# Collagen IV synthesis is restricted to the enteroendocrine pathway during multilineage differentiation of human colorectal epithelial stem cells

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## SUMMARY

**The human large intestine is lined by a rapidly renewing epithelial monolayer where cell loss is precisely balanced with cell production. The continuous supply of new cells is produced by undifferentiated multipotent stem cells via a coordinated program of proliferation and differentiation yielding three epithelial lineages: absorptive, goblet and enteroendocrine. Cell-matrix interactions have been suggested to be regulators of the multilineage differentiation program of the colorectal crypt but the expression of matrix proteins or their receptors does not appear to have the subtlety expected for this task.**

**We have developed an in vitro model system of intestinal epithelial stem cells to facilitate the direct analysis of stem cells undergoing lineage commitment and differentiation. Using this culture system, we can now directly investigate the role of cell-matrix signalling in stem-cell decisions. In this study, collagen-IV synthesis has been followed in monolayers of multipotent cells that have been induced to differentiate into absorptive, goblet and enteroendocrine cells. Our experiments demonstrate that commitment to the enteroendocrine lineage is specifically accompanied by the expression of type-IV collagen that remains enteroendocrine-cell associated. Undifferentiated cells, absorptive cells and goblet cells do not express collagen IV. To confirm that the differential lineage-specific expression of collagen IV observed in the model system was representative of the in vivo situation, collagen-IV synthesis was analysed in isolated human colorectal crypts and tissue sections using immunocytochemistry and in situ hybridisation. These studies confirmed the in vitro findings, in that implementation of the enteroendocrine differentiation program involves synthesis and accumulation of a collagen-IV matrix. Thus, human colorectal enteroendocrine cells are unique in the colorectal crypt in that they assemble a cell-associated collagen-IV-rich matrix not observed on other colorectal epithelial cells.**

**This study provides the first evidence for differential matrix synthesis between colorectal epithelial lineages in human colorectal epithelium. The specialised pericellular environment of the enteroendocrine cells might explain some of the unique phenotypic characteristics of this cell lineage. Furthermore, these findings suggest a potential mechanism whereby individual epithelial cells could modulate their cell-matrix signalling even while rapidly migrating in heterogeneous sheets over a shared basement membrane.**

Key words: Intestinal epithelium, Extracellular matrix, Collagen IV, Stem cell, Enteroendocrine, Lineage commitment

## INTRODUCTION

The epithelial lining of the adult human large intestine is organised as a flat, lumenal surface with numerous invaginations (crypts) embedded in connective tissue. The colorectal epithelium undergoes continuous, rapid renewal by multipotent stem cells, which remain anchored at or near the crypt base. Several studies suggest that a single stem cell is responsible for renewing the entire population of the crypt (Ponder et al., 1985; Griffiths et al., 1988; Winton et al., 1988). Multipotent stem cells divide to produce daughter cells that proliferate rapidly and then commit to three colorectal epithelial lineages (absorptive, goblet and enteroendocrine) as they travel from crypt base to lumenal surface (Cheng and Leblond, 1974; Andrew et al., 1982; Potten et al., 1997). Although stem cells remain anchored at the crypt base, differentiating cells migrate in coherent multilineage sheets with little lateral movement of cells (Wilson et al., 1985), predicting similar turnover times for the component lineages. However, kinetic studies have shown that, although absorptive and goblet cells have a similar turnover time (Cheng and Leblond, 1974), enteroendocrine cells have a longer lifespan (Tsubouchi and Leblond, 1979; Thompson et al., 1990; DeBruine et al., 1992) and therefore cannot comigrate with other lineages. These observations indicate that the adhesive phenotype of the anchored multipotential stem cell differs markedly from its migratory differentiating progeny and might be lineage specific. This complex stem-cell program of proliferation, migration and lineage commitment is executed while all cells remain attached to a basement membrane assembled co-operatively by both epithelial cells and underlying mesenchymal cells

(Simon-Assmann et al., 1988; Weiser et al., 1990; Perreault et al., 1998).

Cell-matrix interactions, mediated via integrin receptors, are known regulators of epithelial differentiation (Adams and Watt, 1993), epithelial stem cell fate (Zhu et al., 1999) and cell migration (Lauffenburger and Horwitz, 1996; Palecek et al., 1997). In the intestine, extracellular matrix (ECM) proteins are present at the epithelial-mesenchymal interface early in development (Simon-Assmann et al., 1995) and changes in spatial distribution are associated with morphogenetic events (Simo et al., 1991). In the adult small intestine, broad differential expression of laminin chains between the crypt (proliferative) and villous (differentiated) basement membranes (Beaulieu and Vachon, 1994; Simon-Assmann et al., 1994) have indicated a role for laminin in intestinal differentiation. This has been confirmed using in vitro studies with the Caco-2 cell line, where enterocytic differentiation requires laminin synthesis (DeArcangelis et al., 1996). Therefore, epithelial matrix protein synthesis regulates epithelial phenotype. In vitro studies have further shown that intestinal epithelial cells bind preferentially to collagen IV (Moore et al., 1994) and that collagen synthesis promotes intestinal epithelial cell migration (Moore et al., 1992; Goke et al., 1996; Wilson and Gibson, 1997).

As intestinal epithelial phenotype is regulated by interactions with ECM proteins, this has led to speculation that specific regional differences in the biochemical composition of the ECM and/or matrix receptor expression might specify epithelial cell behaviour in intestinal epithelium. However, the intestinal basement membrane has a slower turnover than the epithelium (Trier et al., 1990) and a relatively uniform composition. The few reports of differential expression of matrix molecules or receptors have described broad regional differences between crypt and villus in the small intestine (Beaulieu, 1992; Beaulieu and Vachon, 1994; Simon-Assmann et al., 1994) and crypt and lumenal surface in the colon (Koretz et al., 1991; Koukoulis et al., 1993). Although such reports strongly indicate a role for matrix molecules in intestinal epithelial biology, the patterns of expression do not reflect the complexity of the multilineage differentiation program. For example, there have been no reports of microheterogeneity in matrix protein or receptor expression that would be compatible with stem-cell- or lineage-specific cell-matrix signalling (Potten et al., 1997). Consequently, regions of the colorectal crypt that appear to have a uniform basement membrane composition and epithelial matrix receptor expression contain a panoply of colorectal epithelial phenotypes (including anchored or migratory and undifferentiated or lineage committed). Therefore, it is not clear how cell-matrix signalling is specified at the single-cell level to generate the observed phenotypic diversity.

