Original research

Iqgap3-Ras axis drives stem cell proliferation in the stomach corpus during homoeostasis and repair

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INTRODUCTION

Characterisation of the mechanisms underlying the proliferation, plasticity and lineage commitment of adult stem cells is necessary for understanding the preneoplastic events leading to gastric cancer. In the stomach corpus, the gastric gland can be subdivided into four zones: the pit, the isthmus, the neck and the base. Classical radioactive tracer experiments indicated rapidly proliferating cells in the isthmus, which were revealed by electron microscopy to be granule-free stem cells.1 The isthmus region is therefore accepted to be the active stem cell zone of the corpus. Zymogenic chief cells located at the base of the stomach corpus are fully differentiated, postmitotic cells. Interestingly, in 2010, various studies reported that chief cells reacquire proliferative capacity, suggesting that

ABSTRACT

Objective Tissue stem cells are central regulators of organ homoeostasis. We looked for a protein that is exclusively expressed and functionally involved in stem cell activity in rapidly proliferating isthmus stem cells in the stomach corpus.

Design We uncovered the specific expression of Iqgap3 in proliferating isthmus stem cells through immunofluorescence and in situ hybridisation. We performed lineage tracing and transcriptomic analysis of Iqgap3+isthmus stem cells with the Iqgap3-2A-tdTTomato mouse model. Depletion of Iqgap3 revealed its functional importance in maintenance and proliferation of stem cells. We further studied Iqgap3 expression and the associated gene expression changes during tissue repair after tamoxifen-induced damage. Immunohistochemistry revealed elevated expression of Iqgap3 in proliferating regions of gastric tumours from patient samples.

Results Iqgap3 is a highly specific marker of proliferating isthmus stem cells during homoeostasis. Iqgap3+isthmus stem cells give rise to major cell types of the corpus unit. Iqgap3 expression is essential for the maintenance of stem potential. The Ras pathway is a critical partner of Iqgap3 in promoting strong proliferation in isthmus stem cells. The robust induction of Iqgap3 expression following tissue damage indicates an active role for Iqgap3 in tissue regeneration.

Conclusion IQGAP3 is a major regulator of stomach epithelial tissue homoeostasis and repair. The upregulation of IQGAP3 in gastric cancer suggests that IQGAP3 plays an important role in cancer cell proliferation.
chief cells may be progenitors for the preneoplastic spasmyotic polypeptide-expressing metaplasia. In 2013, Troy + chief cells were reported to serve as quiescent stem cells, which can be induced to proliferate during tissue damage to regenerate the entire gastric unit. A different view emerged in 2015, when Mist1 + quiescent stem cells were identified in the isthmus and proposed to be the origin of gastric cancer. Notwithstanding this finding, Choi et al used targeted expression of oncogenic Kras in Mist1 + chief cells to contend that metaplasia arises from differentiation or dedifferentiation of chief cells. Leushacke et al identified the expression of established stem cell factor Lgr5 in a subset of chief cells and observed that Kras (G12D) induction in Lgr5 + chief cells, in the presence of injury, led to metaplastic lesions. Together with the analysis of Lgr5-expressing human gastric cancer tissues, they proposed that gastric cancer originated from Lgr5-expressing chief cells.

In 2017, we uncovered strong activity of an enhancer element of Runx1 (eR1), a haematopoietic and human hair follicle stem cell factor, in proliferating isthmus stem cells, as well as a small number of chief cells. Kras (G12D) expression in eR1 + isthmus stem cells induced foveolar hyperplasia and elimination of parietal cells. However, Kinoshita et al detected induction of Lgr5 in isthmus stem cells after tissue injury and proposed that isthmus stem cells and cells from the neck lineage, instead of chief cells, contributed to metaplasia. Arguing that the contradictory data may have stemmed from the specificity of the chief cell markers, Hata et al identified Gpr30 as a specific marker of chief cells, with no expression in isthmus stem cells. Expression of Kras (G12D) in Gpr30 + chief cells did not result in metaplasia but, instead, led to reduced chief cell population and a compensatory expansion of neck lineage, derived from Kitl + isthmus stem cells. It was further argued that since most chief cells are lost during metaplasia, they are unlikely to be involved in gastric carcinogenesis. Certainly, after acute oxyntic injury, the increased activity of Lrig1-expressing isthmus stem cells has been shown to contribute to tissue regeneration. During Helicobacter pylori-induced chronic injury, it was reported that increased proliferation and accelerated differentiation of Lrig1-expressing cells gave rise to surface mucous cells and chief cells, which subsequently produced spasmyotic polypeptide-expressing metaplasia. The origin of metaplasia continues to be highly debated, in part due to the expression of some markers in both isthmus and chief cell populations, progenitor cell plasticity, effects from stem cell niche as well as injury.

Two models have been suggested for the homeostasis of the corpus: (1) long-lived stem cells supported by the stem cell niche at the isthmus zone is proposed to maintain the gastric epithelium and, thereby, many of these markers were expressed in other cell types and, thus not specific for proliferating isthmus stem cells. Actively cycling isthmus stem cells were also studied via their expression of proliferation markers Stathmin1 (Stmn1) and Mki67. Several signalling pathways, such as Notch, Sonic hedgehog and AMP-dependent protein kinase, were reported to govern proliferation of gastric epithelial cells. In particular, isthmic stem cell proliferation was found to be driven by the Notch pathway.

