6 expression by enhancing the binding of cavolin-1 to β-catenin at the Wnt6 promoter, which in turn boosts cell survival. As such, targeted knockdown of Wnt6 reduces GC cell survival *via* increased caspase-3 induction (Yuan *et al.*, 2013). While it is likely that Wnt6$^+$-resistant cells represent gCSCs, this was not formally demonstrated. More recently, investigations to identify cell-surface proteins that mark chemoresistant and self-renewing GC cells following chemotherapy (cisplatin) reveal enrichment for the cell-surface glycoprotein PMP22 (Cai *et al.*, 2017). PMP22, a Wnt target gene, is expressed in patients that had undergone perioperative chemotherapy, highlighting a strong correlation between PMP22 expression and tumour recurrence. GC cells and tumour xenograft sensitivity to

cisplatin were significantly increased when combined with pharmacological inhibition of PMP22 (Cai *et al.*, 2017). The cell-surface water channel protein aquaporin-3 (AQP3) is overexpressed in GC tissues and promotes invasion and metastasis *via* EMT (Chen *et al.*, 2014); however, its role in chemoresistance has only recently been investigated. GC cells expressing high levels of AQP3 are refractory to treatment with cisplatin, but when combined with targeted AQP3 knockdown, GC cells show increased sensitivity to chemotherapy (Dong *et al.*, 2016). Of note, an independent study revealed that modulation of β-catenin-dependent signalling, *via* GSK3B inhibition or Axin stabilization, is sufficient to regulate the abundance and behaviour of $AQP3^+$ GC cells (Zhou *et al.*, 2016), which implies that targeted modulation of Wnt signalling may represent a way to combat therapy resistant GC cells. This observation of Wnt-regulated chemoresistance has also been observed in other cancer types including medulloblastoma and colon cancer, in which Wnt regulation of the DNA repair enzyme O6-methylguanine-DNA methyltransferase, restored chemo-sensitivity *in vitro* and in tumour xenografts (Wickstrom *et al.*, 2015). Collectively, these data demonstrate that Wnt signalling could be an attractive target to inhibit gastric CSC activity, which will affect tumour growth and recurrence.

Evidence for deregulated Wnt signalling in gastric cancer

The role of β-catenin-dependent Wnt signalling in GC is now well established, with approximately 10–30% of human gastric tumours displaying deregulated Wnt signalling (Wang *et al.*, 2014; Cristescu *et al.*, 2015), with the latest TCGA study confirming significant Wnt pathway mutations in GCs (Cancer Genome Atlas Research, 2014). Functional evidence also demonstrates that deregulated Wnt signalling can trigger tumourigenesis in the stomach, as deletion of *Gsk3b*, a component of the β-catenin degradation complex, resulted in gastric tumour formation (Radulescu *et al.*, 2012), as does deletion of *Apc* in muscle, intestine and stomach expression-1 positive ($Mist1^+$) cells of the gastric epithelium (Hayakawa *et al.*, 2017). Several Wnt genes are up-regulated in GC including ***WNT1*** (Mao *et al.*, 2014), ***WNT5A*** (Boussioutas *et al.*, 2003; Kurayoshi *et al.*, 2006) and *WNT6* (Yuan *et al.*, 2013). These recent discoveries provide a timely backdrop to review the potential of targeting the Wnt pathway for the treatment of GC, as resistance to current chemotherapy is common, and the exact role of each component of the pathway and its potential as a therapeutic target is described in the sections below.

Genetic lesions of the β-catenin destruction complex

Somatic mutation is a common mechanism to facilitate Wnt pathway deregulation in many solid cancers, including GC. Loss-of-function (LOF) mutations of multiple downstream Wnt pathway components such as *APC*, *AXIN* or activating mutations of ***CTNNB1*** (gene encoding β-catenin) feature in the initiation and progression in both intestinal-type and diffuse-type GCs (Cancer Genome Atlas Research, 2014; Wang *et al.*, 2014). This section will review strategies targeting these three components of the destruction complex in GC.

Axin

GCs positive for microsatellite instability (MSI) comprise one of several molecular subgroups identified by large-scale molecular characterization studies (Cancer Genome Atlas Research, 2014). Frameshift mutations of cancer-associated genes with mono- or dinucleotide repeats in the coding sequences are a feature of gastric tumours positive for MSI (Simpson *et al.*, 2001). The scaffold protein Axin serves as a critical rate-limiting protein in the assembly of the β-catenin destruction complex (Lee *et al.*, 2003), highlighting its role as a negative regulator of Wnt signalling and tumour suppressor protein (Li *et al.*, 2012). As such, approximately 30% of MSI-high human GCs harbour frameshift mutations in *AXIN2* (Kim *et al.*, 2009). The *AXIN2* frameshift mutation identified by Kim *et al.* is predicted to introduce a premature stop of amino acid synthesis in the C-terminus of Axin2 protein and hence resemble a typical LOF mutation (Kim *et al.*, 2009). This frameshift mutation (p.Gly665Alafs24) would eliminate a C-terminal half of PP2Ac-binding domain and the entire Axin-binding domain. In addition, missense mutations in Axin have been reported in gastric adenocarcinomas (http://www.cbioportal.org/); however, their functional significance has not been properly investigated. Recent work in *Drosophila* and human cells has identified missense mutations in *AXIN1* that disrupt the conserved core of the N-terminal Axin regulator of G-protein signalling domain, which is necessary for binding *APC* (Anvarian *et al.*, 2016). Cells with a non-functioning Axin scaffold gain paraneoplastic properties by forming protruding nanoscale aggregates, which engage with atypical signal transducers to confer cell-growth advantages (Anvarian *et al.*, 2016). Indeed, *Axin1* null mice develop liver tumours, confirming its role as a tumour suppressor *in vivo* (Feng *et al.*, 2012). Whether this same mutation-aggregate mechanism occurs in GCs harbouring missense mutations to *AXIN* remains to be addressed.

