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Manuscript Draft

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Title: Glucagon-like petide-2 acts on colon cancer myofibroblasts to stimulate proliferation, migration and invasion of both myofibroblasts and cancer cells via the IGF pathway

Article Type: Research Paper

Keywords: glucagon like peptide-2;
insulin like growth factor;
myofibroblast;

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Corresponding Author's Institution: University of Liverpool

First Author: Marianne Shawe-Taylor

Order of Authors: Marianne Shawe-Taylor; J.Dinesh Kumar; Whitney Holden; Steven Dodd; Akos Varga; Olivier Giger; Andrea Varro; Graham J. Dockray

Response to Reviewers: Reviewer #1:

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this study. If no big differences, perhaps a sentence indicating that this was the case?

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We thank the reviewer for this suggestion and have edited this section (p 15, l 375).

Cover Letter

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The editor, *Peptides*

21 Dec 2016

Dear Karl,

We have pleasure in submitting the attached manuscript (**Glucagon-like petide-2 acts on colon cancer myofibroblasts to stimulate proliferation and migration of both myofibroblasts and cancer cells via the IGF pathway**) for publication in *Peptides.* The work has not been published previously and is not under consideration by another journal. All the authors have approved the submitted version of the manuscript.

In spite of considerable interest in the action of GLP-2 on intestinal growth, there has been rather little work done on its possible role in cancer and in particular on the way that GLP-2 might modify the cancer microenvironment by acting on myofibroblasts. As far as we know nobody has previously studied the action of GLP-2 on cancer derived myofibroblasts.

The most obviously distinguished workers in this area are Dan Drucker, Jens Holst and Patricia Brubaker; we think it unlikely that Dan or Jens would have sufficient time to act as reviewers (although we would be happy if they did): we have therefore recommended Patricia as a potential reviewer together with Kay Lund (who is distinguished for her work on the intestinal IGF system and has also worked on GLP-2 and fibroblasts), together with Yash Mahida (who is distinguished for his pioneering studies on gut myofibroblasts).

Best wishes as ever

Graham J Dockray FRCP (Hon), FMedSci, FRS

A member of the
Russell Group

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We have pleasure in submitting a revised version of the attached manuscript (**Glucagon-like petide-2 acts on colon cancer myofibroblasts to stimulate proliferation, migration and invasion of both myofibroblasts and cancer cells via the IGF pathway**) for publication in *Peptides.*

We would like to thank the reviewers for their constructive comments. We have revised the manuscript to deal with most of the these comments, although there are a few instances where we have dealt with issues in our point-by-point response.

Best wishes as ever

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Highlights

- GLP-2 stimulates proliferation and invasion of primary colonic cancer-derived myofibroblasts to a greater extent than those from adjacent tissue; GLP-2 conditioned medium from myofibroblasts stimulated proliferation, migration and invasion of intestinal epithelial cells.
- An inhibitor of IGF receptor signalling blocks the effect of GLP-2 on colonic myofibroblasts.
- GLP-2 increases IGF-1 and -2 transcript abundance in myofibroblasts and stimulates degradation of IGF binding proteins in myofibroblasts medium, compatible with increased bioavailability of IGF.
- GLP-2 may influence the cancer microenvironment via actions on stromal cells such as myofibroblasts.

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*Manuscript
Click here to download Manuscript: GLP2~12~20~16FINAL.doc
Click here to view linked References

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Department of Cellular and Molecular Physiology, Institute of Translational Medicine, University of Liverpool, Liverpool UK.

Short title: GLP-2 actions on colonic myofibroblasts

Key words: GLP-2, IGF, IGFBP, myofibroblast migration, proliferation.

Address correspondence to:

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Abbreviations: ATMs, adjacent tissue myofibroblasts; CAMs, cancer associated myofibroblasts; GLP-2, glucagon-like peptide 2; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; MMP, matrix metalloproteinase.

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Highlights

- GLP-2 stimulates the migration and proliferation of colonic cancer myofibroblasts as well as intestinal epithelial cells.
- An inhibitor of IGF receptor signalling blocks the effect of GLP-2 on colonic myofibroblasts.
- GLP-2 increases IGF-1 and -2 transcript abundance in myofibroblasts and stimulates degradation of IGF binding proteins in myofibroblasts medium, leading to increased bioavailability of IGF.
- GLP-2 may influence the cancer microenvironment via actions on stromal cells such as myofibroblasts.

Abstract

Glucagon-like peptide (GLP)-2 stimulates intestinal epithelial proliferation by acting, in part, via IGF release from sub-epithelial myofibroblasts. The response of myofibroblasts to GLP-2 remains incompletely understood. We studied the action of GLP-2 on myofibroblasts from colon cancer and adjacent tissue, and the effects of conditioned medium from these cells on epithelial cell migration and proliferation. In response to GLP-2, myofibroblasts from cancer and adjacent tissue exhibited increased proliferation, migration and invasion; these responses were inhibited by the IGF receptor inhibitor, AG1024. Conditioned medium from GLP-2 treated myofibroblasts increased proliferation, migration and invasion of SW480, HT29, LoVo epithelial cells and these responses were inhibited by AG1024; GLP-2 alone had no effect on these cells. In addition, when myofibroblasts and epithelial cells were co-cultured in Ibidi chambers there was mutual stimulation of migration in response to GLP-2. The latter increased both IGF-1 and IGF-2 transcript abundance in myofibroblasts. Moreover, a number of IGF binding proteins (IGFBP-4, -5, -7) were identified in myofibroblast medium and in the presence of GLP-2 there was increased abundance of the cleavage products of these proteins suggesting activation of a degradation mechanism increasing IGF bioavailability. The data suggest that GLP-2 stimulates cancer myofibroblast proliferation and migration; GLP-2 acts indirectly on epithelial cells partly via increased IGF expression in myofibroblasts and partly by increased bioavailability through degradation of IGFBPs.

Introduction

Mucosal morphology throughout the gastrointestinal tract is determined by interactions between epithelial cells and underlying stromal cells which release a variety of growth factors [26]. These interactions are also a feature of tumour growth where it is now clear that stromal cells provide a supportive and stimulatory niche for cancer cells [11, 27]. A key stromal cell type is the myofibroblast which physiologically lies in close proximity to the basal membrane of epithelial cells while in cancer these cells are an important component of the stroma that may constitute a high proportion of tumour volume. There is now clear evidence that cancer-associated myofibroblasts (CAMs, often considered to be a subset of cancer-associated fibroblasts) are functionally distinct from myofibroblasts recovered from tissue adjacent to the tumour (ATMs), and from normal tissue [13, 14].

A range of hormonal signals from epithelial enteroendocrine cells (EECs) influence growth of the gastrointestinal tract, including gastrin, cholecystokinin and glucagon-like peptide (GLP)-2. The latter is generated from the glucagon precursor by post-translational cleavage; there are different patterns of processing in pancreatic alpha-cells and L-cells of the ileum and colon. In particular, the main products in L-cells are GLP-2, GLP-1 (which is an insulin secretagogue) and oxyntomodulin (which is a C-terminally extended variant of glucagon). A link between glucagon gene expression and intestinal hypertrophy has been known for several decades, and it is now recognised that GLP-2 is a crucial mediator [7]. Interestingly, GLP-2 receptors are not expressed on intestinal epithelial cells, with the possible exception of some EECs, and as a consequence GLP-2 does not have direct effects on intestinal epithelial cell proliferation. Instead there is evidence that GLP-2 acts indirectly via sub-epithelial cells notably myofibroblasts and neurons [2, 4, 8, 25]. Thus, the evidence suggests that GLP-2 releases growth factors such as IGF, KGF and VEGF-A which in turn act on epithelial cells to stimulate cell proliferation [5, 9, 25].

Gene array data indicate that a wide variety of gastrointestinal myofibroblasts produce both IGF-1 and IGF-2 as well as a number of IGF binding proteins (IGFBPs) including -3, -4, -5, -6 and -7 [3]. The IGFBPs act to sequester extracellular IGF which can be liberated by IGFBP cleavage, for example by matrix metalloproteinase (MMP)-7 produced by epithelial cells [12]. In the upper gastrointestinal tract, the IGFs act on both epithelial cells and the myofibroblasts themselves to stimulate proliferation as well as migration and invasion. The response of intestinal myofibroblasts to GLP-2 (aside from release of IGF) remains uncertain. While there have been studies using a CCD18 myofibroblast cell line, which is derived from normal intestine of a human infant, the possible effects of GLP-2 on cancer myofibroblasts are unclear. In the present study we have examined the hypothesis that GLP-2 acts on myofibroblasts from colon cancer and adjacent tissue to influence colon cancer cells lines, and have examined the role of IGF in mediating these effects.

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Materials and Methods

Cells. Colon cancer cells (HT29, Lovo, SW480) and CCD18 colonic myofibroblasts were obtained from American type culture collection (VA, US). Colonic cancer derived myofibroblasts (CAMs) had been generated from tumour and adjacent tissue (ATMs) of a patient with colon cancer using previously described methods; they have been shown to be positive for α-smooth muscle actin and vimentin and negative for desmin [19]. Colon cancer cells and myofibroblasts were cultured as previously described [14].

Conditioned media. Myofibroblasts (1.5 x 10^6 cells) were plated in T-75 falcon flasks and maintained at 37°C in a 5% v/v CO_2 atmosphere for 24h in full media (FM). Cultures were then washed 3 times with sterile PBS and incubated in 15ml serum free (SF) media with or without GLP-2 (AnaSpec, Freemont, CA, USA) for 24h. Conditioned medium (CM) was collected, centrifuged (7 min, 800 x g, 4°C) and aliquots were stored at -80°C until further use.

EdU incorporation and cell *Counting Kit-8* assays

For EdU incorporation assays, colonic myofibroblasts and colon cancer cells (2.5x10^4 per well) were seeded and incubated overnight in FM followed by serum starvation for 48 h. Cells then were treated with GLP-2 or CM as appropriate for 24h. Proliferation was assessed by incorporation of 5-ethynyl-2'-deoxyuridine (EdU, 10 μM, 16h) and processing of samples using Click-iT (Invitrogen, Paisley, UK) and Alexa 568-azide. EdU positive cells were visualised on a Zeiss AxioCam HRM fluorescence microscope (Carl Zeiss, Welwyn Garden City, UK) on a 40x objective lens counting the total number of cell nuclei in 10 different fields using DAPI (Vector Laboratories, Peterborough, UK) (blue) and EdU positive nuclei.

Cell Counting Kit-8 (CeCo)(Dojindo Laboratories, Munich, Germany) assays on colonic myofibroblasts and cancer cells (8x10^3 per well) were performed in 96 well plates on cells

incubated overnight in FM followed by incubation with GLP-2 or CM medium in phenol red free SF medium for 72h. On the last day 10µl CeCo reagent was added to the wells and incubated for 2-4h as optimised for each cell type. Readings were taken at 450nm using a GenioPlus plate reader (Tecan, Zurich, Switzerland).

Cell migration and invasion assays. Transwell migration and invasion assays were performed using BD inserts (Corning, New York, USA) as previous described (2.5 x 10^4 cells per insert) [31]. GLP-2 or CM were added in the lower well together with AG1024 (Calbiochem, Darmstadt, Germany) as appropriate. Ibidi chamber (Ibidi GmbH, Martinsried, Germany) migration assays were performed in 24 well plates. Ibidi culture inserts were placed at the bottom of the wells followed by seeding of 10^5 cancer cells and $5x10^4$ myofibroblasts on separate sides of the insert. After 24h the insert was removed, leaving a 500µm wound; cells were then treated with GLP-2 up to 42h and images taken with a Hamamatsu Camera (Hamamatsu Photonics, Hamamatsu City, Japan) at 0, 6, 24, 32, 42h using a Leica DMIRE2 microscope on a heated stage, humidified chamber (Solent Scientific, Portsmouth, UK); cells in the wound were counted in 5-8 fields per insert.

qPCR. RNA was extracted in 1.25 ml Tri-Reagent (Sigma, Dorset, UK) according to the manufacturer's instructions from control and GLP-2 treated colonic myofibroblasts. RNA pellets were re-suspended in 50 µl of nuclease free water and 4 µg of RNA reverse transcribed with avian myeloblastosis virus reverse transcriptase and oligo-dT primers (Promega). Real time PCR was carried out using an ABI7500 platform (Applied Biosystems, Warrington, UK) using TaqMan primer/probe sets (human IGF-1, IGF-2, GAPDH), Precision Plus 2x real time PCR master mix (Primer Design, Southampton, UK) and 5'-FAM, 3'-TAMRA double dye probes (Eurogentec, Southampton, UK). All values were standardized to GAPDH. Assays included a no template control (NTC), and a standard curve as previously

described [22]. Primers and probes were designed using Primer Express v3.0 (Applied Biosystems) and were purchased from Eurogentec (Seraing, Belgium). Probes for detection of human GAPDH, IGF-1 and IGF-2 cDNA were intron-spanning and were: GAPDH: 5'-GCT CCT CCT GTT CGA CAG TCA-3'(forward), 5'-ACC TTC CCC ATG GTG TCT GA-3' (reverse), 5'-CGT CGC CAG CCG AGC CAC A-3' (probe); IGF-1: 5'-TGT ATT GCG CAC CCC TCA A-3' (forward), 5'-CT CCC TCT ACT TGC GTT CTT CA-3' (reverse), 5'-ACA TGC CCA AGA CCC AGA AGG AAG TAC A-3' (probe); IGF-2, 5'-CCG TGC TTC CGG ACA ACT T-3' (forward), 5'-GGA CTG CTT CCA GGT GTC ATA TT-3' (reverse), 5'-CCC AGA TAC CCC GTG GGC AAG TTC-3' (probe).

Western blotting. Cell extracts were prepared in RIPA buffer and media samples were concentrated with StrataClean resins (Agilent Technologies Ltd, Santa Clara, CA, USA) and processed for Western blotting as previously described [22] using antibodies to IGFBP-3, 4, 5 and -7, MMP-1, -2, -3 and -10 (R&D Systems) and GAPDH (Biodesign, Maine, USA).

Statistics. Results were calculated as mean ± standard error of means (SEM). Student t-test and ANOVA were performed on the data as appropriate with significance at $p<0.05$ using Systat Software Inc. (London, UK) unless otherwise stated.

Results

GLP-2 stimulates myofibroblast proliferation. In initial studies we showed that GLP-2 produced a dose-related increase in EdU incorporation into CCD18 myofibroblasts (Fig 1A,B). We then showed GLP-2 also stimulated EdU incorporation by both colonic CAMs and ATMs although basal and stimulated incorporation was greater in the CAMs (Fig 1C). In a CeCo assay of cell growth, GLP-2 also stimulated both CAM and ATM growth (Fig 1D).

GLP-2 stimulates myofibroblast migration and invasion. We then examined the actions of GLP-2 on CAM and ATM migration and invasion using Boyden chambers (Fig 2A,B). In both cell types, GLP-2 at a concentration that stimulated proliferation also stimulated migration and these responses were abolished by the inhibitor of IGF receptor tyrosine kinase, AG1024. Similarly there was IGF-dependent stimulation of invasion although the CAM response was significantly greater than that of ATMs.

Myofibroblast conditioned medium stimulates epithelial cell proliferation. There was no change in EdU incorporation (Fig 3A) by two of the epithelial cell lines used in the present study (SW480 and LoVo) in response to GLP-2 applied directly to the cells; there was a small effect on HT29 cells (Fig 3B). In the case of SW480 cells there was a small increase in EdU incorporation in response to CAM CM that was enhanced by pre-treatment of myofibroblasts with GLP-2; there were greater increases in EdU incorporation by HT29 and LoVo cells in response to CAM CM, and in both cases there were small further increases using CM from GLP-2-treated myofibroblasts. In all three intestinal cell lines, the responses to control CM and to GLP-2 CM were suppressed by AG1024 (Fig 3C). In CeCo assays of cell growth, there were robust responses by all three intestinal epithelial lines to control myofibroblast CM and small but significantly increases in response to GLP-2 pretreatment of myofibroblasts (Fig 3D).

Myofibroblast conditioned medium stimulates epithelial cell migration and invasion. When GLP-2 was applied directly to epithelial cells (SW480, HT29, LoVo) there was no effect on migration or invasion in Boyden chambers (Fig 4A). However, there was strong stimulation of migration of all three cell lines in response to control CAM CM and there were small but significant further increases in response to CM from GLP-2 treated myofibroblasts (Fig 4B). In each case the response was inhibited by AG1024. There were relatively modest increases in invasion of HT29 and LoVo cells in response to control myofibroblast CM but the response was strongly enhanced by GLP-2-treated CM; all responses were inhibited by AG1024 (Fig 4C).

GLP-2 stimulates migration in co-cultures of epithelial cells and myofibroblasts. In view of the potential for cross-talk between epithelial cells and myofibroblasts we then asked whether the response to GLP-2 was preserved when these cells were co-cultured. For this purpose we used Ibidi chambers with myofibroblasts in one chamber and epithelial cells in the other so that subsequent removal of the insert yielded cultures of the two cells separated by 500 μm (Fig 5A). Over a period of 42 h after removing the insert there was progressive migration of both cell types toward each other (Fig 5A). The numbers of migrating epithelial cells were roughly 10 times higher than those of myofibroblasts. Nevertheless at each time point examined from 6 – 42 h the presence of GLP-2 increased the migration of each of the epithelial cell lines and of the co-cultured CAMs (Fig 5B).

GLP-2 increases IGF-1 and IGF-2 transcript abundance. In view of the evidence that GLP-2 might act via IGF we first examined microarray data from myofibroblasts which indicated that both IGF-1 and IG-2 were expressed in colonic CAMs and ATMs; the relative abundance of IGF-1 in colonic myofibroblasts was higher compared to IGF-2, while in gastric myofibroblasts IGF-2 was dominant [3]. We then used qPCR for both IGF-1 and IGF-2 to assess responses to GLP-2. At a concentration of GLP-2 (10 nM) that increased

myofibroblast migration and proliferation, there was 1.8 ± 0.2 fold higher IGF-1 transcript abundance and 1.5 ± 0.2 fold higher IGF-2 transcript abundance.

GLP-2 promotes IGFBP degradation in myofibroblasts medium. Since there is evidence that GLP-2 might act via IGFBP-4 [1] we examined by Western blot the profile of IGFBP-4 and also of IGFBP-3, -5 and -7 in CAMs treated with GLP-2. In control media there were clear bands corresponding to intact IGFBP-4, -5 and -7 (Fig 6A), while IGFBP-3 was undetectable (not shown). There were also minor bands corresponding to degradation products of IGFBP4 (15 kD) and IGFBP-5 (10-15 kD). In the presence of GLP-2 the bands corresponding to intact IGFBP-4, and -5 were maintained but there was increased abundance of the degradation products. In the presence of an inhibitor of MMP activity (GM6001, 10 μM) the action of GLP-2 in promoting degradation of IGFBP4 and -5 was inhibited (Fig 6A). There was also an increase in IGFBP-4 and -5 in cell extracts in response to GLP-2 compatible with increased expression (Fig 6B). Multiple proMMPs are secreted by myofibroblasts (MMP-1, -2, -3 and -10) [13]. Of these, MMP-1 was increased in abundance in GLP-2 treated cells and media (Fig 6C,D) although there was little or no change in MMP-2, -3 and -10.

