Review Article

Hereditary diffuse gastric cancer: A manifestation of lost cell polarity

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Hereditary diffuse gastric cancer is a cancer syndrome caused by germline mutations in the gene for the cell adhesion protein E-cadherin (*CDH1*). E-cadherin plays a central role in the maintenance of cell polarity and its loss during tumorigenesis is associated with poorly differentiated cancers and a poor prognosis. Hereditary diffuse gastric cancer is dominated by diffuse-type gastric adenocarcinoma, often with signet ring cell morphology. Large numbers of stage T1a signet ring cell carcinomas exist in the stomachs of *CDH1* mutation carriers from a young age, and these foci sometimes show enrichment to the transition zone between the body and antrum. Generally these signet ring cell carcinomas are hypoproliferative, lack Wnt pathway activation, and are relatively indolent. However, a small proportion of the T1a foci contain cells that are poorly differentiated, display mesenchymal features, and express activated c-Src and its downstream targets. These same features are observed in more advanced stages of hereditary diffuse gastric cancer progression, suggesting that an epithelial–mesenchymal transition is required for tumor invasion beyond the muscularis mucosae. Hereditary diffuse gastric cancer initiation requires somatic down-regulation of the second *CDH1* allele, which in most cases is caused by DNA promoter hypermethylation. Subsequent to *CDH1* down-regulation, lost polarity in gastric stem or progenitor cells would be predicted to interfere with mitotic spindle orientation and the segregation of cell fate determinants. We predict that this disruption of cell division results in daughter cells being deposited in the lamina propria where their population expands and partially differentiates, resulting in the formation of foci of signet ring cells. (*Cancer Sci* 2009; 100: 1151–1157)

E-cadherin is a homophilic cell-to-cell adhesion protein that is expressed ubiquitously at the adherens junction of epithelial tissue and effectively acts as a bridge between the cytoskeletons of adjacent cells. Abrogation of its expression leads to a loss of apical–basal cell polarity and consequent disruption of the numerous fundamental processes that require spatial asymmetry such as specialized membrane function, cytoskeletal organization, intracellular vesicle trafficking, stem cell division, and cell migration.

Somatic mutation or down-regulation of the E-cadherin gene (*CDH1*) is common in sporadic tumors and is associated with a poorly differentiated phenotype and poor clinical outcome. Its loss is typified by weakly adherent cells that do not form glands and are often observed in isolation, small clusters, or ribbons.[1] E-cadherin loss is generally considered to be a late event in tumorigenesis; hence, the protein is frequently described as a suppressor of invasion and metastasis.[2] However, the identification of germline mutations in *CDH1* and the characterization of the associated cancer syndrome, hereditary diffuse gastric cancer (HDGC), have demonstrated an etiological role for E-cadherin loss in tumor initiation. Here, we describe HDGC's molecular and clinical characteristics and review data pertinent to E-cadherin's role in the initiation of gastric cancer.

Germline *CDH1* Mutation Spectrum

To date, more than 100 different germline *CDH1* mutations have been described in HDGC families. Of the identified mutations, about half are frameshift or nonsense.[3] Functional assays have also confirmed the deleterious effect of several mis-sense changes.[4,5] In addition to the coding sequence mutations, specific polymorphisms in the *CDH1* promoter (-160A) and intron 2 regulatory region (163 + 37235A) are also associated with an increased incidence of diffuse gastric cancer.[6,7] In the homozygous state, these low penetrance alleles increase relative risk by between two and 20-fold.

Although inactivating germline *CDH1* mutations have been observed in a diverse range of ethnic groups, it is notable that they appear to occur less frequently in countries with high sporadic gastric cancer rates such as Japan, Korea, Italy, and Portugal. This observation may relate to chance clusters of sporadic gastric cancer obscuring the identity of the families with an inherited susceptibility. Alternatively, the effect may be explained by the population frequencies of low penetrance alleles such as those discussed above; conceivably, the co-existence of low penetrance *CDH1* polymorphisms and inactivating germline *CDH1* mutations may be associated with reduced embryonic viability in these populations.

