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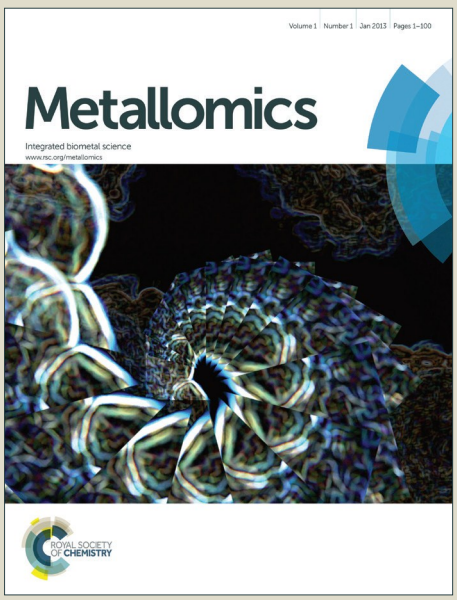

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Activation by zinc of the human gastrin gene promoter in colon cancer cells *in vitro* and *in vivo*.

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Abstract:

Over-expression of growth factors can contribute to the development and progression of cancer, and gastrins in particular have been implicated in accelerating the development of gastrointestinal cancers. Previously our group showed that hypoxia, cobalt chloride (a hypoxia mimetic) and zinc chloride could activate the expression of the gastrin gene *in vitro*. To characterise activation of the gastrin promoter by zinc ions further *in vivo,* TALEN technology was used to engineer a luciferase reporter construct into the endogenous human gastrin gene promoter in SW480 colon cancer cells. Gastrin promoter activity in the resultant $Gast^{luc}$ SW480 colon cancer cells was then measured by bioluminescence in cell culture and in tumour xenografts in SCID mice. Activation of intracellular signalling pathways was assessed by Western blotting. Activation of the gastrin promoter by zinc ions was concentration dependent *in vitro* and *in vivo*. Zinc ions significantly stimulated phosphorylation of ERK1/2 (MAPK pathway) but not of Akt (PI3K pathway). We conclude that the endogenous gastrin promoter is responsive to zinc ions, likely via activation of the MAPK pathway.

Introduction

The hormone gastrin was originally identified as a stimulant of acid secretion [1]. The circulating forms of 17 and 34 residues share the same C-terminal tetrapeptide amide as the related hormone cholecystokinin (CCK), and C-terminal amidation is essential for secretory activity. Gastrin is synthesized as a 101-residue precursor (*preprogastrin*) which, on removal of a 21-residue signal peptide, yields *progastrin* (80 residues). Proteolytic processing in the antral G cell generates a number of intermediate, non-amidated progastrin-derived peptides, including *glycine-extended gastrin*$_{17}$ (Ggly), and amidation of glycine-extended gastrins yields *amidated gastrin* (Gamide). Gamide, acting through the cholecystokinin 2 receptor (CCK2R), is the major hormonal regulator of gastric acid secretion. Gamide, Ggly and progastrin have all been implicated in accelerating the development of cancers of the stomach or colon [2, 3].

We demonstrated for the first time that hypoxia up-regulated gastrin gene expression in the human gastric adenocarcinoma cell line AGS [4]. The increase in gastrin was independent of hypoxia-inducible factor 1 (HIF1), but could be reproduced by the hypoxia mimetic cobalt chloride. Comparison of the effect of other metal ions led us to the unanticipated but remarkable discovery that exogenous Zn^{2+} ions also induced gastrin gene expression by as much as 90-fold in carcinoma cell lines of various origins including gastric (AGS) and colorectal (DLD1, HCT116, HT29, SW480) [5]. As gastrin is a major regulator of gastric acidity, this observation raised the possibility that zinc ions may regulate gastric acidity via induction of gastrin expression. Previously the zinc chelator TPEN has been used to show that acidity within the tubulovesicular and luminal compartments of the gastric gland may be regulated, at least in part, by zinc ions [6, 7]. However, other workers have reported that treatment with Zn^{2+} ions may either increase [8] or reduce gastric acid secretion [9, 10].

To decipher the mechanism responsible for increased gastrin gene expression by Zn^{2+} ions previously we used a traditional reporter gene technology, with transient transfection of plasmids expressing the luciferase gene activated by variants of the human gastrin promoter [5]. However some of these vectors can give rise to aberrant reporter activity because of the presence of cryptic transcription factor binding sites within the plasmid [11], and *in vivo* work requires the creation of stably transfected cells.

An alternative to using a reporter vector is to replace the endogenous gene of interest with a reporter gene at the original locus [12]. Advantages of this approach include the absence of variation in gene copy number or site of integration effects, as well as control over the precise genomic location of the promoter, which will contain its own regulatory elements that may not always be seen with cloned gene promoters in vectors [13]. In the present study gastrin reporter Gast^{luc} SW480

cells were generated by knocking-in a firefly luciferase reporter coding sequence at the translation start site of the endogenous gastrin gene in wild type SW480 colon cancer cells. The aim of these experiments was to use the $Gast^{luc}$ SW480 cells to investigate the role of zinc in gastrin promoter activation *in vitro* and most importantly *in vivo* in mice with established xenografts. The intracellular mediators of the effects of zinc have also been assessed.

Material and Methods:

Cell culture

The human colorectal cancer cell line SW480 was obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Carlsbad, CA, USA) supplemented with 7.5% heat-inactivated fetal bovine serum (FBS, Thermo Scientific, Waltham, MA, USA) and 2 mM of L-Glutamine (Thermo Scientific). Cells were grown at 37°C in a humidified incubator with 95% air and 5% carbon dioxide.

TALEN construction

TALEN sequences were designed using the ZiFiT Targeter software package (http://zifit.partners.org/ZiFiT/). TALENs were constructed using the Joung Lab TAL Effector Engineering Reagents TALEN kit available from ADDgene (www.addgene.org/TALEN/) combined with the REAL (Restriction Enzyme and Ligation) assembly method [14, 15]. Supplemental Fig. 1 details the construction method used.

Targeting plasmid construction

The 3' targeting arm was cloned into the pBSII ks+ plasmid. The luciferase gene was connected to the 5' targeting arm by PCR and subsequently cloned into the 3' arm-pBSII ks+ plasmid. LoxP-puro-loxP was added as a removable selection marker (Supplemental Table 1 lists primers used for cloning).

Generation of Gast^{luc} SW480 cells

TALEN plasmids encoding the 3' and 5' targeting arms were co-transfected with the targeting plasmid at equal concentrations (500 ng each) into SW480 cells (1.5 x 10^6) in 100 µl total volume using the Neon Transfection System (Life Technologies). The electroporated cells were diluted into 15 ml of DMEM and seeded into 150 x 20 mm culture dishes, which were incubated at 37°C for 24 hrs before selection with puromycin (0.5 µg/ml) (Gibco, Life Technologies). Selected clones were screened by PCR for random integration (primers: P1 and P2) or targeted integration (primers: P1 and P3) (Supplemental Table 1) and integration was confirmed by Southern blot using Probe A (Fig. 1). Probe A comprised of 401 base pairs located upstream from exon 1 of the human gastrin gene on chromosome 17:39,867,075-39,867,475 (UCSC genome browser Feb 2009 GRCh37/hg19). The human gastrin gene, including UTRs is located on chromosome 17:

39,868,578-39,872,221. The gastrin gene promoter is located on chromosome 17: 39,867,277-39,868,577.

