Gastrokines: stomach-specific proteins with putative homeostatic and tumor suppressor roles

Trevelyan R. Menheniott, Bayzar Kurklu, and Andrew S. Giraud
Murdoch Childrens Research Institute, Royal Children’s Hospital, Parkville, Melbourne, Victoria, Australia

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Menheniott TR, Kurklu B, Giraud AS. Gastrokines: stomach-specific proteins with putative homeostatic and tumor suppressor roles. Am J Physiol Gastrointest Liver Physiol 304: G109–G121, 2013. First published November 15, 2012; doi:10.1152/ajpgi.00374.2012.—During the past decade, a new family of stomach-specific proteins has been recognized. Known as “gastrokines” (GKNs), these secreted proteins are products of gastric mucus-producing cell lineages. GKNs are highly conserved in physical structure, and emerging data point to convergent functions in the modulation of gastric mucosal homeostasis and inflammation. While GKNs are highly prevalent in the normal stomach, frequent loss of GKN expression in gastric cancers, coupled with established antiproliferative activity, suggests putative tumor suppressor roles. Conversely, ectopic expression of GKNs in reparative lesions of Crohn’s disease alludes to additional activity in epithelial wound healing and/or repair. Modes of action remain unsolved, but the recent demonstration of a GKN2-trefoil factor 1 heterodimer implicates functional interplay with trefoil factors. This review aims to provide a historical account of GKN biology and encapsulate the rapidly accumulating evidence supporting roles in gastric epithelial homeostasis and tumor suppression.

gastrokine; trefoil factor; gastric cancer; Helicobacter pylori; BRICHOS

NEARLY 30 years ago, during the seminal cDNA cloning of the stomach hormone gastrin, a second unknown cDNA clone was isolated but not characterized. This ambiguous clone was later revisited and found to encode a previously uncharacterized 18-kDa antral mucosal protein (AMP-18) that shares strong sequence homology in the human, mouse, and pig. AMP-18 protein was found to be highly expressed in the distal (antral) stomach but was absent in other tissues (65). In the same study, AMP-18 was informally dubbed a “gastrokine” (GKN) on the premise of the observed growth factor or “cytokine-like” activity toward gastric epithelial cells (65). The term gastrokine was adopted formally by a second group that independently identified the same molecule (50, 51). Since approval by the HUGO Gene Nomenclature Committee (HGNC), this protein, which was previously known as AMP18, FOVEOLIN, CA11, BRICD1, and TFIZ2, is formally named GKN1 (HGNC no. 32317) (39, 50, 64, 71). While gastrokines are highly conserved in physical structure, and emerging data point to convergent functions in the modulation of gastric mucosal homeostasis and inflammation. GKN1 and GKN2, which are functional, GKN3 exists in humans as an inactive pseudogene. Nonetheless, GKN3 is conserved and fully functional in a large number of other mammals, including mice (Fig. 1), in which it was initially described. On this basis, we recently proposed inactivation of GKN3 in ancestral humans after the divergence from other mammalian lineages (42). Exclusive attention is devoted to the unusual evolutionary biology of human GKN3 (see below). Unless stated otherwise, all references to GKN3 henceforth pertain to the functional mouse ortholog.

Definitive physiological functions have not been formally ascribed to GKNs; however, the limited published evidence suggests fundamental roles in regulating gastric epithelial homeostasis, the host immune response to Helicobacter pylori (H. pylori), and, given their demonstrated antiproliferative activity (15, 16, 42, 72, 74), tumor suppression. Particularly resonant is the known physical interaction between GKN2 and the prototypical stomach-specific tumor suppressor protein trefoil factor (TFF) 1 (32, 40, 71). Here we provide a detailed review of GKN biology and present an evidence-based framework for future research directions. We encapsulate what is known of the GKNs in terms of their molecular structure, evolution, expression, and biological activities in normal physiology and disease. Furthermore, we discuss their interaction.
and functional interplay with specific members of the TFF family. Finally, we consider some of the unresolved questions pertaining to GKN biology and allude to future research initiatives that might be employed to answer them.

Genomic Organization and Evolutionary Conservation

Three members of the GKN family are known, and this number is likely to be exhaustive, since bioinformatic analysis of available vertebrate genomes does not reveal the existence of additional family members (18, 42). GKN orthologs are highly conserved among eutherian mammals but have not been described in fish or insects; therefore, we surmise that GKNs are an evolutionary innovation of higher vertebrates (Fig. 1). At the genomic level, the three GKN gene paralogs display strict conserved linkage, being clustered tightly together in all mammalian species examined thus far, including humans (42). The human GKN genes are located on chromosome 2p13.3 and are arranged within a 60-kb genomic interval (Fig. 1). GKN2 is located centrally in the cluster and transcribed in the centromere-to-telomere orientation. GKN1 and GKN3 loci are located at the centromeric and telomeric ends of the cluster, respectively, and their transcription (human GKN3 is not transcribed) occurs in the opposite direction to GKN2. Such tightly linked and highly conserved clustering is likely functionally significant, which supports the notion that GKNs are subject to coordinate transcriptional control. This arrangement might also indicate expansion of the existing GKNs from a common ancestral progenitor sequence, most likely by the process of gene duplication (20). The GKN structural genes exhibit near-identical genomic architecture, with each containing six short exons separated by relatively short introns, and, by definition, are relatively compact, with the three loci each occupying a total genomic span of <7 kb (Fig. 1). Gene compactness is, almost universally, a characteristic of highly expressed genes (5, 67). This genomic feature may be mechanistically related to the extremely high mRNA abundance reported for all three GKNs in the stomach (39, 42, 50, 51).

Protein Structure and BRICHOS Domain

GKN genes encode small (181- to 184-amino acid) proteins, which are produced by gastric mucus-secreting cells. Besides having similar molecular weight, GKN proteins also exhibit a number of unifying structural features, which are briefly discussed. Most conspicuous is the enigmatic BRICHOS domain and a COOH-terminal segment that shows considerable divergence between the GKN paralogs but strong amino acid sequence conservation, even between relatively distant orthologs (Fig. 2). GKNs contain a hydrophobic NH2-terminal signal peptide, the processing of which is predicted to generate mature ~160-amino acid proteins with molecular mass of ~18 kDa (18, 42). The GKNs also possess four absolutely invariant, position-conserved cysteine residues that putatively secure tertiary structure (Fig. 2).

