HGF/Met signalling promotes PGE2 biogenesis via regulation of COX-2 and 15-PGDH expression in colorectal cancer cells

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Evidence points towards a pivotal role for cyclooxygenase (COX)-2 in promoting colorectal tumorigenesis through increasing prostaglandin E2 (PGE2) levels. PGE2 signalling is closely associated with the survival, proliferation and invasion of colorectal cancer cells. Recently, a reduction in PGE2 inactivation, a process mediated by the nicotinamide adenine dinucleotide (NAD+)-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH), has also been shown to promote tumoral PGE2 accumulation. The hepatocyte growth factor (HGF) receptor, Met, is frequently over-expressed in colorectal tumours and promotes cancer growth, metastasis and resistance to therapy, although the mechanisms for this have not been fully elucidated. Here, we report that HGF/Met signalling can promote PGE2 biogenesis in colorectal cancer cells via COX-2 up-regulation and 15-PGDH down-regulation at the protein and messenger RNA level. Pharmacological inhibition of MEK and PI3K suggested that both extracellular signal-regulated kinase (ERK) and AKT signalling are required for COX-2 protein up-regulation and 15-PGDH down-regulation downstream of Met. Notably, inhibition of Met with the small molecule inhibitor SU11274 reduced COX-2 expression and increased 15-PGDH expression in high Met-expressing cells. We also show that hypoxia potentiated HGF-driven COX-2 expression and enhanced PGE2 release. Furthermore, inhibition of COX-2 impeded the growth-promoting effects of HGF, suggesting that the COX-2/PGE2 pathway is an important mediator of HGF/Met signalling. These data reveal a critical role for HGF/Met signalling in promoting PGE2 biogenesis in colorectal cancer cells. Targeting the crosstalk between these two important pathways may be useful for therapeutic treatment of colorectal cancer.

Introduction

Colorectal cancer remains a major cause of cancer death, and one important player in colorectal tumorigenesis is cyclooxygenase (COX)-2, a key enzyme in prostanoïd synthesis found over-expressed in the majority of colorectal cancers (1–3). COX-2 inhibitors have potent antitumour effects both in vivo and in vitro (reviewed in ref. 4). Indeed, studies have revealed that genetic deletion or pharmacological inhibition of COX-2 in the APCMin/+ mouse model for intestinal tumorigenesis significantly reduces tumour burden in vivo (5,6), supporting a key role for COX-2 activity in colorectal tumour initiation and maintenance.

The most abundant COX-2 metabolite in colorectal tumour tissue is prostaglandin E2 (PGE2) (7,8). The COX-2/PGE2 pathway influences most, if not all, of the hallmarks of cancer (reviewed in ref. 9). These effects are thought to result at least in part from PGE2-dependent activation of pathways such as PI3K/AKT, Ras-mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) (10), Wnt/β-catenin (11) and epidermal growth factor receptor (EGFR) signalling (12–14). Such pathways also promote COX-2 expression, highlighting the positive feedback mechanisms important for maintaining COX-2 expression during colorectal tumorigenesis (4,15–17).

The tumour microenvironment promotes COX-2 expression, and recently, COX-2 was identified as a direct target of hypoxia-inducible factor-1 in colorectal tumour cells (18). The tumour microenvironment also comprises extracellular growth factors, including hepatocyte growth factor (HGF) (19,20). HGF signalling via the Met receptor up-regulates COX-2 expression in different cell types (21–23), and animal models have also revealed a role for COX-2 as a mediator of Met-driven hepatocellular tumour progression (24) and HGF-induced angiogenesis in vivo (25). Surprisingly, given the importance of COX-2 in colorectal tumorigenesis, the regulation of COX-2 by HGF/Met signalling in colorectal cancer cells has never been reported.

HGF is a stromal factor important in cancer cell invasion and metastasis (26). In many solid tumours, Met is over-expressed, usually as a result of deregulated growth factor or oncogenic signalling (27–30) or via stimulation by microenvironmental factors such as hypoxia (31). Increased Met expression is evident in a large proportion of colorectal tumours (32,33) and correlates with a poor prognosis (34,35). The relevance of Met as a therapeutic target was recently highlighted with the discovery that a significant number of lung cancer patients acquire resistance to the EGFR inhibitor gefitinib through amplification of Met (36). Furthermore, silencing Met expression in tumours using an inducible short hairpin RNA impairs tumour growth and metastasis and promotes regression of established tumours (37). Thus, there is growing interest in targeting HGF/Met signalling therapeutically (38). However, the mechanisms by which HGF/Met signalling promote colorectal tumorigenesis are still not fully understood.

