Zinc ions upregulate the hormone gastrin via an E-box motif in the proximal gastrin promoter

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Abstract

Gastrin and its precursors act as growth factors for the normal and neoplastic gastrointestinal mucosa. As the hypoxia mimetic cobalt chloride upregulates the gastrin gene, the effect of other metal ions on gastrin promoter activity was investigated. Gastrin mRNA was measured by real-time PCR, gastrin peptides by RIA, and gastrin promoter activity by dual-luciferase reporter assay. Exposure to Zn^{2+} ions increased gastrin mRNA concentrations in the human gastric adenocarcinoma cell line AGS in a dose-dependent manner, with a maximum stimulation of 55 ± 14 -fold at $100 \mu\text{M}$ ($P < 0.05$). Significant stimulation was also observed with Cd^{2+} and Cu^{2+} , but not with Ca^{2+} , Mg^{2+} , Ni^{2+} , or Fe^{3+} ions. Activation of MAPK and phosphatidylinositol 3-kinase pathways is necessary but not sufficient for gastrin induction by Zn^{2+} . Deletional mutation of the gastrin promoter identified an 11 bp DNA sequence, which contained an E-box motif, as necessary for Zn^{2+} -dependent gastrin induction. The fact that E-box binding transcription factors play a crucial role in the epithelial–mesenchymal transition (EMT), together with our observation that Zn^{2+} ions upregulate the gastrin gene in AGS cells by an E-box-dependent mechanism, suggests that Zn^{2+} ions may induce an EMT, and that gastrin may be involved in the transition.

Key Words

- ▶ gastrin
- ▶ cobalt
- ▶ zinc
- ▶ iron
- ▶ cadmium
- ▶ transcription
- ▶ promoter
- ▶ E-box
- ▶ MAPK
- ▶ PI3K

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Introduction

The peptide hormone gastrin is secreted by G cells in the gastric antrum and regulates gastric acid secretion in the stomach. Gastrin is synthesized as a large prohormone of 101 amino acids, which yields progastrin (80 amino acids) after cleavage of the signal peptide. Further processing of progastrin results in the generation of the glycine-extended gastrins (termed Ggly) and amidation of glycine-extended gastrins gives rise to amidated gastrins (termed gastrin or Gamide). The cholecystokinin 2 receptor (CCK2R) mediates the acid-stimulatory and proliferative effects of gastrin and is a member of the

G-protein-coupled seven transmembrane receptor family. Several candidates, including annexin II (Singh *et al.* 2007) and the F1 subunit of the mitochondrial ATPase (Kowalski-Chauvel *et al.* 2012), have been identified as possible receptors for progastrin and glycine-extended gastrin respectively. Progastrin and progastrin-derived peptides including Ggly and gastrin are biologically active and have growth-promoting effects on normal and cancer-derived gastrointestinal cells *in vitro* and *in vivo* (Chen *et al.* 2000, Aly *et al.* 2001, 2004, Baldwin *et al.* 2001, Pannequin *et al.* 2002, Ferrand *et al.* 2005, Takaishi *et al.* 2009).

Upregulation of the expression of several growth factors/hormones including gastrin in cancers has been demonstrated previously and their role in tumor progression and metastasis is now well-documented. A key remaining question is how the growth factors are upregulated in tumors. In the case of the gastrin promoter, various transcription factors including Sp1 (Chupreta *et al.* 2000), p73 (Tomkova *et al.* 2006), β -catenin/TCF4 (Chakladar *et al.* 2005), and AP1 are involved in the upregulation. In contrast, the transcription factors NF- κ B (Chakravorty *et al.* 2009) and RINZF/ZBTB10 (Tillotson 1999) have been shown to decrease gastrin expression. A dual role of the zinc finger transcription factor ZBP-89 has been proposed as, depending on the conditions, it can either stimulate or repress gastrin expression (Merchant 2000, Holley-Guthrie *et al.* 2005). The observation that various proteins mediate gastrin transcription via interaction with the Sp1 transcription factor indicates that Sp1 plays a central regulatory role in gastrin expression (Merchant *et al.* 1995, Tillotson 1999, Mensah-Osman *et al.* 2011). Interestingly, gastrin acting via the CCK2R can upregulate its own transcription, and this upregulation is independent of Sp1 binding (Kovac *et al.* 2010).

A previous study from our group demonstrated for the first time that hypoxia upregulated the promoter activity of the gastrin gene in AGS cells, and that this upregulation was HIF-independent (Xiao *et al.* 2012). Moreover, the upregulation induced by the commonly used hypoxia mimetic cobalt chloride (CoCl_2) differed from true hypoxia (1% O_2), as the magnitude of gastrin induction at all three levels of regulation (promoter activity, mRNA, and protein) was much higher in the case of CoCl_2 than with 1% O_2 (Xiao *et al.* 2012). The study was therefore extended to investigate whether other metal ions were able to regulate gastrin expression and the possible mechanisms of upregulation.

Subjects and methods

Cell culture

Human gastric (AGS) and prostate (LNCaP) adenocarcinoma cells were cultured in RPMI 1640 medium. The mouse colorectal cancer (MoCR) cell line used was harvested from a dimethylhydrazine-induced colon carcinoma in a CBA mouse (Kuruppu *et al.* 1996). Colon adenocarcinoma cells (DLD1, HCT116, HT29, and SW480) and MoCR cells were cultured in DMEM (Invitrogen). The media were supplemented with 8% FBS, 100 U/ml penicillin, 100 pg/ml streptomycin, and 10 mM HEPES

(Invitrogen). All cells were maintained at 37 °C in a humidified incubator with 95% air and 5% CO_2 . All human cell lines were obtained from ATCC (American Type Culture Collection (ATCC), Manassas, VA, USA).