The most distinctive structural feature of GKN proteins is the BRICHOS domain, a rare and phylogenetically ancient ~100-amino acid motif shared by a small number of unrelated proteins. Although they are few in number, BRICHOS proteins are clinically significant and have demonstrable associations with human pathologies such as dementia, respiratory disease, and cancer (18, 63). Available genome sequence data encompassing...
multicellular organisms indicate the existence of 12 BRICHOS paralog groups (18); 10 of these, including the GKNs, have numerous mammalian orthologs (18, 42). Comparison of paralogous BRICHOS domains reveals divergence in primary amino acid sequence but strong conservation of secondary and tertiary structure (18). Common to all BRICHOS domains are a pair of strictly position-conserved cysteine residues that putatively secure tertiary architecture via intramolecular disulfide bonding (18, 42) and an aspartic acid residue of unknown significance.

The relevance of the BRICHOS domain to the function of the GKNs is not well defined. Nevertheless, studies of BRICHOS domains in other proteins showing links to human disease have begun to elucidate these roles, including intracellular trafficking, propeptide processing, chaperonin function/protein folding, and secretion (63). The latter is the most obvious of these roles, since all BRICHOS proteins, including GKNs, are secreted or processed to release small secreted peptides (not necessarily including the BRICHOS domain itself), with the NH2-terminal hydrophobic region acting as a transmembrane anchor and/or signal peptide (18). BRICHOS domain tertiary structure is highly conserved and, therefore, likely functionally important. Accordingly, in mutagenesis studies of GKN3, replacement of BRICHOS cysteine residues (which likely secure the tertiary structure via a disulfide linkage) with glycine prevented secretion of the mature protein, leading to retention of the unprocessed precursor in the cytoplasm (42). These studies establish a direct relationship between correct BRICHOS domain (disulfide-linked) tertiary structure and in vivo function.

Emerging data argue that BRICHOS domains possess intrinsic chaperone activity. For example, mutagenesis studies of lung surfactant protein (SP)-C show that in-frame internal deletions within the BRICHOS coding sequence cause incorrect folding of the mature protein, leading to endoplasmic reticulum stress, aggregate formation, and proinflammatory cytokine release.

Fig. 2. GKN protein structures and sequence homology. A: protein structural predictions for human GKN1 and GKN2 and mouse GKN3. Protein structures were predicted using the Phyre_v2.0 server (http://www.sbg.bio.ic.ac.uk/phyre2). B: protein sequence alignment of human GKN1 and GKN2 and predicted open reading frame for human GKN3 as published previously (42). Position of the premature stop codon substituting the ancestral tryptophan residue at position 59 (W59X) of GKN3 is indicated by arrowhead. BRICHOS domains are shown by yellow shading, and transmembrane or hydrophobic regions are shown by light-blue shading. Position-conserved cysteine residues are numbered 1–4. Unique cysteine 38 residue (GKN2 only) is indicated by *. Alignments were generated using the Clustal Omega server (http://www.ebi.ac.uk/Tools/msa/clustalo). In sequence lines, asterisks denote residues identical in all sequences, colons mark conservative substitutions, and dots mark semiconservative substitutions.
Further evidence supporting a chaperone function comes from studies showing that BrtII/TM2B and proSP-C BRICHOS can inhibit amyloid aggregation and fibril formation (21, 49, 55). The proSP-C BRICHOS domain has been shown to simultaneously bind and induce α-helix formation of the transmembrane region of the mature SP-C protein (21). Therefore, the SP-C BRICHOS domain functions as a chaperone that directs folding of an aggregation-prone transmembrane region. While disease-associated BRICHOS somatic mutations have not been reported in any of the GKNs, transcriptional repression of GKN1 and GKN2 is well described in gastric cancer (15, 16, 44, 47, 50, 64, 76, 77). Therefore, disruption of BRICHOS domain function, by mutation or loss of expression, might lead to protein misfolding and/or compromised secretion.

Despite sharing significant structural homology overall, individual GKN paralogs possess a small number of protein structural refinements. Although not proven empirically, these structural variations conceivably might impact GKN function. Most prominent in this regard is the existence of a unique fifth, unpaired cysteine residue (position 38) near the NH2 terminus of GKN2 (71). Cysteine 38 is conserved in all mammalian GKN2 orthologs (T. R. Menheniott, unpublished data) but is not represented in GKN1 or GKN3 (Fig. 2). Protein biochemistry experiments by Westley et al. (71) deduced a putative role for cysteine 38 in mediating disulfide-linked heterodimerization of GKN2 and the TFF1 protein (see below for discussion of the GKN2-TFF1 heterodimer).

Two GKN1 protein isoforms, differing only in the substitution of the NH2-terminal-most amino acid (asparagine or aspartic acid) of the processed mature protein, have been identified (39, 77). The significance of these two isoforms has not been deduced. However, although present in equal abundance in disease-free stomach, specific loss of the asparagine, but not the aspartic acid, variant isoform has been reported in Helicobacter pylori-infected individuals (48). These two isoforms might therefore play differential roles in the mucosal response to H. pylori. Alternative GKN2 and GKN3 isoforms have not been reported, although an upstream in-frame methionine codon at the GKN3 locus gives potential for a second, NH2-terminal extended isoform (42).

GKN1 and GKN3, but not GKN2, are likely subject to posttranslational modification by glycosylation (42, 53). A consensus asparagine (N)-linked glycosylation (Asn-X-Thr/Ser) sequon occurs adjacent to the signal peptide cleavage site in GKN3 and likely forms the NH2 terminus of the processed mature protein. Consistent with this expectation, secreted GKN3 protein shows evidence of complex glycosylation (42; T. R. Menheniott, unpublished data). Since mucus are extensively glycosylated (43), we speculated that glycosylation of GKN3 (and, potentially, GKN1) might enhance solubility or anchoring in gastric mucus. Further work to delineate glycosylation of GKNs would undoubtedly elucidate their roles in vivo.

