# Matrix metalloproteinase-7-catalyzed release of HB-EGF mediates deoxycholyltaurine-induced proliferation of a human colon cancer cell line

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ABSTRACT

Prior evidence indicates that bile acids stimulate colon cancer cell proliferation by muscarinic receptor-induced transactivation of epidermal growth factor receptors (EGFR). To explore further the mechanism underlying this action, we tested the hypothesis that bile acids activate a matrix metalloproteinase (MMP) that catalyzes release of an EGFR ligand. Initial studies showed that non-selective MMP inhibitors blocked the actions of deoxycholyltaurine (DCT), thereby indicating a role for MMP-catalyzed release of an EGFR ligand. DCT-induced cell proliferation was reduced by increasing concentrations of EGFR kinase inhibitors, by antibodies to the ligand binding domain of EGFR, by neutralizing antibodies to heparin binding-EGF-like growth factor (HB-EGF) and by CRM197, an inhibitor of HB-EGF release. These findings and our observations with more selective MMP inhibitors suggested that MMP-7, an enzyme known to release HB-EGF, plays a key role in mediating bile acid-induced H508 colon cancer cell proliferation. We observed that recombinant HB-EGF and MMP-7 mimicked both the signaling and proliferative actions of bile acids. Strikingly, reducing MMP-7 expression with either neutralizing antibody or small interfering RNA attenuated the actions of DCT. MMP-7 expression in H508 cells was confirmed using quantitative reverse transcription PCR. DCT stimulated a greater than 10-fold increase in MMP-7 gene transcription. Co-localization of pro-MMP-7 and pro-HB-EGF at the cell surface (immunofluorescence microscopy) was demonstrated, indicating proximity of the enzyme to its substrate. These findings provide strong evidence that in H508 human colon cancer cells, DCT-induced transactivation of EGFR is mediated by MMP-7-catalyzed release of the EGFR ligand HB-EGF.

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## 1. Introduction

Population and animal studies indicate that fecal secondary bile acids are important co-factors in colon carcinogenesis, particularly with regard to right-sided (cecum and ascending colon) cancer [1–3]. In rodents treated with carcinogens, direct instillation or other means of increasing fecal bile acids augments the development of colon cancer [4–6] (Cheng and

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Raufman, unpublished observations) and reducing fecal deoxycholic acid concentrations by ursodeoxycholic acid feeding decreases colon tumor formation [7,8]. Likewise, in humans with inflammatory bowel disease, modulating fecal bile acids with oral ursodeoxycholic acid treatment reduces the risk of colorectal dysplasia and cancer [9]. Nonetheless, the molecular mechanisms underlying the proliferative actions of bile acids on colonic neoplasia have been elusive.

Paradoxically, in colon cancer cell lines, rather than stimulating cell proliferation, secondary bile acids like lithocholic and deoxycholic acids are commonly reported to stimulate apoptosis [10,11]. Nonetheless, more recent reports indicate that relatively low concentrations of deoxycholic acid (5–50 $\mu$M) stimulate radiolabeled thymidine incorporation [12]. These changes were associated with nuclear translocation of $\beta$-catenin and activation of cyclin D1, known promoters of colon cancer cell proliferation [12]. Thus, whereas high micromolar concentrations ($\sim$500 $\mu$M) of secondary bile acids are pro-apoptotic [10,11], low micromolar concentrations (5–50 $\mu$M) of these agents appear to be pro-proliferative [12]. Moreover, in human colon cancer cells the ability of bile acids to induce proliferation or apoptosis is strongly correlated with their hydrophobicity [13]. That is, equimolar concentrations of conjugated bile acids are less likely to induce apoptosis than their more hydrophobic parent molecules [13,14]. Recently, we confirmed that low micromolar concentrations of more hydrophilic glycine and taurine conjugates of secondary bile acids stimulate human colon cancer cell proliferation [15,16]. Furthermore, as measured by activation of caspase-3 [17], incubation for up to 5 days with concentrations of conjugated secondary bile acids that stimulate colon cancer cell proliferation does not result in apoptosis [15,16].

Proliferative actions of various ligands that interact with G-protein coupled receptors (GPCR) (e.g. $M_3$ muscarinic receptors ($M_3R$)) are dependent on activation of receptor tyrosine kinases (RTK) (e.g. epidermal growth factor receptors (EGFR)). This phenomenon, commonly designated transactivation, is a required mechanism for the physiological actions of many GPCR ligands. Pai et al. showed that in Caco-2 and HT-29 colon cancer cells prostaglandin $E_2$-induced thymidine incorporation, a marker of cell proliferation, is dependent on Src activation, the release of transforming growth factor-$\alpha$ (TGF-$\alpha$) and the consequent transactivation of EGFR [18]. EGFR activation is recognized to play a pivotal role in colon carcinogenesis and provides an important therapeutic target [19,20]. In cell lines derived from human colon cancers, like the H508 cell line that abundantly expresses $M_3R$ [21], interaction of acetylcholine (ACh) and conjugated secondary bile acids with $M_3R$ results in transactivation of EGFR and activation of pro-proliferative post-receptor signaling [16,22,23]. In H508 cells, inhibition of EGFR activation using specific inhibitors or antibodies prevents both ACh- and bile acid-induced colon cancer cell proliferation [16,23]. Bile acid-induced transactivation of EGFR activates post-receptor signaling cascades that involve phosphorylation of p44/42 mitogen activated protein kinase (p44/42 MAPK, also referred to as ERK 1/2) and p90 ribosomal S kinase (p90RSK), a nuclear response protein downstream of p44/42 MAPK [16,24]. Blocking EGFR activation and downstream phosphorylation of p44/42 MAPK and p90RSK inhibits the proliferative actions of bile acids [16].

A frequent mechanism underlying EGFR transactivation involves metalloprotease-mediated release of an EGFR ligand [25]. The EGFR ligand family comprises six members, including EGF, TGF-$\alpha$, amphiregulin, heparin binding-EGF-like growth factor (HB-EGF), betacellulin and epiregulin [25,26]. EGFR ligands are synthesized as pro-ligands that are anchored to cell membranes. A family of secreted and membrane-anchored matrix metalloproteinases (MMP) cleaves pro-EGFR ligands, thereby releasing active ligands from the cell surface [27–29]. Activation of a particular metalloprotease results in release, sometimes referred to as 'shedding', of the corresponding EGFR ligand [30]. For example, in human cholangiocytes, bile acids stimulate matrix metalloprotease-3 (MMP-3)-mediated release of TGF-$\alpha$ with consequent phosphorylation (activation) of EGFR [31]. Pretreatment of cholangiocytes with neutralizing antibodies against TGF-$\alpha$ blocks EGFR phosphorylation [31].

Our previous observation that antibodies to the ligand binding domain of EGFR inhibit the signaling and proliferative actions of bile acids on H508 colon cancer cells implicated the release of an EGFR ligand in this process [16]. This finding stimulated us to elucidate the mechanism of EGFR ligand release and to identify the EGFR ligand responsible for these actions. The initial strategy employed in the present study was to determine the requirement for matrix metalloprotease activation in mediating bile acid-induced transactivation of EGFR. We hypothesized that if the proliferative actions of bile acids depend on activation of a particular matrix metalloprotease, then identification of this agent(s) would provide clues to candidate EGFR ligands that mediate these actions. We examined H508 colon cancer cells because the proliferative actions of bile acids on this colon cancer cell line has been studied extensively and these cells abundantly co-express $M_3R$ and EGFR [15,16,23]. As demonstrated by the data presented here, using a variety of methods in the H508 human colon cancer cell line, we provide novel evidence that activation of MMP-7 and the release of HB-EGF mediate, at least in part, the proliferative actions of conjugated secondary bile acids.