COX-2 activity is physiologically antagonized by the (nicotinamide adenine dinucleotide)-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH), which biochemically inactivates prostaglandins by oxidizing the 15(S)-hydroxyl group into a keto group (39). 15-PGDH is a tumour suppressor in gastrointestinal, breast and lung cancers (40–42), and genetic ablation of 15-PGDH increases intestinal tumour formation in the APCMin/+ mouse model (43). Furthermore, evidence suggests that 15-PGDH is down-regulated in human colorectal tumours (40,43,44). Given the importance of PGE2 in colorectal tumorigenesis, gaining further knowledge of how both COX-2 and 15-PGDH are regulated may be critical in order to develop effective target-based therapies to replace or to complement current therapeutic regimens.

One mechanism by which PGE2 is thought to stimulate colorectal cancer cell invasion is via EGFR-dependent transactivation of Met (45). While this suggests important crosstalk between PGE2 and Met, the connection between HGF/Met signalling and PGE2 synthesis and degradation in colorectal cancer has not been defined. Indeed, there have been no reports in any cell system examining whether HGF/Met signalling regulates 15-PGDH. With this in mind, we hypothesized that HGF might regulate COX-2 and/or 15-PGDH and that PGE2 may play a role in the pro-tumorigenic effects of HGF/Met signalling in colorectal tumours. Here, we report for the first time that HGF/Met signalling increases PGE2 production in colorectal cancer cells via up-regulation of COX-2 and down-regulation of 15-PGDH protein and messenger RNA (mRNA) expression. Investigation into the mechanism

Abbreviations: COX, cyclooxygenase; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; HGF, hepatocyte growth factor; PGE2, prostaglandin E2; 15-PGDH, 15-hydroxyprostaglandin dehydrogenase; MAPK, mitogen-activated protein kinase; mRNA, messenger RNA; MTT, 3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCR, polymerase chain reaction; siRNA, small interfering RNA.

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of COX-2 and 15-PGDH regulation revealed that HGF up-regulates COX-2 and down-regulates 15-PGDH by signalling via both Ras-MAPK/ERK and PI3K/AKT pathways. To our knowledge, this is the first report implicating PI3K/AKT signalling in the repression of 15-PGDH expression. Crucially, the small molecule Met inhibitor SU11274 decreased COX-2 expression and up-regulated 15-PGDH in high Met-expressing HCA7/C29 cells, implicating a role for Met inhibition in the reduction of PGE2 biogenesis. In addition, hypoxia potentiated HGF-driven COX-2 expression and led to synergistic PGE2 release, whereas specific inhibition of COX-2 attenuated HGF-stimulated cell growth. This suggests that COX-2/PGE2 signalling is required for HGF/Met-driven growth stimulation. These data highlight a critical role for HGF/Met signalling in the regulation of PGE2 biogenesis in colorectal cancer cells.

Materials and methods

Cell culture and reagents

Colorectal adenoma–carcinoma-derived cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with glucose (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and 20 or 10% foetal bovine serum, respectively. In addition, 20% foetal bovine serum Dulbecco’s modified Eagle’s medium was supplemented with insulin (0.2 U/ml). The colorectal carcinoma cell lines CaCo2, HT29, HCT116, HCT15, LS174T, SW480 and SW620 were obtained from the American Type Culture Collection (Rockville, MD). LDL-1 were a kind gift from Dr B. Vogelstein (Johns Hopkins University), and HCA7 colony 29 (HCA7/C29) cells were a kind gift from Dr S. Kirkland (Imperial College London, UK). The adenoma-derived cell lines AA/C1 and RG/C2 were derived in this laboratory and are anchorage dependent and non-tumorigenic in athymic mice (46–48). AA/C1/SB10C (SB10C) represents an in vitro-transformed variant of AA/C1 derived following treatment with sodium butyrate and the carcinogen N-methyl-N’-nitrosoguanidine (48). These cells are anchorage independent and tumorigenic in athymic mice at high passage numbers. For hypoxia treatment, cells were placed in a modular incubator in an atmosphere composed of 94% N2, 5% CO2 and 1% O2. The COX-2 inhibitor NS398 was obtained from Sigma (Poole, UK); HGF from R&D Systems (Minneapolis, MN); LY294002 and SU11274 from Calbiochem (La Jolla, CA) and PD184352 was a kind gift from Dr S. Cook (Babraham Institute, Cambridge, UK).

3,4-Dimethylthiazol-2-yl)-2,5-diphenyterrazolium bromide assay

Cell growth was measured using the 3,4-dimethylthiazol-2-yl)-2,5-diphenyterrazolium bromide (MTT) assay. Cells were seeded in 96-well plates and then pre-incubated with NS398 for 4 h before addition of HGF or vehicle for 24 h. Following treatment, cells were incubated in serum-free Dulbecco’s modified Eagle’s medium containing 0.5 mg/ml solubilized MTT (Sigma) at 37°C for 4 h. Formazan crystals were then solubilized in dimethyl sulfoxide (VWR, Lutterworth, Leicester, UK) for 20 min, and spectrophotometric readings were taken at a wavelength of 595 nm.