GKN3 is the only member of the GKN family identified on the basis of protein sequence homology (42), whereas GKN1 and GKN2 were detected in differential expression analysis (16, 64). This testifies to the high degree of structural relatedness displayed by the GKN proteins. Nonetheless, GKN3 has unique structural features that set it apart from the other GKNs. Mammalian GKN3 orthologs contain a highly conserved tract of hydrophobic amino acids at the NH2 terminus that likely functions as a transmembrane anchor (42). This hydrophobic region is contained almost entirely within the NH2-terminal signal peptide sequence and is predicted to undergo proteolytic cleavage prior to secretion of the mature protein (42). GKN1 and GKN2 lack this transmembrane region, although both proteins contain a corresponding region of weak hydrophobicity (42). In summary, strong structural homology shared by the GKNs signifies closely related roles in vivo; however, the attribution of unique features to individual family members also suggests the evolutionary acquisition of subtle functional specializations.

Expression in Normal Tissues

The major site of GKN expression is the stomach (16, 39, 42). Expression of GKNs in other organ systems is largely undetectable, although there are exceptions (see below). Within the stomach, GKN expression is confined to the gastric epithelium, where individual family members manifest striking cell-type-specific localization, both overlapping and complementary, among different mucus-secreting epithelial lineages (39, 42, 50, 53). The gastric epithelium is formed by reiterated glandular units, each composed of several specialized epithelial cell types (24). Sites of expression for individual GKNs in relation to the overall structure of a typical gastric glandular unit are shown in Fig. 3.

Early work showed abundant GKN1 mRNA expression in the normal stomach, reportedly accounting for ~1% of total mRNA, thus ranking among the most abundant transcripts known (39, 50, 51). GKN1 expression is almost exclusive to the stomach, except for trace levels in the uterus and placenta and scattered expression in the duodenum (9, 39). Tissue localization of GKN1 protein and mRNA by immunohistochemistry and in situ hybridization, respectively, confirmed de novo synthesis in surface mucous cells (SMCs) of the gastric antrum and fundus in all species examined, including human (39, 44, 47, 48, 50, 76), mouse (39), and chicken (19). Similarly, GKN2 shows stomach-restricted expression, although trace GKN2 mRNA levels (reportable by quantitative RT-PCR only) have been detected in mouse lung (3). Whether the latter observation reflects marginal transcription throughout the lung or abundant transcription at highly discrete sites within the lung is unknown. Later studies confirmed the stomach-restricted expression of GKN2, also demonstrating specific localization of GKN2 protein in gastric SMCs and its high prevalence in gastric juice (3, 30, 53) of humans and mice.

Because GKN3 is nonfunctional in humans, studies of GKN3 expression have instead been conducted in mice. Akin to other GKNs, GKN3 mRNA and protein were found to be abundantly expressed in the gastric fundus and antrum but were not detected in all other tissues examined. Allied in situ expression studies revealed GKN3 protein and mRNA in antral gland cells (AGCs), a subpopulation of fundic mucous neck cells (MNCs), and Brunner’s glands of the duodenum (42).
Key differences in tissue localization are noted between individual GKNs (Fig. 3). GKN1 and GKN2 are specifically expressed by SMCs of the antrum and fundus, but not by other epithelial lineages situated deeper within the glands marked by GKN3 expression (40, 50, 53). Conversely, GKN3 is absent from all gastric SMCs. These contrasting profiles mirror those manifested by members of the TFF and gastric mucin families (26). Comparisons between expression profiles for the GKNs, TFFs, and mucins are summarized in Fig. 3. Two distinct profiles are evident: 1) TFF1 and MUC5AC expression overlaps GKN1/GKN2 expression in SMCs, while 2) TFF2 and MUC6 coincide with GKN3 in MNCs, AGCs, and Brunner’s glands. These two profiles segregate according to the bidirectional outward migration of immature epithelial cells from the isthmic stem cell zone, suggesting provenance from divergent differentiation programs. Additionally, spatial restriction to the surface (GKN1/GKN2), neck, or base region (GKN3) of gastric glands, away from rapidly proliferating progenitor cells of the isthmus, indicates that mitotically quiescent differentiated epithelial cells are the predominant or possibly sole sources of GKNs.

As discussed above, GKNs are predicted to function as secreted proteins acting primarily in the extracellular and/or luminal environment. Accordingly, results from numerous studies support active secretion of GKNs. Immunogold-labeling experiments have demonstrated the presence of GKN1 protein in gastric mucous cell secretory granules, while analysis of canine gastric mucus (65) or culture supernatants from transfected cell lines (38) suggests that GKN1 protein is secreted apically. Similar approaches have also been employed to demonstrate the presence of GKN2 protein specifically in secretory granules of gastric SMCs in humans and mice (53). Secretion of mouse GKN3 is supported by several lines of evidence. GKN3 protein has been localized in secretory granules of AGCs by immunohistochemistry and detected in gastric juice by immunoblotting. Moreover, signal peptide processing and rapid accumulation in growth medium of mature GKN3 protein were observed following its overexpression in gastric cell lines (42). Collectively, these data argue that GKNs are actively secreted into the mucus gel overlying the gastric epithelium.

Expression in Gastric Neoplasia

GKN1 and GKN2 were independently discovered as genes strongly downregulated in gastric cancer by differential expression studies (16, 50, 51, 64, 77), prompting recognition of the GKNs as putative stomach-specific tumor suppressor genes.
(TSGs) and implying their potential utility in clinical prediction/diagnosis. The expression differential of GKNs in gastric cancer compared with normal stomach is large, likely spanning several orders of magnitude (T. R. Menheniott, unpublished data). For example, GKN1 mRNA is one of the most abundant transcripts known in the normal stomach [reportedly accounting for ∼1% of total gastric mRNA (50)], yet it is frequently undetected in gastric tumors. Loss of GKN1 expression was first described in differential display analysis that compared gastric tumors with disease-free gastric mucosal tissue (77) and, subsequently, in 82% (41 of 50 cases) of a modest-sized cohort without association to tumor subtype, lymph node metastasis, or clinical stage (64). By serial analysis of gene expression, Oien et al. (50, 51) independently identified GKN1 as downregulated in gastric cancer and reported loss in 100% of cancers examined (8 of 8 cases, low numbers noted), including intestinal and diffuse subtypes. The finding of GKN1 mRNA and protein expression loss has since been replicated in several other human gastric tumors (35, 37, 47, 76).