HDGC Clinical Features and Pathology

The clinical history of HDGC families is dominated by diffuse-type gastric adenocarcinoma (Lauren classification[8]), often with signet ring cell (SRC) morphology.[3] The mean age at diagnosis of advanced gastric cancer, prior to the recent introduction of endoscopic surveillance programs and prophylactic surgery in HDGC families, has been 40 years (range 14–85 years).[3]

The anatomical mapping of stomachs from patients with HDGC who have undergone total gastrectomy has shown that almost all patients have multiple independent foci of stage T1a signet ring cell carcinoma (SRCC).[9,10] These foci, here termed 'early HDGC' (eHDGC),[11] have penetrated through the basement membrane into the lamina propria but have not invaded the underlying muscularis mucosae (Fig. 1). In one New Zealand series, the average number of eHDGC foci per stomach was in excess of 100, with the largest number (487) being observed in a 16-year-old female (Charlton *et al.*;[9] A. Charlton and V. Blair, unpubl. data, 2008). The number of foci per stomach was not

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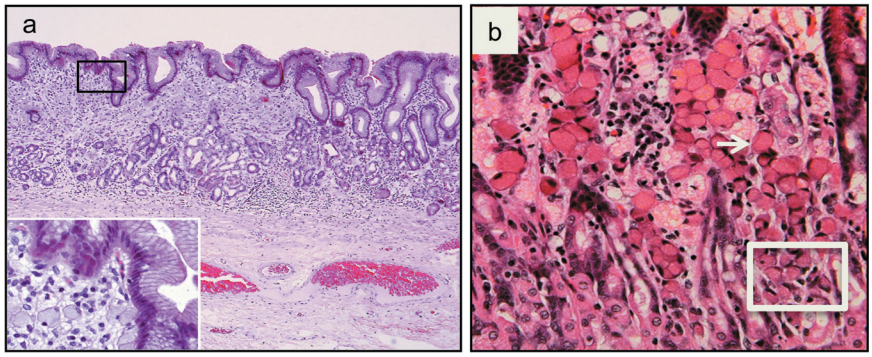

Fig. 1. Stage T1a signet ring cell carcinomas (SRCC) in *CDH1* germline mutation carrier. (a) A 9 mm focus (the left two thirds of the frame) occupies the full thickness of the mucosa under an intact epithelium (hematoxylin–eosin stain, original magnification 40 ×). Inset frame shows signet ring cells in the lamina propria (original magnification 400 ×). Adapted from Charlton *et al*.[9] (b) A SRCC showing typical SRCs in the luminal part (one SRC is marked with an arrow) and smaller, less-differentiated cells at the base of the foci (box). Adapted from Humar *et al*.[11]