RNA preparation and quantitative PCR

Cells were seeded at a density of 2.5×10^5 per well in six-well plates in growth media 1 day before the treatment. Treatments were carried out in serum-free medium for the indicated times. After treatment, total RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from isolated RNA with the SuperscriptII First Strand Synthesis System (Invitrogen), and was then used for real-time PCR (RT-PCR) amplification using an ABI PRISM 7700 Sequence Detector (Applied Biosystems) and TaqMan chemistry. The following primers were used: hGastrin forward, 5'-CCGCAGTGCTGAGGATGAG-3'; hGastrin reverse, 5'-GGAGGTGGCTAGGCTCTGAA-3'; hGastrin MGB probe, 5'-CTAACAATCCTAGAACCAAG-3'. Gene expression was normalized to 18S rRNA and is presented as ΔCt values. For each sample, the mean of the ΔCt values was calculated, and relative gene expression was normalized to controls.

RIA

Amidated gastrin (antiserum 1296) and its precursors glycine-extended gastrin (antiserum 7270) and progastrin (antiserum 1137) were measured with the indicated region-specific gastrin antisera using RIA as described previously (Ciccotosto *et al.* 1995). The cross-reactivity of antisera 7270 and 1137 for amidated gastrin is <0.1%.

Plasmid constructs

The gastrin-promoter Luciferase vector (1300pGASLuc), which contains 1300 bp of the upstream promoter region and the first exon of the human gastrin gene, was a kind gift from Prof. J Merchant (University of Michigan, Ann Arbor, MI, USA). Constructs with further deletions and mutations of the gastrin promoter were generated as described previously (Kovac *et al.* 2010) in the pGL4.10 luciferase vector. Deletional mutagenesis was performed using overlap extension PCR (Ho *et al.* 1989).

Gastrin promoter assays

Cells were transfected using a Neon cell transfection system (Invitrogen), according to the manufacturer's instructions.

Briefly, 2.0×10^6 cells were transfected with 3.5 μ g gastrin promoter luciferase construct and 0.7 μ g control pGL4.74(hRLuc/TK) vector expressing hRLuc luciferase protein. Treatment was carried out 24 h post transfection. Promoter activity was determined using a luciferase assay kit (Promega). Briefly, cells were lysed with 60–70 μ l/well Reporter Lysis Buffer, and Firefly and Renilla luciferase activity were measured with a FLUOstar Optima plate reader (BMG Labtech, Victoria, Australia). As Renilla luciferase activity from the control vector pRL-TK is induced by zinc (data not shown), the use of Renilla luciferase would not be

valid in assays where cells were treated with zinc and in these experiments relative luciferase activity was therefore normalized to the total protein content of each well (Shifera & Hardin 2010) as determined with a Bradford protein assay kit (Bio-Rad). Whenever possible Firefly luciferase activity was normalized to Renilla luciferase activity.

Western blot analysis

Cells were washed once with ice-cold PBS and lysed with 0.1–0.2 ml pre-boiled SDS lysis buffer, followed by protein separation using SDS-PAGE. Proteins were transferred onto Hybond-C Extra nitrocellulose membrane (GE Healthcare, Rydalmere, NSW, Australia). Phospho-ERK1/2, total ERK1/2, phospho-AKT, and total AKT antibodies were purchased from Cell Signaling (Danvers, MA, USA). As a loading control, blots were incubated with a HRP-conjugated rabbit anti- β -actin or anti-GAPDH antibody (Santa Cruz Biotechnology). Bands were visualized in a LAS 3000 Image Reader (Fujifilm, Brookvale, NSW, Australia), with an ECL Advance Western Blotting Detection Kit (GE Healthcare). Densitometric analysis of the protein bands was performed with MultiGauge Software (Fujifilm).

Statistical analysis

Data are presented as means $\pm$ S.E.M. Statistical significance for single comparisons of normally distributed data was determined by Student's *t*-test or for data that were not normally distributed by Mann-Whitney *U* rank sum test. For multiple comparisons, one-way ANOVAs followed by Bonferroni's correction were performed. All statistics were analyzed with the program SigmaStat (Jandel Scientific, San Rafael, CA, USA).

Results

Zinc and cadmium ions activate gastrin transcription

Previously, we have shown that CoCl_2 increased gastrin promoter activity in a dose-dependent manner in the human gastric cancer cell line AGS to a maximum of 2.4 ± 0.3 -fold at 300 μ M (Xiao *et al.* 2012). The data presented in Fig. 1 indicate that Zn^{2+} and Cd^{2+} ions also activated the gastrin promoter in a dose-dependent manner in AGS cells. A maximal stimulation of gastrin promoter activity of 11 ± 1.5 -fold (Fig. 1A) was measured with a gastrin promoter-luciferase construct (365pGASLuc), following treatment of AGS cells with 100 μ M ZnCl_2 for 16 h. Correspondingly treatment with 100 μ M ZnCl_2 induced

Figure 1

ZnCl_2 increases gastrin promoter activity and mRNA expression. (A) Gastrin promoter activity was measured in AGS cells transfected with the gastrin promoter construct 365pGASLuc following treatment with increasing concentrations of ZnCl_2 in serum-free medium for 16 h. The promoter activity was calculated as the fold increase in relative luciferase activity compared with 0 μ M control. Values are expressed as the mean $\pm$ S.E.M. of at least three separate treatments. (B) Gastrin mRNA expression in AGS cells following treatment with increasing doses of ZnCl_2 for 16 h was measured using real-time PCR and normalized to the amount of 18S rRNA. Values are expressed as the fold increase compared with untreated cells, and are the mean $\pm$ S.E.M. of at least three separate treatments. (C) Gastrin promoter activity was measured in AGS cells transfected with the gastrin promoter construct 365pGASLuc following treatment with increasing concentrations of CdCl_2 in serum-free medium for 16 h. (D) Gastrin mRNA expression was measured in AGS cells following treatment with increasing doses of CdCl_2 for 16 h. (E) Gastrin promoter activity was measured in AGS cells transfected with the gastrin promoter construct 365pGASLuc following treatment with various metals (150 μ M) in serum-free medium for 16 h. (F) Gastrin mRNA expression was measured in various cell lines following treatment with 50 μ M ZnCl_2 for 16 h. * $P < 0.05$ vs untreated control.

