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RESEARCH PAPER



Serine protease modulation of Dependence Receptors and EMT protein expression

Kara McNair^a, Caroline M. Forrest^a, Maria C. J. Vincenten^a, L. Gail Darlington^b, and Trevor W. Stone^{a,c}

^aCollege of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, UK; ^bDepartment of Internal Medicine, Ashted Hospital, Ashted, UK; ^cThe Kennedy Institute, University of Oxford, Oxford UK

ABSTRACT

Expression of the tumour suppressor Deleted in Colorectal Cancer (DCC) and the related protein neogenin is reduced by the mammalian serine protease chymotrypsin or the bacterial serine protease subtilisin, with increased cell migration. The present work examines whether these actions are associated with changes in the expression of cadherins, β -catenin and vimentin, established markers of the Epithelial-Mesenchymal Transition (EMT) which has been linked with cell migration and tumour metastasis. The results confirm the depletion of DCC and neogenin and show that chymotrypsin and subtilisin also reduce expression of β -catenin in acutely prepared tissue sections but not in human mammary adenocarcinoma MCF-7 or MDA-MB-231 cells cultured in normal media, or primary normal human breast cells. A loss of β -catenin was also seen in low serum media but transfecting cells with a *dcc*-containing plasmid induced resistance. E-cadherin was not consistently affected but vimentin was induced by low serum-containing media and was increased by serine proteases in MCF-7 and MDA-MB-231 cells in parallel with increased wound closure. Vimentin might contribute to the promotion of cell migration. The results suggest that changes in EMT proteins depend on the cells or tissues concerned and do not parallel the expression of DCC and neogenin. The increased cell migration induced by serine proteases is not consistently associated with the expression of the EMT proteins implying either that the increased migration may be independent of EMT or supporting the view that EMT is not itself consistently related to migration. (241).

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DCC; neogenin; serine proteases; dependence receptors; chymotrypsin; subtilisin; catenin; cadherin

Introduction

The tumour suppressor protein Deleted in Colorectal Cancer (DCC) has been linked with a variety of cancer cells from which it is either absent or in which its concentration or activity is substantially below that in non-cancerous tissues.^{1–5} Its loss or dysfunction by genetic, epi-genetic or acute disruption may represent one of the several factors required for a normal cell to undergo transformation into a cancerous cell.⁶ We have recently reported that the cellular expression of DCC, as well as the structurally and functionally related molecule neogenin^{7–9} both of which are receptors for the secreted family of netrin proteins¹⁰ can be depleted by the mammalian serine protease chymotrypsin, which is present in the gastro-intestinal tract and systemic circulation, and by the bacterial chymotryptic enzyme subtilisin.^{11,12} The removal of DCC and neogenin was accompanied by increased motility and migration in several transformed cell lines including human neuroblastoma (SH-SY5Y) cells and the mammary adenocarcinoma lines MCF-7 and MDA-MB-231. Since chymotrypsin levels in the intestine and circulation are increased with obesity, and since subtilisin is present in processed meat products and agricultural probiotics, it was suggested that these effects might contribute to the associations between a meat-based diet, obesity and carcinogenesis.^{11–13} The effects of chymotrypsin and subtilisin were prevented by Bowman-Birk inhibitors found in many fruit and vegetables, consistent with the hypothesis that

a plant-rich diet would be protective against cancer^{11,12} as concluded by several recent epidemiological studies.^{14,15}

The present work probes further into the interactions between DCC and serine proteases by assessing their effects on markers of Epithelial-Mesenchymal Transition (EMT). E-cadherin is central to the formation of *adherens* junctions between cells and its presence reflects the existence of a stable, resting colony of cells. E-cadherin is a key regulator of EMT initiation and its expression falls as cells lose adherence and adopt a more aggressive, motile phenotype associated with the EMT phase of migration and the formation of distant metastases.^{16–21} β -catenin normally exists in association with E-cadherin in junctional complexes so that a fall in E-cadherin expression results in an increase in unbound β -catenin levels in the cytoplasm from where it can enter the nucleus and can – as a component of the wingless (Wnt) transduction system – activate transcription factors that promote increased proliferation and cell migration.^{22–32} Any β -catenin which is phosphorylated in the cytoplasm remains trapped there until it is transported to the proteasome for degradation and disposal.^{25,33,34} Small molecule inhibitors of β -catenin are able to suppress carcinogenesis.³⁵

Vimentin is a microtubular component involved in the regulation of cytoskeletal function relevant to the changes in intercellular adhesion and EMT.^{36–39} The expression of vimentin is increased as cells lose adherence and transition from a resting, epithelial phenotype to a migratory behaviour.

CONTACT T. W. Stone  Trevor.Stone@kennedy.ox.ac.uk  The Kennedy Institute, Roosevelt Drive, University of Oxford, OX3 7FY, UK.

Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/kcbt.

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These aspects of E-cadherin, β -catenin and vimentin function have resulted in their being linked to a range of cancers, with a fall in E-cadherin levels and increased free β -catenin and vimentin concentrations viewed as characteristic of early malignancy, whereas N-cadherin expression – where it occurs – declines. If this relationship is valid, we predicted that the increased cell migration induced by serine proteases should be accompanied by a reduction in E-cadherin expression and increased β -catenin and vimentin. The objectives of the study were firstly to examine the effects of chymotrypsin and subtilisin on the expression of these proteins to assess whether any effects could contribute to the functional changes in cell behaviour.¹¹ A second objective was to generate cells possessing exogenous, transfected DCC to determine whether this would modify the expression of EMT markers and, if so, whether they would be susceptible to change by chymotrypsin and subtilisin.

An additional reason for clarifying the relationships between the tumour suppressors and EMT markers is that the widely accepted view of EMT as being fundamental to cell migration and metastasis has been challenged by studies which indicate a greater role in determining cell susceptibility to toxic factors including chemotherapeutic drugs.⁴⁰ The results may, therefore, be relevant to treating cancer resistance to chemotherapy and the development of drugs which are less likely to induce or encounter resistance.

Materials and methods

Since we have found that the commonly used housekeeping genes such as actin can be affected by the serine proteases, we have used the cytoskeletal protein RhoA in most cases as a control for consistency of protein content and immunoblotting efficiency.¹¹ This protein is unaffected by serine proteases at concentrations which affect the proteins of primary interest in this study.

Tissue slices

As in our previous work initial experiments were performed on normal mature adult tissue using 450 μ m thick sections of adult rat hippocampus which can be maintained for several hours in a simple, oxygenated physiological solution of defined composition analogous to natural cerebrospinal fluid (CSF) and without the need for serum or other additives, conditions under which they exhibit physiologically normal electrical activity and neuronal communication. These slices are exactly similar to those used routinely for the electrophysiological recording of synaptic potentials and which have been used to compare the physiological and molecular effects of various compounds.

Male Wistar rats (sourced from Harlan Olac, UK) between 100–150 g in weight (4–8 weeks old) were terminally anaesthetised with a 25% aqueous solution of urethane (5 ml/kg) and then killed by cervical dislocation. The brain was rapidly removed into ice-cold artificial CSF of composition (in mM): NaCl 115; KH₂PO₄ 2.2; KCl 2; MgSO₄ 1.2; NaHCO₃ 25; CaCl₂ 2.5; glucose 10, gassed with 5%CO₂ in air. The hippocampi were rapidly dissected

out and chopped into 450 μ m transverse slices using a McIlwain tissue chopper. The slices were held in ice-cold artificial CSF and transferred to mini-petri dishes (diameter 35 mm) in an incubation chamber where they were maintained in the water-saturated atmosphere of 5%CO₂ in air at room temperature (~ 20°C). After a recovery period of 1–2 h, compounds were added to the slices for 4 h, at which time the solution was withdrawn by pipette and replaced by fresh, drug-free solution. This replenishment was repeated twice more until, after 45 minutes, individual slices were washed in fresh solution and transferred to Eppendorf tubes pre-cooled to –20°C in dry ice and stored at –80°C until used for immunoblotting.

Immunoblotting

Hippocampal slices were homogenised in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% Triton X-100, 1% IGEPAL, with a Roche complete protease inhibitor tablet) and centrifuged at 15000 g for 5 min at 4°C. Cell cultures were briefly washed in ice cold PBS, scraped in ice cold RIPA buffer and the cell suspension centrifuged at 15000 g for 5 min at 4°C. Supernatants from tissue and cells were collected for protein concentration determination using the Bio-Rad protein assay (Bio-Rad, Hemel Hempstead, UK). Samples were normalised to 10 μ g and prepared as follows: 65% protein sample, 25% sample buffer and 10% reducing agent (Life Technologies, Paisley, UK) and heated at 70°C for 10 min. The protein samples were loaded onto NuPAGE Novex 4–12% Bis-Tris (1 mM) 15 or 17 lane gels (Life Technologies, Paisley, UK) and run at 175 volts for 70 min to separate proteins according to their molecular weight. SeeBlue pre-stained standard (10 μ L)(Life Technologies, Paisley, UK) was included on each gel as a molecular weight marker. The separated proteins were then blotted onto Invitrolon polyvinylidene difluoride) membranes (Life Technologies, Paisley, UK) at 35 V for 70 min. After rinsing well with distilled water, membranes were blocked for 1 h in 5% non-fat dried milk solution in Tris-buffered saline containing 0.05% Tween (TBST) before overnight incubation at 4°C with the appropriate primary antibody (diluted in 5% milk-TBST). Membranes were then washed 3 times for 15 min with TBST and incubated with the appropriate horseradish peroxidase (HRP) conjugated secondary antibody (prepared in 5% milk-TBST) for 1 h at room temperature. Following secondary antibody incubation, blots were washed 3 times for 15 min with TBST then visualised using a Pierce Enhanced Chemiluminescence 2 detection kit (Fisher Scientific, Loughborough, UK).

Western blot analysis was carried out using the following primary antibodies:-

From Santa Cruz, Insight Biotechnology, Wembley, UK:- Neogenin (goat polyclonal, sc-6536, 1:1000); E-cadherin (mouse monoclonal, sc-8426, 1:1000 dilution); N-cadherin (mouse monoclonal, sc-271386; 1:1000 dilution); pan-cadherin (mouse monoclonal, sc-59876; 1:1000 dilution); vimentin (mouse monoclonal, sc-7557, 1:1000 dilution); RhoA (mouse monoclonal, sc-418, 1:5000 dilution); β 3-tubulin (mouse monoclonal, sc-51670, 1:1000 dilution);

From BD Pharmingen, Oxford, UK):- DCC (mouse monoclonal, 554223, 1:5000 dilution);

From Abcam, Cambridge, UK):- β -catenin (rabbit monoclonal, ab32572, 1:5000 dilution).

The following secondary HRP-conjugated antibodies were used at a 1:5000 dilution: donkey anti-goat HRP (sc-2020); goat anti-mouse (sc-2005); donkey anti-rabbit HRP (sc-2313) (from Santa Cruz, Insight Biotechnology, Wembley, UK).