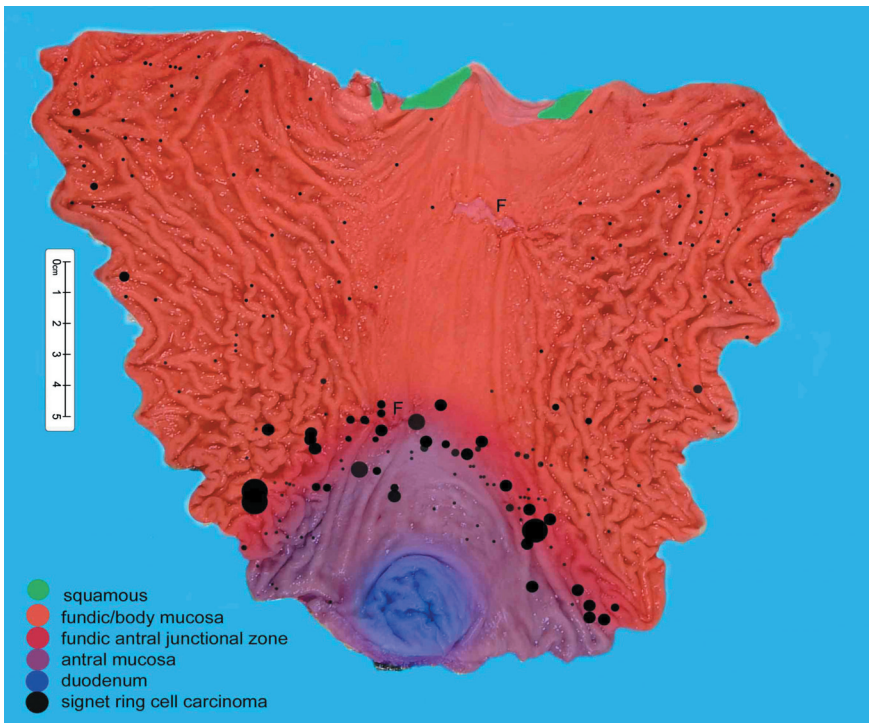

Fig. 2. Stomach map showing size and location of foci of SRCC and mucosal zones in a 28-year-old-male *CDH1* germline mutation carrier. Foci are to scale, except foci less than 1 mm are shown as 1 mm for visibility. This patient had 214 foci with a strong clustering in the transition zone. Adapted from Charlton *et al*.[9]

correlated with the age of the patient (V. Blair, pers. comm., 2008), suggesting that a proportion of these foci are transient. The mapped foci ranged in diameter from 0.1 to 10 mm, with most being less than 1 mm.

In addition to the multifocal T1a SRCC, *in situ* SRCCs and two layered structures composed of an inner layer of benign mucous cells and an outer layer of SRCs (Pagetoid spread) are also occasionally observed in the gastric glands of germline *CDH1* mutation carriers.[12] The relationship between these *in situ* carcinomas and the T1a foci has not yet been established.

In general, eHDGC lack *Wnt* pathway activation[13] and are hypo-proliferative, as evidenced by the relative absence of mitotic cells and lower Ki67 expression than surrounding non-malignant tissue.[11] This apparent indolence is supported by the differentiated state of the SRCs[11] and the observation that, despite the near 100% penetrance of multifocal disease at a young age, the lifetime risk of advanced diffuse gastric cancer is only around 70%. Thus, the vast majority of T1a carcinomas fail to progress to an advanced state.

In some,[9] but not all[10,12,14,15] mutation carriers' stomachs, the foci of eHDGC are larger and occur at highest density in the transition zone between the antrum and body of the stomach (Fig. 2). Transition zone enrichment has been observed previously in gastric cancer models: dogs administered with the carcinogen N-ethyl-N′-nitro-N-nitrosoguanidine (ENNG) for three months developed SRCCs exclusively in the antral mucosa immediately adjacent to the transition zone.[16] In mice with conditional inactivation of the bone morphogenetic protein receptor *Bmpr1a*, tumors develop specifically at both the gastric squamocolumnar and gastrointestinal transition zones.[17] Similarly, mice that have been exposed to the carcinogen N-nitroso-N-butylurea[18] or have a specific *Smad4* mutation[19] also develop tumors which are observed primarily in the gastrointestinal transition zone. It is not yet known why this region is particularly sensitive to tumorigenesis, although it may relate to its higher proliferation rate.[17] Alternatively, the indecision in cell fate commitment in this zone may correspond to a state of epigenetic plasticity that renders the cells vulnerable to epigenetic dysregulation.

