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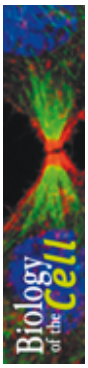
Research article



Integrin α8β1 regulates adhesion, migration and proliferation of human intestinal crypt cells via a predominant RhoA/ROCK-dependent mechanism

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Background. Integrins are transmembrane αβ heterodimer receptors that function as structural and functional bridges between the cytoskeleton and ECM (extracellular matrix) molecules. The RGD (arginine-glycine-aspartate tripeptide motif)-dependent integrin α8β1 has been shown to be involved in various cell functions in neuronal and mesenchymal-derived cell types. Its role in epithelial cells remains unknown.

Results. Integrin α8β1 was found to be expressed in the crypt cell population of the human intestine but was absent from differentiating and mature epithelial cells of the villus. The function of α8β1 in epithelial crypt cells was investigated at the cellular level using normal HIECs (human intestinal epithelial cells). Specific knockdown of α8 subunit expression using an shRNA (small-hairpin RNA) approach showed that α8β1 plays important roles in RGD-dependent cell adhesion, migration and proliferation via a RhoA/ROCK (Rho-associated kinase)-dependent mechanism as demonstrated by active RhoA quantification and pharmacological inhibition of ROCK. Moreover, loss of α8β1, through RhoA/ROCK, impairs FA (focal adhesion) complex integrity as demonstrated by faulty vinculin recruitment.

Conclusions. Integrin α8β1 is expressed in epithelial cells. In intestinal crypt cells, α8β1 is closely involved in the regulation of adhesion, migration and cell proliferation via a predominant RhoA/ROCK-dependent mechanism. These results suggest an important role for this integrin in intestinal crypt cell homoeostasis.

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Key words: cell migration, cell proliferation, crypt cell, integrin, RhoA.

Abbreviations used: BrdU, bromodeoxyuridine; Cdc42, cell division cycle 42; CNS, control non-silencing; DAPI, 4′,6-diamidino-2-phenylindole; DPPIV, dipeptidyl peptidase IV; ECM, extracellular matrix; EV, empty vector; FA, focal adhesion; FX, focal complex; GST, glutathione transferase; HIEC, human intestinal epithelial (cells); LSC, laser scanning cytometry; PFA, paraformaldehyde; RGD, arginine-glycine-aspartate tripeptide motif; RNAi, RNA interference; ROCK, Rho-associated kinase; RPLP0, ribosomal protein, large, P0; RT–PCR, reverse transcription–PCR; shRNA, small-hairpin RNA; TNfn3, third type-III fibronectin repeat of tenascin-C; TRITC, tetramethylrhodamine β-isothiocyanate; VSMC, vascular smooth muscle cell.

Introduction

Integrins are a superfamily of transmembrane receptors that form heterodimers of non-covalently associated α- and β-subunits. In mammals, more than 24 integrin receptors have been reported after the identification of 18 α- and nine β-subunits. Integrin receptors act as structural and functional bridges between the cytoskeleton and ECM (extracellular matrix) components and mediate intracellular

signal transduction (Clark and Brugge, 1995; Bershadsky et al., 2006). Several integrins are present in epithelia throughout the organism, such as $\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$ and $\alpha6\beta4$. The presence of these integrins at the surface of epithelial cells allows recognition and interaction with specific ligands including laminins, collagens and other glycoproteins (Mercurio, 1995; Sheppard, 1996; Beaulieu, 1997). The integrin-dependent connection between the ECM and the cytoplasm promotes the regulation of cell functions such as adhesion, migration, proliferation, apoptosis and differentiation (Giancotti, 1997; Reddig and Juliano, 2005; Lock et al., 2008).