Data analysis

All Western blots were quantified using the Image J software and comparisons were made statistically between groups of slices or cultures using ANOVA followed by the Bonferroni *post hoc* multiple comparison test for selected datasets. The analyses were performed with InStat 3.0 software (GraphPad, San Diego, USA). A probability value of 0.05 was adopted as the criterion for significance. Actual *P*-values are indicated when provided by the InStat software.

Immunocytochemistry

Cells were passaged into 24-well plates containing poly D-lysine (50 μ g/ml, Sigma Aldrich, UK; product P7280) coated glass coverslips. Following individual experimental protocols, cells were fixed with 500 μ l of 4% paraformaldehyde (PFA) for 30 minutes at 4°C and were then rinsed 3 times for 3 min with PBS before being incubated overnight at 4°C in primary antibody made up in PBS with 0.3% Triton X (250 μ l per well). The following day, after 3 \times 3 minute PBS rinses, cells were incubated in the appropriate secondary fluorescent antibody at 1:200 dilution in PBS with 0.3% Triton X (250 μ l per well) for 1 hr at room temperature. This was followed by 3 \times 3 minute PBS rinses before mounting the coverslips with Vectashield® fluorescent mounting medium (Vector Laboratories, California; product H-1000) on microscope slides where they were sealed and subsequently stored at -20°C prior to image analysis.

Primary antibodies were as noted under Western blots, used at dilutions of: DCC 1:500; neogenin 1:500; β -catenin 1:1000. Secondary antibodies were obtained from Molecular Probes, Life Technologies, Paisley, UK; AlexaFluor 488 goat anti-rabbit #A11008; used at 1:200 dilution.

Cell cultures

The MCF-7 cell line is an adherent, human Caucasian breast adenocarcinoma cell line purchased at passage 15 (ECACC, Wiltshire, UK, #86012803). We routinely used this cell line only in the first 30 passages following resuscitation. The medium used consisted of Minimum Essential Medium Eagle (EMEM); 2 mM glutamine; 1% penicillin/streptomycin; 1% Non-Essential Amino Acids (NEAA) and 10% Fetal Bovine Serum (FBS). The cells were maintained at 37°C in 5%CO₂. Cells were plated at an initial density of 1 \times 10⁵ cells/ml unless otherwise stated. This cell line was passaged once per week and fed every 2–3 days by a 50% media exchange.

The MDA-MB-231 cell line is an adherent, human Caucasian breast adenocarcinoma cell line purchased at passage 40 (ECACC, Wiltshire, UK, #92020424). This line was used for a limited number of passages (not more than 30 from

initial passage number). The medium used was L15: 1% penicillin/streptomycin, 2 mM glutamine and 15% FBS unless otherwise indicated. The cells were maintained at 37°C in a humidified CO₂-free environment. Cells were plated at an initial density of 1 \times 10⁵ cells/ml unless otherwise stated. This cell line was passaged twice per week and fed every 2–3 days, when required, with a 50% media change.

The SH-SY5Y cell line (ECACC, Wiltshire, UK, #94030304) is an adherent, human neuroblastoma cell line obtained at a passage number of 17. This cell line was only used up to passage 30 as beyond this passage the line begins to lose its neuronal characteristics as measured by β -tubulin protein expression using Western blot analysis. The medium used for this cell line was HAM's F-12: EMEM (1:1): 2 mM glutamine, 1% penicillin/streptomycin, 1% NEAA and 15% FBS (reduced to 10% once cultures were established) unless otherwise indicated. The cells were maintained at 37°C in a humidified atmosphere containing 5%CO₂. Cells were plated at an initial density of 5 \times 10⁴ cells/ml unless otherwise stated. This cell line was passaged once per week and fed every 2–3 days with a 50% media change.

Normal adult human primary mammary epithelial cells (HMEpC) (ECAC, Wiltshire, UK, #06090747; Cell Applications #830-85 A) were obtained at 5th passage and were maintained in culture for up to 16 doublings. The cells were grown in Human Mammary Epithelial Cell Growth Medium (Cell Applications #815-500, Sigma). This is a defined medium which does not require serum supplementation. HMEpC were maintained at 37°C in 5% CO₂. The primary cells were passaged once per week and seeded at a density of 5000 cells/cm². Cultures were fed every 2–3 days with a 50% media change. During passaging, the trypsin was inactivated by adding media containing 10% FBS. The serum was then removed by centrifuging and re-suspending the pellet in serum free media complete growth medium.

All cell lines were passaged using a 0.25% trypsin-EDTA solution (Sigma, T4049) added to the cells briefly before being removed. Cells were then incubated at 37°C for 2–4 minutes until all cells detached from the flask. The trypsin was then neutralised by the addition of medium containing FBS. Again, for the purposes of the primary cell line, following neutralisation, the cells were gently spun down and the medium containing FBS was removed and cells re-suspended in HMEpC medium before cell densities were calculated by counting on a haemocytometer and appropriate dilution in media.

All media components were obtained from Sigma-Aldrich UK, with product codes as follows: HAM's F-12: N4888; EMEM: #M2279; Human Mammary Epithelial Cell Growth Medium (#815K-500); NEAA: M7145; L-glutamine: G7513; Penicillin/Streptomycin: P0781; FBS: F9665.

Agarose spot migration

As a test of chemotactic migration we used the agarose spot method described by Wiggins & Rappoport⁴¹ as in our previous work.¹¹ Low melting point agarose (LMA) (Sigma-Aldrich, Poole, UK, #A9045) was made up to a 0.5% solution in Dulbecco's PBS (DPBS) (Life Technologies, Paisley, UK, product #14190-094) by boiling until fully dissolved. The

solution was then cooled to 40°C ready for use. Human netrin-1 (R&D systems, Abingdon UK, product #1254-N4) was added to the LMA solution at 1000 ng/ml. Control LMA solution contained DPBS only. A spot of 10 µl of LMA solution was pipetted carefully into the centre of each well of a 12-well plate and allowed to set for at least 1 hour in a refrigerator at 4°C.

Cells were passaged in full serum media and 1.5 ml of cell suspension was pipetted into each well containing an LMA spot. They were incubated at 37°C in 5% CO₂ for 4 hours when the medium was changed to a migrating medium containing 0.1% serum with dissolved compounds to be added to the cells. After 24 hours the wells were photographed at x4 magnification on an Olympus IX50 inverted microscope attached to an Olympus DP50 camera, utilising Cell software from Soft Imaging System (EMSIS GmbH, Munster, Germany). In total, 6 images were taken per well, corresponding roughly to 12, 2, 5, 6, 7 and 10 o'clock positions. A minimum of 4 different experiments from 4 different passages were used for each experiment.

Data analysis

Image analysis for the spot assay was carried out using Image J software (<http://rsb.info.nih.gov/ij/>). Cells that had crossed into the spot were counted, and the average number of cells per spot was calculated per treatment.

Wound healing assay

For assessing spontaneous migration potential, the wound or scratch healing assay was used.^{11,42,43} Cells were plated at a density of 2×10^5 cells/ml in 6-well plates in normal media. These plates were previously marked with a grid reference aid by scoring lines 2 mm apart on the bottom of the plate. After attaining confluence a scratch wound was made down the centre of each well (perpendicular to the grid reference) with a sterile 200 µl pipette tip. The cells were then washed twice with 1 ml of medium after which the cells were replenished fully with 10% serum medium. Photographs were taken immediately at 0 h at 3 points along the wound using an Olympus DP50 camera attached to an Olympus IX50 inverted microscope and Cell software at x4 magnification (Soft Imaging Systems, EMSIS GmbH, Munster, Germany). These three points were identified using the grid reference allowing the same three points to be imaged at subsequent time points. Following the initial image at $t = 0$ drugs of interest were added. Photographs were subsequently taken after 24 h, 48 h and 72 h and wound closure was quantified by measuring open wound area, using the T-scratch software.

Data analysis

The percentage difference of open wound area between 0 h and the later time points was calculated and comparisons between control and treated groups were made using *t*-tests based on the results obtained using the T-scratch software. The analyses were performed with InStat 3.0 software (GraphPad, San Diego, USA). A probability value of 0.05 was adopted as the criterion for significance. Actual *P*-values are indicated when provided by the software.

Proliferation:bromodeoxyuridine (brdu)assay

Proliferation was assessed in the MCF-7 and MDA-MB-231 breast adenocarcinoma cell lines and in the primary (HMEpC) breast cells using a colorimetric BrdU ELISA kit (ab126556, Abcam, Cambridge, UK). Each assay was performed in triplicate. MCF-7 cells were plated at a density of 2×10^5 cells/ml in 96-well plates and left to attach for 24 hours. The cells were then treated with subtilisin and α -chymotrypsin for 2, 6 or 24 hours. BrdU reagent was added to the cells at 2, 6 and 24 hours to evaluate proliferation. A series of wells on each plate were left untreated with BrdU as a background reading. The cells were fixed and the DNA denatured before assaying. Briefly, cells were washed, incubated with detector antibody for 1 hour, washed again and then incubated with peroxidase conjugate secondary antibody for 1 hour. Following a final wash step, cells were incubated for 30 mins with TMB peroxidase substrate. Stop solution was added to the wells and plates read in a microplate reader (Opsys MR, Dynex Technologies, Worthing, UK) at dual wavelengths of 450/550 nm. Proliferation was expressed as the mean optical density of BrdU positively labelled cells/BrdU negative cells.

Data analysis

Statistical analyses were performed with InStat 3.0 software (GraphPad, San Diego, USA), with a probability of 0.05 adopted as the criterion for significance. Actual *P*-values are indicated when provided by the InStat software.

Transfection of dcc plasmid

A pCMV *dcc* plasmid (#16459) was obtained from Addgene courtesy of Dr. B. Vogelstein. This plasmid contains the Cytomegalovirus (CMV) promoter, a strong constitutive promoter, the *dcc* gene flanked by 2 XhoI restriction sites, a polyadenylation signal (Poly A), Ampicillin resistance gene and Neomycin (Neo) resistance gene.

DH5alpha *E.coli* cells containing the plasmid were grown on agar plates containing 75 µg/ml ampicillin and the plates were incubated overnight at 37°C. Individual colonies of bacteria were picked with a sterile loop and used to inoculate 6 ml aliquots of Luria-Bertani medium containing ampicillin and grown overnight in a shaking incubator at 37°C and 180 rpm. An aliquot of bacterial culture containing plasmid was frozen down in 15% glycerol stocks and stored at -80°C. The remaining bacterial medium was used to extract pCMV *dcc* plasmid DNA using Pure yield plasmid miniprep system (A1223, Promega UK, Southampton, UK) following manufacturer's instructions.