To elucidate the molecular mechanisms regulating intestinal stem-cell lineage commitment and differentiation, we have developed an in vitro stem-cell model. This model uses a cloned human rectal epithelial cell line, HRA-19, that has tripotential progenitor cell characteristics (Henderson and Kirkland, 1996) and can be induced to differentiate into all colorectal epithelial lineages (i.e. absorptive, goblet and enteroendocrine) (Henderson and Kirkland, 1996). Differentiation requires transfer of HRA-19 cells to serum-free medium (Henderson and Kirkland, 1996) but does not need specialised ECM-coated substrates. The model facilitates the direct analysis of multipotent colorectal epithelial cells undergoing commitment in the absence of basement-membrane proteins or mesenchymal cells. Thus, epithelial matrix synthesis, a regulator of epithelial phenotype, can be investigated as cells execute their differentiation program.

In this study, we have chosen to investigate type-IV-collagen synthesis during HRA-19 differentiation as collagen-IV substrates promote enteroendocrine lineage commitment in the HRA-19 model (S.C.K. and K.H., unpublished). In addition, ascorbic acid, an essential cofactor for collagen biosynthesis, promotes enteroendocrine- and goblet-lineage commitment of HRA-19 cells (Henderson and Kirkland, 1996). Furthermore, several studies have indicated that collagen-IV synthesis is required for intestinal-cell migration (Moore et al., 1992). This study describes the analysis of type-IV-collagen synthesis in human colorectal epithelial cells as they differentiate along enteroendocrine, goblet and absorptive lineage pathways.

## MATERIALS AND METHODS

### HRA-19 cell line

The HRA-19 cell line was established from a primary human rectal adenocarcinoma (Kirkland and Bailey, 1986). Experiments in this study were performed with a clone of the cell line designated HRA-19a1.1 between passages 20 and 50 (Kirkland, 1988). The cells were free from *Mycoplasma* contamination.

Differentiating monolayers were obtained by trypsinising HRA-19 cells from T25 flasks and seeding into eight-chamber Permanox multislides in serum-free medium. The serum-free medium used in all experiments was Dulbecco's Eagle's medium (Gibco, Paisley, UK) containing sodium pyruvate (110 μg $ml^{-1}$), kanamycin (100 μg $ml^{-1}$), insulin (2 μg $ml^{-1}$), transferrin (2 μg $ml^{-1}$) and ascorbic acid (10 μg $ml^{-1}$). Monolayers were fixed at day 3 or 4 for immunocytochemistry.

### Isolation of normal human colonic crypts

Normal colonic mucosa was obtained from resections for colorectal carcinoma at a site distant from the tumour. Colonic crypts were liberated from the mucosa by a modification of the method described by Whitehead et al. (1987). Mucosa was incubated for 1.5 hours in the following solution: NaCl (8 g $l^{-1}$), KCl (0.2 g $l^{-1}$), $Na_2HPO_4{\cdot}12H_2O$ (2.9 g $l^{-1}$), $KH_2PO_4$ (0.2 g $l^{-1}$) EDTA (0.2 g $l^{-1}$), 0.5% phenol red (1.5 ml), followed by vigorous shaking to liberate mainly intact crypts. Extended incubations in this solution reduced collagen staining. Isolated crypts were dried onto microscope slides overnight then immunostained.

### Antibodies

#### Type-IV collagen and prolyl-4-hydroxylase antibodies

PCO: rabbit polyclonal to type-IV collagen (Eurodiagnostica, Euro-Path, Cornwall, UK). pα1: rabbit polyclonal raised against monomeric collagen α1 (IV) globular domain (Butlowski et al., 1989) (a kind gift from J. Wieslander and T. Hellmark, Lund, Sweden). ZIB7214: rabbit polyclonal against human type-IV collagen (TCS Biologicals, Botolph Claydon, UK). 00-401-106: rabbit polyclonal against type-IV collagen (Rockland, Lorne Lab., Reading, UK). CIV 22: mouse monoclonal to human collagen IV (DAKO, Denmark). Monoclonal antibody to human prolyl-4-hydroxylase β subunit (3-2B12, TCS Biologicals, Botolph Claydon, UK).

#### Cell lineage markers

Mouse monoclonal to human chromogranin (LK2H10, Boehringer). Rabbit polyclonal to PYY (Cambridge Research Biochemicals,

Cambridge, UK). PR4D4 is a monoclonal antibody that specifically reacts with goblet-cell mucus (Richman and Bodmer, 1987). PR4D4 does not stain absorptive or enteroendocrine cells (Richman and Bodmer, 1987). PR2D3 is a monoclonal antibody that reacts with pericryptal mesenchymal cells (Richman et al., 1987).

### Secondary antibodies

Peroxidase-linked secondary antibodies (DAKO, Denmark). Rhodol-Green-conjugated goat anti-mouse IgG (H+L) (Molecular Probes, Cambridge Bioscience, Cambridge, UK). Cy3-conjugated affinity purified donkey anti-rabbit IgG (H+L) and Cy3-conjugated Goat anti-mouse IgG (H+L) (Jackson Immunoresearch, Stratech Scientific Ltd, Luton, UK).

## Immunocytochemistry

### Cell monolayers

Immunocytochemistry was performed essentially as described previously (Henderson and Kirkland,1996) using primary antibodies at the following concentrations: PCO, 1:50; pα1, 1:1000; ZIB 7214, 1:300; 600-410-106, 1:500; CIV22, 1:50; prolyl-4-hydroxylase, 1:50. Rhodol-Green-conjugated goat anti-mouse IgG and Cy3-conjugated donkey anti-rabbit IgG were used as secondary antibodies at 1:200.