Yet, these studies did not reveal the presence of an exclusive and definitive stem cell marker in proliferative isthmus stem cells. In this study, we show that eR1 + isthmus stem cell proliferation is driven by Iqgap3, a member of the Iqgap (IQ motif containing GTPase activating protein) cytoskeletal scaffold family that was reported to be necessary and sufficient for cell proliferation. Knockdown of Iqgap3 in undifferentiated cell lines, such as embryonal carcinoma NTERA-2 and gastric carcinoma HGC-27, resulted in reduction of stem cell-associated NANOG and OCT4 gene expression, as well as induction of differentiation. Therefore, Iqgap3 represents a functionally indispensable stem cell specific factor, which regulates stem cell function in homeostasis and tissue damage repair. Our work further revealed that Iqgap3 induction during tissue repair is associated with the acquisition of oncogenic traits. Finally, we found a strong correlation between Iqgap3 expression and proliferating cancer cells in tumours isolated from gastric cancer patients. Together, these data reveal a mechanism by which Ras is hyperactivated in preneoplasia and identify a role for Iqgap3 in lineage plasticity and proliferation in cancer initiation.

MATERIAL AND METHODS

Mice and treatment

Wild-type (WT) C57BL/6JInv mice were obtained from InVivos Pte Ltd. The eR1-enhanced green fluorescent protein mice and the eR1-CreERT2;Rosa-Lox-Stop-Lox (LSL)-tdTomato (eR1-CreERT2;Rosa-tdTomato) mice were described previously. To induce tdTomato expression in eR1-CreERT2;Rosa-tdTomato mice for short lineage tracing experiments, 6–8 weeks old mice were given a single intraperitoneal injection of 2 mg of tamoxifen (Merck) diluted in corn oil (Merck). The mice were analysed at 16 or 24 hours post-tamoxifen injection. Iqgap3-2A-tdTomato mice were generated to monitor Iqgap3 expression in the stomach (Cyagen). The 2A-tdTomato sequence was inserted before the stop codon in exon 38 (online supplemental figure S1A). Iqgap3-2A-CreERT2 mice were generated for inducing or repressing expression of the gene of interest in Iqgap3 + cells (Cyagen). The 2A-CreERT2 sequence was inserted before the stop codon in exon 38 (online supplemental figure S2A). Iqgap3-2A-CreERT2 mice were bred to Rosa-tdTomato mice to generate Iqgap3-2A-CreERT2;Rosa-tdTomato mice. To induce tdTomato expression, 6–8 weeks old mice were given a single intraperitoneal injection of 2 mg of tamoxifen. The mice were analysed 1-day, 3-month, 6-month and 1-year post-tamoxifen injections. Iqgap3-2A-CreERT2 mice were also bred to LSL-Kras (G12D/12D) mice to generate Iqgap3-2A-CreERT2;Kras (G12D/12D) mice. To induce Kras (G12D) expression, 6–8 weeks old mice were given a single intraperitoneal injection of 2 mg of tamoxifen. The mice were analysed 3 months post-tamoxifen injection. To induce tissue damage in the murine stomach, 6–8 weeks mice were given a single intraperitoneal injection of a high dose of tamoxifen (HDT) (5 mg per 20 g body weight). The mice were analysed at indicated time points post-tamoxifen injection.

Detailed materials and methods are described in online supplemental methods.

RESULTS

Iqgap3 is specifically expressed in rapidly proliferating isthmus stem cells

Using immunofluorescence staining and RNA in situ hybridisation (ISH), we found that Iqgap3 is strongly coexpressed with the proliferation marker Ki67 (figure 1A–D), thereby identifying Iqgap3 as a marker of rapidly proliferating cells at the isthmus zone. Using an Iqgap3-2A-tdTomato mouse, we found...
Figure 1  Expression of Iqgap3 in the isthmus of corpus epithelium. (A) Schematic diagram of a gastric unit in the corpus of the mouse stomach. (B) Immunofluorescence (IF) staining for Iqgap3, Ki67 and E-cadherin (E-cad) on the corpus of wild-type (WT) mouse stomach (n=3). (C) Quantification of Iqgap3+cells in isthmus or base (n=3). Error bar represents SD from 2025 Iqgap3+cells of 3 mice. The Ki67+ region was defined as the isthmus zone. (D) In situ hybridisation (ISH) for Iqgap3 (green) and Mki67 (red) on the corpus of WT mice (n=2). (E) IF staining for tdTomato and Ki67 on the corpus of Iqgap3-2A-tdTomato mice (n=3). (F) Flow cytometry to isolate tdTomato/Iqgap3 high expression epithelial cell fraction (Iqgap3high) and tdTomato/Iqgap3 low or negative expression epithelial cell fraction (Iqgap3low/neg) from stomach of Iqgap3-2A-tdTomato reporter mice (n=5). (G) qPCR for Cdh1, tdTomato, Iqgap3, Mki67, Stathmin1 (Stmn1), Lgr5, Bliha15 (Mist1) and Tnfrsf19 (Troy) mRNA from isolated tdTomatohigh (Iqgap3high) and tdTomato(low/neg) (Iqgap3low/neg) gastric epithelial cells. mRNA expression was normalised by Gapdh expression (n=3). Error bars represent SD scale bar=100 µm. qPCR, quantitative PCR.