The intestinal epithelium is a valuable system for understanding the relationship between cell state and interactions with the ECM. The functional unit of the small intestinal epithelium is organized as two distinct compartments, the crypts and villi, where proliferative and differentiated epithelial cell populations are located respectively (Arsenault and Menard, 1989; Ménard and Beaulieu, 1994; Babyatsky and Podolsky, 1999). The crypt–villus axis ensures the constant renewal of the epithelial barrier via a tight equilibrium between cell growth, migration and differentiation of mature enterocytes and apoptosis (Babyatsky and Podolsky, 1999; Ménard et al., 2006). In this context, these cell functions are susceptible to regulation by specific interactions between integrins and ECM components (Beaulieu, 1997, 1999; Zargham et al., 2007a). Besides the laminin- and collagen-binding integrins common to most epithelial cells, intestinal epithelial cells have been found to express most other members of the $\beta1$ integrin repertoire at one time or another. For example, integrin $\alpha9\beta1$, which can bind tenascin-C (Yokosaki et al., 1998) and a cryptic domain of osteopontin (Smith et al., 1996), is transiently expressed in epithelial cells of the intestinal crypts in an EGF (epidermal growth factor)-dependent manner (Desloges et al., 1998) and is re-expressed in colon cancer cells (Basora et al., 1997), whereas expression of the fibronectin receptor $\alpha5\beta1$ and the αv-containing integrin(s) was found to be related to intestinal cell proliferation (Beaulieu et al., 1992; Vachon et al., 1995; Zhang et al., 2003).

Integrin $\alpha8\beta1$ was first identified in the chick neuronal system prior to the characterization of its human counterpart (Bossy et al., 1991; Schnapp et al., 1995a, 1995b). So far, $\alpha8\beta1$ has been shown to be associated with FA (focal adhesion) points where it participates in the regulation of spreading, adhesion, growth and survival in different neuronal and mesenchymal-derived cell types (Muller et al., 1995; Schnapp et al., 1995b; Levine et al., 2000; Bieritz et al., 2003; Wagner et al., 2003; Farias et al., 2005; Zargham and Thibault, 2005; Zargham et al., 2007a). Integrin $\alpha8\beta1$ has been shown previously to modulate actin stress fibre assembly (Zargham and Thibault, 2006) and to promote RhoA GTPase activation in VSMCs (vascular smooth muscle cells) (Zargham et al., 2007a, 2007b). However, the existence of an analogous mechanism in an epithelial context has not yet been reported.

In the present study, we report that $\alpha8\beta1$ is expressed in the crypt cell population of the human small intestine where it represents one of the major RGD (arginine-glycine-aspartate tripeptide motif)-binding integrins and is a central RhoA/ROCK (Rho-associated kinase)-dependent modulator of cell migration and proliferation, two fundamental functions of intestinal crypt cells.

Results

Expression of the integrin α8 subunit in human intestinal epithelial cells

Previous analyses of gene expression profiles in intestinal cells failed to identify integrin subunits that displayed statistically significant levels of differential expression between proliferating and differentiating cells (Tremblay et al., 2006). Rescreening of these experimental models by RT–PCR (reverse transcription–PCR) revealed $\alpha8$ to be a strongly differentially expressed α-subunit. The $\alpha8$-subunit was not expressed by differentiating villus-like Caco-2/15 cells but was present in undifferentiated crypt-like HIEC (human intestinal epithelial cells) at the transcript (Figure 1A) and protein levels (Figure 1B). To emphasize a potential relationship between $\alpha8\beta1$ expression and the undifferentiated phenotype, we analysed $\alpha8$ expression in HIEC cells overexpressing GATA-4, a transcription factor well known for its ability to trigger enterocytic differentiation. Induction of the differentiation marker DPPIV (dipeptidyl peptidase IV) in HIEC/GATA-4 cells was accompanied by a dramatic decrease in the $\alpha8$ subunit level (Figure 1C), suggesting that $\alpha8\beta1$ integrin expression was restricted to undifferentiated progenitor epithelial cells. To further study this

Figure 1 | Expression of the integrin α8 subunit in intestinal cells is lost with differentiation

(**A**) Representative RT–PCR, indicating strong expression of the α8 subunit (Int α8) transcript in the progenitor HIEC cells and its absence in differentiating Caco-2/15 cells at various stages of confluence (0, +5 and +10 days post-confluence) as monitored by sucrase-isomaltase (SI) expression. (**B**) Confirmation of α8 expression in HIEC at the protein level was made by Western-blot analysis. (**C**) Representative RT–PCR of α8 subunit transcript expression in stable HIEC cell lines with and without ectopic expression of GATA-4. RPLP0 was used as a normalizing gene and DPPIV as a differentiation marker.

