ENDOGLIN/CD105 is expressed in KIT positive cells in the gut and in gastrointestinal stromal tumours

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Abstract

ENDOGLIN/CD105 (ENG) is a transmembrane glycoprotein and an auxiliary unit of the transforming growth factor-β (TGF-β) receptor, expressed predominantly in vascular endothelium. Noteworthy, Eng mRNA expression has been reported also in Kit⁺ interstitial cells of Cajal (ICC) in the mouse intestine. Gastrointestinal stromal tumours (GIST) are thought to derive from ICC. Here we have investigated Eng expression in the KitK641E mouse GIST model, in human GIST and in the Ba/F3 cell model. In wild type (WT) mouse antrum, Eng immunoreactivity (ir) was detected in CD34⁻/CD31⁺ endothelium and in Kit⁺ ICC. In KitK641E mice, hyperplasia of Kit⁺ cells made Eng⁻ ir even more evident. Quantitative PCR confirmed the increased expression of Eng transcript in KitK641E mice. On human GIST TMA, 26/49 cases stained positive for ENG. Strong ENG staining was associated with malignant and high-risk tumours. ENG negative cases were predominantly of the epithelioid type or harboured PDGFRA mutation. In vitro, Eng mRNA was up-regulated in Ba/F3 cell lines stably expressing various oncogenic Kit mutations (K641E, del559, del814). This effect appeared to be independent of Kit activation, as neither the stimulation of WT Kit by its ligand SCF, nor the inhibition of Kit autophosphorylation by imatinib mesylate in oncogenic mutants, altered Eng expression. Elevated Eng expression in Kit oncogenic mutants appeared rather to be indirectly mediated by DNA hypomethylation, because treatment with the demethylating agent 5-Aza/dC increased Eng mRNA expression in KitWT cells. ENG expression in ICC and in GIST deserves further consideration as ENG is emerging as a potential target for cancer therapy.

Keywords: ENDOGLIN • KIT • gastrointestinal stromal tumour • interstitial cells of Cajal

Introduction

ENDOGLIN/CD105 (ENG) is a 180 kD homodimeric transmembrane glycoprotein, expressed predominantly on vascular endothelial and haematopoietic cells. ENG is an auxiliary protein of the transforming growth factor β (TGF-β) receptor complex involved in cellular proliferation, differentiation and migration [1]. Two ENG isoforms, differing in the length of their cytoplasmic domain, have been characterized in human and mouse tissues [2, 3]. The predominant L-ENG isoform contains in its cytosolic domain a consensus PDZ-binding motive, which is lacking in the barely expressed S-ENG isoform. L-ENG has been shown to be involved in epidermal carcinogenesis, whereas no influence of S-ENG could be demonstrated during carcinogenesis in vivo or in vitro [3]. ENG extracellular domain can undergo proteolytic cleavage and shed from the cell membrane into the bloodstream, giving rise to soluble ENG [4]. Elevated levels of soluble ENG have been reported in pre-eclampsia [5, 6] while studies in cancer patients remain conflicting, some studies reporting increased levels [7–9] whereas others reported a decrease [4] or no change [10–12] in circulating soluble ENG.
ENG expression counteracts the potent inhibitory effect of TGF-β on cell proliferation in several cell lines [13, 14]. Mutations in the ENG gene are responsible for the hereditary haemorrhagic telangiectasia type 1 [HHT1, Osler-Weber-Rendu syndrome (OMIM 187300)]. ENG has been largely investigated in hypoxic responses and as a putative diagnostic, prognostic and therapeutic target in tumoural neoangiogenesis. Conversely, ENG expression in non-endothelial cells has so far attracted little attention [1, 15]. Endoglin/CD105 was found to be expressed in non-endothelial cells of various histotypes, including Kit+ interstitial cells of Cajal (ICC) in the mouse small intestine [16]. ICC are specialized mesenchymal cells located within the muscularis propria of the gastrointestinal tract, where they play major roles in coordinating peristalsis through intrinsic pacemaker function and network formation [17]. Gastrointestinal stromal tumours (GIST) are the most common sarcoma of the gastrointestinal tract and are thought to derive from the ICC lineage. Approximately 85% of GIST harbour oncogenic KIT mutations and 7% contain oncogenic platelet-derived growth factor receptor alpha (PDGFRα) mutations. Kit and PDGFRα are members of the receptor tyrosine kinase family type III. Oncogenic mutations lead to their constitutive autophosphorylation, ligand-independent activation of the downstream signal transduction pathways and subsequent deregulation of cell proliferation, survival and migration [18]. KIT K642E, an oncogenic KIT mutation originally identified in sporadic GIST [19], has also been encountered as germ-line mutation in a family with hyperplasia of the ICC layer and GIST formation [20]. Transgenic mice harbouring Kit K641E, the murine homologue of human KIT K642E, have been generated by a knock-in gene targeting strategy, providing an in vivo GIST model with massive hyperplasia of Kit+ cells, especially in antrum and caecum [21, 22].