Gastrin promoter assays

Expression of firefly luciferase was driven by the endogenous gastrin gene promoter. Briefly gastrin wild type $Gast^{wt}$ SW480 and luciferase expressing $Gast^{luc}$ SW480 cells were separately seeded into a 24 well plate (1.0×10^5 cells in 1 ml) and treated with $CoCl_2$ (Sigma-Aldrich, Sydney, Australia) or $ZnCl_2$ (Sigma-Aldrich) in serum free medium for 16 hrs. Luciferase activity was determined using the Dual Luciferase® reporter assay system (Promega). Cells were lysed with 80 µl/well 1X reporter lysis buffer and firefly luciferase activity was measured with a Fluostar Optima plate reader (BMG Labtech, Mornington, Australia). Relative luciferase activity was normalized to the total protein content of each well determined with a Bradford protein assay kit (Bio-Rad, Gladesville, Australia). LY294002 (Sigma-Aldrich) and UO126 (Cell Signaling, Danvers, MA, USA) were used to inhibit the PI3 kinase and MAP kinase pathways, respectively. Cells were incubated with inhibitors for 30 min prior to metal ion treatment (10 µM) for 16 hrs.

Protein quantification

Cells (4×10^5/well) were seeded in 2 ml/well in a 6 well plate and treated with inhibitors or metal ions as described above. Western blots were performed as described previously [5]. The indicated antibodies (Cell Signaling) were diluted 1:2000 in Tris buffered saline tween 20 (TBST)-5% BSA for pMAPK (9101S, p-p42/44 MAPK), tMAPK (9102L, p44/42 MAPK (ERK1/2)), pAkt (4060S, S473 DgE mAb), tAkt (4685, pan 11E7) and 1:20,000 in TBST-5% BSA for GAPDH (2118L).

In vivo bioluminescence

Animal care and experiments followed the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes endorsed by the National Health and Medical Research Council. All procedures were approved by the Animal Ethics Committee (Austin Health, Melbourne, Australia, project number 2014/5203). Xenografts were initiated by subcutaneous injection of 5×10^6 $Gast^{luc}$ SW480 cells in 100 µl Dulbecco`s phosphate buffered saline (DPBS) in the right flank of SCID mice. Tumours were grown to an average volume of 30-50 mm^3 prior to treatment. Mice were then treated with saline (control), 5 mg/kg $ZnCl_2$, 10 mg/kg $ZnCl_2$ or 30 mg/kg $CoCl_2$ on days 2, 8 and 10, and imaged on days 1, 3, 9 and 11. Bioluminescence arising from stimulation of the gastrin promoter was detected using the IVIS® Spectrum for Bioluminescence (Thermofisher Scientific). For imaging, mice were injected i.p. with D-luciferin (150 mg/kg body weight,

ThermoFisher Scientific) prior to induction of anaesthesia with isoflurane. The mice were then transferred onto the imaging platform IVIS and nose cones were placed on the animals to maintain anaesthesia with an isoflurane/oxygen mixture. Images were taken 6 min post luciferin injection for 16 min, with an image every 2 min, to obtain optimal signal data. Data was acquired and analysed with Living Image 4.4 software (Thermofisher Scientific). Bioluminescent counts were converted to radiant units, and expressed as a percent increase compared to untreated control for each treatment group. Our preliminary studies included a dose of 30 mg/kg zinc chloride, however it adversely affected the health of the mice and therefore was not used further.

MTT cell proliferation assay

Cell proliferation was measured using a colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Briefly, 5×10^4 cells/well were plated into two 24 well plates in DMEM with 7.5% FBS. Next day a MTT assay was performed on one plate to determine the relative cell numbers prior to the start of zinc treatment. The values were recorded as the 0 hr absorbance reading. Cells in the second plate were treated with various concentrations of zinc either in the presence or absence of serum and incubated for a further 24 hrs, after which a second MTT assay was performed. Following the solubilization of formazan crystals in acidified isopropanol the absorbance, which is directly proportional to cell numbers, was measured at a wavelength of 570 nm with background subtraction at 620 nm. Data are expressed as a percentage of the 0 hr reading taken just prior to zinc treatment.

Zn^{2+} quantification

Relative zinc concentrations in tissue and serum were determined using a zinc quantification kit (Abcam, Melbourne, Australia) according to the manufacturer's instructions. In brief, tumours (50 mg) were sonicated in 250 µl EDTA-free lysis buffer: 7% TCA (1:1) or 50 µl of 7% TCA was added to 100 µl of serum. Samples were centrifuged and 50 µl of each sample was transferred to a 96 well plate. Zinc reaction mix (100 µl/well) was added and the plate incubated for 10 min prior to reading at 570 nm on the Fluostar Optima Plate Reader (BMG Labtech, Australia).

Intracellular free Zn^{2+} measurements

$Gast^{luc}$ SW480 cells (2.5×10^3 cells in 100 µl/well) were seeded onto black 96 well plates and incubated in 5% CO_2 for 24 hrs at 37°C. $ZnCl_2$ was added and cells were incubated for an additional 0, 2 or 24 hrs. FluoZin-3 (AM, cell permeant, F-24195, Life Technologies) was added to a final concentration of 5 µM (50 µl/well) and the 96 well plates were covered with foil to protect them

from light. Samples were equilibrated for 30 min before the dye was removed and replaced by Hank's balanced salt solution (HBSS) for 15 min. The resulting fluorescence was recorded on a MicroLumi XS luminometer (Harta Instruments, Gaithersburg, MA, USA). The minimum (F_{min}) and maximum (F_{max}) fluorescence was determined by incubating with zinc chelator N,N,N',N'-tetrakis(2-pyridylmethyl)ethane-1,2-diamine (TPEN) (10 μM) or $ZnCl_2$ (500 μM), respectively. The dissociation constant (K_d) of FluoZin-3 for zinc is 15 nM according to the manufacturer's instruction manual. Free zinc (nM) was calculated as:

$$\frac{(F - Fmin)}{(Fmax - F)} \times 15$$

Statistics

Data are presented as the average of the mean ± SEM. One way ANOVA and student's t- test were used to determine significance as appropriate using SigmaStat and graphed using SigmaPlot (Jandel Scientific, San Rafael, CA, USA).

Results

Generation of the knock-in gastrin reporter Gast^{luc} SW480 colon cancer cell line.