Adenomatous polyposis coli

Since its discovery as a major tumour suppressor in colorectal cancer (Kinzler *et al.*, 1991; Su *et al.*, 1992; Korinek *et al.*, 1997), LOF mutations to *APC* have been commonly reported in many other epithelial cancers, including GC (Sano *et al.*, 1991). Sequencing of human gastric tumours showed several different mutations to *APC*; however, the small sample size of this study makes it difficult to conclude any correlation between *APC* mutation status and tumour sub-type (Nakatsuru *et al.*, 1992). Recent large-scale genomic characterization of gastric tumours reveals frequent somatic mutations to *APC* in non-hypermutated chromosomal instable GCs (Cancer Genome Atlas Research, 2014), which is further supported by an independent patient dataset reporting an even higher incidence of somatic mutations to *APC* (Cristescu *et al.*, 2015). Likewise, in gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS) patients, mutation analysis and Sanger sequencing successfully mapped point

mutations in *APC* promoter 1B, which was shown to reduce binding of the Yin Yang 1 (YY1) transcription factor and impaired the activity of *APC* 1B promoter (Li *et al.*, 2016). Importantly, allelic imbalance of *APC* is detectable in patient blood and saliva samples, serving as an excellent biomarker for prospective GAPPS patients (Li *et al.*, 2016). In support, GC cell lines reveal mutations at codon 1450 of *APC* that encodes a truncated form of *APC* that fails to negatively regulate β-catenin, which causes constitutive activation of Wnt signalling (Sasaki *et al.*, 2001). Importantly, several reports have demonstrated that conditional deletion or loss-of-heterozygosity of wild type *Apc* is sufficient to drive gastric hyperplasia and subsequent adenoma formation (Tomita *et al.*, 2007; Radulescu *et al.* 2012; Powell *et al.*, 2014; Sarkar *et al.*, 2016). Collectively, patient genomics and preclinical mouse models highlight the relevance of *APC* mutations in driving gastric tumourigenesis, which offer an attractive therapeutic target to treat GC patients. As such, the application of tankyrase inhibitors (discussed in detail below) has been shown to be efficacious in colorectal cancer (CRC) cell lines and mouse models with *APC* mutations, which suggests that a similar therapeutic benefit could be observed in preclinical models of GC harbouring *APC* mutations (Chen *et al.*, 2009; Huang *et al.*, 2009; Waaler *et al.*, 2011; Table 1).

CTNNB1 (β-catenin)

Nuclear β-catenin, a hallmark of active Wnt signalling, is detected in approximately 30% of GC tumours, identifying β-catenin as a suitable target for therapeutic intervention (Clements *et al.*, 2002). Gastric tumours displaying nuclear β-catenin frequently harbour mutations at exon3 of *CTNNB1* (Clements *et al.*, 2002; Woo *et al.*, 2001). Activating mutations at exon3 alter targeted serine-threonine phosphorylation sites by GSK3B, which confer resistance to phosphorylation and lead to the accumulation of cytoplasmic and nuclear β-catenin and subsequent changes in expression of genes that regulate proliferation (*cyclin D1*, *D2* and *E*) (Akama *et al.*, 1995; Takano *et al.*, 2000; Liang *et al.*, 2003; Arici *et al.*, 2009). Genetic association analysis investigating the correlation between tagged single nucleotide polymorphisms (SNPs) spanning *CTNNB1* and GC incidence and survival showed that SNPs rs1880481, rs4135385, rs11564475 and rs2293303 were significantly associated with GC susceptibility (Wang *et al.*, 2012b). In addition, the rs4135385 AG/AA genotypes were associated with a 0.74-fold reduced adjusted hazard ratio for favourable overall 5 year survival of non-cardia GC (Chiurillo, 2015; Wang *et al.*, 2012b). A recent proof-of-principle study demonstrated that conditional mutation of exon3 in *CTNNB1* is sufficient to induce intestinal-type gastric adenomas in the antral stomach of adult mice and increased activation of Wnt signalling (Radulescu *et al.*, 2012). As such, targeted siRNA knockdown of β-catenin in human GC cells leads to inhibition of Wnt target gene transcription, decreased cell proliferation and an increase in apoptosis of GC cells (Jiang *et al.*, 2010). The expression of **survivin (BIRC5)**, a Wnt target gene, was deregulated following β-catenin knockdown, suggesting that elevated Wnt signalling might inhibit apoptosis by regulating *survivin* during GC (Jiang *et al.*, 2010). A complementary gene-targeted approach demonstrated that recombinant adenovirus carrying the p53 up-regulated modulator of apoptosis (PUMA) under the control of β-catenin/TCF-responsive promoter (AdTOP-PUMA) selectively targeted and killed AGS GC cells with active Wnt signalling (Dvory-Sobol *et al.*, 2007). Synergistic cell killing was observed when AdTOP-PUMA was used in combination with standard chemotherapeutic agents (5-florouracil, doxorubicin and paclitaxel) highlighting the potential of adjuvant Wnt-targeted therapies in GC (Dvory-Sobol *et al.*, 2007). Of note, even in GCs with no detectable mutations to *CTNNB1* or *APC*, the abundance of β-catenin

Table 1

Wnt mutations and modifications in gastric cancers

Gene name	Mutation/ modification type	Suitable class of drug(s)	Reference
APC	Truncation, point mutation	Tankyrase inhibitors, inhibitors of β-catenin transcription	Wang *et al.*, 2014, Li *et al.*, 2016
AXIN2	Missense mutation	Inhibitors of β-catenin transcription	Kim *et al.*, 2009
CTNNB1	Exon3 mutation, SNPs	Tankyrase inhibitors, inhibitors of β-catenin transcription	Radulescu *et al.*, 2012, Wang *et al.*, 2012b
DKK1–3	Promoter methylation	Porcupine inhibitors, FZD-blocking antibodies	Yu *et al.*, 2009
FZD_1, FZD_2 and FZD_7 receptors	Overexpression	FZD-blocking antibodies	Zhao *et al.*, 2014
RNF43	Truncation, missense	Porcupine inhibitors, FZD-blocking antibodies	Wang *et al.*, 2014
sFRP1–5	Promoter methylation	Porcupine inhibitors, FZD-blocking antibodies	Nojima *et al.*, 2007
TCF7	Missense mutation	Tankyrase inhibitors, inhibitors of β-catenin transcription	Kim *et al.*, 2009
Wnt-1, Wnt-2b, Wnt-3, Wnt-5a and Wnt-6	Overexpression	Porcupine inhibitors, FZD-blocking antibodies	Mao *et al.*, 2014, Zhao *et al.*, 2014 Yuan *et al.*, 2013

mRNA is greatly enhanced (Ebert *et al.*, 2002), and although post-translational rather than transcriptional regulation of β-catenin is at the core of active Wnt signalling, increased β-catenin protein production may suggest that upstream components of the Wnt pathway are deregulated, thereby activating Wnt signalling in GC.

Genetic lesions of the Wnt receptor complex

Aberrant activation of the Wnt pathway can also occur at the level of Wnts and/or FZD receptors. Genetic and/or epigenetic events that alter the function of Wnt regulators, thereby deregulating the expression of Wnt and/or FZD receptors resulting in pathway activation, have been identified in several cancers, including GC, and also represent a target for cancer therapy which is reviewed in this section.