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robust *tdTomato* expression in Ki67+cells at the isthmus (figure 1E; online supplemental figure S1A,B). We next isolated *Iqgap3* tdTomatohigh EpCAMhigh epithelial cells by flow cytometry (figure 1F). Quantitative PCR (qPCR) showed that *tdTomato*, *Iqgap3* and *Mki67* mRNA were strongly enriched in the *Iqgap3* tdTomatohigh EpCAMhigh epithelial cells, relative to the *Iqgap3* tdTomato*low/neg* population (figure 1G, upper panel). Strong enrichment of *Stmn1* mRNA—previously shown to mark cycling isthmus stem cells—further confirmed the proliferative nature of *Iqgap3* tdTomatohigh cells (figure 1G, lower panel). *Lgr5* and *Toyro* expression were low in *Iqgap3* tdTomatohigh cells, suggesting that *Iqgap3*+ cells are stem cells distinct from *Lgr5*+ cells (figure 1G, lower panel). Similarly, *Mist1* expression—earlier reported to mark chief cells and quiescent isthmus stem cells in the corpus—was relatively low in *Iqgap3* tdTomatohigh cells (figure 1G, lower panel). We had previously shown that the activity of the Runx1 enhancer element, *eR1*, could be used to identify adult stem cells in stomach tissues. 9 *eR1-CreERT2;Rosa-tdTomato* mice were treated with tamoxifen to examine *eR1* and *Iqgap3*+ cells in *vivo*. After 16 hours of short lineage tracing, *eR1*+ cells partially coincided with *Iqgap3*—expressing cells at the isthmus, likely because of the variegated expression intrinsic to the mouse model (online supplemental figure S1C). This indicated that *Iqgap3*+ cells harbour isthmus stem cells.

The *Igap* family comprises three genes, namely *Igap1*, 2 and 3. 25 Unlike *Igap1*, the expression of *Igap1* and 2 were not confined to the isthmus, but observed throughout the gastric unit (online supplemental figure S1D–G). Therefore, *Igap3*—alone in the *Igap* family—plays a unique role in driving proliferation of isthmus stem cells.

### Iqgap3+ isthmus stem cells are multipotent and responsible for core homeostasis of corpus glands

We next used *Iqgap3* 2A CreERT2;Rosa-tdTomato mice to perform lineage tracing of *Iqgap3*+ cells (figure 2A,B; online supplemental figure S2A,B). One-day post-tamoxifen injection revealed strong coexpression of *Mki67* and *tdTomato* at the isthmus (figure 2C; online supplemental figure S2B); at 3-month, 6-month and 1-year post-tamoxifen injection, we observed progressive expansion of *tdTomato*+ cells that span almost the entire gastric gland (figure 2C). *tdTomato* expression overlapped with markers of various corpus lineages, including mucous pit, neck, parietal and chief cells (figures 1A and 2D,E). Analysis of the lineage tracing events at 6-month post-tamoxifen injection revealed that ~50% of the glands showed *tdTomato* positivity spanning from the pit to neck region, while ~40% showed *tdTomato* positivity from the pit to the transition regions (see #1 and #2 in figure 2F). About 10% of the glands showed *tdTomato*+ cells spanning from the pit to base region (see #3 in figure 2F). At 1-year post-tamoxifen injection, *tdTomato* positivity spanning the pit to base region had increased to ~55% of the glands (figure 2F). XZ-plane imaging further confirmed *tdTomato*+ cells in the neck (GS-II+/Gif+), transition (GS-II+/Gif+) and chief cell populations (GS-II+/Gif+) (online supplemental figure S2C). The self-replicating nature of chief cells may be the reason why not all chief cells were labelled with *tdTomato*, even after 1 year of tracing. Nevertheless, our time course data indicated that lineage tracing initiated at the isthmus and that *tdTomato*+ cells are multipotent stem cells.

*tdTomato*+ isthmus cells from corpus units isolated from *Iqgap3* 2A CreERT2;Rosa-tdTomato mice readily formed organoids, further confirming the stem potential of *Iqgap3*+ isthmus cells (figure 2G). Induction of differentiation via removal of Wnt3a and R-spondin1 from the organoid culture media (termed ENFG as opposed to WENFRG) 26 led to decreased *Iqgap3* mRNA, accompanied by a sharp increase in differentiation marker *Muc5ac* (online supplemental figure S2D,E). *Iqgap3* expression was therefore specific to stem cells and shows rapid reduction on differentiation in ENFG media. *Iqgap3*+/tdTomato+ organoids showed upregulation of Muc5ac protein on differentiation (online supplemental figure S2F). The organoids derived from *Iqgap3*+ cells possessed PgC+/GS-II+, PgC+/GS-II– and PgC–/GS-II+ cells, as well as Gif+/GS-II+ and Gif+/GS-II– cells, demonstrating that *Iqgap3*+ isthmus cells differentiated to the chief cell, mucus-neck cell and transition cell lineages in our organoid culture system (online supplemental figure S2G,H). In addition, the *Iqgap3*+ cell-derived organoids also showed expression of *Ki67*, but not H, K-ATPase (online supplemental figure S2J). *Iqgap3*+/tdTomato+ cells can therefore generate pit, neck and chief cells in *vivo*.

To understand the function of *IQGAP3* in maintaining stemness, we performed siRNA-mediated knockdown of *IQGAP3* in a well-established model for pluripotency and differentiation, the human embryonal carcinoma NTERA-2 cell line. Depletion of *IQGAP3* led to significant reductions in mRNA and protein expression levels of stem cell factors such as *NANOG*, *OCT4* and *KLF4* (figure 2H,I). We also observed an increase of the glial fibrillary acidic protein, which is expressed on differentiation to astrocytes (figure 2H,I). This proof-of-concept experiment indicates that *IQGAP3* is necessary for the maintenance of stem cell gene expression signature and not just a marker for proliferation.