### Colonic sections

Normal colonic mucosa was obtained from resections for colorectal carcinoma at a site distant from the tumour and fixed in neutral buffered formalin overnight before routine processing and sectioning. Colon sections for type-IV collagen staining required prior digestion for 30 minutes at 37°C with 0.1% protease (Sigma P-4789) in PBS. Sections were stained with primary antibodies at the following concentrations: PCO, 1:10; pα1, 1:100; ZIB 7214, 1:50; 600-410-106, 1:100; CIV22, 1:100; prolyl-4-hydroxylase, 1:400. Serial sections were stained alternately with pα1 (1:100) and either rabbit polyclonal antibody to PYY (1:400) or a monoclonal antibody to human chromogranin (1:200) (LK2H10).

Frozen sections were allowed to dry overnight and fixed for 10 minutes in acetone at room temperature before immunostaining. Peroxidase-linked secondary antibodies (Dako) and development in a DAB/Nickel solution (Henderson and Kirkland, 1996) were used to detect primary antibody binding.

### Isolated crypts

Isolated crypts were fixed in ethanol for 10 minutes at room temperature using the same antibody concentration used for monolayers except 600-401-106 (1:150), which was used to detect type-IV collagen in double-stained preparations. On whole-crypt preparations, Rhodol-Green-conjugated goat anti-mouse IgG secondary antibody was used at 1:300.

## In situ hybridisation

Normal human colonic epithelium was fixed in 4% paraformaldehyde in DEPC-treated PBS for 6 hours at 4°C before routine processing and sectioning onto silanised slides. Sections were dewaxed in xylene (three times for 2 minutes each), rehydrated in distilled water for 5 minutes and air dried for 5 minutes. In situ hybridisation was performed using reagents supplied in a custom-made kit from Biognostik (TCS Biologicals, Botolph Claydon, UK). Sections were prehybridised in 50 μl Hybribuffer for 3 hours at 30°C and then incubated overnight at 30°C with 50 μl Hybribuffer containing 50 pmol of double-FITC-labelled oligonucleotide probe. The collagen-IV probe was a 29-base oligonucleotide (ATG GCC AAG TAT CTC ACC TGG ATC ACC CT) representing the reverse complement of bases 589-617 of the total sequence of the α1 chain of human collagen-IV gene (Brazel et al., 1987). Following hybridisation, sections were washed in DEPC-treated SSC (twice for 30 seconds each and once for 5 minutes) and then 0.1% SSC (twice for 7 minutes each). Sections were then incubated in 1:20 normal rabbit serum for 10 minutes at room temperature, then in 1:50 peroxidase-linked rabbit anti-FITC (DAKO) for 1 hour at room temperature. Sections were washed in PBS (three times for 5 minutes each). Peroxidase was visualised by development in Dab/Ni solution for 10 minutes (Henderson and Kirkland, 1996).

Controls included omission of probe and a reverse-control double-FITC probe of 29 bases supplied by Biognostik.

## Combined in situ hybridisation/immunocytochemistry

For in situ hybridisation and immunocytochemistry on a single section, chromogranin antibody (1:200) was added to the probe mixture. Following the SCC washes, sections were incubated in PBS containing 1:20 rabbit serum and 1:20 goat serum. Cy3-conjugated goat anti-mouse IgG (Jackson Immunoresearch, Stratech Scientific Ltd, Luton, UK) at 1:300 was included in the anti-FITC solution.

# RESULTS

## Collagen-IV expression accompanies colorectal enteroendocrine lineage commitment in vitro

The human colorectal epithelial cell line HRA-19 forms polarised epithelial sheets when grown in culture medium supplemented with 10% foetal calf serum, but cells within these monolayers do not express colorectal epithelial lineage specific markers (Table 1; Fig. 1A). The transfer of HRA-19 cells to serum-free conditions induces cells to differentiate along three colorectal epithelial lineages so that induced monolayers are composed of undifferentiated cells, absorptive cells, enteroendocrine cells (Fig. 1B) and goblet cells (Table 1) (Henderson and Kirkland, 1996). To determine whether type-IV collagen was expressed during the colorectal epithelial multilineage differentiation program, immunocytochemistry for type-IV collagen was performed on control uninduced monolayers (serum-containing medium) and induced monolayers (serum-free medium) containing multiple lineages. Whereas uninduced monolayers were negative for type-IV-collagen staining (Fig. 1C), induced monolayers contained a scattered cell population with strong positive staining for type-IV collagen (Fig. 1D). This experiment was repeated with a further four type-IV-collagen antibodies (three polyclonals and one monoclonal) (Table 2). For each antibody, staining was performed at least three times and, on all occasions, collagen-IV staining was found to be negative on undifferentiated monolayers whereas differentiating monolayers contained a scattered subpopulation of collagen-IV-positive cells but no evidence of collagen IV deposited onto the plastic substratum (Table 2). Some of the collagen-IV-positive subpopulation displayed the

**Table 1. Effect of culture environment on lineage commitment in HRA-19 cells**

| | Cell lineages (lineage marker) | | |
|---|---|---|---|
| | Enteroendocrine (chromogranin) | Goblet (mucous) | Absorptive (villin) |
| Culture medium | | | |
| DMEM+10%FCS* | – | – | – |
| Serum-free medium‡ | + | + | + |

*, Dulbecco's Eagles medium supplemented with 10% fetal calf serum.
‡, Dulbecco's Eagles medium supplemented with insulin, transferrin and ascorbic acid.
+, cells expressing lineage marker present; –, negligible expression of lineage markers.