Stable transfection was performed in MCF-7 cells and transient transfection in MDA-MB-231 cells passaged in normal media, 24 h prior to transfection. To promote the efficiency of transfection, cells were plated at a density sufficient to result in approximately 60–70% confluency 24 h after passage. At this time, normal medium was removed from each well and replaced with antibiotic free medium for approximately 1 h. The transfection reagent Lipofectamine 2000 was used (Life Technologies, Paisley, UK, #11668-027)

for successful transfection of the DCC plasmid according to the manufacturer's instructions using a ratio of 1:2 DNA: Lipofectamine 2000. Briefly, DNA (1000 ng for MCF-7 cells, 1500 ng for MDA-MB231 cells) was combined with Lipofectamine 2000 in the presence of OptiMem (Life Technologies, Paisley, UK product 31985-062), incubated at room temperature for 15 minutes and then added dropwise to each well containing cells in antibiotic-free medium (250 μ l in 6-well plates). Approximately 4-5 h after the introduction of DNA, medium was removed and replaced with medium containing antibiotics and test agents as required.

To generate the stable MCF-7 cell line, 48 hours post transfection, cells were trypsinised, diluted 1:2 and plated in 6 well plates with antibiotic G418 at a concentration of 300 μ g/ml (determined from a previous kill curve) before transfer to 24-well plates where the cells were fed every 3 to 4 days with G418 containing media. Islands of surviving cells were isolated using cloning cylinders, where they were then grown and collected for further use as a stable cell line. Transfer of a single cell from these resistant colonies were transferred to 96 well plates to confirm antibiotic resistance.

In experiments where MDA-MB-231 cells were only transiently transfected with *dcc*, cells were either harvested for Western blot analysis or used for further experiments 48 h after the initial introduction of the plasmid.

Sources

From Sigma-Aldrich

Subtilisin A (#P3580); α -chymotrypsin (#C4129); chymostatin (#C7268); Protease Inhibitor Cocktail (P2714); *From Cayman Chemicals*: carfilzomib (#17554); Epoxomicin (#1000 7806); *From Tocris Chemicals*: MG132 (#1748); *R&D Systems*: Human recombinant netrin-1 (#6419-N1); hr-netrin-4 (#1254).

The protease inhibitor cocktail (PIC) provided AEBBSF hydrochloride 100 μ M (inhibits serine proteases); aprotinin 15 nM (serine proteases and esterases); bestatin 6 μ M (aminopeptidases); E-64 50 nM (cysteine proteases); EDTA 50 μ M (metalloproteases); leupeptin 50 nM (cysteine and tryptic proteases)

Results

Tissue slices

An acknowledged limitation of cell lines is that they are already abnormal, so that an examination of factors that cause, or are associated with, cancerous behaviour are based on cells which may already have undergone changes in the signalling pathways under investigation. As in our previous study¹¹ we have therefore attempted to circumvent this problem by using fresh tissue from adult laboratory rats in addition to human cancer-derived and human primary mammary cells. Brain sections were used as fresh intact tissue since they retain higher concentrations of DCC in adult rats than most other tissues and their use does not require molecular manipulation before use: brain slices are prepared acutely and used immediately in a simple chemically defined medium (artificial cerebrospinal fluid, aCSF, see Methods) lacking

added serum, enzymes or growth factors. Such slices retain most of their biochemical and physiological properties for many hours and are well established as a method of preserving physiological levels of neuronal excitability and communication. The system therefore provides a valuable means of comparing results with cell cultures which, while being more amenable to molecular interference and being available for study over several days, need to be maintained in relatively complex media with highly specific and relatively artificial additions such as serum. This adds growth factors and other nutritive components including trace elements and undefined levels of endogenous enzymes and inhibitors which are difficult to define absolutely.

As seen in **Figure 1**, the addition of chymotrypsin (CT, 1 μ M) or subtilisin (sub, 100 nM) reduced the expression of DCC (**Figure 1A**) and neogenin (**Figure 1B**) in tissue slices. The concentrations used were based on our previous studies of concentration-effect relationships.¹¹ While both enzymes are defined as chymotryptic in view of their overall substrate specificity, the Protease Inhibitor Cocktail (PIC, see Methods – Sources) – significantly inhibited only chymotrypsin and not subtilisin (**Figure 1A,B**). The cytoskeletal protein RhoA was unaffected at the same concentrations of the proteases (**Figure 1C**), allowing its use as a housekeeping control for protein loading as noted above.

Expression of β -catenin in the tissue was also reduced significantly by chymotrypsin (**Figure 1D**) and subtilisin (**Figure 1E**) and both these effects were blocked by PIC, consistent with the loss of DCC and neogenin. In addition, the effects of chymotrypsin and subtilisin on β -catenin expression were blocked by the highly selective serine protease inhibitor chymostatin (**Figure 1D,E**) as we have shown previously for DCC and neogenin and confirming their substantially similar chymotryptic substrate specificity.

Since β -catenin is normally ubiquitinated and transported to the proteasome for chymotryptic degradation, it was of interest to determine whether the loss of β -catenin produced by chymotrypsin was mediated through the chymotryptic activity of the proteasome. This seems not to be the case since the selective 20S proteasome inhibitors carfilzomib (50 nM), epoxomicin (1 μ M) and MG132 (10 μ M) did not modify the activity of chymotrypsin on β -catenin expression (**Figure 1F**), producing a similar pattern to that reported previously for DCC and neogenin.¹¹ However, all three inhibitors showed a clear tendency to increase the resting levels of β -catenin, the effect of MG132 being statistically significant, consistent with a continuous ongoing removal of β -catenin by proteasomal activity and consistent with similar observations on DCC and neogenin in earlier work.¹¹ A pan-cadherin antibody indicated no overall effect of chymotrypsin or the proteasome inhibitors on total cadherin expression (**Figure 1G**) but in subsequent experiments E-cadherin and N-cadherin were examined separately.

Cell cultures

MCF-7 mammary cancer cells

In the MCF-7 line of human mammary adenocarcinoma cells, normal resting cells expressed neogenin but not DCC. In normal 10% serum-containing medium, 48 h treatment with chymotrypsin (CT 1000 nM) or subtilisin (sub 30, 100 nM)

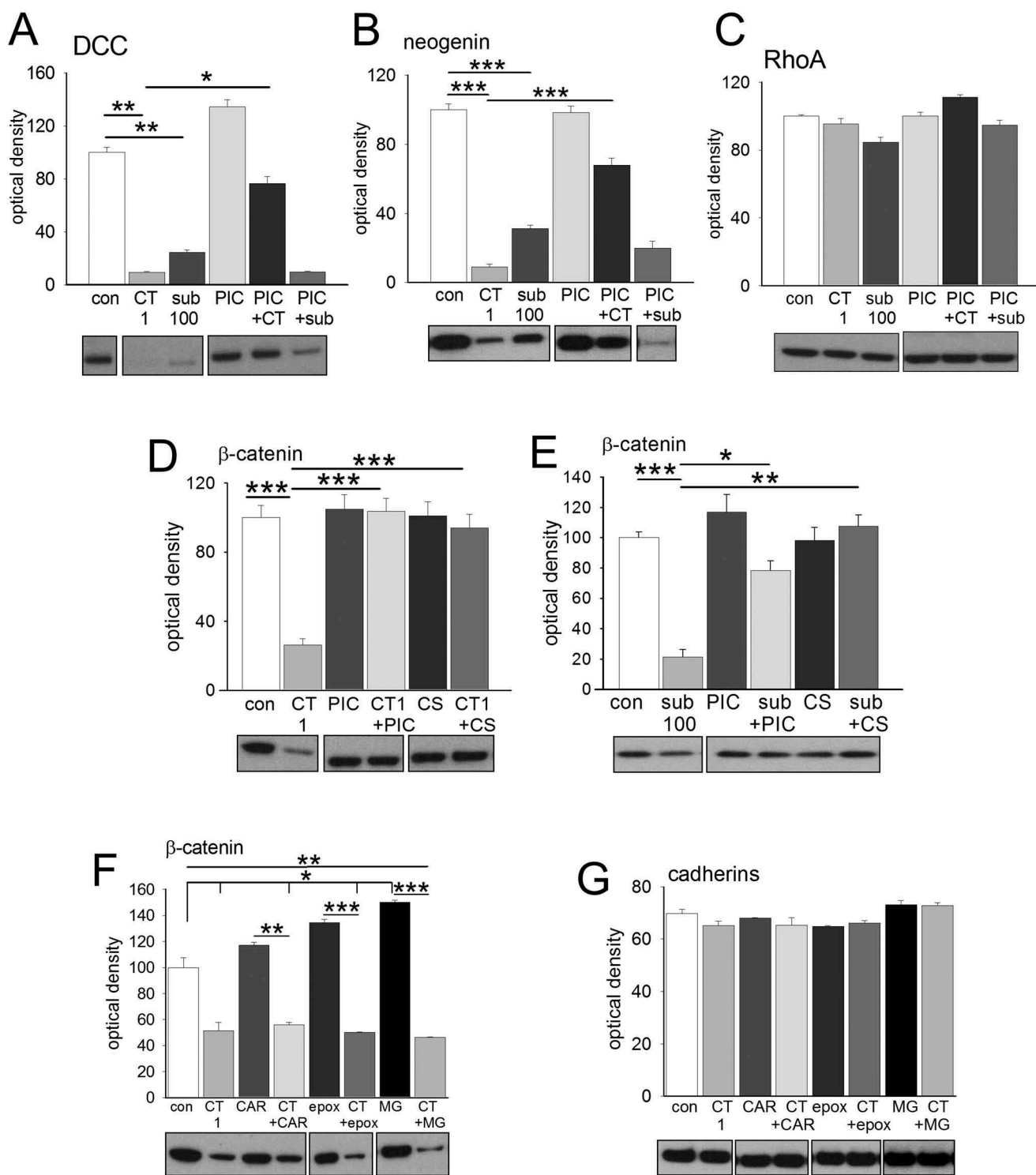


Figure 1. Effects of serine proteases on dependence receptor expression in tissue.

Bar charts showing the quantified Western blot densities of proteins expressed in rat brain slices. The charts indicate expression in normal tissue (con), or after 4 h incubation with chymotrypsin (CT) 1 μ M or subtilisin (sub) 100 nM alone or with the Protein Inhibitor Cocktail (PIC). Expression is shown of (A) DCC, (B) neogenin, (C) RhoA. Panels (D) and (E) summarise the expression of β -catenin, its suppression by CT 1 μ M (D) or sub 100 nM (E) and the blockade of these enzymes by PIC and chymostatin 30 μ M (CS). (F) and (G) quantify the expression of β -catenin (F) and cadherins (G) showing the effects of CT 1 μ M alone or in combination with carfilzomib 50 nM (CAR), epoxomicin 1 μ M (epox) or MG132 10 μ M (MG).