Here we have identified for the first time ENG immunoreactivity (-ir) in Kit+ ICC by immunofluorescence (IF). In the normal mouse gut, in the Kit K641E GIST mouse model, as well as in human GIST. Oncogenic Kit mutations up-regulated Eng mRNA expression in the murine Ba/F3 cell model in vitro. This effect appeared however to be independent of Kit phosphorylation/activation, as neither the stimulation of WT Kit by its ligand SCF, nor the inhibition of Kit autophosphorylation by imatinib mesylate in oncogenic mutants, altered Eng expression.

Materials and methods

The Kit K641E murine GIST model

The knock-in Kit K641E murine GIST model has been previously described [21, 22]. Two-week-old (P14) Kit K641E/K641E, Kit WT/K641E and Kit WT/WT wild-type littermates were used. Mice were bred and experiments performed in compliance with protocol #LA1230331/214N approved by the ethics committee for animal well-being of the Faculty of Medicine, Université Libre de Bruxelles, Brussels, Belgium.

Mice were killed by cervical dislocation. The gastrointestinal tract was carefully removed immediately after killing. The antrum, delineated by visual land marks, was harvested together with pieces of mid small intestine, liver and descending colon. Tissues were subsequently processed as described later.

Ba/F3 cell model

Generation and characterization of Ba/F3 cells stably expressing the full-length mouse Kit cDNA (accession number XM 132123.1) (KitWT) or the oncogenic mutants Kit del559, Kit K641E and Kit del814 have been reported previously [20, 23]. Untransfected (original, ORI) Ba/F3 and Ba/F3-Kit WT cells were cultured at 37°C in RPMI 1640 medium (GIBCO, CA, USA) supplemented with 10% foetal bovine serum (FBS), 2% penicillin/streptomycin, 1% glutamine and 2.5% supernatant of WEHI cell culture (referred to as ‘IL-3 conditioned medium’). The constitutively active Ba/F3-Kit WT, Ba/F3-Kit del559 and Ba/F3-Kit del814 mutants were maintained in medium without IL-3 conditioned medium.

Sustained activation of KitWT signalling pathways was achieved by culturing Ba/F3-KitWT cells in medium without IL3 containing 100 ng/ml recombinant rmSCF (Sigma-Aldrich, St. Louis, MO, USA) for 48 hrs. Inhibition of Kit phosphorylation was achieved by culturing Ba/F3-Kit WT, Ba/F3-Kit del559 and Ba/F3-Kit del814 cells in IL-3 conditioned medium containing 10 μM imatinib mesylate (Novartis, Annandale, NJ, USA) for 48 hrs. The methyltransferase inhibitor 5’-AzA-2’-Deoxycytidine (5-Aza-2C) (Sigma-Aldrich) was dissolved in 0.2% DMSO at 0.1 μM and applied for 72 hrs. DMSO-treated cells were used as control.

Human GIST882 cell line

The human GIST cell line GIST882 [24] was kindly provided by Dr. Jonathan A. Fletcher, Harvard Medical School, Boston, MA, USA. Cells were cultured at 37°C in DMEM (GIBCO, CA, USA) supplemented with 10% FBS, 2% penicillin/streptomycin.

Flow cytometry analysis (FACS)

FACS was used to detect the expression of Kit receptor and presence of phospho-Tyrosine (p-Tyr) in Ba/F3 cells. 2 × 10^5 cells were fixed and permeabilized using BD cytofix/cytoperm (BD Pharmingen, San Diego, CA, USA) for 20 min. on ice. After washing twice with BD Perm/Wash buffer (BD Pharmingen), the cells were incubated with R-phycocerythrin (R-PE)-conjugated ACK45 antibody directed against c-kit/CD117 and p-Tyr Alexa Fluor 647 conjugated antibodies (Table 2) for 30 min. in the dark on ice. After incubation, the cells were resuspended in 250 μl PBS–BSA 0.1% and analysed by a laser flow cytometer FACSCalibur system (BD Biosciences) and data were analysed with WinMDi 2.8 software (BD Biosciences). Contaminants were removed by forward and side scatter gating. Kit-PE fluorescence was detected through the FL-1 channel, p-Tyr-Alexa647 fluorescence was assessed using the FL-4 channel and data were acquired and analysed using the CellQuest® application on a Power Macintosh G3 computer. For each sample, at least 10,000 individual cells were collected and the mean fluorescence intensity was evaluated.

Real-time quantitative PCR (qPCR)

qPCR was performed as described [21]. Briefly RNA was extracted from mouse antrum using RNeasy Mini Kit (Qiagen, Valencia, CA, USA).
was reverse transcribed with M-MLV Reverse Transcriptase (Invitrogen, Eugene, OR, USA) in a reaction containing random primers (Amersham Bioscience, Piscataway, NJ, USA). Specific primers for long (L-Eng), short (S-Eng) and total (T-Eng) Eng transcripts were used for amplification using SYBR Green chemistry on a 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Transcriptional quantification relative to glyceraldehyde 3-phosphate dehydrogenase (Gapdh) and housekeeping genes for mouse tissue or Gapdh with hypoxanthine-guanine phosphoribosyl transferase (Hprt) for Ba/F3 cells lysate was calculated relative normalized quantities of mRNA expression (NRQ) [25]. In antrum, the Eng mRNA expression was then normalized by the endothelial marker CD31 mRNA level. Primer sequences are summarized in Table 1.