PGE2 quantification

To determine PGE2 levels released into the culture medium following treatment, cells were stimulated with 47 μM arachidonic acid (Sigma) for 45 min before aliquots of the medium sample frozen in liquid nitrogen (~196°C) and stored at -70°C. A competitive enzyme immunoassay for PGE2 (Cayman Europe) was then used according to the manufacturer’s protocol.

Western blotting and immunoprecipitation

Whole-cell lysates were recovered by scraping in ice-cold lysis buffer (Cell Signaling Technology, Danvers, MA) supplemented with protease inhibitors (Roche Diagnostics, East Sussex, UK). Lysates were cleared by centrifugation and protein concentration determined using the Bio-Rad DC protein assay kit (Bio-Rad, Hertfordshire, UK). Equal amounts of protein were separated using sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Western blot analysis was performed using antibodies to p-Met (Tyr1234/1235), p-ERK1/2 (Thr202/Tyr204), p-AKT (Ser473), Met, ERK1/2 and AKT (Cell Signaling Technology); COX-2, COX-1 and phosphorylated tyrosine (PY99; Santa Cruz, Santa Cruz, CA); hypoxia-inducible factor-1α (BD Biosciences, Oxford, UK) and 15-PGDH (Abcam, Cambridge, UK). After incubation with the appropriate secondary antibody, proteins were detected using an enhanced chemiluminescence detection kit (KPL, Gaithersburg, MD). Blots were subsequently reprobed for α-tubulin (Sigma) to control for equal protein loading. For immunoprecipitation experiments, 500 μg protein lysate was cleared with 1 μg antibody coupled to protein A-sepharose, and western blotting was performed as described above using anti-phospho-tyrosine (PY99) antibody (Santa Cruz). The same blot was then stripped and reprobed for total Met.

Quantitative real-time polymerase chain reaction

Following appropriate treatment, total RNA was extracted from cells using TRI-Reagent (Sigma), chloroform and isopropanol precipitation. After treatment with DNase I (Ambion, Cambridgeshire, UK), complementary DNA was reverse transcribed using Moloney marble leukemia virus reverse transcriptase (Promega, Madison, WI). Comparative quantitative real-time polymerase chain reaction (PCR) was performed using a QuantiTect SYBR Green PCR Kit and QuantiTect primers for COX-2, 15-PGDH, TBP and hypoxanthine guanine phosphoribosyl transferase (QIAGEN, Crawley, West Sussex, UK) in a Stratagene MX3005P QPCR cycler (La Jolla, CA). Transcripts were normalized to the housekeeping genes TBP or hypoxanthine guanine phosphoribosyl transferase as indicated.

Reduction of 15-PGDH protein expression using small interfering RNA

Small interfering RNAs (siRNAs) were obtained from Ambion. Cells were transfected with siRNA targeted against human 15-PGDH (50 nM), a validated negative control siRNA or a BAD (BCL-2 antagonist of cell death) siRNA shown previously to reduce BCL-2 antagonist of cell death expression (49) as a positive control for knock down (data not shown). Cells were grown in standard growth medium for 24 h before transfection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for 6 h in OptiMEM (Invitrogen). Following this, the medium was replaced with standard growth medium and cells harvested at the indicated time point.

Statistical analysis

Statistical analyses were carried out using Student’s t-test and expressed as: *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001.

Results

Expression of Met and COX-2 in colorectal tumour cell lines

The expression of both Met and COX-2 is increased during colorectal tumorigenesis (2,3,32). To examine this in vitro, we investigated the expression of total Met, active (phosphorylated) Met (p-Met) and COX-2 protein in a panel of colorectal tumour cell lines (Figure 1). Western blotting for total Met revealed a band of 145 kDa representing the native Met β-chain and a band of 170 kDa indicating the single-chain pro-Met precursor, which was detected at varying levels in all the cell lines (9/19). In agreement with in vivo reports (32), Met expression was detected in both carcinoma and adenoma cells,

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**Fig. 1.** Western blot analysis for expression of total Met, phosphorylated Met (p-Met; Tyr1234/1235) and COX-2 in a panel of colorectal tumour cell lines. Protein lysates were obtained from subconfluent colorectal carcinoma (6), transformed adenoma (1) and adenoma (2) cell lines grown in standard growth medium as described in Materials and Methods. For total Met, two hypoxia-inducible factor-1α (BD Biosciences, Oxford, UK) and 15-PGDH (Abcam, Cambridge, UK). After incubation with the appropriate secondary antibody, proteins were detected using an enhanced chemiluminescence detection kit (KPL, Gaithersburg, MD). Blots were subsequently reprobed for α-tubulin (Sigma) to control for equal protein loading.

