The human colorectal epithelium is maintained by multipotent stem cells which give rise to absorptive, mucous and endocrine lineages. Recent evidence suggests that human colorectal cancers are likewise maintained by a minority population of so-called cancer stem cells. We have previously established a human colorectal cancer cell line with multipotent characteristics (HRA-19) and developed a serum free medium that induces endocrine, mucous and absorptive lineage commitment by HRA-19 cells in vitro. In this study, we investigate the role of the β1 integrin family of cell surface extracellular matrix receptors in multilineage differentiation by these multipotent human colorectal cancer cells. We show that endocrine and mucous lineage commitment is blocked in the presence of function blocking antibodies to β1 integrin. Function blocking antibodies to α2 integrin also blocked both HRA-19 endocrine lineage commitment and enterocytic differentiation by Caco-2 human colon cancer cells, both effects being abrogated by the MEK inhibitor, PD908059, suggesting a role for ERK signalling in α2 mediated regulation of colorectal cancer cell differentiation. To further explore the role of α2 integrin in multilineage differentiation, we established multipotent cells expressing high levels of wild type α2 integrin or a non-signalling chimeric α2 integrin. Over-expression of wild-type α2 integrin in HRA-19 cells significantly enhanced endocrine and mucous lineage commitment, whilst cells expressing the non-signalling chimeric α2 integrin had negligible ability for either endocrine or mucous lineage commitment. This study indicates that the collagen receptor α2β1 integrin is a regulator of cell fate in human multipotent colorectal cancer cells. A small population of multipotent epithelial stem cells maintains the integrity and function of the adult intestinal epithelium (1) Colorectal epithelial stem cells proliferate slowly giving rise to daughter cells that undergo a phase of rapid proliferation and then differentiate into absorptive, mucous and endocrine cells. Homeostasis requires a precise balance between stem cell renewal and generation of lineage-committed cells; processes regulated by the Wnt,TGF-β, Hedgehog and Notch pathways (2). Dysregulation of the Wnt signalling pathway, a critical regulator of normal stem cell renewal, is commonly present in colorectal cancer as the result of well described mutations in Wnt signalling components(3). This suggests that signalling cascades which promote normal colorectal epithelial stem cell renewal persist in colorectal cancer cells. Indeed there is growing support for the idea that human cancers, including colorectal cancer, are diseases of stem cells(4,5). It has been shown that only a small minority of tumour cells, termed cancer stem cells, are able to initiate tumour growth. Furthermore putative human colorectal cancer stem cells have been isolated on the basis of their expression of epithelial cell adhesion molecule and CD44 (6) or CD133(7,8). However the relationship between cancer stem cells and their normal counterparts remains to be elucidated. This will require a greater understanding of the mechanisms which balance self-renewal and differentiation in colorectal epithelial stem cells and colorectal cancer cells. Maintenance of stem cells is thought to require a specialized tissue microenvironment known as a stem cell niche. The intestinal stem cell niche, like the intestinal stem cell, remains poorly defined but it seems probable that intestinal stem cell behaviour will be specified by the integration of signalling pathways triggered by soluble factors and stem cell adhesion to other cell types or extracellular matrix proteins(9,10). Extracellular matrix is a powerful regulator of stem cell function(11,12). Cell-matrix interactions...
are mediated, to a large extent, by the integrin family of transmembrane receptors (13). Integrins mediate bi-directional signalling between the extracellular milieu and intracellular pathways. Integrins are heterodimers (one α and one β subunit (14)), without intrinsic catalytic activity. That signal by association with a diverse range of proteins including cytoskeletal proteins and kinases. Integrins can activate growth factor signalling pathways (15) and regulate many cell functions including proliferation, differentiation and matrix assembly.

Elevated αβ1 integrin expression is a hallmark of skin (16), prostate (17) and neural stem cells (18); and β1 integrins regulate epidermal (19), neural (20) and embryonic (21) stem cell fate. β1 integrins are also candidate intestinal stem cell regulators as they are highly expressed in the stem cell region and epithelial cells with high β1 expression show enhanced clonogenicity in vitro (22). Conditional deletion of β1 integrin in intestinal epithelium did not decrease adhesion as expected but instead increased proliferation, reduced differentiation and increased expression of the putative stem cell marker Musashi-1 (23) suggesting that β1 integrins regulate intestinal stem cell fate. What is not clear is whether stem cell regulation is mediated entirely by the β1 integrin chain or in the context of a particular αβ heterodimer.