## 2.    Materials and methods

### 2.1.    Chemicals and reagents

The materials used were purchased as follows: PD168393, GM6001, NC-GM6001, MMP inhibitors-II and -III and MMP-3 inhibitors-II and -IV were from Calbiochem; transforming growth factor-$\alpha$ (TGF-$\alpha$) and recombinant human MMP-1 from Oncogene; recombinant human MMP-7 from R&D Systems; FITC- and Tritc-conjugated secondary antibodies and Hoechst stain for immunofluorescence microscopy, CRM197 and deoxycholyltaurine (DCT) were from Sigma. All other chemicals were obtained from Sigma or Fisher.

### 2.2.    Cell culture

H508 human colon cancer cell lines (ATCC) were grown in RPMI 1640 (GIBCO) supplemented with 10% fetal bovine serum

(FBS) (Biowhittaker). Adherent cultures were passaged weekly at subconfluence after trypsinization. Cultures were maintained in incubators at 37 °C in an atmosphere of 5% $CO_2$ and 95% air.

## 2.3. Cytotoxicity assays

Before proceeding with these studies, we evaluated potential cytotoxic actions of bile acids on H508 cells by trypan blue exclusion (Sigma assay kits). At concentrations used in the following experiments none of these agents altered trypan blue exclusion.

## 2.4. Antibodies and immunoblotting

Rabbit polyclonal anti-MAPK, mouse monoclonal antiphospho-MAPK, rabbit polyclonal antiphospho-EGFR and rabbit polyclonal antiphospho-p90RSK were from Cell Signaling (Beverly, MA, USA). TGF-$\alpha$ antibody was from Oncogene; EGF and EGFR antibodies from UBI (Lake Placid, NY, USA); HB-EGF antibody from Santa Cruz Biotechnology. Phosphorylation of p44/42 MAPK, EGFR and p90RSK was determined by methods described previously [32]. Briefly, cells were subcultured in six-well plates ($10^6$ cells/well). After a 24 h incubation at 37 °C, the cells were serum-starved for an additional 24 h, washed with PBS and allowed to recover in PBS for 30 min at 37 °C before adding test agents. Following a 10 min incubation with test agents, the reaction was terminated by adding lysis buffer [150 mM NaCl, 10 mM Tris/HCl, 1% (w/v) deoxycholic acid, 1% (v) Nonidet-40, 0.1% (w/v) SDS, 4 mM EDTA, 1 mM $Na_3VO_4$, 20 $\mu$g/ml leupeptin, 20 $\mu$g/ml aprotinin, 250 $\mu$g/ml nitrophenylphosphate and 1 mM phenylmethylsulphonyl fluoride, pH 8]. When inhibitors (e.g. GM6001) were used, they were added 30 min before test agents. When antibodies (e.g. anti-HB-EGF and anti-TGF-$\alpha$) were used, they were added 2 h before test agents. Cell lysates were subjected to SDS-PAGE (10% gel, Invitrogen). Resolved proteins were transferred electrophoretically to nitrocellulose membranes (Micron Separations) and probed with anti-phospho-p44/42 MAPK, anti-phospho-EGFR or anti-phospho-p90RSK. Bound antibody was detected by chemiluminescence (Supersignal kit, Pierce). To verify that equal amounts of protein were added to each lane, the blots used with anti-phospho-p44/42 MAPK were stripped and re-probed with anti-p42.

## 2.5. Cell proliferation assay

Cell proliferation was determined using the validated sulforhodamine B (SRB) colorimetric assay [33]. Cells were seeded in 96-well plates (Corning Glass Works, Corning, NY, USA) at approximately 10% confluence and allowed to attach for 24 h. The growth medium was removed and fresh medium without FBS and containing the indicated concentration of test agent was added. Cells were incubated for 5 days at 37 °C in an atmosphere of 5% $CO_2$ and 95% air without further additions of test agents. After incubation, cells were treated for 30 min with 0.4% (w/v) SRB dissolved in 1% acetic acid. Protein-bound dye was extracted with unbuffered 10 mM Tris base. Absorbance was measured at 560 nm using

a computer-interfaced, 96-well microtiter plate reader (SpectraMax384).

## 2.6. Quantitative real-time PCR

Total cellular RNA was isolated from cells using Trizol reagent (Invitrogen). First strand cDNAs were synthesized using the Superscript III First Strand Synthesis System for RT-PCR (Invitrogen). Primers for RT-PCR were designed using on-line software (http://www.genscript.com/ssl-bin/app/primer). Real-time PCR was performed using 7900HT Fast System (ABI) with Power SYBR Green master mix (ABI), cDNA was synthesized from 50 ng total RNA with 20 ng of each primer. PCR conditions included 5 min at 95 °C, followed by 35 cycles of 95 °C for 15 s, 60 °C for 15 s, 72 °C for 30 s. PCR data were analyzed using ABI instrument software SDS 2.1. Specificity of the amplifications was confirmed by melting-curve analysis. Relative levels of mRNA expression were calculated according to the standard $\Delta\Delta Ct$ method. Individual expression values were normalized by comparison with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Oligonucleotides used in PCR from different exons of MMP-7 were: forward primer within exon 4, 5′-GGAGATGCTCACTTCGATGA-3′ and reverse primer within exon 5, 5′-ATACCCAAAGAATGGCCAAG-3′. The length of the MMP-7 PCR product is 104 bp. PCR primers used for GAPDH were: forward primer, 5′-CCCCATGGTGTCT-GAGCG-3′ and reverse primer, 5′-CGACAGTCAGCCG-CATCTT-3′. The length of the GAPDH product is 67 bp.

## 2.7. Immunofluorescence microscopy

H508 cells were subcultured in four-well Lab-Tek II chamber slides ($5 \times 10^4$ cells/well), and incubated for 24 h at 37 °C. After washing with PBS, PBS/2 M NaCl, cells were kept on ice, fixed with cold MeOH for 10 min, treated with 0.1% TX-100 for an additional 10 min, and blocked for 30 min with PBS/5% serum derived from the same animal species as the secondary antibody. Cells were then incubated at 4 °C overnight with the primary antibodies. After incubation, cells were washed in PBS, incubated with secondary FITC- or/and Tritc-conjugated antibodies at room temperature for 30 min and washed. Cell nuclei were visualized with Hoechst staining. To determine the cellular and subcellular distribution of pro-MMP-7 and pro-HB-EGF, slides were analyzed using both standard (Nikon Eclipse 80i) and confocal immunofluorescence microscopy (Zeiss LSM 510).

## 2.8. Small interfering RNA transfection

H508 cells were seeded in 96-well plates at approximately 10% confluence and incubated at 37 °C for 24 h. Four pooled small interfering RNA (siRNA) duplex oligos (Dharmacon) targeting human MMP-7 or a non-specific duplex oligo negative control (fluorescein-conjugated from Cell Signaling) were transfected into H508 cells with transfection reagent (Mirus) according to the manufacturer's recommendations. Two days following transfection, cells were incubated for an additional 5 days in FBS-free medium containing 50 $\mu$M DCT at 37 °C in an atmosphere of 5% $CO_2$ and 95% air. Cell proliferation was determined using the SRB assay as described above.