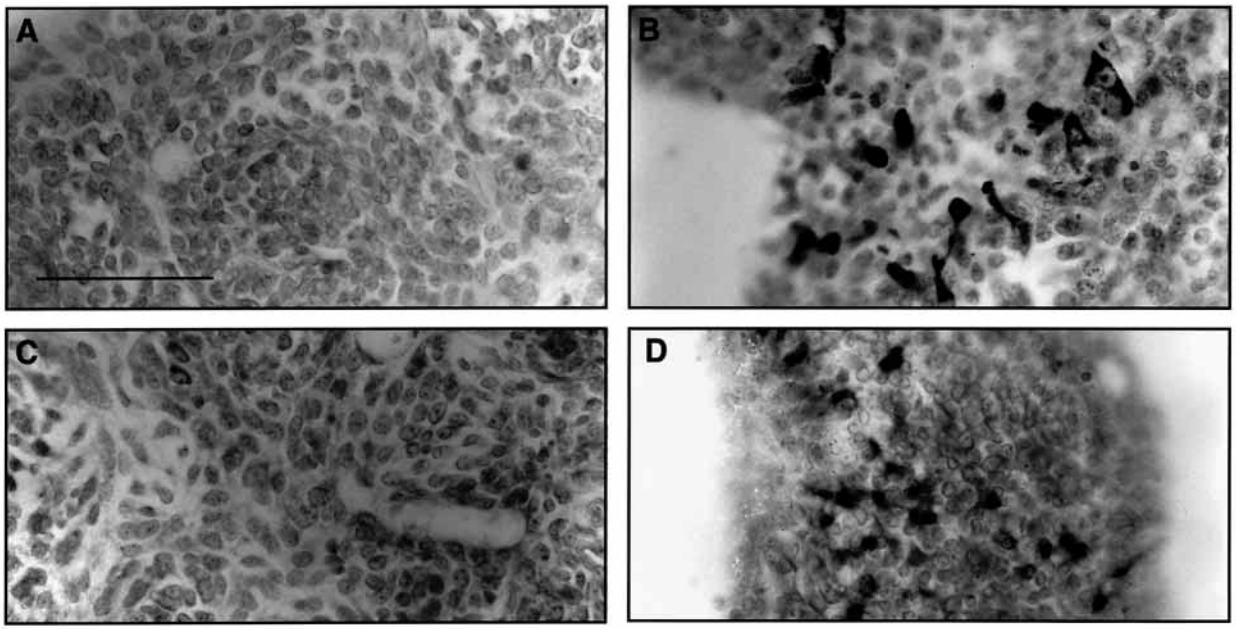


**Fig. 1.** HRA-19 monolayers grown for 4 days in culture medium containing 10% foetal calf serum-containing (A,C) or serum-free medium (B,D) and stained using immunocytochemistry for either chromogranin A (A,B) or type-IV collagen (C,D). Scattered chromogranin-A-positive endocrine cells are observed only under serum-free conditions (B) in similar numbers to collagen-IV-positive endocrine cells (D), which are also only present in serum-free conditions. Bar, 100 μm.

characteristic elongated morphology of enteroendocrine cells, which have been widely studied in the HRA-19 model using endocrine lineage markers such as chromogranin (Fig. 1B). This led us to speculate that the collagen-IV cells were enteroendocrine cells, and this was confirmed by double immunofluorescent immunocytochemical staining with a monoclonal antibody to chromogranin A (a specific endocrine cell marker (Lloyd and Wilson, 1983)) (Fig. 2A) and a polyclonal antibody to collagen IV (PCO) (Fig. 2B; Table 2). Identical results were obtained with double immunofluorescent staining using a polyclonal antibody specific to the α1 chain of type-IV collagen (pα1) and chromogranin (Table 2). These results suggest that type-IV-collagen synthesis is an inherent feature of the colorectal enteroendocrine lineage, but we sought to corroborate this finding further by demonstrating that enteroendocrine cells contain crucial elements of the collagen biosynthetic pathway such as prolyl-4-hydroxylase.

## Prolyl-4-hydroxylase expression accompanies colorectal enteroendocrine lineage commitment in vitro

Prolyl-4-hydroxylase, a key post-translational modifying enzyme in collagen synthesis, catalyses the formation of 4-hydroxyproline, which is essential for triple-helix formation

**Table 2. Collagen IV staining in human colorectal epithelial cells**

| | HRA-19 monolayers | | Human colonic epithelium | | |
|---|---|---|---|---|---|
| | Control | Differentiating | Wax sections | Frozen sections | Isolated crypts |
| Collagen IV antibody | | | | | |
| Polyclonal | | | | | |
| PCO | – | +e (dc) | * | * | +e (dc) |
| pα1 | – | +e (dc) | +e (s(c+p)w) | * | n.d. |
| ZIB 7214 | – | + | * | n.d. | +e (dc) |
| 600-401-106 | – | + | * | * | +e (dc) |
| Monoclonal | | | | | |
| CIV22 | – | + | * | * | n.d. |

+, strong positive staining of scattered cell population compatible with enteroendocrine cell number (i.e. 1% of total colorectal epithelial cells); +e, strong positive staining of endocrine cells confirmed with double immunocytochemistry (d) or serial sections (s) with antibodies to chromogranin (c) or PYY (p); w, weak basement membrane staining; *, intense basement membrane staining masking epithelial staining; –, negative; n.d., not done.

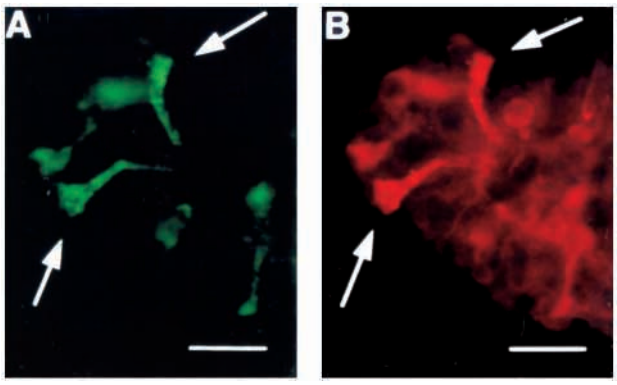


**Fig. 2.** Double immunocytochemical staining of an intact monolayer of HRA-19 cells. (A) Chromogranin-A-positive endocrine cells (arrows). (B) Type-IV-collagen staining in the same cells as (A) (arrows). Notice that the staining is restricted to individual cells. Faint staining over the whole monolayer is non-specific background staining, indistinguishable from the no-primary-antibody controls. Bar, 50 μm.

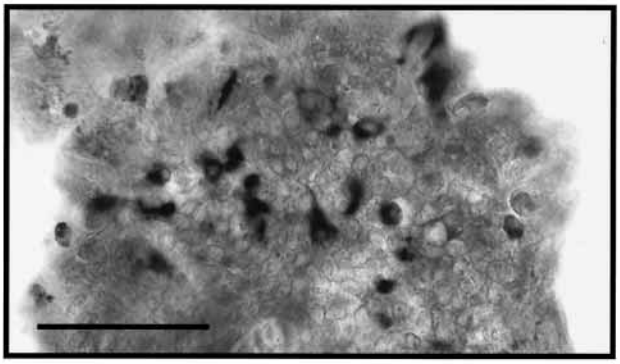

**Fig. 3.** Immunocytochemical staining for prolyl-4-hydroxylase in an HRA-19 epithelial monolayer grown under serum-free conditions. Positive cells (black) are scattered throughout the monolayer. Bar, 100 μm.