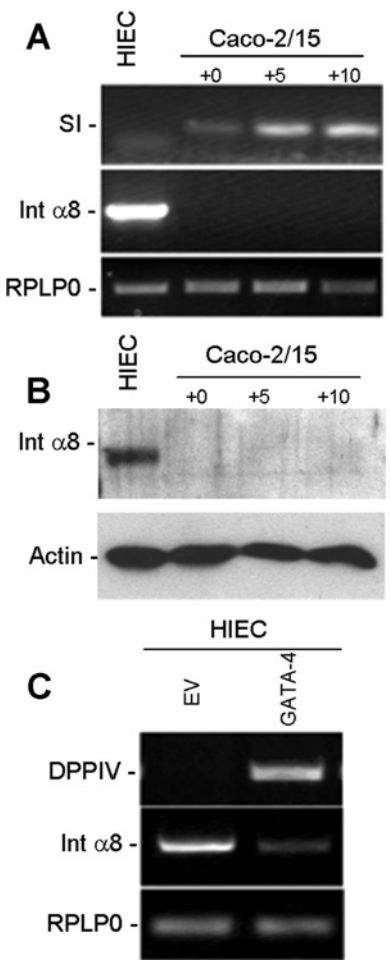
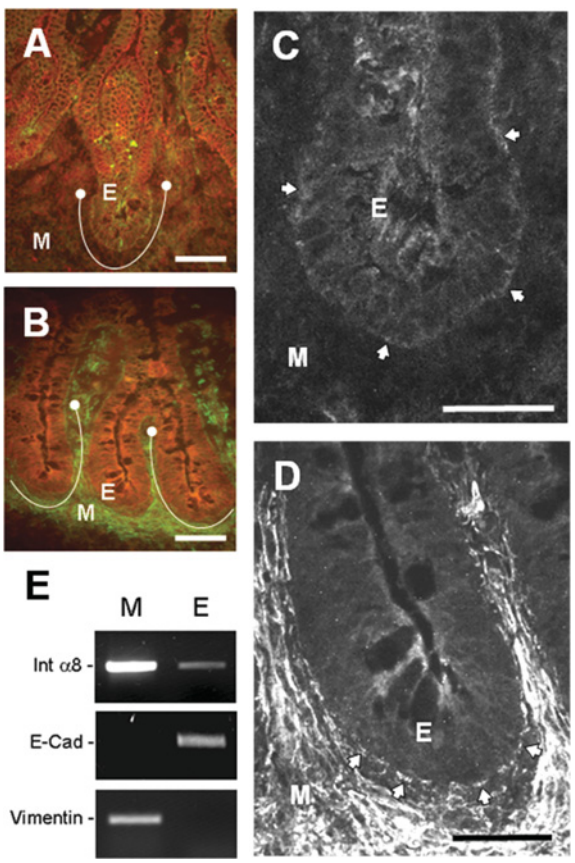


Figure 2 | Expression and distribution of the α8 integrin subunit in the human small intestine