We previously established a multipotent human colorectal cancer cell line, HRA-19 (24). Clones of this cell line execute a multilineage differentiation program forming absorptive, mucous and endocrine cells in xenografts (24) and in vitro upon transfer to serum free medium (25). This study investigates the role of cell surface αβ1 integrin matrix receptors in lineage commitment in these multipotent human colon cancer cells in vitro. We report that α2 integrin regulates cell fate in human colorectal cancer cells.

**Experimental Procedures**

**Materials-Azide free antibodies (Abs) to β1 (JB1A LM534), α1 (FB12), α2 (P1E6), α4 (P1H4), α5 (P1D6), α6 (NKI-GoH3); MAb to α2β1 integrin (MAB1998); Ab against α1 integrin cytoplasmic domain (AB1934) and MAb to human chromogranin (MAB5268) (Chemicon). α2 integrin MAb (611016) (BD Transduction). Mucous MAb, PR4D4 (kind gift from George Elia, CRUK). Integrin Abs used in this study have previously been shown to block integrin function:**

**β1 (JB1A (26)), α1 (FB12 (27)), α2 (P1E6 (28)), α3 (P1B5 (29)), α3 (P1H4 (30)), α5 (P1D6 (29)), α6 (NKI-GoH3 (31)), α1 (P3G8 (32)).**

**Endocrine and Mucous Lineage commitment assay-** Twice cloned HRA-19a1.1. cells (24) were used in this study. Multiplex PCR analysis performed at the ECACC (Porton Down) confirmed the cells to have a unique profile. Lineage commitment experiments were performed as previously described (25) or with minor modifications. Briefly, cells were seeded into 8 Chamber plastic slides (Nunc) at a dilution equivalent to a 1:5 split ratio (approximately 1.2x10^4 cells/0.5 ml/chamber) (cells are transferred as a mixture of cell clumps and single cells: single cell suspension is not possible without major cell damage) in DMEM with 10%FCS. On Day 3 cells were transferred to serum free medium (ITA): DMEM with 2mM glutamine, ascorbic acid (10µg/ml), Insulin/Transferrin/Selenium (ITS-X: Invitrogen) and incubated at 37°C. Monolayers were stained for endocrine cells (chromogranin) on Day 5 and mucous cells (PR4D4) on Day 6 (33) by immunocytochemistry. G418 was omitted during lineage commitment assay as it inhibited differentiation (unpublished observations). Values were normalized for cell number using the WST-1 reagent (Roche) as described by supplier.

**Immunofluorescence-** Immunofluorescence used ethanol fixed cells with Abs to chromogranin (LK2H10: Chemicon) or α2 integrin (BD Transduction) followed by Alexa488-Rb anti-Ms Immunoglobulins (Invitrogen).

**Cell Adhesion Assay-** Multiwell plates (Nunc Maxisorp) were coated with Collagen I or IV (6µg/ml) overnight at 4°C, washed and blocked with 1% BSA in DPBS. 10^5 cells per well were incubated for 2hr at 37°C to allow attachment. Adherent cell number was measured using crystal violet staining, absorbance read at 595nm. Blank values from BSA coated wells (typically less than 5% of maximal adhesion) were subtracted from test values. Antibodies were incubated with cells for 15min at 37°C before adding to the matrix-coated wells.

**Plasmid constructs and transfection-** Integrin constructs in the PAWneo2 expression vector were a kind gift from Dr J. Ivaska. Constructs were checked by sequencing and transfected into cells in 10cm dishes using 10µg DNA and 37.5 µl of Fugene 6 (Roche Diagnostics) prepared in OptiMem medium (Invitrogen) and overnight incubation at 37°C. Cells were transferred to
DMEM with 10%FCS for 24 hours, then G418 200µg/ml (Invitrogen) was added. G418 resistant colonies were selected with cloning cylinders. Cells grew very slowly in G418 and selection took many months. Transfected cells were maintained in DME/10%FCS supplemented with 2mM glutamine and 200µg/ml G418.