Loss-of-function mutations to RNF43

Mammalian tissues that undergo constant renewal, such as the skin, blood and the gut, rely on tightly controlled Wnt signalling to maintain stem cell populations that fuel the replenishment of exhausted cells. For example, $LGR5^+$ intestinal stem cells, which are exquisitely sensitive to Wnt, sustain the constant turnover of the intestinal epithelium (Barker *et al.*, 2007). More recently, the potent Wnt agonist **R-spondin** (*RSPO1*) was shown to be a ligand for the receptor LGR5 and sufficient to potentiate β-catenin-dependent signalling (Kazanskaya *et al.*, 2004; Kim *et al.*, 2008; de Lau *et al.*, 2011; Carmon *et al.*, 2012). Following the discovery of **R-spondin** as the ligand for LGR5, independent investigations identified two highly related transmembrane E3 ubiquitin ligases, zinc-ring finger 3 (ZNRF3) and ring finger 43 (RNF43), as negative regulators of Wnt signalling that are integral to **R-spondin**/LGR5 Wnt potentiation (Hao *et al.*, 2012; Koo *et al.*, 2012). Extensive biochemical experiments reveal that ZNRF3 and RNF43 regulate Wnt signalling by promoting the ubiquitylation, internalization and degradation of FZD receptor-LRP5/6 complexes following Wnt activation, thereby limiting the duration and intensity of Wnt signalling (Hao *et al.*, 2012). These findings support the following model: in the absence of **R-spondin**, ZNRF3/RNF43 ubiquitylates the FZD receptor *via* the DEP domain of Dvl and promotes the degradation of the FZD receptor-LRP5/6 complex (Jiang *et al.*, 2015), thus keeping Wnt signalling to low levels. However, in the presence of **R-spondin**, an interaction between LGR5 and ZNRF3/RNF43, *via* R-spondin furin domains (1&2) leads to the clearance of ZNRF3/RNF43, allowing FZD receptor/LRP5/6 complexes to accumulate at the membrane to enhance β-catenin-dependent Wnt signalling (Hao *et al.*, 2012; Peng *et al.*, 2013).

Recent large-scale genomic data have identified frequent *RNF43* LOF mutations, predominantly truncating or missense alterations, in >50 and 4.8% of MSI and microsatellite stable gastric tumours respectively (Cancer Genome Atlas Research, 2014; Wang *et al.*, 2014). A recent examination of the progressive genomic and transcriptomic alterations from early-stage gastric adenomas through to later-stage disease validate the recurrent mutations to *RNF43* previously described (Min *et al.*, 2016). Furthermore, the frequency of *RNF43* mutations in early-mid stage gastric tumours identifies deregulated Wnt signalling as a critical driver and potential biomarker of early gastric tumourigenesis. Thus, determining if a gastric tumour has *RNF43* mutations will help stratify which patients are more likely to benefit from therapies targeted to the Wnt receptor complex or the production of Wnts (Min *et al.*, 2016). To date, the efficacy of antibodies that block FZD receptors and/or inhibitors of Wnt secretion has not been thoroughly tested in preclinical models of GC. However, one study has shown significant cell growth arrest and Wnt pathway inhibition using an inhibitor of Wnt secretion, IWP-2 (Chen *et al.*, 2009), on human intestinal-type gastric adenocarcinoma cells (MKN28); however, there have been no reports of *RNF43* mutations in this cell line (Mo *et al.*, 2013).

Encouragingly, other preclinical models of solid cancers harbouring *RNF43* mutations such as pancreatic, colon and breast cancer treated with inhibitors directed to the receptor complex (inhibitors of Wnt secretion or FZD receptor-blocking antibodies) have yielded potent anti-tumourigenic effects, especially when used in combination with standard chemotherapeutics (Gurney *et al.*, 2012; Jiang *et al.*, 2013; Steinhart *et al.*, 2017).

Epigenetic silencing of Wnt antagonists

Until relatively recently, it was considered that constitutive Wnt activation triggered by mutation to *APC*, *CTNNB1* or *AXIN* was impervious to further regulation from upstream Wnt components, that is, ligands and receptors. However, it has been demonstrated that the secreted frizzled-related protein (sFRP) family of Wnt negative regulators are frequently silenced *via* promoter hypermethylation in a variety of cancers, including GC (Caldwell *et al.*, 2004; Suzuki *et al.*, 2004; Cheng *et al.*, 2007). The family of sFRP glycoproteins is comprised of five family members, which can bind directly to Wnt *via* its cysteine-rich domain (CRD), the Wnt-FZD receptor binding interface (Janda *et al.*, 2012), thereby competing with FZD receptors for Wnt binding. Given that sFRPs can bind directly to Wnt, they are able to inhibit both β-catenin-dependent and β-catenin-independent signalling. In normal gastric mucosa, the expression of **sFRP1**, **sFRP2** and **sFRP5** is readily detected. However, in primary gastric tumours and GC cell lines, the expression of sFRP is absent, which is attributed to significant DNA methylation within the promoter-associated cytosine-phosphate-guanine islands (Cheng *et al.*, 2007; Nojima *et al.*, 2007; Zhao *et al.*, 2009). The silencing of *sFRP via* methylation is detected in pre-neoplastic gastric tissue, suggesting that this is a mechanism of tumour initiation in the stomach and thus could be used as a biomarker to screen for patients with an enhanced risk of developing GC (Cheng *et al.*, 2007). Much like the studies performed in CRC cell lines (Caldwell *et al.*, 2004; Suzuki *et al.*, 2004; Caldwell *et al.*, 2006), transfection of *sFRP-1,-2 or -5* successfully suppressed Wnt signalling, which is sufficient to block proliferation and induce apoptosis in GC cell lines harbouring *APC* or *CTNNB1* mutations (Nojima *et al.*, 2007). Xenografts have also been used to demonstrate that transfecting mice with *sFRP2* could block tumour growth, thus illustrating the potential for sFRP2 to act as a functional tumour suppressor (Cheng *et al.*, 2007). The ability of sFRP to

attenuate Wnt signalling is dependent on a functional Wnt-binding CRD as sFRP constructs lacking a functional CRD failed to inhibit proliferation and induce apoptosis (Nojima *et al.*, 2007).