### Iqgap3 drives stem cell proliferation by promoting the Ras-extracellular signal-regulated kinase signalling pathway

To identify the transcriptional programmes integral to the stem cell state, we next isolated *Iqgap3* tdTomatohigh and *Iqgap3* tdTomato*low/neg* expressing cells by flow cytometry (figure 3A). RNA-sequencing revealed that *Iqgap3* tdTomatohigh cells were highly enriched in *Iqgap3*, *Mki67*, *Stmn1*, *Kit* mRNA, while showing low expression levels of chief cell markers *PgC* and *Gpr30* (online supplemental figure S3A). Gene set enrichment analysis (GSEA) confirmed that *Iqgap3* tdTomatohigh stem cells are observed from *Lgr5*+ cells (figure 3B; online supplemental figure S3B). The transcriptional profile of *Iqgap3* tdTomatohigh cells mapped closely with the short-term haematopoietic stem cell gene signature ST-HSC (online supplemental figure S3C,D). The enriched expression of HSC self-renewal—and asymmetric cell division-associated genes in the *Iqgap3* tdTomatohigh fraction further reinforced the notion that *Iqgap3*+ cells possess stem cell properties (online supplemental figure S3E). Notch, Hedgehog and Wnt pathways were not significantly upregulated (online supplemental figure S3F). Conversely, the strong upregulation of Myc-target, E2F-target and Ras signalling genes in *Iqgap3* tdTomatohigh cells identified these pathways as core programmes driving isthmus stem cell proliferation (figure 3C).

Notably, key components of the Ras signalling pathway, namely Erbb2, Erbb3, Fgfr2, Fgfr3, Met and Ras, were enriched in *Iqgap3* tdTomatohigh stem cells (figure 3D).

We, therefore, investigated the activity of the Ras pathway in isthmus stem cells in *vivo*. Immunostaining showed that *Hras*, *Nras* and *Kras* were all expressed in the corpus, partially overlapping with *Ki67* expression (figure 3E; online supplemental figure S4A,B). Her2 is known to promote Ras pathway activation. 27,28 Immunostaining indicated the expression of Her2 and Ras downstream effector phosphorylated Erk (p-Erk) in the...
Figure 2  The Iqgap3-expressing cells in the isthmus are multipotent stem cells. (A) Iqgap3-2A-CreERT2;Rosa-tdTomato mouse model. (B) Experimental strategy for lineage tracing time course. (C) IF staining for Ki67 and tdTomato on the corpus of Iqgap3-2A-CreERT2;Rosa-tdTomato mice at 1 day (1 d), 3 months (3 m), 6 months (6 m) and 1 year (1 y) post-tamoxifen induction (p.i.) (n=3). Lineage tracing, LT. (D, E) IF staining for tdTomato and markers of major stomach differentiated cells (Muc5ac, H,K-ATPase, GS-II and Gif) on the corpus of Iqgap3-2A-CreERT2;Rosa-tdTomato mice at 1 year post-tamoxifen induction (n=3). (F) Quantification of lineage tracing on the corpus of Iqgap3-2A-CreERT2;Rosa-tdTomato mice at 6 months and 1 year post-tamoxifen induction. (n=3). The tdTomato+lineage tracing glands were categorised into pit to neck (LT #1: Pit - Neck), pit to mucus-neck/chief cell transition (LT #2: Pit-Transition) and pit to base (LT #3: Pit – Base). A total of 167 tdTomato+glands from three mice (6 months) or 172 tdTomato+glands from three mice (1 year) were counted. Error bars represent SD data sets were analysed by one-way ANOVA. ***P<0.001. (G) tdTomato expression in the isolated corpus gastric units from Iqgap3-2A-CreERT2;Rosa-tdTomato mice at 20–24 hours post-tamoxifen administration (top). Corpus organoids were generated from tdTomato +cells (n=2). (H) qPCR for IqGAP3, NANOG, OCT4, KLF4, cMYC, SOX2, CD44v9 and GFAP mRNA from IqGAP3 knockdown embryonic stem cell line NTERA-2. mRNA expression was normalised by GAPDH expression (n=3). Error bars represent SD data sets were analysed by Student’s t-test. *P<0.05, **p<0.01. (I) Immunoblot for IqGAP3, Nanog, Oct4, KLF4, CD44v9, GFAP and GAPDH from knockdown embryonic stem cell line NTERA-2 (n=3). Scale bar=100 μm. ANOVA, analysis of variance; GFAP, glial fibrillar acidic protein; IF, immunofluorescence; qPCR, quantitative PCR.
Figure 3  Igappa3 regulates stem cell proliferation via Ras-ERK pathway. (A) Average of RNA expressions in sorted Igappa3\textsuperscript{high} and Igappa3\textsuperscript{low/neg} cell fractions are shown. (B) Gene set enrichment analysis (GSEA) showing enrichment of Lgr5-negative (LGR5neg) and—high (LGR5pos) corpus epithelial cell gene signature from public datasets (GSE86603) in Igappa3\textsuperscript{high} and Igappa3\textsuperscript{low/neg} cell fractions. P values determined by a weighted Kolmogorov–Smirnov-like statistic and adjusted for multiple hypothesis testing. (C) GSEA showing enrichment of Myc target gene signature, E2F targets gene signature, Ras pathway gene signature in Igappa3\textsuperscript{high} and Igappa3\textsuperscript{low/neg} cell fractions. (D) Heat MAP showing expression of representative Ras-ERK pathway genes in Igappa3\textsuperscript{high} and Igappa3\textsuperscript{low/neg} cell fractions. Gene expression levels are shown in Z-score of CPM of RNA-sequencing. (E–G) IF staining for HRAS, Ki67, E-cad, HER2 and phosphorylated ERK (p-Erk) on the corpus of wild-type (WT) mice (n=3). (H) Immunoprecipitation for the interaction of Venus-tagged Igappa3 (Venus-Igappa3) with Myc-tagged HRAS (Myc-Hras) or Myc-tagged Hras\textsuperscript{G12V} (Myc-Hras\textsuperscript{G12V}) and immunoblot for cell lysate from Venus-Igappa3/Myc-Hras/Myc-Hras\textsuperscript{G12V} expressed 293T cells. (I) Experimental strategy to suppress Igappa3 expression by shRNA in organoids from WT mice. (J) Quantification of size of Igappa3 knockdown organoids from 0 passage (6 day post doxycycline (Dox) treatment, before passage), first passage (8 days postpassage) and second passage (7 days postpassage) (n=2). Error bars represent SD from each population. Data sets were analysed by one-way ANOVA. Scale bar=100 µm. ANOVA, analysis of variance; CPM, counts per million; ERK, extracellular signal-regulated kinase; NES, normalised enrichment score.
Ki67 +proliferating cells at the isthmus (figure 3F,G). Her2high and Her2low-expressing cells isolated by flow cytometry revealed enrichment of Erbb2, Igqgap3 and Mki67 mRNA in Her2high cells, and elevated Lgr5 and Mist1 mRNA in Her2low cells (online supplemental figure S4C,D). Kras, Hras and Nras mRNA were detected in both Her2high and Her2low cells, with Kras and Hras showing significantly higher expression in Her2high cells (online supplemental figure S4E). Igqgap3 expression, therefore, positively correlates with the activity of Erk signalling cascade. Indeed, immunoprecipitation revealed that the interaction of Igqgap3 with the constitutively active HrasG12V mutant was associated with increased p-Erk in the total cell lysate (figure 3H). To ascertain if the Igqgap3-Ras-Erk axis contributes to organoid growth, we subjected organoids to shRNA mediated depletion of Igqgap3 (figure 3I). Depletion of Igqgap3 using three different shRNAs (online supplemental figure S5A) resulted in drastically smaller organoids with reduced ability to be serially passaged (figure 3J; online supplemental figure S5B–D). Corpus glands were treated with two different inhibitors of the Erk pathway, U0126 and MK-8863. Immunostaining of the organoids revealed that both inhibitors resulted in severe depletion of p-Erk (online supplemental figure S5E). After 96 and 72 hours, organoid growth was strongly inhibited in a concentration-dependent manner by U0126 and MK-8833, respectively (online supplemental figure S5F,G).