**Immunoblotting**-Lysates were prepared with non-reducing SDS lysis buffer (Cell Signalling). Equal amounts of protein (RC-DC assay:Biorad) were separated on 3-8%Tris-Acetate gels (Invitrogen) and blotted onto nitrocellulose. Blots were blocked with 5% milk solution, rinsed in wash buffer (10mM Tris-HCl, 0.1M NaCl, 0.1%Tween 20) and incubated overnight with antibodies (α2 integrin; mAb 1965(Chemicon): or β1 integrin; α2 integrin (611016). in blot wash. Blots were washed and incubated in 1hr rabbit anti-mouse antibodies (Dako) in blot wash for 1hr at room temperature, washed and developed using ECL-Plus (Amersham).

**Biotinylation & Immunoprecipitation**-Cells were surface biotinylated in 1mg/ml Sulfo-NHS-Biotin (freshly prepared) (Pierce) in DPBS for 30 min. at room temperature with gentle shaking. Cells were washed and lysed in 1% Triton X-100, 2mM EDTA, 0.15M NaCl, 50mM Tris-HCl, 1mM PMSF. For immunoprecipitation, lysates were precleared with protein-G agarose (Roche) then incubated with antibodies to β1(1965), α2(PIE6) or α2β1 (1998) for 4hr at 4°C. Immune complexes were collected onto Protein-G agarose. Following electrophoresis and blotting, biotinylated proteins were detected with Streptavidin HRP(Pierce). Immunoprecipitation of α2α1 integrin was performed with AB1934 to the cytoplasmic domain of α1 integrin and detected on blots with α2 integrin antibody.

**Alkaline phosphatase activity in Caco-2 cells**-Subconfluent cells were transferred to serum free medium (TSG) containing 0.2%BSA, transferrin (5µg/ml), sodium selenite (5 ng/ml) and 1mM glutamine for 24hr then harvested with 0.0125% trypsin/versene and added to an equal volume of soy bean trypsin inhibitor (0.5mg/ml). Cells were plated at 0.6 x 10⁴ cells/well in TSG medium into 96 well plates either untreated or coated with α2 integrin antibody (AK7) or MsIgG control (Biologend).After 72hr, cell number was estimated with WST-1 reagent(Roche). Alkaline phosphatase activity was measured using p-nitrophenyl-phosphate (Chemicon), the reaction product p-nitrophenol was measured at 405nm. Absorbance was normalised using the WST-1 values.

**RESULTS**

**Endocrine lineage commitment is regulated by α2β1 integrin-To** determine whether β1 integrins were involved in lineage commitment, HRA-19 cells were transferred to serum free medium to induce endocrine lineage commitment in the presence of a β1 antibody (JB1A) which blocks cell adhesion (26) and signalling (34). JB1A reduced endocrine lineage commitment to 2% of control values (Fig 1A). Furthermore LM534, another β1 antibody which binds to the extracellular domain of β1 integrin, also reduced endocrine lineage commitment significantly (Fig 1A). Function blocking β1 integrin antibody,JB1A, also blocked mucous lineage commitment in HRA-19 cells (Fig. 1B) while addition of equivalent amounts of isotype control antibody did not affect mucous cell numbers. In addition, total cell number was unaffected by treatment with JB1A or isotype control indicating that the antibodies had not affected attachment, proliferation or survival. (Fig 1B). These results indicate a role for the β1 integrins in regulating endocrine and mucous lineage commitment in HRA-19 cells. However, integrins are heterodimers and modulation of β1 chain function could potentially be affecting all members of the β1 integrin family. Therefore we sought to identify which β1 integrin heterodimer(s) was involved in blocking endocrine/mucous lineage commitment.

HRA-19 endocrine lineage commitment was induced in the presence of function blocking antibodies to a range of alpha integrin chains known to form heterodimers with β1 integrin. Only antibodies to α2 integrin were shown to markedly reduce the ability of HRA-19 cells to generate endocrine cells while other alpha chain antibodies had no effect (Fig 2A). The α2 chain antibody gave a dose-responsive inhibition of endocrine lineage commitment while an antibody recognizing the α1 chain of another collagen receptor, α1β1 integrin, had no effect at the same doses (Fig 2B). Previous work has shown that α2 integrin only partners β1 integrin to form the α2β1 heterodimer (35), therefore these experiments suggest that α2β1 integrin regulates cell fate.