A knock-in cell line was constructed using wild type human SW480 colon cancer cells to monitor gastrin expression. The gene targeting strategy is shown in Fig. 1A. The coding region of the firefly luciferase reporter protein was expressed at the start site of translation of the endogenous gastrin gene using specifically designed TALENs (Supplemental Fig. 1). This strategy eliminated chromosomal position effects and copy number concerns in cells stably transfected with a reporter vector. Targeting specificity was determined by PCR and Southern blot. Both PCR using external and internal primers (Fig. 1B) and Southern blot (Fig. 1C) with an external probe produced bands consistent with correct targeting event. The removable loxP-puromycin-loxP cassette contained its own promoter and stop site and was used as a selection marker. The remaining portion of the gastrin gene had no promoter or start site and was out of frame, and therefore not expressed.

Zinc and cobalt induce gastrin promoter activity in the Gast^{luc} SW480 colon cancer cells

The induction of the luciferase reporter gene driven by the endogenous gastrin promoter in the gastrin knock-in SW480 colon cancer cells (Gast^{luc} SW480) was examined by assaying luciferase activity [4]. Consistent with our previous report that the gastrin promoter can be stimulated by the divalent metal ions zinc and cobalt [4, 5], treatment of Gast^{luc} SW480 cells with zinc chloride for 16 hrs increased the relative luciferase activity, which is directly proportional to gastrin promoter activation. Maximal stimulations of 22 ± 3-fold and 68 ± 7-fold were observed following treatment of Gast^{luc} SW480 cells with 50 μM $ZnCl_2$ (Fig. 2A) or 250 μM cobalt chloride (Fig. 2B), respectively. Zinc at 50 μM was approximately 4 times more potent than cobalt chloride at the same dose.

$ZnCl_2$ mediated gastrin promoter activation is dependent on the MAPK pathway

Activation of the gastrin promoter by zinc was dependent on the MAP kinase and PI3 kinase pathways in gastric cancer AGS cells [5]. In order to establish which signalling pathway was involved in the stimulation of gastrin expression by Zn^{2+} ions in colon cancer cells, the gastrin promoter activity was measured after pre-treatment of Gast^{luc} SW480 cells for 30 min with either the PI3K inhibitor LY294002 (10 μM) or the MAPK inhibitor U0126 (10 μM), followed by treatment with either 50 μM $ZnCl_2$ or 250 μM $CoCl_2$ for 16 hrs in the presence of the same inhibitors. As shown in Fig. 3A treatment with the MAPK inhibitor U0126 (10 μM) significantly reduced the zinc- or cobalt-stimulated gastrin promoter activity to 31 ± 9 % and 14 ± 3 %, respectively, compared to cells treated with zinc or cobalt only (100 %). In contrast, the PI3K

inhibitor LY294002 had no effect on cobalt-stimulated gastrin promoter activity, but reduced the zinc-stimulated gastrin promoter activity to only 73 ± 2 % compared to cells treated with zinc 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 $Gast^{luc}$ SW480 cells, the expression of phospho-Akt and phospho-ERK1/2 was measured by Western blotting. Treatment of $Gast^{luc}$ SW480 cells with 50 μM $ZnCl_2$ increased phosphorylation of ERK1/2 (Fig. 3B) in a time-dependent manner, with a significant decrease in the expression of phospho-Akt (Fig. 3C).

Serum reduces the stimulation of gastrin promoter activity by zinc

Many metals including zinc bind to serum albumin [16, 17] and the biological effects of extracellular Zn^{2+} ions are significantly reduced in the presence of serum in cell culture media [18]. Consistent with this observation, the 16 ± 6-fold increase in gastrin promoter activity stimulated by 50 μM $ZnCl_2$ was completely abolished in the presence of 7.5% FBS (Fig. 4A). Interestingly treatment of $Gast^{luc}$ SW480 cells with 100 μM $ZnCl_2$ in serum-free medium resulted in only a 7 ± 3-fold increase in gastrin promoter activity due to significant toxicity. However, the presence of 7.5% FBS not only abolished toxicity induced by 100 μM $ZnCl_2$, but also significantly increased the gastrin promoter activity by 25 ± 3-fold. $ZnCl_2$ at 200 μM was toxic to the $Gast^{luc}$ SW480 cells either in the presence or absence of 7.5% FBS (Fig. 4A). As observed previously [18], serum albumin may provide an important extracellular "zinc buffer" which in turn mitigates zinc toxicity whilst providing safe free zinc concentrations for cellular uptake .

Effect of zinc on cell proliferation

To determine whether exposure of $Gast^{luc}$ SW480 cells to zinc had any effect on proliferation, relative cell numbers were determined by MTT cell proliferation assay following the incubation of gastrin reporter $Gast^{luc}$ SW480 cells with various concentrations of zinc for 24 hrs. As seen in Figure 4B, the relative cell numbers in either serum free or 7.5% FBS -containing medium increased to 217 ± 5 % and 281 ± 8 %, respectively, compared to the numbers at 0 h (100 %). The 143 ± 5 % increase in the relative cell numbers following the treatment of $Gast^{luc}$ SW480 with 50 μM $ZnCl_2$ for 24 hrs in the absence of serum suggests that cells are still able to proliferate in the presence of 50 μM $ZnCl_2$ albeit at a slower rate than untreated cells (217 ± 5 %). This observation suggests that the induction of the gastrin promoter by 50 μM $ZnCl_2$ is independent of cell death induced by heavy metal toxicity. Interestingly only 12 ± 2 % cells survived following the treatment of $Gast^{luc}$ SW480 with 100 μM $ZnCl_2$ for 24 hrs in the absence of serum. However, when serum was included with the zinc treatment the relative cell numbers increased to 176 ± 7 %.

Measurement of free zinc in Gast^{luc} SW480 cells

To understand the relationship between intracellular Zn^{2+} and activation of the gastrin promoter, the increase in intracellular free Zn^{2+} was measured using FluoZin-3 in Gast^{luc} SW480 cells incubated with 50 μM $ZnCl_2$ in culture medium in the presence or absence of 7.5% FBS (Fig. 4C). Incubation of Gast^{luc} SW480 cells for 2 hrs in serum-free medium containing 50 μM $ZnCl_2$ led to a 7 ± 1-fold increase in intracellular free Zn^{2+} concentration compared to untreated cells. In contrast after incubation of Gast^{luc} SW480 cells for 2 hrs in medium containing 7.5% FBS and 50 μM $ZnCl_2$, the increase in intracellular free Zn^{2+} concentration was only 3 ± 1-fold. Surprisingly there was no significant change in the intracellular free Zn^{2+} following the incubation of Gast^{luc} SW480 cells in 50 μM $ZnCl_2$ for 24 hrs either in the presence of absence of serum compared to untreated cells.