## 2.9. Statistical analysis

All figures (e.g. immunoblots) show data representative of at least three independent experiments. All graphs show the mean and standard error of the mean of at least three independent experiments. Statistical calculations were performed using the Student's two-tailed unpaired t-test assuming normal distribution with equal variance. Statistical significance is given by the number of *asterisks* ($^{*}P < 0.05$; $^{**}P < 0.005$) and a P value of less than 0.05 was considered statistically significant.

## 3. Results

### 3.1. Dose–response and time-course for the signaling and proliferative actions of deoxycholyltaurine on H508 colon cancer cells

To select appropriate bile acid concentrations and incubation times for the experiments that follow, we examined both the dose–response curves and time-courses for the actions of deoxycholyltaurine on p44/42 MAPK phosphorylation (activation) and on cell proliferation. We selected DCT as the test bile acid for these experiments because previous studies indicated that this agent interacts with $M_3$ muscarinic receptors on H508 colon cancer cells, and that this interaction results in transactivation of EGFR, thereby activating post-receptor MAPK signaling and stimulating cell proliferation [16,23,34].

As shown in Fig. 1A, DCT caused dose-dependent phosphorylation (activation) of p44/42 MAPK that was detectable with 10 μM and was maximal at the highest DCT concentration tested, 300 μM. Based on these findings, to readily detect differences in the degree of protein phosphorylation, we selected 300 μM DCT as the test concentration for experiments involving assays for p44/42 activation. To demonstrate clearly inhibitor effects on p44/42 MAPK phosphorylation, we sometimes used submaximal concentrations of DCT.

As shown in Fig. 1B, increases in cell proliferation were stimulated over the same range of DCT concentrations that activated p44/42 MAPK. Cell proliferation was maximal with 50 μM DCT and decreased slightly with higher concentrations of the bile acid (Fig. 1B). Hence, we used 50 μM DCT as the test concentration for experiments involving cell proliferation. Unless indicated otherwise, the concentrations of inhibitors and antibodies selected for study did not alter basal values for either MAPK phosphorylation or cell proliferation.

Previous extensive work with conjugated secondary bile acids indicates that DCT-induced H508 colon cancer cell proliferation is maximal after 5 days incubation [15,16]. Hence, we selected 5 day incubations to study changes in cell proliferation. In contrast, activation of post-receptor signaling cascades is rapid. As shown in Fig. 1D, DCT-induced activation of p44/42 MAPK was detected within 1–2 min and was
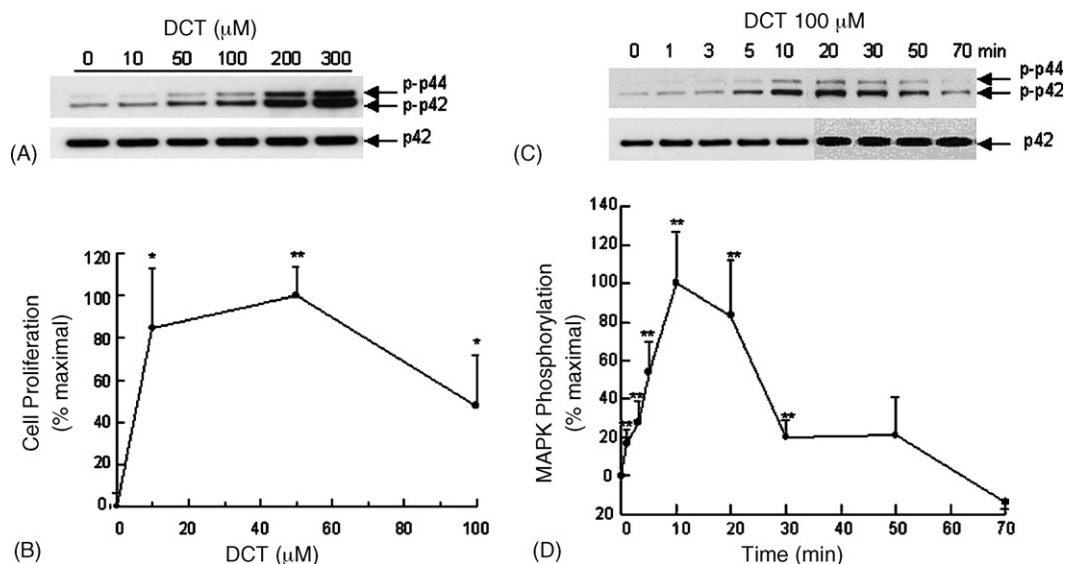


Fig. 1 – Dose–response and time-course for the signaling and proliferative actions of DCT on H508 colon cancer cells. (A) Dose–response for DCT-induced p44/42 MAPK phosphorylation. H508 cells were treated with the indicated concentrations of DCT for 10 min at 37 °C. p44/42 MAPK activity was determined by immunoblotting with antibodies specific for phosphorylated p44/42 MAPK. The quantity of protein added was verified by immunoblotting with antibodies specific for total p42 MAPK. Results are representative of five separate experiments. (B) Dose–response for DCT-induced cell proliferation. H508 cells were incubated for 5 days at 37 °C with the indicated concentrations of DCT. Cell proliferation was determined by the sulforhodamine B (SRB) colorimetric assay [33]. Results are expressed as mean ± S.E.M. of at least five separate experiments. $^{*}P < 0.05$ and $^{**}P$ 0.005, respectively, vs. unstimulated cells. (C) Time-course for DCT-induced p44/42 MAPK phosphorylation. H508 cells were treated with 100 μM DCT for 70 min at 37 °C and p44/42 MAPK activity was determined by immunoblotting at the indicated times with antibodies specific for phosphorylated p44/42 MAPK. The quantity of protein added was verified by immunoblotting with antibodies specific for total p42 MAPK. A representative immunoblot is shown and the graph (D) depicts quantitative densitometric analysis of at least five immunoblots. Results are expressed as mean ± S.E.M. $^{**}P < 0.005$ vs. unstimulated cells.

(A)

| DCT (300 μM) | − | + | + | + | + | + |
| GM6001 (μM) | − | − | 20 | 40 | − | − |
| NC GM6001 (μM) | − | − | − | − | 20 | 40 |

(B)

| DCT (50 μM) | − | − | − | + | + | + |
| GM6001 (0.2 μM) | − | + | − | + | + | − |
| NC GM6001 (0.2 μM) | − | − | + | − | + | + |

(C)

| DCT (300 μM) | − | + | + | + | + | − | + | + | + | + |
| MMP Inhibitor II (μM) | − | − | 10 | 100 | − | − | − | − | − | − |
| MMP Inhibitor III (μM) | − | − | − | − | 10 | − | − | − | − | − |
| MMP-3 Inhibitor II (μM) | − | − | − | − | − | − | 300 | 400 | − | − |
| MMP-3 Inhibitor IV (μM) | − | − | − | − | − | − | − | − | 100 | 200 |

(D)