gastrin mRNA expression by 55 ± 14 -fold (Fig. 1B) when measured by RT-PCR. Treatment of AGS cells with $10 \mu\text{M}$ CdCl_2 stimulated the gastrin promoter by 4.0 ± 0.4 -fold (Fig. 1C) and gastrin mRNA expression by 50 ± 12 -fold (Fig. 1D). Although $50 \mu\text{M}$ CdCl_2 stimulated gastrin promoter and gastrin mRNA expression, significant cell toxicity was observed at this concentration.

To determine whether the increase in gastrin promoter activity was specific to zinc, cobalt, and cadmium ions, gastrin promoter activity was measured following the treatment of AGS cells with various metal ions for 16 h. Calcium, iron (III) in the form of ferric citrate, magnesium, and nickel ions at $150 \mu\text{M}$ had no effect on gastrin promoter activity (Fig. 1E) or on gastrin mRNA expression (data not shown). Treatment of AGS cells with either lower ($50 \mu\text{M}$) or higher ($300 \mu\text{M}$) doses of calcium, ferric, magnesium, and nickel ions had no effect on gastrin promoter activity (data not shown). Although treatment with copper ions at $150 \mu\text{M}$ resulted in a statistically significant stimulation of gastrin promoter activity, the increase was only 1.4 ± 0.1 -fold compared with 7.3 ± 2.0 -fold stimulation by $150 \mu\text{M}$ ZnCl_2 .

Transition metal ions have been shown to generate reactive oxygen species and subsequent oxidative stress in cells. To determine if oxidative stress played any role in gastrin induction by the metal ions, the gastrin promoter activity in the presence of H_2O_2 was determined. The observation that treatment of AGS cells with $100 \mu\text{M}$ H_2O_2 did not stimulate gastrin promoter activity (data not shown) ruled out the involvement of reactive oxygen species.

To confirm that zinc-induced stimulation of gastrin expression was a general phenomenon and not cell-specific, gastrin mRNA expression was measured in various cell lines including the human colon cancer cell lines DLD1, HCT116, HT29, and SW480, and the human prostate carcinoma cell line LNCaP after treatment with $50 \mu\text{M}$ ZnCl_2 for 16 h. Treatment with Zn^{2+} ions induced gastrin mRNA expression in all cell lines, although the fold increase varied widely from a maximum of 101 ± 37 -fold in HCT116 cells to a minimum of 6 ± 1 -fold in SW480 cells (Fig. 1F). Furthermore, the observation that treatment of the mouse colon cancer cell line (MoCR) with $50 \mu\text{M}$ ZnCl_2 for 16 h increased gastrin expression by 18 ± 1 -fold indicated that gastrin induction by Zn^{2+} ions was not species-specific.

Zinc ions stimulate expression of both amidated and non-amidated forms of gastrin

To confirm that the Zn-induced increase in transcription led to an increase in cellular expression and secretion of

gastrin peptides, the concentrations of amidated Gamide and non-amidated Ggly and progastrin in cell extracts and in conditioned media of AGS cells were measured following treatment with $50 \mu\text{M}$ ZnCl_2 for 16 h (Fig. 2). In agreement with the observed increase in gastrin mRNA, the concentration in cell extracts and conditioned media of all measured forms of gastrin-derived peptides increased following ZnCl_2 treatment. Gamide in cell extracts increased from 3.3 ± 0.5 fmol/million cells in the untreated control cells to 7.6 ± 2.6 fmol/million cells following ZnCl_2 treatment (Fig. 2A). Similarly, the concentration of Ggly in AGS cell extracts increased from 3.4 ± 0.6 fmol/million cells in untreated AGS cells to 9.2 ± 2.9 fmol/million cells following ZnCl_2 treatment (Fig. 2C). The most dramatic increases of nearly fourfold in cell extracts and 50-fold in conditioned medium were seen in the concentration of progastrin following treatment with Zn^{2+} ions. The concentration of progastrin in AGS cell extracts increased from 10.4 ± 1.9 fmol/million cells in the untreated control to 45.4 ± 7.2 fmol/million cells after treatment with $50 \mu\text{M}$ ZnCl_2 (Fig. 2E). Similarly, in the conditioned

Figure 2

ZnCl_2 increases gastrin peptide expression. Concentrations of amidated gastrin (Gamide; A and B), glycine-extended gastrin (Ggly; C and D), and progastrin (E and F) in cell extracts (A, C and E) and conditioned medium (B, D and F) following treatment of AGS cells with $50 \mu\text{M}$ ZnCl_2 for 16 h were measured by RIA. Values are expressed as the mean $\pm$ s.e.m. of at least three separate experiments. * $P < 0.05$ vs untreated control.