Dose-dependent effect of zinc on gastrin promoter activation in vivo

To investigate whether zinc ions induce gastrin promoter activity *in vivo,* Gast^{luc} SW480 cells were established as xenografted tumours in SCID mice. SCID mice bearing Gast^{luc} SW480 tumours were injected intraperitoneally with either saline or $ZnCl_2$ at three different doses (5 mg/kg, 10 mg/kg and 30 mg/kg) or with $CoCl_2$ at 30 mg/kg. Bioluminescence after injection of D-luciferin was then measured on the IVIS as described in the Methods section. Preliminary treatment of mice with 30 mg/kg $ZnCl_2$ was toxic and this treatment was not studied further. As shown in Fig. 5B, there was a 700 ± 140 % increase in gastrin promoter activity following a single injection of 10 mg/kg $ZnCl_2$. In contrast $CoCl_2$ at 30 mg/kg induced a smaller increase of 249 ± 64 % in gastrin promoter activity compared to the saline control. Injecting the mice twice more with either 10 mg/kg $ZnCl_2$ or 30 mg/kg $CoCl_2$ did not result in any further increase in gastrin promoter activity. However, three injections of $ZnCl_2$ at 5 mg/kg did not activate gastrin promoter activity. Gastrin promoter activity in mice treated with $ZnCl_2$ at 10 mg/kg was still present up to 5 days post treatment (Fig. 5C). Relative zinc concentrations were determined in xenografted tumours of Gast^{luc} SW480 cells and in serum 24 hrs post treatment of SCID mice with 10 mg/kg $ZnCl_2$ or saline. As shown in Fig. 5D, there was no significant difference in zinc concentrations in either the xenografted tumours or the serum 24 hrs post injection and this finding is consistent with the *in vitro* data (Fig. 4C).

Discussion

Although gene expression studies using promoter reporter constructs transfected either transiently or stably have been critical for our understanding of physiological and pathophysiological processes, a number of studies have raised concerns regarding their use [11, 19, 20]. Unfortunately in stable cell lines established using these plasmids random genomic integration might not create an accurate read out for the activation of the gastrin gene expression as vector incorporation site and the number of integration event within the genome may results in differential expression of gastrin compared to expression from its endogenous locus. Thus a more precise approach is required. Gene targeting in mammalian cells is constrained by two limitations: the low rate of homologous recombination in the commonly used somatic human cancer cells and the high rate of random (non-targeted) integration of the vector DNA [21]. Furthermore the presence of repetitive sequences as in the human gastrin gene makes targeting difficult. However with the use of genome editing technologies such as TALEN or CRISPR it is now possible to generate knock-out or knock-in mutants of commonly used cancer cells [22]. In this study TALENs have been used to generate a knock-in gastrin reporter $Gast^{luc}$ SW480 colon cancer cell line in which the endogenous human gastrin gene sequence has been replaced by the firefly luciferase coding sequence.

Previously we had shown that treatment of wild type SW480 cells with exogenous zinc induced the expression of gastrin mRNA [5]. In the present study we have confirmed that gastrin promoter activity is induced by both zinc (Fig. 2A) and cobalt (Fig. 2B) in $Gast^{luc}$ SW480 colon cancer cells. Induction of the gastrin gene at the mRNA and promoter level was abrogated to a similar extent by inhibitors of both MAPK (U0126) and PI3K (LY294002) pathways in AGS gastric cancer cells [5]. However in $Gast^{luc}$ SW480 colon cancer cells inhibition of MAPK pathway led to a more potent inhibition of zinc induced gastrin promoter activation as compared to the inhibition of PI3K pathway. The reduced effect of the PI3K inhibitor is consistent with the observation that zinc ions were unable to significantly induce phosphorylation of Akt (a downstream target of PI3K pathway) in these cells.

The finding that serum blocked the zinc-induced activation of the gastrin promoter is consistent with the hypothesis that the increase in intracellular free zinc ions is indispensable for activation of gastrin promoter activity. The observation that the intracellular increase in free zinc ions following the treatment of $Gast^{luc}$ SW480 colon cancer cells with exogenous zinc ions was significantly lower in the presence of serum (Fig. 4C) is in agreement with a previously published study by Haase and co-workers in which the uptake of exogenous zinc ions was reduced in the

presence of 1% FBS and was completely abrogated in the presence of 10% FBS [18]. Interestingly, in our study the increase in intracellular free zinc ions was only transient as the 7-fold difference in intracellular free zinc ions observed between zinc-treated and untreated cells at 2 hours completely disappeared by 24 hours (Fig. 4C), although the activation of gastrin promoter was still apparent 24 hours following the addition of zinc ions (Fig. 5). The transient nature of the increase in free zinc ions may be due to an increased expression of zinc transporters, leading to an increase in efflux of zinc ions [23, 24].

In vitro studies have now convincingly confirmed that serum reduces the zinc-mediated biological effects such as the increase in phosphorylation of p38 [18] or activation of the gastrin promoter (Fig. 4A) by decreasing the zinc uptake. These studies have raised the possibility that blood proteins such as serum albumin may decrease zinc uptake and in turn reduce the zinc-mediated biological effects *in vivo.* Low doses of zinc may not reach the biological threshold and, at higher doses, zinc may become ineffective due to zinc toxicity (Fig. 4A, 4B). To determine whether there was a biologically relevant relationship between zinc dose and promoter response *in vivo*, xenografts of the gastrin reporter $Gast^{luc}$ SW480 cells were established in SCID mice. A single i.p. injection of zinc ions (10 mg/kg) produced a 7 fold increase in promoter activity while $CoCl_2$ at 30 mg/kg resulted in a smaller 2.5 fold increase as measured by whole animal bioluminescence imaging. Both the timing and amount of zinc ions appear to be critical as three injections of zinc at 5 mg/kg, cumulating to a total of 15 mg/kg, failed to elicit activation of the gastrin promoter, while one injection of zinc at 10 mg/kg elicited maximal gastrin promoter activity after 24 hours. These observations highlight the fact that the individual dose of zinc ions, as well as the time at which the end effect (biological activity) is measured, are both very important factors to consider when investigating zinc-dependent effects *in vivo*.

Even though there was no significant difference in relative zinc concentrations in either the xenografted tumour or the serum 24 hrs post saline or zinc injection, the stimulation of gastrin promoter activity in mice treated with 10 mg/kg $ZnCl_2$ was still present up to 5 days after zinc treatment, even though the luciferase protein is stable for only 3-4 hours (Promega). Therefore, while the initial activation of the gastrin promoter is directly dependent on an increase in intracellular free zinc ions, in the longer term zinc ions may also activate other growth factors or proteins which then in turn sustain the longer term activation of gastrin promoter activity in an autocrine positive feed forward mechanism [25, 26].