Discussion

The present study provides evidence that GLP-2 acts on cancer-derived myofibroblasts to stimulate their proliferation, migration and invasion. The latter effects are blocked by an inhibitor of IGF1 receptor tyrosine kinase and since there is an increase in IGF-1 and -2 transcript abundance as well as increased degradation of IGFBP-4 and -5, the data support the idea that the actions of GLP-2 on myofibroblasts are at least partly mediated by IGF. Consistent with previous findings, GLP-2 had little or no effect on three intestinal epithelial cell lines, but conditioned medium from CAMs stimulated proliferation, migration and invasion of these cells, and to varying degrees GLP-2 treatment of myofibroblasts enhanced the responses in an IGF-receptor dependent mechanism. The data add to previous work in this area by indicating (a) that GLP-2 might regulate myofibroblast numbers and motility, and (b) that myofibroblasts in cancer are putative targets for GLP-2. In view of the importance of myofibroblasts in determining the tumour microenvironment the data raise the prospect that GLP-2 might influence cancer progression.

The importance of myofibroblasts as targets for the trophic action of GLP-2 in the intestine has been clear for some time [5, 25]. Previous work has made use of CCD18 myofibroblasts that are derived from normal infant intestine [5], mixed intestinal cell cultures [9] and intestinal sub-epithelial fibroblasts [23]. The present findings extend these studies to include myofibroblasts derived from colon cancer and adjacent tissue. For the most part previous studies have focussed on the mechanisms by which myofibroblasts influence epithelial cell growth. While the growth factor responses of the myofibroblasts themselves have received attention, less has been given to other aspects of myofibroblasts biology. The observation that GLP-2 increases myofibroblast cell number, and very likely cell position (via actions on migration/invasion), suggests a more dynamic system than previously supposed.

It is recognised that cancer-derived fibroblasts and myofibroblasts differ from their counterparts in normal tissue [6, 14, 24]. The different properties of CAMs may, at least to some extent, reflect epigenetic changes [17]. The fact that these cells retain the ability to respond to GLP-2 provides a mechanism by which GLP-2 might influence cancer progression [18]. The therapeutic value of GLP-2 in treatment of short bowel syndrome is now widely appreciated, but it would be as well to keep in mind that in some circumstances GLP-2 might have deleterious effects in influencing cellular microenvironments. A particularly clear example is provided by gastrointestinal stromal tumours that have been reported to express GLP-2R [21]; the possibility that GLP-2 may also act on colon cancers via their stromal component should now be considered.

A number of growth factors have been proposed to mediate the actions of GLP-2 on myofibroblasts, including IGF [23], VEGF-A [5] and KGF [25]. It is important to be clear that these need not be mutually exclusive; nevertheless the evidence is particularly strong for a role for the IGF system. Our data suggest that colonic CAMs stimulate epithelial cell growth, migration and invasion by an IGF-receptor dependent mechanism [29] that is enhanced to varying degrees by treatment of myofibroblasts with GLP-2. The increase in epithelial proliferation was modest, the migratory response was stronger and the invasive responses in two cells lines were very strong. High constitutive expression of IGF has already been reported [23] and would account for the present findings on intestinal epithelial cell proliferation and migration. In the case of the strong invasion response of these cells we suggest that increased bioavailability of IGF as well as increased MMP-activity [13] might interact to enhance the response.

There was increased transcript abundance of IGF-1 and IGF-2 in response to GLP-2 which would provide one mechanism to account for the biological responses of both epithelial cells and myofibroblasts. However, our data suggest that there are also likely to be other

mechanisms. Secreted IGF is sequestered by IGFBPs. In gastrointestinal myofibroblasts the main IGFBPs include IGFBP-4, -5 and -7. Cleavage of IGFBP-5 by MMP-7 released by epithelial cells has previously been shown to increase the bioavailability of IGF-2 which may stimulate both epithelial and stromal cells [12]. In addition to regulating IGF bioavailability, some IGFBPs or their fragments, may exert independent biological activities [10, 15]. Recently, a role for IGFBP-4 in mediating the effects of GLP-2 was indicated by the observation that in mice null for IGFBP-4 the effect of GLP-2 was inhibited [1]. The precise cellular mechanisms remain uncertain, but it is interesting that in our studies GLP-2 treatment of myofibroblasts increased the abundance of IGFBP-4 degradation products seen in western blot; there was a similar effect on IGFBP-5 but not IGFBP-7. Moreover, there was an increase in cellular abundance of IGFBP-4 and -5 compatible with stimulation of expression by GLP-2 as previously reported [1]. The GLP-2 stimulated degradation of IGFBP-4 and -5 was blocked by a broad spectrum inhibitor of MMP activity; in addition, GLP-2 increased the abundance in media and cell extracts of proMMP-1 (although not proMMP-2, -3 and -10). We suggest therefore, that GLP-2 increases the expression in myofibroblasts of IGFBP-4 and -5, and proMMP-1, and that following secretion there is increased bioavailability of IGF through MMP-mediated degradation of its binding proteins.

The maintenance of tissue architecture is presumed to involve two-way interactions in vivo between myofibroblasts and epithelial cells. We attempted to design a simple in vitro model to establish whether the actions of GLP-2 on myofibroblasts, and those of myofibroblasts on epithelial cells, were preserved when the two cell types were in co-culture. The data obtained using Ibidi chambers indicate that the migratory responses observed when the two cells were cultured separately were preserved in co-cultures. Interestingly, though, the magnitude of the migratory responses by epithelial cells was considerably greater than that of myofibroblasts. Nevertheless the data allow us to conclude that any feedback mechanism

from intestinal cells that might exist to limit the myofibroblast response to GLP-2 is relatively modest.

Alongside an appreciation of the therapeutic benefits of GLP-2, there has also been an appreciation that GLP-2 might have growth promoting effects with deleterious consequences in cancer [28]. In two mouse models of carcinogen-induced colon cancer (dimethylhydrazine, azoxymethane) administration of GLP-2 increased tumour size [16, 30], although in other models eg $APC^{min-/-}$ mice, it had no effect [20]. Taken as a whole the present data raise the prospect that GLP-2 acts on colon cancer-derived myofibroblasts to trigger pathways influencing myofibroblast number and motility, as well as their secretion of growth factors and MMPs that exacerbate cancer cell responses. The potential of GLP-2 to aggravate human colon cancer progression should therefore be kept under review.

References

[1] Austin K, Imam NA, Pintar JE, Brubaker PL. IGF binding protein-4 is required for the growth effects of glucagon-like peptide-2 in murine intestine. Endocrinology. 2015;156:429-36.
[2] Austin K, Markovic MA, Brubaker PL. Current and potential therapeutic targets of glucagon-like peptide-2. Current opinion in pharmacology. 2016;31:13-8.
[3] Balabanova S, Holmberg C, Steele I, Ebrahimi B, Rainbow L, Burdyga T, et al. The neuroendocrine phenotype of gastric myofibroblasts and its loss with cancer progression. Carcinogenesis. 2014;35:1798-806.
[4] Bjerknes M, Cheng H. Modulation of specific intestinal epithelial progenitors by enteric neurons. Proceedings of the National Academy of Sciences of the United States of America. 2001;98:12497-502.
[5] Bulut K, Pennartz C, Felderbauer P, Meier JJ, Banasch M, Bulut D, et al. Glucagon like peptide-2 induces intestinal restitution through VEGF release from subepithelial myofibroblasts. European journal of pharmacology. 2008;578:279-85.
[6] De Wever O, Van Bockstal M, Mareel M, Hendrix A, Bracke M. Carcinoma-associated fibroblasts provide operational flexibility in metastasis. Seminars in cancer biology. 2014;25:33-46.
[7] Drucker DJ, Erlich P, Asa SL, Brubaker PL. Induction of intestinal epithelial proliferation by glucagon-like peptide 2. Proceedings of the National Academy of Sciences of the United States of America. 1996;93:7911-6.
[8] Drucker DJ, Yusta B. Physiology and pharmacology of the enteroendocrine hormone glucagon-like peptide-2. Annual review of physiology. 2014;76:561-83.
[9] Dube PE, Forse CL, Bahrami J, Brubaker PL. The essential role of insulin-like growth factor-1 in the intestinal tropic effects of glucagon-like peptide-2 in mice. Gastroenterology. 2006;131:589-605.
[10] Firth SM, Baxter RC. Cellular actions of the insulin-like growth factor binding proteins. EndocrRev. 2002;23:824-54.
[11] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144:646-74.
[12] Hemers E, Duval C, McCaig C, Handley M, Dockray GJ, Varro A. Insulin-like growth factor binding protein-5 is a target of matrix metalloproteinase-7: implications for epithelial-mesenchymal signaling. Cancer Res. 2005;65:7363-9.
[13] Holmberg C, Ghesquiere B, Impens F, Gevaert K, Kumar JD, Cash N, et al. Mapping proteolytic processing in the secretome of gastric cancer-associated myofibroblasts reveals activation of MMP-1, MMP-2, and MMP-3. Journal of proteome research. 2013;12:3413-22.
[14] Holmberg C, Quante M, Steele I, Kumar J, Balabanova S, Duval C, et al. Release of TGFbetaig-h3 by gastric myofibroblasts slows tumor growth and is decreased with cancer progression. Carcinogenesis. 2012;33:1553-62.
[15] Hwa V, Oh Y, Rosenfeld RG. The insulin-like growth factor-binding protein (IGFBP) superfamily. Endocr Rev. 1999;20:761-87.
[16] Iakoubov R, Lauffer LM, Trivedi S, Kim YI, Brubaker PL. Carcinogenic effects of exogenous and endogenous glucagon-like peptide-2 in azoxymethane-treated mice. Endocrinology. 2009;150:4033-43.
[17] Jiang L, Gonda TA, Gamble MV, Salas M, Seshan V, Tu S, et al. Global hypomethylation of genomic DNA in cancer-associated myofibroblasts. Cancer Res. 2008;68:9900-8.

[18] Kannen V, Garcia SB, Stopper H, Waaga-Gasser AM. Glucagon-like peptide 2 in colon carcinogenesis: possible target for anti-cancer therapy? Pharmacol Ther. 2013;139:87-94.
[19] Kemeny LV, Schnur A, Czepan M, Rakonczay Z, Jr., Gal E, Lonovics J, et al. Na+/Ca2+ exchangers regulate the migration and proliferation of human gastric myofibroblasts. American journal of physiology Gastrointestinal and liver physiology. 2013;305:G552-63.
[20] Koehler JA, Harper W, Barnard M, Yusta B, Drucker DJ. Glucagon-like peptide-2 does not modify the growth or survival of murine or human intestinal tumor cells. Cancer research. 2008;68:7897-904.
[21] Korner M, Rehmann R, Reubi JC. GLP-2 receptors in human disease: high expression in gastrointestinal stromal tumors and Crohn's disease. Molecular and cellular endocrinology. 2012;364:46-53.
[22] Kumar JD, Steele I, Moore AR, Murugesan SV, Rakonczay Z, Venglovecz V, et al. Gastrin stimulates MMP-1 expression in gastric epithelial cells: putative role in gastric epithelial cell migration. American journal of physiology Gastrointestinal and liver physiology. 2015;309:G78-86.
[23] Leen JL, Izzo A, Upadhyay C, Rowland KJ, Dube PE, Gu S, et al. Mechanism of action of glucagon-like peptide-2 to increase IGF-I mRNA in intestinal subepithelial fibroblasts. Endocrinology. 2011;152:436-46.
[24] Orimo A, Gupta PB, Sgroi DC, renzana-Seisdedos F, Delaunay T, Naeem R, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell. 2005;121:335-48.
[25] Orskov C, Hartmann B, Poulsen SS, Thulesen J, Hare KJ, Holst JJ. GLP-2 stimulates colonic growth via KGF, released by subepithelial myofibroblasts with GLP-2 receptors. Regul Pept. 2005;124:105-12.
[26] Powell DW, Adegboyega PA, Di Mari JF, Mifflin RC. Epithelial cells and their neighbors I. Role of intestinal myofibroblasts in development, repair, and cancer. AmJPhysiol GastrointestLiver Physiol. 2005;289:G2-G7.
[27] Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. Nature medicine. 2013;19:1423-37.
[28] Rowland KJ, Brubaker PL. The "cryptic" mechanism of action of glucagon-like peptide-2. American journal of physiology Gastrointestinal and liver physiology. 2011;301:G1-8.
[29] Rowland KJ, Trivedi S, Lee D, Wan K, Kulkarni RN, Holzenberger M, et al. Loss of glucagon-like peptide-2-induced proliferation following intestinal epithelial insulin-like growth factor-1-receptor deletion. Gastroenterology. 2011;141:2166-75 e7.
[30] Thulesen J, Hartmann B, Hare KJ, Kissow H, Orskov C, Holst JJ, et al. Glucagon-like peptide 2 (GLP-2) accelerates the growth of colonic neoplasms in mice. Gut. 2004;53:1145-50.
[31] Varro A, Noble PJ, Pritchard DM, Kennedy S, Hart CA, Dimaline R, et al. Helicobacter pylori induces plasminogen activator inhibitor 2 (PAI-2) in gastric epithelial cells through NF-kB and RhoA: implications for invasion and apoptosis Cancer Res. 2004;64:1695-702.

Legends

Fig 1. GLP-1 stimulates myofibroblast proliferation. A, Representative image of EdU labelled myofibroblast (filled arrow; unlabelled cells, open arrows). B, dose-response relationship for GLP-2 stimulation of EdU incorporation by CCD-18 cells. C, GLP-2 (10 nM) stimulates EdU incorporation into both cancer-derived myofibroblasts (CAM) and adjacent tissue myofibroblasts (ATM). D, GLP-2 also increases cell growth measured by CeCo assay. Horizontal arrows, $p<0.05$, ANOVA or t test.

Fig 2. Stimulation of myofibroblast migration by GLP-2. A, GLP-2 (10nM) increases migration of both CAMs and ATMs in Boyden chamber chemotaxis assays and the response is reversed by an inhibitor of IGF1 receptor tyrosine kinase (AG1024, 2μM). B, similar data for invasion assays.

Fig 3. GLP-2 enhances the increase in intestinal epithelial cell proliferation in response to myofibroblast conditioned media. A, Representative images of EdU (red) labelled HT29, SW480 and LoVo cells (nuclei stained blue with DAPI). B, GLP-2 has no direct effect on proliferation of SW480 or LoVo cells and a small effect on HT29 cells. C, Conditioned media from myofibroblasts stimulates EdU incorporation in SW480, HT29 and LoVo cells: the responses are enhanced by previous treatment of myofibroblasts with GLP-2 and are blocked by the IGF receptor inhibitor AG1024. D, in CeCo assays myofibroblast CM stimulates growth of SW480, HT29 and LoVo cells and the effect is enhanced by prior treatment of myofibroblasts with GLP-2; GLP-2 alone has no effect.

Fig 4. GLP-2 enhances the increase in intestinal epithelial cell migration and invasion in response to myofibroblast conditioned media. A, GLP-2 alone (10nM) has no effect on migration (left) or invasion (right) of SW480, HT29 or LoVo cells in Boyden chamber chemotaxis assays. B, Conditioned media (CM) from myofibroblasts stimulates migration of SW480, HT29 and LoVo cells: the responses are enhanced by previous treatment of myofibroblasts with 10nM GLP-2 (GLP-2 CM) and are blocked by the IGF receptor inhibitor AG1024. C, similar results for invasion assays. Horizontal bars, $p<0.05$, ANOVA.

Fig 5. Mutual stimulation of migration by epithelial cells and myofibroblasts in co-cultures treated with GLP-2. A, Representative images of Ibidi chambers with CAMs on one side and either SW480, HT29 or LoVo cells on the other side; on the left is a schematic illustrating the experimental design; thereafter are images taken at 0, 6, 24, 32 and 42 h. At each time point epithelial cells are shown on the left and CAMs on the right; the box indicates the area in which cells were quantified. B, time course of responses measured as numbers of cells in the defined area migrating towards the opposite side in either control co-cultures, or treated with GLP-1. The data are shown for co-cultures of SW480 and CAMs (left), HT29 and CAMs (centre), and LoVo cells and CAMs (right). In each case the data for epithelial cells are in the upper panel and CAMs from the corresponding co-culture in the lower panel. Horizontal bars, $p<0.05$, ANOVA.

Fig 6. Western blot showing IGFBP-4, -5 and –7, and MMP-1, -2, -3 and -10 after GLP-2 treatment of myofibroblasts. A, IGFBP-4, -5 and –7 in media of CAMs treated with GLP-2 (10nM, 24h) with or without the MMP-inhibitor GM6001 (10 μM). B, similar data including GAPDH for corresponding cell extracts. C, MMP-1, -2, -3 and -10 in media of CAMs treated with GLP-2. D, MMP-1 and GAPDH in the corresponding cell extracts.

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Glucagon-like petide-2 acts on colon cancer myofibroblasts to stimulate proliferation, migration and invasion of both myofibroblasts and cancer cells via the IGF pathway

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Abbreviations: ATMs, adjacent tissue myofibroblasts; CAMs, cancer associated myofibroblasts; GLP-2, glucagon-like peptide 2; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; MMP, matrix metalloproteinase.

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Highlights

- GLP-2 stimulates proliferation and invasion of primary colonic cancer-derived myofibroblasts to a greater extent than those from adjacent tissue; GLP-2 conditioned medium from myofibroblasts stimulated proliferation, migration and invasion of intestinal epithelial cells.
- An inhibitor of IGF receptor signalling blocks the effect of GLP-2 on colonic myofibroblasts.
- GLP-2 increases IGF-1 and -2 transcript abundance in myofibroblasts and stimulates degradation of IGF binding proteins in myofibroblasts medium, compatible with increased bioavailability of IGF.
- GLP-2 may influence the cancer microenvironment via actions on stromal cells such as myofibroblasts.

Abstract

Glucagon-like peptide (GLP)-2 stimulates intestinal epithelial proliferation by acting, in part, via IGF release from sub-epithelial myofibroblasts. The response of myofibroblasts to GLP-2 remains incompletely understood. We studied the action of GLP-2 on myofibroblasts from colon cancer and adjacent tissue, and the effects of conditioned medium from these cells on epithelial cell proliferation, migration and invasion. GLP-2 stimulated proliferation, migration and invasion of myofibroblasts and the proliferative and invasive responses of cancer-associated myofibroblasts were greater than those of myofibroblasts from adjacent tissue. The responses were inhibited by an IGF receptor inhibitor, AG1024. Conditioned medium from GLP-2 treated myofibroblasts increased proliferation, migration and invasion of SW480, HT29, LoVo epithelial cells and these responses were inhibited by AG1024; GLP-2 alone had no effect on these cells. In addition, when myofibroblasts and epithelial cells were co-cultured in Ibidi chambers there was mutual stimulation of migration in response to GLP-2. The latter increased both IGF-1 and IGF-2 transcript abundance in myofibroblasts. Moreover, a number of IGF binding proteins (IGFBP-4, -5, -7) were identified in myofibroblast medium; in the presence of GLP-2 there was increased abundance of the cleavage products of IGBBP-4 and IGFBP-5 suggesting activation of a degradation mechanism that might increase IGF bioavailability. The data suggest that GLP-2 stimulates cancer myofibroblast proliferation, migration and invasion; GLP-2 acts indirectly on epithelial cells partly via increased IGF expression in myofibroblasts and partly, perhaps, by increased bioavailability through degradation of IGFBPs.