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although generally higher expression was evident in carcinomas than adenomas. An exception was the SW480 cell line, which is reported to express low Met levels (33). Using an anti-p-Met antibody that recognizes Met phosphorylated in the intrinsic kinase domain (Tyr1234/1235), we only readily detected constitutive p-Met in HCA7/C29 cells, which have very high total Met levels, and in SW620 cells, which are derived from a lymph node metastasis (Figure 1). However, immunoprecipitation for Met and western blotting for phosphorylated tyrosine (p-Tyr) later revealed that basal p-Met could be detected in other cell lines including HT29 cells (Figure 3A), albeit at a low level. Blotting for COX-2 revealed a band at ~72 kDa, and occasionally, a higher molecular weight non-specific band could also be detected (marked with an asterisk). Overall, a simple correlation in the expression of COX-2 and Met was not evident in most of the colorectal tumour cell lines examined. However, HCA7/C29 cells expressed the highest level of COX-2, Met and p-Met of all the cell lines analysed. Furthermore, Met expression has been shown to correlate with an invasive phenotype in colorectal cancer (33,35), and interestingly, the in vitro-transformed tumorigenic adenoma cell line AA/C1/SB10C (SB10C) (48) expressed higher Met and COX-2 levels than parental non-tumorigenic AA/C1 adenoma cells (Figure 1), indicating a possible role for both Met and COX-2 in the progression from benign to malignant tumours.

HGF up-regulates COX-2 expression and promotes PGE2 accumulation in colorectal cancer cells

Given the importance of COX-2 and Met in cell survival, proliferation and invasion during colorectal tumorigenesis, we hypothesized that HGF regulates COX-2 in colorectal tumour cells. To investigate this hypothesis, we used HT29 colorectal carcinoma cells as they expressed moderate levels of both Met and COX-2 protein under basal conditions (refer to Figure 1). HGF treatment of HT29 cells revealed a dose-dependent induction of COX-2, and maximum induction of COX-2 was achieved with 30 ng/ml HGF (Figure 2A). This dose was therefore used for subsequent experiments using HT29 cells. Further analysis demonstrated COX-2 induction from as early as 4 h, which was sustained for 16 h (Figure 2B).

In order to establish whether COX-2 was induced by HGF in other colorectal cancer cell lines, we treated two low COX-2-expressing cell lines (HCT116 and HCT15) and a high COX-2-expressing cell line (HCA7/C29) with HGF and investigated COX-2 protein expression using western blotting. In all three cell lines, COX-2 was up-regulated after 16 h HGF treatment (Figure 2C), confirming that HGF can regulate COX-2 protein expression in colorectal cancer cells, even when endogenous COX-2 levels are very low or very high.

A growing body of evidence points towards a crucial role for the COX-2 metabolite PGE2 in the promotion of colorectal tumorigenesis (9,10,18,50). Having demonstrated that COX-2 is up-regulated by HGF in colorectal cancer cells, we investigated whether PGE2 levels also increased with HGF treatment. Treatment with HGF significantly increased PGE2 release from HT29 cells in a COX-2-dependent manner, given that this increase was inhibited in the presence of the COX-2 inhibitor NS398 at 10 μM (Figure 2D). This dose was previously reported to selectively inhibit COX-2 without inhibiting cell growth (51). Furthermore, no change in expression of COX-1 was observed with HGF treatment (Figure 2E), suggesting that COX-2-dependent synthesis is important in HGF-driven PGE2 production.

HGF up-regulates COX-2 via Ras-MAPK/ERK- and PI3K/AKT-signalling pathways

Having shown that COX-2 expression and function is up-regulated by HGF, we further investigated the mechanism for COX-2 up-regulation, focussing on two pathways key to colorectal cancer cell survival that classically couple to HGF/Met signalling, namely the Ras-MAPK/ERK and PI3K/AKT survival signalling cascades. Previous reports have demonstrated that HGF/Met signalling...
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up-regulates COX-2 in gastric and squamous cell carcinoma cells via MAPK signalling (21,23), although in endothelial cells HGF stimulates COX-2 expression in a PI3K-dependent fashion (25). With this in mind, we investigated whether HGF activated these survival pathways in colorectal cancer cells. Activation of Met by HGF in HT29 cells was first confirmed by immunoprecipitation for total Met and western blot analysis for phosphorylated tyrosine (p-Tyr; Figure 3A). Although not detectable by western blotting with the phosphorylation-specific Met antibody (refer to Figure 1), basal tyrosine phosphorylated Met was detected in HT29 cells and was increased by HGF treatment (Figure 3A). Furthermore, time course analysis and western blotting confirmed that phosphorylation of both ERK and AKT was increased by HGF (Figure 3B), suggesting activation of the Ras-MAPK/ERK and PI3K/AKT pathways, as previously reported (52,53). This activation was maximal at 2 h and still detectable at 8 h, although returned to control levels by 16 h (Figure 3B).