Taken together the current study using colonic cells SW480 and our previously published study [5] using gastric AGS cells demonstrate that the induction of gastrin gene expression by Zn^{2+} ions is both a reproducible and a general phenomenon. However, the relationship between Zn^{2+} ions and gastric acidity is unclear, as Zn^{2+} ions have been reported to either increase [8] or reduce gastric acid secretion [9, 10]. Using the zinc chelator TPEN it has been shown that acidity within the tubulovesicular and luminal compartments of the gastric gland may be regulated, at least in part, by zinc ions [6, 7]. Our observation that treatment with Zn^{2+} increases both intracellular and secreted gastrin in the gastric carcinoma cell line AGS [5], if replicated in antral G cells *in vivo*, would be expected to result in a CCK2R-mediated increase in gastric acid secretion. However, Kirchhoff and co-workers have shown direct inhibitory effects of Zn^{2+} on gastric acid secretion [10] and therefore further work will be required to establish the relative importance of Zn^{2+}-induced gastrin expression and of the direct inhibitory effects of Zn^{2+} on the parietal cell.

A key mechanism of a cell's ability to adapt to low oxygen (hypoxia) involves a set of hypoxia-inducible transcription factors (HIFs) that induce more than 200 functionally diverse genes involved in cell survival [27]. $CoCl_2$ is often used as a hypoxia-mimicking agent, by virtue of its ability to induce the expression of HIFs including HIF1α and HIF2α. However, chemical hypoxia induced by $CoCl_2$ appears to differ from true hypoxia [28] as, although both Co^{2+} and hypoxia (1% O_2) induce HIF1α and HIF2α, the overlap in gene expression profiles is very limited [28, 29]. Interestingly our previous studies have shown that the induction of gastrin expression by either Co^{2+} or Zn^{2+} ions is independent of either HIF1α or HIF2α induction [4, 5] and is mediated by an as yet unidentified E-box binding transcription factor. These findings taken together suggest that the effect of $CoCl_2$ on gene expression may be mediated at least in part by intracellular zinc ions. A critical implication of these results is that data obtained using $CoCl_2$ may need to be re-examined to determine if results previously attributed to HIFs could, in part, be due to Zn^{2+}.

In summary our study using luciferase reporter $Gast^{luc}$ SW480 colon cancer cells have shown that both zinc and cobalt ions activate the endogenous gastrin gene promoter *in vitro* and *in vivo* and that activation is dependent on the MAPK but not the PI3K pathway. The biological roles of zinc are of similar significance to those of iron and calcium [30]. Zinc-initiated changes in gene expression have been implicated in the etiology of a number of diseases including diabetes, Alzheimer's and cancer [31], and a better understanding of the zinc-gastrin axis is highly relevant in this context.

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Figure Legends

Figure 1. Targeting and screening for a gastrin promoter-linked luciferase. (A) Targeting plan for incorporating the firefly luciferase gene at the gastrin translation start site at the 5' end of exon 2 to be translated by the gastrin promoter. Black boxes represent gastrin exons and hatched boxes represent repetitive DNA that makes targeting the human gastrin gene more difficult. TALENs (not to scale) were designed to cleave the gastrin gene at the start of exon 2, promoting DNA repair with the targeting vector. Targeted clones were generated with puromycin selection. Luciferase has a stop site to prevent any random tags being added. The removable loxP-puro-loxP cassette contains its own promoter and stop site. The remaining portion of the gastrin gene has no promoter and is out of frame, and will therefore not be expressed. (B) PCR screening was used to determine random, R (828 bp, primers P1 and P2), or targeted, T (1526 bp, primers P1 and P3), integration of the vector using the primers P1-P3 (arrows, not to scale) in panel A. PCR products were only derived from the cDNA of luciferase ($Gast^{luc}$) clones and not from the cDNA of wild type ($Gast^{wt}$) cells. (C) Southern blot of genomic DNA restriction digested with XbaI from $Gast^{wt}$ (wt) and $Gast^{luc}$ (luc) cells. Probe A (panel A) used in combination with XbaI genomic DNA digestion detects a single band of 14.6 kb from wild type $Gast^{wt}$ SW480 cells and a second band of 7.6 kb from $Gast^{luc}$ targeted SW480 cells.

Figure 2. Activation of the endogenous gastrin promoter. $Gast^{luc}$ SW480 cells that express luciferase under the control of the endogenous gastrin promoter were stimulated with the indicated concentrations of (A) $ZnCl_2$ or (B) $CoCl_2$ for 16 hrs. Both treatments resulted in concentration-dependent activation of the gastrin promoter, shown as the fold increase in luciferase activity normalised to that of untreated cells (0 μM $ZnCl_2$ or $CoCl_2$). The average $\pm$ SEM are shown, $n \geq 3$ independent experiments with multiple replicates; ***, $p < 0.001$.

Figure 3. Gastrin promoter activation by $ZnCl_2$ is dependent on the MAPK pathway.
(A) Gastrin promoter activity was measured in $Gast^{luc}$ SW480 colon cancer cells following treatment with 50 μM $ZnCl_2$ for 24 hrs in the presence of either a PI3K inhibitor (10 μM LY294002) or a MAPK inhibitor (10 μM U0126). $ZnCl_2$ and $CoCl_2$ stimulated the gastrin promoter activity by 22 ± 3-fold and 68 ± 7-fold, respectively, compared with untreated control. Phosphorylation of ERK1/2 (B), a mediator of the MAPK pathway, and of Akt (C), a mediator of the PI3K pathway, was measured by Western blot as described in Materials and Methods following the treatment of $Gast^{luc}$ SW480 cells with 50 μM $ZnCl_2$ for the times indicated. Band densities were

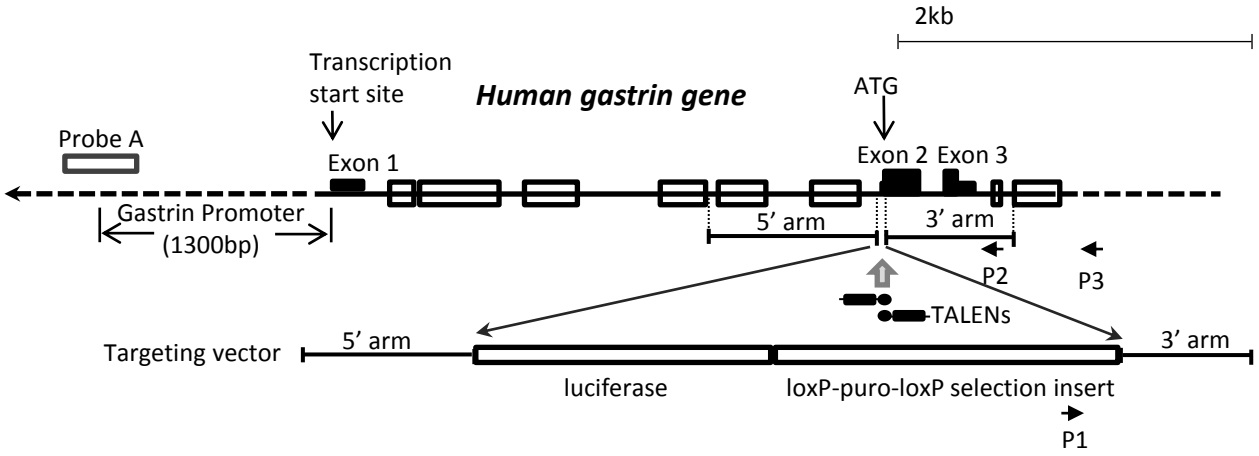
determined and are presented as the ratio of densities of phosphorylated to total protein. The average ± SEM are shown, n ≥ 3 independent experiments with multiple replicates; *, $p < 0.05$; ***, $p < 0.001$ compared to control (no inhibitor).