Figure 3

Gastrin induction by ZnCl₂ is dependent on MAPK and PI3K pathways. Gastrin promoter activity (A) and gastrin mRNA expression (B) were measured in AGS cells following treatment with 50 μ M ZnCl₂ for 16 h in the presence of either a PI3K inhibitor (10 μ M LY294002) or a MAPK inhibitor (10 μ M U0126). * P < 0.05 vs 50 μ M ZnCl₂. Treatment of AGS cells with 50 μ M ZnCl₂ stimulated the gastrin promoter activity by 4.1 ± 0.8 and gastrin mRNA expression by 12.5 ± 2.1 -fold compared with untreated control. Phosphorylation of ERK1/2 (C), a mediator of the MAPK pathway, and of

AKT (D), a mediator of the PI3K pathway, was measured by western blot as described in 'Subjects and methods' following the treatment of AGS cells with 50 μ M ZnCl₂ for the time indicated. * P < 0.05 vs 0 min control. Phosphorylation of ERK1/2 (E) and AKT (F) was measured by western blot following the treatment of AGS cells with 50 μ M ferric citrate for the time indicated. Band densities were determined and are presented as the ratio of densities of phosphorylated protein:total protein. * P < 0.05 vs 0 min control.

medium the concentration of progastrin increased from 0.15 ± 0.09 fmol/ml per million cells in the untreated control to 5.5 ± 1.1 fmol/ml per million cells after treatment with 50 μ M ZnCl₂ (Fig. 2F).

MAPK and phosphatidylinositol 3-kinase pathways are involved in gastrin stimulation by zinc ions

Treatment with extracellular Zn²⁺ ions has been shown to activate both the MAPK and phosphatidylinositol 3-kinase

(PI3K) pathways (Kim *et al.* 2000, Azriel-Tamir *et al.* 2004, Hershfinkel *et al.* 2007). In order to establish which signaling pathway was involved in the stimulation of gastrin expression by Zn²⁺ ions, the expression of gastrin mRNA and gastrin promoter activity was measured after pretreatment of AGS cells for 30 min with either the PI3K inhibitor LY294002 (10 μ M) or the MAPK inhibitor U0126 (10 μ M), followed by treatment with 50 μ M ZnCl₂ for 16 h in the presence of the same inhibitors. Treatment with the PI3K inhibitor LY294002 reduced the gastrin promoter

activity (Fig. 3A) and the corresponding gastrin mRNA expression (Fig. 3B) to 35 ± 10 and $22 \pm 2\%$, respectively, compared with cells treated with $50 \mu\text{M}$ ZnCl_2 only (100%). Similarly, the MEK inhibitor (U0126) reduced the gastrin promoter activity (Fig. 3A) and the corresponding gastrin mRNA expression (Fig. 3B) to 29 ± 7 and $23 \pm 5\%$, respectively, compared with cells treated with $50 \mu\text{M}$ ZnCl_2 only (100%).

To confirm that Zn^{2+} ions activate phosphorylation of AKT (a downstream target of PI3K) and of ERK1/2 (a downstream target of MAPK) in AGS cells, the expression of phospho-AKT and phospho-ERK1/2 was measured by western blotting. Treatment of AGS cells with $50 \mu\text{M}$ ZnCl_2 increased phosphorylation of both ERK1/2 (Fig. 3C) and AKT (Fig. 3D) in a time-dependent manner. The phosphorylation of ERK1/2 in response to zinc was biphasic; the early phase of activation peaked at 30 min (4.8 ± 1.5 -fold), and the later phase of activation was sustained for at least 16 h (26 ± 6 -fold) (Fig. 3C). In contrast, the phosphorylation of AKT peaked at 4 h (9.5 ± 3.3 -fold), and gradually decreased thereafter (Fig. 3D).

Although the increase in phosphorylation of ERK1/2 and AKT in AGS cells after treatment with Zn^{2+} ions is necessary for induction of gastrin expression (Fig. 3A and B), the question of whether it was sufficient was next investigated. As seen in Fig. 3E, incubation of AGS cells with $50 \mu\text{M}$ ferric citrate caused an 11 ± 4 -fold increase in the phosphorylation of ERK1/2 after 10 min. Similarly, AKT phosphorylation was increased by 6 ± 1 -fold following treatment of AGS cells with $50 \mu\text{M}$ ferric citrate for 30 min (Fig. 3F). However, the observation that treatment with ferric citrate did not induce gastrin promoter activity (Fig. 1E) clearly indicated that activation of ERK1/2 and AKT was not sufficient for induction of gastrin expression.

To determine whether activation of PI3K was upstream of the activation of MAPK, AGS cells were treated with $50 \mu\text{M}$ ZnCl_2 for 4 h in the presence of either the PI3K inhibitor (LY294002) or the MEK inhibitor (U0126) and the phosphorylation of AKT and ERK1/2 was measured by western blotting. As shown in Fig. 4A, the 8 ± 2 -fold increase in the phosphorylation of ERK1/2 in response to Zn^{2+} ions was reduced to 3.2 ± 1.1 -fold in the presence of PI3K inhibitor. In contrast, there was no statistically significant difference in the phosphorylation of AKT in response to Zn^{2+} ions in the absence (6 ± 1 -fold) or presence (5.5 ± 0.5 -fold) of the MEK inhibitor (Fig. 4B). These observations are consistent with the conclusion that activation of PI3K is upstream of the activation of MAPK. Treatment of AGS cells with $300 \mu\text{M}$ CoCl_2 for 4 h also

Figure 4

Phosphorylation of AKT in response to ZnCl_2 is upstream of phosphorylation of ERK1/2. (A) Phosphorylation of ERK1/2 was measured by western blot as described in 'Subjects and methods' following the treatment of AGS cells with $50 \mu\text{M}$ ZnCl_2 for 4 h in the presence of either a PI3K inhibitor (10 μM LY294002) or a MAPK inhibitor (10 μM U0126). (B) Phosphorylation of AKT was measured by western blot following the treatment of AGS cells with $50 \mu\text{M}$ ZnCl_2 for 4 h in the presence of either a 10 μM LY294002 or 10 μM U0126. Band densities were determined and are presented as the ratio of densities of phosphorylated protein:total protein. * $P < 0.05$ vs untreated control and # $P < 0.05$ vs $50 \mu\text{M}$ ZnCl_2 .

stimulated phosphorylation of AKT and ERK1/2 by 3.0 ± 0.3 - and 2.0 ± 0.3 -fold respectively.