In addition to the stomach, germline *CDH1* mutation carriers have developed cancers at other diverse sites including the breast, lung, salivary gland, and colorectum,[3,20] but only lobular breast cancer occurs at a frequency significantly above the risk of the general population.[21,22] Analogous to diffuse gastric cancer, lobular breast cancer does not form glands and is marked by

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isolated cells or small clusters and ribbons of poorly differentiated malignant cells.[1]

It is unclear why germline *CDH1* mutation predisposes predominantly to gastric cancer. One possibility is increased genetic and epigenetic damage occurring in the gastric epithelium due to higher carcinogen exposure, relative hypoxia, chronic inflammation, or *Helicobacter pylori* infection. A second possibility relates to the gastric mucosa's inherently high cellular turnover and capacity for tissue remodeling or repair; in this active setting, fewer mutational or epi-mutational events may be required to shift a cell to a poorly controlled invasive, proliferative state. Finally, as discussed later, proteolytic enzyme(s) produced specifically by gastric cells may be required for the penetration of the early, E-cadherin-deficient carcinomas through the epithelial basement membrane.

The Second *CDH1* Hit

Both the multifocal eHDGC and late stage diffuse gastric tumors observed in HDGC have low E-cadherin expression compared to the surrounding non-malignant mucosa, demonstrating that the non-mutated (second) *CDH1* allele has also been down-regulated or lost.[11] Decreased E-cadherin expression in eHDGC is associated with reduced membranous staining of other proteins that form part of the adherens junction, providing evidence that loss of the second *CDH1* allele precipitates the disintegration of the adherens junction and the subsequent loss of normal cell polarity. Studies in both advanced HDGC tumors[5,23] and eHDGC[13] have shown that this 'second hit' is caused by promoter hypermethylation in at least 50% of cases. Mutation and LOH-mediated second hits are reported to occur less frequently.[24] Of interest, the methylation patterns observed in the eHDGCs are monoallelic and specific for each focus,[13] indicating that the second hit has affected a single cell that has expanded clonally. This places epigenetic silencing of the *CDH1* promoter at the beginning of eHDGC evolution. Notably, promoter methylation appears to also be a major mechanism underlying *CDH1* down-regulation in sporadic diffuse gastric cancers[25] and lobular breast carcinomas.[26]

Promoter hypermethylation is likely to be just one mechanism in a hierarchy of inter-related epigenetic silencing events implicated in HDGC. In particular, histone modifications are also likely to induce the second *CDH1* hit, even if only transiently. The known transcriptional repressors of *CDH1* (Snail, Slug, ZEB1, ZEB2,[27] and probably also Twist and E47[28]) all act through histone deacetylase activity, and over-expression of the Polycomb repression complex protein EZH2 causes transcriptional silencing of *CDH1* by trimethylation of lysine 27 on histone 3 (H3K27).[29] Repression of *CDH1* transcription by these factors may decrease E-cadherin levels below the critical threshold required for functionality. Since active transcription is crucial to maintaining CpG islands in an unmethylated state,[30–32] short-term, reversible *CDH1* repression may eventually become 'fixed' through DNA methylation. This possibility is supported by recent studies with the vHMEC-ras breast cancer cell line model.[33] Culturing vHMEC-ras cells in serum-rich media induces an epithelial–mesenchymal transition (EMT) that is associated with the up-regulation of Snail and the down-regulation of *CDH1*. *CDH1* down-regulation appears to be mediated by histone deacetylation in the early passages after serum exposure and by promoter methylation in later passages. The promoter methylation is permanent and heritable, remaining in place following the withdrawal of serum.

Triggers for the Second *CDH1* Hit

Factors which promote the second *CDH1* hit in germline *CDH1* mutation carriers are likely to trigger SRC formation and contribute to the variable penetrance of HDGC. Several physiological and pathological processes are capable of inducing sustained E-cadherin down-regulation in human tissue, including repair processes,[34,35] *H. pylori* infection,[36] the inflammatory response,[27,37] and hypoxia.[38,39] Perhaps surprisingly, *H. pylori* infection, which is equally associated with the diffuse and intestinal types of gastric cancer,[40] and severe gastritis are not obvious features of HDGC patients at the time of gastrectomy. This suggests that either these mechanisms are not major triggers of the second *CDH1* hit, or that transient episodes of *H. pylori* infection or gastritis are sufficient to provoke stable DNA methylation.