**HRA-19 cells express α2β1 integrin**-Immunoblotting was used to analyse integrin expression in HRA-19 cells. Lysates contained two β1 integrin bands representing the immature (smaller band) and the mature glycosylated forms.
(Fig. 3A) (36). α2 integrin expression was also demonstrated (Fig. 3B). α2β1 integrin was demonstrated at the cell surface by biotinylation of live cells and immunoprecipitation with MAb to β1 integrin (Fig. 3C). Only the fully glycosylated β1 integrin band is seen at the cell surface along with a β1 integrin associated protein which co-migrates with α2 integrin (Fig 3C). Immunoprecipitation with antibodies to α2 integrin and α2β1 integrin complex also revealed two biotinylated protein bands corresponding in molecular weight to α2 and β1 integrin (Fig 3C).

α2β1 integrin is a collagen receptor in HRA-19 cells -α2β1 is a major collagen receptor (37) in many cell types. Cell adhesion experiments were used to establish whether α2β1 mediated collagen binding in HRA-19 cells. Attachment to collagen I and IV was blocked by antibodies to β1 and α2 integrin (Fig. 4) indicating that α2β1 integrin is a receptor for both Collagen I and IV in HRA-19 cells. Antibodies recognizing other α chains did not significantly reduce cell binding to either Collagen I or Collagen IV (Fig. 4).

Integrin α2 cytoplasmic tail is required for endocrine and mucous lineage commitment-To support a role for the α2 integrin chain in cell fate regulation we generated HRA-19 transfectants over expressing either wild-type α2 integrin or a non-signalling chimeric protein composed of the extracellular and transmembrane domain of α2 integrin and the cytoplasmic domain of α1 integrin (Fig. 5A). Cell colonies were analysed for their expression of α2 integrin (Fig. 5B) and α2α1 integrin (Fig. 5C). Two colonies, α2B and α2F, were chosen for further study as they showed markedly higher α2 integrin expression than the parent cell line (Fig 5B). The chimeric protein was immunoprecipitated using an antibody to the cytoplasmic tail of α1 integrin and then detected on western blots using an Ab to the extracellular region of the α2 chain (Fig.5C). The α2 band was not observed in α1 immunoprecipitates of parent cells or α2 transfectants (α2B or α2F) but was present in chimeric transfectants α2α1B and α2α1E cells (Fig 5C), which were selected for use in subsequent experiments. In the parent HRA-19 cells α2 integrin is primarily localized at cell-cell contacts (Fig 5D), as shown previously in the intestine (38), a localization retained by cells transfected with either wild-type α2 integrin (α2F) or chimeric α2α1 integrin (α2α1E) (Fig 5D).

Parent cells, α2 and α2α1 transfectants were induced to undergo lineage commitment by growth in serum free (ITA) medium. Endocrine cell numbers were much higher in the wild-type α2 transfectants, α2B and α2F, than in the chimeric transfectants α2α1B and α2α1E which showed little endocrine lineage commitment (Figure 6A & 6B). α2F and α2α1E cells had the highest expression of α2 and α2α1 proteins respectively (Fig. 5B&C) and these colonies showed the most extreme phenotypes with α2F cells showing 10.5 fold higher endocrine lineage commitment than the parent cells while α2α1E cells show only 2% of parent endocrine cell lineage commitment. Immunofluorescence staining of HRA-19 monolayers for chromogranin shows differential endocrine lineage commitment (Fig 6B) between parent cells and transfectants. Phase contrast images are included to show that cells are present in the α2α1E monolayers but endocrine lineage commitment is negligible. α2F cells contain many typical chromogranin positive endocrine cells with long processes (Fig 6B-white arrow).

To further investigate the lineage commitment program of the transfectants, we examined the ability of transfectants to generate mucous cells when transferred to serum free medium (ITA). Again we found that α2F cell monolayers contained 9.8 fold parent cell mucous cell numbers whilst α2α1E cells contained only 4% of parent cell mucous numbers (Fig.6C). These results strongly suggest that α2 integrin regulates colorectal epithelial cell fate by a mechanism requiring signalling via the α2 cytoplasmic tail.

α2 integrin regulates Caco-2 enterocytic differentiation- To examine the wider significance of α2 integrin mediated effects in human colon cancer cells, enterocytic differentiation was investigated in the well differentiated Caco-2 cell line. Caco-2 cells were shown to express the enterocytic differentiation marker, alkaline phosphatase when grown for several days in serum free medium (TSG). Growth of cells on surfaces coated with an α2 integrin antibody increased cell proliferation (Fig 7A) and reduced alkaline phosphatase expression (Fig 7B). These results show that α2 integrin regulates differentiation in other colorectal carcinoma cells and can modulate enterocytic as well as endocrine and mucous lineage commitment.
α2 integrin regulates stem cell behaviour via the ERK signalling pathway.