Zinc ion-induced gastrin expression is independent of the zinc receptor GPR39

A number of groups have proposed that binding of Zn^{2+} ions to the zinc receptor (GPR39) triggers intracellular signaling pathways that regulate key cell functions (Hershinkel *et al.* 2001, 2007, Cohen *et al.* 2012). To determine whether GPR39 has any role in the stimulation of gastrin transcription by Zn^{2+} ions, the effect of GPR39

Figure 5

Gastrin induction by ZnCl₂ is independent of the zinc receptor GPR39 and is mediated intracellularly. (A) Gastrin promoter activity was measured in AGS cells co-transfected with the 365pGASLuc luciferase construct and siRNA against GPR39, following treatment with 50 μM ZnCl₂ and 300 μM CoCl₂ for 16 h. Values are expressed as the mean ± S.E.M. of at least three separate experiments. **P* < 0.05 vs control. (B) Expression of GPR39 was measured by western blot using antibody ab39227 (Abcam, Cambridge, UK) following the transfection of AGS cells with siRNA against either GPR39 or Lamin A/C as a control. Values are expressed as the mean ± S.E.M. of two separate experiments each in triplicate. **P* < 0.05 vs Lamin A/C siRNA. (C) Phosphorylation of ERK1/2 was measured by western blot as described in 'Subjects and methods' following treatment of AGS cells with 50 μM ZnCl₂ or ferric citrate for 10 min in the presence or absence of TPEN. Intracellular loading of TPEN was achieved by the incubation of AGS cells with 500 μM TPEN for 60 min. Following the incubation, extracellular TPEN was removed by washing before treating the cells with 50 μM ZnCl₂ or ferric citrate for 10 min. Band densities were determined and are presented as the ratio of densities of phosphorylated protein:total protein **P* < 0.05 vs 50 μM ZnCl₂ and #*P* < 0.05 vs 50 μM ferric citrate.

knockdown on gastrin promoter activity was investigated. A previously validated siRNA sequence CCATGGAGTTC-TACAGCATtt which was effective in knocking down GPR39 expression in the neuronal SHSY-5Y cell line (Chorin *et al.* 2011) and in HT29 colonocytes (Cohen *et al.* 2012) was used. AGS cells were co-transfected with a gastrin promoter luciferase construct (365pGASLuc) and

either 100 nM GPR39 siRNA or 100 nM Lamin A/C siRNA as a control. As there was no difference in the gastrin promoter activity of the two transfectants following treatment with 50 μM ZnCl₂ (Fig. 5A), we concluded that the zinc receptor GPR39 does not play a major role in the stimulation of gastrin transcription by zinc. As seen in Fig. 5B, transfection of AGS cells with siRNA against GPR39 resulted in nearly 50% knockdown of GPR39 protein compared with cells transfected with control Lamin A/C siRNA as determined by western blot using an anti-GPR39 antibody.

To investigate whether the effect of Zn²⁺ ions on gastrin expression was mediated intracellularly or extracellularly, we determined whether the increase in the phosphorylation of ERK1/2 by Zn²⁺ and ferric ions was inhibited by a cell-permeable zinc-selective chelator, *N,N,N',N'*-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN). Previously, it has been shown that chelating intracellular Zn²⁺ ions does not change the activation of the MAPK pathway by extracellular Zn²⁺ ions (Hansson 1996, Hershinkel *et al.* 2001). As shown in Fig. 5C, ERK1/2 phosphorylation was completely abolished in the cells treated with either 50 μM ZnCl₂ or ferric citrate for 10 min in the presence of 500 μM TPEN. Next, we determined whether intracellular chelation of Zn²⁺ or ferric ions was able to abolish activation of ERK1/2 following the treatment of AGS cells with 50 μM ZnCl₂ or ferric citrate. Intracellular loading of cells was carried out by incubating AGS cells with 500 μM TPEN for 60 min and removing the extracellular TPEN by washing. Following the removal of TPEN, cells were treated with 50 μM ZnCl₂ or ferric citrate for 10 min. The observation that intracellular chelation of Zn²⁺ or ferric ions by intracellular TPEN attenuated the phosphorylation of ERK1/2 in response to 50 μM ZnCl₂ or ferric citrate indicated that ERK1/2 was activated at least in part by intracellular permeation of metal ions (Fig. 5C).

Characterization of the zinc response element within the gastrin promoter

Several approaches were investigated to define the zinc response element (ZRE) within the gastrin promoter. The observation that shortening of the gastrin promoter from 365 bp (365pGASLuc) to 109 bp (109pGASLuc) completely abolished gastrin promoter activation by Zn²⁺ or Co²⁺ ions indicated that the zinc/cobalt response element lay between 365 and 109 bp upstream from the transcription start site (Fig. 6A). Neither of the two GC boxes that function as binding sites for the transcription factor Sp1 within this region was required for the activation of the

Figure 6

Gastrin induction by ZnCl_2 is mediated exclusively by the 11 bp sequence located between -120 and -109 bp in the proximal promoter. (A) Gastrin promoter activity was measured in AGS cells transfected with the 365pGASLuc, 365pGASLuc double GC mutant, and 109pGASLuc luciferase constructs following treatment with $50 \mu\text{M}$ ZnCl_2 (black bar) or $300 \mu\text{M}$ CoCl_2 (grey bar) for 16 h. Firefly luciferase activity and Renilla luciferase activity (as a transfection control) were determined as described in 'Subjects and methods'. Values are expressed as the mean $\pm$ S.E.M. of at least three separate experiments. * $P < 0.05$ vs untreated control.