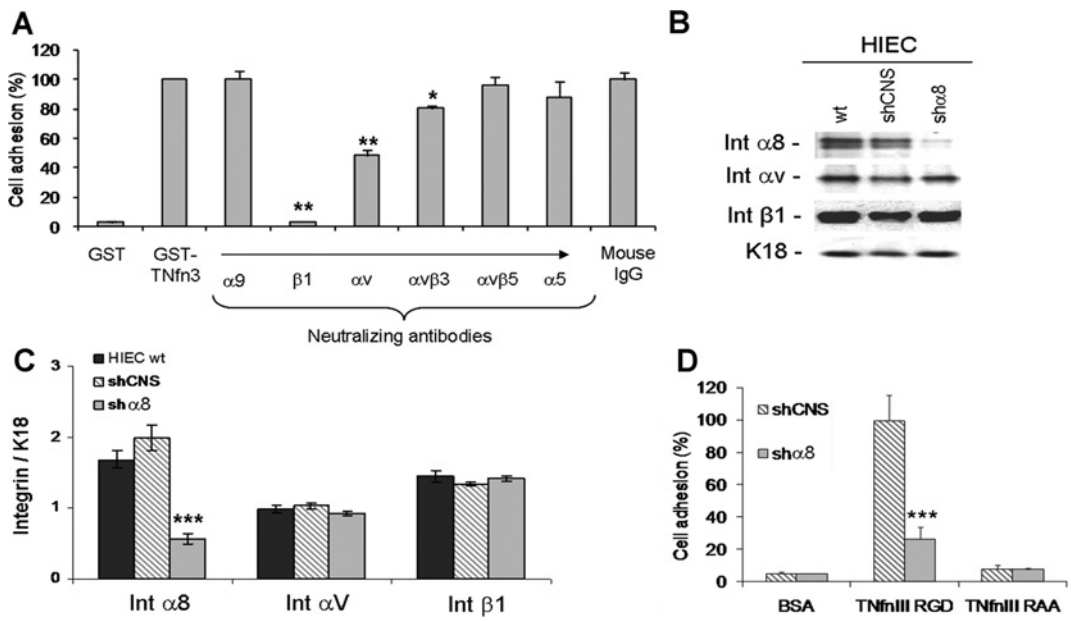
Representative immunofluorescence micrographs of human jejunum cryosections at 14 weeks (**A**, **C**) and 20 weeks of gestation (**B**, **D**) stained with an anti-α8 integrin subunit antibody. In both stages, the α8 subunit was detected in the crypt regions (defined by the brackets in **A** and **B**). Higher magnifications of the crypt regions showed that the α8 subunit was present at the basolateral surface (arrows) of epithelial crypt cells (E) at both 14 and 20 weeks (**C**, **D**). Weak staining was observed in the mesenchyme (M) at 14 weeks, whereas at 20 weeks, specimens show more prominent staining with the differentiation of smooth muscle cells. Scale bars: (**A**, **B**) 50 μm and (**C**, **D**) 25 μm. (**E**) RT–PCR experiments confirm the presence of the α8 transcript (Int α8) in the 18–20 week jejunal epithelium accompanied by strong expression in the mesenchyme. Epithelial (E) and mesenchymal (M) fraction purity was validated by the absence of vimentin and E-cadherin (E-Cad) respectively.



correlation, we analysed α8 subunit expression *in vivo* on 14- and 20-week human fetal jejunum specimens by indirect immunofluorescence. In 14-week specimens, the α8 subunit was detected at the base of the proliferative epithelial cells located in the intervillous area (Figures 2A and 2C). At 20 weeks of gestation, the α8 subunit persisted at the base of the proliferative epithelial cell population located in the newly formed crypts, and strong expression of α8 was now also present in the stromal cell population

Figure 3 | Integrin α8β1 and RGD-dependent adhesion of human intestinal crypt cells

(**A**) Short-term (1 h) adhesion assays of HIEC cells plated on GST or RGD-containing GST–TNfn3 coating. Cells were treated or untreated with neutralizing antibodies directed against RGD-binding integrins expressed by HIEC. Mouse IgG was used as a negative control ($n \geqslant 3$; $^{*}P < 0.05$; $^{**}P < 0.01$, compared with mouse IgG). (**B**) Assessment of α8 (Int α8), αv (Int αv) and β1 (Int β1) protein expression levels in wt (wild-type) HIEC and stable cell populations of HIEC/shCNS and HIEC/shα8 by Western blotting. (**C**) Relative amounts of α8, αv and β1 were detected for each group according to the loading control keratin 18 (K18) ($n = 3$; $^{***}P < 0.0001$). (**D**) Long-term (24 h) adhesion assays with HIEC/shCNS and HIEC/shα8 cells plated on GST, RGD-containing GST–TNfn3 or RAA-mutated GST–TNfn3 coating. Cycloheximide (10 μg/ml) was added to the adhesion medium to avoid endogenous fibronectin secretion/deposition ($n = 4$; $^{***}P < 0.0001$).



(Figures 2B and 2D). Integrin α8β1 expression in human fetal intestinal epithelial cells was further confirmed by RT–PCR of pure epithelial and mesenchymal fractions isolated from 18–20-week jejunum specimens. As expected, α8 mRNA was readily detected in the mesenchyme, as well as in the epithelial fraction (Figure 2E). Fraction purity was verified by assessing the presence of specific markers for epithelium and mesenchyme, E-cadherin and vimentin respectively (Figure 2E). Taken together, these results showed that integrin α8β1 was specifically expressed in intestinal crypt cells. We then investigated the role of this integrin using HIEC cells.