In order to test whether Met, Ras-MAPK/ERK and/or PI3K/AKT signalling were required for HGF-driven COX-2 up-regulation in colorectal cancer cells, we employed a pharmacological approach using SU11274, a specific ATP-binding competitive inhibitor for Met (54), PD184352, a potent and selective MEK inhibitor (55), and LY294002, a PI3K inhibitor (56). HT29 cells were pretreated with the inhibitors for 2 h and then stimulated with HGF or vehicle for 16 h; the results are summarized in Figure 3C. Inhibition of MAPK and PI3K signalling with PD184352 and LY294002, respectively, was confirmed by blotting for p-ERK and p-AKT, and combining the inhibitors resulted in a large decrease in basal COX-2 expression in unstimulated cells (lane 2). A slight decrease in basal COX-2 expression was also detected following incubation with SU11274 (lane 3). Addition of HGF up-regulated COX-2 (lane 4), and inhibition of Met with SU11274 completely inhibited HGF-mediated COX-2 up-regulation (compare lane 8 with lane 4), suggesting that HGF up-regulates COX-2 via activation of the Met receptor. Furthermore, addition of either PD184352 or LY294002 partially inhibited HGF-mediated COX-2 up-regulation (compare lanes 5 and 6 with lane 4), and in the presence of both PD184352 and LY294002, HGF-driven COX-2 up-regulation was completely prevented, such that COX-2 expression was equivalent in HGF-stimulated and unstimulated cells (compare lane 7 with lane 2; Figure 3C). Thus, these data suggest that COX-2 up-regulation by HGF is Met-dependent and requires signalling via both Ras-MAPK/ERK and PI3K/AKT pathways.

COX-2 is an inducible gene responsive to a number of transcription factors (reviewed in ref. 4). In order to establish whether the up-regulation of COX-2 protein by HGF involved an increase in COX-2 mRNA, quantitative real-time PCR was carried out at the indicated time points (Figure 3D). Indeed, COX-2 mRNA levels increased over 4-fold after 8 h HGF stimulation; although by 16 h, the levels of COX-2 mRNA had almost returned to control levels (Figure 3D), correlating with the kinetics of AKT and ERK activation following HGF stimulation (Figure 3B). These data suggest that HGF/Met signalling up-regulates COX-2 expression at least in part via increased COX-2 mRNA expression.

**Inhibition of COX-2 attenuates HGF-stimulated colorectal cancer cell growth**

Previous studies have identified a role for PGE₂ in promoting colorectal tumour cell proliferation and survival (10,50,57). Having shown that HGF/Met signalling promotes COX-2 up-regulation and PGE₂ release, we investigated the significance of COX-2 up-regulation during HGF-stimulated cell growth using the MTT assay. After 24 h,
HGF increased growth of HT29 cells, and this was significantly inhibited in the presence of NS398 (10 μM; Figure 4A) at a dose that had previously been shown to inhibit COX-2 without inhibiting cell growth (51). This suggests that at least in part, HGF-stimulated colorectal cancer cell growth is dependent on the increase in PGE2 brought about by COX-2 up-regulation.

**Hypoxia potentiates HGF-dependent COX-2 up-regulation and PGE2 release**

We recently identified hypoxia as a stimulus of the COX-2/PGE2 pathway in colorectal tumour cells (18). With this in mind, we investigated whether HGF was able to synergize with a hypoxic microenvironment to promote COX-2 expression and activity. While hypoxia (1% O2) alone induced COX-2 up-regulation and a small but significant increase in PGE2 (P = 0.003; Student’s t-test), 16 h treatment with HGF (30 ng/ml) in hypoxia resulted in a striking increase in COX-2 expression (Figure 4B) and synergistic PGE2 release (Figure 4C). Again, the increase in PGE2 was reduced by NS398, demonstrating that this increase was derived from COX-2 activity (Figure 4C). These data suggest that HGF/Met signalling can synergize with a hypoxic tumour microenvironment to promote high levels of COX-2 expression and PGE2 release in colorectal cancer cells.

**HGF reduces expression of 15-PGDH protein and mRNA**

Having shown that HGF/Met signalling is a key stimulant of COX-2 expression and function, it was of interest to determine whether HGF/Met signalling might also stimulate PGE2 accumulation through down-regulation of 15-PGDH, the enzyme that controls the rate-limiting step in PGE2 inactivation. Initial screening of a panel of colorectal carcinoma cell lines revealed low expression of 15-PGDH protein in most cells grown in standard serum-containing medium (Figure 5A). Highest expression was evident in DLD-1 and SW480 cells; interestingly, expression of 15-PGDH was almost undetectable in SW620 cells, the metastatic derivative of SW480. This inversely correlated with expression of Met in these cells, which was much higher in SW620 than SW480 (refer to Figure 1).