Introduction

Mucosal morphology throughout the gastrointestinal tract is determined by interactions between epithelial cells and underlying stromal cells which release a variety of growth factors [34]. These interactions are also a feature of tumour growth where it is now clear that stromal cells provide a supportive and stimulatory niche for cancer cells [16, 35]. A key stromal cell type is the myofibroblast which normally lies in close proximity to the basal membrane of epithelial cells while in cancer these cells are an important component of the stroma that may constitute a high proportion of tumour volume. There is now clear evidence that cancer-associated myofibroblasts (CAMs, often considered to be a subset of cancer-associated fibroblasts) are functionally distinct from myofibroblasts recovered from tissue adjacent to the tumour (ATMs), and from normal tissue [18, 19].

A range of hormonal signals from epithelial enteroendocrine cells (EECs) influence growth of the gastrointestinal tract, including gastrin, cholecystokinin and glucagon-like peptide (GLP)-2. The latter is generated from the glucagon precursor by post-translational cleavage; there are different patterns of processing in pancreatic alpha-cells and L-cells of the ileum and colon. In particular, the main products in L-cells are GLP-2, GLP-1 (which is an insulin secretagogue) and oxyntomodulin (which is a C-terminally extended variant of glucagon). A link between glucagon gene expression and intestinal hypertrophy has been known for several decades, and it is now recognised that GLP-2 is a crucial mediator [10]. Interestingly, GLP-2 receptors are not expressed on intestinal epithelial cells, with the possible exception of some EECs, and as a consequence GLP-2 does not have direct effects on intestinal epithelial cell proliferation. Instead there is evidence that GLP-2 acts indirectly via sub-epithelial cells notably myofibroblasts and neurons [2, 5, 11, 32]. Thus, the evidence suggests that GLP-2 releases growth factors such as IGF, KGF, VEGF-A and EGF-family members which in turn act on epithelial cells to stimulate cell proliferation [7, 13, 32, 40].

Gene array data indicate that a wide variety of gastrointestinal myofibroblasts produce both IGF-1 and IGF-2 as well as a number of IGF binding proteins (IGFBPs) including -3, -4, -5, -6 and -7 [4]. The IGFBPs act to sequester extracellular IGF which can be liberated by IGFBP cleavage, for example by matrix metalloproteinase (MMP)-7 produced by epithelial cells [17]. In the upper gastrointestinal tract, the IGFs act on both epithelial cells and the myofibroblasts themselves to stimulate proliferation as well as migration and invasion. The response of intestinal myofibroblasts to GLP-2 remains relatively unexplored. While there have been studies using a CCD18 myofibroblast cell line, which is derived from normal intestine of a human infant, the possible effects of GLP-2 on cancer myofibroblasts are unclear. The question is of interest in the context of colon cancer, because GLP-2 stimulates normal colonic, as well as small intestinal, growth [30] and reduces injury in a mouse model of colitis [12]; moreover in some mouse models of colon cancer there is evidence that GLP-2 may exert growth-promoting effects [38]. A recent review hypothesized that GLP-2 might act via cancer associated fibroblasts [23]. However, direct studies of the action of GLP-2 on stromal cells from colorectal carcinoma have been neglected. In the present study we have examined the hypothesis that GLP-2 acts on myofibroblasts from colon cancer and adjacent tissue to influence colon cancer cells lines, and have examined the role of IGF in mediating these effects.

Materials and Methods

Cells. Colon cancer cells (HT29, Lovo, SW480) and CCD18 colonic myofibroblasts were obtained from American type culture collection (VA, US). Myofibroblasts were generated from a colon tumour (CAMs) and adjacent tissue (ATMs) of an 85 year old female patient (T3N2M0; post-operative survival 2 months) as previously described [19, 24]; these cells were positive by immunohistochemistry for α-smooth muscle actin and vimentin, and were negative for desmin and cytokeratin. Cancer cells and myofibroblasts were cultured as previously described [19].

Conditioned media. Myofibroblasts (1.5 x 10^6 cells) were plated in T-75 falcon flasks and maintained at 37°C in a 5% v/v CO_2 atmosphere for 24h in full media (FM). Cultures were then washed 3 times with sterile PBS and incubated in 15ml serum free (SF) media with or without GLP-2 (AnaSpec, Freemont, CA, USA) for 24h. Conditioned medium (CM) was collected, centrifuged (7 min, 800 x g, 4°C) and aliquots were stored at -80°C until further use.

EdU incorporation and *Cell Counting Kit-8* assays

For EdU incorporation assays, colonic myofibroblasts and colon cancer cells (2.5x10^4 per well) were seeded and incubated overnight in FM followed by serum starvation for 48 h. Cells then were treated with GLP-2 or CM as appropriate for 24h. Proliferation was assessed by incorporation of 5-ethynyl-2'-deoxyuridine (EdU, 10 µM, 16h) and processing of samples using Click-iT (Invitrogen, Paisley, UK) and Alexa 568-azide. EdU positive cells were visualised on a Zeiss AxioCam HRM fluorescence microscope (Carl Zeiss, Welwyn Garden City, UK) on a 40x objective lens counting the total number of cell nuclei in 10 different fields using DAPI (Vector Laboratories, Peterborough, UK) (blue) and EdU positive nuclei.

1 148 Cell Counting Kit-8 assays (Dojindo Laboratories, Munich, Germany; the manufacturers use
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3 149 the abbreviation "CCK8" to describe these kits, but to avoid confusion with cholecystokinin
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6 150 octapeptide which shares the same abbreviation, we use the alternative contraction "CeCo"
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8 151 assays here) were performed on colonic myofibroblasts and cancer cells ($8x10^3$ per well) in
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10 152 96 well plates incubated overnight in FM followed by incubation with GLP-2 or CM medium
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12 153 in phenol red free SF medium for 72h. On the last day 10µl CeCo reagent was added to the
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14 154 wells and incubated for 2-4h as optimised for each cell type. Readings were taken at 450nm
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17 155 using a GenioPlus plate reader (Tecan, Zurich, Switzerland).
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22 157 **Cell migration and invasion assays.** Transwell migration and invasion assays were
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24 158 performed using BD inserts (Corning, New York, USA) as previous described (2.5×10^4 cells
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27 159 per insert) [39]. GLP-2 or CM were added in the lower well together with AG1024
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29 160 (Calbiochem, Darmstadt, Germany) as appropriate. Ibidi chamber (Ibidi GmbH, Martinsried,
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31 161 Germany) migration assays were performed in 24 well plates. Ibidi culture inserts were
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34 162 placed at the bottom of the wells followed by seeding of 10^5 cancer cells and $5x10^4$
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36 163 myofibroblasts on separate sides of the insert. After 24h the insert was removed, leaving a
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38 164 500µm wound; cells were then treated with GLP-2 up to 42h and images taken with a
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40 165 Hamamatsu Camera (Hamamatsu Photonics, Hamamatsu City, Japan) at 0, 6, 24, 32, 42h
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42 166 using a Leica DMIRE2 microscope on a heated stage, humidified chamber (Solent Scientific,
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45 167 Portsmouth, UK); cells in the wound were counted in 5-8 fields per insert.
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49 169 ***qPCR.*** RNA was extracted in 1.25 ml Tri-Reagent (Sigma, Dorset, UK) according to the
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51 170 manufacturer's instructions from control and GLP-2 treated colonic myofibroblasts. RNA
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54 171 pellets were re-suspended in 50 µl of nuclease free water and 4 µg of RNA reverse
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56 172 transcribed with avian myeloblastosis virus reverse transcriptase and oligo-dT primers
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58 173 (Promega). Real time PCR was carried out using an ABI7500 platform (Applied Biosystems,
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Warrington, UK) using TaqMan primer/probe sets (human IGF-1, IGF-2, GAPDH), Precision Plus 2x real time PCR master mix (Primer Design, Southampton, UK) and 5'-FAM, 3'-TAMRA double dye probes (Eurogentec, Southampton, UK). All values were standardized to GAPDH. Assays included a no template control (NTC), and a standard curve as previously described [28]. Primers and probes were designed using Primer Express v3.0 (Applied Biosystems) and were purchased from Eurogentec (Seraing, Belgium). Probes for detection of human GAPDH, IGF-1 and IGF-2 cDNA were intron-spanning and were: GAPDH: 5'-GCT CCT CCT GTT CGA CAG TCA-3'(forward), 5'-ACC TTC CCC ATG GTG TCT GA-3' (reverse), 5'-CGT CGC CAG CCG AGC CAC A-3' (probe); IGF-1: 5'-TGT ATT GCG CAC CCC TCA A-3' (forward), 5'-CT CCC TCT ACT TGC GTT CTT CA-3' (reverse), 5'-ACA TGC CCA AGA CCC AGA AGG AAG TAC A-3' (probe); IGF-2, 5'-CCG TGC TTC CGG ACA ACT T-3' (forward), 5'-GGA CTG CTT CCA GGT GTC ATA TT-3' (reverse), 5'-CCC AGA TAC CCC GTG GGC AAG TTC-3' (probe).

Western blotting. Cell extracts were prepared in RIPA buffer and media samples were concentrated with StrataClean resins (Agilent Technologies Ltd, Santa Clara, CA, USA) and processed for Western blotting as previously described [28] using antibodies to IGFBP-3, 4, 5 and -7, MMP-1, -2, -3 and -10 (R&D Systems) and GAPDH (Biodesign, Maine, USA).

Statistics. Results were calculated as mean ± standard error of means (SEM). Student t-test and ANOVA were performed on the data as appropriate with significance at $p<0.05$ using Systat Software Inc. (London, UK) unless otherwise stated.

Results

GLP-2 stimulates myofibroblast proliferation. In initial studies we showed that GLP-2 produced a dose-related increase in EdU incorporation into CCD18 myofibroblasts (Fig 1A,B). We then studied EdU incorporation by colonic CAMs and ATMs. As previously reported for gastric CAMs [19], basal EdU incorporation was greater in the colonic CAMs compared with the corresponding ATMs. In the presence of GLP-2 there was stimulation of EdU incorporation into both CAMs and ATMs and the response in the former was greater than the latter (Fig 1C). In a CeCo assay of cell growth, GLP-2 also stimulated colonic CAM growth and to a lesser extent that of ATMs (Fig 1D).

GLP-2 stimulates myofibroblast migration and invasion. We then examined the actions of GLP-2 on CAM and ATM migration and invasion using Boyden chambers (Fig 2A,B). In both cell types, GLP-2 at a concentration that stimulated proliferation also stimulated migration and these responses were similar in CAMs and ATMs; moreover in both cases they were abolished by AG1024 which selectively inhibits IGF-1 receptor tyrosine kinase activity at the concentrations used [33]. Similarly, there was IGF-dependent stimulation of CAM invasion and in this case the response was significantly greater than that of ATMs.

Myofibroblast conditioned medium stimulates epithelial cell proliferation. Consistent with a previous report that epithelial cells lines lack the capacity to respond to GLP-2 [25], there was no change in EdU incorporation (Fig 3A) by two of the epithelial cell lines used in the present study (SW480 and LoVo) in response to GLP-2 applied directly to the cells while there was a small effect on HT29 cells (Fig 3B). However, there was a small increase in EdU incorporation in response to CAM CM in SW480 cells that was enhanced by pre-treatment of myofibroblasts with GLP-2 and there were greater increases in EdU incorporation by HT29 and LoVo cells in response to CAM CM, and in both cases there were small further increases using CM from GLP-2-treated myofibroblasts. In all three intestinal cell lines, the

responses to control CM and to GLP-2 CM were suppressed by AG1024 (Fig 3C). In CeCo assays of cell growth, there were robust responses by all three intestinal epithelial lines to control myofibroblast CM and small but significantly increases in response to GLP-2 pretreatment of myofibroblasts (Fig 3D).

Myofibroblast conditioned medium stimulates epithelial cell migration and invasion. When GLP-2 was applied directly to epithelial cells (SW480, HT29, LoVo) there was no effect on migration or invasion in Boyden chambers (Fig 4A). However, there was strong stimulation of migration of all three cell lines in response to control CAM CM and there were small but significant further increases in response to CM from GLP-2 treated myofibroblasts (Fig 4B). In each case the response was inhibited by AG1024. There were relatively modest increases in invasion of HT29 and LoVo cells in response to control myofibroblast CM but the response was strongly enhanced by GLP-2-treated CM; all responses were inhibited by AG1024 (Fig 4C).

GLP-2 stimulates migration in co-cultures of epithelial cells and myofibroblasts. In view of the potential for cross-talk between epithelial cells and myofibroblasts we then asked whether the response to GLP-2 was preserved when these cells were co-cultured. For this purpose we used Ibidi chambers with myofibroblasts in one chamber and epithelial cells in the other so that subsequent removal of the insert yielded cultures of the two cells separated by 500 μm (Fig 5A). Over a period of 42 h after removing the insert there was progressive migration of both cell types toward each other (Fig 5A). The numbers of migrating epithelial cells were roughly 10 times higher than those of myofibroblasts. Nevertheless at each time point examined from 6 – 42 h the presence of GLP-2 increased the migration of each of the epithelial cell lines and of the co-cultured CAMs (Fig 5B).

GLP-2 increases IGF-1 and IGF-2 transcript abundance. In view of the evidence that GLP-2 might act via IGF we first examined microarray data from myofibroblasts which indicated that both IGF-1 and IGF-2 were expressed in colonic CAMs and ATMs; the relative abundance of IGF-1 in colonic myofibroblasts was higher compared to IGF-2, while in gastric myofibroblasts IGF-2 was dominant [4]. We then used qPCR for both IGF-1 and IGF-2 to assess responses to GLP-2. At a concentration of GLP-2 (10 nM) that increased myofibroblast migration and proliferation, there was 1.8 ± 0.2 fold higher IGF-1 transcript abundance and 1.5 ± 0.2 fold higher IGF-2 transcript abundance.

GLP-2 promotes IGFBP degradation in myofibroblasts medium. Since there is evidence that GLP-2 might act via IGFBP-4 [1] we examined by Western blot the profile of IGFBP-4 and also of IGFBP-3, -5 and -7 in CAMs treated with GLP-2. In control media there were clear bands corresponding to intact IGFBP-4, -5 and -7 (Fig 6A), while IGFBP-3 was undetectable (not shown). There were also minor bands corresponding to degradation products of IGFBP4 (15 kD) and IGFBP-5 (10-15 kD). In the presence of GLP-2 the bands corresponding to intact IGFBP-4, and -5 were maintained but there was increased abundance of the degradation products. In the presence of an inhibitor of MMP activity (GM6001, 10 μM) the action of GLP-2 in promoting degradation of IGFBP4 and -5 was inhibited (Fig 6A). There was also an increase in IGFBP-4 and -5 in cell extracts in response to GLP-2 compatible with increased expression (Fig 6B). Multiple proMMPs are secreted by myofibroblasts (MMP-1, -2, -3 and -10) [18]. Of these, MMP-1 was increased in abundance in GLP-2 treated cells and media (Fig 6C,D) although there was little or no change in MMP-2, -3 and -10.

Discussion

The present study provides evidence that GLP-2 acts on cancer-derived myofibroblasts to stimulate their proliferation, migration and invasion. These effects are blocked by an inhibitor of IGF1 receptor tyrosine kinase and since there is an increase in IGF-1 and -2 transcript abundance as well as increased degradation of IGFBP-4 and -5, the data support the idea that the actions of GLP-2 on myofibroblasts are at least partly mediated by IGF. Consistent with previous findings, GLP-2 had little or no effect on three intestinal epithelial cell lines, but conditioned medium from CAMs stimulated proliferation, migration and invasion of these cells, and to varying degrees GLP-2 treatment of myofibroblasts enhanced the responses in an IGF-receptor dependent mechanism. The data add to previous work in this area by indicating (a) that GLP-2 might regulate myofibroblast numbers and motility, and (b) that myofibroblasts in cancer are putative targets for GLP-2. In view of the importance of myofibroblasts in determining the tumour microenvironment the data raise the prospect that GLP-2 might influence cancer progression.

The importance of myofibroblasts as targets for the trophic action of GLP-2 in the intestine has been clear for some time [7, 32]. Previous work has made use of CCD18 myofibroblasts that are derived from normal infant intestine [7], mixed intestinal cell cultures [13] and intestinal sub-epithelial fibroblasts [29]. The present findings extend these studies to include myofibroblasts derived from colon cancer and adjacent tissue. For the most part previous studies have focussed on the mechanisms by which myofibroblasts influence epithelial cell growth. While the growth factor responses of the myofibroblasts themselves have received attention, less has been given to other aspects of myofibroblasts biology. The observation that GLP-2 increases myofibroblast cell number, and very likely cell position (via actions on migration/invasion), suggests a more dynamic system than previously supposed.

It is recognised that cancer-derived fibroblasts and myofibroblasts differ from their counterparts in normal tissue [9, 19, 31]. The different properties of CAMs may, at least to some extent, reflect epigenetic changes [22]. The differences are important given the emerging evidence that stromal cells in general [16], and myofibroblasts in particular, stimulate cancer progression [8]. Previous work has shown that in both gastric and oesophageal cancer, CAMs exhibit increased proliferation, migration and invasion compared with ATMs [19, 27]. The present study has shown that colonic CAMs similarly exhibit a more aggressive phenotype than colonic ATMs i.e. increased basal and stimulated proliferation and invasion in response to GLP-2. The fact that these cells retain the ability to respond to GLP-2 provides a mechanism by which GLP-2 might influence cancer progression [23]. The therapeutic value of GLP-2 in treatment of short bowel syndrome is now widely appreciated, but it would be as well to keep in mind that in some circumstances GLP-2 might have deleterious effects in influencing cellular microenvironments. A particularly clear example is provided by gastrointestinal stromal tumours that have been reported to express GLP-2R [26]; the possibility that GLP-2 may also act on colon cancers via their stromal component should now be considered.

A number of growth factors have been proposed to mediate the actions of GLP-2 on myofibroblasts, including IGF [29], VEGF-A [7], KGF [32] and EGF-family members [3, 40]. It is important to be clear that these need not be mutually exclusive. Our data suggest that colonic CAMs stimulate epithelial cell growth, migration and invasion by an IGF-receptor dependent mechanism [37] that is enhanced to varying degrees by treatment of myofibroblasts with GLP-2. The increase in epithelial proliferation was modest, the migratory response was stronger and the invasive responses in two cells lines were very strong. High constitutive expression of IGF has already been reported [29] and would account for the present findings on intestinal epithelial cell proliferation and migration. In the case of the

strong invasion response of these cells we suggest that increased bioavailability of IGF as well as increased MMP-activity [18] might interact to enhance the response.