Proposed Mechanism of HDGC Initiation

The simultaneous occurrence of up to several hundred foci of diffuse gastric cancer in the stomachs of germline *CDH1* mutation carriers argues that specific mutations in other genes are unlikely to be required for HDGC initiation, although more widespread epigenetic dysregulation is probable. Therefore, eHDGCs may be regarded as simple neoplasms whose initial existence owes more to the inherent characteristics of the tissue than to widespread mutation and disorganization of the genome.

Lineage labeling experiments with gastric differentiation markers and the proliferation marker Ki67 are consistent with an initial development of eHDGC from the upper isthmus of the neck region of the gastric gland.[11,13] The neck region is thought to be the location of the gastric stem and progenitor cells.[41] Initiation appears to be followed by movement towards the luminal surface and expression of mucin markers characteristic of differentiated surface pit cells.[11,13] A comparable pattern of SRCC development has been described by Sunagawa *et al*. in the ENNG-induced canine gastric cancer model[16] and by Sugihara *et al*. in human sporadic SRCC.[42] Both groups also noted the formation of an early double-layered structure in the gastric glands as described in HDGC families by Carneiro *et al*.[12]

We propose that the initiation of eHDGC is caused by the abrogation of E-cadherin's critical role in cell polarity and epithelial tissue architecture. E-cadherin-mediated cell-to-cell adhesion provides a spatial cue that promotes cellular asymmetry and polarity.[43,44] Without this correct spatial organization, the fundamental processes that regulate cell division, such as the orientation of the mitotic spindle, are disrupted. Since the plane of cell division is parallel to the plane of the mitotic spindle, the orientation of the spindle directs the positioning of the daughter cells. Fleming *et al*.[45] recently used microtubule fluorescence imaging of the intestinal tissue of adult mice to predict that an apically located spindle with an angle greater than 30° to the apical surface would be likely to produce one daughter cell that had lost contact with the basement membrane. In *Drosophila*, the mitotic spindle of the dividing *pIIa* cell in the developing sensory organ of the dorsal cortex rotates to align with the *Drosophila* E-cadherin (DE-cadherin) complex, ensuring the daughter cells retain the orientation of the mother cell.[46] However, partial loss of DE-cadherin disrupts orientation of the spindle, leading to irregularity in the orientation of cell division and displacement of daughter cells out of the epithelial plane. Similarly, in the developing mouse embryo, knockout of α-catenin (an intracellular component of the adherens junction) results in randomization of spindle alignment and mis-oriented cell division in epithelial cells of the skin.[47]

Degradation of the basement membrane would be required before misaligned daughter cells could be deposited in the lamina propria (Fig. 3a). Membrane degradation may be induced by the over-expression of specific matrix metalloproteinases (MMPs). Down-regulation of E-cadherin is correlated with up-regulation of the type IV collagenases MMP-2 and MMP-9.[48,49] Although eHDGC does not strongly express these proteases (B. Humar, unpubl. data, 2007), limited secretion of these MMPs from the basolateral surface of unpolarized, *CDH1*-deficient cells may be