We investigated whether HGF regulated 15-PGDH in HT29, HCT15 and HCA7/C29 cells as they expressed moderate, low and very low levels of 15-PGDH, respectively (Figure 5A). Cells were treated in serum-free medium to avoid the influence of growth factors in serum, and 48 h HGF treatment resulted in a dose-dependent reduction in 15-PGDH expression in HCT15 (Figure 5B) and HCA7/C29 (see Figure 6A) cells. HT29 cells were treated for 24 h due to their sensitivity to serum deprivation, but interestingly, no consistent regulation of 15-PGDH was detectable in these cells following HGF treatment (Figure 5B). Furthermore, HT29 cells displayed no 15-PGDH regulation at later time points in serum-containing medium (data not shown). A similar phenomenon, whereby 15-PGDH regulation varies between cell lines, has been previously reported with epidermal growth factor-dependent 15-PGDH down-regulation in both colorectal and lung cancer cell lines (44,58). This highlights that growth factor-mediated 15-PGDH repression may depend on the cellular context.

Having observed HGF-mediated 15-PGDH protein down-regulation in HCT15 cells, we went on to examine the expression of 15-PGDH mRNA. Quantitative real-time PCR demonstrated a reduction in 15-PGDH mRNA expression after 48 h HGF treatment (Figure 5C). Furthermore, although HCT15 and HT29 cells differed in their response to HGF in terms of 15-PGDH regulation, assaying for PGE2 release demonstrated that PGE2 was increased to a similar level in both cell lines (400–500 pg/10^6 cells) (Figure 5D). Therefore, although HGF only stimulated COX-2-dependent PGE2 synthesis in HT29 cells, a similar level of PGE2 was achieved in HCT15, which displayed HGF-mediated stimulation of PGE2 synthesis via COX-2 up-regulation and repression of degradation via decreased 15-PGDH.

In agreement with a previous report (59), decreased expression of

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**Fig. 4.** NS398 inhibits HGF-driven cell growth and hypoxia potentiates HGF-driven COX-2 expression and PGE2 release in colorectal carcinoma cells. (A) HT29 cells were pre-incubated with vehicle or 10 μM NS398 for 4 h before treatment with 30 ng/ml HGF for 24 h. Cell yield was assessed by MTT assay. Data represent the percentage increase in cell yield normalized to the relevant untreated control. Columns indicate mean of four independent experiments performed in sextuplicate; bars indicate SEM. (B) Hypoxia potentiates HGF-driven COX-2 expression. HT29 cells were treated with HGF (30 ng/ml) for 16 h in either normoxia (21% O2) or hypoxia (1% O2) and COX-2 and hypoxia-inducible factor (HIF)-1α protein expression assessed by western blot. α-Tubulin confirmed equal protein loading. (C) Effect of hypoxia on HGF-stimulated PGE2 release. HT29 cells were treated with 30 ng/ml HGF for 16 h in normoxia or hypoxia with or without 4 h preincubation with 10 μM NS398. PGE2 levels in the culture medium were subsequently analysed using a commercial enzyme-linked immunosorbent assay and standardized to cell number. Columns indicate data from one experiment representative of three individual experiments performed in triplicate; bars indicate standard deviation (***P < 0.001; **P < 0.01; Student’s t-test).
15-PGDH using siRNA resulted in an increase in PGE2 release from HCT15 cells without altering COX-2 expression, confirming the biological significance of 15-PGDH repression (Figure 5E). Therefore, HGF/Met-mediated 15-PGDH repression is likely to contribute to an increase in PGE2 accumulation in HCT15 cells in addition to the contribution of COX-2-dependent synthesis.
p-Met in HT29 cells compared with HCA7/C29 (refer to Figure 1). (Figure 3C), perhaps reflecting the lower expression of Met and COX-2 was observed following SU11274 treatment of HT29 cells. Met altered basal COX-2 and 15-PGDH expression. Treatment with high Met- and p-Met-expressing cell line HCA7/C29. Interestingly, HGF-driven COX-2 expression was markedly potentiated in hypoxia, further enhancing PGE2 accumulation. Given the side effects sometimes associated with high-dose/long-term COX-2 inhibition, alternative mechanisms to reduce tumoral PGE2 are highly sought after. We report that the Met inhibitor SU11274 reduced COX-2 expression and increased 15-PGDH expression and propose that targeting Met may significantly reduce PGE2 accumulation in vivo, thus, representing an attractive alternative or adjuvant to COX-2 inhibitors alone.

Evidence suggests that Met may be a good candidate for target-based therapy (38). PF2341066—a recently developed small molecule Met inhibitor—is currently in Phase I/II clinical trials, is well tolerated, and has both antitumour and anti-angiogenic properties (60). We propose that the COX-2/PGE2 pathway is a critical mediator of HGF-driven tumorigenesis and that inhibition of Met may have such anti-tumour effects by reducing PGE2 biogenesis. In vivo evidence supports this hypothesis, since COX-2 is a key mediator of HGF/Met-driven hepatocellular carcinoma formation and metastasis (24) and given that COX-2 inhibition prevents HGF-driven angiogenesis in mice (25). Our study compliments previous reports in other cell types including gastric epithelial cells (21), squamous cell carcinoma cells (23) and lung carcinoma cells (22) and identifies COX-2 as a target of HGF/Met-driven hepatocellular carcinoma formation and metastasis (24). Our study compliments previous reports in other cell types including gastric epithelial cells (21), squamous cell carcinoma cells (23) and lung carcinoma cells (22) and identifies COX-2 as a target of HGF/Met-driven hepatocellular carcinoma formation and metastasis (24).