GKN2, although researched less intensively than GKN1, appears equally vulnerable to downregulation during gastric cancer development. GKN2 was initially identified with respect to profound expression loss revealed by subtractive hybridization between gastric tumors and disease-free tissues (16). May et al. (40) subsequently reported ubiquitous loss of GKN2 (TFIZ1) expression in a small number of gastric cancers (15 of 15 cases) and also found significant association with lymph node metastasis in combination with high TTF1 expression. A large (155 cases) cohort study by Moss et al. (44) is unique in having investigated the combined prognostic effects of GKN1 and GKN2. The authors revealed GKN1 expression loss in 78% of diffuse-type and 42% of the more prevalent intestinal-type gastric cancers. Frequencies of GKN2 loss were similar, affecting 85% and 54% of diffuse- and intestinal-type cancers, respectively. Overall, expression loss of GKN1 was positively correlated with that of GKN2. Next, Moss et al. examined the predictive utility of combined GKN1/GKN2 expression loss finding significant association with poor prognosis after interventional surgery. Additionally, GKN2 loss was correlated with shorter survival time in intestinal-type gastric cancer by multivariate analysis (44). Therefore, combined GKN1/GKN2 expression loss occurs at high frequency in gastric cancer (Table 1) and may correlate with shorter overall survival in the intestinal subtype. Echoing the cautionary sentiment of Moss et al., the true applicability of GKN1 and GKN2 as prognostic markers, used alone or combined, awaits validation in appropriately powered clinical trials.

In addition to solid tumors, universal loss of GKN expression has been reported in gastric cancer cell lines, including MKN1, MKN28, MKN45, MKN74, NUGC-4, and KATO III (77). These observations have been confirmed and extended by subsequent studies (39, 50, 51). Complete loss of GKN2 expression has been confirmed in several gastric cancer cell lines, including AGS, KATO III, and HCT-1 (71). On the basis of these findings, it is tempting to speculate that loss of GKNs is a prerequisite for the oncogenic transformation of gastric epithelial cells. Then again, these data are explained equally well by a model in which cancer lineages expand predominantly from cell types not normally expressing GKN1 or GKN2.

While there is no doubt that expression of GKNs is altered in gastric cancer, causal molecular mechanisms have been neither widely sought nor well defined. Cross cytogenetic aberrations, including duplications, translocations, deletions, or loss of heterozygosity encompassing human chromosome 2p14, where the GKN genes are located, have not been found in gastric tumors (7, 52, 54). It is also noteworthy that chromosome 2 anomalies associated with gastric cancer in general are extremely uncommon (25, 27, 54). These observations argue that mechanisms other than chromosome anomalies disrupt the expression of GKNs in gastric cancer. Accordingly, somatic mutations have been sought in the coding regions of GKN1 and GKN2, yet none were found (76). Recently, using qualitative methylation-specific PCR, Yoon et al. (76) identified hypermethylation of the GKN1 promoter in a small subset of gastric cancers (2 of 25 cases), suggesting that epigenetic mechanisms are involved in some instances of cancer-related GKN expression loss. They also reported frequent (>50%), but highly variable, decreases in GKN1 copy number that correlated to some extent with mRNA, but not protein, levels (76). While unifying molecular mechanisms have not emerged, altered DNA copy number and epigenetic modification may

Table 1. Altered expression of GKNs in human and animal gastrointestinal disease models

<table>
<thead>
<tr>
<th>Disease/Model</th>
<th>Expression Change</th>
<th>Species</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastric adenocarcinoma</td>
<td>GKN1, GKN2</td>
<td>Human</td>
<td>16, 35, 40, 44, 47, 50, 64, 75–77</td>
</tr>
<tr>
<td>Gastric cancer cell lines</td>
<td>GKN1, GKN2</td>
<td>Human</td>
<td>16, 35, 50, 64, 71, 72, 77</td>
</tr>
<tr>
<td>Helicobacter pylori infection/CAG</td>
<td>GKN1, GKN2</td>
<td>Human</td>
<td>36, 47, 48, 60</td>
</tr>
<tr>
<td>Intestinal metaplasia/dysplasia</td>
<td>GKN1, GKN2</td>
<td>Human</td>
<td>35, 44, 50</td>
</tr>
<tr>
<td>NSAID treatment (aspirin, celecoxib/meloxicam)</td>
<td>GKN1</td>
<td>Human</td>
<td>36, 38</td>
</tr>
<tr>
<td>Hypertrophy (H+/K+-ATPase β-subunit−/−)</td>
<td>GKN1, GKN2</td>
<td>Mouse</td>
<td>42</td>
</tr>
<tr>
<td>Fundic atrophy/mucus metaplasia (gp130fl/flTff2−/−)</td>
<td>GKN1, GKN2</td>
<td>Mouse</td>
<td>42</td>
</tr>
<tr>
<td>Antral tumorigenesis (gp130fl/fl)</td>
<td>GKN1, GKN2</td>
<td>Mouse</td>
<td>42, 56</td>
</tr>
<tr>
<td>Gastric NSAID injury (indomethacin)</td>
<td>GKN1</td>
<td>Mouse</td>
<td>65</td>
</tr>
<tr>
<td>Gastric nematode challenge</td>
<td>GKN3</td>
<td>Sheep</td>
<td>46</td>
</tr>
<tr>
<td>Gastric metaplasia (duodenum)</td>
<td>GKN2</td>
<td>Human</td>
<td>3</td>
</tr>
<tr>
<td>Gastric metaplasia (ovarian mucinous tumor)</td>
<td>GKN1</td>
<td>Human</td>
<td>50</td>
</tr>
<tr>
<td>Gastric metaplasia (Barrett’s esophagus)</td>
<td>GKN1</td>
<td>Human</td>
<td>50</td>
</tr>
<tr>
<td>Crohn’s disease/UACL</td>
<td>GKN1, GKN2</td>
<td>Human</td>
<td>50, 53</td>
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</table>