Impact of integrin α8β1 on RGD-dependent cell adhesion

Adhesion assays were performed on a GST (glutathione transferase) fusion peptide containing the TNfn3 (third type-III fibronectin repeat of tenascin-C), which possesses a functional RGD-binding domain for α8β1. Wild-type HIEC cells adhered efficiently to this RGD-containing domain (Figure 3A), and adhesion was completely abolished in the presence of a soluble RGD-containing peptide (results not shown) or by blocking the β1 integrin subunit. The addition of a neutralizing anti-αv antibody reduced adhesion by approx. 50% and neutralizing antibodies directed against the α5 or α9 subunits did not significantly alter this RGD-dependent adhesion (Figure 3A). By elimination, these results suggested the involvement of an additional RGD-dependent β1 integrin, such as α8β1, but we were unable to test this using this assay as no anti-α8 neutralizing antibody is yet available. We therefore opted for the establishment of α8-knockdown and CNS (control non-silencing) stable HIEC cell lines using shRNA (small-hairpin RNA) technology. By Western blotting,

we confirmed that this strategy caused a ~70% reduction in α8 subunit expression in HIEC/shα8 compared with wild-type HIEC and HIEC/shCNS cells (Figures 3B and 3C). In addition, we confirmed that the expression levels of integrin subunits αv and β1 in the three different cell lines were not affected (Figures 3B and 3C). Using these shRNA cell lines we performed adhesion assays on the RGD-containing TNfn3 matrix, and showed a ~70% decrease in the adhesion of HIEC/shα8 cells compared with control HIEC/shCNS cells (Figure 3D), confirming an important contribution of the α8β1 integrin to RGD-dependent adhesion of intestinal cells. Adhesion on the inactive RAA (arginine-alanine-alanine)-mutated TNfn3 matrix showed only negligible amounts of adherent cells, comparable with assays on control GST-coated plates, for both groups.

Effect of α8 knockdown on focal contact and stress fibre organization

Since integrin α8β1 appeared to be important for the adhesion of epithelial crypt cells, we investigated the impact of α8β1 depletion on focal contact and actin cytoskeleton organization. Control and HIEC/shα8 cells were seeded on serum-coated glass coverslips and analysed by double-labelling immunofluorescence using an anti-vinculin antibody to detect focal contacts and phalloidin to stain actin microfilaments. Knockdown of α8 expression in HIEC led to a reorganization of the actin network, from transverse and parallel stress fibres to a cortical localization (Figures 4A and 4B). Moreover, we observed a significant decrease in the number of vinculin-positive FA points (Figure 4C) and a reduction in spreading in HIEC/shα8 cells, compared with control cells (Figures 4A and 4B), without affecting the overall vinculin expression levels (Figure 4D).

Integrin α8β1 and RhoA GTPase activation

Considering the previously reported relationship between the integrin α8β1, actin network organization and small-GTPase RhoA membrane recruitment/activity (Zargham and Thibault, 2006; Zargham et al., 2007a, 2007b), we treated HIEC cells with the Rho kinase (ROCK 1/2) pharmacological inhibitor Y-27632. ROCK is the principal effector of RhoA GTPase and, once activated, it contributes to cell growth, stress fibre assembly and recruitment of FA components (Loirand et al., 2006). Treatment of wild-type HIEC with Y-27632 at 20 μM for 24 h (Lai et al., 2003) generated a similar phenotype to the HIEC/shα8 cells in terms of stress fibre assembly and vinculin distribution (Figures 5A and 5B). We evaluated RhoA activity in HIEC/shα8 cells by analysing the proportion of membrane-associated RhoA, which is indicative of the GTP-bound active conformation (Zargham et al., 2007a). A significant decrease in membrane-associated RhoA was observed in HIEC/shα8 compared with HIEC/shCNS cells, while the total amount of RhoA remained unchanged between the two groups (Figure 5C and Supplementary Figure S1A at http://www.biolcell.org/boc/101/boc1010695add.htm). We assayed RhoA activity directly by performing a RhoA–GTP pull-down assay using beads coupled with the rhotekin RhoA-binding domain (Cetin et al., 2007). HIEC/shα8 cells demonstrated a significant reduction in the amount of active RhoA compared with HIEC/shCNS (Figure 5D). No significant difference was observed in the total and membrane-associated amounts of Rac1 and Cdc42 (cell division cycle 42) GTPases in HIEC/shCNS and HIEC/shα8 cells (Supplementary Figures S1B and S1C). These observations suggested that integrin α8β1 was central to RhoA functionality in intestinal undifferentiated epithelial cells.