Similar to the sFRP family of Wnt negative-modulators, the dickkopf family of glycoproteins (DKK1–4) are potent inhibitors of Wnt signalling (Niehrs, 2006). However, in contrast to sFRPs, DKK selectively inhibits β-catenin-dependent Wnt signalling through interacting with Lrp *via* the EGF repeat domains within Lrp6 (Mao *et al.*, 2001), thus preventing Wnt and the FZD receptor from forming a ternary complex (MacDonald *et al.*, 2004). DKK can also modulate Wnt by associating with **kremen 1** and **kremen 2** to form a complex that regulates the internalization of Lrp (Niehrs, 2006). Much like sFRPs, the expression of DKK3 is commonly silenced (70%) in GC tissues *via* promoter methylation and is associated with poor patient survival, as shown by multivariate analysis (Yu *et al.*, 2009). Reversal of DNA methylation with demethylating agents or ectopic expression of DKK3 is sufficient to restore DKK3 expression and subvert GC cell growth and Wnt signalling (Yu *et al.*, 2009). More recently, conventional adenoviral gene therapy has been utilized to deliver functional **DKK1** to inhibit Wnt signalling and attenuate gastric tumourigenesis (Wang *et al.*, 2012a). Following infection with the chimeric Ad5/35-DKK1 adenovirus, the number of $CD44^+$ GC stem cells and volume of tumour xenografts was significantly reduced (Wang *et al.*, 2012a). While the authors report successful infection of GC cells *in vitro*, issues surrounding delivery, tissue penetration and immunological response currently limit the feasibility of gene therapy technologies in GC patients (Sutter and Fechner, 2006).

Collectively, the silenced expression of sFRP and/or DKK inhibitors in GC undoubtedly contributes to unrestrained Wnt pathway activation at the level of the receptor complex. As such, patients with DNA-promoter modifications to sFRP and/or DKK negative modulators, which can be detected in pre-neoplastic tissue, might benefit from Wnt-targeted therapies at the level of Wnt receptors and/or Wnt secretion.

Overexpression of Wnt ligands and FZD receptors

While the incidence of genetic lesions to genes that encode for Wnts and FZD receptors is low, deregulated expression of Wnts and FZD receptors is a common feature of GCs, which can be attributed to the Wnt pathway mutations described previously. This suggests that GCs with abundant Wnts and FZD receptors can be targeted therapeutically. For instance, Wnt5a, a prototypical β-catenin-independent Wnt known to inhibit β-catenin-dependent Wnt signalling and promote cell migration and invasion (Moon, 2002), is overexpressed and correlated with aggressive GC phenotypes (Kurayoshi *et al.*, 2006). Infiltrating macrophages secrete Wnt5a following *H. pylori* infection, which induces the migration and invasion of GC cells *via* CXCR4 chemokine receptors (Zhao *et al.*, 2013). In support, a recent preclinical investigation has identified that Wnt5a produced by innate lymphoid cells supports diffuse GC progression by inhibiting anoikis, which enables anchorage-independent growth of GC cells (Hayakawa *et al.*, 2015). Indeed, chimeric mice transplanted with bone marrow from $Wnt5a^{flox/flox}$ mice (Wnt5a deficient) exhibit significantly fewer signet-ring foci than wild-type bone marrow recipient chimeras (Hayakawa *et al.*, 2015). Furthermore, therapeutic targeting of Wnt5a using a novel anti-Wnt5a polyclonal antibody (pAb5a-5) has been shown to reduce the migration and invasion of GC cells *in vitro* and *in vivo* by blocking receptor complex internalization, which is necessary to activate target gene expression required for cell motility (Hanaki *et al.*, 2012). Originally identified as a gene that preferentially integrates into mouse mammary tumour virus proviral DNA (Nusse and Varmus, 1982), Wnt-1 is overexpressed in GC tissues and has been shown to induce the expression of CSC genes (Oct4 and Cd44), which is associated with disease progression and poor outcome (Mao *et al.*, 2014). Modified AGS GC cells that overexpress Wnt-1 confer increased GC cell proliferation and Wnt target gene expression *in vitro* and in xenograft tumours (Mao *et al.*, 2014). Treatment of AGS-Wnt-1 overexpressing cells with the antibacterial potassium ionophore salinomycin, which was shown to inhibit β-catenin-dependent signalling by inducing the degradation of LRP6 (Lu *et al.*, 2011), effectively reduces tumour growth and the associated elevated expression of CSC and Wnt target genes (Mao *et al.*, 2014).

Similar to their cognate ligands, the frizzled family of Wnt receptors is frequently deregulated in GCs, which is often associated with poor clinical outcome (Schmuck *et al.*, 2011; Phesse *et al.*, 2016). Of the 10 family members, multiple reports have identified *Fzd7* to be overexpressed in GC tissues, which are associated with various stages of disease progression (Kirikoshi *et al.*, 2001; Schmuck *et al.*, 2011; Zhao *et al.*, 2014). The FZD_7 receptor is unique among other FZD members as it is one of the few FZD receptors shown to transduce all major signalling branches of the Wnt pathway and is associated with maintaining proliferation in adult stem cell populations (Fernandez *et al.*, 2014; Flanagan *et al.*, 2015; Phesse *et al.*, 2016). This places the FZD_7 receptor in a unique position to mediate cell proliferation as well as tumour dissemination and metastasis (Vincan *et al.*, 2005; Vincan *et al.*, 2007; Ueno *et al.*, 2009). A side population (SP) of cells, which display cancer stem cell properties, isolated from human GC cell lines reveal increased expression of *Fzd7* and other genes associated with CSCs, which supports a role for *Fzd7* in promoting GC (Schmuck *et al.*, 2011). Moreover, our unpublished data demonstrates that targeted molecular inhibition of *Fzd7* or conditional deletion of *Fzd7* is sufficient to block the growth of human GC cells and in mouse models of intestinal-type GC. Similarly, siRNA knockdown of the **FZD_2** receptor, which is structurally related to the **FZD_1** and FZD_7 receptors (Sagara *et al.*, 1998; Fredriksson *et al.*, 2003), reduced cell proliferation and invasion in MKN45 and MKN74 GC cells (Tomizawa *et al.*, 2015). In contrast, GC cell lines (AGS and SGC7901) reveal transcriptional silencing of ***Fzd6*** by miRNA-21 (Yan *et al.*, 2016). Re-introduction of *Fzd6* expression represses GC cell proliferation by antagonizing β-catenin-dependent Wnt signalling, which can be blocked by siRNA-targeted *Fzd6* knockdown or pharmacological inhibition of miRNA-21 (Yan *et al.*, 2016). Together, these data demonstrate that aberrant expression of Wnts and FZD receptors contributes to gastric tumourigenesis, and thus, they represent a therapeutic

target for GC patients with tumours showing elevated Wnt activity.