**Table 1 Table of primers**

| Primer   | Sequence                        |
|----------|---------------------------------|
| T:L-ENG F | CAGTCTCCATGCGCCTGAAC            |
| T:Eng R  | GGTGATACCCGAGTACAGGGGCA         |
| L-ENG R  | CTACGGCCATGCTGGTG               |
| S-ENG R  | GGAAATGGGGTGAGGGCTT             |
| CD/31 F  | GGTGATACCCGAGTACAGGGGCA         |
| CD/31 R  | TCTCTGGCTCTCGTCCTCCC            |
| Gapdh F  | TGTGTCGTCGTTGATCTGTA            |
| Gapdh R  | CCGCTCACCACCTCCTGTA             |
| Hprt F   | GCTACTGTGAATGATCGTACCCGGG      |
| Hprt R   | AAGCTTTCACCTTACATTTCG           |
| β-Actin F | AACGCTGAAAAAGATGCCACAGAT       |
| β-Actin R | GCCCTGATGGCTACTGACATG          |

**Immunofluorescence staining**

Specimens were processed as described [21]. Briefly, slides were brought to RT, rinsed in 10 mM Tris (Merck-Belgolabo, Overijse, Belgium) and 0.15 M sodium chloride, pH 7.4 TBS, containing 0.1% (v/v) Triton-X 100 (TBS-TX), and incubated for 1 hr in 10% normal horse serum (NHS) (Hormonologie Laboratoire, Marloie, Belgium) and TBS-TX. The slides were incubated overnight at RT with the primary antibodies (Table 2), rinsed twice in TBS and incubated in the dark for 1 hr at RT in TBS containing the secondary antibodies (Table 2). Slides were then rinsed twice in TBS and incubated in the dark for 1 hr at RT with streptavidin-coupled NorthernLight™ 557 (NL557) (R&D Systems, Abingdon, Oxon, UK).

The optimal working dilution has been determined empirically for each antibody. The protocol used for double IF staining did not modify the distribution or the intensity of each individual labeling observed in corresponding single procedures. Omission of the primary or the secondary antibodies resulted in the absence of the respective fluorescent signal. Nuclei were stained with 5 μM Hoechst in Tris-HCl (Merck-Belgolabo) 0.05 M (pH 7.4), containing 0.5 mg/ml type 1-AS Ribonuclease A for 5 min. in the dark at RT.

**Confocal microscopy**

After three rinses in TBS, cover slips were mounted with Fluor Save™ Reagent anti-fade mounting medium (Calbiochem, Nottingham, UK) before viewing under a LSM510 NLO multiphoton confocal microscope fitted on an Axiovert M200 inverted microscope equipped with a C-Apochromat 40 x/1.2 N.A. water immersion objective (Zeiss, Jena, Germany).

The 488 nm excitation wavelength of the Argon/2 laser, a main dichroic HFT 488 and a band-pass emission filter (BP500–550 nm) were used for selective detection of the green fluorochrome. The 543 nm excitation wavelength of the HeNe1 laser, a main dichroic HFT 488/543/633 and a long-pass emission filter (LP560 nm) were used for selective detection of the red fluorochrome. The nuclear stain Hoechst was excited in multiphotonic mode at 760 nm with a Mai Tail® tunable broad-band laser (Spectra-Physics, Darmstadt, Germany) and detected using a main dichroic HFT KP650 and a band-pass emission filter (BP435–485 nm). Single optical sections, 2 μm thick, were collected for each fluorochrome sequentially. The images generated (pixel size: 0.1 μm) were merged and displayed using the Zeiss LSM510 software. Fluorescence intensity line plots were generated using the same software. Images were exported in .jpg image. All figures show single optical sections across the regions of interest.

**Western blotting (WB)**

Tissue samples were lysed in sucrase lysis buffer containing 20 mM Tris-HCl (pH 7.9), 250 mM sucrose, 0.01% NaN3, 0.1% Nonidet P-40 and 20 μg/ml proteases inhibitor mixture (Roche, Mannheim, Germany). The antral muscularis propria was cleaned up by sharp dissection and the mucosa was discarded before lysis.

Proteins were solubilized in sample buffer, heated at 95°C for 5 min., separated by SDS-PAGE on 10% polyacrylamide gel and transferred on a 0.2 μm nitrocellulose membrane. Primary antibodies raised in different species and secondary antibodies coupled with different fluorochromes (Table 2), were sequentially combined to specifically label one marker in green (800 DyLight), the other in red (680 DyLight). Gapdh was used as loading control. Liver was used as positive control for Eng expression. The Odyssey™ imaging system (LI-COR Biotechnology, Lincoln, NE, USA) was used to quantify the signals.