There was increased transcript abundance of IGF-1 and IGF-2 in response to GLP-2 which would provide one mechanism to account for the biological responses of both epithelial cells and myofibroblasts. However, our data suggest that there are also likely to be other mechanisms. Secreted IGF is sequestered by IGFBPs. In gastrointestinal myofibroblasts the main IGFBPs include IGFBP-4, -5 and -7. Cleavage of IGFBP-5 by MMP-7 released by epithelial cells has previously been shown to increase the bioavailability of IGF-2 which may stimulate both epithelial and stromal cells [17]. In addition to regulating IGF bioavailability, some IGFBPs or their fragments, may exert independent biological activities [14, 20]. Recently, a role for IGFBP-4 in mediating the effects of GLP-2 was indicated by the observation that in mice null for IGFBP-4 the effect of GLP-2 was inhibited [1]. The precise cellular mechanisms remain uncertain, but it is interesting that in our studies GLP-2 treatment of myofibroblasts increased the abundance of IGFBP-4 degradation products seen in western blot; there was a similar effect on IGFBP-5 but not IGFBP-7. Moreover, there was an increase in cellular abundance of IGFBP-4 and -5 compatible with stimulation of expression by GLP-2 as previously reported [1]. The degradation of IGFBP-4 in other systems is attributable to PAPP-A which is a member of the pappalysin group of metzincin metalloproteinase [6]. The GLP-2 stimulated degradation of IGFBP-4 and -5 was blocked by a broad spectrum inhibitor of MMP activity, GM6001, which is reported to have little effect on pappalysins [15]. We found GLP-2 increased the abundance in media and cell extracts of proMMP-1 (although not proMMP-2, -3 and -10). We suggest therefore, that GLP-2 increases the expression in myofibroblasts of IGFBP-4 and -5, and proMMP-1. Further work is now needed to determine the biological significance of the degradation products of IGFBP-4 and -5, particularly with respect to IGF binding and any potential biological activities independent of IGF.

The maintenance of tissue architecture is presumed to involve two-way interactions in vivo between myofibroblasts and epithelial cells. We attempted to design a simple in vitro model to establish whether the actions of GLP-2 on myofibroblasts, and those of myofibroblasts on epithelial cells, were preserved when the two cell types were in co-culture. The data obtained using Ibidi chambers indicate that the migratory responses observed when the two cells were cultured separately were preserved in co-cultures. Interestingly, though, the magnitude of the migratory responses by epithelial cells was considerably greater than that of myofibroblasts. Nevertheless the data allow us to conclude that any feedback mechanism from intestinal cells that might exist to limit the myofibroblast response to GLP-2 is relatively modest.

Alongside an appreciation of the therapeutic benefits of GLP-2, there has also been an appreciation, based on data from animal models, that GLP-2 might have growth promoting effects with deleterious consequences in cancer [36]. Thus in two mouse models of carcinogen-induced colon cancer (dimethylhydrazine, azoxymethane) administration of GLP-2 increased tumour size [21, 38], although in other models eg APC$^{min-/-}$ mice, it had no effect [25]. Whether or not GLP-2 aggravates human colon cancer progression needs to be kept under review. The case for further studies of GLP-2 and human colon cancer is supported by the present data which raise the prospect that GLP-2 acts on human colon cancer-derived myofibroblasts to trigger pathways influencing myofibroblast number and motility and, indirectly, cancer cell function. It is worth stressing, that since the present data indicate that GLP-2 targets the IGF system in colonic myofibroblasts this system would presumably be susceptible to novel therapeutic strategies targeted at IGF-responsive cells in colon cancer [41].

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References

[1] Austin K, Imam NA, Pintar JE, Brubaker PL. IGF binding protein-4 is required for the growth effects of glucagon-like peptide-2 in murine intestine. Endocrinology. 2015;156:429-36.

[2] Austin K, Markovic MA, Brubaker PL. Current and potential therapeutic targets of glucagon-like peptide-2. Current opinion in pharmacology. 2016;31:13-8.

[3] Bahrami J, Yusta B, Drucker DJ. ErbB activity links the glucagon-like peptide-2 receptor to refeeding-induced adaptation in the murine small bowel. Gastroenterology. 2010;138:2447-56.

[4] Balabanova S, Holmberg C, Steele I, Ebrahimi B, Rainbow L, Burdyga T, et al. The neuroendocrine phenotype of gastric myofibroblasts and its loss with cancer progression. Carcinogenesis. 2014;35:1798-806.

[5] Bjerknes M, Cheng H. Modulation of specific intestinal epithelial progenitors by enteric neurons. Proceedings of the National Academy of Sciences of the United States of America. 2001;98:12497-502.

[6] Boldt HB, Conover CA. Pregnancy-associated plasma protein-A (PAPP-A): a local regulator of IGF bioavailability through cleavage of IGFBPs. Growth Horm IGF Res. 2007;17:10-8.

[7] Bulut K, Pennartz C, Felderbauer P, Meier JJ, Banasch M, Bulut D, et al. Glucagon like peptide-2 induces intestinal restitution through VEGF release from subepithelial myofibroblasts. European journal of pharmacology. 2008;578:279-85.

[8] De Wever O, Demetter P, Mareel M, Bracke M. Stromal myofibroblasts are drivers of invasive cancer growth. IntJCancer. 2008;123:2229-38.

[9] De Wever O, Van Bockstal M, Mareel M, Hendrix A, Bracke M. Carcinoma-associated fibroblasts provide operational flexibility in metastasis. Seminars in cancer biology. 2014;25:33-46.

[10] Drucker DJ, Erlich P, Asa SL, Brubaker PL. Induction of intestinal epithelial proliferation by glucagon-like peptide 2. Proceedings of the National Academy of Sciences of the United States of America. 1996;93:7911-6.

[11] Drucker DJ, Yusta B. Physiology and pharmacology of the enteroendocrine hormone glucagon-like peptide-2. Annual review of physiology. 2014;76:561-83.

[12] Drucker DJ, Yusta B, Boushey RP, DeForest L, Brubaker PL. Human [Gly2]GLP-2 reduces the severity of colonic injury in a murine model of experimental colitis. The American journal of physiology. 1999;276:G79-91.

[13] Dube PE, Forse CL, Bahrami J, Brubaker PL. The essential role of insulin-like growth factor-1 in the intestinal tropic effects of glucagon-like peptide-2 in mice. Gastroenterology. 2006;131:589-605.

[14] Firth SM, Baxter RC. Cellular actions of the insulin-like growth factor binding proteins. EndocrRev. 2002;23:824-54.

[15] Garcia-Castellanos R, Tallant C, Marrero A, Sola M, Baumann U, Gomis-Ruth FX. Substrate specificity of a metalloprotease of the pappalysin family revealed by an inhibitor and a product complex. Archives of biochemistry and biophysics. 2007;457:57-72.

[16] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144:646-74.

[17] Hemers E, Duval C, McCaig C, Handley M, Dockray GJ, Varro A. Insulin-like growth factor binding protein-5 is a target of matrix metalloproteinase-7: implications for epithelial-mesenchymal signaling. Cancer Res. 2005;65:7363-9.

[18] Holmberg C, Ghesquiere B, Impens F, Gevaert K, Kumar JD, Cash N, et al. Mapping proteolytic processing in the secretome of gastric cancer-associated myofibroblasts reveals activation of MMP-1, MMP-2, and MMP-3. Journal of proteome research. 2013;12:3413-22.
[19] Holmberg C, Quante M, Steele I, Kumar J, Balabanova S, Duval C, et al. Release of TGFbetaig-h3 by gastric myofibroblasts slows tumor growth and is decreased with cancer progression. Carcinogenesis. 2012;33:1553-62.
[20] Hwa V, Oh Y, Rosenfeld RG. The insulin-like growth factor-binding protein (IGFBP) superfamily. Endocr Rev. 1999;20:761-87.
[21] Iakoubov R, Lauffer LM, Trivedi S, Kim YI, Brubaker PL. Carcinogenic effects of exogenous and endogenous glucagon-like peptide-2 in azoxymethane-treated mice. Endocrinology. 2009;150:4033-43.
[22] Jiang L, Gonda TA, Gamble MV, Salas M, Seshan V, Tu S, et al. Global hypomethylation of genomic DNA in cancer-associated myofibroblasts. Cancer Res. 2008;68:9900-8.
[23] Kannen V, Garcia SB, Stopper H, Waaga-Gasser AM. Glucagon-like peptide 2 in colon carcinogenesis: possible target for anti-cancer therapy? Pharmacol Ther. 2013;139:87-94.
[24] Kemeny LV, Schnur A, Czepan M, Rakonczay Z, Jr., Gal E, Lonovics J, et al. Na+/Ca2+ exchangers regulate the migration and proliferation of human gastric myofibroblasts. American journal of physiology Gastrointestinal and liver physiology. 2013;305:G552-63.
[25] Koehler JA, Harper W, Barnard M, Yusta B, Drucker DJ. Glucagon-like peptide-2 does not modify the growth or survival of murine or human intestinal tumor cells. Cancer research. 2008;68:7897-904.
[26] Korner M, Rehmann R, Reubi JC. GLP-2 receptors in human disease: high expression in gastrointestinal stromal tumors and Crohn's disease. Molecular and cellular endocrinology. 2012;364:46-53.
[27] Kumar JD, Holmberg C, Kandola S, Steele I, Hegyi P, Tiszlavicz L, et al. Increased expression of chemerin in squamous esophageal cancer myofibroblasts and role in recruitment of mesenchymal stromal cells. PloS one. 2014;9:e104877.
[28] Kumar JD, Steele I, Moore AR, Murugesan SV, Rakonczay Z, Venglovecz V, et al. Gastrin stimulates MMP-1 expression in gastric epithelial cells: putative role in gastric epithelial cell migration. American journal of physiology Gastrointestinal and liver physiology. 2015;309:G78-86.
[29] Leen JL, Izzo A, Upadhyay C, Rowland KJ, Dube PE, Gu S, et al. Mechanism of action of glucagon-like peptide-2 to increase IGF-I mRNA in intestinal subepithelial fibroblasts. Endocrinology. 2011;152:436-46.
[30] Litvak DA, Hellmich MR, Evers BM, Banker NA, Townsend CM, Jr. Glucagon-like peptide 2 is a potent growth factor for small intestine and colon. Journal of gastrointestinal surgery : official journal of the Society for Surgery of the Alimentary Tract. 1998;2:146-50.
[31] Orimo A, Gupta PB, Sgroi DC, renzana-Seisdedos F, Delaunay T, Naeem R, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell. 2005;121:335-48.
[32] Orskov C, Hartmann B, Poulsen SS, Thulesen J, Hare KJ, Holst JJ. GLP-2 stimulates colonic growth via KGF, released by subepithelial myofibroblasts with GLP-2 receptors. Regul Pept. 2005;124:105-12.
[33] Parrizas M, Gazit A, Levitzki A, Wertheimer E, LeRoith D. Specific inhibition of insulin-like growth factor-1 and insulin receptor tyrosine kinase activity and biological function by tyrphostins. Endocrinology. 1997;138:1427-33.
[34] Powell DW, Adegboyega PA, Di Mari JF, Mifflin RC. Epithelial cells and their neighbors I. Role of intestinal myofibroblasts in development, repair, and cancer. AmJPhysiol GastrointestLiver Physiol. 2005;289:G2-G7.
[35] Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. Nature medicine. 2013;19:1423-37.

[36] Rowland KJ, Brubaker PL. The "cryptic" mechanism of action of glucagon-like peptide-2. American journal of physiology Gastrointestinal and liver physiology. 2011;301:G1-8.
[37] Rowland KJ, Trivedi S, Lee D, Wan K, Kulkarni RN, Holzenberger M, et al. Loss of glucagon-like peptide-2-induced proliferation following intestinal epithelial insulin-like growth factor-1-receptor deletion. Gastroenterology. 2011;141:2166-75 e7.
[38] Thulesen J, Hartmann B, Hare KJ, Kissow H, Orskov C, Holst JJ, et al. Glucagon-like peptide 2 (GLP-2) accelerates the growth of colonic neoplasms in mice. Gut. 2004;53:1145-50.
[39] Varro A, Noble PJ, Pritchard DM, Kennedy S, Hart CA, Dimaline R, et al. Helicobacter pylori induces plasminogen activator inhibitor 2 (PAI-2) in gastric epithelial cells through NF-kB and RhoA: implications for invasion and apoptosis Cancer Res. 2004;64:1695-702.
[40] Yusta B, Holland D, Koehler JA, Maziarz M, Estall JL, Higgins R, et al. ErbB signaling is required for the proliferative actions of GLP-2 in the murine gut. Gastroenterology. 2009;137:986-96.
[41] Zhong H, Fazenbaker C, Chen C, Breen S, Huang J, Yao X, et al. Overproduction of IGF-2 drives a subset of colorectal cancer cells, which specifically respond to an anti-IGF therapeutic antibody and combination therapies. Oncogene. 2017;36:797-806.

Legends

Fig 1. GLP-2 stimulates myofibroblast proliferation. A, Representative image of EdU labelled myofibroblast (filled arrow; unlabelled cells, open arrows). B, dose-response relationship for GLP-2 stimulation of EdU incorporation by CCD-18 cells (n = 4). C, GLP-2 (10 nM) stimulates EdU incorporation into both cancer-derived myofibroblasts (CAM, n = 6) and adjacent tissue myofibroblasts (ATM, n = 3). D, GLP-2 also increases cell growth measured by CeCo assay (n = 4). Horizontal arrows * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Fig 2. Stimulation of myofibroblast migration by GLP-2. A, GLP-2 (10nM) increases migration of both CAMs and ATMs in Boyden chamber chemotaxis assays and the response is reversed by an inhibitor of IGF1 receptor tyrosine kinase (AG1024, 20μM)(n = 3 – 6). B, similar data for invasion assays (n = 3). See Legend to Fig 1 for further details.

Fig 3. GLP-2 enhances the increase in intestinal epithelial cell proliferation in response to myofibroblast conditioned media. A, Representative images of EdU (red) labelled HT29, SW480 and LoVo cells (nuclei stained blue with DAPI). B, GLP-2 (10 nM) has no direct effect on proliferation of SW480 or LoVo cells and a small effect on HT29 cells (n = 3). C, Conditioned media from myofibroblasts stimulates EdU incorporation in SW480, HT29 and LoVo cells: the responses are enhanced by previous treatment of myofibroblasts with GLP-2 (10 nM) and are blocked by the IGF receptor inhibitor AG1024 (20 μM)(n = 3 – 6). D, in CeCo assays myofibroblast CM stimulates growth of SW480, HT29 and LoVo cells and the effect is enhanced by prior treatment of myofibroblasts with GLP-2 (10 nM); GLP-2 alone has no effect (n = 3 – 4). See Legend to Fig 1 for further details.

Fig 4. GLP-2 enhances the increase in intestinal epithelial cell migration and invasion in response to myofibroblast conditioned media. A, GLP-2 alone (10nM) has no effect on migration (left) or invasion (right) of SW480, HT29 or LoVo cells in Boyden chamber chemotaxis assays (n = 3). B, Conditioned media (CM) from myofibroblasts stimulates migration of SW480, HT29 and LoVo cells: the responses are enhanced by previous treatment of myofibroblasts with 10nM GLP-2 (GLP-2 CM) and are blocked by the IGF receptor inhibitor AG1024 (20 μM)(n = 3 – 6). C, similar results for invasion assays (n = 3). See Legend to Fig 1 for further details.

Fig 5. Mutual stimulation of migration by epithelial cells and myofibroblasts in co-cultures treated with GLP-2. A, Representative images of Ibidi chambers with CAMs on one side and either SW480, HT29 or LoVo cells on the other side; on the left is a schematic illustrating the experimental design; thereafter are images taken at 0, 6, 24, 32 and 42 h. At each time point epithelial cells are shown on the left and CAMs on the right; the box indicates the area in which cells were quantified. B, time course of responses measured as numbers of cells in the defined area migrating towards the opposite side in either control co-cultures, or treated with GLP-2 (10 nM). The data are shown for co-cultures of SW480 and CAMs (left), HT29 and CAMs (centre), and LoVo cells and CAMs (right). In each case the data for epithelial cells are in the upper panel and CAMs from the corresponding co-culture in the lower panel (n = 3). See Legend to Fig 1 for further details.

Fig 6. Western blots showing IGFBP-4, -5 and –7, and MMP-1, -2, -3 and -10 after GLP-2 treatment of myofibroblasts. A, IGFBP-4, -5 and –7 in media of CAMs treated with GLP-2

1 546 (10nM, 24h) with or without the MMP-inhibitor GM6001 (10 μM). B, similar data including
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3 547 GAPDH for corresponding cell extracts. C, MMP-1, -2, -3 and -10 in media of CAMs treated
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5 548 with GLP-2. D, MMP-1 and GAPDH in the corresponding cell extracts. Representative
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Glucagon-like petide-2 acts on colon cancer myofibroblasts to stimulate proliferation and migration of both myofibroblasts and cancer cells via the IGF pathway

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Abbreviations: ATMs, adjacent tissue myofibroblasts; CAMs, cancer associated myofibroblasts; GLP-2, glucagon-like peptide 2; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; MMP, matrix metalloproteinase.

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Highlights

- GLP-2 stimulates proliferation and invasion of primary colonic cancer-derived myofibroblasts to a greater extent than those from adjacent tissue; GLP-2 conditioned medium from myofibroblasts stimulated proliferation, migration and invasion of intestinal epithelial cells.
- An inhibitor of IGF receptor signalling blocks the effect of GLP-2 on colonic myofibroblasts.
- GLP-2 increases IGF-1 and -2 transcript abundance in myofibroblasts and stimulates degradation of IGF binding proteins in myofibroblasts medium, compatible with increased bioavailability of IGF.
- GLP-2 may influence the cancer microenvironment via actions on stromal cells such as myofibroblasts.

Abstract

Glucagon-like peptide (GLP)-2 stimulates intestinal epithelial proliferation by acting, in part, via IGF release from sub-epithelial myofibroblasts. The response of myofibroblasts to GLP-2 remains incompletely understood. We studied the action of GLP-2 on myofibroblasts from colon cancer and adjacent tissue, and the effects of conditioned medium from these cells on epithelial cell proliferation, migration and invasion. GLP-2 stimulated proliferation, migration and invasion of myofibroblasts and the proliferative and invasive responses of cancer-associated myofibroblasts were greater than those of myofibroblasts from adjacent tissue. The responses were inhibited by an IGF receptor inhibitor, AG1024. Conditioned medium from GLP-2 treated myofibroblasts increased proliferation, migration and invasion of SW480, HT29, LoVo epithelial cells and these responses were inhibited by AG1024; GLP-2 alone had no effect on these cells. In addition, when myofibroblasts and epithelial cells were co-cultured in Ibidi chambers there was mutual stimulation of migration in response to GLP-2. The latter increased both IGF-1 and IGF-2 transcript abundance in myofibroblasts. Moreover, a number of IGF binding proteins (IGFBP-4, -5, -7) were identified in myofibroblast medium; in the presence of GLP-2 there was increased abundance of the cleavage products of IGBBP-4 and IGFBP-5 suggesting activation of a degradation mechanism that might increase IGF bioavailability. The data suggest that GLP-2 stimulates cancer myofibroblast proliferation, migration and invasion; GLP-2 acts indirectly on epithelial cells partly via increased IGF expression in myofibroblasts and partly, perhaps, by increased bioavailability through degradation of IGFBPs.