(Kivirikko and Myllyharju, 1998). Differentiating and non-differentiating HRA-19 monolayers were stained using immunocytochemistry for prolyl-4-hydroxylase. Although uninduced monolayers were negative, differentiating monolayers contained a scattered population of prolyl-4-hydroxylase-positive cells with the characteristic enteroendocrine morphology (Fig. 3). These studies further suggest that enteroendocrine lineage commitment is associated with the biosynthesis of type-IV collagen.

To understand the association between collagen-IV synthesis further, prolyl-4-hydroxylase expression and commitment to the enteroendocrine lineage, a time-course experiment was performed following the induction of differentiation by transfer of cells to serum-free conditions. From day 3 to day 6, monolayers were evaluated daily in quadruplicate for the presence of two colorectal epithelial lineages by scoring the proportion of chromogranin-positive cells (enteroendocrine cells) and colonic-mucus-positive cells (goblet cells). Replicate monolayers were also scored for the proportion of type-IV-collagen-positive cells and prolyl-4-hydroxylase-positive cells. After three days in serum-free medium, chromogranin-positive cells can be demonstrated in HRA-19 monolayers (33.75 cells in each chamber) whereas only a few prolyl-4-hydroxylase-positive cells are seen (5.25 cell in each chamber) and collagen-IV-positive or mucus-positive cells are not detected (Fig. 4). These results confirm previous findings that the commitment to the enteroendocrine lineage precedes other lineage commitment in this in vitro stem-cell model (day 3, 33.75±20.36 enteroendocrine cells, 0 goblet cells; day 5, 1162±247endocrine cells, 33±7.52 goblet cells). Prolyl-4-hydroxylase-expressing cells were detected in HRA-19 monolayers following chromogranin expression, but the rate of endocrine-cell commitment was mirrored by the rate of appearance of prolyl-4-hydroxylase-positive cells following this lag phase. Collagen-IV-expressing cells were first detected on day 4 and their numbers increased rapidly on days 5 and 6. Goblet cells were detected in significant numbers on day 5 but were present in much lower numbers than the collagen-IV-positive cells. These results indicate that chromogranin expression is an early event in enteroendocrine lineage commitment that is followed by the acquisition of collagen biosynthetic elements (prolyl-4-hydroxylase) and then by the accumulation of a cell-associated collagen-IV matrix. All studies described here were performed on HRA-19 cells grown on uncoated tissue-culture plastic in the absence of mesenchymal cells and so enteroendocrine-cell-associated type-IV collagen does not require either mesenchymal regulation or interaction with a native colorectal basement membrane, although such factors might modulate the collagen IV expression in vivo.

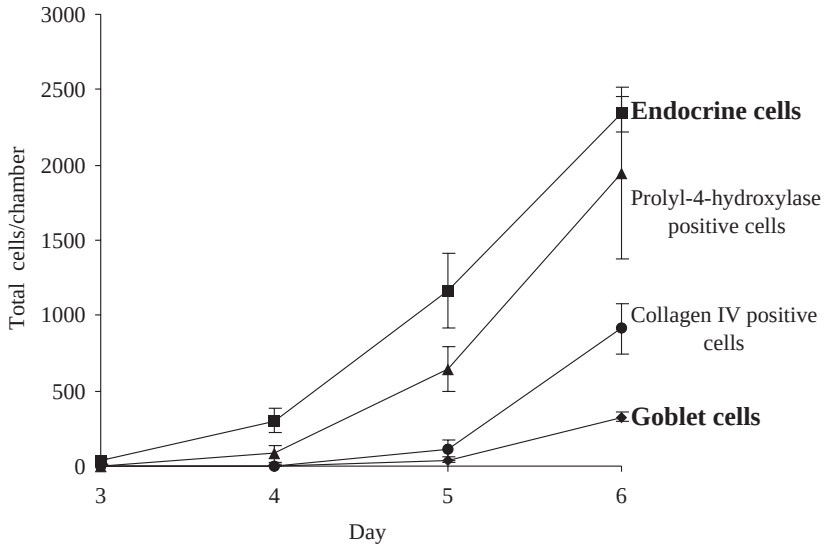


**Fig. 4.** Time-course analysis of endocrine- and goblet-lineage commitment in HRA-19 cells following the transfer of cells at day 0 to serum-free medium. Total number of cells expressing prolyl-4-hydroxylase and collagen IV in each replicate monolayer can be seen in relation to the numbers of endocrine (chromogranin-A positive) and goblet (colonic-mucus positive) cells.

### Collagen-IV expression accompanies colorectal enteroendocrine lineage commitment in vivo

To confirm that the expression of type-IV collagen was also a feature of normal human colorectal enteroendocrine cells in vivo, paraffin and frozen sections from normal human colonic epithelium were stained with a variety of monoclonal and polyclonal antibodies to type-IV collagen. As expected, most antibodies gave intense basement membrane staining for type-IV collagen that masked any epithelial staining (Table 2). However, one polyclonal antibody to type-IV collagen raised against monomeric α1 collagen-IV globular domain (Butlowski et al., 1989) (kindly provided by Dr J. Wieslander, Lund, Sweden) reacted only weakly with type-IV collagen once assembled into intestinal basement membrane and formalin fixed. Colonic sections stained with this antibody showed that, in addition to the weakly stained basement membrane, scattered cells within the epithelium were also stained (Fig. 5). The distribution, morphology and number of these cells indicated that they were endocrine cells. Enteroendocrine cells represent ~1% of the total colorectal