**Human GIST tissue microarrays (TMA)**

A cohort of formalin-fixed, paraffin-embedded human GIST samples was retrieved from the pathology files of the Cleveland Clinic, Cleveland, OH. The collection and analysis of human tissue samples was approved by the Cleveland Clinic Institutional Review Board (IRB 06-977). Consent was not obtained from any participants as it was waived by the IRB because it was a retrospective study involving only collection of tissue cores from paraffin blocks used in the diagnosis of patient tumour samples.

Clinico-pathological characterization of GIST used is summarized in Table 3. Risk assessment was performed using the criteria of Miettinen and Lasota [26], as recommended by the National Cancer Care Network [27]. Mutations in Kit exons 9, 11, 13 and 17 and PDGFRA exons 12 and 18 were examined as described previously [28]. This material was used to construct a TMA with 1 mm cores using a TMarrayer™ (P/N 02110016) semi-automated tissue arrayer (Pathology Devices, Westminster, MD, USA). Each case was present in duplicate in this array.
Immunohistochemistry (IHC) on human material

After rehydration through phenol and graded alcohol solutions, TMA slides were heated at 96°C in 1 mM EDTA, 0.05% Tween20 (pH 8.0) antigen retrieval solution for 20 min. to achieve epitope unmasking. Slides were then cooled for 10 min. before being rinsed in TBS. The staining with primary Eng antibody and secondary rabbit biotinylated antibody (Table 2) was performed as mentioned earlier. Then sections were incubated in ABC solution (ABC kit standard PK-4000; Vector Laboratories, Burlingame, CA, USA) for 90 min. at room temperature and peroxidase activity revealed for 5–10 min. with nickel-enhanced DAB (DAB-Ni), resulting in a black precipitate. The DAB-Ni solution was prepared by dissolving 0.2 g of nickel ammonium sulphate (Fluka, Buchs, Switzerland) and 7.5 mg of DAB (Sigma-Aldrich) in 50 ml of 0.05 M Tris/HCl, pH 8. Immediately before use, 10 μl of 30% H2O2 (Merck, Darmstadt, Germany) was added.

GIST tissue present in each core was scored as strong (comparable to intensity of vascular endothelial staining), weak (weaker then staining of vascular endothelia and clearly distinguishable from background), negative (intensity comparable to background) or non-contributive because of poor tissue quality.

### Table 2 Table of antibodies

| Antibody | Cat. No. | Supplier | Host | Dilution |
|----------|----------|----------|------|----------|
| **IF** | | | | |
| Primary antibodies | | | | |
| Endoglin/CD105 | BAF1320 | R&D Systems, Minneapomis, MN | Goat | 1/1000 |
| Kit/CD117 | A4502 | Dako North America, Carpinteria, CA | Rabbit | 1/500 |
| Kit (C-14) | sc-1494 | Santa Cruz Biotechnology, Santa Cruz, CA | Goat | 1/500 |
| CD/31 | 550274 | BD Pharmingen, San Diego, CA | Rat | 1/1000 |
| CD34 | 14-0341 | eBioscience, San Diego, CA | Rat | 1/500 |
| **Secondary antibodies** | | | | |
| Goat Alexa 448 | A11055 | Invitrogen, Eugene, OR | Donkey | 1/200 |
| Rabbit biotinylated | 711065152 | Jackson Immunoresearch, Cambridge, UK | Donkey | 1/200 |
| Rat FITC | 712095150 | Jackson Immunoresearch, Cambridge, UK | Donkey | 1/200 |
| **IHC** | | | | |
| Primary antibodies | | | | |
| Endoglin (H-300) | sc-20632 | Santa Cruz Biotechnology, Santa Cruz, CA | Rabbit | 1/100 |
| **Secondary antibodies** | | | | |
| Rabbit biotinylated | 711065152 | Jackson Immunoresearch, Cambridge, UK | Donkey | 1/200 |
| **WB** | | | | |
| Primary antibodies | | | | |
| Endoglin/CD105 (mice tissue) | BAF1320 | R&D Systems, Minneapomis, MN | Goat | 1/1000 |
| Endoglin (H-300) (GIST882 cells) | sc-20632 | Santa Cruz Biotechnology, Santa Cruz, CA | Rabbit | 1/500 |
| Gapdh | #2118L | Cell Signaling Technology, Danvers, MA | Rabbit | 1/1000 |
| **Secondary antibodies** | | | | |
| Goat DyLight™ 680 | 35518 | Pierce, Thermo Fisher Scientific, Erembodegem, BE | 1/10000 |
| Rabbit DyLight™ 800 | 35571 | Pierce, Thermo Fisher Scientific, Erembodegem, BE | 1/10000 |
| **FACS** | | | | |
| p-Tyr (Alexa Fluor™ 641 conjugated) | #9415 | Cell Signaling Technology Inc., Danvers, MA | 1/50 |
| Kit/CD117 (R-PE conjugated) | #9415 | BD Pharmingen, San Diego, CA | 1/50 |
Statistical analysis

Student’s t-test for independent samples was performed to compare mean values of qPCR and WB data. Fisher’s exact test was used to assess a possible relationship between ENG expression status and clinicopathological parameters, KIT-ir IHC or mutation status of GIST specimens. Student’s t-test for independent samples was used to compare means of tumour size and mitotic counts between ENG+ and ENG- GIST. Statistical significance was set at a P value of less than 0.05.