**Igqgap3 is strongly induced following tissue injury**

We next examined the expression of Igqgap3 during tissue injury. Parietal cell protonophores such as tamofoxifen, DMP-777 and L635 have been used to study the development of metaplasia. Tamofoxifen was reported to cause the back wash of acid into parietal cells, resulting in parietal cell death. In addition to induction of reversible atrophy, tamofoxifen treatment has been associated with increased p-Erk in the total cell lysate (figure 3H). To ascertain if the Igqgap3-Ras-Erk axis contributes to organoid growth, we subjected organoids to shRNA mediated depletion of Igqgap3 (figure 3I). Depletion of Igqgap3 using three different shRNAs (online supplemental figure S5A) resulted in drastically smaller organoids with reduced ability to be serially passaged (figure 3J; online supplemental figure S5B–D). Corpus glands were treated with two different inhibitors of the Erk pathway, U0126 and MK-8863. Immunostaining of the organoids revealed that both inhibitors resulted in severe depletion of p-Erk (online supplemental figure S5E). After 96 and 72 hours, organoid growth was strongly inhibited in a concentration-dependent manner by U0126 and MK-8833, respectively (online supplemental figure S5F,G).

**HDT treatment promotes stem cell activity and neoplastic features**

Immunostaining of the injured tissue showed reduction of neck and chief cell markers, such as G5-II and PgC (figure 5A); mRNA of differentiated cell markers Atp4b, PgC, Gif, and Muc6 were reduced as well (figure 5B). HDT-associated depletion of chief cells has been reported previously. Taken together with the observations by Radyk et al., the decreased expression of chief cell markers in our work suggests that the injury-induced Igqgap3+Ki67+cells at the base were stem-like cells possibly derived from reprogramming of chief cells. Conversely, Hata et al. had reported that chief cells do not dedifferentiate after HDT and that the compensatory response from neck progenitors contributed to the replacement of chief cells. Our work indicated that two complementary events, namely the expansion of Igqgap3-expressing cells from the isthmus zone and the Igqgap3-associated dedifferentiation of chief cells to stem-like cells, accelerated tissue regeneration.