Knockdown of the integrin α8 subunit affects vinculin, but not paxillin, recruitment to FAs

To further characterize the effect of α8 silencing on FA formation we plated control HIEC and HIEC/shα8 on different substrata and quantified the number of vinculin- and paxillin-positive FAs that were generated (Table 1 and Supplementary Figure S2 at http://www.biolcell.org/boc/101/boc1010695add.htm). In wild-type HIEC and HIEC/shCNS cells plated on a serum coating, which provided the RGD-containing fibronectin and vitronectin, vinculin and paxillin displayed abundant (~70–80 per cell) and broad (mature) FAs in a sparse peripheral pattern (Table 1 and Supplementary Figures S2A and S2B). HIEC/shα8 cells plated on a serum coating resulted in a severe decrease in the number of vinculin-positive FAs, whereas paxillin staining was not affected (Table 1 and Supplementary Figure S2C). Similar observations were made for Y-27632-treated wild-type HIEC cells (Table 1 and

Table 1 | Quantification of vinculin- and paxillin-marked FAs

Results shown (means ± S.E.M.) are the number of vinculin- and paxillin-positive FAs per cell in wild-type (wt), shCNS-, shα8- and Y-27632-treated HIEC plated on to RGD-containing substratum and HIEC/shCNS on to type I collagen ($n \geqslant 8$).

	Number of FAs per cell				
	RGD-containing substratum				Collagen
	HIEC/wt	HIEC/shCNS	HIEC/shα8	HIEC/Y-27632	HIEC/shCNS
Vinculin	70.83 ± 2.24	78.90 ± 9.76	20.33 ± 1.19	15.13 ± 1.11	18.63 ± 2.00
Paxillin	69.75 ± 5.89	73.88 ± 4.38	77.25 ± 6.84	73.38 ± 12.27	76.00 ± 7.45

Figure 4 | Integrin α8β1 regulates focal contact and stress fibre organization

Representative micrographs of vinculin immunofluorescence (green) and TRITC–phalloidin staining of actin stress fibres (red) on HIEC/shCNS (**A**) and HIEC/shα8 (**B**) 24 h after seeding ($n = 4$; scale bars: 50 μm). (**C**) Quantification of vinculin-stained FA per cell in HIEC/shCNS and HIEC/shα8 ($n = 8$; $^{***}P < 0.0001$). (**D**) Vinculin expression levels in both cell populations were assayed by Western blot ($n = 3$).

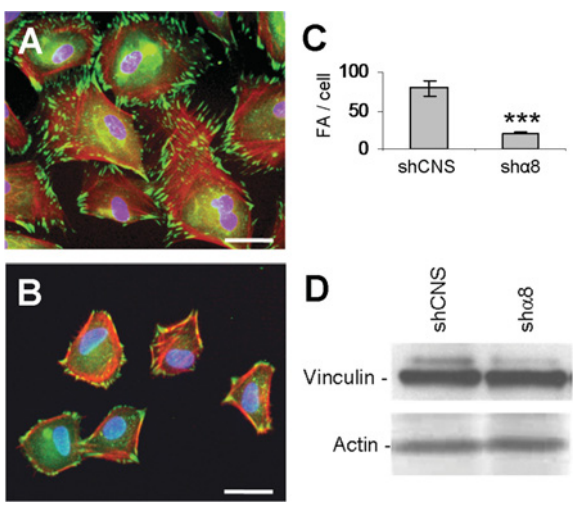
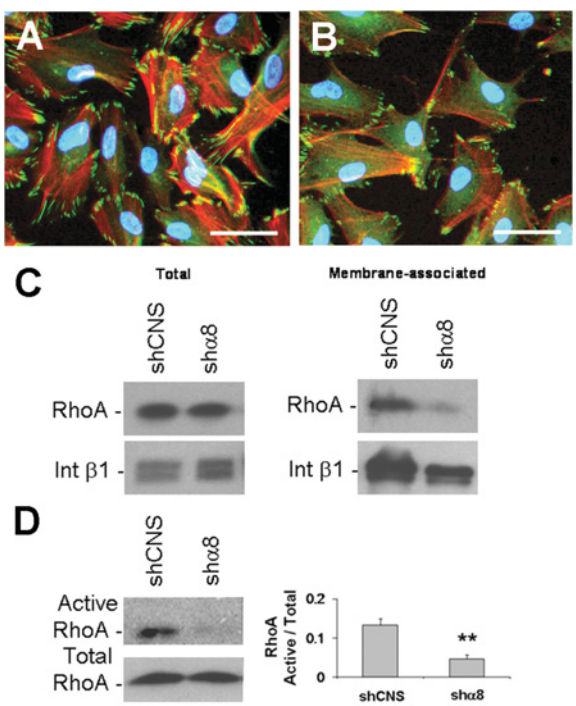