Infection and innervation influence Wnt activation in gastric cancer

The aetiology of GC is further complicated by complex interactions between bacteria, host and environmental factors (Gravaghi *et al.*, 2008). Infection with the bacterial carcinogen *H. pylori* is the greatest risk factor for GC (Hardbower *et al.*, 2014), with ~75% of the global GC burden attributed to *H. pylori*-induced inflammation and associated tumourigenesis (Parkin *et al.*, 2005). Following successful colonization of the stomach epithelium, *H. pylori* drives superficial gastritis, which progresses to chronic inflammation (Correa, 1996). *H. pylori*-driven inflammation is sufficient to induce the expression of caudal-related homeobox transcription factor 2 (*CDX2*) and mucin 2 (*MUC2*), which enables the transdifferentiation of gastric cells to adopt an intestinal cell phenotype (intestinal metaplasia). This is characterized by the loss of parietal cells and presence of Paneth and goblet cells. The resulting intestinal metaplasia (predominantly in the antrum) or spasmolytic polypeptide-expressing metaplasia (predominantly in corpus) progresses to dysplasia and ultimately cancer. *H. pylori* delivers bacterial virulence factors that modulate epithelial biology and inflammatory responses for its own benefit. Of the virulence factors produced by *H. pylori* associated with GC development, cytotoxin-associated gene product (CagA) and its associated type IV secretion system have been shown to activate Wnt signalling and promote gastric tumourigenesis and progression (Amieva and Peek, 2016; Neal *et al.*, 2013).

Gerbils infected with a carcinogenic strain of *H. pylori* develop rapid and highly penetrant changes in the gastric mucosa of recipient animals that progress to gastric adenocarcinoma (Franco *et al.*, 2005). The induction of gastric dysplasia following *H. pylori* infection was associated with increased expression of nuclear β-catenin, which was shown to be CagA-dependent (Franco *et al.*, 2005). Research using transgenic zebrafish has extended previous findings, demonstrating that activation of Wnt and epithelial changes triggered by CagA are downstream or parallel to the β-catenin destruction complex but upstream of Tcf-4 (Neal *et al.*, 2013). In addition, gastric cells co-cultured with *H. pylori* induce phosphorylation of Lrp6 within 30 min, which is sufficient to stabilize β-catenin where it can mediate β-catenin target gene transcription (Gnad *et al.*, 2010). The success of *H. pylori* to induce changes to the gastric epithelium hinge on its capacity to attach to and transform gastric stem cells. Adult gastric stem cells, marked by the Wnt target gene *Lgr5*, are not only reliant on Wnt signalling for their maintenance but are also the cell-of-origin in gastric tumourigenesis following *Lgr5*$^+$-targeted Wnt activation (Barker *et al.*, 2010a). Adult murine *Lgr5*$^+$ gastric stem cells infected with *H. pylori* increase proliferation and the expression of Wnt target genes, which is sufficient to transform *Lgr5*$^+$ gastric stem cells and their progeny (Sigal *et al.*, 2015). Strains of *H. pylori* with defects in chemotaxis or that are CagA-deficient are unable to induce transformation of *Lgr5*$^+$ gastric stem cells and subsequent hyperplastic changes (Sigal *et al.*, 2015). A complementary approach using *ex vivo* human gastric organoids also demonstrated the transformation of human gastric stem cells following infection with *H. pylori* (Bartfeld *et al.*, 2015). Taken together, these studies suggest that small molecule inhibitors targeted to the interaction between β-catenin and Tcf or other co-factors required for Wnt-driven transcription might abolish the increased activation of Wnt signalling observed following infection and thus have therapeutic value in patients infected with carcinogenic strains of *H. pylori*.

Several recent studies from Timothy Wang's group have elegantly shown that a functional nervous system innervation is required at all stages of GC in a Wnt-dependent manner. The studies collectively reveal a nerve growth factor/M_3 receptor signalling axis that activates Wnt signalling *via* promoting YAP/β-catenin complexes (Hayakawa *et al.*, 2017) in gastric stem cells, which in turn fuels gastric tumour growth (Hayakawa *et al.*, 2015; Zhao *et al.*, 2014). Furthermore, pharmacological inhibition of cholinergic signalling with botulinum toxin was sufficient to block tumour growth in mouse models of GC, associated with down-regulation of Wnt target genes (Zhao *et al.*, 2014).

These data suggest that Wnt signalling is rate limiting for cholinergic signalling-associated GC. Indeed, gastric tumours that developed in the antrum following *Apc* truncation in Mist1$^+$ cells of *Mist1CreERT2*; *Apc*$^{flox/flox}$ mice were significantly reduced when the M_3 receptor was co-deleted (Hayakawa *et al.*, 2017). In addition to demonstrating that Wnt signalling is rate limiting for M_3 receptor-driven gastric tumours, these data also highlight that the antrum is more sensitive to Wnt-driven tumourigenesis than the corpus, since Mist1$^+$ cells are found in both areas of the stomach (Hayakawa *et al.*, 2017).

Suitable inhibitors of Wnt signalling for gastric cancers

Aberrant Wnt signalling in GC can be achieved through either mutational or non-mutational alterations. As such, molecular blockade of Wnt signalling is sufficient to inhibit tumour growth in several preclinical models of solid cancers, prompting the recent development of Wnt-targeted inhibitors, several of which are in early-stage clinical trials. However, it is clear that the advancement of anti-Wnt drug candidates is slow both in numbers of candidates entering the trials and in the stage of advancement through the phase 1–2–3 clinical trials. Pharmacological modulation of Wnt signalling can be divided into compounds that modulate the ligand/receptor interface, stabilize the degradation complex or interfere with β-catenin-dependent gene transcription (Figure 2A, B) (Tai *et al.*, 2015). Note that the pharmacology and anti-tumour effects of Wnt inhibitors described below have not been validated in preclinical models of GC, although there is now sufficient biological evidence to suggest they may be of therapeutic benefit for the treatment of GC in the future.