In addition, factorial analysis of multiple correspondences (FAMC), followed by an ascending hierarchic classification (AHC) was applied to the subset (n = 34) of GIST cases for which full information was available, using the SPAD version 7.0 statistical software (Coheris, Suresnes, France). These methods provide a graphic representation of the relations between variables, modalities and subjects, close positions indicating similarities [29]. Multiple modalities that could interact with each other were simultaneously analysed and their respective influence was addressed by FAMC, whereas AHC allowed building homogeneous clusters of subjects. By comparing the position of these clusters in the plane with the position of the modalities, subjects belonging to a cluster can be characterized according to these modalities. A test of proportions was performed. Statistical significance was set at a P value of less than 0.05.

Results

Endoglin expression in Kit\(^{\text{K641E/K641E}}\) mouse antrum

Expression of long (L-Eng), short (S-Eng) and total (T-Eng) Eng transcripts in Kit\(^{\text{WT/WT}}\) and Kit\(^{\text{K641E/K641E}}\) antrum was investigated (Fig. 1A). To estimate the proportion of Eng expression in Kit+ cells and not in endothelium, Eng mRNA level was normalized against the endothelial marker CD31 mRNA level (NRQ = 1 and 2.7 for Kit\(^{\text{WT/WT}}\) and Kit\(^{\text{K641E/K641E}}\), respectively). Relative expression of T-Eng and L-Eng in Kit\(^{\text{K641E/K641E}}\) antrum was significantly increased, compared to Kit\(^{\text{WT/WT}}\) littermates (NRQ = 2.8; \(P = 0.04\) and NRQ = 3.4; \(P = 0.006\), respectively). S-Eng expression in WT antrum was very low compared to L-Eng (0.79 versus 34.49 arbitrary units, respectively) and its relative change in Kit\(^{\text{K641E/K641E}}\) antrum compared to Kit\(^{\text{WT/WT}}\) was not statistically significant (\(P = 0.22\)) (Fig. 1B).

A single band at the expected molecular weight of ~90 kD, representing the predominant L-ENG form was detected by WB on protein extracts of P14 Kit\(^{\text{K641E/K641E}}\) and Kit\(^{\text{WT/WT}}\) mice antrum. No statistically significant difference between genotypes was observed. In the liver, used as control, the L-ENG, S-ENG and soluble Eng isoforms were detected by WB (Fig. S1).

Endoglin is expressed in endothelial cells but also in Kit+ cells in the mouse gut wall

Eng-ir was observed in CD34+/CD31+ endothelium, as expected. Eng-ir was also detected, albeit fainter than in endothelium, in many, but not all, Kit+ ICC (Fig. 2A and C). The same features were

| Primary tumour site         | Total (n) |
|-----------------------------|-----------|
| Gastric                     | 34        |
| Small bowel                 | 9         |
| Colon                       | 2         |
| Rectovaginal                | 1         |
| Disseminated                | 3         |

| Tumour morphology          | Total (n) |
|-----------------------------|-----------|
| Spindle                     | 35        |
| Epithelioid                 | 14        |

| Risk category              | Total (n) |
|-----------------------------|-----------|
| Malignant                   | 7         |
| High risk                   | 10        |
| Moderate risk               | 13        |
| Low risk                    | 8         |
| Very low risk               | 6         |
| No risk                     | 5         |

| Mitotic figures            | Total (n) |
|-----------------------------|-----------|
| \(\leq 5/5 \text{ mm}^2\)   | 29        |
| \(>5/5 \text{ mm}^2\)       | 13        |
| N.A.                        | 7         |

| Mutation status            | Total (n) |
|-----------------------------|-----------|
| KIT mutation                | 25        |
| KIT Exon 9 AY 502-503 duplication | 4       |
| KIT exon 11 duplication      | 3         |
| KIT exon 11 deletion        | 8         |
| KIT exon 11 V559D           | 6         |
| KIT exon 11 V560D           | 2         |
| KIT exon 11 W557G           | 1         |
| KIT exon 13 K642E           | 1         |

| PDGF mutation               | 9         |
| PDGFR\(^{\text{A}}\) exon 12 – V561D | 2       |

| PDGFR\(^{\text{A}}\) exon 12 deletion | 1         |
| PDGFR\(^{\text{A}}\) exon 18 D842V   | 4         |
| PDGFR\(^{\text{A}}\) exon 18 D842Y   | 1         |
| PDGFR\(^{\text{A}}\) exon 18 Ins-Del | 1         |

| Wild type                   | 11        |
| N.A.                        | 4         |

| KIT-ir                      | Total (n) |
|-----------------------------|-----------|
| Positive                    | 44        |
| Negative                    | 4         |
| N.A.                        | 1         |

N.A.: data not available.