Introduction

Mucosal morphology throughout the gastrointestinal tract is determined by interactions between epithelial cells and underlying stromal cells which release a variety of growth factors [34]. These interactions are also a feature of tumour growth where it is now clear that stromal cells provide a supportive and stimulatory niche for cancer cells [16, 35]. A key stromal cell type is the myofibroblast which normally lies in close proximity to the basal membrane of epithelial cells while in cancer these cells are an important component of the stroma that may constitute a high proportion of tumour volume. There is now clear evidence that cancer-associated myofibroblasts (CAMs, often considered to be a subset of cancer-associated fibroblasts) are functionally distinct from myofibroblasts recovered from tissue adjacent to the tumour (ATMs), and from normal tissue [18, 19].

A range of hormonal signals from epithelial enteroendocrine cells (EECs) influence growth of the gastrointestinal tract, including gastrin, cholecystokinin and glucagon-like peptide (GLP)-2. The latter is generated from the glucagon precursor by post-translational cleavage; there are different patterns of processing in pancreatic alpha-cells and L-cells of the ileum and colon. In particular, the main products in L-cells are GLP-2, GLP-1 (which is an insulin secretagogue) and oxyntomodulin (which is a C-terminally extended variant of glucagon). A link between glucagon gene expression and intestinal hypertrophy has been known for several decades, and it is now recognised that GLP-2 is a crucial mediator [10]. Interestingly, GLP-2 receptors are not expressed on intestinal epithelial cells, with the possible exception of some EECs, and as a consequence GLP-2 does not have direct effects on intestinal epithelial cell proliferation. Instead there is evidence that GLP-2 acts indirectly via sub-epithelial cells notably myofibroblasts and neurons [2, 5, 11, 32]. Thus, the evidence suggests that GLP-2 releases growth factors such as IGF, KGF, VEGF-A and EGF-family members which in turn act on epithelial cells to stimulate cell proliferation [7, 13, 32, 40].

Gene array data indicate that a wide variety of gastrointestinal myofibroblasts produce both IGF-1 and IGF-2 as well as a number of IGF binding proteins (IGFBPs) including -3, -4, -5, -6 and -7 [4]. The IGFBPs act to sequester extracellular IGF which can be liberated by IGFBP cleavage, for example by matrix metalloproteinase (MMP)-7 produced by epithelial cells [17]. In the upper gastrointestinal tract, the IGFs act on both epithelial cells and the myofibroblasts themselves to stimulate proliferation as well as migration and invasion. The response of intestinal myofibroblasts to GLP-2 remains relatively unexplored. While there have been studies using a CCD18 myofibroblast cell line, which is derived from normal intestine of a human infant, the possible effects of GLP-2 on cancer myofibroblasts are unclear. The question is of interest in the context of colon cancer, because GLP-2 stimulates normal colonic, as well as small intestinal, growth [30] and reduces injury in a mouse model of colitis [12]; moreover in some mouse models of colon cancer there is evidence that GLP-2 may exert growth-promoting effects [38]. A recent review hypothesized that GLP-2 might act via cancer associated fibroblasts [23]. However, direct studies of the action of GLP-2 on stromal cells from colorectal carcinoma have been neglected. In the present study we have examined the hypothesis that GLP-2 acts on myofibroblasts from colon cancer and adjacent tissue to influence colon cancer cells lines, and have examined the role of IGF in mediating these effects.

Materials and Methods

Cells. Colon cancer cells (HT29, Lovo, SW480) and CCD18 colonic myofibroblasts were obtained from American type culture collection (VA, US). Myofibroblasts were generated from a colon tumour (CAMs) and adjacent tissue (ATMs) of an 85 year old female patient (T3N2M0; post-operative survival 2 months) as previously described [19, 24]; these cells were positive by immunohistochemistry for α-smooth muscle actin and vimentin, and were negative for desmin and cytokeratin. Cancer cells and myofibroblasts were cultured as previously described [19].

Conditioned media. Myofibroblasts (1.5 x 10^6 cells) were plated in T-75 falcon flasks and maintained at 37°C in a 5% v/v CO_2 atmosphere for 24h in full media (FM). Cultures were then washed 3 times with sterile PBS and incubated in 15ml serum free (SF) media with or without GLP-2 (AnaSpec, Freemont, CA, USA) for 24h. Conditioned medium (CM) was collected, centrifuged (7 min, 800 x g, 4°C) and aliquots were stored at -80°C until further use.

EdU incorporation and *Cell Counting Kit-8* assays

For EdU incorporation assays, colonic myofibroblasts and colon cancer cells (2.5x10^4 per well) were seeded and incubated overnight in FM followed by serum starvation for 48 h. Cells then were treated with GLP-2 or CM as appropriate for 24h. Proliferation was assessed by incorporation of 5-ethynyl-2'-deoxyuridine (EdU, 10 μM, 16h) and processing of samples using Click-iT (Invitrogen, Paisley, UK) and Alexa 568-azide. EdU positive cells were visualised on a Zeiss AxioCam HRM fluorescence microscope (Carl Zeiss, Welwyn Garden City, UK) on a 40x objective lens counting the total number of cell nuclei in 10 different fields using DAPI (Vector Laboratories, Peterborough, UK) (blue) and EdU positive nuclei.

Cell Counting Kit-8 assays (Dojindo Laboratories, Munich, Germany; the manufacturers use the abbreviation "CCK8" to describe these kits, but to avoid confusion with cholecystokinin octapeptide which shares the same abbreviation, we use the alternative contraction "CeCo" assays here) were performed on colonic myofibroblasts and cancer cells ($8x10^3$ per well) in 96 well plates incubated overnight in FM followed by incubation with GLP-2 or CM medium in phenol red free SF medium for 72h. On the last day 10µl CeCo reagent was added to the wells and incubated for 2-4h as optimised for each cell type. Readings were taken at 450nm using a GenioPlus plate reader (Tecan, Zurich, Switzerland).

Cell migration and invasion assays. Transwell migration and invasion assays were performed using BD inserts (Corning, New York, USA) as previous described (2.5×10^4 cells per insert) [39]. GLP-2 or CM were added in the lower well together with AG1024 (Calbiochem, Darmstadt, Germany) as appropriate. Ibidi chamber (Ibidi GmbH, Martinsried, Germany) migration assays were performed in 24 well plates. Ibidi culture inserts were placed at the bottom of the wells followed by seeding of 10^5 cancer cells and $5x10^4$ myofibroblasts on separate sides of the insert. After 24h the insert was removed, leaving a 500µm wound; cells were then treated with GLP-2 up to 42h and images taken with a Hamamatsu Camera (Hamamatsu Photonics, Hamamatsu City, Japan) at 0, 6, 24, 32, 42h using a Leica DMIRE2 microscope on a heated stage, humidified chamber (Solent Scientific, Portsmouth, UK); cells in the wound were counted in 5-8 fields per insert.

qPCR. RNA was extracted in 1.25 ml Tri-Reagent (Sigma, Dorset, UK) according to the manufacturer's instructions from control and GLP-2 treated colonic myofibroblasts. RNA pellets were re-suspended in 50 µl of nuclease free water and 4 µg of RNA reverse transcribed with avian myeloblastosis virus reverse transcriptase and oligo-dT primers (Promega). Real time PCR was carried out using an ABI7500 platform (Applied Biosystems,

Warrington, UK) using TaqMan primer/probe sets (human IGF-1, IGF-2, GAPDH), Precision Plus 2x real time PCR master mix (Primer Design, Southampton, UK) and 5'-FAM, 3'-TAMRA double dye probes (Eurogentec, Southampton, UK). All values were standardized to GAPDH. Assays included a no template control (NTC), and a standard curve as previously described [28]. Primers and probes were designed using Primer Express v3.0 (Applied Biosystems) and were purchased from Eurogentec (Seraing, Belgium). Probes for detection of human GAPDH, IGF-1 and IGF-2 cDNA were intron-spanning and were: GAPDH: 5'-GCT CCT CCT GTT CGA CAG TCA-3'(forward), 5'-ACC TTC CCC ATG GTG TCT GA-3' (reverse), 5'-CGT CGC CAG CCG AGC CAC A-3' (probe); IGF-1: 5'-TGT ATT GCG CAC CCC TCA A-3' (forward), 5'-CT CCC TCT ACT TGC GTT CTT CA-3' (reverse), 5'-ACA TGC CCA AGA CCC AGA AGG AAG TAC A-3' (probe); IGF-2, 5'-CCG TGC TTC CGG ACA ACT T-3' (forward), 5'-GGA CTG CTT CCA GGT GTC ATA TT-3' (reverse), 5'-CCC AGA TAC CCC GTG GGC AAG TTC-3' (probe).

Western blotting. Cell extracts were prepared in RIPA buffer and media samples were concentrated with StrataClean resins (Agilent Technologies Ltd, Santa Clara, CA, USA) and processed for Western blotting as previously described [28] using antibodies to IGFBP-3, 4, 5 and -7, MMP-1, -2, -3 and -10 (R&D Systems) and GAPDH (Biodesign, Maine, USA).

Statistics. Results were calculated as mean ± standard error of means (SEM). Student t-test and ANOVA were performed on the data as appropriate with significance at $p<0.05$ using Systat Software Inc. (London, UK) unless otherwise stated.

Results

GLP-2 stimulates myofibroblast proliferation. In initial studies we showed that GLP-2 produced a dose-related increase in EdU incorporation into CCD18 myofibroblasts (Fig 1A,B). We then studied EdU incorporation by colonic CAMs and ATMs. As previously reported for gastric CAMs [19], basal EdU incorporation was greater in the colonic CAMs compared with the corresponding ATMs. In the presence of GLP-2 there was stimulation of EdU incorporation into both CAMs and ATMs and the response in the former was greater than the latter (Fig 1C). In a CeCo assay of cell growth, GLP-2 also stimulated colonic CAM growth and to a lesser extent that of ATMs (Fig 1D).

GLP-2 stimulates myofibroblast migration and invasion. We then examined the actions of GLP-2 on CAM and ATM migration and invasion using Boyden chambers (Fig 2A,B). In both cell types, GLP-2 at a concentration that stimulated proliferation also stimulated migration and these responses were similar in CAMs and ATMs; moreover in both cases they were abolished by AG1024 which selectively inhibits IGF-1 receptor tyrosine kinase activity at the concentrations used [33]. Similarly, there was IGF-dependent stimulation of CAM invasion and in this case the response was significantly greater than that of ATMs.

Myofibroblast conditioned medium stimulates epithelial cell proliferation. Consistent with a previous report that epithelial cells lines lack the capacity to respond to GLP-2 [25], there was no change in EdU incorporation (Fig 3A) by two of the epithelial cell lines used in the present study (SW480 and LoVo) in response to GLP-2 applied directly to the cells while there was a small effect on HT29 cells (Fig 3B). However, there was a small increase in EdU incorporation in response to CAM CM in SW480 cells that was enhanced by pre-treatment of myofibroblasts with GLP-2 and there were greater increases in EdU incorporation by HT29 and LoVo cells in response to CAM CM, and in both cases there were small further increases using CM from GLP-2-treated myofibroblasts. In all three intestinal cell lines, the

responses to control CM and to GLP-2 CM were suppressed by AG1024 (Fig 3C). In CeCo assays of cell growth, there were robust responses by all three intestinal epithelial lines to control myofibroblast CM and small but significantly increases in response to GLP-2 pretreatment of myofibroblasts (Fig 3D).

Myofibroblast conditioned medium stimulates epithelial cell migration and invasion. When GLP-2 was applied directly to epithelial cells (SW480, HT29, LoVo) there was no effect on migration or invasion in Boyden chambers (Fig 4A). However, there was strong stimulation of migration of all three cell lines in response to control CAM CM and there were small but significant further increases in response to CM from GLP-2 treated myofibroblasts (Fig 4B). In each case the response was inhibited by AG1024. There were relatively modest increases in invasion of HT29 and LoVo cells in response to control myofibroblast CM but the response was strongly enhanced by GLP-2-treated CM; all responses were inhibited by AG1024 (Fig 4C).

GLP-2 stimulates migration in co-cultures of epithelial cells and myofibroblasts. In view of the potential for cross-talk between epithelial cells and myofibroblasts we then asked whether the response to GLP-2 was preserved when these cells were co-cultured. For this purpose we used Ibidi chambers with myofibroblasts in one chamber and epithelial cells in the other so that subsequent removal of the insert yielded cultures of the two cells separated by 500 μm (Fig 5A). Over a period of 42 h after removing the insert there was progressive migration of both cell types toward each other (Fig 5A). The numbers of migrating epithelial cells were roughly 10 times higher than those of myofibroblasts. Nevertheless at each time point examined from 6 – 42 h the presence of GLP-2 increased the migration of each of the epithelial cell lines and of the co-cultured CAMs (Fig 5B).

GLP-2 increases IGF-1 and IGF-2 transcript abundance. In view of the evidence that GLP-2 might act via IGF we first examined microarray data from myofibroblasts which indicated that both IGF-1 and IGF-2 were expressed in colonic CAMs and ATMs; the relative abundance of IGF-1 in colonic myofibroblasts was higher compared to IGF-2, while in gastric myofibroblasts IGF-2 was dominant [4]. We then used qPCR for both IGF-1 and IGF-2 to assess responses to GLP-2. At a concentration of GLP-2 (10 nM) that increased myofibroblast migration and proliferation, there was 1.8 ± 0.2 fold higher IGF-1 transcript abundance and 1.5 ± 0.2 fold higher IGF-2 transcript abundance.

GLP-2 promotes IGFBP degradation in myofibroblasts medium. Since there is evidence that GLP-2 might act via IGFBP-4 [1] we examined by Western blot the profile of IGFBP-4 and also of IGFBP-3, -5 and -7 in CAMs treated with GLP-2. In control media there were clear bands corresponding to intact IGFBP-4, -5 and -7 (Fig 6A), while IGFBP-3 was undetectable (not shown). There were also minor bands corresponding to degradation products of IGFBP4 (15 kD) and IGFBP-5 (10-15 kD). In the presence of GLP-2 the bands corresponding to intact IGFBP-4, and -5 were maintained but there was increased abundance of the degradation products. In the presence of an inhibitor of MMP activity (GM6001, 10 μM) the action of GLP-2 in promoting degradation of IGFBP4 and -5 was inhibited (Fig 6A). There was also an increase in IGFBP-4 and -5 in cell extracts in response to GLP-2 compatible with increased expression (Fig 6B). Multiple proMMPs are secreted by myofibroblasts (MMP-1, -2, -3 and -10) [18]. Of these, MMP-1 was increased in abundance in GLP-2 treated cells and media (Fig 6C,D) although there was little or no change in MMP-2, -3 and -10.

Discussion

The present study provides evidence that GLP-2 acts on cancer-derived myofibroblasts to stimulate their proliferation, migration and invasion. These effects are blocked by an inhibitor of IGF1 receptor tyrosine kinase and since there is an increase in IGF-1 and -2 transcript abundance as well as increased degradation of IGFBP-4 and -5, the data support the idea that the actions of GLP-2 on myofibroblasts are at least partly mediated by IGF. Consistent with previous findings, GLP-2 had little or no effect on three intestinal epithelial cell lines, but conditioned medium from CAMs stimulated proliferation, migration and invasion of these cells, and to varying degrees GLP-2 treatment of myofibroblasts enhanced the responses in an IGF-receptor dependent mechanism. The data add to previous work in this area by indicating (a) that GLP-2 might regulate myofibroblast numbers and motility, and (b) that myofibroblasts in cancer are putative targets for GLP-2. In view of the importance of myofibroblasts in determining the tumour microenvironment the data raise the prospect that GLP-2 might influence cancer progression.

The importance of myofibroblasts as targets for the trophic action of GLP-2 in the intestine has been clear for some time [7, 32]. Previous work has made use of CCD18 myofibroblasts that are derived from normal infant intestine [7], mixed intestinal cell cultures [13] and intestinal sub-epithelial fibroblasts [29]. The present findings extend these studies to include myofibroblasts derived from colon cancer and adjacent tissue. For the most part previous studies have focussed on the mechanisms by which myofibroblasts influence epithelial cell growth. While the growth factor responses of the myofibroblasts themselves have received attention, less has been given to other aspects of myofibroblasts biology. The observation that GLP-2 increases myofibroblast cell number, and very likely cell position (via actions on migration/invasion), suggests a more dynamic system than previously supposed.

It is recognised that cancer-derived fibroblasts and myofibroblasts differ from their counterparts in normal tissue [9, 19, 31]. The different properties of CAMs may, at least to some extent, reflect epigenetic changes [22]. The differences are important given the emerging evidence that stromal cells in general [16], and myofibroblasts in particular, stimulate cancer progression [8]. Previous work has shown that in both gastric and oesophageal cancer, CAMs exhibit increased proliferation, migration and invasion compared with ATMs [19, 27]. The present study has shown that colonic CAMs similarly exhibit a more aggressive phenotype than colonic ATMs i.e. increased basal and stimulated proliferation and invasion in response to GLP-2. The fact that these cells retain the ability to respond to GLP-2 provides a mechanism by which GLP-2 might influence cancer progression [23]. The therapeutic value of GLP-2 in treatment of short bowel syndrome is now widely appreciated, but it would be as well to keep in mind that in some circumstances GLP-2 might have deleterious effects in influencing cellular microenvironments. A particularly clear example is provided by gastrointestinal stromal tumours that have been reported to express GLP-2R [26]; the possibility that GLP-2 may also act on colon cancers via their stromal component should now be considered.

A number of growth factors have been proposed to mediate the actions of GLP-2 on myofibroblasts, including IGF [29], VEGF-A [7], KGF [32] and EGF-family members [3, 40]. It is important to be clear that these need not be mutually exclusive. Our data suggest that colonic CAMs stimulate epithelial cell growth, migration and invasion by an IGF-receptor dependent mechanism [37] that is enhanced to varying degrees by treatment of myofibroblasts with GLP-2. The increase in epithelial proliferation was modest, the migratory response was stronger and the invasive responses in two cells lines were very strong. High constitutive expression of IGF has already been reported [29] and would account for the present findings on intestinal epithelial cell proliferation and migration. In the case of the

strong invasion response of these cells we suggest that increased bioavailability of IGF as well as increased MMP-activity [18] might interact to enhance the response.