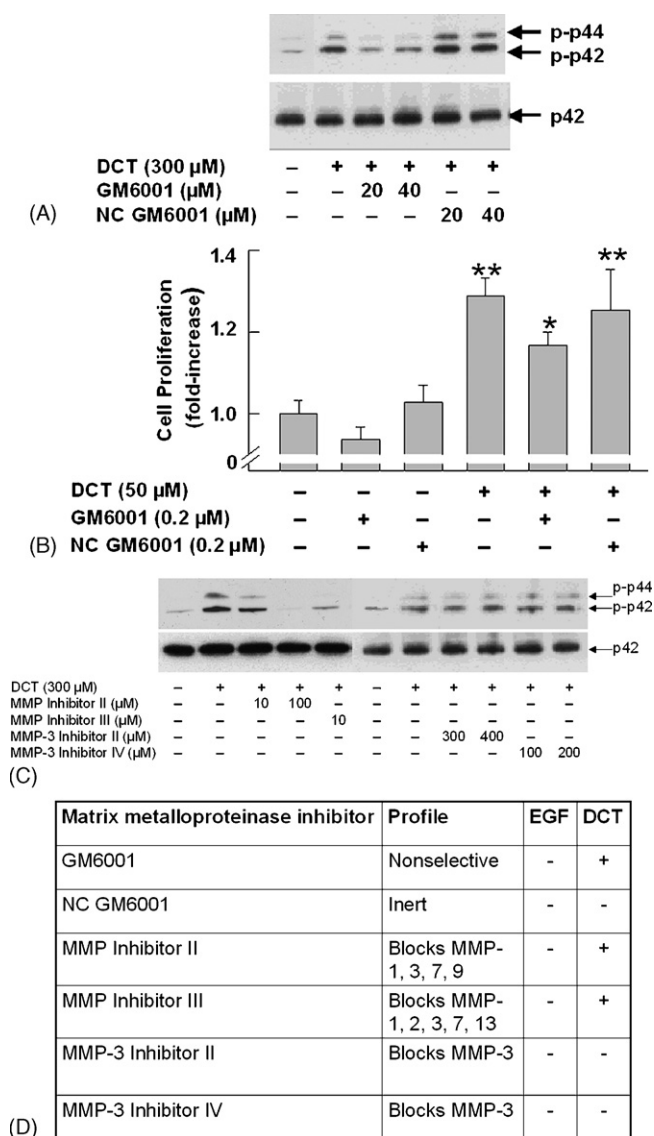| Matrix metalloproteinase inhibitor | Profile | EGF | DCT |
| --- | --- | --- | --- |
| GM6001 | Nonselective | − | + |
| NC GM6001 | Inert | − | − |
| MMP Inhibitor II | Blocks MMP-1, 3, 7, 9 | − | + |
| MMP Inhibitor III | Blocks MMP-1, 2, 3, 7, 13 | − | + |
| MMP-3 Inhibitor II | Blocks MMP-3 | − | − |
| MMP-3 Inhibitor IV | Blocks MMP-3 | − | − |

**Fig. 2 – Actions of matrix metalloproteinase (MMP) inhibitors on bile acid-induced MAPK phosphorylation and stimulation of H508 cell proliferation. (A) MAPK phosphorylation. H508 cells were treated with the indicated concentration of DCT for 10 min at 37 °C, alone or with two concentrations of GM6001 and NC GM6001. p44/42 MAPK activity was determined by immunoblotting with antibodies specific for phosphorylated p44/42 MAPK. The quantity of protein added was verified by immunoblotting with antibodies specific for total p42 MAPK. Results are representative of three separate experiments. (B) Cell proliferation. H508 cells were incubated with DCT, alone and with GM6001 or NC GM6001 for 5 days at 37 °C. Cell proliferation was determined by the sulforhodamine B (SRB) colorimetric assay [33]. Results are expressed as mean ± S.E.M. of at least three experiments. $^*P < 0.05$ vs. cells stimulated with DCT alone. $^{**}P < 0.005$ vs. unstimulated cells. (C) Actions of MMP inhibitors-II and -III and MMP-3 inhibitors-II and -IV on p44/42 MAPK phosphorylation. H508 cells were treated with 300 μM DCT for 10 min at 37 °C, alone or with the indicated concentrations of MMP inhibitors-II and -III, and**

maximal at 10 min. The level of p44/42 MAPK phosphorylation declined after 10 min and returned to baseline by 70 min (Fig. 1D). Based on these results, to study MAPK activation we examined 10 min incubations with test agents.

### 3.2. Matrix metalloproteinase inhibitors block DCT-induced p44/42 MAPK phosphorylation

A common mechanism for release of EGFR ligands, involves activation of secreted or cell membrane-anchored MMP's [30]. We used non-selective and selective MMP inhibitors as tools to determine whether the proliferative actions of bile acids are dependent on release of an EGFR ligand. GM6001, a non-selective hydroxamate MMP inhibitor [35], blocked DCT-induced p44/42 MAPK phosphorylation (Fig. 2A). In contrast, NC GM6001, an inert control agent, did not alter the actions of DCT (Fig. 2A). To determine the functional correlate of inhibiting MMP action with GM6001, we examined the effect of the inhibitor and control agent on DCT-induced cell proliferation. As show in Fig. 2B, the addition of GM6001, but not NC GM6001, caused a significant reduction in DCT-induced H508 cell proliferation.

To define more clearly which MMP mediates the actions of DCT, we examined the actions of more selective MMP inhibitors. MMP inhibitors-II and -III both blocked the actions of DCT (Fig. 2C). Within the MMP family, the spectrum of MMP inhibition by GM6001, MMP inhibitor-II, and MMP inhibitor-III, limits the likely candidates to MMP-1, -3 and -7 [30] (Fig. 2D). We used MMP-3 inhibitors-II and -IV to evaluate the possibility that MMP-3, which catalyzes release of TGF-α, mediates the actions of DCT. Addition of these MMP-3 inhibitors did not alter DCT-induced p44/42 MAPK phosphorylation (Fig. 2C). To exclude non-specific effects of the MMP inhibitors, control experiments showed that none of these agents altered EGF-induced p44/42 MAPK phosphorylation (not shown). These control experiments indicate that MMP inhibitors block release of EGFR ligands but do not block EGFR activation per se (that is, EGF stimulates p44/42 MAPK phosphorylation in the absence or presence of MMP inhibitors).

The activity profile for the MMP inhibitors used in these studies limits candidate MMP's mediating the actions of bile acids to MMP-1 and MMP-7 (Fig. 2D). A selective MMP inhibitor that can distinguish between MMP-1 and MMP-7 activity is not currently available. Overall, these data provide additional evidence in support of the hypothesis that bile acids stimulate the release from H508 cells of a ligand that binds to EGFR and activates post-receptor signaling. Moreover, the inhibitory actions of GM6001 and MMP inhibitors-II and -III on DCT-induced signaling support the hypothesis that bile acids activate EGFR by indirect mechanisms. That is,

**MMP-3 inhibitors-II and -IV. The quantity of protein added was verified by immunoblotting with antibodies specific for total p42 MAPK. Results are representative of three separate experiments. (D) Profiles and actions of MMP inhibitors on EGF- and bile acid-induced phosphorylation of p44/42 MAPK in H508 human colon cancer cells. (−) indicates the inhibitor had no effect; (+) indicates inhibition by the inhibitor.**

one would not expect MMP inhibitors to block cell proliferation if this was a consequence of direct interaction of DCT with EGFR. Narrowing candidate MMP's that mediate bile acid actions to MMP-1 and/or MMP-7 limits the relevant reported non-structural extracellular matrix component substrates for these enzymes to TGF-α and HB-EGF [30,36].