RNA-sequencing and GSEA of epithelial and non-epithelial cells from tissues isolated from HDT-treated mice revealed that mitotic genes, E2F- and Myc-target genes were upregulated, reflecting increased proliferation state of the HDT-treated cells (online supplemental figure S7A,B). Myc expression itself was upregulated (2.5-fold increase) after tissue damage (online supplemental figure S7B). Myc induction was reported to activate an embryonic stem cell (ESC)-like transcriptional signature associated with diverse epithelial cancers. We observed that the ESC-like transcriptional programme, together with transcriptional programmes associated with Kras transformation, early gastric cancer and inflammation were significantly upregulated after HDT-induced injury (figure 5C,D; online supplemental figure S7E,F). An earlier study by Leushacke et al. reported upregulation of matrix metalloproteinase-7 and downregulation of sclerostin domain containing 1 after injury, which they suggested might amplify Wnt signalling to drive tissue regeneration. While we detected upregulation of various Wnt-related genes (eg, Axin2, Notch1 and Myc), there was no statistically significant trend of Wnt activation (online supplemental figure S7D,E). Instead, our data suggest that a combination of dysregulated Myc and Kras signalling—driven by injury-induced Igqgap3 expression—contributed to a less differentiated state, and a more proliferative, stem-like and neoplastic phenotype. Importantly, HDT-treated tissues showed elevated p-Erk staining labelled by tdTomato, indicating expression of Igqgap3 in chief cells (figure 4H). qPCR showed that the induction of Mki67 mRNA reflected that of Igqgap3 during tissue injury, whereas Lgr5 showed moderate increase in expression (figure 4I). There was a population of Ki67 +cells near the gland bottom, in the area outside of the epithelium (figure 4G). These CD45 +Ki67+E-cad-cells (online supplemental figure S6E,F) are CD45 +leucocytes, most likely macrophages as described previously. To investigate whether cells with robust expression of Igqgap3 are involved in the repair of HDT-damaged tissue, Igqgap3-2A-CeERT2,Rosa-tdTomoato mice were treated with HDT. At 14 days post HDT, the tissue morphology, confined Ki67 staining at the isthmus, and regeneration of H, K-ATPase-producing parietal cells indicated near complete repair (figure 4J,K; online supplemental figure S6G). The regenerated gastric glands showed patches of tDTomato-labelled from the bottom to the top, suggesting that Igqgap3 +cells, including Lgr5+chief cells, gave rise to the regenerated parietal cells as well as multiple lineages during repair (online supplemental figure S6G).

**Stomach**

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Figure 4  Iqgap3-expressing cells drive corpus epithelial regeneration post-tissue damage. (A) H&E staining on untreated and high dose tamoxifen (HDT) treated WT corpus at 48 hours post-tamoxifen administration. (B) IF staining for Ki67 and E-cad on untreated and 48 hours post-HDT treated WT corpus. (C–E) ISH for Iqgap3(green) and Lgr5(red) on untreated, 24 hours and 48 hours post-HDT treated WT corpus. Boxes indicate enlarged regions. (F) IF staining for Muc5ac and Ki67 on 48 hours post-HDT treated WT corpus. (G, H) IF staining for tdTomato, Ki67 and Gif on 48 hours post-HDT treated corpus from Iqgap3-2A-tdTomato mice. (I) qPCR for Mki67, Iqgap3 and Lgr5 from isolated WT corpus tissue of untreated and 48 hours post-HDT treated mice (n=3). Error bars represent SE of mean. Data were analysed by Student’s t-test. (J) H&E staining on HDT treated corpus at 14 days post-tamoxifen administration. (K) IF staining of Ki67, tdTomato and H,K-ATPase on 14 days post-HDT treated corpus of Iqgap3-2A-CreERT2,Rosa-tdTomato mice. Scale bars=50 µm. IF, immunofluorescence; ISH, in situ hybridisation; LT, lineage tracing; qPCR, quantitative PCR; WT, wild-type.
Figure 5  HDT-induced tissue damage promotes stem cell activity and neoplastic characteristics. (A) IF staining for PGC, Ki67 and GS-II on untreated and 48 hours post-HDT treated WT corpus. (B) qPCR for Atp4b, Pgc, Gif, Muc6 and Chga from isolated corpus tissue of untreated or 48 hours post-HDT treated WT mice (n=4, expressed as Log2 scale). Data were analysed by Student’s t-test. *P<0.05. NS, not significant. (C) GSEA showing enrichment of embryonic stem cell gene signature, early gastric cancer gene signature and neoplastic transformation KRAS gene signature in 48 hours post-HDT treated WT corpus (n=2). P values determined by a weighted Kolmgorov-Smirnov-like statistic and adjusted for multiple hypothesis testing. (D) Heat MAP showing top 20 genes upregulated in (C) based on RNA-sequencing data from untreated and 48 hours post-HDT corpus tissue. (E) IF staining for p-Erk, Ki67 and E-cad on 48 hours post-HDT treated WT corpus. (F) Experimental strategy to generate corpus organoids from HDT treated mice. (G) Microscopic image of corpus organoids derived from untreated and 48 hours post-HDT treated WT mice. (H, I) Organoid growth efficiency and diameter of corpus organoids derived from untreated or 48 hours post-HDT treated WT mice at 7 days of organoid culture (n=3). Data were analysed by Student’s t-test. Scale bars=50 µm (A), 100 µm (E), 500 µm (G). Error bars represent SEM. GSEA, gene set enrichment analysis; HDT, high dose of tamoxifen; IF, immunofluorescence; p-Erk, phosphorylated extracellular signal-regulated kinase; qPCR, quantitative PCR; SEM, SE of mean; WT, wild-type.
at Ki67 enriched regions, thereby confirming that Ras activation is associated with proliferation during tissue repair (figure 5E). The increased efficiency of organoid formation following injury further confirmed the increased stemness and proliferative potential of the regenerating cells (figure 5F–I).