(B) Gastrin promoter activity was measured in AGS cells transfected with 365pGASLuc or with one of the five mutants with block deletions of ~ 50 bp generated from the 365pGASLuc construct. Values are expressed as the mean $\pm$ S.E.M. of at least three separate experiments. * $P < 0.05$ vs 365pGASLuc. (C) Gastrin promoter activity was measured in AGS cells transfected with 163pGASLuc, 153pGASLuc, 132pGASLuc, 120pGASLuc, or 109pGASLuc gastrin promoter luciferase constructs. Values are expressed as the mean $\pm$ S.E.M. of at least three separate experiments. * $P < 0.05$ vs 163pGASLuc.

gastrin promoter by Co^{2+} ions (Xiao *et al.* 2012). Similarly, the observation that mutation of both GC boxes had no effect on activation of the gastrin promoter by Zn^{2+} ions (Fig. 6A) strongly suggested that Sp1 was not involved in

the zinc-dependent activation of the gastrin gene. As the region between 365 and 109 bp in the proximal gastrin promoter did not contain the consensus binding site, (TGC(G/C)CNC(G)), for the zinc-responsive transcription

Figure 7

Basal gastrin promoter activity in AGS cells is regulated by an 11 bp sequence. (A) Basal gastrin promoter activities were determined following the transfection of AGS cells with 365pGASLuc or with one of the five mutants with block deletions of ~50 bp generated from the 365pGASLuc construct. The firefly luciferase activity of each construct was normalized to the control Renilla luciferase activity and expressed as a percentage relative to the value for the corresponding 365pGASLuc construct. Values are expressed as the mean $\pm$ S.E.M. of at least three separate experiments.

* $P < 0.05$ vs 365pGASLuc. (B) Basal gastrin promoter activities were determined following the transfection of AGS cells with 163pGASLuc, 153pGASLuc, 132pGASLuc, 120pGASLuc, or 109pGASLuc constructs. The firefly luciferase activity of each construct was normalized to the control Renilla luciferase activity and expressed as a percentage relative to the value of the corresponding 163pGASLuc construct. Values are expressed as the mean $\pm$ S.E.M. of at least three separate experiments. * $P < 0.05$ vs 163pGASLuc.

factor MTF1 which induces expression of genes involved in metal homeostasis including metallothioneins in response to heavy metals such as Zn^{2+} and Cd^{2+} (Majumder *et al.* 2003), the possibility of a direct involvement of MTF1 in gastrin induction was ruled out. However, to test for an indirect effect we showed that downregulation of MTF1 expression by transient transfection of AGS cells with MTF1 shRNA did not inhibit activation of gastrin promoter activity by Zn^{2+} ions (data not shown).

To narrow down the ZRE still further, plasmids with additional deletions of the region between 365 and 109 bp were constructed. To rule out the possibility that multiple elements in the promoter region might control the activation of gastrin transcription by Zn^{2+} ions (Plevy *et al.* 1997), five mutants with block deletions of ~50 bp were generated from the 365pGASLuc luciferase reporter construct (Fig. 6B). Although treatment of AGS cells transfected with 365pGASLuc with 50 μM ZnCl_2 or 300 μM CoCl_2 significantly stimulated the gastrin

Figure 8

Mutation of the E-box in the proximal gastrin promoter completely abolished induction by zinc or cobalt ions. (A) Diagram of the WT 365pGASLuc gastrin promoter construct and the ΔE-box 365pGASLuc construct with a mutated E-box. (B) Gastrin promoter activities were determined in AGS cells transfected with either the WT 365pGASLuc or the mutant ΔE-box 365pGASLuc constructs following treatment with 50 μM ZnCl₂ or 300 μM CoCl₂ for 16 h. The firefly luciferase activity of each construct was expressed as a ratio to the corresponding value for WT 365pGASLuc. Values are expressed as the mean ± s.e.m. of at least three separate experiments. [#]*P* < 0.05 vs WT 365pGASLuc.

promoter activity by 6.2 ± 0.7 - and 2.4 ± 0.9 -fold, respectively, and individual deletion of blocks 1–4 did not significantly reduce the stimulation, deletion of the sequence between –163 and –109 bp (block 5) from the gastrin promoter completely abolished the response to Zn²⁺ or Co²⁺ ions (Fig. 6B). As the zinc regulatory region is within –163 and –109 bp, shorter gastrin promoter luciferase constructs named 163pGASLuc, 153pGASLuc, 132pGASLuc, and 120pGASLuc were generated. The reduction of Zn²⁺ ion-stimulated gastrin promoter activity from 3.9 ± 0.2 -fold in AGS cells transfected with the 120pGASLuc construct to 1.5 ± 0.2 -fold in AGS cells transfected with the 109pGASLuc construct indicated that the ZRE lay within the 11 nucleotides between –120 and –109 bp in the gastrin promoter (Fig. 6C). The cobalt response element lay within the same region (Fig. 6C).

The ZRE regulates basal gastrin promoter activity in AGS cells

To investigate whether the ZRE regulates basal gastrin promoter activity, the ratio of firefly:Renilla luciferase activity was calculated for each of the gastrin promoter constructs in the untreated cells and expressed as a percentage of the value observed for 365pGASLuc. Deletion of the ~50 bp sequence (block 5) between –163 and –109 bp in the gastrin promoter reduced the basal non-stimulated gastrin promoter activity to $19 \pm 3\%$ of the value for 365pGASLuc (100%) (Fig. 7A). Furthermore, although there was no significant difference between the basal gastrin promoter activity measured for the deletion of constructs 163pGASLuc, 153pGASLuc, or 132pGASLuc, the basal gastrin promoter activity measured for the 109pGASLuc ($18 \pm 2\%$) gastrin promoter construct was significantly lower than the value ($109 \pm 8\%$) for the 120pGASLuc construct (Fig. 7B). This observation indicated that basal gastrin expression was also regulated by the ZRE consisting of the 11 nucleotides between –120 and –109 bp.