The Extracellular-signal-regulated kinase (ERK MAPK) signalling pathway is important in intestinal epithelial differentiation (39,40) and it’s dysregulation in colorectal cancer is thought to play a part in progression of this disease (41). The MEK inhibitor PD98059, which blocks ERK signalling, was used to determine whether α2 integrin regulation of lineage commitment was mediated via this signalling pathway. PD98059 abrogated the α2 integrin mediated reduction in endocrine lineage commitment in HRA-19 cells (Fig 8A) and enterocytic differentiation in Caco-2 cells (Fig 8B) without a change in cell number (Fig. 8A).

DISCUSSION

The β1 integrin family of cell surface extracellular matrix receptors are known stem cell regulators but their role in intestinal epithelial stem cell fate has yet to be established. To define the role of β1 integrins in cell fate decisions in multipotent human colorectal cancer cells, we induced lineage commitment in the presence of β1 integrin function blocking antibodies. Endocrine and mucous lineage commitment were inhibited in the presence of β1 integrin Ab JB1A which blocks β1 integrin mediated adhesion and signalling (34). No change in morphology or cell adhesion was observed during antibody treatment suggesting that the effects were on intracellular signalling rather than cell adhesion. Conditional knock-out of β1 integrin in adult mouse intestine results in enhanced proliferation and decreased differentiation suggesting perturbation of stem cell behaviour(23). Somewhat surprisingly, β1 integrin knock-out did not appear to modulate intestinal cell adhesion suggesting that a signalling, rather than an adhesive, function of β1 integrin was involved in specifying stem cell fate. Likewise, in this study, β1 integrin antibodies did not change cell morphology or perturb cell adhesion but markedly inhibited the ability of cells to undergo endocrine or mucous lineage commitment suggesting that β1 integrin signalling is also involved in regulating the balance between cell renewal and lineage commitment in human colorectal cancer cells. These function blocking experiments suggested a role for β1 integrin in regulating cell fate however β1 integrin partners with one of at least 12 alpha integrin chains to form matrix specific heterodimers. Therefore we sought to establish whether the observed effects of β1 integrin blockade were due to modulation of a specific αβ1 heterodimer(s). Endocrine lineage commitment was induced in HRA-19 cells in the presence of function blocking antibodies to α integrin chains known to associate with β1 integrin. We show that a function blocking antibody to the α2 integrin chain specifically and efficiently blocked endocrine lineage commitment by HRA-19 cells. As α2 integrin is only known to associate with β1 integrin, this finding suggests that α2β1 integrin is a regulator of stem cell fate. α2 integrin MAb and β1 integrin Mab gave similar blockade of endocrine lineage commitment suggesting that α2β1 integrin is the sole member of the β1 integrin family involved in cell fate determination. Our results support the lack of involvement of β1 integrins: α1β1, α4β1, α5β1 and αvβ1.

We next investigated α2β1 integrin expression in HRA-19 cells and showed α2 and β1 integrin expression by immunoblotting. Surface biotinylation following by immunoprecipitation demonstrated that α2β1 integrin is present on the HRA-19 cell surface and is the major β1 integrin heterodimer. Adhesion assays confirmed that α2β1 integrin was a collagen receptor mediating HRA-19 binding to Collagen I and Collagen IV.