There was increased transcript abundance of IGF-1 and IGF-2 in response to GLP-2 which would provide one mechanism to account for the biological responses of both epithelial cells and myofibroblasts. However, our data suggest that there are also likely to be other mechanisms. Secreted IGF is sequestered by IGFBPs. In gastrointestinal myofibroblasts the main IGFBPs include IGFBP-4, -5 and -7. Cleavage of IGFBP-5 by MMP-7 released by epithelial cells has previously been shown to increase the bioavailability of IGF-2 which may stimulate both epithelial and stromal cells [17]. In addition to regulating IGF bioavailability, some IGFBPs or their fragments, may exert independent biological activities [14, 20]. Recently, a role for IGFBP-4 in mediating the effects of GLP-2 was indicated by the observation that in mice null for IGFBP-4 the effect of GLP-2 was inhibited [1]. The precise cellular mechanisms remain uncertain, but it is interesting that in our studies GLP-2 treatment of myofibroblasts increased the abundance of IGFBP-4 degradation products seen in western blot; there was a similar effect on IGFBP-5 but not IGFBP-7. Moreover, there was an increase in cellular abundance of IGFBP-4 and -5 compatible with stimulation of expression by GLP-2 as previously reported [1]. The degradation of IGFBP-4 in other systems is attributable to PAPP-A which is a member of the pappalysin group of metzincin metalloproteinase [6]. The GLP-2 stimulated degradation of IGFBP-4 and -5 was blocked by a broad spectrum inhibitor of MMP activity, GM6001, which is reported to have little effect on pappalysins [15]. We found GLP-2 increased the abundance in media and cell extracts of proMMP-1 (although not proMMP-2, -3 and -10). We suggest therefore, that GLP-2 increases the expression in myofibroblasts of IGFBP-4 and -5, and proMMP-1. Further work is now needed to determine the biological significance of the degradation products of IGFBP-4 and -5, particularly with respect to IGF binding and any potential biological activities independent of IGF.

The maintenance of tissue architecture is presumed to involve two-way interactions in vivo between myofibroblasts and epithelial cells. We attempted to design a simple in vitro model to establish whether the actions of GLP-2 on myofibroblasts, and those of myofibroblasts on epithelial cells, were preserved when the two cell types were in co-culture. The data obtained using Ibidi chambers indicate that the migratory responses observed when the two cells were cultured separately were preserved in co-cultures. Interestingly, though, the magnitude of the migratory responses by epithelial cells was considerably greater than that of myofibroblasts. Nevertheless the data allow us to conclude that any feedback mechanism from intestinal cells that might exist to limit the myofibroblast response to GLP-2 is relatively modest.

Alongside an appreciation of the therapeutic benefits of GLP-2, there has also been an appreciation, based on data from animal models, that GLP-2 might have growth promoting effects with deleterious consequences in cancer [36]. Thus in two mouse models of carcinogen-induced colon cancer (dimethylhydrazine, azoxymethane) administration of GLP-2 increased tumour size [21, 38], although in other models eg $APC^{min-/-}$ mice, it had no effect [25]. Whether or not GLP-2 aggravates human colon cancer progression needs to be kept under review. The case for further studies of GLP-2 and human colon cancer is supported by the present data which raise the prospect that GLP-2 acts on human colon cancer-derived myofibroblasts to trigger pathways influencing myofibroblast number and motility and, indirectly, cancer cell function. It is worth stressing, that since the present data indicate that GLP-2 targets the IGF system in colonic myofibroblasts this system would presumably be susceptible to novel therapeutic strategies targeted at IGF-responsive cells in colon cancer [41].

References

[1] Austin K, Imam NA, Pintar JE, Brubaker PL. IGF binding protein-4 is required for the growth effects of glucagon-like peptide-2 in murine intestine. Endocrinology. 2015;156:429-36.
[2] Austin K, Markovic MA, Brubaker PL. Current and potential therapeutic targets of glucagon-like peptide-2. Current opinion in pharmacology. 2016;31:13-8.
[3] Bahrami J, Yusta B, Drucker DJ. ErbB activity links the glucagon-like peptide-2 receptor to refeeding-induced adaptation in the murine small bowel. Gastroenterology. 2010;138:2447-56.
[4] Balabanova S, Holmberg C, Steele I, Ebrahimi B, Rainbow L, Burdyga T, et al. The neuroendocrine phenotype of gastric myofibroblasts and its loss with cancer progression. Carcinogenesis. 2014;35:1798-806.
[5] Bjerknes M, Cheng H. Modulation of specific intestinal epithelial progenitors by enteric neurons. Proceedings of the National Academy of Sciences of the United States of America. 2001;98:12497-502.
[6] Boldt HB, Conover CA. Pregnancy-associated plasma protein-A (PAPP-A): a local regulator of IGF bioavailability through cleavage of IGFBPs. Growth Horm IGF Res. 2007;17:10-8.
[7] Bulut K, Pennartz C, Felderbauer P, Meier JJ, Banasch M, Bulut D, et al. Glucagon like peptide-2 induces intestinal restitution through VEGF release from subepithelial myofibroblasts. European journal of pharmacology. 2008;578:279-85.
[8] De Wever O, Demetter P, Mareel M, Bracke M. Stromal myofibroblasts are drivers of invasive cancer growth. IntJCancer. 2008;123:2229-38.
[9] De Wever O, Van Bockstal M, Mareel M, Hendrix A, Bracke M. Carcinoma-associated fibroblasts provide operational flexibility in metastasis. Seminars in cancer biology. 2014;25:33-46.
[10] Drucker DJ, Erlich P, Asa SL, Brubaker PL. Induction of intestinal epithelial proliferation by glucagon-like peptide 2. Proceedings of the National Academy of Sciences of the United States of America. 1996;93:7911-6.
[11] Drucker DJ, Yusta B. Physiology and pharmacology of the enteroendocrine hormone glucagon-like peptide-2. Annual review of physiology. 2014;76:561-83.
[12] Drucker DJ, Yusta B, Boushey RP, DeForest L, Brubaker PL. Human [Gly2]GLP-2 reduces the severity of colonic injury in a murine model of experimental colitis. The American journal of physiology. 1999;276:G79-91.
[13] Dube PE, Forse CL, Bahrami J, Brubaker PL. The essential role of insulin-like growth factor-1 in the intestinal tropic effects of glucagon-like peptide-2 in mice. Gastroenterology. 2006;131:589-605.
[14] Firth SM, Baxter RC. Cellular actions of the insulin-like growth factor binding proteins. EndocrRev. 2002;23:824-54.
[15] Garcia-Castellanos R, Tallant C, Marrero A, Sola M, Baumann U, Gomis-Ruth FX. Substrate specificity of a metalloprotease of the pappalysin family revealed by an inhibitor and a product complex. Archives of biochemistry and biophysics. 2007;457:57-72.
[16] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144:646-74.
[17] Hemers E, Duval C, McCaig C, Handley M, Dockray GJ, Varro A. Insulin-like growth factor binding protein-5 is a target of matrix metalloproteinase-7: implications for epithelial-mesenchymal signaling. Cancer Res. 2005;65:7363-9.

[18] Holmberg C, Ghesquiere B, Impens F, Gevaert K, Kumar JD, Cash N, et al. Mapping proteolytic processing in the secretome of gastric cancer-associated myofibroblasts reveals activation of MMP-1, MMP-2, and MMP-3. Journal of proteome research. 2013;12:3413-22.
[19] Holmberg C, Quante M, Steele I, Kumar J, Balabanova S, Duval C, et al. Release of TGFbetaig-h3 by gastric myofibroblasts slows tumor growth and is decreased with cancer progression. Carcinogenesis. 2012;33:1553-62.
[20] Hwa V, Oh Y, Rosenfeld RG. The insulin-like growth factor-binding protein (IGFBP) superfamily. Endocr Rev. 1999;20:761-87.
[21] Iakoubov R, Lauffer LM, Trivedi S, Kim YI, Brubaker PL. Carcinogenic effects of exogenous and endogenous glucagon-like peptide-2 in azoxymethane-treated mice. Endocrinology. 2009;150:4033-43.
[22] Jiang L, Gonda TA, Gamble MV, Salas M, Seshan V, Tu S, et al. Global hypomethylation of genomic DNA in cancer-associated myofibroblasts. Cancer Res. 2008;68:9900-8.
[23] Kannen V, Garcia SB, Stopper H, Waaga-Gasser AM. Glucagon-like peptide 2 in colon carcinogenesis: possible target for anti-cancer therapy? Pharmacol Ther. 2013;139:87-94.
[24] Kemeny LV, Schnur A, Czepan M, Rakonczay Z, Jr., Gal E, Lonovics J, et al. Na+/Ca2+ exchangers regulate the migration and proliferation of human gastric myofibroblasts. American journal of physiology Gastrointestinal and liver physiology. 2013;305:G552-63.
[25] Koehler JA, Harper W, Barnard M, Yusta B, Drucker DJ. Glucagon-like peptide-2 does not modify the growth or survival of murine or human intestinal tumor cells. Cancer research. 2008;68:7897-904.
[26] Korner M, Rehmann R, Reubi JC. GLP-2 receptors in human disease: high expression in gastrointestinal stromal tumors and Crohn's disease. Molecular and cellular endocrinology. 2012;364:46-53.
[27] Kumar JD, Holmberg C, Kandola S, Steele I, Hegyi P, Tiszlavicz L, et al. Increased expression of chemerin in squamous esophageal cancer myofibroblasts and role in recruitment of mesenchymal stromal cells. PloS one. 2014;9:e104877.
[28] Kumar JD, Steele I, Moore AR, Murugesan SV, Rakonczay Z, Venglovecz V, et al. Gastrin stimulates MMP-1 expression in gastric epithelial cells: putative role in gastric epithelial cell migration. American journal of physiology Gastrointestinal and liver physiology. 2015;309:G78-86.
[29] Leen JL, Izzo A, Upadhyay C, Rowland KJ, Dube PE, Gu S, et al. Mechanism of action of glucagon-like peptide-2 to increase IGF-I mRNA in intestinal subepithelial fibroblasts. Endocrinology. 2011;152:436-46.
[30] Litvak DA, Hellmich MR, Evers BM, Banker NA, Townsend CM, Jr. Glucagon-like peptide 2 is a potent growth factor for small intestine and colon. Journal of gastrointestinal surgery : official journal of the Society for Surgery of the Alimentary Tract. 1998;2:146-50.
[31] Orimo A, Gupta PB, Sgroi DC, renzana-Seisdedos F, Delaunay T, Naeem R, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell. 2005;121:335-48.
[32] Orskov C, Hartmann B, Poulsen SS, Thulesen J, Hare KJ, Holst JJ. GLP-2 stimulates colonic growth via KGF, released by subepithelial myofibroblasts with GLP-2 receptors. Regul Pept. 2005;124:105-12.
[33] Parrizas M, Gazit A, Levitzki A, Wertheimer E, LeRoith D. Specific inhibition of insulin-like growth factor-1 and insulin receptor tyrosine kinase activity and biological function by tyrphostins. Endocrinology. 1997;138:1427-33.
[34] Powell DW, Adegboyega PA, Di Mari JF, Mifflin RC. Epithelial cells and their neighbors I. Role of intestinal myofibroblasts in development, repair, and cancer. AmJPhysiol GastrointestLiver Physiol. 2005;289:G2-G7.
[35] Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. Nature medicine. 2013;19:1423-37.

[36] Rowland KJ, Brubaker PL. The "cryptic" mechanism of action of glucagon-like peptide-2. American journal of physiology Gastrointestinal and liver physiology. 2011;301:G1-8.
[37] Rowland KJ, Trivedi S, Lee D, Wan K, Kulkarni RN, Holzenberger M, et al. Loss of glucagon-like peptide-2-induced proliferation following intestinal epithelial insulin-like growth factor-1-receptor deletion. Gastroenterology. 2011;141:2166-75 e7.
[38] Thulesen J, Hartmann B, Hare KJ, Kissow H, Orskov C, Holst JJ, et al. Glucagon-like peptide 2 (GLP-2) accelerates the growth of colonic neoplasms in mice. Gut. 2004;53:1145-50.
[39] Varro A, Noble PJ, Pritchard DM, Kennedy S, Hart CA, Dimaline R, et al. Helicobacter pylori induces plasminogen activator inhibitor 2 (PAI-2) in gastric epithelial cells through NF-kB and RhoA: implications for invasion and apoptosis Cancer Res. 2004;64:1695-702.
[40] Yusta B, Holland D, Koehler JA, Maziarz M, Estall JL, Higgins R, et al. ErbB signaling is required for the proliferative actions of GLP-2 in the murine gut. Gastroenterology. 2009;137:986-96.
[41] Zhong H, Fazenbaker C, Chen C, Breen S, Huang J, Yao X, et al. Overproduction of IGF-2 drives a subset of colorectal cancer cells, which specifically respond to an anti-IGF therapeutic antibody and combination therapies. Oncogene. 2017;36:797-806.

Legends

Fig 1. GLP-2 stimulates myofibroblast proliferation. A, Representative image of EdU labelled myofibroblast (filled arrow; unlabelled cells, open arrows). B, dose-response relationship for GLP-2 stimulation of EdU incorporation by CCD-18 cells (n = 4). C, GLP-2 (10 nM) stimulates EdU incorporation into both cancer-derived myofibroblasts (CAM, n = 6) and adjacent tissue myofibroblasts (ATM, n = 3). D, GLP-2 also increases cell growth measured by CeCo assay (n = 4). Horizontal arrows * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Fig 2. Stimulation of myofibroblast migration by GLP-2. A, GLP-2 (10nM) increases migration of both CAMs and ATMs in Boyden chamber chemotaxis assays and the response is reversed by an inhibitor of IGF1 receptor tyrosine kinase (AG1024, 20μM)(n = 3 – 6). B, similar data for invasion assays (n = 3). See Legend to Fig 1 for further details.

Fig 3. GLP-2 enhances the increase in intestinal epithelial cell proliferation in response to myofibroblast conditioned media. A, Representative images of EdU (red) labelled HT29, SW480 and LoVo cells (nuclei stained blue with DAPI). B, GLP-2 (10 nM) has no direct effect on proliferation of SW480 or LoVo cells and a small effect on HT29 cells (n = 3). C, Conditioned media from myofibroblasts stimulates EdU incorporation in SW480, HT29 and LoVo cells: the responses are enhanced by previous treatment of myofibroblasts with GLP-2 (10 nM) and are blocked by the IGF receptor inhibitor AG1024 (20 μM)(n = 3 – 6). D, in CeCo assays myofibroblast CM stimulates growth of SW480, HT29 and LoVo cells and the effect is enhanced by prior treatment of myofibroblasts with GLP-2 (10 nM); GLP-2 alone has no effect (n = 3 – 4). See Legend to Fig 1 for further details.

Fig 4. GLP-2 enhances the increase in intestinal epithelial cell migration and invasion in response to myofibroblast conditioned media. A, GLP-2 alone (10nM) has no effect on migration (left) or invasion (right) of SW480, HT29 or LoVo cells in Boyden chamber chemotaxis assays (n = 3). B, Conditioned media (CM) from myofibroblasts stimulates migration of SW480, HT29 and LoVo cells: the responses are enhanced by previous treatment of myofibroblasts with 10nM GLP-2 (GLP-2 CM) and are blocked by the IGF receptor inhibitor AG1024 (20 μM)(n = 3 – 6). C, similar results for invasion assays (n = 3). See Legend to Fig 1 for further details.

Fig 5. Mutual stimulation of migration by epithelial cells and myofibroblasts in co-cultures treated with GLP-2. A, Representative images of Ibidi chambers with CAMs on one side and either SW480, HT29 or LoVo cells on the other side; on the left is a schematic illustrating the experimental design; thereafter are images taken at 0, 6, 24, 32 and 42 h. At each time point epithelial cells are shown on the left and CAMs on the right; the box indicates the area in which cells were quantified. B, time course of responses measured as numbers of cells in the defined area migrating towards the opposite side in either control co-cultures, or treated with GLP-2 (10 nM). The data are shown for co-cultures of SW480 and CAMs (left), HT29 and CAMs (centre), and LoVo cells and CAMs (right). In each case the data for epithelial cells are in the upper panel and CAMs from the corresponding co-culture in the lower panel (n = 3). See Legend to Fig 1 for further details.

Fig 6. Western blots showing IGFBP-4, -5 and –7, and MMP-1, -2, -3 and -10 after GLP-2 treatment of myofibroblasts. A, IGFBP-4, -5 and –7 in media of CAMs treated with GLP-2

(10nM, 24h) with or without the MMP-inhibitor GM6001 (10 μM). B, similar data including GAPDH for corresponding cell extracts. C, MMP-1, -2, -3 and -10 in media of CAMs treated with GLP-2. D, MMP-1 and GAPDH in the corresponding cell extracts. Representative images from 4 independent experiments.