Zinc ions induce gastrin expression via an E-box motif

Sequence analysis of the ZRE consisting of the 11 nucleotides between –120 and –109 bp indicated the presence of a putative E-box consensus sequence (CANNTG). To investigate whether the putative E-box sequence regulates gastrin expression in response to treatment with Zn²⁺ ions, the E-box in the 365pGASLuc construct was mutated to generate the mutant ΔE-box 365pGASLuc construct (Fig. 8A). AGS cells were transfected with the plasmids 365pGASLuc or ΔE-box 365pGASLuc, and gastrin promoter activity was measured by luciferase assay. Mutation of the E-box sequence completely abrogated the stimulation of gastrin promoter activity by either 50 μM ZnCl₂ or 300 μM CoCl₂ (Fig. 8B). Further studies are needed to identify the transcription factor that binds the E-box and upregulates gastrin expression in response to Zn²⁺ ions.

Discussion

In the current study, we have demonstrated for the first time that treatment with Zn²⁺ ions induces gastrin gene expression at the promoter, mRNA, and protein levels in the human gastric adenocarcinoma cell line AGS. Furthermore, Zn²⁺-induced gastrin expression is not limited to gastric cells (AGS) as Zn²⁺ ions were able to induce gastrin

expression in carcinoma cell lines of various origin including colon (DLD1, HCT116, HT29, and SW480) and prostate (LNCaP). Analysis of the GEO database (NCBI) revealed that a twofold increase in gastrin mRNA in non-malignant HPR1, but not in malignant PC3 prostate cells, has been demonstrated previously in response to Zn^{2+} treatment (Lin *et al.* 2006). Furthermore, induction of gastrin expression by Zn^{2+} ions in the mouse colon cell line MoCR indicates that the induction is not limited to human cells. We conclude that induction of gastrin gene expression by Zn^{2+} ions is likely to be a general phenomenon.

Induction of the gastrin gene at the mRNA and promoter level is abrogated by inhibitors of the MAPK (U0126) and PI3K (LY294002) pathways in AGS cells. Furthermore, western blots revealed that Zn^{2+} (Fig. 3) and Co^{2+} (data not shown) ions induce phosphorylation of ERK1/2 and AKT in a time-dependent manner, in agreement with a previous study which showed that Zn^{2+} -stimulated proliferation of mouse embryonic stem cells involves both AKT and ERK1/2 phosphorylation (Ryu *et al.* 2009). In contrast to the ability of the PI3K inhibitor to inhibit Zn^{2+} -induced ERK1/2 phosphorylation, the failure of a MEK1 inhibitor to abrogate Zn^{2+} -induced activation of AKT phosphorylation indicates that Zn^{2+} -induced AKT phosphorylation is upstream of ERK1/2 phosphorylation in AGS cells. Our results are consistent with a previous study in which treatment of neuroblastoma SH-SY5Y cells with the PI3K inhibitor LY294002 completely abolished the Zn^{2+} -stimulated phosphorylation of AKT and ERK1/2, although neither the NF- κ B inhibitor (BAY11-7082) nor the MEK inhibitor U0126 affected AKT activity (Zhou *et al.* 2011). Previously, Tang & Shay (2001) have demonstrated that, in contrast to Zn^{2+} , neither Ca^{2+} nor Mg^{2+} ions increased Ser-473 phosphorylation of AKT in 3T3-L1 fibroblasts and adipocytes. The observation that neither Ca^{2+} nor Mg^{2+} ions increased gastrin promoter activity in our study indicates that the ability of Zn^{2+} to induce Ser-473 phosphorylation of AKT in AGS cells and to increase gastrin expression is not a property of all metal ions.

Although an increase in phosphorylation of ERK1/2 and AKT is necessary (Fig. 3A and B), it is not a sufficient condition for the induction of gastrin expression in AGS cells after treatment with metal ions. This conclusion was reached from a comparison of the effects of Zn^{2+} (Fig. 3C and D) and ferric (Fig. 3E and F) ions. Phosphorylation of both ERK1/2 and AKT was increased, although the time courses were completely different. Hence, the observation that treatment with ferric ions did not induce gastrin

promoter activity (Fig. 1E) clearly indicates that activation of ERK1/2 and AKT is not sufficient for the induction of gastrin expression.

Previously, GPR39 has been identified as a zinc-sensing receptor that is specifically activated by extracellular Zn^{2+} at physiological concentrations (Hershinkel *et al.* 2001, 2007). Binding of Zn^{2+} to GPR39 triggers the release of Ca^{2+} from intracellular stores via the inositol 1,4,5-trisphosphate (IP3) pathway, and the Ca^{2+} ions in turn stimulate downstream signaling via the MAPK and PI3K pathways. Interestingly, our study demonstrates that Zn^{2+} treatment induces gastrin expression independently of GPR39. In agreement with that conclusion, we have demonstrated that activation of MAPK and PI3K pathways is dependent on the intracellular permeation of Zn^{2+} and Fe^{+3} ions.

The metal-responsive transcription factor 1 (MTF1) plays an important role in the transcriptional regulation of genes. Heavy metal ions like zinc, cadmium, copper, mercury, gold, silver, cobalt, nickel, and bismuth ions are known to induce expression of metallothioneins via MTF1 (Majumder *et al.* 2003). Interestingly, Zn^{2+} ions have been shown to activate MTF1 directly, whereas other metals probably activate it by mobilizing the intracellular Zn^{2+} pool (Palmiter 1994). The release of Zn^{2+} ions from the oxidation of metallothioneins by H_2O_2 has been shown to upregulate MTF1 activity (Zhang *et al.* 2003). The observation that calcium, copper, magnesium or nickel ions, or H_2O_2 itself, had little or no effect on gastrin promoter activity (Fig. 1) suggested that MTF1 was not involved in gastrin activation by Zn^{2+} ions. This hypothesis was confirmed by the demonstration that downregulation of MTF1 expression did not inhibit activation of gastrin promoter activity by Zn^{2+} ions. The Sp1 transcription factor, which plays a central role in gastrin expression (Merchant *et al.* 1995, Tillotson 1999, Mensah-Osman *et al.* 2011), was also eliminated from contention in the current study.