### 3.3. HB-EGF release and interaction with EGFR plays a role in mediating bile acid-induced p44/42 MAPK phosphorylation and proliferation of H508 colon cancer cells

Based on the results of our experiments with MMP inhibitors, to identify the EGFR ligand that mediates DCT-induced transactivation of EGFR we used antibodies to three common EGFR ligands: HB-EGF, EGF and TGF-α (Fig. 3). TGF-α is the most common EGFR ligand in the intestines and is reported to mediate carbachol- and bile acid-induced EGFR transactivation in T84 colon cancer cells [29,37] and in a cholangiocyte cell line [31], respectively.

Strikingly, in H508 colon cancer cells, neutralizing antibody to HB-EGF abolished the actions of DCT and HB-EGF, but not those of EGF (Fig. 3A). Neutralizing antibody to EGF reduced the actions of EGF but not those of DCT (Fig. 3B), whereas neutralizing TGF-α antibody reduced the actions of TGF-α but did not alter the actions of DCT or EGF (Fig. 3C). Collectively, the MMP inhibitor profile (Fig. 2) and the results with neutralizing antibodies to EGFR ligands (Fig. 3) indicated that HB-EGF was a promising candidate for the EGFR ligand that mediates the proliferative actions of DCT.

As shown in Fig. 3D, both neutralizing antibody to HB-EGF, and a non-toxic mutant form of diphtheria toxin that prevents HB-EGF release, CRM197, attenuated the proliferative actions of DCT. Control experiments with both anti-EGF and anti-TGFα antibodies showed that these agents inhibited EGF- and TGFα-induced cell proliferation ($P < 0.05$ and $P < 0.005$), respectively, but did not alter DCT-induced cell proliferation (not shown). Likewise, anti-EGF antibody did not block the proliferative effects of TGFα and anti-TGFα antibody did not block the proliferative effects of EGF. Overall, these data support the hypothesis that, in H508 colon cancer cells, MMP-catalyzed release of the EGFR ligand HB-EGF, but not EGF or TGF-α, plays a role in mediating bile acid-induced cell signaling and proliferation.

### 3.4. Recombinant HB-EGF-induced activation of p44/42 MAPK is inhibited by EGFR and MEK inhibitors and by antibody to the ligand binding domain of EGFR

To confirm that HB-EGF activates EGFR and post-receptor signaling in H508 cells, we examined the actions of increasing concentrations of the EGFR ligand on p44/42 MAPK phosphorylation (Fig. 4A). p44/42 MAPK phosphorylation was detected with 0.2 and maximal with 20 ng/ml HB-EGF (Fig. 4A). As expected for the actions of an EGFR ligand, an antibody to the ligand binding domain of EGFR, an inhibitor of EGFR activation (PD168393) and an inhibitor of MEK (MAPK kinase) (PD98059) all inhibited HB-EGF-induced p44/42 MAPK phosphorylation (Fig. 4B). Regarding effects on cell proliferation, addition of either EGFR inhibitors or antibody to the ligand binding
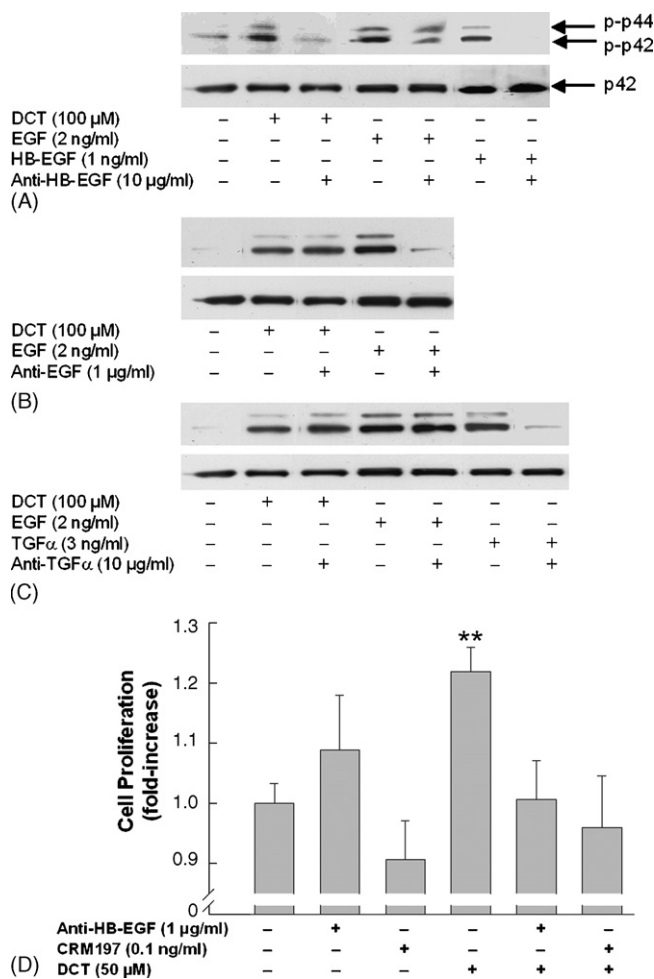


Fig. 3 – Effect of adding neutralizing EGFR ligand antibodies on DCT-induced p44/42 MAPK phosphorylation and H508 colon cancer cell proliferation. (A–C) Effect of adding HB-EGF, EGF and TGF-α antibodies on p44/42 MAPK phosphorylation. H508 cells were treated with DCT, EGF, HB-EGF and TGF-α for 10 min at 37 °C, alone or with the EGFR ligand antibodies shown. p44/42 MAPK activity was determined by immunoblotting with antibodies specific for phosphorylated MAPK. The quantity of protein added was verified by immunoblotting with antibodies specific for total p42 MAPK. Results are representative of three separate experiments. (D) Effect of adding HB-EGF antibodies and CRM197 on cell proliferation. H508 cells were incubated with DCT, alone and with antibody to HB-EGF or with CRM197 (0.1 ng/ml) for 5 days at 37 °C. Cellular proliferation was determined by the sulforhodamine B (SRB) colorimetric assay [33]. Results are mean ± S.E.M. of three to five experiments. **$P < 0.005$ vs. unstimulated cells.

domain of EGFR attenuated HB-EGF-induced cell proliferation ($P < 0.005$ for both) (not shown).

### 3.5. Recombinant MMP-7 mimics the actions of bile acids and antibody to MMP-7 blocks these actions

Based on the spectrum of action of MMP inhibitors used for these experiments (Fig. 2), we concluded that activation of
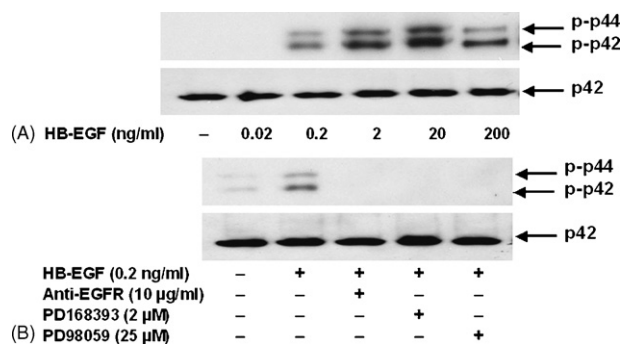
**Fig. 4 – Actions of recombinant HB-EGF on p44/42 MAPK phosphorylation in H508 colon cancer cells. (A) Actions of increasing concentration of HB-EGF on p44/42 MAPK phosphorylation. H508 cells were treated with increasing concentrations of HB-EGF for 10 min at 37 °C. p44/42 MAPK activity was determined by immunoblotting with antibodies specific for phosphorylated MAPK. The quantity of protein added was verified by immunoblotting with antibodies specific for total p42 MAPK. Results are representative of three separate experiments. (B) Antibodies to the ligand binding domain of EGFR, and EGFR (PD168393) and MEK (PD98059) inhibitors block HB-EGF-induced p44/42 MAPK phosphorylation. H508 cells were treated with the indicated concentration of HB-EGF for 10 min at 37 °C, alone or in the presence of the indicated concentrations of antibodies and inhibitors. p44/42 MAPK activity was determined by immunoblotting with antibodies specific for phosphorylated MAPK. The quantity of protein added was verified by immunoblotting with antibodies specific for total p42 MAPK. Results are representative of three separate experiments.**