GKN, gastrokine; CAG, chronic atrophic gastritis; NSAID, nonsteroidal anti-inflammatory drug; UACL, ulcer-associated cell lineage. ↓, Decreased or loss of expression; ↑, increased or ectopic (e) expression.
Resnick et al. (60) found that GKN2 expression in H. pylori-infected gastric mucosal tissue (36, 47, 48, 60). Also relevant are reports of decreased GKN1 expression in the context of gastric mucosal aspirin injury (36, 38), suggesting that gastric inflammation, rather than bacterial presence, is the critical factor mediating transcriptional repression of GKNs. A microarray study of paired gastric mucosal biopsies collected from H. pylori-infected patients before and after antibiotic eradication therapy (60) provides further evidence that GKNs participate in the host response to H. pylori eradication. Resnick et al. (60) found that GKN2 mRNA was the most strongly upregulated in the gastric transcriptome after H. pylori eradication, while GKN1 was similarly upregulated. Concomitant with restored expression of GKNs, the infection-related genes, IL-8, REG3α, and β-defensin, were downregulated, consistent with the cessation of proinflammatory responses. Findings in other gut inflammatory models suggest that GKNs exert cytoprotective and/or anti-inflammatory activities and may even be subject to transcriptional regulation by inflammatory stimuli (36, 38, 65, 69). GKNs might therefore subdue low-level incidental inflammation under normal conditions, a process that appears to be compromised by H. pylori infection. Whether decreased expression of GKNs is mediated directly by H. pylori for the purposes of infection or, alternatively, represents a host strategy to potentiate immune responses against the bacterium remains to be proven.

Expression in Other Pathologies

Unlike the TFFs, which display altered expression in neoplasias within and outside the gastrointestinal tract (1, 2, 4, 6, 22, 29, 41, 56, 66), altered expression of GKNs is associated almost exclusively with gastric cancer. Exceptions have been noted. For example, ectopic GKN2 expression has been described in gastric metaplasia of the duodenum (3). Similarly, ectopic GKN1 expression has been found in gastric metaplasia of Barrett’s esophagus and ovarian neoplasias (50). Finally, ectopic expression of GKN1 and GKN2 was reported in the ulcer-associated cell lineage of reductive intestinal epithelium in Crohn’s disease (50, 53), sites where ectopic TFF1 and TFF2 expression has also been found (26). Taken together, these studies suggest broader roles for GKNs in regulating not only gastric epithelial homeostasis, but also gastrointestinal wound healing and/or repair, possibly in conjunction with TFFs.

Expression in Animal Models of Gastric Disease

Mouse models of gastric disease have been underutilized as a resource for studies of GKN expression and function. Nevertheless, the limited literature identifies several mouse models that accurately recapitulate the transcriptional responses typi-


cally exhibited by GKNs in corresponding human pathologies (Table 1). For example, Gkn1 and Gkn2 show decreased expression in mouse genetic models of gastric glandular atrophy, metaplasia, hypertrophy (42), tumorigenesis (56), and H. pylori infection (T. R. Menheniott, unpublished data). Reduced Gkn1 expression was observed in a mouse model of gastric indomethacin injury (65), again correlating with the findings of analogous studies in humans (36, 38).

Animal models have provided valuable insights into the possible function(s) of GKN3, which, because of its acquired pseudogene status, cannot be investigated directly in humans (42). We observed Gkn3 to be upregulated in murine gastric atrophy and mucous cell metaplasia (42), which are precancerous lesions known typically to be associated with H. pylori infection in mice and humans (17). Upregulation of Gkn3 expression was also seen following nematode infection in genetically susceptible sheep compared with resistant sheep (46). In summary, animal models have been instrumental in revealing differential responses of individual GKNs to gastric disease. Generally, Gkn1 and Gkn2 are downregulated, while Gkn3, characteristically, is upregulated. Understanding of the functional significance of these responses awaits transgenic overexpression or loss-of-function experiments in mouse models of perturbed gastric epithelial homeostasis or inflammation. Published data describing altered expression of GKNs in human disease and relevant animal models are summarized in Table 1.

Transcriptional Regulation

Mechanisms regulating transcription of the GKNs are not well understood, and this area remains one of the most intriguing, yet least studied, aspects of GKN biology. For example, it is unclear how the lineage-specific expression of GKNs or their altered expression in gastric disease is controlled at the molecular level. Of possible relevance, the GKN genes show strict conserved linkage at the genomic level, suggesting coordinate regulation by shared cis elements. Although there is no doubt that such elements exist, current literature indicates neither their location nor the identity of cognate transcription factors that bind them.

A relatively small number of unrelated transcription factors are known to regulate differentiation of the major gastric epithelial lineages (23, 28, 31, 58, 68), yet none of these have proven critical for regulating GKNs. For example, the forkhead domain transcription factor FOXQ1 is required for the correct differentiation and function of gastric SMCs, the major site of GKN1 and GKN2 expression. Mice homozygous for the Foxq1<sup><b>Δ/Δ</b></sup> (radiation-induced Foxq1 mutant) allele displayed definitive SMC differentiation and lacked expression of the diagnostic marker Muc5ac, yet Gkn1 (and Tff1) expression was unaffected (68). Therefore, FOXQ1 is not likely required for expression of GKNs (or TFFs) in SMCs.

In accordance with their profound suppression in H. pylori infection, GKNs are the most upregulated genes in the human transcriptome following H. pylori eradication and cessation of associated gastritis (60). This seminal observation invokes the hypothesis that proinflammatory cytokines (also immune-related transcription factors) might influence, or even regulate, transcription of the GKNs. In recognition of this possibility, Baus-Loncar et al. (3) investigated the effects of proinflammato-
GASTROKINES IN STOMACH HOMEOSTASIS AND DISEASE

In summary, mechanisms governing coordinated and lineage-specific transcription of the GKNs are not well understood. Lessons learned from studies of similarly expressed genes, such as the TFFs, provide a blueprint on which future transcriptional studies of the GKNs could be based. Emerging data suggest that transcription of GKN2 (and, potentially, GKN1) might be negatively regulated by proinflammatory cytokines, likely by activation of cytokine-dependent signaling cascades. If this notion is proven correct, transcriptional repression by cytokines will establish a mechanistic link between inflammatory pathways (74).