**Elevated Iqgap3 expression is associated with proliferation in gastric cancer**

The Iqgap3-2A-CreERT2;KrasG12D/+ mouse model was used to investigate the effects of oncogenic Ras activation in Iqgap3+ cells (figure 6A,B). Three months after tamoxifen injection to induce Kras expression, we observed the development of pseudopyloric metaplasia, which was characterised by a massive induction of Muc5ac+ surface mucous cells and a reduction of parietal cells (figure 6C–E). The Ki67+ proliferative cell zone was restricted to the lower neck zone (figure 6F). We observed cancer stem cell marker CD44v10 expression at the base of the metaplastic gland (figure 6G). Pdx1, frequently expressed in pseudopyloric glands and intestinal metaplasia,18 was also detected in corpus units with oncogenic Ras signalling (figure 6H). We also note the induction of Tif2 throughout the aberrant gland (compare figure 6I with the U-shaped Muc5ac staining pattern in figure 6E). The induced characteristics of the Iqgap3-2A-CreERT2;KrasG12D/+ mouse model resembled to an extent, Menetrier’s disease. Menetrier’s disease has been attributed to TGFα and receptor tyrosine kinase RTK EGFR.39 It could be that the specific expression of hyperactivated Ras in isthmus stem cells elicited features that mimicked Menetrier’s disease, which has been tenuously linked to gastric cancer.40 Together, our findings suggest that induction of oncogenic signalling in Iqgap3+ cells can give rise to hyperproliferative disorders and perhaps, preneoplastic lesions.

We, therefore, investigated the expression of IQGAP3 in human cancer. Similar to the mouse, we detected coexpression of IQGAP3 with Ki67 at the isthmus of the normal human stomach corpus (figure 7A). Microarray and RNA-sequencing showed significantly higher IQGAP3 expression levels in gastric cancer tissues obtained from Singapore patients (figure 7B). Tissue microarray analysis of gastric tumour tissues showed that although the tumour and adjacent normal tissues showed IQGAP3 expression, IQGAP3 levels were specifically elevated in neoplastic regions, relative to the adjacent normal tissue (figure 7C; online supplemental figure S8A,B). IQGAP3 expression was elevated in intestinal and mixed gastric cancers (figure 7D). We examined the effects of depleting IQGAP3 in the undifferentiated human gastric carcinoma cell line HGC-27. siRNA-mediated knockdown of IQGAP3 in HGC-27 resulted in drastic reduction of stem cell factors such as NANOG, OCT4, SOX2 and an increase of chief cell marker PGC (see mRNA and protein levels in online supplemental figure S8C,D). Expectedly, KLF4 was significantly increased following IQGAP3 knockdown. Despite its designation as a Yamanaka factor, KLF4 is associated with differentiation in the stomach—the work by Miao et al indicated that KLF4 repressed isthmus stem cell proliferation and induced differentiation to pit and parietal cells.15 Interestingly, cancer stem cell marker CD44v9 was also reduced after IQGAP3 depletion (online supplemental figure S8C,D). Our data, therefore, suggested that IQGAP3 expression contributed to a stem-like state and that its depletion promoted a more differentiated state in HGC-27.

The Cancer Genome Atlas database showed that IQGAP3 expression positively correlated with proliferation markers MKI67 and PCNA in gastric cancer (online supplemental figure S8E). We examined whether IQGAP3 also regulates proliferation of cancer stem cells. We found that the relationship between IQGAP3, Ki67 and CD44v9 expression varied across human cancer tissues. Whereas IQGAP3 invariably coexpressed with Ki67 in all examined human gastric cancer tissues, there was partial overlap of IQGAP3 and CD44v9 expression (figure 7E–G; online supplemental figure S9A–C). Importantly, IQGAP3 was expressed in all CD44v9+Ki67+ double positive cells in the tumour samples examined. Our observations suggested that IQGAP3 was expressed in activated, but not quiescent, cancer stem cells.

**DISCUSSION**

Here, we established Iqgap3 as a novel stem cell factor that is required not only for rapid proliferation of isthmus stem cells, but also for the maintenance of stem cell properties. Although Iqgap3 expression pattern reflected closely that of Ki67, the fact that Iqgap3 depletion in NTERA-2 cells resulted in reduced transcription of several pluripotency-related genes (ie, KLF4, NANOG and OCT4) and the onset of differentiation indicate a complex role for Iqgap3 in regulating stem cell behaviour. Likewise, we found that IQGAP3 expression contributes to a stem-like state in the gastric cancer cell line HGC-27. It is tempting to speculate that IQGAP3 may be therapeutically targeted to induce differentiation, or an irreversible exit from cell cycle, and thereby improve gastric cancer treatment.

Iqgap3 belongs to a three-membered cytoskeletal, scaffold protein family known to regulate diverse cellular processes such as signal transduction, cell-cell adhesion, cell motility and cytokinesis. The multiple protein binding domains in the Iqgap3 protein afford the tethering of signalling pathway components, which increases efficiency of complex formation and signalling intensity.25 41 Many key components of cell signalling, such as calmodulin, β-catenin, calmodulin and ERK, have been identified as Iqgap3 binding partners.25 Of note, Iqgap3 specifically interacts with the active, GTP-bound form of Ras, and through Ras-dependent Erk activation, constitutes an essential aspect of cell proliferation.23 Moreover, Iqgap3, along in the Iqgap3 family, is indispensable for cell proliferation and motility during zebrafish embryonic development.42 IQGAP3 functions downstream of FGFR1-Ras signalling to regulate proliferation during zebrafish embryogenesis.42 Here, we show that the promotion of the Ras-Erk signalling pathway by Iqgap3 is a key underlying mechanistic basis for stem cell proliferation in the corpus. Khurana et al had previously showed that ERK signalling regulates isthmus stem cell proliferation through CD44 signalling.43 Our work, therefore, suggests a cascade involving IQGAP3, ERK and CD44 as main players in the regulation of proliferation at the isthmus zone. Aside from proliferation, the FGF-ERK cascade has also been shown to promote self-renewal in human ESCs.44 45 Erk1/2 signalling can also induce chromatin remodelling in mouse ESCs, underscoring the importance of Erk1/2 signalling in stem cells.45 It is, therefore, tempting to hypothesise that Iqgap3-Ras signalling influences chromatin architecture to direct stem cell-specific transcription programme.