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ ($n = 3$ or 4 ; ANOVA and Bonferroni *post hoc* test for selected datasets.)

reduced the expression of neogenin (Figure 2A), with no effect on RhoA (Figure 2B) at the same concentrations. When the concentration of serum in the incubation medium was reduced from 10% to 1% to reduce interference from serum

components the cells exhibited increased sensitivity to the proteases such that chymotrypsin, even at 300 nM significantly reduced neogenin expression and subtilisin at 100 nM completely eliminated neogenin expression (Figure 2C). There was

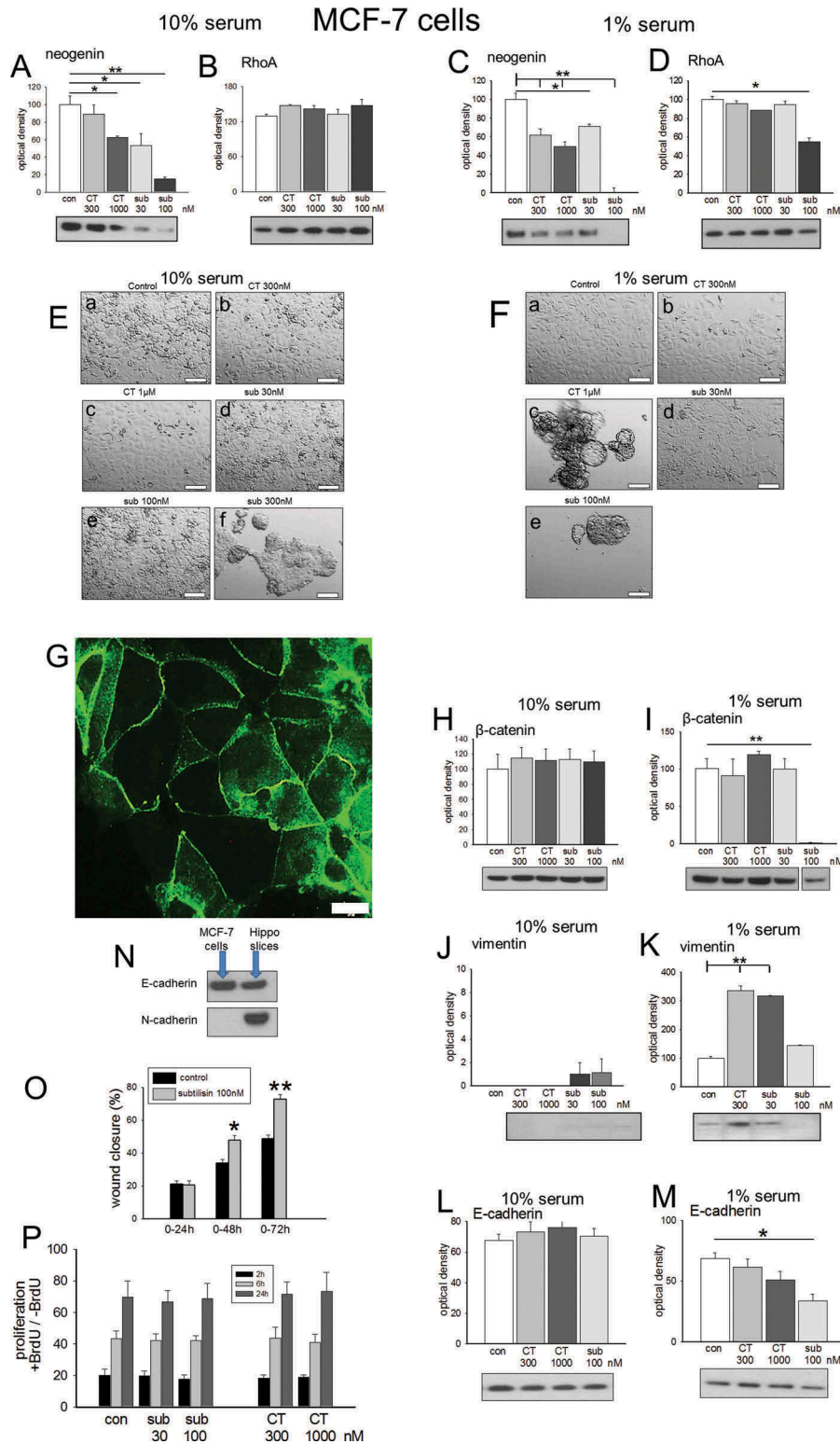


Figure 2. Effects of serine proteases on dependence receptor expression in MCF-7 cells.

Bar charts showing the quantified Western blot densities of proteins expressed in MCF-7 cells. Expression is shown of (A) neogenin and (B) RhoA after incubation in normal medium containing standard 10% FBS. (C) and (D) show expression after culturing in low serum (1%) medium. The charts indicate expression in control normal cells (con), or after 48 h exposure to chymotrypsin (CT) 300 or 1000 nM, or subtilisin (sub) 30 or 100 nM. (E), (F). Micrographs of cultures after 48 h exposure to CT 300 or 1000 nM, or subtilisin 30, 100 or 300 nM under control conditions (E; 10% FBS-containing medium) or low serum (F; 1%) medium. Scale bar 100 μ m. (G) immunocytochemical localisation of β -catenin primarily in the cell membrane, with some cells exhibiting β -catenin in the cytoplasm. (H)–(M) show bar charts of quantified Western blot densities in 10% serum medium (H, J, L), or low 1% serum media (I, K, M) for β -catenin (H, I), RhoA (I, K), vimentin (J, K) and E-cadherin (L, M). The charts indicate expression in normal cells (con), or after exposure to chymotrypsin (CT) 300 or 1000 nM, or subtilisin (sub) 30 or 100 nM. Each chart is accompanied by a sample blot. (N) illustrates Western blots for MCF-7 cells or tissue slices, revealing the presence of E-cadherin but not N-cadherin in MCF-7 cells whereas both are clearly expressed in adult brain tissue. (O) summarises the rate of wound closure 24, 48 and 72 h after wounding cultures under normal (control) conditions (dark columns) or in the presence of subtilisin 100 nM (light columns). (P) Measurement of MCF-7 proliferation rate using the bromodeoxyuridine technique under control conditions (con) or in the presence of subtilisin (sub) 30 or 100 nM, or chymotrypsin (CT) 300 or 1000 nM. Results are shown after 2 h (black columns), 6 h (light shading) or 24 h (heavy shading).

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ ($n = 3$; ANOVA and Bonferroni *post hoc* test for selected datasets).

also a significant reduction ($p = 0.05$) of RhoA (Figure 2D) at 100 nM subtilisin, indicating a degree of sensitivity of the cytoskeleton under these low serum conditions, or an increased susceptibility to toxicity induced by the lack of some serum components. An examination of cell morphology showed that, in 10% serum-containing medium, the cells retained their normal planar cohesive morphology in controls and low protease concentrations (Figure 2E,a–d). At 100 nM subtilisin the cells began to reveal signs of disruption (Figure 2E,e) and at 300 nM this progressed to an obvious loss of adherence with the formation of islands of cells which appeared to be seriously damaged or dead (Figure 2E,f). Under the low serum (1%) conditions (Figure 2F), the cells showed clear signs of disruption and death in chymotrypsin at 1 μ M (Figure 2F,c) or subtilisin 100 nM (Figure 2F,e).

β -catenin was clearly present primarily in the cell membrane region of the MCF-7 cells (Figure 2G). The serine proteases did not affect the expression of β -catenin or RhoA in normal medium (Figure 2H) but in 1% media serum β -catenin was reduced by 100 nM subtilisin (Figure 2I) possibly indicating non-specific damage related to their combination with the deficiency in serum components.

No significant vimentin expression was seen in MCF-7 cells cultured in normal 10% serum medium (Figure 2J), an observation that has been made previously.⁴⁴ There was, however, a suggestion (not significant) of expression in the presence of subtilisin (Figure 2J). This trend became highly significant during incubation in 1% serum medium which induced a clear baseline expression of vimentin (Figure 2K) which was further increased by chymotrypsin (300 nM) and the low (30 nM) concentration of subtilisin.

E-cadherin showed no significant loss of expression after incubation with chymotrypsin or subtilisin in normal medium (Figure 2L) but a clear tendency for a reduction by chymotrypsin at 1 μ M and a significant reduction at 100 nM subtilisin in low serum conditions (Figure 2M). Although E-cadherin was present in MCF-7 cells, N-cadherin was not detected. This could not be attributed to antibody failure as tissue slices exhibited clear expression of both proteins (Figure 2N).

Using the wound closure assay of spontaneous cell migration subtilisin at 100 nM (in 10% serum medium) increased the amount of migration after 48 or 72 h, confirming that a more aggressively motile phenotype had been induced (Figure 2O). This increase in migration could not be attributed to an artifact of increased cell proliferation as there was no change in proliferation using the BrdU incorporation assay (Figure 2P).

Stable transfection

Since there was no expression of DCC in the MCF-7 cells, as noted by Miyamoto et al.,⁴⁵ the relationship between DCC and other proteins involved in cell migration was studied after stable transfection with a *dcc* plasmid as described previously.¹¹ The transfected cells appeared normal, with the usual planar morphology (Figure 3A,a), and were unaffected by subtilisin at 30 or 100 nM even after 48 h incubation (Figure 3A,b,c). At the higher concentration of 300 nM subtilisin the cells separated into individuals without forming the

clumps noted in naïve, non-transfected cells (Figure 3A,d; compare with Figure 2E,f) and individual cells continued to exhibit a normal morphology suggesting that DCC transfection had reduced sensitivity to the serine proteases.

The transfection generated clear levels of DCC protein (Figure 3B) but 6 h incubation with subtilisin at 100 nM did not reduce expression of this ectopic DCC (Figure 3B) or neogenin (Figure 3C) compared with DCC in adult rat tissue and neogenin in untransfected MCF-7 cells. Only at the higher concentration of 300 nM subtilisin was any loss seen in DCC or neogenin expression. A similar pattern was observed for β -catenin expression (Figure 3D), E-cadherin (Figure 3E) and RhoA (Figure 3F) consistent with a possible degree of toxicity at 300 nM as noted above. No expression of vimentin was seen in these cells, as noted above in naïve MCF-7 cells.

Wound healing assay of cell mobility. To assess the functional effects of DCC transfection, cells were examined in the wound healing assay of intrinsic mobility. The rate of spontaneous migration was lower in the DCC-transfected cells ($P = 0.05$) (Figure 3G) consistent with the view that DCC expression suppresses migration. Chymotrypsin increased the intrinsic mobility although the effect was smaller in the transfected cells than in naïve cells. Subtilisin at 100 nM increased migration after DCC transfection consistent with its increased migratory effect in non-transfected MCF-7 cells.

Spot chemotaxis assay. In the spot chemotaxis assay using netrin-1 (1000 ng/ml) as the chemoattractant, the resting rate of migration of transfected cells was significantly greater than that of naïve cells, but the stimulant effect of netrin was less than in normal cells and did not reach statistical significance (Figure 3H). Indeed, the overall number of cells migrating in response to netrin was significantly less in the transfected cells (Figure 3H).