### Table 3 Clinicopathologic features of cases on the Cleveland Clinic GIST TMA

| Primary tumour site     | Total (n) |
|-------------------------|-----------|
| Gastric                 | 34        |
| Small bowel             | 9         |
| Colon                   | 2         |
| Rectovaginal            | 1         |
| Disseminated            | 3         |

| Tumour morphology       | Total (n) |
|-------------------------|-----------|
| Spindle                 | 35        |
| Epithelioid              | 14        |

| Risk category           | Total (n) |
|-------------------------|-----------|
| Malignant               | 7         |
| High risk               | 10        |
| Moderate risk           | 13        |
| Low risk                | 8         |
| Very low risk           | 6         |
| No risk                 | 5         |

| Mitotic figures         | Total (n) |
|-------------------------|-----------|
| \(\leq 5/5 \text{ mm}^2\) | 29        |
| \(>5/5 \text{ mm}^2\)    | 13        |
| N.A.                    | 7         |

| Mutation status         | Total (n) |
|-------------------------|-----------|
| KIT mutation            | 25        |
| KIT Exon 9 AY 502-503 duplication | 4       |
| KIT exon 11 duplication  | 3         |
| KIT exon 11 deletion     | 8         |
| KIT exon 11 V559D        | 6         |
| KIT exon 11 V560D        | 2         |
| KIT exon 11 W557G        | 1         |
| KIT exon 13 K642E        | 1         |

| PDGF mutation           | 9         |
| PDGFR\(^{\text{A}}\) exon 12 – V561D | 2       |

| PDGFR\(^{\text{A}}\) exon 12 deletion | 1         |
| PDGFR\(^{\text{A}}\) exon 18 D842V   | 4         |
| PDGFR\(^{\text{A}}\) exon 18 D842Y   | 1         |
| PDGFR\(^{\text{A}}\) exon 18 Ins-Del | 1         |

| Wild type                | 11        |
| N.A.                    | 4         |

| KIT-ir                   | Total (n) |
|--------------------------|-----------|
| Positive                 | 44        |
| Negative                 | 4         |
| N.A.                     | 1         |

N.A.: data not available.
observed at different levels (antrum, small intestine, transverse colon) in the WT mouse gastrointestinal tract (Figs S2 and S3).

The higher abundance of Kit+/H11001 cells in KitK641E heterozygotes and homozygotes made Eng-ir more readily discernible than in the KitWT/WT antrum (Fig. 2A).

**Endoglin expression in human GIST**

The antibody used for ENG-ir on GIST TMA was validated using the human GIST882 cell line. Two immunoreactive bands of the expected MW for L-ENG and S-ENG were detected by WB. By IF, GIST882 cells stained positively for ENG-ir, whereas omission of the primary antibodies resulted in the absence of signal (Fig. S4).

In GIST TMA, 26 GIST stained positively for ENG-ir (ENG+/H11001/H11001/H11001) and 23 were negative (ENG-/H11002). Labelling of endothelial cells provided internal positive controls.

Results of correlation between ENG-ir and histopathological GIST characteristics are summarized in Table 4. Median size of the ENG+/H11001/H11001/H11001 and ENG-/H11002 tumours was 5.82 cm (range 0.5–16 cm) and 8.62 (range 0.3–31.5 cm), respectively. Mitotic counts per 5 mm² were significantly higher in ENG+/H11001/H11001/H11001 compared to ENG-/H11002 GIST (P = 0.013). Strongly positive ENG++ staining was associated with malignant or high-risk cases (P = 0.014) (Fig. 3A). ENG− were associated with PDGFRα mutation status (P = 0.001), KIT negative IHC (P = 0.002), epithelioid histological type (P = 0.007), intermediate risk category (P = 0.011), large tumour size (>10 cm) (P = 0.022) and mitotic counts (>5/5 mm²) (P = 0.046) (Fig. S5).

**Endoglin mRNA expression in Ba/F3 cell is increased by Kit oncogenic mutants but is not controlled by Kit phosphorylation**

Compared to Ba/F3-KitWT cells, T-Eng expression was significantly higher in the constitutively activated mutants Ba/F3-Kit K641E (NRQ = 5.5; P = 0.025), Ba/F3-Kitdel559 (NRQ = 8.2; P = 0.019) and Ba/F3-Kitdel814 (NRQ = 5.2; P = 0.001).

Sustained (48 hrs) stimulation of Ba/F3-KitWT by Kit ligand SCF did not modify Eng mRNA expression level (Fig. 4A). Efficiency of SCF treatment was confirmed by the increase of pTyr-ir assessed by flow cytometry. SCF had no effect on pTyr-ir in original Ba/F3 (Fig. S6).