To provide further evidence for a role of α2 integrin in specifying colorectal cancer stem cell fate and gain some mechanistic insight, multipotent colorectal cancer cells with permanent modifications to α2 integrin function were derived. Endocrine and mucous lineage commitment of colorectal cancer cells expressing highly elevated levels of wild-type α2 integrin were compared to parent cells and also cells expressing a non-signalling chimeric α2 integrin. This chimeric α2α1 integrin comprised the extracellular and transmembrane domain of the α2 chain but the cytoplasmic domain, crucial for α2 mediated cell signalling (42,43), was replaced with that from the α1 chain. α1β1 integrin (another collagen receptor) did not appear to be endogenously expressed by HRA-19 cells as cell adhesion to collagen could not be blocked by antibodies to α1 integrin. Furthermore α1 integrin Mab did not modulate lineage commitment in these cells. HRA-19 cells expressing high levels of wild-type α2 integrin demonstrated a marked
increase in both endocrine and mucous lineage commitment under serum free conditions while cells expressing the chimeric protein showed a general failure to execute the colorectal lineage commitment program. These results suggest that α2β1 integrin regulates cell fate in human colorectal epithelial cells via a mechanism requiring the α2 cytoplasmic tail. Elevated α2β1 integrin expression is found on epidermal (16) and prostate stem cells (17). In the intestine, α2 integrin is expressed in the stem/progenitor cell zone and down regulated during normal differentiation(22) suggesting a possible role for α2β1 in stem cell renewal and lineage commitment. β1 integrin is a known stem cell regulator in a variety of stem cells, however the question of which β1 integrin heterodimer(s) is involved has not yet been addressed. Our data raises the possibility that α2β1 integrin is the β1 heterodimer involved in regulating other stem cell types. Elevated α2β1 integrin expression is found on epidermal (16) and prostatic stem cells (17) while collagen, an α2β1 integrin ligand, blocks differentiation of mouse embryonic stem cells (44). Furthermore rare prostate cancer stem cells with self-renewal and differentiation potential have been isolated on the basis of a CD44+, α2β1 integrin+, CD133+ phenotype (45), suggesting shared characteristics between normal and neoplastic prostate epithelial stem cells.

To examine whether α2 integrin signalling was involved more widely in the differentiation of human colorectal cancer cells we investigated the well characterised cell line, Caco-2. Blockade of α2 integrin signalling in Caco-2 cells with function blocking antibody was shown to promote proliferation and inhibit differentiation, again supporting a role for α2β1 integrin in balancing cell-renewal and differentiation. Previous studies have linked α2 integrin function with the ERK signalling pathway (46) in human colon cancer cells. Furthermore normal intestinal stem cells express the MAPK family member ERK1 (47) while loss of ERK activation accompanies intestinal epithelial differentiation in vitro(40). This suggests a role for ERK signalling in maintaining self-renewal in intestinal epithelial stem cells. To determine whether α2β1 integrin mediated effects required ERK signalling we used the MEK signalling inhibitor PD98059, which abrogated the ability of α2 integrin antibodies to block endocrine lineage commitment in HRA-19 cells and enterocytic differentiation in Caco-2 cells. These preliminary results suggest that α2 integrin regulates ERK signalling, but further experiments will be required to confirm this possibility and identify other cell signalling pathways triggered by α2β1 integrin.

Several studies have suggested a link between α2β1 integrin and the development of human cancer. A functional association exists between α2β1 integrin and the EGF receptor (48,49), a kinase whose aberrant signalling is associated with many cancer types including colorectal cancer where anti-EGFR reagents are under investigation as potential therapeutic agents (50). In addition, α2β1 integrin has been implicated as a promoter of malignant phenotype in pancreatic cancer cells (51) and metastasis to bone (52). Finally, it is intriguing that E-cadherin, a tumour suppressor, is found to be a ligand for α2β1 integrin(53). The functional significance of this finding remains uncertain but E-cadherin-α2β1 integrin interaction could be involved in the modulation of Wnt signalling as E-cadherin also binds β-catenin, a pivotal protein in this pathway.

Evidence is accumulating to support the idea that human colorectal cancer is a stem cell disease. Cancer stem cells are thought to initiate tumour growth and generate heterogeneity within tumour cell populations which suggests that successful therapy will depend upon elimination of cancer stem cells. However many questions remain about the role that cancer stem cells play in cancer development (54) and much remains unknown about the molecular mechanisms which balance self-renewal and lineage commitment in normal and neoplastic colorectal epithelial cells.

Our study indicates that α2 integrin regulates cell fate in cloned multipotent human colorectal cancer cells (HRA-19) probably via α2β1 integrin signalling. Previous studies support a role for β1 integrins as stem cell regulators in normal intestinal epithelium, suggesting that colorectal cancer cells retain elements of integrin-regulated cell fate decisions. Identification of the molecular mechanisms that regulate colorectal epithelial cell fate may explain the diminished differentiation that is the hallmark of colorectal cancer and suggest new therapeutic strategies.
REFERENCES

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Figure Legends

Figure 1. β1 integrins regulate endocrine and mucous lineage commitment by HRA-19 cells

(A) HRA-19 cells were seeded into 8 chamber plastic slides in serum free medium with varying dilutions of β1 integrin mAbs JB1A or LM534. On Day 5 cells were fixed and stained for the endocrine lineage marker chromogranin using immunocytochemistry. Data shown are mean ± S.D. (n=7). ** P<0.001. Results are representative of three independent experiments.