The roles of isthmus stem cells and chief cells during tissue repair have long been mired in controversy. Two disparate models, both supported by extensive evidence, have been proposed. One model holds that chief cells acquire plasticity and contribute to repair. The other model contends that quiescent Mist1-expressing isthmus stem cells are solely responsible for repair of the entire pit. Our work not only shows that rapidly
Figure 6  
Iqgap3-2A-CreERT2;Kras^{G12D/+} mice present pseudopyloric metaplasia. (A) Schematic representation of the genetic construct used to establish the Iqgap3-2A-CreERT2;Kras^{G12D/+} mouse model. (B) Experimental strategy for inducing Iqgap3-driven active Kras^{G12D} expression. (C) H&E staining of the lesser and greater curvature on the corpus of Iqgap3-2A-CreERT2;Kras^{G12D/+} mouse. (D, E) IF staining for Muc5ac and H,K-ATPase on the corpus of control and Iqgap3-2A-CreERT2;Kras^{G12D/+} mouse. (F–I) IF staining for Muc5ac/Ki67 (F), CD44v10 (G), Pdx1/E-cad (H) and Tff2/Ki67/E-cad (I) on the corpus of Iqgap3-2A-CreERT2;Kras^{G12D/+} mice (n=3). Box indicates enlarged region. Scale bars=50 µm. IF, immunofluorescence; p.i, post-tamoxifen induction.
Figure 7  IQGAP3 is coexpressed with Ki67 in human gastric cancer. (A) IF staining for IQGAP3, Ki67 and E-cad on normal corpus in the human stomach (n=3). (B) Microarray and RNA-sequencing analysis for IQGAP3 expression in gastric tumour and normal stomach tissue (Singapore cohort). Error bars for microarray (tumour=185, normal=89) and RNA-sequencing (tumour=27, normal=18) represent SEM from each population. Data were analysed by two-tailed Wilcoxon RANK sum test. (C) IQGAP3 tissue microarray (TMA) of gastric cancer and paired-adjacent normal stomach tissue (n=237). (D) Box plot for comparing IQGAP3 TMA score in adjacent normal stomach, intestinal type gastric cancer, diffuse type gastric cancer and mixed type gastric cancer. Error bars represent SEM from each population. Data sets were analysed by one-way ANOVA. (E) H&E staining on human gastric tumour (n=3). (F) IF staining for IQGAP3, Ki67 and E-cad on the human gastric tumour (n=5). (G) IF staining for IQGAP3, Ki67 and CD44v9 on the human gastric tumour (n=7). Scale bar=100 µm. ANOVA, analysis of variance; IF, immunofluorescence; SEM, SE of mean.
proliferating isthmus stem cells contribute to the repair process, but also clearly supports the self-replicating and plasticity properties of chief cells. However, we cannot rule out the contribution of Mist1-expressing isthmus quiescent stem cells. We posit that expression of Iqgap3, be it in isthmus or chief cells, bestows stem-like behaviour to drive tissue repair. It is interesting that pERK was also detected in the Iqgap3-expressing cells after HDT. In view of the findings by Khurana et al., it is tempting to propose that an Iqgap3-ERK-CyD44 axis drives stem cell proliferation during homeostasis and tissue repair.

Interestingly, Iqgap3 induction during tissue repair was associated with the upregulation of c-Myc and genes associated with ESC. The abilities of c-Myc to increase cancer stem cell population and induce dedifferentiation suggest its involvement in tumour initiation. Moreover, our transcriptomic analysis reveals the enrichment of gene expression signatures associated with early gastric cancer, Kras-linked neoplastic transformation in HDT treated cells. Our results suggest that Iqgap3 is not simply a proliferation factor; rather, the injury-related induction of Iqgap3 and associated gene signatures represent the initial step of cancer development. Accordingly, we found that IQGAP3 is expressed in rapidly proliferating regions of gastric cancer. Since multiple cell types respond to HDT by expressing Iqgap3, we were not able to specify the cell of origin of cancer following injury. Nevertheless, by using the mouse strain harbouring Iqgap3-2A-CreERT2 to express KrasG12D in Iqgap3-expressing cells, we observed rapid generation of pseudopyloric metaplasia. aberrant activation of Ras in Iqgap3-expressing cells is thus likely to be responsible for induction of early stage of cancer development.

The expression of Iqgap3 in isthmus or chief cells may be necessary to trigger Ras-driven cell proliferation and subsequent metaplastic transformation. Moreover, IQGAP3 ablation has been shown to reduce proliferation of breast and gastric cancer cell lines. We propose that an IQGAP3-Ras-associated mechanism might be conserved for proliferation of cancer cells.

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**Contributors** YI, JM and ST conceived the study. YI supervised the study. YI, JM and DD designed the experiments. JM, DD, KM, AT, NNM, DH, SC, NAM, NN and KK performed experiments. YL, SWTH, NL, HKL, JMC and HP performed bioinformatic analysis. PT, SS, TM, JBY, WP, K-GY provided and analysed human clinical samples. ST, AT and MA provided reagents and conceptual advice. All authors commented on the results and discussed implications. YI, LSHC and JM analysed the data and wrote the manuscript.

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**Data availability statement** All data relevant to the study are included in the article or uploaded as online supplemental information. DNA-sequencing datasets generated in this study are deposited in Gene Expression Omnibus (GEO) database under accession codes GSE161443 for Iqgap3-2A-Tomato reporter mice and GSE161442 for HDT treatment respectively. All supporting data are available from the corresponding authors upon reasonable request.

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