Figure

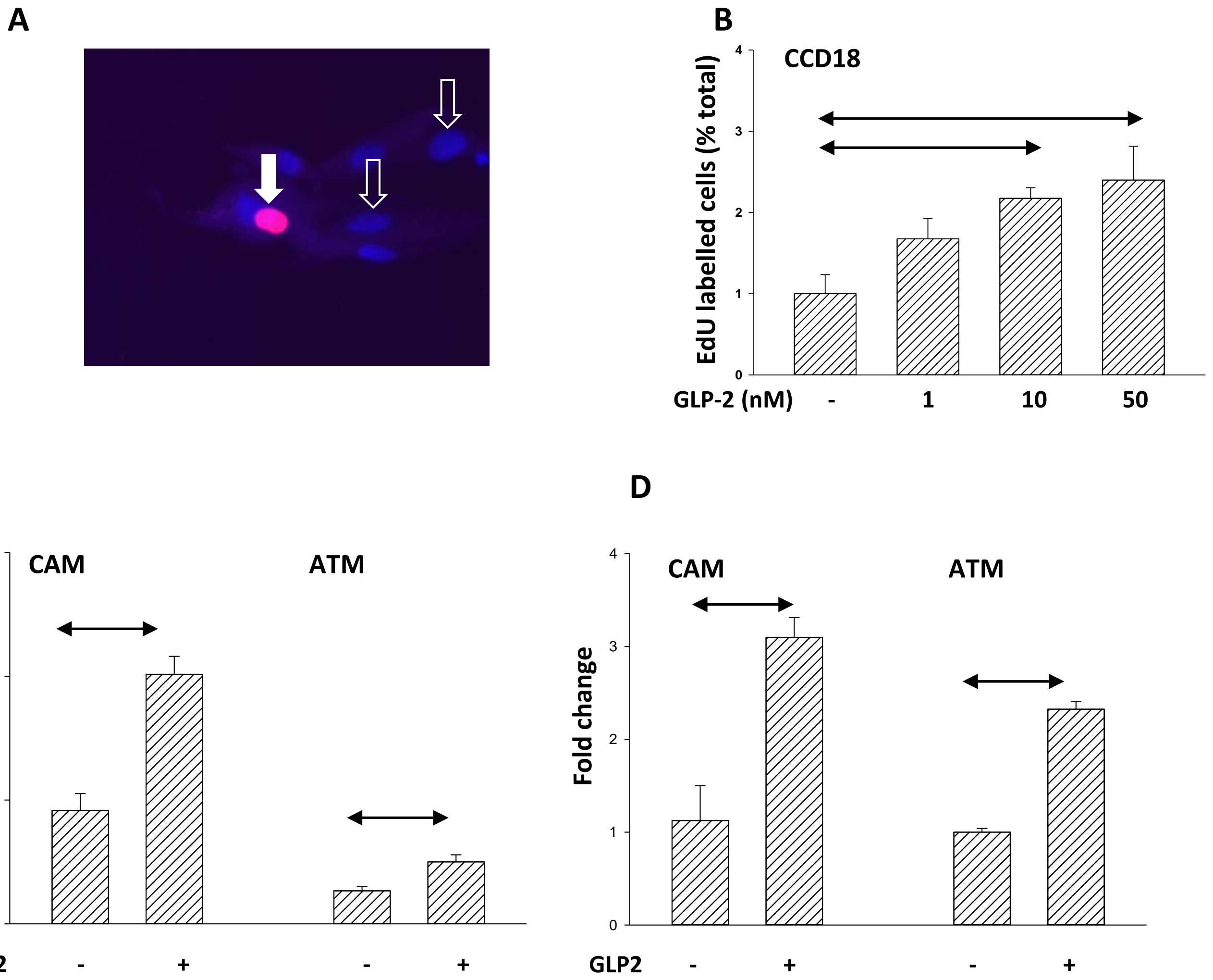


Figure 1

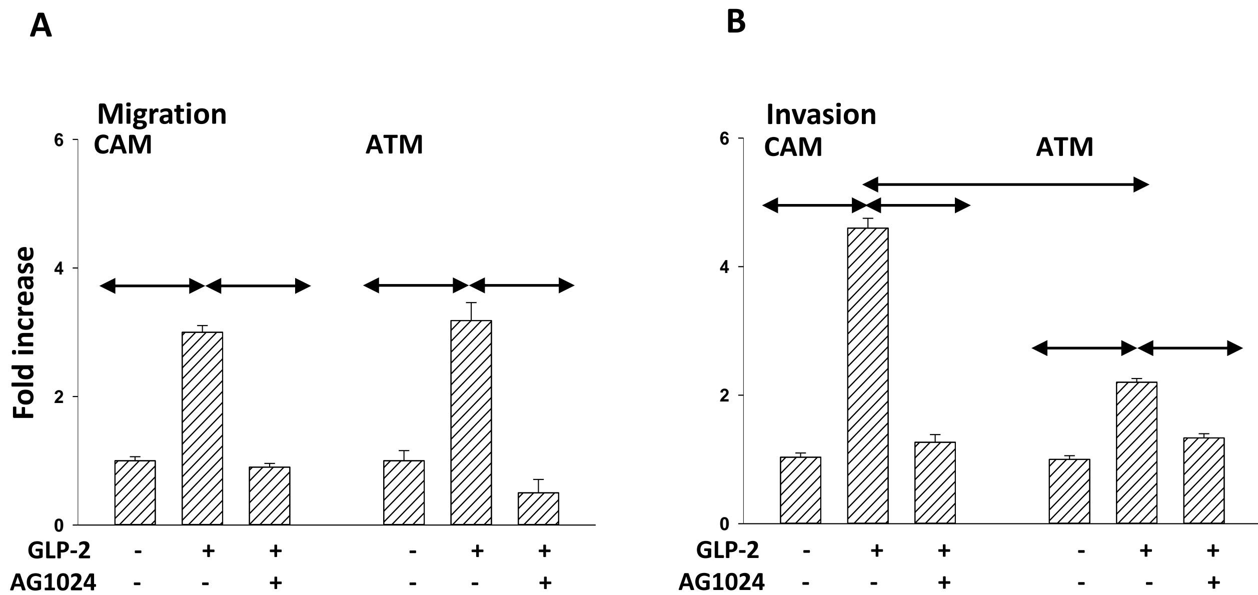
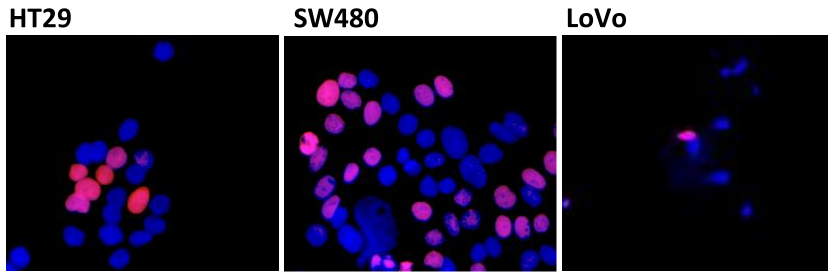
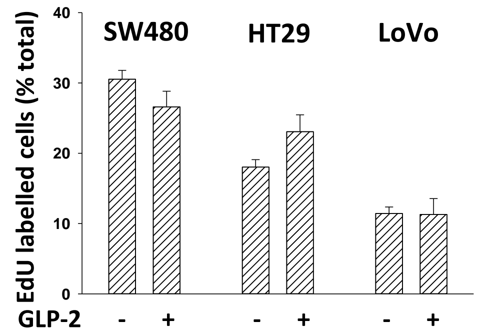
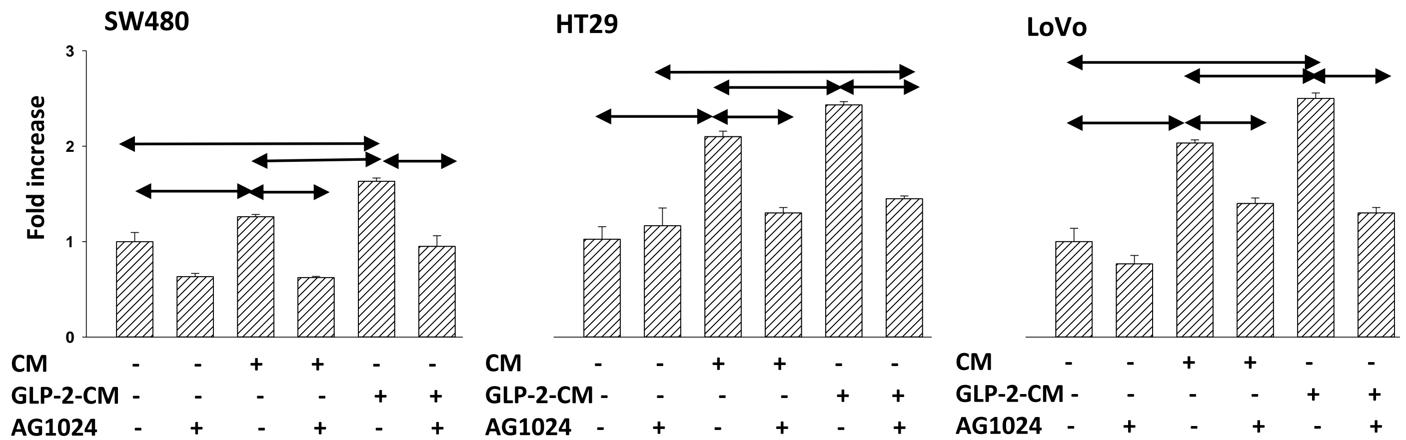


Figure 2







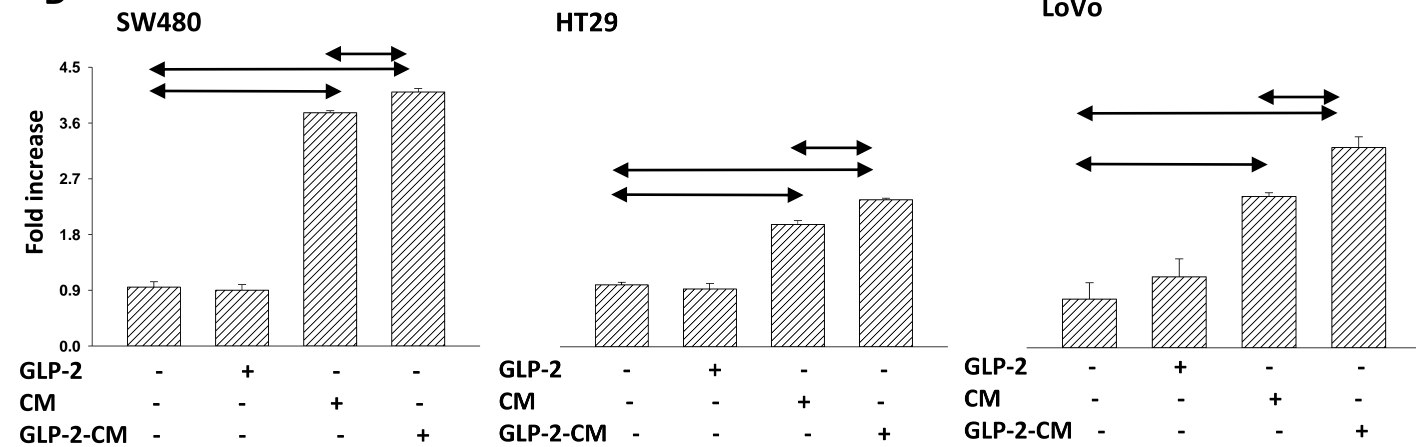


Figure 3

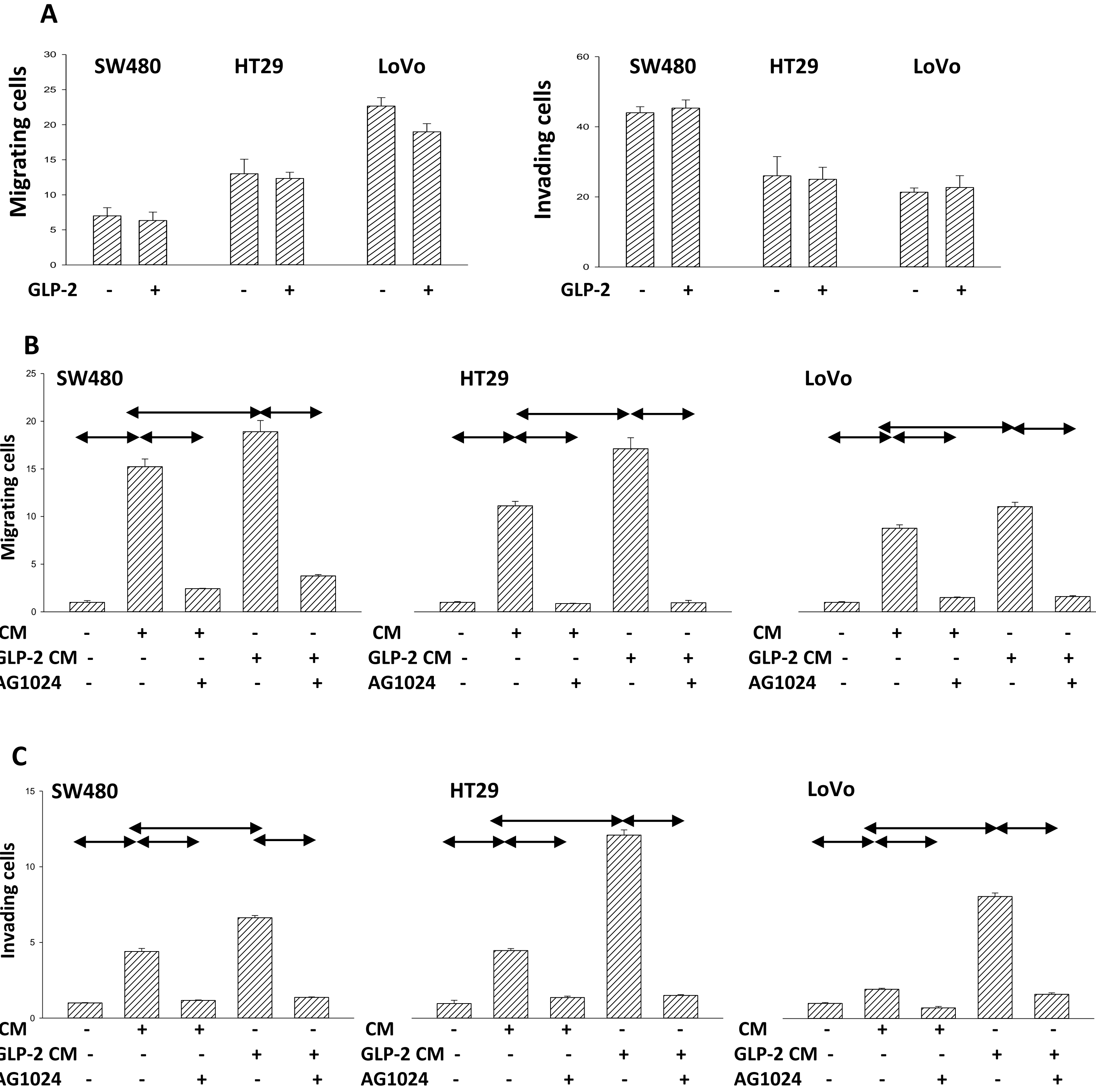
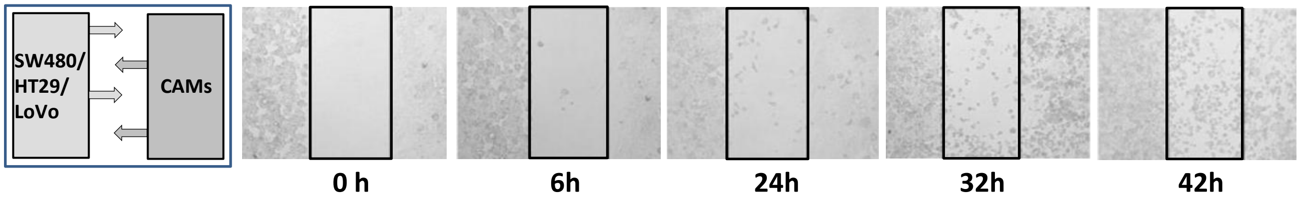