either MMP-1 or MMP-7 may play a role in mediating the actions of DCT. Previous reports indicate that MMP-7 (also known as matrilysin; EC3.4.24.23) catalyzes release of HB-EGF [36]. Moreover, in clinical studies, expression of MMP-7 increases progressively with more advanced grades of colonic epithelial dysplasia and cancer [38–40] and, in a rodent model, the formation of intestinal tumors is reduced in *Min* mice that do not express MMP-7 [41]. Hence, experimental data support a role for MMP-7 in facilitating colon carcinogenesis. Our data suggest that bile acid-induced activation of the enzyme with consequent release of the EGFR ligand HB-EGF represents the molecular mechanism underlying these observations.

To resolve further the role of matrix metalloproteinases in mediating the observed actions of bile acids, we examined the effect of adding exogenous human recombinant MMP-1 and MMP-7 to H508 cells. As shown in Fig. 5A, MMP-7 but not MMP-1 (both 0.1 μg/ml) stimulated p44/42 MAPK phosphorylation. Adding an EGFR inhibitor (PD168393), neutralizing antibodies to HB-EGF or antibodies directed to the ligand binding domain of EGFR blocked the actions of MMP-7 (Fig. 5A).

As shown in Fig. 5B, the addition of increasing concentrations of MMP-7 and HB-EGF, but not of MMP-1, stimulated H508 cell proliferation. Over the range of concentrations tested, HB-EGF was both more potent and more efficacious than MMP-7. Moreover, as shown in Fig. 5B, the magnitude of

MMP-7- and HB-EGF-induced cell proliferation overlapped with that observed with a maximally efficacious concentration of DCT. The increase in H508 cell proliferation observed with 50 μM DCT was achieved with approximately 100 ng/ml MMP-7 or 0.2 ng/ml HB-EGF. These findings support the hypothesis that the actions of DCT are mediated by MMP-7-catalyzed release of HB-EGF with consequent autocrine stimulation of EGFR. Furthermore, the findings in Fig. 5A and B clearly exclude the possibility that MMP-1 plays any role in these actions.

To test further the hypothesis that activation of MMP-7 mediates the proliferative actions of DCT, we examined the effects of reducing MMP-7 expression with neutralizing antibody and siRNA. As shown in Fig. 5C, MMP-7 antibody blocked both MMP-7- and DCT-induced H508 cell proliferation. Likewise, as shown in Fig. 5D, transfecting cells with increasing concentrations of MMP-7, but not control, siRNA progressively inhibited DCT-induced H508 cell proliferation. Fluorescein imaging with labeled control siRNA verified the efficiency of transfection (Fig. 5D top). These data provide strong evidence that MMP-7 mediates the proliferative actions of the bile acid.

### 3.6. PCR confirms MMP-7 expression and induction by DCT, and immunofluorescence microscopy reveals plasma membrane co-localization of pro-HB-EGF and pro-MMP-7 expression in H508 colon cancer cells

To confirm that H508 human colon cancer cells express both pro-HB-EGF and pro-MMP-7 and to determine the subcellular localization of these molecules, we used quantitative reverse transcription-PCR and immunofluorescence microscopy. As shown in Fig. 6A, in H508 cells, PCR confirmed both that MMP-7 is expressed and that DCT induces time-dependent MMP-7 gene transcription. GAPDH gene transcription is shown as a control. As shown in Fig. 6B, adding 100 μM DCT stimulates a >10-fold induction of MMP-7 gene transcription (compared to basal MMP-7 mRNA level at each time point). This effect is maximal at 4–6 h and MMP-7 mRNA levels return to near basal values by 24 h. For immunohistochemistry, cells were incubated with goat anti-human pro-HB-EGF antibody and mouse anti-human pro-MMP-7 antibody. This was followed by a second incubation with FITC-conjugated rabbit anti-goat antibody and Tritc-conjugated goat anti-mouse antibody. Control cells incubated with only the second antibody did not reveal cellular immunofluorescence (not shown). As shown in Fig. 6C, phase contrast microscopy of H508 cells revealed many pleomorphic cells with multiple nuclei and an increased nuclear to cytoplasmic ratio typical of cancer cells. Cell treatment with both the primary antibody and the second antibody conjugated to the fluoroprobe revealed that human H508 cells express both pro-HB-EGF (green) and pro-MMP-7 (red). Moreover, Hoechst nuclear staining along with a merged image of both pro-HB-EGF and pro-MMP-7 immunostaining revealed cytoplasmic co-localization of pro-HB-EGF and pro-MMP-7 (Fig. 6C, extreme right panel).

To determine the subcellular localization of pro-HB-EGF (green) and pro-MMP-7 (red), we used confocal microscopy. As shown in Fig. 6D, confocal immunofluourescence studies showed a prominent co-localization of pro-HB-EGF and pro-
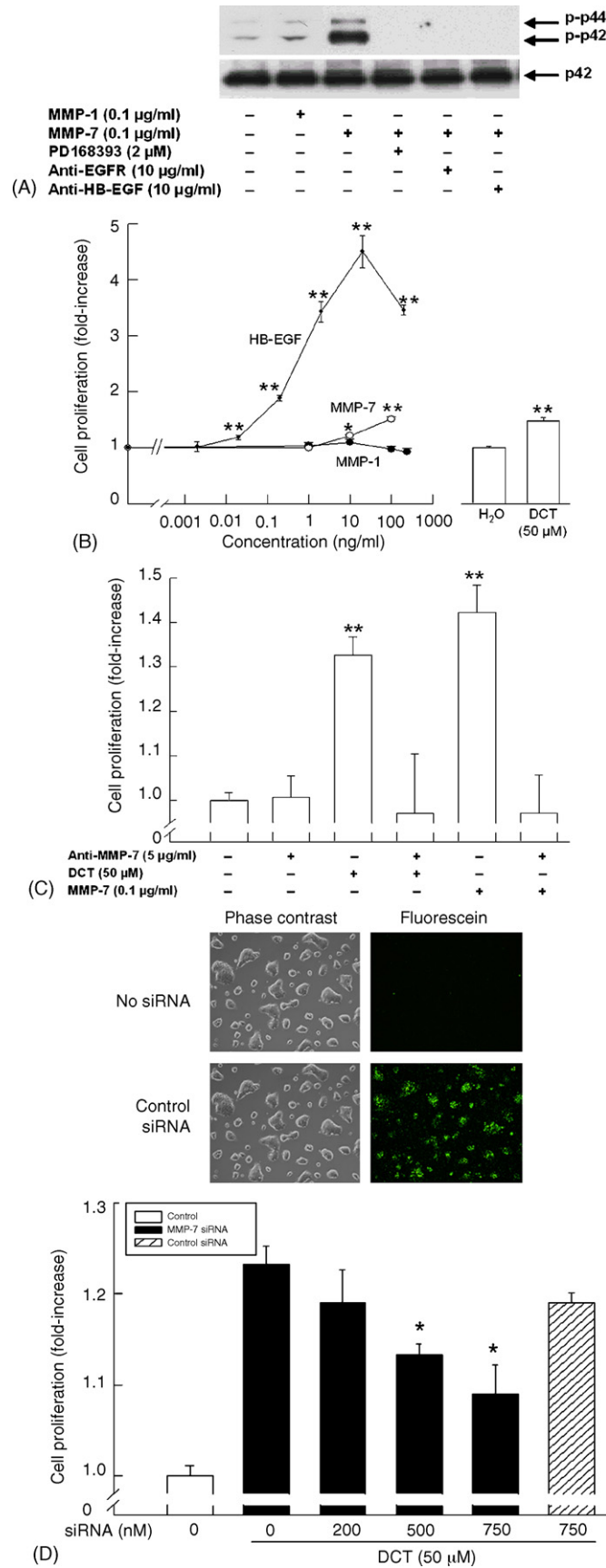
Fig. 5 – MMP-7 mimics the actions of bile acids on H508 colon cancer cells. (A) Effect of MMP-1 and MMP-7, alone or in the presence of an EGFR inhibitor and antibodies to EGFR and HB-EGF, on p44/42 MAPK phosphorylation. H508 cells were