Similarly, inhibition of Kit phosphorylation by imatinib mesylate for 48 hrs or 7 days did not alter Eng mRNA expression in the oncogenic Kit mutants Ba/F3-KitK641E, Ba/F3-Kitdel559 and in imatinib resistant clone Ba/F3-Kitdel814 (Figs 4A and S7). Efficiency of imatinib treatment was confirmed in Ba/F3-KitK641E in Ba/F3-Kitdel559 and in Ba/F3-Kitdel814 by the drop of pTyr-ir, assessed by flow cytometry (Fig. S8).
Expression of Endoglin mRNA can be activated in Ba/F3<sup>WT</sup> cells by the DNA methyltransferase inhibiting agent 5-Aza/dC

We wondered whether DNA hypomethylation could be involved in the increase of Eng expression in Ba/F3-Kit<sup>K641E</sup>, BaF3-Kit<sup>del559</sup> and BaF3-Kit<sup>del814</sup> cells. Treatment with the DNA methyltransferase inhibitor 5-Aza/dC significantly increased Eng mRNA expression in Ba/F3-Kit<sup>WT</sup> (P < 0.003) and in Ba/F3-Kit<sup>WT</sup> cells stimulated with SCF for 48h (P = 0.003). Level of Eng expression in Ba/F3-Kit<sup>WT</sup> after 5-Aza/dC treatment was comparable to Eng expression in Kit oncogenic mutants. Conversely, 5-Aza/dc did not have a statistically significant effect on Eng mRNA expression in Ba/F3-Kit<sup>K641E</sup>, BaF3-Kit<sup>del559</sup> and BaF3-Kit<sup>del814</sup> cells (P = 0.5, 0.09 and 0.2, respectively) (Fig. 5).

Another possible mechanism for the transcriptional activation of Eng by the oncogenic Kit mutants could be the up-regulation of an upstream transcription factor. Expression of Hypoxia inducible
| Cohort (n = 49) | ENG⁺⁺ | ENG⁺ | Total | ENG⁻ | P value* |
|----------------|------|------|-------|------|---------|
| Mean tumour size | 5.82 (cm) | 8.62 (cm) | 0.07 |
| Primary site |     |     |       |     |         |
| Gastric | n (%) | n (%) | n (%) | n (%) |         |
| Small bowel | n (%) | n (%) | n (%) | n (%) |         |
| Colon |     |     |       |     |         |
| Rectovaginal |     |     |       |     |         |
| Disseminated |     |     |       |     |         |
| Total | n (%) | n (%) | n (%) | n (%) | 0.08 |
| Mitotic figures |     |     |       |     |         |
| ≤5/5 mm² | n (%) | n (%) | n (%) | n (%) |         |
| >5/5 mm² | n (%) | n (%) | n (%) | n (%) |         |
| Total | n (%) | n (%) | n (%) | n (%) | 0.013 |
| Histological type |     |     |       |     |         |
| Epithelioid | n (%) | n (%) | n (%) | n (%) |         |
| Spindle | n (%) | n (%) | n (%) | n (%) |         |
| Total | n (%) | n (%) | n (%) | n (%) | 0.02 |
| Mutation Status |     |     |       |     |         |
| KIT mutation | n (%) | n (%) | n (%) | n (%) |         |
| PDGF mutation | n (%) | n (%) | n (%) | n (%) |         |
| Wild type | n (%) | n (%) | n (%) | n (%) |         |
| Total | n (%) | n (%) | n (%) | n (%) | 0.027 |
| Risk classification |     |     |       |     |         |
| Malignant–high risk | n (%) | n (%) | n (%) | n (%) |         |
| Moderate–low risk | n (%) | n (%) | n (%) | n (%) |         |
| Very low–no risk | n (%) | n (%) | n (%) | n (%) |         |
| Total | n (%) | n (%) | n (%) | n (%) | 0.014 |

*Student's t-test.
†Fisher's exact test.
factor 1α (Hif1α), a well-characterized transcription factor upstream of Eng, did not differ in Ba/F3-Kit^{WT} cells, compared to the constitutive active mutants BaF3-Kit^{K641E}, BaF3-Kit^{del559}, and BaF3-Kit^{del814}. Similarly, relative expression of Hif1α in Kit^{K641E/WT} antrum did not differ from expression in Kit^{WT/WT} littermates (Fig. S9).

**Discussion**

We first focused on Eng-ir in the murine gut as Eng mRNA expression had been reported in Kit^{+} ICC isolated by FACS from mouse small intestine [16]. Here, we have demonstrated, for the first time, by IF the presence of Eng-ir in the Kit^{+} ICC of the normal mouse gut.

As GIST are thought to derive from the Kit^{+} ICC lineage [18], we next investigated Eng expression in the Kit^{K641E} GIST model. The higher abundance of Kit^{+} cells in Kit^{K641E} heterozygous and homozygous antrum made Eng-ir even more readily discernible than in Kit^{WT/WT} ICC. However, WB (this study) and microarrays [21] performed on the entire antrum wall did not demonstrate any significant increase of Eng expression in Kit^{K641E} mice. Similarly, up-regulation of ENG has not been reported in microarray studies comparing human GIST and normal tissue. This may illustrate an intrinsic limitation of techniques using homogenates of whole tissues, as the robust ENG expression in endothelium could easily overshadow the weaker signal arising from the Kit^{+} cells. In line with that interpretation, qPCR data, when normalized against the endothelial marker CD31, revealed a significantly higher expression of T-Eng and L-Eng mRNA in Kit^{K641E/K641E} antrum of a magnitude (two- to threefold), similar to the increase of Kit mRNA expression observed in this model [21]. Expression of S-Eng transcript was very low and S-Eng protein was hardly detectable on Western blot. Eng antibodies available do not discriminate L-Eng and S-Eng isoforms, precluding study of their respective distribution by IF. S-Eng may play some regulatory role [3], but its distribution could not be resolved in this study.