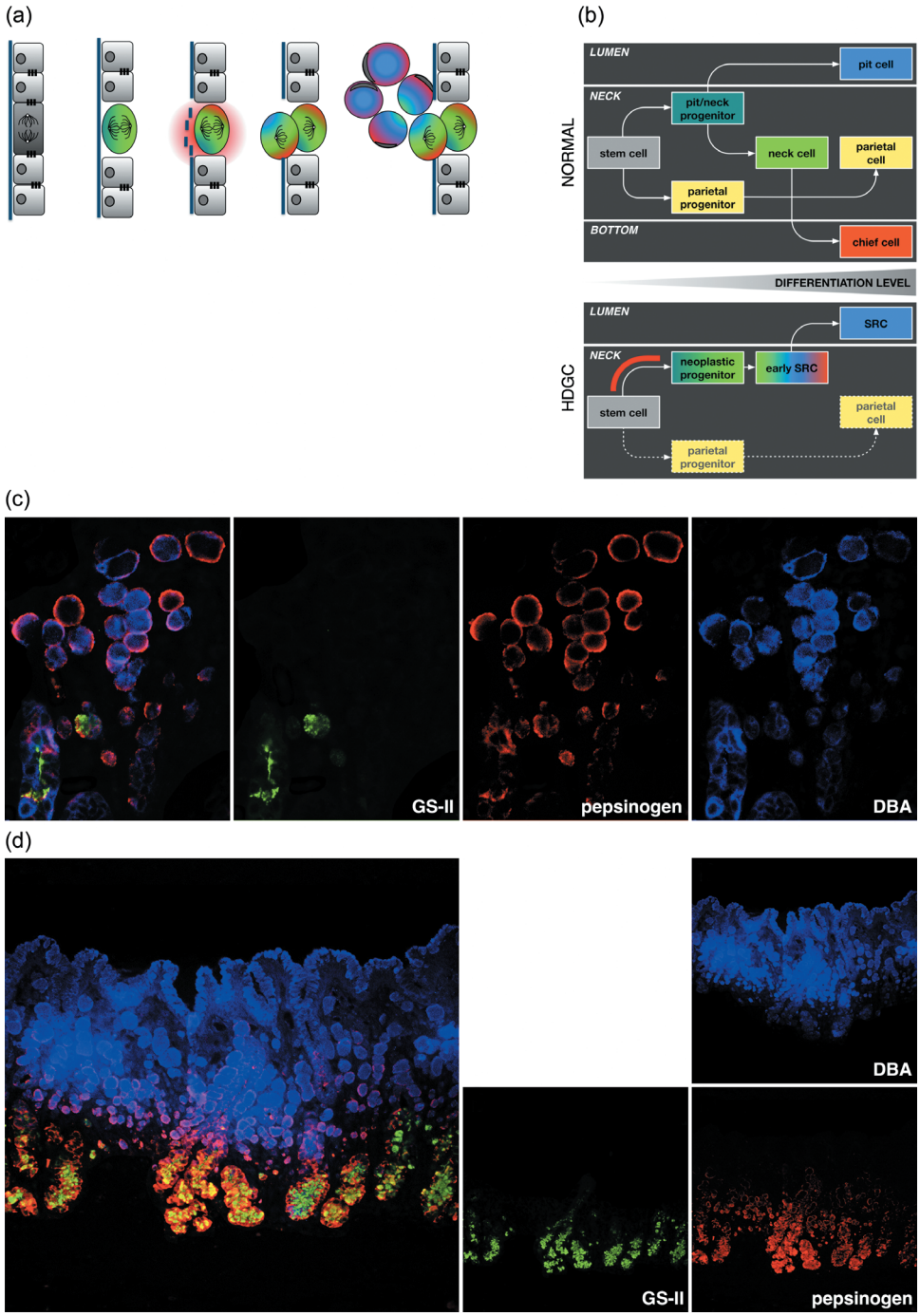

Fig. 3. Model of the early development and differentiation of eHDGC. (a) Mis-orientation of the mitotic spindle and enzymatic digestion of the basement membrane may result in daughter cells dividing out of the epithelial plane and accumulating in the lamina propria. Colors refer to the different gastric lineages as detailed in (b–d). (b) Developmental program of gastric epithelium. The gastric stem cell is thought to reside at the upper isthmus (the upper part of the gastric proliferative zone, the mucous neck region) and give rise to two precursor cells: the mucous neck cell/surface pit cell progenitor and the parietal progenitor. The parietal progenitor produces the lineage of terminally differentiated, acid-secreting parietal cells found mostly around the neck region of gastric glands. The neck/pit progenitor gives rise to two separate lineages: the terminally differentiated surface pit cells located at the gastric lumen, and the proliferative mucous neck cells at the gastric neck. The neck cells further mature into pepsinogen-secreting, terminally differentiated chief cells located at the bottom of gastric units (upper). In HDGC, E-cadherin deficiency causes loss of polarity and, as a consequence, mis-distribution of gastric cell fate factors (lower). Accordingly, gastric lineages often do not become separated, with neoplastic SRCs expressing markers of up to three distinct cell types including the co-expression of differentiation markers normally observed at the lumen (pit) or the bottom (chief) of gastric units. Red bar indicates approximate developmental stage at which the second hit can occur. The fate of the parietal lineage during HDGC development is not known. Either the second hit does not affect the gastric stem cell, or E-cadherin deficiency is not compatible with the survival of the parietal lineage. Notably, diffuse gastric cancer is characterized by a lack of parietal-type cells. (c) Fluorescence image showing the co-expression of up to three gastric lineage markers in individual SRCs (GSII/green, mucous neck cells; pepsinogen/red, chief cells; DBA/blue, pit cells).[11] Note simultaneous expression of pit and chief cell markers in the large, luminal SRCs and the peripheral, unpolarized expression of pepsinogen in the SRCs. (d) Birth of intramucosal SRCs from the proliferating region (green) of the gastric antrum. The gastric antrum lacks the typical bottom part of gastric glands enriched with chief cells. Note expression of pepsinogen in the luminal, pit-type SRCs.