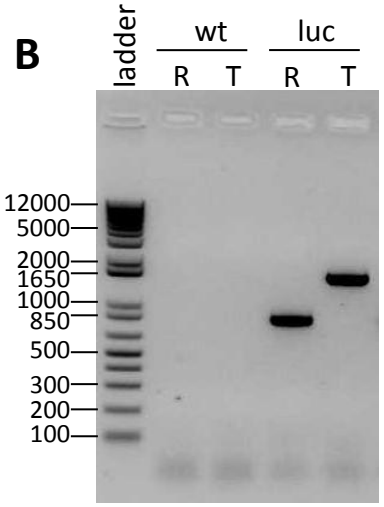
Figure 4. Impact of serum on zinc-induced gastrin promoter activation and intracellular increases in Zn^{2+}. (A) Gastrin promoter activity was measured in $Gast^{luc}$ SW480 colon cancer cells following treatment with 50, 100 or 200 μM $ZnCl_2$ in culture medium with or without fetal bovine serum (FBS). (B) Relative cell numbers were determined using the MTT assay following the incubation of $Gast^{luc}$ SW480 with various concentrations of zinc either in the presence or absence of serum for 24 hrs. Following the solubilization of formazan crystals in acidified isopropanol the absorbance, which is directly proportional to cell numbers, was measured at a wavelength of 570 nm with background subtraction at 620 nm. Data are expressed as a percentage of the 0 hr reading taken just prior to zinc treatment. All data are shown as means ± SEM of n ≥ 3 independent experiments. (C) Intracellular free zinc (Zn^{2+}) was determined using the Fluozin3 fluorescent probe in cells treated with 50 μM $ZnCl_2$ in culture medium with or without fetal bovine serum (FBS). Fluorescence was measured and the free Zn^{2+} concentration (nM) was calculated as described in Material and Methods. Minimum (Fmin) and maximal (Fmax) fluorescence was determined by addition of 10 μM TPEN and 500 μM $ZnCl_2$ respectively. The average ± SEM are shown, n ≥ 3 independent experiments with multiple replicates; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 5. $ZnCl_2$ stimulates the gastrin promoter *in vivo*. SCID mice bearing $Gast^{luc}$ SW480 xenografted tumours were given intraperitoneal injections of $ZnCl_2$ (5 or 10 mg/kg) or $CoCl_2$ (30 mg/kg) on days 2, 8 and 10 and imaged for bioluminescence the following day. Animals received no treatments prior to imaging on day 1. (A) Representative images of bioluminescence observed in mice before and after the first injection. (B) Relative luciferase activity was determined as a percent increase from the untreated control for each treatment group. (C) Luciferase levels were stable up to 5 days post zinc stimulation, as there was no decrease in activity after the last injection on day 10. The average ± SEM are shown, n ≥ 5 mice per treatment; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, compared to untreated on day 1 in the same treatment group, set to 100%. #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$, compared to the control group on the same day. Mice treated with $ZnCl_2$ (10 mg/kg) had significantly more bioluminescence than mice treated with 5 mg/kg $ZnCl_2$ or 30 mg/kg $CoCl_2$ on days 3 and 9, and more bioluminescence than mice treated with 5 mg/kg $ZnCl_2$ on day 11. (D) Relative zinc concentrations were determined in tumour and serum 24 hrs after injection with

vehicle (control) or 10 mg/kg $ZnCl_2$ using an Abcam zinc quantification kit (Ab102507). No significant difference was observed between control and zinc-treated mice. The average $\pm$ SEM are shown, $n \geq 5$ mice per treatment.