Assessment of proliferation. In the BrdU assay of cell proliferation, DCC-transfected cells showed no difference from naïve cells in the rate of proliferation after 6 h (Figure 3I) or 24 h (Figure 3J) incubation. Neither subtilisin (30 nM or 100 nM) nor chymotrypsin (300 or 1000 nM) affected the proliferation of MCF-7 cells, normal or transfected (Figure 3I, J), indicating that changes in cell proliferation were not likely to have contributed to the changes in apparent cell mobility.

MDA-MB-231 mammary adenocarcinoma cells

Since MCF-7 cells are generally regarded as being relatively quiescent, we also examined the more aggressive human breast adenocarcinoma line of MDA-MB-231 cells. MDA-MB-231 cells are basal type B epithelial cells, indicating their high level of invasiveness relative to type A.^{46,47} They are post-mitotic cells derived from human metastatic adenocarcinoma,⁴⁸ with low ability to form intercellular adhesions.⁴⁹ They have been reported to express several EMT markers including vimentin and low levels of differentiated epithelial markers such as E-cadherin.²¹

In these cells 48 h treatment with chymotrypsin (1 μ M) failed to affect neogenin expression and a high concentration

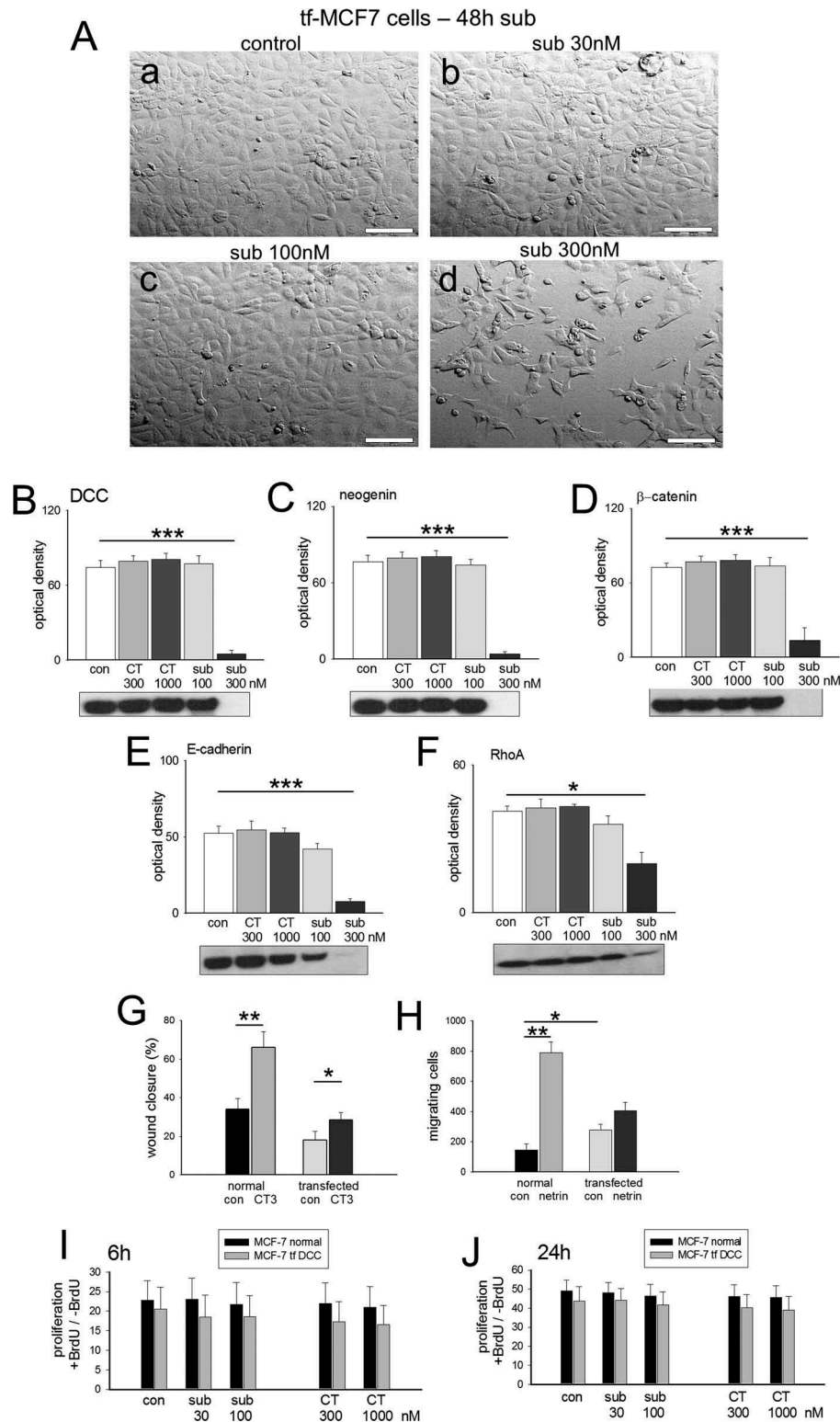


Figure 3. Effects of serine proteases on dependence receptor expression in DCC stable-transfected MCF-7 cells.

(A) illustrates cultures of MCF-7 cells after stable transfection with the *dcc* plasmid. Cultures are shown under control conditions or after 48 hr incubation with subtilisin (sub) 30, 100 or 300 nM. Only at the highest concentration is there any disruption, with a loss of intercell adhesion but no sign of toxicity or clumping. Scale bar = 100 μm. (B)-(F) Bar charts showing the quantified Western blot densities of proteins expressed in *dcc*-transfected MCF-7 cells. Expression is shown of (B) DCC, (C) neogenin, (D) β-catenin, (E) E-cadherin and (F) RhoA showing the effects of 6 h treatment with chymotrypsin (CT) 300 or 1000 nM and subtilisin (sub) 100 or 300 nM in 10% FBS. Each chart is accompanied by a sample blot. (G) the rate of scratch closure 24 h after wounding cultures under normal control conditions (con) or in the presence of chymotrypsin 3 μM (CT3) in normal or stably *dcc*-transfected cells. (H) measurement of cell chemotactic migration to a netrin-1 containing agarose spot. The chart shows the number of normal or *dcc*-transfected MCF-7 cells entering the spot over 24 h under control conditions (con) or with the chemotactic protein netrin-1 (1000 ng/ml) in the spot. Transfected cells showed greater spontaneous movement but were less attracted to the netrin target. (I, J) Comparison of proliferation rates of normal MCF-7 cells (black columns) and cells transfected with a *dcc* plasmid (light columns), using the bromodeoxyuridine technique under control conditions (con) or in the presence of subtilisin (sub) 30 or 100 nM, or chymotrypsin (CT) 300 or 1000 nM. Results are shown after 6 h (I) and 24 h (J). There were no significant differences in proliferation rate between normal and transfected cells, or between control and protease-treated cultures.

*P < 0.05, **P < 0.01, ***P < 0.001 (n = 3; ANOVA and Bonferroni *post hoc* test for selected datasets.)

of 300 nM subtilisin was required to do so in the normal serum (15%)-containing medium (Figure 4A). In low serum (1%) medium sensitivity to the proteases was enhanced substantially, with chymotrypsin (1 μ M) and subtilisin even at 100 nM producing over 90% depletion (Figure 4B), changes qualitatively similar to those seen in MCF-7 cells.

In normal medium, resting MDA-MB-231 cultures consisted of a mixture of multipolar and spindle-shaped cells ("resting cells"), together with more spherical cells which had undergone temporary de-differentiation from the normal phenotype in preparation for the movement and migration ("active cells") which is typical of this line⁵⁰ (Figure 4C,a). Treatment with the serine proteases for 48 h increased the proportion of active profiles (Figure 4C,b–f) at concentrations which reduced neogenin expression, but with a good representation of resting cells even at 300 nM subtilisin (Figure 4,f). This change in profile was exaggerated in 1% serum medium, in which cells appeared normal in control medium (Figure 4D,a), but with no resting profiles visible at 1 μ M chymotrypsin (Figure 4D,c) or 100 and 300 nM subtilisin (Figure 4D,e,f).

β -catenin showed quite a different localisation in the MDA-MB-231 cells (a basal cell-derived line) compared with that observed above in the luminal epithelium-derived MCF-7 cells (Figure 4E, compare with Figure 2G). The MDA-MB-231 cells showed a more generalised cytoplasmic distribution of the protein (Figure 4E) compared with the largely peripheral, membrane-associated localisation in MCF-7 cells. In MDA-MB-231 cells, β -catenin protein expression was only affected by subtilisin at 300 nM in normal medium (Figure 4F) but became more sensitive in low serum, responding to chymotrypsin 1 μ M and subtilisin 100 nM (Figure 4G). E-cadherin (Figure 4H,I) and RhoA (Figure 4J,K) showed similar patterns of changes being unaffected in normal media and becoming more sensitive in low serum media.

A low level of vimentin expression was observed in naïve MDA-MB-231 cells (normal 15% medium) (Figure 4L) but, as in the MCF-7 cells, low serum medium (1%) itself induced a significant increase in vimentin expression (Figure 4L). Chymotrypsin (1 μ M) and subtilisin (100 nM) inhibited vimentin expression very significantly in low serum medium.

For an examination of MDA-MB-231 migratory cell behaviour in the wound closure assay, cells were cultured in a medium with reduced serum content (0.1%). This was necessary to suppress spontaneous proliferation to a level which would not interfere with the assay as MDA-MB-231 cells can proliferate to close a scratch wound within a few hours, making it difficult to quantify drug-induced changes on migration. Subtilisin increased cell migration (Figure 4M) although the concentrations required (1–3 nM) were lower than those required in normal media, consistent with the view expressed above that normal serum contains components inhibiting the enzyme activity. In the BrdU proliferation assay neither chymotrypsin nor subtilisin affected proliferation of the MDA-MB-231 cells in normal medium (Figure 4N) after a short (2 h) or prolonged (24 h) incubation.

Transient transfection of DCC

To examine the effect of DCC on MDA-MB-231 cell sensitivity to serine proteases, cells were transiently transfected with

the ectopic *dcc* plasmid. This generated high levels of the corresponding protein (Figure 5A) which remained sensitive to depletion by subtilisin in normal 15% serum medium (Figure 5A). The expressions of endogenous neogenin (Figure 5B), β -catenin (Figure 5C) and vimentin (Figure 5D) were not changed by DCC transfection and these proteins showed a similar sensitivity to subtilisin in normal and transfected cells. RhoA expression was not affected significantly by the serine proteases (Figure 5E) with or without transfection.