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sufficient to breach the basement membrane. Alternatively, degradation may be the result of deregulated zymogen secretion. Mucous neck cells, the presumed origin of eHDGC,[11] are precursors of pepsinogen-producing chief cells (Fig. 3b) and already transcribe pepsinogen mRNA.[50] Early neoplastic cells may secrete sufficient pepsinogen from the basolateral surface for focal perforation of the basement membrane. Accordingly, we have observed a belt of pepsinogen surrounding unpolarized SRCs in eHDGC (Fig. 3c). Focal degradation of type IV collagen α chains has been observed previously in other subtypes of minimally invasive gastric intramucosal neoplastic lesions.[51]

In addition to the failing in spindle orientation, abrogated cell polarity also leads to the disruption of cell fate determination.[47,52] Somatic stem cells have the defining capacities of self-renewal and differentiation. Self-renewal describes the stem cell's ability to preserve itself by either 'symmetrical division' in which both daughter cells are stem cells, or 'asymmetrical division' in which one daughter cell remains a stem cell and the other becomes a 'progenitor cell' that is locked into a differentiation pathway. The fate of the daughter cells from asymmetric divisions is governed by proteins that migrate and accumulate asymmetrically in response to cues established within polarized cells. Following cell division, the presence or absence of these cell fate proteins in the daughter cells dictates either self-renewal capability or a pathway to differentiation. This process of fate determination has been well illustrated in *Drosophila* neuroblasts, where the tumor suppressor proteins Numb, Brat, and the homeobox transcription factor Prospero segregate asymmetrically in the polarized neuroblast and inhibit self-renewal in one of the two daughter cells.[53] Numb is a repressor of the Notch signaling pathway, Brat represses the transcription factor Myc, and Prospero acts to repress cell cycles mediators including cyclin A and E, as reviewed in Wodarz and Gonzalez[54]. Together, these proteins ensure this daughter cell exits the cell cycle and moves into a pathway that generates terminally differentiated neurons. In mutants that lack asymmetrical segregation of Numb, Brat, or Prospero, neither daughter cell is repressed, resulting in proliferation and the development of large metastasizing brain tumors, as reviewed in Knoblich[55]. In mammalian neuroepithelial cells, deletion of Numb causes dispersion of neuronal progenitors, along with a mislocalization of E-cadherin and the disorganization of adherens junctions, resulting in the formation of structures similar to those found in primitive neuroepithelial human tumors. Importantly, silencing of *Cdh1* in the neuroepithelium results in a phenotype similar to that of Numb mutants.[56]