Figure 1





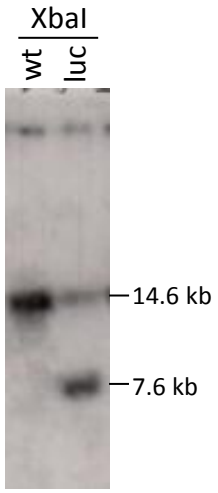


Figure 2

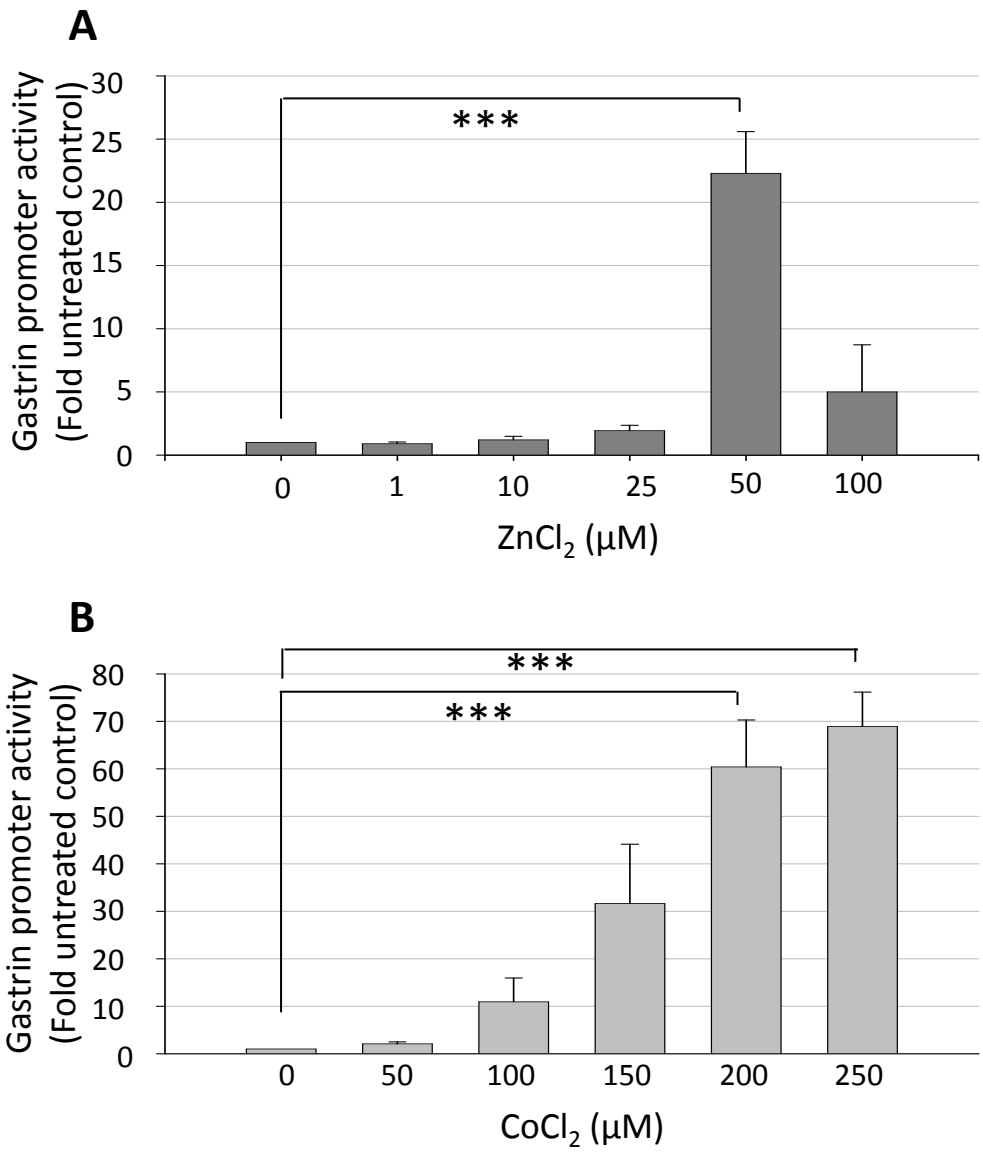
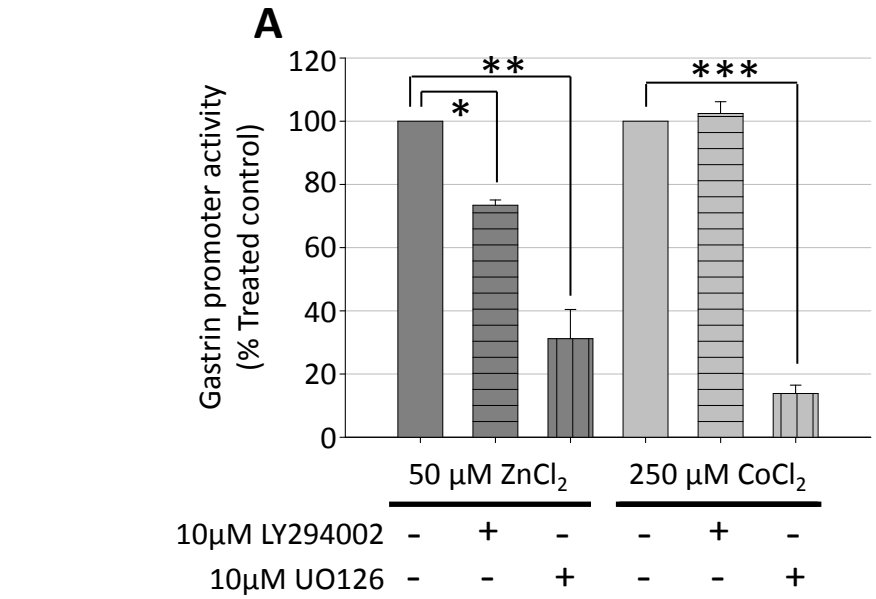
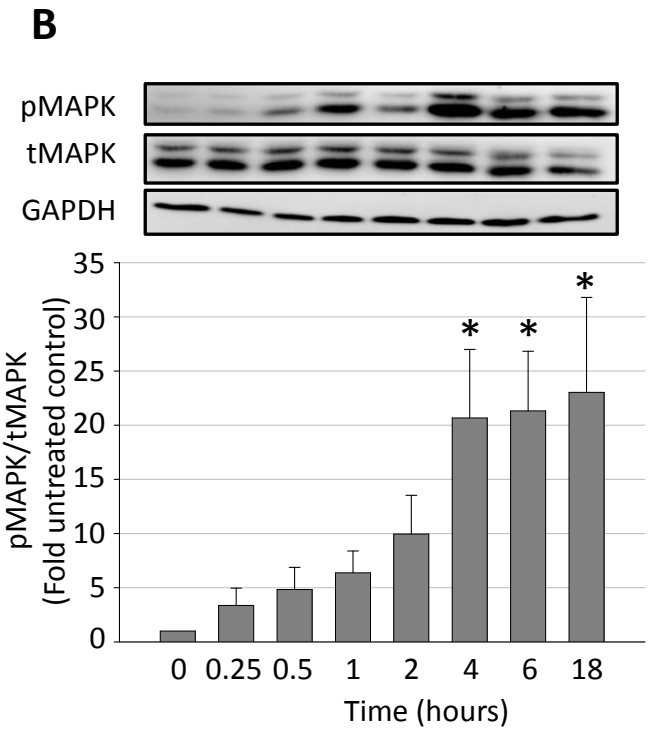