Primary mammary epithelial cells

To compare normal cells with the transformed lines used above human primary mammary epithelial cells (HMEpC) were examined. These cells are maintained in the media supplied, with no added serum. No DCC was detectable in these cells, as previously noted by Kenny et al.⁵¹ Subtilisin at 10 nM for 6h was sufficient to reduce substantially the expression of neogenin (Figure 6A) with no significant effect on β -catenin (Figure 6B). There was a loss of vimentin expression (Figure 6C) with 10 nM subtilisin, and a reduction in N-cadherin with 10 nM subtilisin (Figure 6D) but no effect on E-cadherin (Figure 6E) or Rho expression (Figure 6F). These protein changes paralleled morphological features of the cells which normally form monolayer cultures (Figure 6G,a). At 10 nM subtilisin, the cultures retain the appearance of single cells or small groups of loosely adherent cells (Figure 6G,b) despite the loss of neogenin, β -catenin, vimentin and N-cadherin but with clear areas appearing devoid of cells and suggesting possible toxicity on these cells. At 100 nM subtilisin the cells showed clear signs of damage with clumping into batches separated by large cell-free areas (data not shown).

In the wound closure assay, low concentrations of both chymotrypsin (3 nM) and subtilisin (3 nM) increased the rate of scratch closure over a 24 h period, indicating an increase in cell migration (Figure 6H). At the same concentrations there were no changes in cell proliferation (Figure 6I).

SH-SY5Y cells

The human neuroblastoma line of SH-SY5Y cells was included as these cells normally have detectable levels of endogenous DCC. Exposure to subtilisin 300 nM was required to reduce the expression of DCC (Figure 7A) or neogenin (Figure 7B), while there was no effect of chymotrypsin at 1 μ M. Expression of β -catenin (Figure 7C) and E-cadherin (Figure 7D) were also reduced at 300 nM subtilisin while Rho showed a non-significant trend for reduction (Figure 7E). The serine proteases tended to increase the expression of vimentin (Figure 7F) with a significant effect using subtilisin 300 nM, as noted for MCF-7 cells above.

In the wound closure assay of cell migration, incubation with chymotrypsin at 3 μ M or subtilisin at 30 nM for 72 h increased the rates of cell migration (Figure 7G).

Discussion

DCC and neogenin are 'dependence receptors' regulating cell viability and survival depending on the presence of their

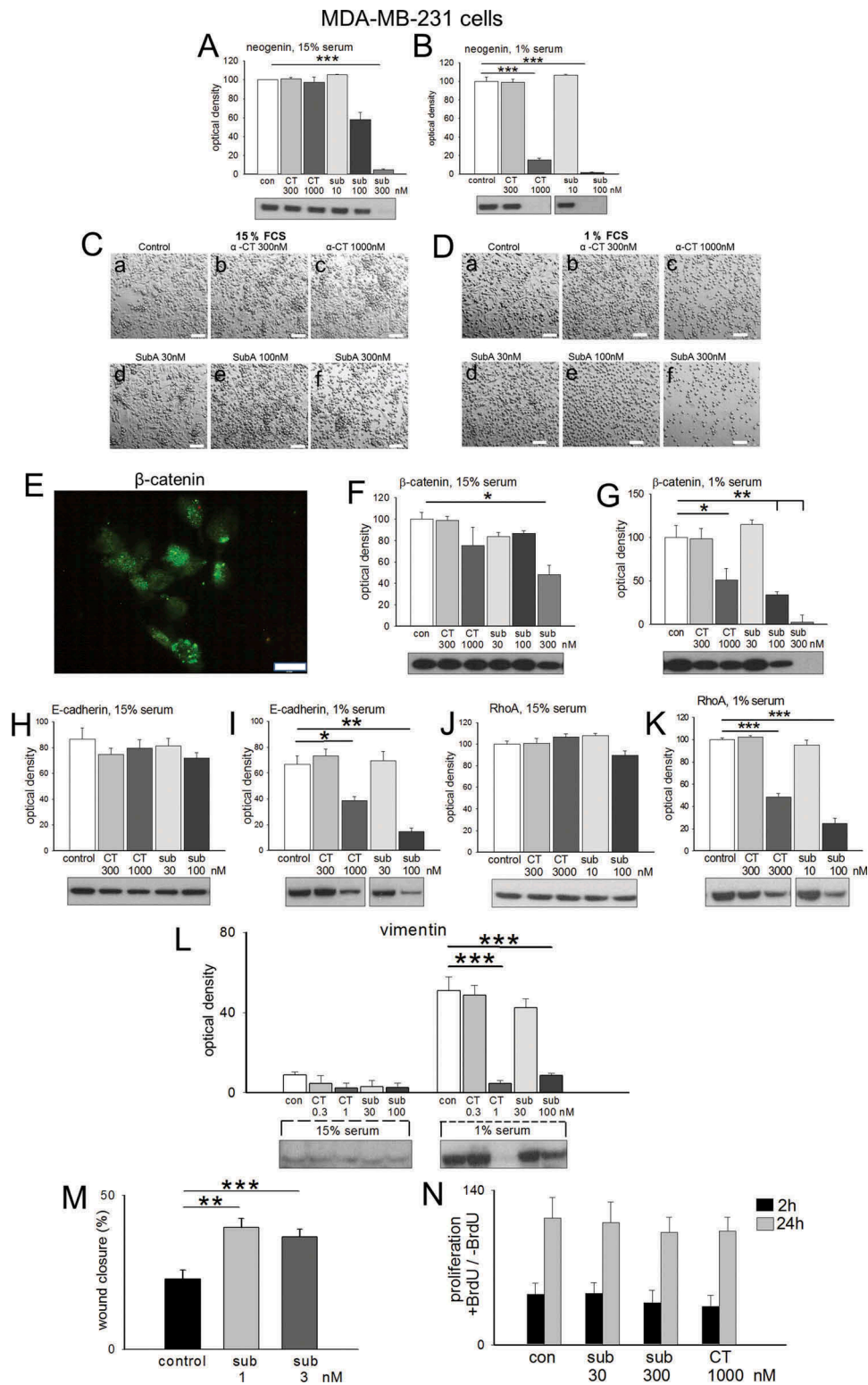


Figure 4. Effects of serine proteases on dependence receptor expression in MDA-MB-231 cells.

(A, B): Bar charts showing the quantified Western blot densities of proteins expressed in MDA-MB-231 cells. Expression is shown of neogenin after incubation in normal medium containing the standard 15% FBS (A) or after incubation in low serum (1%) medium (B). The charts indicate expression in normal control cells (con), or after 48 h exposure to chymotrypsin (CT) 300 or 1000 nM, or subtilisin (sub) 10, 100 or 300 nM. Each chart is accompanied by a sample blot. (C,D) Micrographs of cultures after exposure to CT 300 or 1000 nM, or subtilisin 30, 100 or 300 nM under control conditions (15% FBS-containing medium) (C) or low serum (1%) medium (D). Scale bar = 100 μm. (E) immunocytochemical localisation of β-catenin primarily in the cytoplasm. (F-L) show bar charts of quantified Western blot densities for β-catenin (F, G), E-cadherin (H, I), RhoA (J, K) and vimentin (L) in normal 1%-serum media (F, H, J, L) or after incubation in low serum (1%) media (G, I, K, L). Both high and low serum data are illustrated together to emphasise the increase in expression induced by low serum (L). The charts indicate expression in normal control cells (con), or after exposure to chymotrypsin (CT) 300 or 1000 nM, or subtilisin (sub) 30, 100 or 300 nM. Each chart is accompanied by a sample blot. (M) the rate of scratch closure 24 h after wounding cultures under normal (control) conditions (black column) or in the presence of subtilisin 1 nM (light grey column) or 3 nM (dark grey column). (N) Measurement of proliferation rate using the bromodeoxyuridine technique under control conditions (con) or in the presence of subtilisin (sub) 30 or 300 nM, or chymotrypsin (CT) 1000 nM. Results are shown after 2 h (black columns) or 24 h (light grey columns).

*P < 0.05, **P < 0.01, ***P < 0.001 (n = 3; ANOVA and Bonferroni *post hoc* test for selected datasets.)

MDA-MB-231 cells – transfected

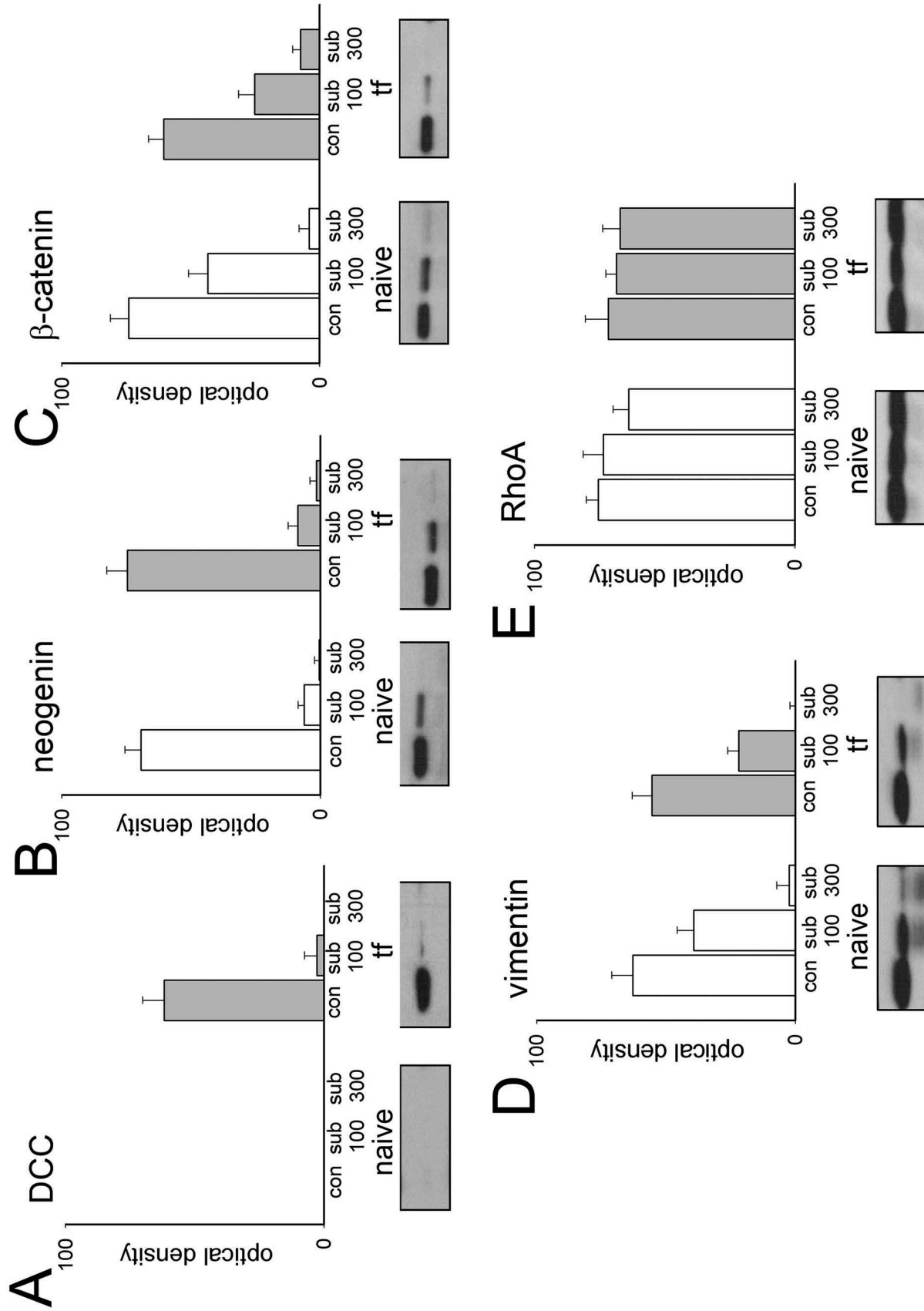


Figure 5. Protein expression in normal and *ddc*-transfected MDA-MB-231 cells. Bar charts showing the quantified Western blot densities of proteins expressed in MDA-MB-231 cells following transient transfection with the *ddc* plasmid. Expression is shown of (A) DCC, (B) neogenin, (C) beta-catenin, (D) vimentin and (E) RhoA. The charts indicate expression after 48 h incubation with subtilisin (sub) 100 nM or 300 nM in normal cells (naive) or transfected cells (tf). Each chart is accompanied by a sample blot. Each column indicates the mean expression from n = 3 experiments.