MMP-7 (light orange) at the cell surface, presumably corresponding to the plasma membrane. These experiments confirmed both the expression and plasma membrane co-localization of pro-HB-EGF and pro-MMP-7 in H508 human colon cancer cells.

## 4.    Discussion

To implicate activation of a particular MMP in the promotion of colon cancer cell proliferation, several criteria must be achieved; the candidate MMP must be expressed in the cell being studied at a pericellular location, at the right time and concentration, and it must be activated or inhibited appropriately. Previously, we presented evidence for the functional interaction of conjugated secondary bile acids with muscarinic receptors and the resulting transactivation of EGFR [15,16,34]. Novel evidence favoring important roles for MMP-7 and HB-EGF in mediating the proliferative actions of bile acids include the following observations: the pattern of activity of MMP inhibitors is consistent with a role for MMP-7; recombinant MMP-7 mimics both the signaling and proliferative actions of HB-EGF and DCT; PCR confirms MMP-7 expression and induction by DCT; immunofluorescence microscopy demonstrates pro-MMP-7 localization at the cell surface in association with the pro-HB-EGF substrate; neutralizing HB-EGF antibody inhibits the effects of DCT; neutralizing MMP-7 antibody inhibits both MMP-7- and DCT-induced cell proliferation; and finally, cell transfection with siRNA to reduce MMP-7 expression inhibits DCT-induced cell proliferation.

Upregulation of EGFR expression and activation plays a pivotal role in colon cancer cell proliferation (reviewed in ref. [25]). Like other mammalian EGFR ligands, HB-EGF is synthesized as type I transmembrane protein (pro-ligand) [29]. EGFR pro-ligand cleavage is dependent on the actions of endopeptidases, primarily matrix metalloproteases, which regulate release of the active proliferative factor [29]. Active matrix metalloproteases are secreted or activated as part of a membrane-anchored pro-ligand/enzyme complex [29,36], and target a variety of cellular and extracellular protein substrates, including EGFR pro-ligands [30]. Collectively, the data presented herein provide compelling evidence that bile acid-induced proliferation of colon cancer cells is mediated by MMP-7-catalyzed release of HB-EGF, followed by activation of EGFR and downstream signaling cascades. The latter events alter gene transcription and result in cellular proliferation [29,42].

The matrix metalloprotease family comprises at least 22 members that are grouped by structure and cellular location (reviewed in refs. [30,43]). Of eight structural groups, five are secreted into the extracellular space and three are tethered to the cell membrane by covalent linkages [30,43]. MMP-7 (matrilysin; EC3.4.24.23), a secreted 'minimal domain' MMP that is associated with the cell surface, contains three major structural components; an amino-terminal sequence that directs it to the endoplasmic reticulum, a sequence containing a zinc-interacting thiol group that maintains MMP-7 as an inactive zymogen and a zinc binding catalytic domain [30]. As confirmed in the present study by immunofluorescence microscopy, cell surface association increases MMP-7 stability and proximity to potential plasma membrane substrates (e.g. pro-HB-EGF) [30,36,44].

MMP-7-mediated cleavage of pro-HB-EGF (206 amino acids) and release of HB-EGF (86 amino acids), is reported in several cancer cell types [30,36]. Studies conducted more than a decade ago, reported MMP-7 expression in approximately 80% of human colon cancers [38] and in the CaR-1 human rectal carcinoma cell line [45]. Moreover, in a murine cancer model, MMP-7 gene knockout reduces intestinal tumor formation [41]. In the present report, we contribute the novel observations that H508 human colon cancer cells express MMP-7, that a bile acid induces a substantial increase in MMP-7 expression, and that MMP-7-catalyzed release of HB-EGF plays a key role in mediating the proliferative actions of DCT.

Experiments, beyond the scope of the present communication, are needed in human colon cancer cells to validate the role of MMP-7 in mediating bile acid signaling and to place it in the context of other enzymes that can release EGFR ligands. Demonstrating expression and bioactivity of MMP-7 in colon cancer cells is necessary, but not sufficient to prove that this is the only enzyme that mediates bile acid-induced