(B) HRA-19 cells were grown in 8 chamber slides for 3 days and then transferred to serum free medium. On Day 7, monolayers were stained with the colonic mucous antibody, PR4D4 using immunocytochemistry. Data shown are mean±S.D. (n=4) **, P<0.001. Cell number was determined in replicate wells using WST1 reagent (Absorbance 450/620nm) (n=4) Results are representative of three independent experiments.

Figure 2. α2 integrin regulates endocrine lineage commitment in HRA-19 cells

(A) HRA-19 cells were seeded into 8 chamber slides in serum free medium with and without antibodies to α2 (P1E6), α4 (P1H4), α5 (P1D6) and αv (P3G8) integrin, all at 500ng/ml. Experiments were also attempted with α6 antibody (NKI-GoH3) but cell attachment was severely affected and therefore data could not be collected. Data shown are mean±S.D. (n=3). ** P<0.0001. Results are representative of a series of experiments performed with control and antibody treated cells: α2 mAb P1E6 (5 independent experiments), α4 mAb P1H4 (3 independent experiments), α5 mAb P1D6 (2 independent experiments), αv mAb P3G8 (3 independent experiments).

(B) Cells were seeded into 8 chamber slides in the presence of differing doses of antibodies to α1 integrin (FB12) or α2 integrin (P1E6). Data are mean±S.D. (n=4). ** P<0.0001. This experiment was representative of 2 independent experiments. Values presented as % control for comparison.
Figure 3. Integrin expression in HRA-19, human colorectal cancer cells.
Western blot analysis of β1 (A) and α2 (B) integrin expression in lysates of HRA-19 cells. (C) Surface expression of α2β1 integrin in HRA-19 cells, demonstrated by biotinylation, lysis and immunoprecipitation with antibodies to β1, α2 and α2β1 integrin. Biotinylated proteins were detected with Streptavidin-HRP.

Figure 4. α2β1 integrin is a collagen receptor in HRA-19, human colorectal cancer cells.
Equal numbers of HRA-19 cells were seeded into wells coated with either Human Collagen I or IV and allowed to attach for 2 hours at 37°C. Attached cell number was measured using crystal violet staining and measurement at 595nm: α1 2 mg/ml α2 0.37 mg/ml; α3 1.25 mg/ml; α5 2 mg/ml; α6 0.25 mg/ml; β1 2253 5 mg/ml. Data are mean ± S.D. (n=3). ** P<0.001 * P<0.005. Results are representative of a series of independent experiments performed on Collagen I and Collagen IV always including control wells and a range of antibodies. α1 (2 experiments), α2 (4 experiments), α3 (3 experiments), α5 (3 experiments), α6 (2 experiments), β1 (5 experiments).

Figure 5. Expression of α2 integrin constructs in HRA-19, human colorectal cancer cells
(A) Wild-type α2 and chimeric α2α1 integrin constructs transfected into HRA-19 cells. (B) α2 integrin expression in α2 and α2α1 transfectants and HRA-19 cells. Experiment performed twice (C) α2α1 integrin expression. α2α1 integrin was immunoprecipitated using an α1 cytoplasmic domain antibody then detected using western blot with an α2 extracellular domain antibody. Chimeric protein band was found only in α2α1 transfected colonies, α2α1B and α2α1E. Experiment performed 5 times. (D) α2 integrin localisation was examined by immunofluorescence in α2F, HRA-19 and α2α1E cells. Bar =100 µm.

Figure 6. α2 integrin regulates colorectal epithelial stem cell fate.
(A) Endocrine lineage commitment (chromogranin expression) in cells after 48 hours in serum free medium. Mean ± S.D. Experiment was performed 3 times. Endocrine and mucous cell numbers were normalized to an Absorbance of 1 obtained with the WST-1 cell proliferation reagent to eliminate variation in cell number. Cells used were α2 transfected colonies α2B and α2F, chimeric α2α1 transfected colonies α2α1B and α2α1E and the parent non-transfected cell line HRA-19. (B) Chromogranin expression in α2F, α2α1E cells and HRA-19 cells after 48 hours in serum free medium. Images obtained using a confocal microscope. White arrow shows typical endocrine cell with long process. Phase contrast images of the same fields. Bar = 100 µm. (C) Mucous lineage commitment in parent and transfected cell colonies detected with mucous antibody PR4D4 after 72 hours in serum free medium. Mean ± S.D. Experiment was performed 3 times.