The epithelial-mesenchymal transition (EMT) in cancer cells endows them with invasiveness and stem cell characteristics which in turn make them more aggressive and resistant to chemotherapy. Although many transcription factors that can trigger EMT have been identified, the three major groups of transcription factors that orchestrate EMT are the ZEB, Snail, and Twist families (Sanchez-Tillo *et al.* 2012). Although these three groups of transcription factors interact with an E-box motif in the proximal promoters of EMT marker genes (Peinado *et al.* 2007, Vandewalle *et al.* 2009), it is still unclear how the transcription factors themselves are

activated. Yamashita *et al.* (2004) have shown that zinc deficiency caused by knockdown of the zinc transporter Zip6 prevented the EMT, and on that basis proposed the hypothesis that Zn^{2+} ions may induce EMT through phosphorylation of AKT (Farrell *et al.* 2011). Our result that Zn^{2+} ions induce gastrin expression through the activation of an E-box binding transcription factor via the phosphorylation of AKT raises the possibility that Zn^{2+} may induce the EMT through direct activation of E-box-binding transcription factors. Although further studies of the transcription factors involved are warranted, in the light of evidence that many known (Longo *et al.* 2008, Terragni *et al.* 2011) and as yet uncharacterized transcription factors (Biggs *et al.* 2007) can bind E-boxes it is beyond the scope of the current study to identify one single transcription factor which activates gastrin expression in response to Zn^{2+} .

What does our finding that gastrin is induced by Zn^{2+} ions imply about normal gastric physiology? The relationship between Zn^{2+} ions and gastric acidity is unclear as Zn^{2+} ions have been reported to either increase (Yamaguchi *et al.* 1980) or reduce gastric acid secretion (Cho *et al.* 1976, Kirchhoff *et al.* 2011). Our observation that treatment with Zn^{2+} increases both intracellular and secreted Gamide in the gastric carcinoma cell line AGS, if replicated in antral G cells, would result in a CCK2R-mediated increase in gastric acid secretion. However, any effect of Gamide might be counterbalanced by the direct inhibitory effects of Zn^{2+} on gastric acid secretion reported by Kirchhoff *et al.* (2011) in isolated gastric glands, and further work will be required to establish the relative importance of the two opposing contributions (Kirchhoff *et al.* 2011).

The medical significance of our finding that treatment with Zn^{2+} ions induces gastrin expression lies in the observation that Zn^{2+} ions exert a gastro-protective effect (Cho & Ogle 1978). Indeed Zn^{2+} ions, or compounds containing Zn^{2+} ions (such as polaprezinc), are used as anti-ulcer drugs and accelerate gastric ulcer healing in humans (Watanabe *et al.* 1995, Opoka *et al.* 2010). However, the precise mechanisms behind the effects of zinc-containing compounds on mucosal integrity, gastro-protection, and ulcer healing remain unclear. Recently, Opoka *et al.* have shown that administration of Zn^{2+} ions to rats inhibited gastric secretion and increased gastrin expression in the plasma. The authors postulated that elevated gastrin may enhance cell proliferation and differentiation to accelerate ulcer healing (Opoka *et al.* 2010). Interestingly, this group observed a divergence between the inhibition of gastric secretion and the

increase in gastrin expression in vehicle- and Zn^{2+} -treated animals and raised the possibility that the increase in plasma gastrin observed in Zn^{2+} -treated animals could be secondary to the decrease in luminal acidity and that Zn^{2+} -containing compounds could directly influence the G-cells to stimulate production of gastrin (Opoka *et al.* 2010). Our results provide experimental evidence that Zn^{2+} ions do indeed have a direct effect on gastrin expression in gastric cells and highlight the importance of gastrin in the mechanisms of ulcer healing and of the gastro-protective effect exerted by zinc compounds. However, as Zn^{2+} induces both non-amidated and amidated forms of gastrin, and both forms exert trophic effects in the gastrointestinal tract, pancreas, and liver (Koh & Chen 2000), further studies will be required to establish which forms of gastrin play a role in the gastro-protection induced by Zn^{2+} ions.

The role of gastrin in EMT in gastric cancer is well-established. Infection of AGS and MGLVA1 cells by pathogenic *Helicobacter pylori* results in the upregulation of the EMT marker matrix metalloproteinase 7 (MMP7) and a consequent increase in soluble HB-EGF. Both outcomes are partially dependent on gastrin *in vivo* and *in vitro* (Yin *et al.* 2010). Furthermore, gastrin has been shown to increase migration of AGS cells expressing CCK2R receptors via upregulation of MMP7 (Mishra *et al.* 2010). Gastrin is one of the mediators of EMT in gastric cancer and the link between deregulation of zinc homeostasis and gastrin-induced EMT needs further attention.

Several metal-inducible genes have been reported but surprisingly little is known about the transcriptional regulation of gene expression in response to Zn^{2+} or related metal ions. Evidence of altered zinc homeostasis in the etiology of diseases, including diabetes, Alzheimer's and cancer (Fukada *et al.* 2011) highlights the importance of studying Zn^{2+} -regulated gene expression. A better understanding of the zinc–gastrin axis may aid in unraveling the role of zinc signaling in the EMT and in various pathological conditions.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

L X, S K, and M C performed the experiments analyzed, and interpreted the data. A S, G S B, and O P designed the research, analyzed, and interpreted the data. G S B and O P wrote the paper.

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