Recent studies attribute growth suppression by GKN1 to activities of canonical tumor suppressor proteins and signal transduction cascades. Xing et al. (72) found that antiproliferative activities of GKN1 in BGC-823 gastric cancer cells require activation of p16INK4a/retinoblastoma and p21WAF senescence pathways. Furthermore, GKN1-dependent activation of p16INK4a/p21WAF required sustained MAP kinase signaling in gastric cancer cells and a tumor xenograft model (72). Similarly, another study showed sustained activation of the MAP kinases ERK1/2 and JNK1/2, together with reduced activation of PKCβ, in concert with GKN1-dependent growth arrest in SGC-7901 cells (74). Therefore, GKN1 might inhibit the growth of gastric cancer cells by coordinate regulation of specific intracellular protein kinase and senescence pathways (74).

Yan et al. (74) employed a proteomic approach to elucidate global changes underlying GKN1-dependent growth inhibition. They reported significant downregulation of the pronocogenic protein enolase-α (ENO1), among 74 differentially expressed proteins. RNA interference-mediated knockdown of ENO1 was sufficient to induce cell cycle arrest in SGC-7901 gastric cancer cells in the absence of GKN1, while forced expression of ENO1 prevented GKN1-dependent growth inhibition and cell cycle arrest (74). These results identify ENO1 as a possible target and antagonist of GKN1 activity in gastric cancer cells.

### Biological Activity and Signal Transduction

In vivo functions of the GKNs have not been clearly demonstrated. Moreover, progress in understanding their fundamental physiological roles has been hindered, particularly by a lack of mouse genetic models deficient in, or overexpressing, GKNs. Current literature implicates roles in epithelial protection/wound healing, immune modulation, growth promotion/inhibition, and tumor suppression (Table 2). Still, much of this evidence has been captured from in vitro studies using transformed cell lines that were not always of gastric origin. Thus it is not surprising that conflicting data have emerged. Many known effects are attributed to GKN1, but whether the other GKNs serve or share these activities has not been extensively tested.

Numerous studies have described antiproliferative activity for GKNs in gastric epithelial cells, which, together with frequent GKN expression loss in cancer, adds weight to long-suspected roles as stomach-specific TSGs. GKN1 overexpression and/or recombinant protein has been shown to inhibit growth of gastric cancer cells by several independent groups (35, 64, 72, 74, 76). Similarly, GKN2 and GKN3 overexpression inhibits growth of SGC-7901 and MKN28 gastric cancer cells, respectively (15, 16, 42).

Table 2. Reported biological activities of GKNs

<table>
<thead>
<tr>
<th>Activity</th>
<th>GKN</th>
<th>Species/Cell Line/Model</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antiproliferative</td>
<td>GKN1, GKN2, GKN3</td>
<td>Human gastric cancer, AGS, MKN28, SGC-7901, BGC-823</td>
<td>15, 16, 35, 42, 64, 72, 74–76</td>
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<tr>
<td>Mitogenic</td>
<td>GKN1</td>
<td>Human gastric normal, GES1, rat intestinal, IEC18</td>
<td>39, 72</td>
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<tr>
<td>Apoptosis</td>
<td>GKN1</td>
<td>Human gastric cancer, AGS, BGC-823</td>
<td>35, 72</td>
</tr>
<tr>
<td>Migration/resistion</td>
<td>GKN1</td>
<td>Human gastric cancer, AGS, BGC-823</td>
<td>35, 72</td>
</tr>
<tr>
<td>Anti-EMT/invasion</td>
<td>GKN1</td>
<td>Human HAE, rat IEC18 (scratch assays)</td>
<td>56, 69</td>
</tr>
<tr>
<td>TFF interaction</td>
<td>GKN2</td>
<td>Human AGs (Matrigel invasion assays)</td>
<td>75</td>
</tr>
<tr>
<td>Gastric epithelial protection</td>
<td>GKN1</td>
<td>Mouse NSAID injury model (indomethacin)</td>
<td>65</td>
</tr>
<tr>
<td>Intestinal epithelial protection</td>
<td>GKN1</td>
<td>Mouse DSS colitis model, human colonic Caco-2, MDCK</td>
<td>69</td>
</tr>
</tbody>
</table>

TFF, trefoil factor; rhAMP-1877-97 recombinant human 18-kDa antral mucosal protein (synthetic 21-residue peptide derived from the BRICHOS domain of GKN1); DSS, dextran sodium sulfate; EMT, epithelial-mesenchymal transition; MDCK, Madin-Darby canine kidney.

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In contradiction to its proposed tumor suppressor role, GKN1 has been reported to exert mitogenic activity (39, 65, 69). Martin et al. (38, 39) observed proliferative effects of mouse and pig gastric protein extracts in BSC-1 kidney epithelial cells that could be blocked by coincubation with anti-GKN1 (AMP-18)-neutralizing antibodies, but not preimmune serum. In addition, full-length recombinant human GKN1 peptide (rhAMP-18) stimulated proliferation of rat IEC-18 intestinal epithelial cells, which, again, was blocked by coincubation with anti-GKN1 antibodies (39). In allied studies, a bioactive recombinant 21-mer peptide comprising amino acids 77–97 of the mature GKN1 protein (rhAMP1877–97) stimulated proliferation (and migration) of human gastric cancer cell lines and nontransformed IEC-6 rat intestinal epithelial and BSC-1 cells, but not fibroblasts or HeLa cells (39, 65).