Figure 4

A



B

SW480

HT29

LoVo

6h 24h 32h 42h

Migrating cells

500
400
300
200
100
0

CAM

CAM

CAM

Migrating cells

70
60
50
40
30
20
10
0

GLP-2 - + - + - + - +

GLP-2 - + - + - + - +

GLP-2 - + - + - + - +

Figure 5

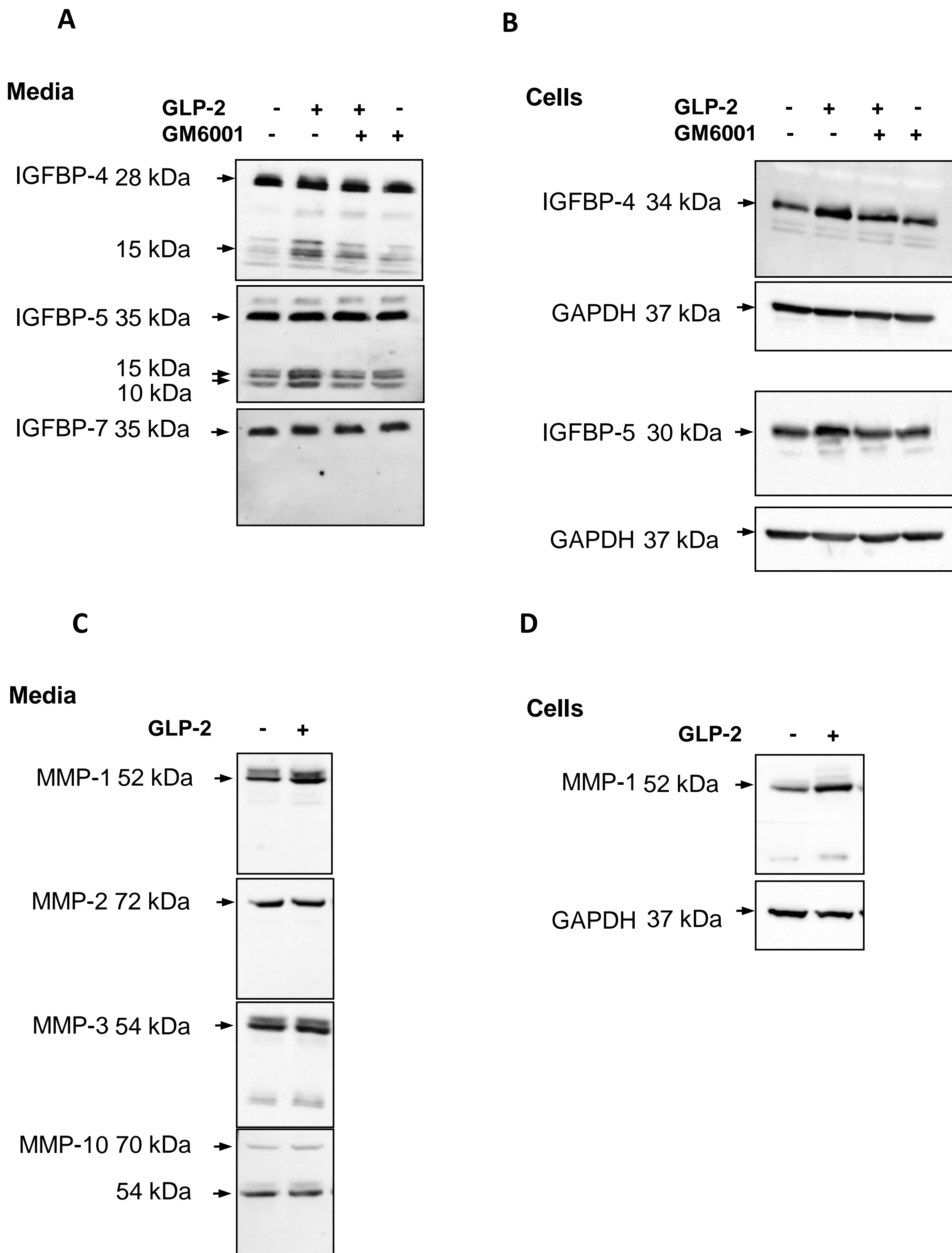


Figure 6

Figure

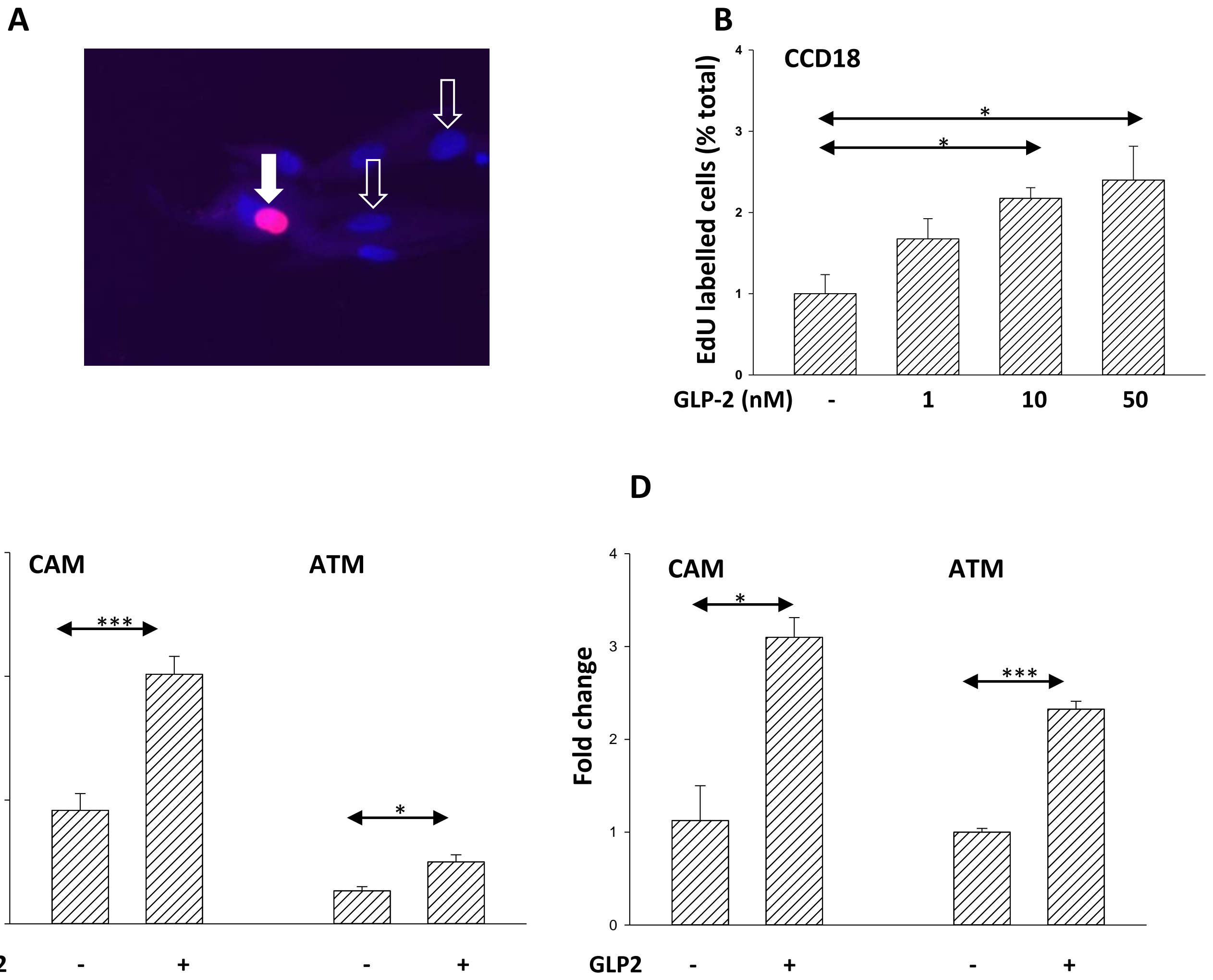


Figure 1

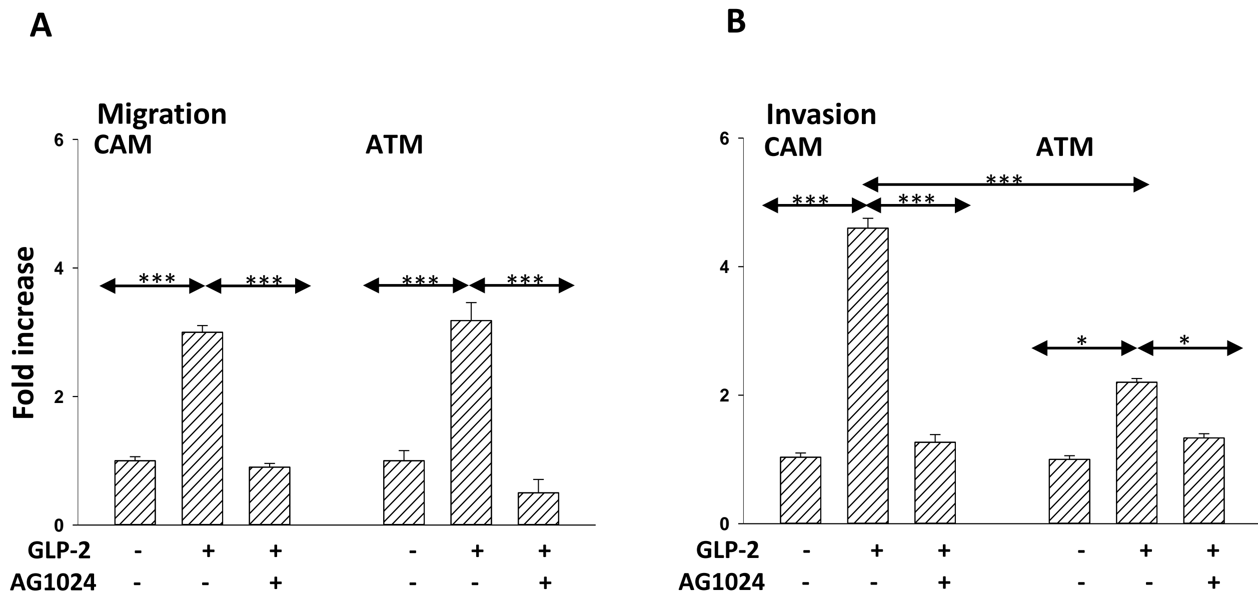
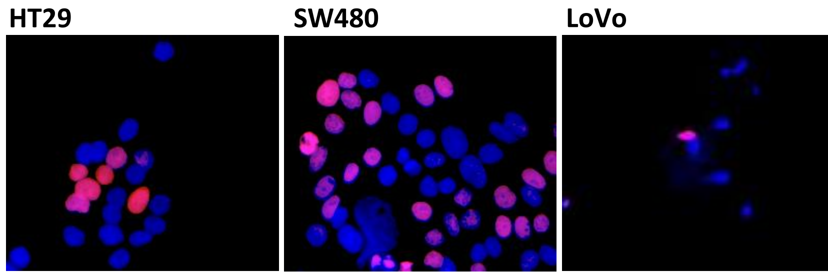


Figure 2

A



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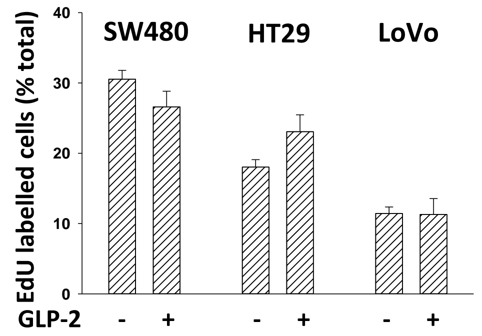

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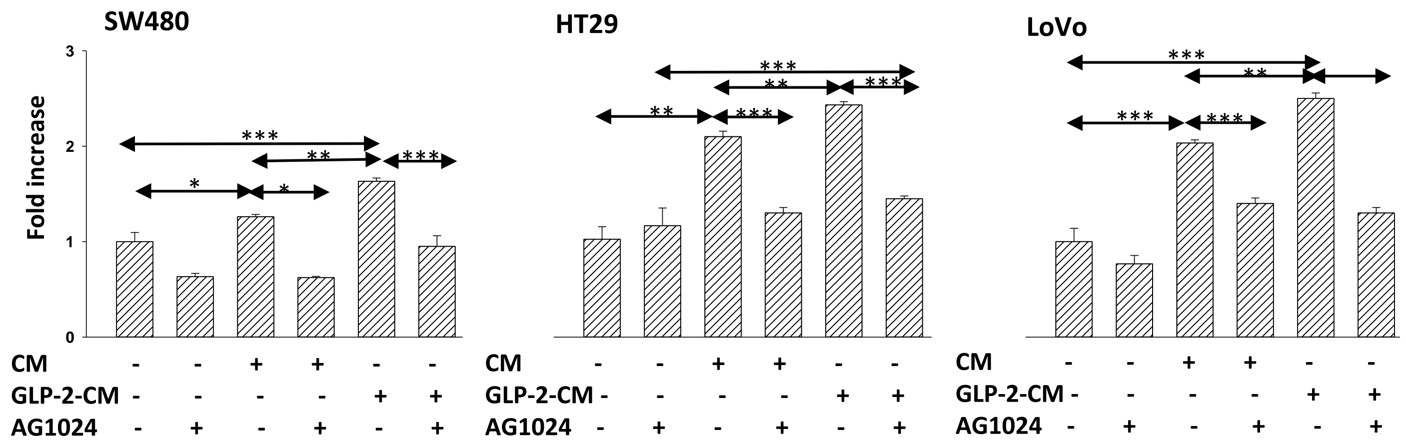

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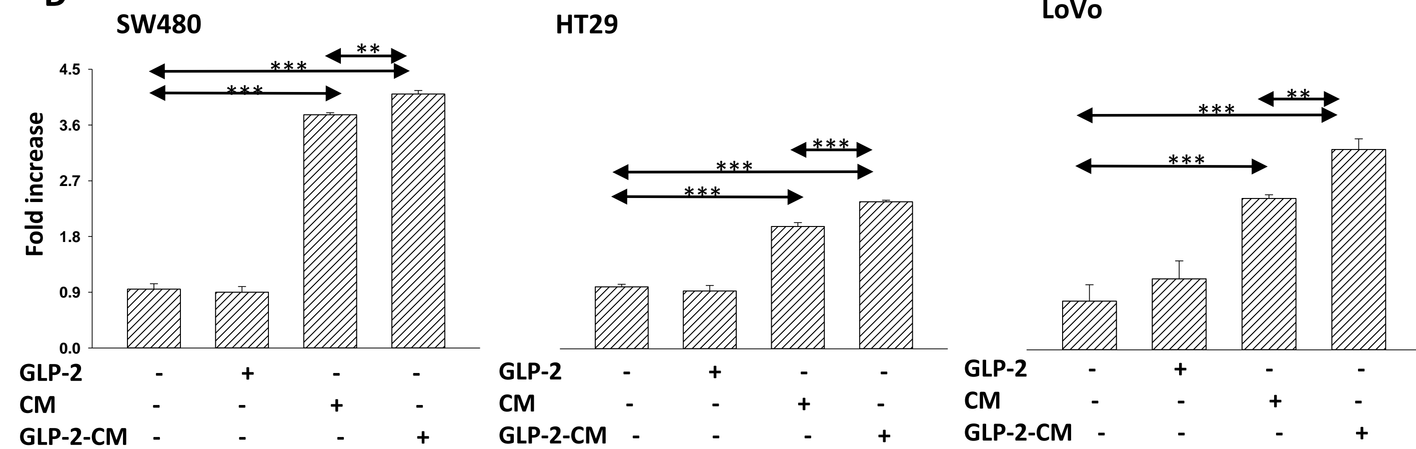


Figure 3

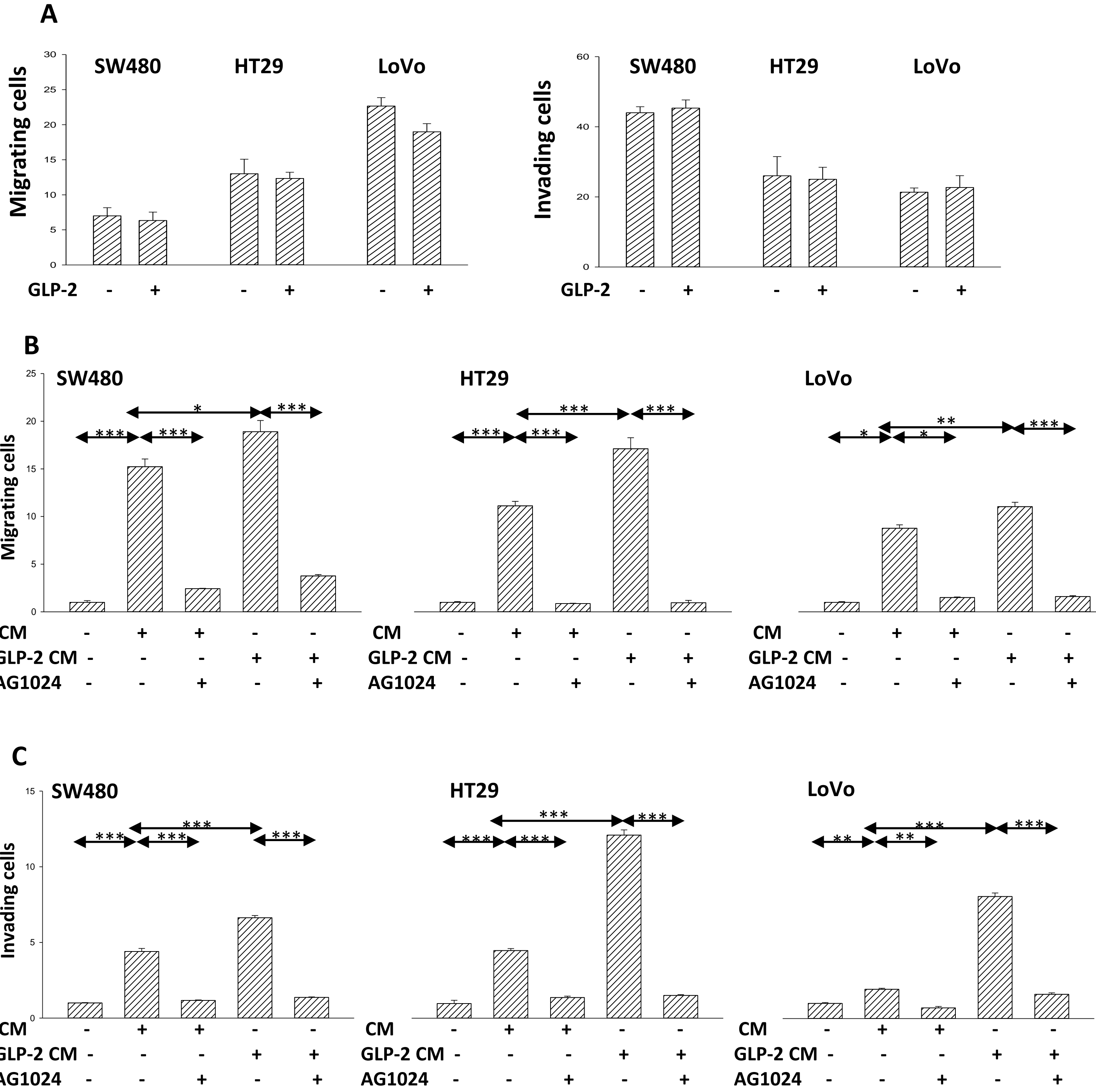
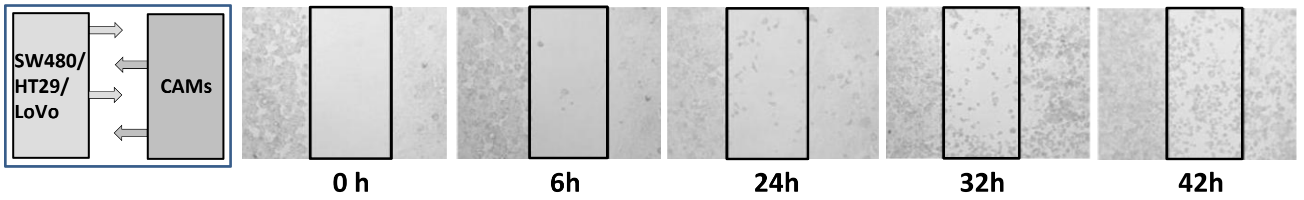


Figure 4

A



B

SW480 HT29 LoVo

6h 24h 32h 42h

Migrating cells

0 100 200 300 400 500

CAM CAM CAM

Migrating cells

0 10 20 30 40 50 60 70

GLP-2 - + - + - + - +

Figure 5

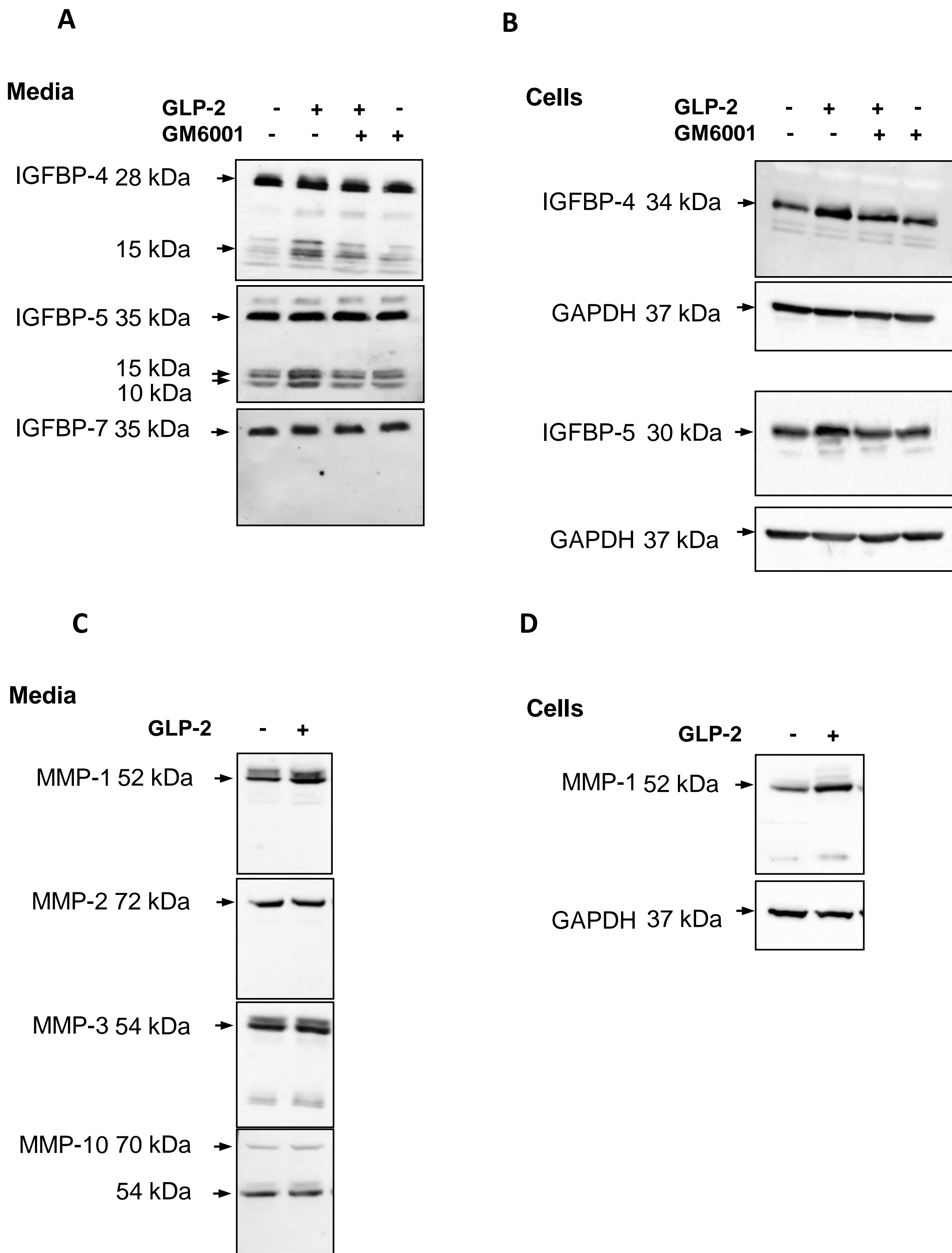


Figure 6