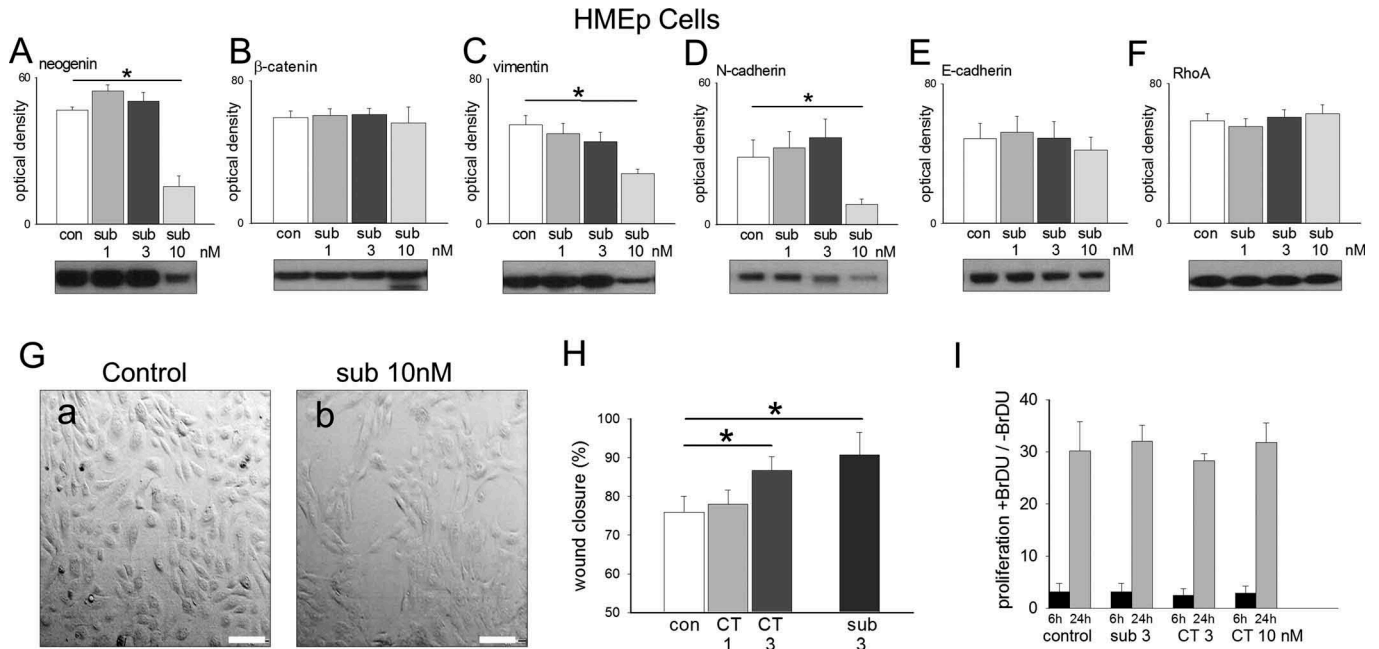


Figure 6. Protein expression in primary human mammary myoepithelial (HMEp) cells. Bar charts showing the quantified Western blot densities of proteins expressed in primary human mammary myoepithelial (HMEp) cells. Expression is shown of (A) neogenin, (B) β-catenin, (C) vimentin, (D) N-cadherin, (E) E-cadherin and (F) RhoA. The charts indicate expression in normal control cells (con), or after exposure to subtilisin (sub) 1, 3 or 10 nM for 6 h. Each chart is accompanied by a sample blot. (G) Micrographs of control cultures (G,a) or after 6 h exposure to subtilisin 10 nM (G, b). Scale bar = 100 μm. (H) Rate of scratch closure 48 h after wounding control cultures (con) or in the presence of chymotrypsin (CT) 1 or 3 nM, or subtilisin 3 nM. (I) Measurement of proliferation rate using the bromodeoxyuridine technique under control conditions (con) or in the presence of subtilisin (sub) 3 nM or chymotrypsin (CT) 3 or 10 nM. Results are shown after 6 h (black columns) or 24 h (light columns). *P < 0.05, (n = 3; ANOVA and Bonferroni *post hoc* test for selected datasets.)

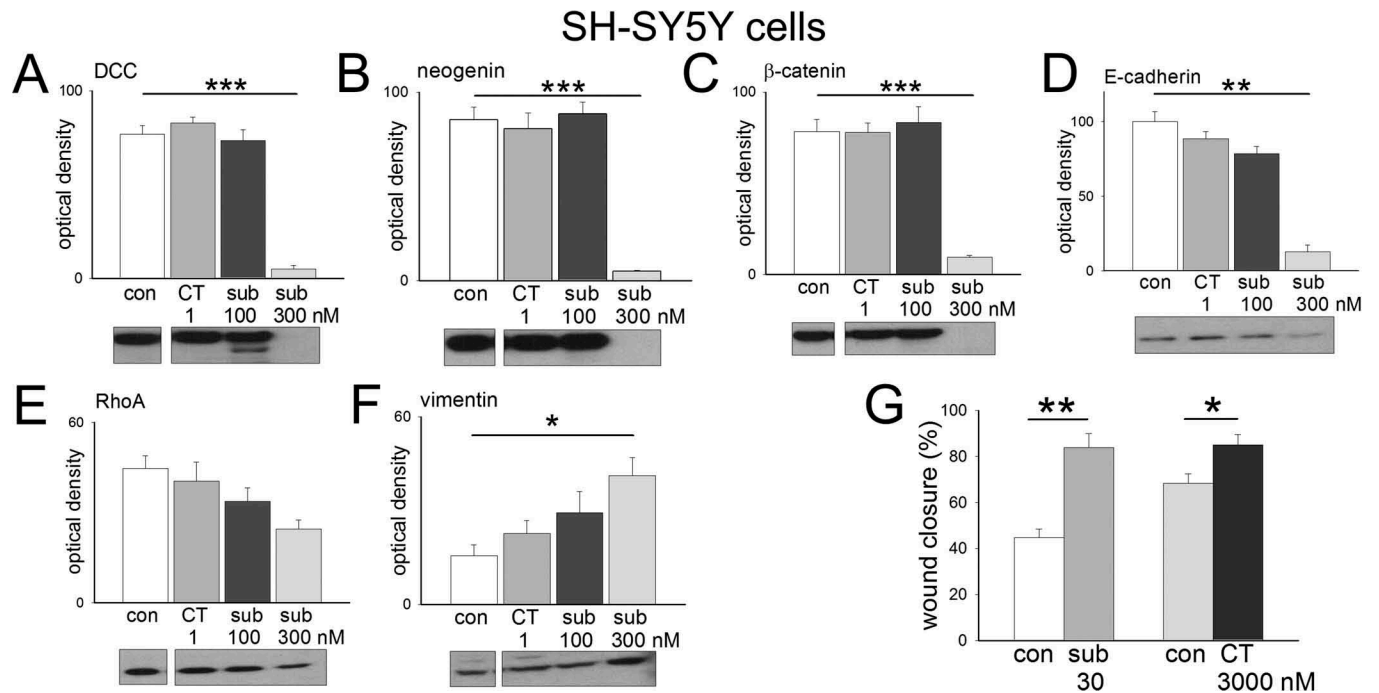


Figure 7. Protein expression in SH-SY5Y human neuroblastoma cells. Bar charts showing the quantified Western blot densities of proteins expressed in SH-SY5Y cells. Expression is shown of (A) DCC, (B) neogenin, (C) β-catenin, (D) E-cadherin, (E) RhoA and (F) vimentin. The charts indicate expression in normal control cells (con), or after exposure to chymotrypsin (CT) 1 μM or subtilisin (sub) 100 or 300 nM for 48 h. Each chart is accompanied by a sample blot. (G) Rate of scratch closure 48 h after wounding control cultures (con) or in the presence of subtilisin (sub) 30 nM or chymotrypsin (CT) 3000 nM. *P < 0.05, (n = 3; ANOVA and Bonferroni *post hoc* test for selected datasets.)

primary ligands, the netrin family of extracellular, secreted proteins.^{52,53} DCC has received the most attention since, compared with normal cells surrounding an early cancerous

growth, the expression of DCC is greatly reduced or absent in several forms of cancer and genetic changes such as gene mutation or loss of heterozygosity have been described by

many groups.^{5,26,54–56} Its insertion into cells can inhibit proliferation and migration.^{3,57–59}

The overall functional role of DCC remains elusive, since it was originally thought to affect proliferation but now appears to be more relevant to cellular migration, metastasis formation and chemoresistance.⁶⁰ Interfering with DCC expression or function, for example, does not always produce changes in proliferation or survival.⁶¹ However, it has a potentially important involvement in differentiation.^{62,63} The protein was detected in differentiated, mucus-producing cells but not un-differentiated cells in the intestine and was absent from colorectal cancer cells which failed to differentiate into mucus-producing cells.⁶⁴ These observations are consistent with the finding that during early development DCC is first expressed in differentiating neurons⁶⁵ and that it is required for the differentiation of neurons induced by Nerve Growth Factor.⁴ It is highly expressed in early stage hyperplastic tissues and then declines in parallel with the extent of metastatic activity and malignancy.^{56,63,66} These authors concluded that DCC expression was associated with maintaining cell differentiation. A parallel interpretation is that DCC levels fall, not before or at the beginning of migration, but during the course of migration and dissemination.^{5,67} This might indicate that DCC or neogenin can modulate the transition of resting cells into the activated, pro-metastatic state of EMT.