The above-described findings are not easily reconciled with the widely described antiproliferative activities of GKN1 (35, 64, 72, 74, 76). However, mitogenic functions attributed to GKN1 in gastric cells are largely with respect to the synthetic rhAMP1877–97 peptide, which has no demonstrated equivalent in vivo, thus questioning its physiological relevance. Similarly, it must be acknowledged that mitogenic effects of full-length rhAMP-18 have been firmly established only in intestinal epithelial cells (39), but not convincingly in any cells of gastric origin. More recently, Xing et al. (72) reported opposing growth effects of GKN1 in two different gastric cell lines. Using full-length recombinant GKN1, they observed dose-dependent growth inhibition of BGC-823 cells, but not GES-1 cells, which instead showed modestly increased growth (72). While the basis of these discrepant responses is unclear, GES-1 cells are SV40-transformed, questioning whether en

While the data are compelling, additional stringently controlled experiments are needed to authenticate the bioactivities of the rhAMP1877–97 peptide as correctly representative of endogenous GKN1 function. Indeed, the notion of GKNs having bioactivity toward intestinal epithelial cells seems at odds with their endogenous synthesis in the stomach, raising the question of how it might be enacted physiologically. Possibly relevant are reports of ectopic GKN1 and GKN2 expression in the ulcer-associated cell lineage of reparative intestinal epithelium in Crohn’s disease (50, 53). Although speculative, induction of GKN2 transcription by the wound-healing-associated cytokine transforming growth factor β1, specifically in colonic, but not gastric, epithelial cells (3), suggests a mechanism by which this might occur.

In summary, GKNs clearly elicit cellular responses (Table 2) and activate signal transduction (72, 74, 75), yet similar to the TFFs (26), firm evidence supporting the existence of cognate receptors is lacking. Efforts to further delineate signaling mechanisms underlying GKN bioactivity are warranted to gain a better understanding of their cell-type specificity, as well as to reveal strategies for exploiting GKN1, or its peptide derivatives, as selective antitumor or mucosal protective therapeutics.

**TFF Interaction**

Formal recognition of GKNs as putative TFF-interacting proteins was garnered by the seminal finding of Westley et al. (71) that GKN2 exists predominantly as a heterodimer with TFF1. Using specific antibodies to each of the native proteins in coimmunoprecipitation assays, the authors characterized the physical interaction of GKN2-TFF1 dimers. Immunoblot analysis revealed a major ∼25-kDa protein under nonreducing conditions fragmented into two lower-molecular-mass (∼18- and ∼7-kDa) proteins when reduced. Since the molecular mass of TFF1 is known to be close to 7 kDa, further work to characterize the 18-kDa band revealed a BRICHOS family protein with 42% sequence similarity to GKN1. This protein, initially named trefoil factor interactions(z) 1 (TFIZ1), was subsequently found to be synonymous with GKN2. GKN2-TFF1 heterodimers were highly resistant to chemical denaturation, proteolysis, or boiling but were readily monomerized by treatment with mucolytic thiol agents (71), conclusively showing linkage of the two proteins by disulfide bonding.

Distinct from other family members, GKN2 contains an additional cysteine residue at position 38 (Fig. 2) (71) that likely stabilizes the heterodimer by disulfide linkage to an unpaired cysteine (cysteine 58) in TFF1. GKN2 is a neutral protein [isoelectric point (pI) = 6.59], whereas TFF1 is highly acidic (pI = 3.94). GKN2-TFF1 heterodimers consequently form a relatively acidic complex (pI = 5.16) (71), which might have functional relevance to mucous integrity or biophysical properties not conducive to microbial colonization.

Aside from the work by Westley et al. (71), subsequent studies have failed to unearth any persuasive evidence of in vivo interactions between other GKNs and TFFs (40, 42, 53). Nevertheless, a noncovalent in vitro interaction between mouse Gkn2 and Tff2 has been described (53): an alkaline phospha-
the conclusions of May et al., discordant GKN2 and TFF1 levels, in tumors of the breast, pancreas, and bile duct (2, 26, 29), sites where GKN2 expression does not occur. To reiterate, GKN2-TFF1 heterodimers are demonstrably prooncogenic (26, 57). This has functional relevance to cancer progression, since equilibrium shifts in favor of TFF1 homodimerization (40).

molecules becomes limiting due to loss of expression, the physiological roles of GKN2-TFF1 heterodimers remain undefined. Nevertheless, strong sequence conservation of human and mouse TFF1 and GKN2 orthologs, including the critical cysteine 38 residue in GKN2 (T. R. Menheniott, unpublished data; 40, 71), supports the existence of Gkn2-Tff1 heterodimers in mice, as does coexpression of GKN2 and TFF1 in gastric SMCs of both species.

The issue of GKN-TFF interaction resurfaced following the discovery of GKN3. Spatial coexpression of Gkn3 and Tff2 in mice led us to hypothesize that Gkn3 represents an unrecognized Tff2 binding protein (42). Nevertheless, mouse Gkn3 and Tff2 proteins were not coimmunoprecipitated after transfection into MKN28 cells or directly from gastric lysates, thus arguing against physical Gkn3-Tff2 interactions in vitro or in vivo (42). In any event, naturally occurring disulfide-linked Gkn3-Tff2 heterodimers are unlikely to exist, because neither protein contains the unpaired cysteine residues required for this mode of covalent bonding (42). We conclude that TFF interaction is not a shared attribute of GKNs and is likely unique to GKN2. Therefore, the terminology “trefoil factor interactions(z)” or “TFIZ” (71) has limited application to GKNs, since GKN2 (TFIZ1) interacts with only one of the three TFFs in vivo, and evidence supporting interactions of GKN1 (TFIZ2) or GKN3 with the TFFs is lacking.

Despite extensive molecular characterization, the salient physiological roles of GKN2-TFF1 heterodimers remain undefined. Nevertheless, heterodimer formation is likely to be essential for the correct function of both proteins. May et al. (40) proposed that relative amounts of the different molecular forms of GKN2 and TFF1 might be determined by the molar ratio of these two proteins. That is, if the number of GKN2 molecules becomes limiting due to loss of expression, the equilibrium shifts in favor of TFF1 homodimerization (40). This has functional relevance to cancer progression, since TFF1 homodimers are demonstrably pronoecogenic (26, 57). Additionally, TFF1 is ectopically expressed, often at high levels, in tumors of the breast, pancreas, and bile duct (2, 26, 29), sites where GKN2 expression does not occur. To reiterate the conclusions of May et al., discordant GKN2 and TFF1 expression in some gastric cancers might explain why TFF1 is reportedly beneficial in normal gastric epithelium (where GKN2 is present) but often regarded as detrimental in tumors (where GKN2 is frequently absent) (26, 40). The data are consistent with the idea that GKN2 functions directly as a tumor suppressor or that heterodimer formation is required for the tumor suppressor activity of TFF1 to be enacted.