treated with MMP-7 (0.1 μg/ml) and MMP-1 (0.1 μg/ml), alone or in the presence of an EGFR inhibitor (PD168393, 2 μM), an anti-EGFR antibody (10 μg/ml) and an anti-HB-EGF neutralizing antibody (10 μg/ml), for 10 min at 37 °C. p44/42 MAPK activity was determined by immunoblotting with antibodies specific for phosphorylated MAPK. The quantity of protein added was verified by immunoblotting with antibodies specific for total p42 MAPK. Results are representative of three separate experiments. (B) Comparison of the efficacy of increasing concentrations of HB-EGF, MMP-7 and MMP-1, with that of DCT for stimulating H508 cell proliferation. Cells were incubated with increasing concentrations of MMP-7, MMP-1 and HB-EGF for 5 days at 37 °C and stimulation of cell proliferation was compared to that observed with water and DCT. Results are mean ± S.E.M. of three experiments. \*P < 0.05 and \*\*P 0.005, respectively, vs. unstimulated cells. The vertical axis (cell proliferation) is the same for the line drawing (left panel) and bar graph (right panel). (C) MMP-7 antibodies inhibit the proliferative actions of MMP-7 and DCT on H508 colon cancer cells. Cells were incubated with DCT and MMP-7, alone and in the presence of antibody to MMP-7, for 5 days at 37 °C. Cellular proliferation was determined by the sulforhodamine B (SRB) colorimetric assay [33]. Results are mean ± S.E.M. of three experiments. \*\*P < 0.005 vs. unstimulated cells. (D) Transfection of cells with MMP-7 siRNA inhibits the proliferative actions of DCT. H508 cells were transfected with siRNA duplex oligos targeting human MMP-7 or a fluorescein-tagged non-specific duplex oligo negative control. Two days following transfection, cells were incubated for an additional 5 days in FBS-free medium containing 50 μM DCT. The inset at the top shows cellular accumulation of fluorescent control siRNA. Cellular proliferation was determined by the sulforhodamine B (SRB) colorimetric assay [33]. Results are mean ± S.E.M. of three experiments. \*P < 0.05 vs. cells incubated with DCT alone.
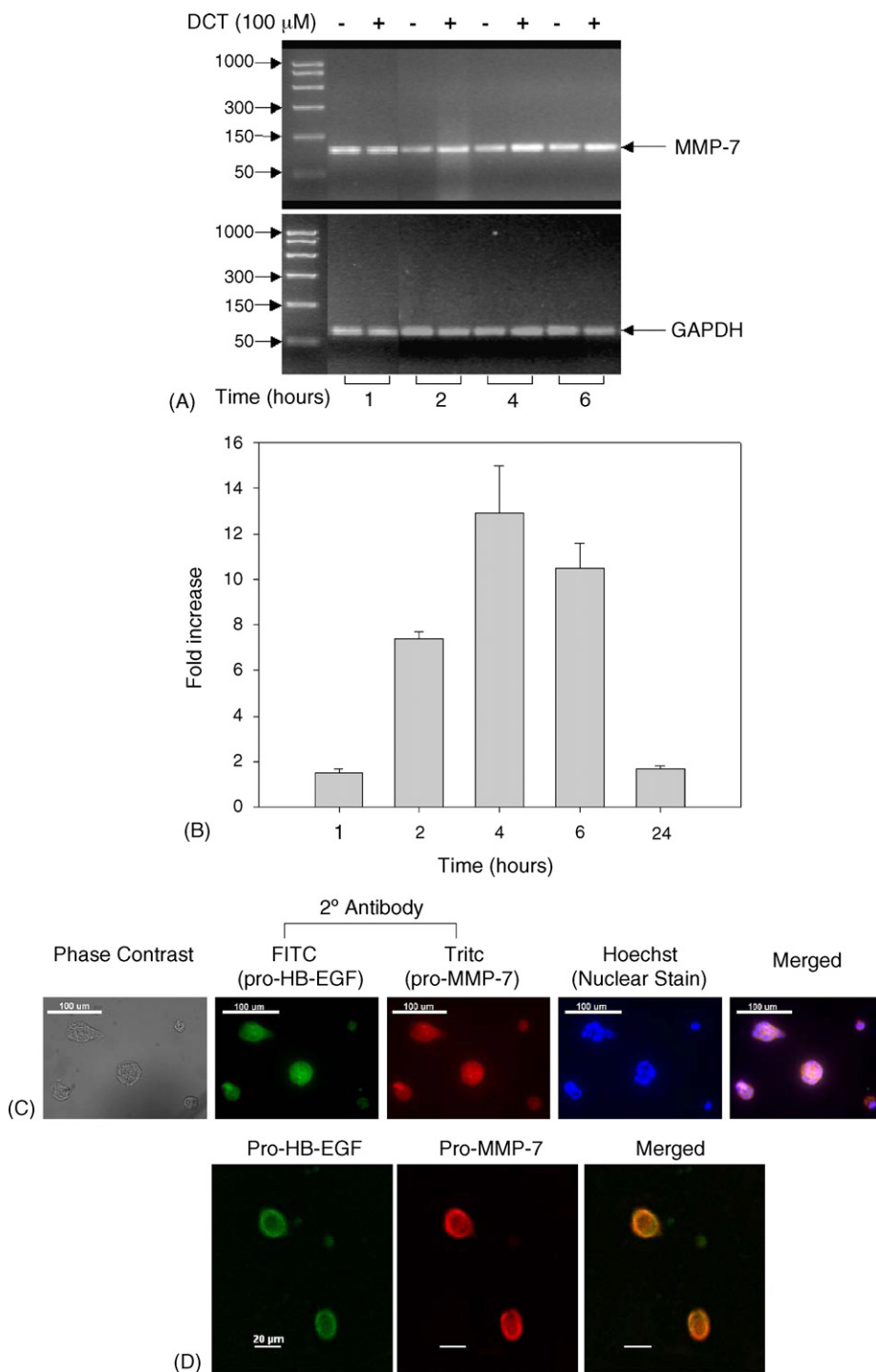
Fig. 6 – Expression of MMP-7 and plasma membrane co-localization of pro-HB-EGF and pro-MMP-7 in H508 human colon cancer cells. (A) Agarose gel (2%) of PCR products from quantitative reverse transcription-PCR in H508 cells confirms MMP-7 gene transcription and induction by DCT. H508 cells were cultured in serum-free media for 24 h prior to DCT (100 μM) treatment. Total RNA was isolated following harvest of cells at the indicated time points. DNA size markers (base pairs) are indicated at left with arrows. Arrows at right indicate PCR product for MMP-7 and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control. (B) DCT induces MMP-7 mRNA expression in H508 colon cancer cells. The level of MMP-7 mRNA was determined by quantitative real-time PCR as described in Section 2. Results are mean ± S.E.M. of three experiments. (C) Immunofluorescence microscopy. (Left panel) Phase contrast microscopy of H508 colon cancer cells. Immunofluorescence localization in H508 colon cancer cells using pro-HB-EGF (FITC, green fluorescence) and pro-MMP-7 (Tritc, red fluorescence) antibodies is shown. Cell nuclei are stained with Hoechst (blue). (Extreme right panel) Merged images of pro-HB-EGF, pro-MMP-7 and cell nuclei. Cytoplasmic co-localization of pro-HB-EGF and pro-MMP-7 is shown by light orange color. Scale bar = 100 μm. (D) Confocal microscopy. Immunofluorescence staining of pro-HB-EGF (left, green

transactivation of EGFR. Although our results using MMP-7 neutralizing antibody and siRNA provide strong evidence that the enzyme plays an important role in mediating the actions of bile acids, enzymes in the metzincin superfamily of metalloproteases, designated 'disintegrin and metalloproteinase' domain metalloproteases (ADAMs), can also catalyze EGFR ligand release [43]. Current data on the expression of ADAM metalloproteases in normal or neoplastic colonic epithelium are limited, and few selective inhibitors of their actions are available. Hence, the possibility that other metalloproteases besides MMP-7 play a role in bile acid-induced HB-EGF release is currently being explored in our laboratory. It is also conceivable that in a given cancer cell line more than one metalloprotease mediates these actions or that different colon cancer cell lines express different bile acid-responsive metalloproteases. A multiplicity of EGFR pro-ligand releasing enzymes could provide additional regulatory control of bile acid-induced cell proliferation. As we showed for MMP-7, beyond demonstrating expression of a particular metalloprotease(s), it is necessary to confirm that the actions of bile acids (e.g. activation of EGFR signaling and stimulation of cell proliferation) are attenuated by pharmacological inhibition, and by molecular approaches that knockout and/ or knockdown the enzyme(s).

Our work now focuses on identifying additional MMPs and/ or ADAMs that are expressed in H508 colon cancer cells. Future studies will elucidate the precise mechanisms underlying bile acid-induced metalloprotease activation and the role of bile acid interaction with muscarinic receptors in initiating this process [16,23]. Based on studies from our laboratory and others, we conclude that conjugated secondary bile acids function as growth factors, thereby contributing to a favorable microenvironment that promotes the survival and proliferation of colon cancer cells. Blocking the proliferative actions of bile acids may retard colon cancer growth and, possibly, tumor invasion and spread. The clinical and therapeutic implications of our work include the possibility of achieving these goals by targeting multiple steps leading from metalloprotease activation to release of HB-EGF and activation of EGFR signaling.

## Acknowledgements

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fluorescence) and pro-MMP-7 (middle, red fluorescence) demonstrates cell surface co-localization (right, light orange fluorescence). Scale bar = 20 μm.

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