HGF/Met signalling reduces 15-PGDH expression via Ras-MAPK/ERK and PI3K/AKT signalling

Given that Met-dependent regulation of COX-2 was dependent on signalling via both Ras-MAPK/ERK and PI3K/AKT pathways, we then went on to investigate whether these pathways also played a role in the HGF-driven reduction in 15-PGDH expression. Stimulation with HGF resulted in a striking decrease in 15-PGDH expression (Figure 5F; compare lane 5 with lane 1). Incubation of HCT15 cells with either PD184352 (a MEK inhibitor) or LY294002 (a PI3K inhibitor) up-regulated basal 15-PGDH expression (lanes 2 and 3), suggesting that both ERK and AKT signalling are involved in the repression of constitutive 15-PGDH expression in colorectal tumour cells (Figure 5F). Furthermore, both PD184352 and LY294002 partially rescued HGF-mediated down-regulation of 15-PGDH (lanes 6 and 7), and in the presence of both PD184352 and LY294002, 15-PGDH down-regulation was completely rescued, such that it was equivalent to that in non-HGF-stimulated cells (compare lanes 4 and 8; Figure 5F). This suggests that both Ras-MAPK/ERK and PI3K/AKT signalling mediate HGF-driven 15-PGDH down-regulation, in addition to mediating HGF-stimulated COX-2 up-regulation (Figure 3C), implicating a critical role for the pathways in stimulating PGE2 biogenesis downstream of HGF/Met signalling.

Met inhibition increases 15-PGDH and reduces COX-2 expression in high Met-expressing HCA7/C29 cells

Having found that HGF could repress 15-PGDH expression, we then examined the effect of HGF and the Met inhibitor SU11274 on the high Met- and p-Met-expressing cell line HCA7/C29. Interestingly, these cells also express very high COX-2 (refer to Figure 1) and low 15-PGDH (refer to Figure 5A). Despite this, HGF could further repress 15-PGDH expression (Figure 6A), consistent with results in HCT15 cells (Figure 5B). Given that HCA7/C29 cells express very high Met and p-Met levels, we went on to test whether inhibition of Met altered basal COX-2 and 15-PGDH expression. Treatment with the Met inhibitor SU11274 resulted in increased 15-PGDH and reduced COX-2 expression at 48 and 96 h time points (Figure 6B). While the reduction in COX-2 expression resulting from SU11274 treatment was easily detected in HCA7/C29, only slight inhibition of COX-2 was observed following SU11274 treatment of HT29 cells (Figure 3C), perhaps reflecting the lower expression of Met and p-Met in HT29 cells compared with HCA7/C29 (refer to Figure 1). These data suggest that Met inhibition may represent an effective way to reduce PGE2 biogenesis in colorectal tumours expressing high Met levels. This study reveals important crosstalk between HGF/Met signalling and PGE2 signalling in colorectal cancer cells, with HGF mediating both up-regulation of COX-2 and down-regulation of 15-PGDH to promote PGE2 biogenesis.

Discussion

While COX-2 inhibitors remain promising candidates for colorectal cancer prevention and therapy, the associated risk of cardiovascular side effects mean that it is important to understand all aspects of PGE2 biogenesis and how this becomes deregulated during tumorigenesis. Here, we report that HGF/Met signalling is an important regulator of the COX-2/PGE2 pathway in colorectal cancer cells, stimulating PGE2 synthesis via COX-2 up-regulation and inhibiting PGE2 degradation via 15-PGDH down-regulation. The critical role for COX-2 as a mediator of HGF/Met signalling was highlighted with the observation that inhibition of COX-2 attenuated HGF-driven colorectal cancer cell growth. Interestingly, HGF-driven COX-2 expression was markedly potentiated in hypoxia, further enhancing PGE2 accumulation. Given the side effects sometimes associated with high-dose/long-term COX-2 inhibition, alternative mechanisms to reduce tumoral PGE2 are highly sought after. We report that the Met inhibitor SU11274 reduced COX-2 expression and increased 15-PGDH expression and propose that targeting Met may significantly reduce PGE2 accumulation in vivo, thus, representing an attractive alternative or adjuvant to COX-2 inhibitors alone.