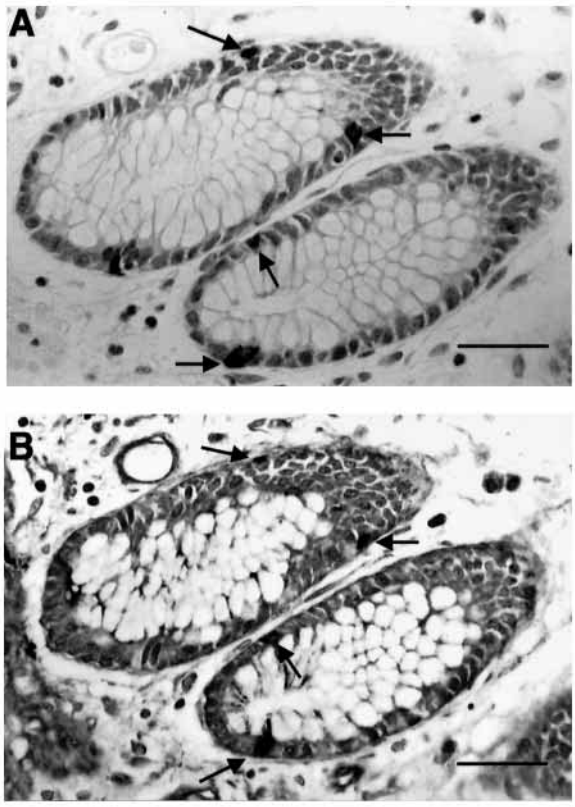


**Fig. 5.** Immunocytochemical staining in two adjacent serial sections of normal human colon. Endocrine cells that are PYY positive (A) (arrows) match cells with type-IV-collagen staining in the adjacent section (B) (arrows). Bar, 25 μm.

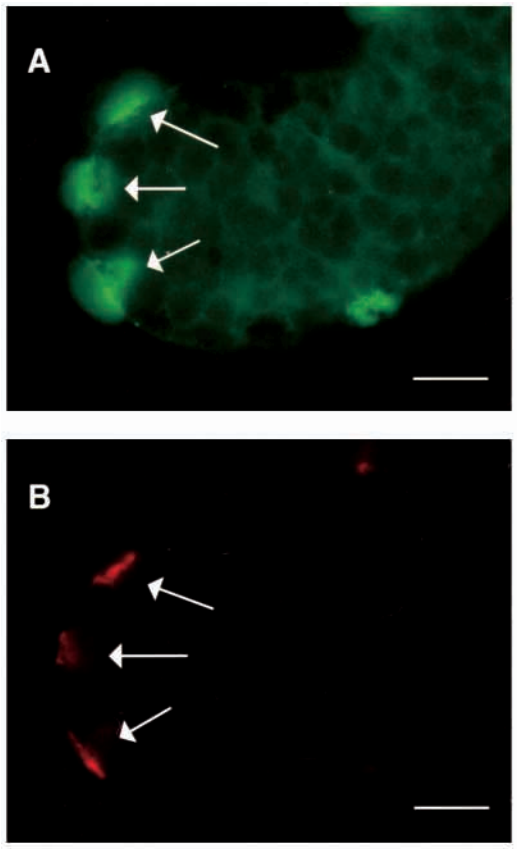


**Fig. 6.** Double immunocytochemical staining of whole colonic crypts for chromogranin A and type-IV collagen. Three chromogranin-A-positive endocrine cells are located at the crypt base (arrows) (A). These cells are also shown to be type-IV-collagen positive (arrows) (B). Bar, 25 μm.

epithelial cell population, the remaining differentiated colorectal epithelial population being composed of absorptive and goblet cells. In addition, enteroendocrine cells have a characteristic distribution within the epithelium, being most frequently found in the lower half of the colorectal crypt. The pattern of staining with the pα1 collagen-IV antibody in human colorectal epithelial sections was entirely compatible with an enteroendocrine cell staining. Staining of isolated crypts with three more collagen-IV antibodies gave staining patterns that were indistinguishable from those obtained with enteroendocrine lineage markers such as chromogranin. The endocrine cell phenotype of collagen-IV-staining cells was further confirmed by immunostaining of serial sections with anti-collagen-IV antibody and antibodies to PYY, a neuropeptide expressed by colonic enteroendocrine cells (Lundberg et al., 1982) or chromogranin. Type-IV collagen was shown by staining to localise to the same cells as PYY (Fig. 5A,B) and chromogranin (data not shown). This indicates that human colorectal enteroendocrine cells are unique among colorectal epithelial cells in that they are collagen-IV positive.

To confirm type-IV collagen expression in enteroendocrine cells with other, more widely available antibodies, we needed to remove the intestinal epithelium from its underlying basement membrane while retaining the epithelial pericellular matrix. To achieve this objective, whole crypts were isolated from normal human colorectal epithelium using a modified EDTA isolation method. These crypts were fixed in buffered formalin, wax embedded, sectioned and stained for collagen but staining was weak and difficult to resolve. Likewise, frozen sections of these crypt preparations were inadequate for the resolution needed in this experiment. Therefore, whole, freshly isolated crypts were dried overnight onto glass slides and immunostained intact. These whole crypts were confirmed to be free of pericryptal fibroblasts by immunocytochemistry with PR2D3 (Richman et al., 1987; S.C.K. and K.H., unpublished) but to retain enteroendocrine cells, as demonstrated by immunostaining for chromogranin A (Fig. 6A). Isolated crypts demonstrated no overall staining for type-IV collagen (Fig. 6B), confirming previous observations that the basement membrane is absent from crypts isolated in this way. Immunostaining for type-IV collagen in these isolated crypts confirmed the presence of scattered type-IV-collagen-positive cells. These collagen-IV-positive cells in freshly isolated normal human colorectal crypts were confirmed as enteroendocrine cells using double immunocytochemistry with each of three polyclonal collagen-IV antibodies and chromogranin (Fig. 6A,B; Table 2). In the isolated crypts, staining for type-IV collagen was not observed in any other cell lineage or in the stem-cell zone with any of the collagen-IV antibodies, thereby confirming in vitro observations that type-IV collagen expression is restricted to the enteroendocrine lineage.