Figure 3





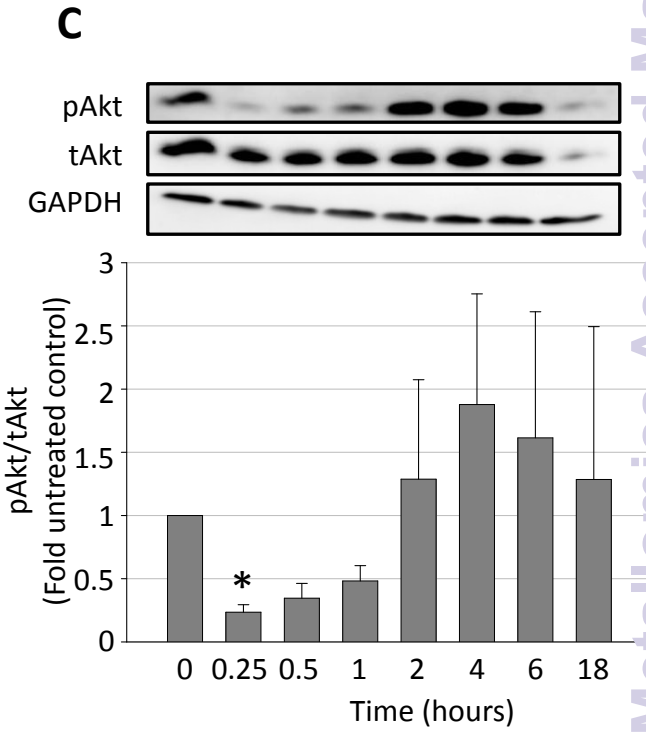


Figure 4

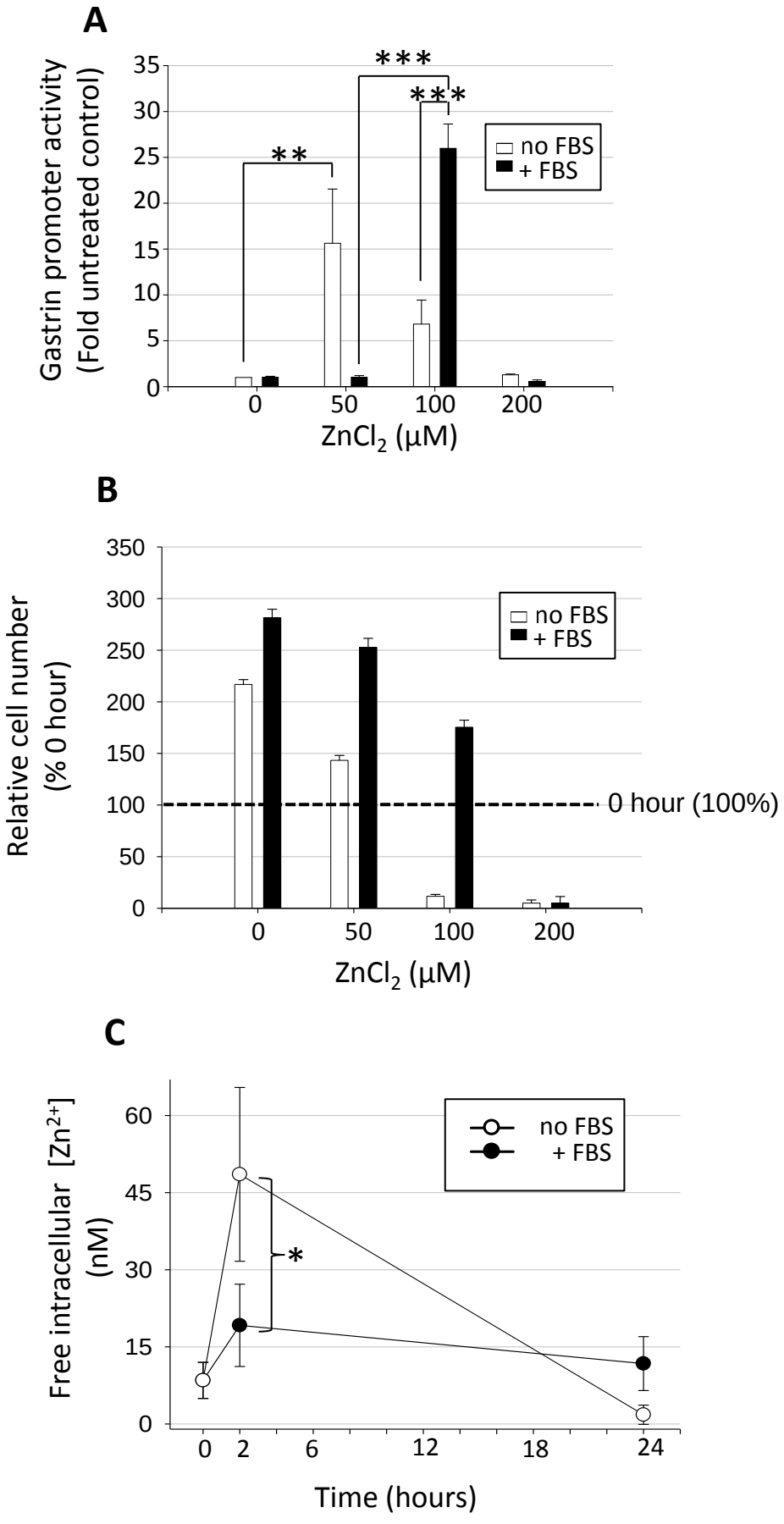


Figure 5

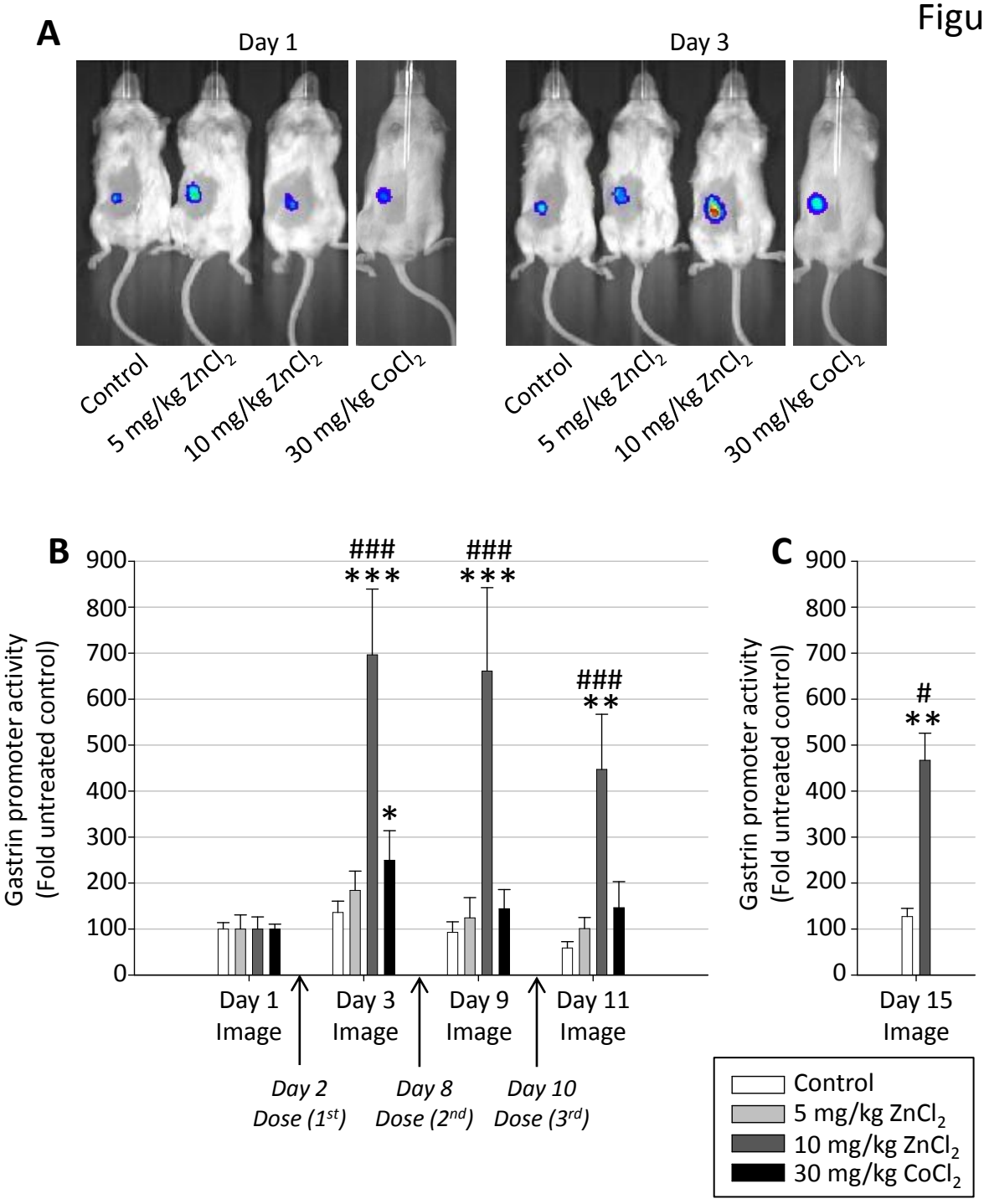


Figure 5D

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