There is also a strong rational for the repositioning of existing FDA approved drugs for the treatment of diseases with deregulated Wnt signalling such as cancer. For example, the anthelmintic agent niclosamide was originally approved

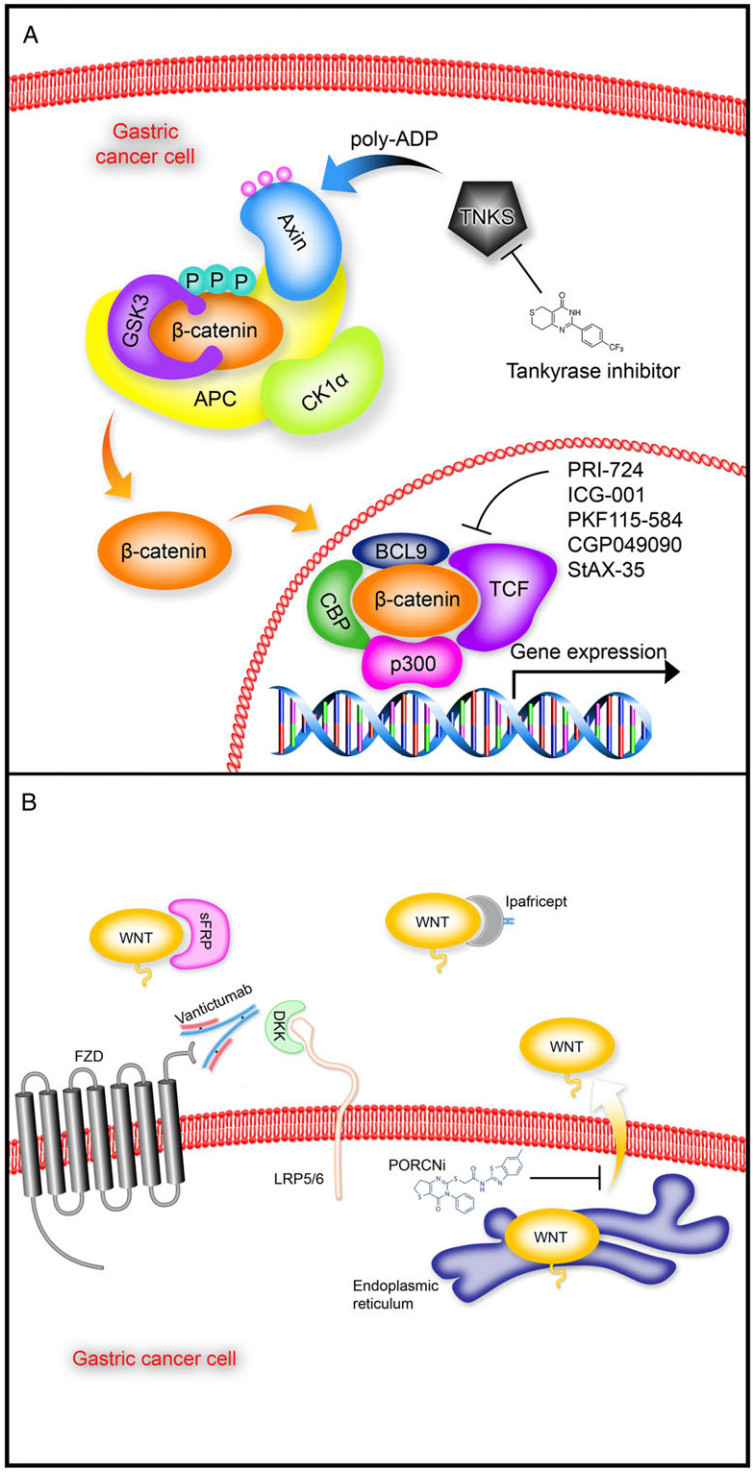

Figure 2

Inhibitors of Wnt signalling. (A) Following Wnt/FZD receptor binding (not shown), recruitment of the scaffolding protein AXIN to the receptor complex leads to inhibition of GSK3B and the β-catenin destruction complex, which allows newly synthesized β-catenin to accumulate and translocate to the nucleus (orange arrows), where it binds with co-factors to form a transcriptionally active complex. Indicated are some of the inhibitors that target various intracellular nodes of Wnt signal transduction that could have therapeutic benefit for gastric cancer patients with *APC*, *AXIN* or *CTNNB1* mutations. (B) Wnt signalling (WNT) can be inhibited at the cell surface by various pathway inhibitors such as sFRPs and DKK, which bind to Wnt and LRP5/6 respectively. Other approaches target Wnt signalling using small molecules that inhibit porcupine (PORCN), which prevent the secretion of Wnt from the endoplasmic reticulum (yellow arrow). In addition, FZD receptor-blocking antibodies (vantictumab) and decoy FZD receptors (ipafricept) inhibit Wnt signalling by blocking FZD receptors and sequestering Wnts respectively. Gastric cancer patients with *RNF43* mutations or overexpression of Wnt and/or FZD receptors would probably benefit from these types of Wnt inhibitors.

for clinical use in treating tapeworm infections but has been shown to inhibit the growth of several cancer cell lines including colon, breast, pancreas, lung and prostate (Mook *et al.*, 2015). Niclosamide is a multi-functional drug and has been shown to inhibit several oncogenic signalling pathways including Notch, mTOR, Stat-3, NFκB and Wnt. Indeed, niclosamide treatment significantly inhibited the growth of *APC* mutant colon cancer xenografts, independently of mTOR and NFκB signalling, which was associated with reduced Wnt signalling. As the stomach is more easily accessible than other organs such as the pancreas and brain, the problem of delivery for anti-cancer treatments is less of an issue and, therefore, drugs such as niclosamide which have a poor bioavailability have a greater chance of success in GCs.

Inhibitors of Wnt signalling at the receptor complex

The gaps in our understanding pertaining to intrinsic Wnt pathway complexity pose technical concerns when considering how best to inhibit β-catenin-dependent and β-catenin-independent Wnt signalling. One approach to block both β-catenin-dependent and β-catenin-independent Wnt signalling is to focus on inhibiting the interaction between Wnts and their cognate FZD receptors, which can be achieved using monoclonal antibodies and decoy receptors. Vantictumab (OMP-18R5, Oncomed Pharmaceuticals) is an anti-FZD receptor blocking antibody, originally identified to bind the FZD_7 receptor, which can functionally bind to five out of 10 mammalian FZD receptors (1, 2, 5, 7 and 8) by binding a series of highly conserved residues spanning the receptors' extracellular cleft (Gurney *et al.*, 2012). Vantictumab can successfully inhibit the ability of several Wnts to activate β-catenin-dependent Wnt signalling, which is accompanied by decreased Wnt transcription (Gurney *et al.*, 2012). Furthermore, when tested on tumour xenografts, vantictumab significantly inhibited the growth of several types of solid human tumours including breast, pancreatic and colon. Significant synergy was observed when vantictumab was combined with standard-of-care chemotherapies, such as paclitaxel and taxol (Gurney *et al.*, 2012). Vantictumab is currently in phase 1b clinical trials for HER2-negative breast cancer (NCT01973309) and advanced pancreatic cancers (NCT02005315) and was well tolerated up to the current dose of 15 $mg{\cdot}kg^{-1}$ every 3 weeks. Reported side effects include fatigue, abdominal pain, constipation and nausea. An interim efficacy assessment has demonstrated an overall response rate of 48% for patients given vantictumab and chemotherapy. Patient-free survival and overall survival data are not yet available for these clinical studies. Of note, significant bone turnover was observed in a subset of patients, which resulted in a temporary hold on three phase 1b clinical trials (Tai *et al.*, 2015). The temporary hold has been lifted after reviewing substantial clinical safety and efficacy data and revised protocols submitted by Oncomed. Other strategies employ soluble decoy receptors that sequester Wnts, akin to sFRPs, thus preventing Wnt–FZD receptor interactions. Ipafricept (OMP-54F28, Oncomed Pharmaceuticals) is a soluble Fc fusion protein that consists of the CRD of the FZD_8 receptor fused to the Fc domain of human IgG1 (http://www.oncomed.com/). Ipafricept inhibits the growth of patient-derived xenografts, which is characterized by reduced CSC frequency and proliferation, as well as promoting tumour cell differentiation. As such, ipafricept has progressed to phase 1b clinical trials and is tested in combination with chemotherapy for the treatment of various solid tumours such as hepatocellular carcinoma, ovarian and pancreatic cancer (clinicaltrials.gov). Interim safety data demonstrate that the combination of ipafricept with chemotherapy was well tolerated and an overall response rate of 39% was observed (Tai *et al.*, 2015).