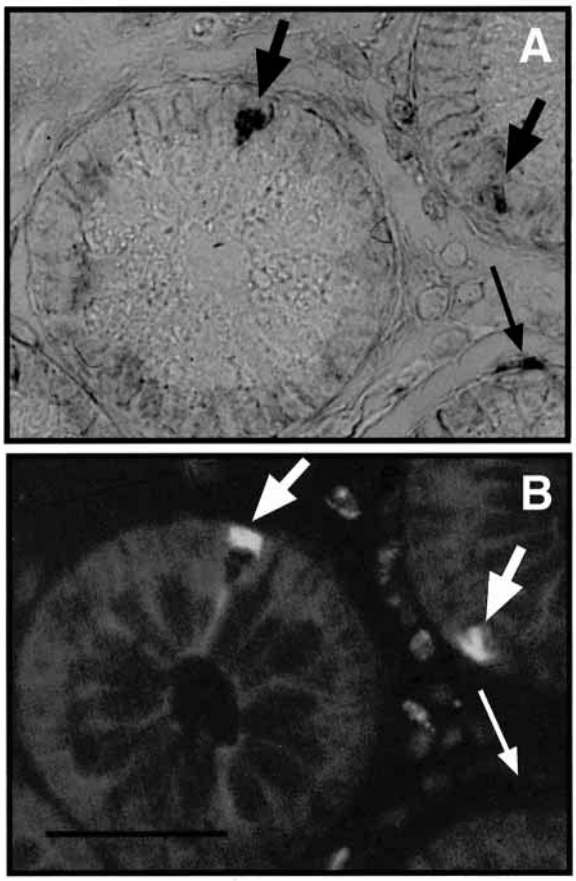


**Fig. 7.** Wax sections of normal human colonic epithelium stained using immunocytochemistry for prolyl-4-hydroxylase. (A) Positive staining is seen in both epithelial cells (wide arrows) and pericryptal mesenchymal cells (thin arrow). (B) Fluorescence microscopy of the same section shows that autofluorescence of endocrine cells colocalises with the epithelial prolyl-4-hydroxylase staining. Bar, 50 μm.

## Prolyl-4-hydroxylase expression accompanies colorectal enteroendocrine lineage commitment in vivo

As prolyl-4-hydroxylase was associated with enteroendocrine lineage commitment in vitro, the expression of this enzyme was studied in sections of normal human colorectal epithelium. Immunocytochemical staining for prolyl-4-hydroxylase revealed a scattered epithelial cell population that was positive for this enzyme (Fig. 7A). Prolyl-4-hydroxylase could also be seen expressed in pericryptal fibroblasts, which synthesise type-IV collagen for assembly into the colorectal basement membrane (Simon-Assmann et al., 1988; Weiser et al., 1990; Perreault et al., 1998). The scattered epithelial cell population expressing prolyl-4-hydroxylase was confirmed as enteroendocrine using endocrine cell autofluorescence combined with immunofluorescent staining for prolyl-4-hydroxylase (Fig. 7A,B). Enteroendocrine cells were shown to be the only population in human colorectal epithelium to express prolyl-4-hydroxylase, a key enzyme in collagen biosynthesis.

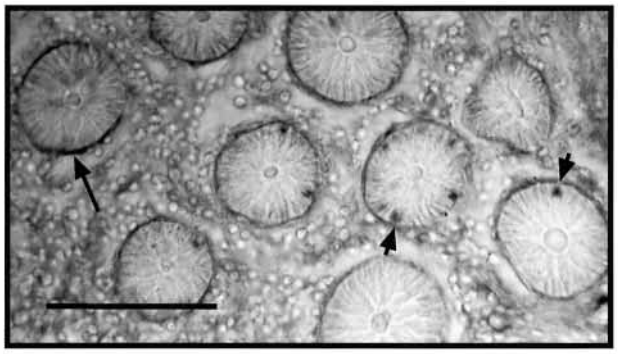

**Fig. 8.** In situ hybridisation for type-IV collagen on wax sections of normal human colon. Pericryptal fibroblasts, the source of basement-membrane collagen IV, stained positively for collagen mRNA (long arrow). Scattered epithelial cells are also positive for collagen mRNA (arrowheads). Bar, 200 μm.

## Colorectal enteroendocrine cells synthesise type-IV collagen

The results of our in vitro and in vivo studies strongly suggest that enteroendocrine cells synthesise collagen IV as part of their differentiation program. In vitro studies particularly support such a conclusion as alternative sources are not available. However, to confirm that enteroendocrine cells are indeed synthesising type IV collagen, in situ hybridisation was performed on wax sections of colorectal epithelium using an oligonucleotide probe specific for type-IV collagen mRNA. Pericryptal fibroblasts, the source of basement-membrane collagen IV, stained positively for collagen mRNA (Fig. 8) and acted as an internal positive control for collagen-IV staining. In addition, a scattered population within the epithelium was also shown to stain positively for collagen-IV mRNA (Fig. 8). These epithelial cells were confirmed as enteroendocrine cells by performing in situ hybridisation for collagen IV and immunocytochemistry for the enteroendocrine-specific

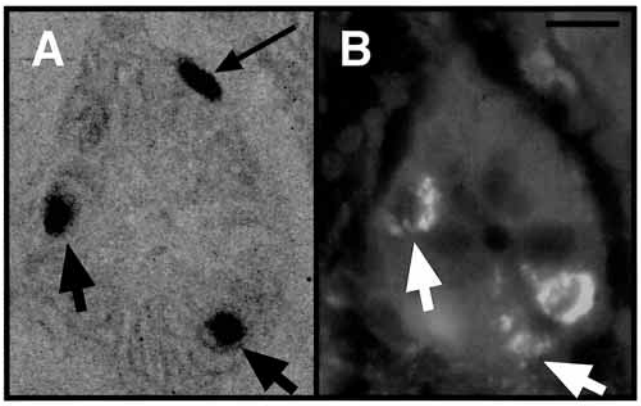


**Fig. 9.** A single section of normal human colonic epithelium stained with in situ hybridisation for collagen IV mRNA (A) and immunocytochemistry for chromogranin A (B). A single crypt is shown to contain two collagen-IV mRNA-positive epithelial cells (short arrow) with an associated positively staining pericryptal fibroblast (long arrow) (A). Both collagen-IV mRNA-positive epithelial cells are shown to be chromogranin A positive (B). Bar, 25 μm.

protein, chromogranin on a single section of human colon (Fig. 9A,B). A control probe with the reversed sequence stained neither enteroendocrine cells nor pericryptal fibroblasts (results not shown). These results confirm that enteroendocrine lineage commitment is accompanied by synthesis of type-IV collagen.