Further evidence for a profound polarity defect in eHDGC comes from the observation that eHDGC cells can aberrantly express markers of normally separate gastric lineages. The gastric stem cell is thought to produce a precursor that gives rise to both the surface pit cell lineage and the mucous neck cell lineage, which further differentiates into zymogenic chief cells (Fig. 3b).[57] Of note, early eHDGC cells can express markers for pit, neck, and chief cells simultaneously, consistent with a defect in the asymmetric distribution of cell fate determinants (Fig. 3d).[11] Moreover, this observation places the timing, in some instances at least, of the initiating second *CDH1* hit at the root of the gastric developmental program, either just before the progenitor cell separates into the pit and the neck lineages, or as early as in the gastric stem cell itself.

In HDGC, the presence of large numbers of indolent, hypoproliferative foci of signet ring cells is consistent with the displacement into the lamina propria of daughter cells that are committed to a differentiated fate.[11] Consequently, these foci of SRCC may be relatively short-lived or transient. However, the unpolarized localization of cell fate determinants and abnormal mitotic spindle orientation may also occasionally result in the displacement of cells with self-renewal capability. The presence of self-renewing cells in the lamina propria may lead to the formation of SRCCs which have the capacity for sustained cell division and the potential to progress. The stochastic nature of these initiating events, and perhaps the requirement for additional mutations, may explain the unpredictable progression of these intramucosal foci to advanced cancer.

Progression of eHDGC Beyond the Gastric Mucosa

Invasion of the indolent eHDGC into extramucosal tissue is associated with a phenotypic shift from signet ring morphology to a poorly differentiated state. A small percentage of eHDGC foci contain an underlying population of smaller, poorly differentiated cells that display features of an EMT. The mechanisms behind this EMT are not known; however, they may involve the kinase c-Src, an established promoter of the mesenchymal state; c-Src and its downstream targets focal adhesion kinase and Stat-3 are active specifically in the intramucosal, poorly differentiated cells, but not in the overlying SRCs.[11] Poorly differentiated cells with activated c-Src increasingly dominate the histology of more advanced (>T1a) disease stages,[11] while the signet ring cells remain concentrated in the region above the neck.

Recent studies have suggested that both normal and neoplastic 'stem-like' cells express markers associated with an EMT.[58,59] This observation would support the contention that the population of eHDGC that displays features of an EMT have self-renewing stem cell characteristics that will enable their survival, expansion, and invasion.

Concluding Remarks

The characterization of HDGC provides several chemopreventative and therapeutic approaches for the clinical management of this cancer syndrome that may have broader implications for sporadic diffuse gastric cancer and lobular breast cancer.[26] Firstly, administration of demethylating agents and/or histone deacetylase (HDAC) inhibitors in a chemopreventative setting would be predicted to maintain expression of the second *CDH1* allele and prevent the loss of cell polarity. This might inhibit the development of SRCC, or reduce the number of existing foci. The DNA demethylating agent 5-aza-2′-deoxycytidine, which has been FDA-approved for the treatment of myelodysplastic syndrome, re-expresses *CDH1* in numerous cell types, and histone deacetylase inhibitors including sodium butyrate[60] and the well-characterized anticonvulsant valproic acid[61] also promote up-regulation of *CDH1*. Interestingly, aspirin has also recently been shown to repress *CDH1* methylation in human gastric epithelia.[62]

Given the likely importance of stem cells, or stem-cell characteristics, in HDGC development, agents that interfere with stem cell pathways might provide therapeutic options for both the hereditary and sporadic forms of diffuse gastric cancer. Of the pathways implicated in stem cell biology, Notch and Hedgehog present promising candidates, because they are thought to be required for both the maintenance of tumor-initiating cells[63,64] and the development of the embryonic stomach.[65] Both the Notch and Hedgehog pathways are over-expressed in diffuse gastric cancer and have been correlated with disease progression.[66,67] Finally, inhibitors of the EMT-associated proteins c-Src, focal adhesion kinase, and Stat-3 may also act to inhibit the progression of SRCC.

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