Evidence suggests that Met may be a good candidate for target-based therapy (38). PF2341066—a recently developed small molecule Met inhibitor—is currently in Phase I/II clinical trials, is well tolerated, and has both antitumour and anti-angiogenic properties (60). We propose that the COX-2/PGE2 pathway is a critical mediator of HGF-driven tumorigenesis and that inhibition of Met may have such anti-tumour effects by reducing PGE2 biogenesis. In vivo evidence supports this hypothesis, since COX-2 is a key mediator of HGF/Met-driven hepatocellular carcinoma formation and metastasis (24). Our study compliments previous reports in other cell types including gastric epithelial cells (21), squamous cell carcinoma cells (23) and lung carcinoma cells (22) and identifies COX-2 as a target of HGF/Met signalling in colorectal cancer cells. However, this is the first report demonstrating that HGF/Met signalling increases PGE2 levels not only by increasing COX-2 expression but importantly by preventing degradation of PGE2 via decreasing 15-PGDH expression. Met-driven down-regulation of 15-PGDH is also likely to be relevant to many other cancer types with deregulated Met signalling and high tumour PGE2 levels. Crucially, Met inhibition not only reduced COX-2 expression but also increased 15-PGDH expression. This suggests that therapeutic targeting of Met may be a useful approach to reduce tumoral PGE2 by reducing COX-2-dependent synthesis and also increasing the degradation of PGE2 present in the tumour microenvironment.

Given that HGF modulates both synthesis and degradation of PGE2, we propose that PGE2 is an important mediator of HGF-driven colorectal cancer cell survival and metastasis. Intriguingly, PGE2 can activate Met signalling independent of HGF (45); therefore, given the data presented here, a positive feedback loop may exist that sustains COX-2 expression and Met activation during colorectal tumorigenesis. We demonstrate that COX-2 inhibition impedes HGF-stimulated cell growth, emphasizing the merit of disrupting this feedback loop therapeutically. These results also imply that COX-2 inhibitors may have particular benefits for short-term therapy in a subset of patients with high tumour Met or HGF expression.

The antitumour role of 15-PGDH has been highlighted in vivo (40–42), therefore, the down-regulation of 15-PGDH by Met is potentially an important mechanism by which Met promotes tumour growth. We propose that drugs which up-regulate 15-PGDH may be more beneficial than COX-2 inhibitors and limit side effects, since 15-PGDH does not metabolize all prostaglandins. For example, PGE2
is a poor substrate for 15-PGDH (39) and is reported to have anticancer properties (61,62). Therefore, rather than blocking synthesis of all COX-2-derived prostaglandins with COX-2 inhibitors, the inhibition of Met may allow more selective targeting of pro-tumorigenic PGE2 via up-regulation of 15-PGDH and down-regulation (but not complete inhibition) of COX-2.

Signalling via both the Ras-MAPK/ERK and PI3K/AKT pathways is clearly important in promoting PGE2 accumulation, given that these pathways could simultaneously promote PGE2 synthesis by up-regulating COX-2 and inhibit degradation by reducing 15-PGDH expression. HGF-driven 15-PGDH protein down-regulation was accompanied by a reduction in mRNA expression, and a small number of reports have identified key transcriptional regulators of the HPGD gene. Epidermal growth factor-mediated 15-PGDH down-regulation has been shown to occur via ERK-dependent stimulation of the transcriptional repressor Snail (63). However, our study is the first to implicate PI3K/AKT signalling in the repression of 15-PGDH. Studies suggest that Snail can also be induced via PI3K/AKT signalling (64,65), and in addition, AKT can inhibit hepatocyte nuclear factor 3β (also known as Foxa2) (66), which is a reported transcriptional activator of the 15-PGDH gene (67). Therefore, increased Snail expression and/or repression of hepatocyte nuclear factor 3β may play a role in HGF-mediated 15-PGDH down-regulation.

Although the role of HGF/Met signalling in the modulation of 15-PGDH expression appears to depend on the cellular context, biologically the overall net effect of HGF treatment was a striking increase in PGE2 release in both HT29 and HCT15 cells. We propose that PGE2 is likely to be stimulating cell growth, although a role for other prostaglandins cannot be excluded. The potentiation of HGF-mediated 15-PGDH down-regulation may play a role in the tumour microenvironment can impact on cellular signalling and gene expression. The resulting high level of COX-2 and PGE2 production may be particularly important for the survival and adaptation to hypoxia, for example by stimulating angiogenesis. Furthermore, other prostaglandins could contribute to hypoxic adaptation, with reports suggesting that HGF and protooncogene 1 may synergize to induce angiogenesis (68).

In summary, this study uncovers a critical role for HGF/Met signalling in the promotion of PGE2 biogenesis in colorectal cancer cells, via increased COX-2 expression and reduced expression of 15-PGDH. The modulation of COX-2 and 15-PGDH expression results in an increase in PGE2 release, and furthermore in a hypoxic environment, HGF-driven COX-2 expression is potentiated, leading to synergistic PGE2 production. COX-2 inhibition impeded HGF-driven cell growth and furthermore, Met inhibition down-regulated COX-2 and up-regulated 15-PGDH expression. Given the key role for PGE2 in stimulating a large number of tumorigenic processes during colorectal carcinogenesis, inhibition of Met may represent a useful clinical alternative or supplement to COX-2 inhibition and result in reduced tumoral PGE2 accumulation.

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