Although less well studied, neogenin expression is involved in cell migration and differentiation in early embryogenesis^{68,69} and is inversely related to tumour activity in several disorders such as breast cancer.^{7–9,70–72} The present study was designed to assess the effects of chymotrypsin and subtilisin on the expression of DCC and neogenin, in parallel with markers associated with EMT.

EMT represents a major reorganisation of cellular metabolism, a “comprehensive reprogramming” of cell function⁷³ and recent results indicate that EMT may be related to the development of drug *resistance* rather than to the promotion of cell migration.^{40,60} Furthermore, the deletion of transcription factors thought to be essential for EMT, such as Snail and Twist, did not reduce cancer cell invasiveness or prevent metastasis.⁴⁰

We have previously reported that two common serine proteases, endogenous mammalian chymotrypsin and bacterial subtilisin, reduce the expression of DCC and neogenin at low physiologically relevant concentrations, accompanied by increased cell migration.¹¹ The present results confirm this finding and extend it to several cancer-derived cell lines and primary human mammary epithelial cells. In most experiments there was selectivity for the depletion of DCC and neogenin with several other proteins being less affected at the same protease concentrations.

Inserting *dcc* increases intercellular adhesion, whereas antisense oligonucleotides increase adhesion to the extracellular surface.⁶⁶ This is consistent with our observation that DCC-transfected cells retain their planar morphology when exposed to the serine proteases at concentrations which induce cell dissociation of naïve cells. The loss of dependence receptors, especially in MDA-MB-231 cells, was accompanied by the generation of increased numbers of separated, isolated, spherical cells. These have been considered to be invasive, colony-forming

cells⁷⁴ with high expression of vimentin and N-cadherin but little or no E-cadherin, a profile typical of cells in EMT.

Cadherin

Under resting conditions most epithelial-derived cells express high levels of E-cadherin with very low expression of N-cadherin. E-cadherin is thought to be a major suppressor of EMT and tumour invasion, EMT being regarded as a primary marker of normal, non-cancerous, differentiated cells.^{16,75,76} A reduction in E-cadherin expression has been linked with other changes involved in de-differentiation, oncogenesis and metastasis.^{20,77,78} There is a moderate expression in invasive carcinomas without metastases but elevated levels in metastatic deposits: it is reduced or absent in many tumours and a decline in expression induces microRNAs which function as tumour suppressors, limiting invasive, aggressive behaviour.^{76,79}

Conversely N-cadherin is absent or poorly expressed in resting cells and its expression rises during EMT. However, the link between E-cadherin and aggression is not a secure one, since E-cadherin has been found in all of 69 specimens of squamous cell carcinomas, more than half of which exhibited higher than normal levels especially in late stage tumours.^{19,80} An alternative explanation of our results is that the DCC/cadherin relationship functions via a non-canonical β -catenin-independent route. Indeed, there are clearly factors affecting E-cadherin expression that remain poorly understood: a low E-cadherin content has been linked to increased susceptibility of Asian patients to develop colorectal cancer with a poor prognosis whereas this association does not seem to apply to European patients.⁸¹

The present data are in agreement with the absence of E-cadherin from MDA-MB-231 cells^{51,82} although it was, as expected, present in the less invasive MCF-7 cells. Our finding that serine proteases reduce E-cadherin expression is consistent with a report that trypsin inhibition increased endothelial E-cadherin levels,⁸³ just as protease inhibition increases DCC and neogenin expression.¹¹

β -catenin

Cellular concentrations of β -catenin and E-cadherin are normally complementary, with an increase in β -catenin expression accompanying a loss of E-cadherin at the start of EMT or during active periods of migration.^{34,76,78,84,85} The effect is believed to follow the dissolution of the *adherens* junctions, the removal of E-cadherin and the consequent release of bound β -catenin into the cytoplasm: E-cadherin exerts a stabilising influence on the levels of β -catenin.^{16,86–88} Altered expression or activity of β -catenin has been implicated in regulating proliferation^{28,84} and oncogenesis^{22,33,75,89–91} being proposed as a ‘co-factor’ in the initiation of EMT.^{92,93} Inhibition of the Wnt/ β -catenin pathway inhibits cancer cell proliferation^{31,94,95} although others have linked β -catenin activation to reduced growth and increased differentiation.⁹⁶ The levels of β -catenin expression may be different in a range of tumour cells types.²⁵

β -catenin has already been linked with DCC function.²⁶ Tumours with low DCC levels were found to have reduced β -catenin expression, leading to the proposal that there might be some common or mutual regulation of expression.^{5,27} The converse pattern is also seen in which induced DCC expression resulted in a reduction of β -catenin expression in parallel with effects on proliferation, adhesion and migration.^{97–99} Several groups have concluded that deletion or interference with the generation of β -catenin had little overall effect on cancer-related behaviours such as proliferation or migration in HCT116 colon cancer cells, although reduced cell adhesion was noted.¹⁰⁰ This may explain why β -catenin levels can appear to be independent of cell proliferation and migration.

The present results reveal β -catenin expression in cells in the virtual absence of DCC, and also show that levels were reduced by the serine proteases (consistent with²⁶) not increased as expected for cells with increased mobility.

Although subtilisin and CT remove DCC and neogenin from cells, increasing cell migration, they reduce rather than promote the expression of β -catenin and vimentin, leading to the conclusion that the sequence of events they induce is not directly related to the EMT and may represent either a non-canonical equivalent process or a completely independent mechanism for increased migration. Indeed, both β -catenin and E-cadherin may have several functions within the cell unrelated to carcinogenesis including tissue regeneration¹⁰¹ and it is possible that changes in their expression induced by serine proteases have a greater effect on some of those alternative activities than on those directly related to oncogenesis.

A different perspective is that the loss of β -catenin and DCC exhibit a distinct but important difference in pharmacology. The loss of β -catenin is prevented by both PIC and chymostatin whereas only the latter compound prevents the removal of DCC and neogenin. Not only does this mean that slight differences exist in the relevant target site protein structure but, more importantly, it indicates that the loss of β -catenin and DCC are very unlikely to be sequential in a common pathway. If there were close functional links, targeting the more distal site in the pathway should affect the cellular function or expression of both proteins.

MCF-7 cells show good levels of β -catenin in the membrane and cytoplasm after stimulation by Wnt ligands, but not in the nucleus.¹⁰² Since these authors found β -catenin in cytoplasmic endosomes, Golgi bodies and coated complexes, they suggested that these might reflect cellular functions of β -catenin unrelated to oncogenesis, such as Golgi body transport. If correct, this could explain some of the inconsistencies of β -catenin behaviour and the possibility that in some situations the balance of functions might give rise to an overall decrease in β -catenin expression, rather than an increase. These observations are consistent with the suggestion that β -catenin might have other functions in the cell independent of any role in proliferation or migration.⁸⁴ This might also account for our finding good levels of β -catenin expression in tumour cells where there is no DCC.

In the absence of DCC, netrin drives cells into EMT, proliferation and migration.^{24,85,103,104} In early embryonic

development, netrin induces a decrease in DCC expression which results in new cell formation and migration to form the developing organs and tissues in mammals and *Drosophila*.¹⁰⁵ The reported ability of netrin to induce E-cadherin degradation leading to EMT and cell migration⁸⁵ might be related to this early loss of DCC. The over-expression of netrins in some human cancers^{106,107} may then be driving tumour formation via the down-regulation of DCC and E-cadherin expression resulting in destabilisation of the differentiated state and the promotion of oncogenesis.

Vimentin

The intermediate filament protein vimentin is the major cytoskeletal protein in mesenchymal cells and has become one of the most widely used markers of EMT²¹ correlating with the relatively aggressive proliferative and migratory activity of cells during embryogenesis and cancer metastasis.^{36–38,79,108–110} In the absence of vimentin, migration is significantly reduced.³⁹ Thus the E-cadherin/vimentin ratio determines the switch from low to high invasion capacity. It has been proposed that a major regulator of EMT – Transforming Growth Factor- β – is responsible for the induction of vimentin expression and the decrease in E-cadherin.¹¹¹

Independent changes in the expression of β -catenin and vimentin have been reported by others. For example, vimentin can be increased in the absence of any changes in β -catenin¹¹² and Messi et al¹¹³ have described aggressively invasive cells which do not possess vimentin. We have observed here that vimentin expression increases when cells are incubated in low serum media, consistent with the report of differentiation being accompanied by an increase in vimentin.¹¹⁴ However, the increased expression of vimentin by chymotrypsin and subtilisin may be sufficient to explain the protease-induced increase of migration in the absence of the normally associated increase of β -catenin, perhaps implying its ability to promote motility without EMT.

Future avenues

An important aspect of any research conclusion is the recognition that it can apply with certainty only to the experimental preparations used and there may be a different spectrum of factors operating in other systems. While information from such alternative systems cannot invalidate the existing data, it may throw further light on the interpretation of results and their general applicability and may yield new insights based on the variations of results obtained in relation to fundamental differences in the biology and behaviour of those systems. In this respect, the differences in rates of cell proliferation, migration and tissue invasiveness between cell types and their origins should allow the functional significance of different results to be used to improve substantially the hypotheses that have been pursued here.

An outstanding example might be the use of primary human glioblastoma cells which exhibit atypically high rates of migration and infiltration accounting for the high mortality associated with them.¹¹⁵ These cells, as primary

cells, do not carry some of the inevitable limitations of studying cell lines which are, by their nature, already transformed and often passaged for an unclearly defined number of times. Another reason for studying glioblastoma cells is that proteases have been clearly linked with their migration and invasive potential, although these have been identified as primarily cysteine proteases, such as cathepsins B and S¹¹⁵⁻¹¹⁷ which are likely to have actions and targets quite different from those of the serine proteases which are the focus of the present work. Nevertheless, future work on aggressive cancer cells of this and similar origins could prove highly informative in the context of the present results.

Conclusions

There are two overall conclusions from this work. Firstly, the relationship between β -catenin, cadherins and vimentin appears to depend on the cell type, culture medium and whether the cells are resting or challenged with serine proteases. β -catenin expression parallels the serine protease-induced changes in the dependence receptors, although with reduced sensitivity. There is no clear or consistent relationship between several of the proteins thought to be associated with EMT, so the concept that EMT can be characterised by a fall in E-cadherin expression coupled with an increase in vimentin, β -catenin and N-cadherin does not entirely account for the increased cell migration induced by serine proteases. This might then imply that a range of cell properties could be reflected in the quantitative, possibly qualitative and, most importantly, *relative* changes in EMT-related protein expression.

Secondly, however, E-cadherin expression is reduced by the proteases and vimentin expression is increased, consistent with conventional views of EMT and leading to the conclusion that among the EMT marker proteins, differential changes can be induced by some treatments such as serine proteases. The events induced by these enzymes may represent either a non-canonical equivalent process to EMT or an independent mechanism for increased migration. More work on these relationships is clearly needed.

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