GKN3: An Example of Advantageous Gene Loss in Humans?

One of the most enigmatic aspects of GKN biology pertains to the functional loss of human GKN3 (42). Unlike most other mammals in which GKN3 is active, the human GKN3 ortholog persists only as a nontranscribed pseudogene. While remarkably conserved at the level of exon structure (Fig. 1), nucleotide sequence, and open reading frame, human GKN3 contains a premature stop codon (nonsense mutation) substituting a tryptophan codon in the ancestral sequence at position 59, described as “W59X” (Fig. 2). Nonsense mutations can result in the expression of truncated or erroneous proteins and are usually eliminated at the transcriptional level by a type of mRNA surveillance known as “nonsense-mediated decay” (NMD).4 We confirmed that absence of GKN3 mRNA expression in humans is due to NMD, possibly acting on the W59X mutation (42).

Homozygosity for W59X is absolute in most human populations, but it is intriguing that a polymorphic ancestral GKN3 “read-through” allele predominates in individuals of sub-Saharan African descent. While the overall picture is still unclear, the population genetic data are consistent with the rapid spread and fixation of nonfunctional W59X alleles among non-Africans during recent evolution (42).

Evolutionary forces underlying the spread of nonfunctional GKN3 alleles are not apparent. However, precedents exist for the rapid spread of nonsense mutations at other human loci by positive selection (33, 62, 70), of which the best-characterized example is CASPASE (CASP) 12 (61, 62). Belonging to the family of inflammatory caspases, which generally promote activation of specific proinflammatory cytokines, CASP12 encodes a functionally distinct enzyme that, instead, attenuates release of these cytokines, including IL-1β, IL-18, and IFNγ (61). The CASP12 gene carries a nonsense single-nucleotide polymorphism that generates a full-length functional proenzyme or a truncated nonfunctional variant. The functional form is common in individuals of African descent and is linked to greater risk of sepsis. Conversely, the nonfunctional CASP12 allele has reached fixation in most other populations, where it correlates with increased responsiveness to bacterial endotoxins and resistance to sepsis (62). In support of these observations, Casp12−/−-deficient mice show enhanced bacterial clearance and resistance to sepsis (61). Current opinion asserts that near-complete fixation of nonfunctional CASP12 (in non-Africans) was an adaptive event driven by positive selection, probably because of the benefits conferred by protection against sepsis (70, 73). By analogy, future phenotype analysis of Gkn3−/−-deficient mice may elucidate whether inactivation of GKN3 in humans conferred physiological advantages on which positive selection could have acted.

4 Nonsense-mediated decay is a cellular mechanism of mRNA surveillance that functions to detect nonsense mutations and block the expression of truncated or erroneous proteins.
Why gene inactivation has specifically affected GKN3, but not other human GKNs, will doubtless be subject to further debate. Without firm data either way, neutral drift due to genetic redundancy seems improbable, since GKN3 is likely to function differently from the other family members (Fig. 3, Table 1) (42). We therefore speculate that GKN3, because of its functional distinction, was uniquely exposed to selective forces ultimately driving its loss in ancestral humans.

Unresolved Questions and Concluding Remarks

Significant advances in knowledge of the GKNs have been made since their discovery during the past decade. The literature reviewed here illustrates that GKNs have major clinical significance: they likely participate in the host mucosal response to H. pylori and, via antiproliferative or epithelial homeostatic activity, may function as stomach-specific tumor suppressors. As evidenced by ectopic expression in reparative lesions of Crohn’s disease, GKNs might also exert cytoprotective and restitutive activities, which, on the basis of in vitro functional data, could extend beyond the stomach to the intestinal epithelium.

Modes of action are becoming increasingly apparent, yet the picture is far from complete. As discussed here, GKNs likely activate intracellular signal transduction, yet cognate cell surface receptors remain elusive. The well-described GKN2-TFF1 physical interaction, at first thought to signify broader interplay between GKNs and TFFs, now seems to be unique among these two protein families. While the functional significance of GKN2-TFF1 heterodimers remains unsubstantiated, discrepant expression of the two proteins in gastric cancer argues strongly for codependent epithelial homeostatic and/or tumor suppressor actions. Not yet proven, GKN2 and TFF1 may have other codependent activities distinct from their homeostatic roles. For example, TFF1 functions as an extracellular chemotractant (8, 59) utilized by motile H. pylori to colonize gastric surface cells. Future studies to discern whether GKN2-TFF1 heterodimers have H. pylori chemotactic activity comparable to or different from TFF1 homodimers may yield insights of clinical importance.

The widely implicated “anticancer” activity of GKNs questions how they might be harnessed for therapeutic benefit. Future research might explore the utility of complementing GKN expression and/or function using recombinant peptides, particularly in advanced gastric preneoplasia, where H. pylori eradication may not halt cancer progression. Also relevant, gastric inflammation and associated proinflammatory cytokine release likely contribute to transcriptional repression of GKNs, which is rapidly reversed following H. pylori eradication. A better understanding of mechanisms underlying these transcriptional responses will be pivotal to the therapeutic potential of GKNs being realized. Thus, as an alternative approach to recombinant peptide delivery, pharmacological targeting of the key transcriptional control mechanisms might enable endogenous GKN expression to be restored in advanced preneoplastic disease to delay or prevent cancer progression. However, the immediate challenges ahead involve the generation and comprehensive phenotype analysis of knockout mice carrying null alleles for each of the GKNs. The marked and highly replicated perturbation of GKNs in gastric pathologies suggests that roles of profound significance will ultimately be proven.

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No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

T.R.M., B.K., and A.S.G. are responsible for conception and design of the research; T.R.M. prepared the figures; T.R.M., B.K., and A.S.G. drafted the manuscript; T.R.M. and A.S.G. edited and revised the manuscript; T.R.M. and A.S.G. approved the final version of the manuscript.

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