## DISCUSSION

In this study, we analysed collagen-IV expression during lineage commitment in human colorectal epithelial cells. Our initial studies were performed with an in vitro model where multipotential cells (HRA-19) were induced to differentiate along three lineage pathways (absorptive, goblet and enteroendocrine). These cells can differentiate under serum-free conditions on a plastic substrate without exogenous matrix proteins or mesenchymal cells. Therefore, any requirement for matrix proteins either to direct or define lineage commitment must be met endogenously within HRA-19 monolayers. Immunocytochemical studies on induced HRA-19 monolayers using a number of specific collagen-IV antibodies demonstrated that commitment to the enteroendocrine lineage was accompanied by collagen-IV expression. This finding was confirmed for enteroendocrine lineage commitment in vivo by investigating collagen-IV expression in isolated human crypts and tissue sections using immunocytochemistry and in situ hybridisation. Thus, both in vitro and in vivo data demonstrate that the enteroendocrine differentiation program directs the synthesis of collagen IV. This finding contrasts with previous studies that have demonstrated that intestinal type-IV collagen synthesis is restricted to mesenchymal elements (Simon-Assmann et al., 1988; Weiser et al., 1990; Perreault et al., 1998), although some epithelial expression of recently described novel type-IV collagen chains has been shown (Beaulieu et al., 1994; Perreault et al., 1998). There are two main reasons why enteroendocrine cell collagen IV might not have been detected in these previous investigations. First, biochemical or molecular biological investigation of total epithelial extracts is unlikely to find either enteroendocrine cell proteins or mRNAs because of the low abundance of these cells in the epithelium. In addition, immunocytochemical staining of intestinal sections for type-IV collagen with most antibodies results in intense basement-membrane staining, which obscures the staining of endocrine cells. These difficulties were circumvented in this study by using isolated human colorectal crypts devoid of basement membrane for immunostaining and corroborating these findings with in situ hybridisation on tissue sections.

Although these techniques have demonstrated the novel synthesis of collagen IV by enteroendocrine cells, they have also confirmed previous studies in that collagen IV synthesis could not be demonstrated in most colorectal epithelial cells with undifferentiated, goblet or absorptive phenotypes (Simon-Assmann et al., 1988; Weiser et al., 1990; Perreault et al., 1998). Therefore, whereas previous studies have described epithelial versus mesenchymal matrix protein contributions, our data provide the first evidence of a new principle in intestinal cell-matrix interactions: that synthesis of matrix proteins can be epithelial-lineage specific rather than simply epithelium specific. Both in vivo and in vitro data show that collagen IV remains tightly associated with enteroendocrine cells: neighbouring cells are type-IV-collagen negative, suggesting that the assembly and retention of a type-IV-collagen-rich microenvironment is part of the enteroendocrine differentiation program. Cell-associated type-IV collagen is observed on enteroendocrine cells in vitro and is therefore an inherent feature of the colorectal enteroendocrine lineage and not dependent on the presence of a native basement membrane or mesenchymal cells.

To define the role of type-IV collagen in enteroendocrine lineage commitment further, type-IV collagen expression was followed as cells differentiated along the enteroendocrine lineage pathway. Our data indicate that progression along the enteroendocrine pathway is characterised by a sequential expression of chromogranin, prolyl-4-hydroxylase and, finally, type-IV collagen. Therefore, collagen IV appears to be synthesised only when cells have already committed to the endocrine lineage, indicating that collagen IV is involved in endocrine cell function rather than lineage commitment decisions. The implementation of the enteroendocrine differentiation program must thus involve specific synthesis of matrix proteins that appears to result in the elaboration of a type-IV-collagen-rich pericellular matrix by enteroendocrine cells. The purpose of the collagen-IV-rich matrix in specifying enteroendocrine cell phenotype has yet to be elucidated but the unique migratory behaviour of this lineage suggests one potential role. Several studies have demonstrated that enteroendocrine cells have a much longer lifespan than other intestinal epithelial cells (Tsubouchi and Leblond, 1979; Thompson et al., 1990; DeBruine et al., 1992), strongly indicating that they have a lower migration rate. Therefore, enteroendocrine cells require a cell-specific mechanism(s) to reduce migration rate relative to neighbouring goblet and absorptive cells in the epithelial sheet migrating from crypt base to lumenal surface. Could the production of collagen IV and assembly of a collagen-IV-rich pericellular matrix alter the migratory phenotype of enteroendocrine cells? This possibility is supported by the finding that collagen synthesis has been shown to be involved in intestinal epithelial migration. Enterocytes preferentially bind and spread on type-IV collagen (Moore et al., 1994) and inhibition of collagen synthesis inhibits intestinal epithelial cell migration in vitro (Moore et al., 1992; Goke et al., 1996). Furthermore in vitro studies with other cell types have shown that matrix protein concentration modulates integrin expression and cell motility (Palecek et al., 1997; Condic and Letourneau, 1997), and also the motility response of cells to growth factors (Ware et al., 1998). High concentrations of ligand lead to strong adhesion with reduced migration (Palecek et al., 1997). Therefore, it is plausible to suggest that the assembly of a type-IV-collagen-rich pericellular environment could reduce enteroendocrine cell motility, enabling absorptive and goblet cells to bypass them in this rapidly migrating epithelial sheet. The concept of a lineage-specific pericellular matrix composition defining patterns of migration warrants further study as it has wide ranging implications for understanding cell anchorage in normal epithelia and the aberrent migratory behaviour of neoplastic cells.

Recent studies have highlighted the importance of cell-surface matrix composition in regulating cell behaviour via cell-matrix signalling (Basbaum and Werb, 1996). Our work indicates that ECM synthesis is also a contributory factor in

defining the cell-surface matrix composition of individual cells. Lineage-specific pericellular matrix composition would explain how single cells could maintain specific cell-matrix signalling even while migrating in heterogeneous sheets over a shared basement membrane. Cell-matrix interactions have been shown to regulate the differentiation of epithelial cells (Roskelley et al., 1995), including those of the intestine (Simon-Assmann et al., 1995). Therefore the ability of colorectal epithelial cells to maintain differential matrix synthesis has important implications for understanding cell-matrix regulation of the epithelial phenotype in the human colorectal crypt.

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