An alternative approach to inhibit Wnt signalling is to block the biogenesis and secretion of Wnt proteins. Wnts undergo two types of known lipid modification. The first is a palmitate to cysteine 77, which is conserved among all Wnt family members (Willert *et al.*, 2003). The second reported modification is the addition of a mono-unsaturated palmitoleate moiety to serine 209, which is required for release of Wnt from the endoplasmic reticulum (ER) and binding to the FZD receptor (Takada *et al.*, 2006). Porcupine (PORC) is an essential non-redundant enzyme that is responsible for the serine 209 modification, and as such, PORC inhibition causes Wnt retention in the ER and thus blocks their secretion and subsequent pathway activation (Takada *et al.*, 2006). However, there are some cells, including $CD8^+$ T-cells and human astrocytes that have a PORC-independent mechanism of Wnt secretion, as treatment with the PORC inhibitor IWP-2 did not prevent Wnt secretion in these cells (Richards *et al.*, 2014). Several small molecule PORC inhibitors have been developed in recent years and have shown promising anti-tumour effects in preclinical models with minimal off-target effects (Chen *et al.*, 2009; Jiang *et al.*, 2013; Madan *et al.*, 2016), which have been extended to phase 1 clinical trials for Wnt ligand-dependent malignancies such as pancreatic adenocarcinoma and proto-oncogene B-Raf-mutant colorectal cancer which are currently ongoing (NCT01351103).

Stabilizers of the β-catenin destruction complex

The scaffolding protein AXIN is the rate-limiting component of the β-catenin destruction complex (Lee *et al.*, 2003) with the levels of AXIN1 and AXIN2 proteins being constantly surveyed and regulated by tankyrases, TNK1 and TNK2. Tankyrases regulate the stability of AXIN1 and AXIN2 through poly(ADP-ribosyl)ation (PARsylation) (Huang *et al.*, 2009), which directs AXIN ubiquitylation by RNF146 and proteasomal degradation (Callow *et al.*, 2011; Zhang *et al.*, 2011).

Recent efforts using cell-based screens have discovered several small-molecule tankyrase inhibitors, which successfully stabilize AXIN1 and AXIN2 by preventing PARsylation, thus promoting β-catenin destruction complex stability and functionality (Huang *et al.*, 2009). First generation tankyrase inhibitors, inhibitors of Wnt response and XAV939, were independently discovered, and both were shown to inhibit β-catenin-dependent signalling and cell viability in *APC* or *CTNNB1* mutant cancer cell lines, highlighting their therapeutic promise for the inhibition of Wnt-dependent cancers (Chen *et al.*, 2009; Huang *et al.*, 2009). Second generation tankyrase inhibitors have been optimized for potency, selectivity and tested in preclinical mouse models of intestinal cancer, which demonstrate stabilization of AXIN

to reduce β-catenin-dependent signalling and subsequent tumour burden (Lau *et al.* 2013; Waaler *et al.*, 2012). The translation of this class of drugs into the clinic has been frustrating as significant intestinal toxicity is observed in preclinical models (Lau *et al.*, 2013; Kahn, 2014). Next generation tankyrase inhibitors are being developed to reduce off-target effects on genes such as PARP1 and are currently being tested in preclinical models (Tai *et al.*, 2015; Nathubhai *et al.*, 2016). As such, it will be of interest to see if these new compounds can be optimized to balance inhibiting Wnt activity while reducing toxicity, which may advance their progression into the clinic.

Inhibitors of β-catenin interaction partners and transcription

A key mechanism of β-catenin-dependent Wnt signalling is the dynamic regulation, and subsequent activation, of a β-catenin-centric complex where β-catenin interacts with and recruits a series of nuclear co-activator proteins such as TCF/LEF, cAMP response element-binding protein (CBP), B-cell chronic lymphocytic leukaemia/lymphoma-9 (BCL-9), histone acetyltransferase p300 and others, to activate target gene transcription (Valenta *et al.*, 2012). Thus, drugging such protein–protein interfaces offers an attractive route for blocking Wnt signalling downstream of common pathway mutations such as *APC*, which would limit the risk of mutational circumvention leading to drug resistances. However, this therapeutic approach is not suitable for oncogenic mutations identified in genes responsible for β-catenin transcriptional activity, such as *TCF7L2* (Kim *et al.*, 2009). To date, a number of small-molecule inhibitors have been identified *via* cell-based high throughput screens that successfully inhibit β-catenin interactions necessary for transcriptional activation, which significantly limit Wnt signalling and subsequent tumourigenesis in preclinical models.

An early approach to detect β-catenin–TCF interactions utilized a high-throughput ELISA screen. Subsequent screening of extensive compound libraries (>45 000 compounds) identified eight compounds that dose-dependently inhibited β-catenin–TCF complex formation (Lepourcelet *et al.*, 2004). Functional inhibition of β-catenin–TCF interaction was confirmed *via* decreased activation of Wnt-specific reporter assays (TOPflash), colon cancer cell proliferation/viability and β-catenin-induced axis duplication in *Xenopus laevis* embryos (Lepourcelet *et al.*, 2004). Of the eight compounds, two structurally related compounds, PKF115-584 and CGP049090, proved the most potent and were also found to disrupt the β-catenin–APC interaction, implicating potential direct binding to β-catenin (Lepourcelet *et al.*, 2004). However, the exact molecular mechanism by which these two compounds inhibit Wnt signalling remains unresolved (Lepourcelet *et al.*, 2004; Kahn, 2014).