Next, we analysed ENG expression in human GIST, using TMA and IHC. ENG-ir was detected in half (26/49) of the cases. Strong ENG expression was significantly associated with malignant or high-risk tumours. Moreover, absence of ENG-ir was observed in 8/9 GIST harbouring PDGFRA mutations, raising the hypothesis of...
a possible link between ENG expression and oncogenic KIT mutants in GIST. Larger prospective series will be needed to confirm the predictive value of ENG-ir in GIST.

Noteworthy, TGF-β/Smad’s signaling pathway plays a central role in the development and differentiation of mesenchymal cells in the digestive tract [30, 31] and lack of Smad3, an intermediate in TGF-β signaling, leads to the lack of ICC in the colonic wall in Smad3-null mice [32]. Taken together, these data indirectly suggest that the TGF-β receptor complex, including ENG, might be involved in the development of the KIT+ ICC in the normal gut and in the malignant behavior of KIT+ GIST, thus expanding the scope of ENG roles beyond its well-established regulatory role in endothelium.

Fig. 4 Ba/F3 cell lines. Eng mRNA expression is independent of Kit phosphorylation in vitro. (A) T-Eng expression was significantly higher in constitutive active mutants Ba/F3-Kitdel314, Ba/F3-KitE641E and Ba/F3-Kitdel559, compared to Ba/F3-KitWT. Sustained (48 hrs) stimulation of Ba/F3-KitWT by SCF, did not modify T-Eng mRNA expression level. Data reported as mean ± S.E.M of three independent experiments and presented as normalized relative quantities (NRQ). Student’s t-test, *P < 0.05, ***P < 0.001. (B) Inhibition of Kit phosphorylation by imatinib mesylate did not significantly alter T-Eng mRNA expression in any of the oncogenic Kit mutants. Data reported as the mean ± S.E.M of three independent experiments and presented as normalized relative quantities (NRQ).

Finally, we analysed the influence of Kit oncogenic mutants on Eng expression in Ba/F3 cell lines, a well-established model to investigate signalling pathways downstream of the Kit receptor tyrosine kinase in vitro [23]. In this model, three different constitutively activated (oncogenic) Kit mutants markedly increased Eng mRNA expression compared to original (untransfected) cells or cells transfected with KitWT. Oncogenic mutations lead to constitutive autophosphorylation, and subsequent ligand-independent activation of the downstream signal transduction. Noteworthy, Eng expression in vitro was not directly dependent of Kit receptor phosphorylation as neither sustained activation of KitWT by its ligand SCF, nor inhibition of Kit phosphorylation by imatinib mesylate in the Kit oncogenic mutants, altered Eng expression level.

Expression of Hypoxia inducible factor 1α (Hif1α), a well-characterized transcription factor upstream of Eng, did not differ in the various cell lines. Elevated Eng expression in Kit oncogenic mutants appeared to be rather indirectly mediated by DNA hypomethylation, because treatment with the demethylating agent 5-Aza/dC increased Eng mRNA expression in KitWT cells to similar level. Aberrant DNA methylation is a frequent phenomenon in cancer, including GIST [33, 34]. The precise mechanism by which Kit oncogenic mutants could lead to alterations of DNA methylation remains however to be determined.
Although important mechanistic issues remain to be elucidated, this study has brought ENG into the spotlight in the field of KIT-ir ICC and GIST. As therapeutic interventions targetingEng are already being considered [1, 15], ENG deserves further evaluation as a putative novel marker and/or therapeutic target for GIST harbouring KIT oncogenic mutations.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Eng protein expression in Kit\textsuperscript{K641E} antrum.

Fig. S2. Eng expression in endothelium and in Kit\textsuperscript{+} ICC in WT mouse small intestine.

Fig. S3. Eng expression in endothelium and in Kit\textsuperscript{+} ICC in WT mouse colon.

Fig. S4. ENG expression in GIST882 cell line.

Fig. S5. Graphic representation of factorial analysis of multiple correspondences based on TMA variables and their modalities.

Fig. S6. Efficiency of SCF treatment was confirmed by the increase of pTyr-ir assessed by flow cytometry.

Fig. S7. Sustained imatinib treatment does not affect Eng mRNA expression in Ba/F3 cells.

Fig. S8. Efficiency of imatinib treatment was confirmed by the drop of pTyr-ir assessed by flow cytometry.

Fig. S9. Expression of Hif1\textalpha in Ba/F3 cells and in mouse antrum.

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