Figure 7. α2 integrin regulates enterocytic differentiation of Caco-2 cells
Caco-2 cells were seeded into control wells or wells coated with either α2 integrin Ab (AK7) or MsIgG (both 5µg/ml) in TSG medium. (A) Cell number after 72 hours in TSG medium measured with WST-1 reagent (B) Alkaline phosphatase expression after 72 hours in TSG medium normalised using WST-1 values. Quadruplicate wells. Values shown are mean ± S.D. **P<0.001 *P<0.005. Experiment was performed 4 times.

Figure 8. α2 integrin effects are mediated via the ERK signalling pathway.
(A) HRA-19 cells were transferred to ITA medium with or without the α2 integrin antibody P1E6 (25ng/ml) and the MEK inhibitor PD98059 (10µM) or DMSO control. Chromogranin positive cells were
detected by immunocytochemistry. Experiment performed 3 times. Quadruplicate chambers were used for each condition. Mean± S.D. *P<0.005. Cell number was determined in replicate wells using the WST1 reagent (Absorbance 450/620nm).

(B) Caco-2 cells were plated in TSG medium onto surfaces coated with either α2 integrin MAb AK7 or MsIgG (10μg/ml) with the MEK inhibitor PD98059 (10μM) or DMSO control. Alkaline phosphatase expression (Abs 405nm) was normalised for cell number with the WST-1 reagent (Abs 450nm/Abs 620nm). Quadruplicate wells were used for each condition Mean ± S.D. Experiment performed 4 times. **P<0.001.
Figure 1

A

Endocrine cells/chamber

% Antibody

JB1A

LM534

B

Mucous cells/chamber

Mucous cells

Absorbance 450/620nm

Total cells

Antibody Concentration (ng/ml)

% Antibody

MslIgG1

JB1A
Figure 2

A

Endocrine cells (%control)

Control  α2  α4  α5  αv

B

Endocrine cells (%control)

Antibody Concentration (ng/ml)

0  50  100  200

α2  α1
Figure 3

A  WB  β1  116

B  WB  α2  116

C  IP  β1  α2  α2β1  116
Figure 4

![Graph showing absorbance at 595nm for Col I and Col IV with different control and treatment labels.]

**Absorbance 595nm**
Figure 5

A

\[ \alpha_2 \text{ construct} \]

\[ \alpha_2 \]

\[ \alpha_2 \]

\[ \text{GFFKRKYEKMTKNPDEIDETTELSS} \]

\[ \alpha_2 \alpha_1 \text{ construct} \]

\[ \alpha_2 \]

\[ \alpha_1 \]

\[ \text{GFFKRPLKKMEK} \]

B

\| \alpha_2 \alpha_2 \alpha_1 \|

\| B \ F \ HRA-19 \| B \ E \|

150

\text{\alpha_2 \ integrin}

50

\text{\beta-actin}

37

C

\| \alpha_2 \alpha_2 \alpha_1 \|

\| B \ F \ HRA-19 \| B \ E \|

D

\[ \alpha_2F \]

\[ \text{HRA-19} \]

\[ \alpha_2\alpha_1E \]
### Figure 6

#### A

<table>
<thead>
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<th>F</th>
<th>E</th>
<th>HRA-19</th>
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<tr>
<td><code>α2α1</code> integrin</td>
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- **Endocrine Cells**
  - Chromogranin
  - Phase-contrast

#### B

- **α2F**
- **α2α1**
- **HRA-19**

#### C

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</table>
Figure 7

(A) Cell Number

(B) Alkaline Phosphatase

Absorbance 450/620nm

Absorbance 405/ WST Abs

Control  α2 integrin MAb  MsIgG
Figure 8

A

Endocrine cells/chamber

+PD98059

Total cells

Abs. 450/620nm

Control  α2 Ab  α2 Ab +PD98059

B

Alkaline Phosphatase

Abs. 405/ WST1 Abs

Control  α2 Ab  α2 Ab +PD98059  MslIgG
Alpha2 Beta1 integrin regulates lineage commitment in multipotent human colorectal cancer cells
Susan C. Kirkland and Huijun Ying

J. Biol. Chem. published online July 29, 2008

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