Using a similar approach, colon cancer cell lines challenged with a secondary structure-templated chemical library revealed a novel low-molecular weight inhibitor of β-catenin-dependent signalling, ICG-001. Characterization of ICG-001 demonstrated potent suppression of TOPflash assays and Wnt target gene transcription. ICG-001 suppressed the Wnt pathway by directly binding to CBP, thus disrupting the interaction between CBP and β-catenin (Emami *et al.*, 2004). ICG-001 selectively induces apoptosis in transformed cells but not in normal colon cells as seen in the $Apc^{Min/+}$ mouse model and xenograft models of colon cancer (Emami *et al.*, 2004). A second generation class of CBP-β-catenin inhibitors, PRI-724, have progressed into phase II and Ib clinical trials for metastatic CRC (NCI-2015-00436 and NCT02413853) and advanced pancreatic adenocarcinoma (NCT01764477) respectively and are currently ongoing.

Implementing 'peptide stapling' technology, which involves the introduction of a synthetic hydrocarbon bridge into an α-helical peptide, both the β-catenin–BCL-9 (Takada *et al.*, 2012) and the β-catenin–T-cell factor-4 (Grossmann *et al.*, 2012) interfaces have been targeted, yielding potent *in vitro* inhibitors (IC_{50} ~10–20 nM), which displayed low micromolar cell-based inhibition. The clinical utility of the stapled peptide class of molecules has yet to be established (Grossmann *et al.*, 2012; Takada *et al.*, 2012; Kahn, 2014). As β-catenin-dependent Wnt signalling is deregulated in GC, with mutations in *APC* and *RNF43* observed (Cancer Genome Atlas Research, 2014; Wang *et al.*, 2014), it will be interesting to see if this class of drugs is also effective in gastric tumours displaying high Wnt signalling.

Concerns surrounding drugging the Wnt pathway

As mechanisms of Wnt deregulation during cancer have been slowly exposed, this has been mirrored by considerable research effort into discovering Wnt-targeted therapeutics (Barker and Clevers, 2006; Kahn, 2014). However, drugging developmental pathways can have potentially devastating effects on embryonic development, adult stem cell populations and the regenerative response following injury (Kahn, 2014). A common concern relating to Wnt pathway therapeutics is the potential for acute toxicity in adult tissues that are maintained by adult stem cells (including intestine, haematopoietic system, skin, bone and hair) regulated by Wnt signalling (Clevers *et al.*, 2014). With respect to the intestinal epithelium, it is encouraging that targeted ablation of intestinal $Lgr5^+$ stem cells, which display exquisite sensitivity to Wnt, does not disrupt intestinal homeostasis and a full recovery of the stem cell compartment is driven by substantial cellular plasticity displayed by differentiated intestinal cell types (Tian *et al.*, 2011; van Es *et al.*, 2012; Buczacki *et al.*, 2013; Tetteh *et al.*, 2016). Additional side effects may include metabolic changes due to the impact of Wnt on liver zonation, bone toxicity and potential neurological effects given the role of Wnt signalling in synapse formation and maintenance in the CNS and PNS (Burke *et al.*, 2009; Liu *et al.*, 2011; Harrison-Uy *et al.*, 2013). Therefore, when considering the targeting of crucial developmental pathways that are utilized by both CSCs as well as normal somatic stem cells, the potential 'Jekyll and Hyde'-like behaviour of this class of therapeutic agents remains an ever-present issue (Kahn, 2014).

In addition, the appropriate dose of a Wnt pathway inhibitor must be taken into account given the pleiotropic effects and varying thresholds of Wnt signalling across many tissues. For example, the level of β-catenin-dependent Wnt signalling

in liver and melanoma oncogenesis is lower than intestinal tumourigenesis (Buchert *et al.*, 2010). Therefore, therapeutic agents that alter Wnt signalling activity may reach an efficacious threshold in one tissue but fail to do so in another. Moreover, within the intestinal epithelium, different Wnt threshold levels distinguish stem and progenitor cells, which could be useful for exclusively targeting CSCs without damaging the renewal capability of the intestine (Tao *et al.*, 2015).

Another question to consider is whether it is best to treat gastric malignancy through antagonizing or activating the Wnt pathway? As mentioned earlier, restoration of *Fzd6* expression in GC cells is sufficient to inhibit β-catenin-dependent Wnt signalling and associated tumour-promoting phenotypes (Yan *et al.*, 2016). Similarly, hyperactivation of Wnt signalling in *Cited1*-deficient mice reduced tumourigenesis in the intestine (Meniel *et al.*, 2013). In contrast, inhibition of Wnt signalling at several different levels of the pathway has been demonstrated to provide anti-tumour effects in several cancers (Polakis, 2007; Anastas and Moon, 2013). Therefore, a greater understanding of how Wnt signalling is controlled in GC tissues is needed to guide which, if any, inhibitors of Wnt signalling should be applied. Another parameter to consider is the complex inter- and intra-tumour heterogeneity observed in GCs (Zhong *et al.*, 2016), which can confound drug responsiveness and lead to acquired resistance. It is therefore imperative to have a greater understanding of individual patient tumour biology and to identify predictive biomarkers of efficacy to aid therapeutic strategy.

Concluding remarks

When taken together, the increasing amount of proof-of-principle studies strongly identify that aberrant Wnt signalling contributes to GC and suggest that interfering with this signal could be an effective treatment for this disease. Although promising therapeutic leads have been advanced and tested in combination with other chemotherapeutics in several solid tumours, the same Wnt-targeted therapy combinations have not been tested in preclinical models of GC. While the potential safety concerns associated with Wnt-targeted therapies are legitimate, similar complications are also relevant with all drugs. If used and integrated correctly (Cancer Genome Atlas Research, 2017), the recent deluge of genomic (Cancer Genome Atlas Research, 2014), epigenomic (Ooi *et al.*, 2016) and functional profiles from cancer patients (van de Wetering *et al.*, 2015) will pave a clear path forward to identify the appropriate patients for a specific Wnt inhibitor.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015a,b,c).

Acknowledgements

The authors wish to thank Gavan Mitchell for helping generate the figures in this manuscript. Funding is gratefully acknowledged from the following; National Health and Medical Research Council of Australia (NHMRC) project grants (566679 and APP1099302 awarded to E.V.); Melbourne Health project grants (605030 and PG-002 awarded to E.V./T.J.P.), and an Early Career Researcher grant (GIA-033) awarded to D.J.F.; Cancer Council of Victoria project grants (CCV, APP1020716) awarded to E.V. and T.J.P., CCV Fellowship awarded to D.J.F. and Cardiff University Research Fellowship awarded to T.J.P.

Conflict of interest

The authors declare no conflicts of interest.

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