Figure 5 | Integrin α8β1 and RhoA GTPase activation in HIEC

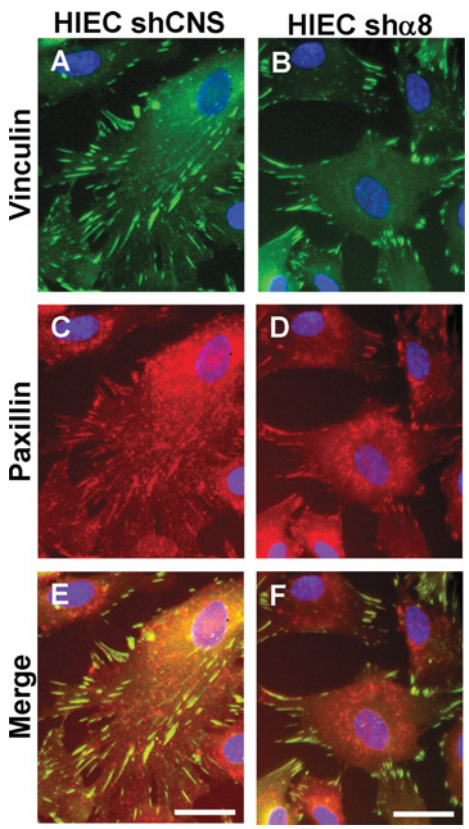
Representative micrograph of vinculin immunofluorescence (green) and TRITC–phalloidin staining of actin stress fibres (red) in normal HIEC (**A**) and Y-27632-treated (20 μM) HIEC (**B**) over a 24 h period after seeding ($n = 3$; scale bar: 50 μm). Western-blot analyses of RhoA in total and membrane fractionated protein extracts (**C**) from HIEC/shCNS and HIEC/shα8 cells ($n = 3$). (**D**) Active RhoA pull-down experiments using beads coupled with the rhotekin RhoA–binding domain with HIEC/shCNS and HIEC/shα8 ($n = 4$; $^{**}P = 0.005$). Int β1, integrin β1.



Supplementary Figure S2D), suggesting that recruitment of vinculin, but not paxillin, to the developing FA was RhoA/ROCK-dependent. Moreover, when HIEC/shCNS cells were seeded on a type-I collagen matrix, a non-RGD substratum, the number of vinculin-positive FAs was significantly reduced compared with paxillin-positive FAs (Table 1 and Supplementary Figure S2E), closely recreating the phenotype of α8-knockdown and Y-27632-treated wild-type HIEC cells. To further document differences in cellular localization between vinculin and paxillin after α8 silencing, co-immunostaining was performed. Similar localization patterns of vinculin and paxillin were observed in the broad mature FAs at the periphery of control cells, with a few paxillin-positive punctate spots detected in the central region (Figures 6A, 6C and 6E). HIEC/shα8 cells displayed paxillin-stained dots localized more centrally that

Figure 6 | Knockdown of the integrin α8 subunit affects vinculin localization but not paxillin in HIEC

(**A**—**D**) Representative micrographs of vinculin (green) (**A**, **B**) and paxillin (red) (**C**, **D**) immunofluorescence detection in HIEC/shCNS (**A**, **C**) and HIEC/shα8 cells (**B**, **D**) 24 h after seeding. (**E**, **F**) Merged images of vinculin and paxillin for HIEC/shCNS (**E**) and HIEC/shα8 (**F**) illustrate the differential cellular localization of these two proteins under α8-knockdown conditions ($n = 3$; scale bar: 25 μm).



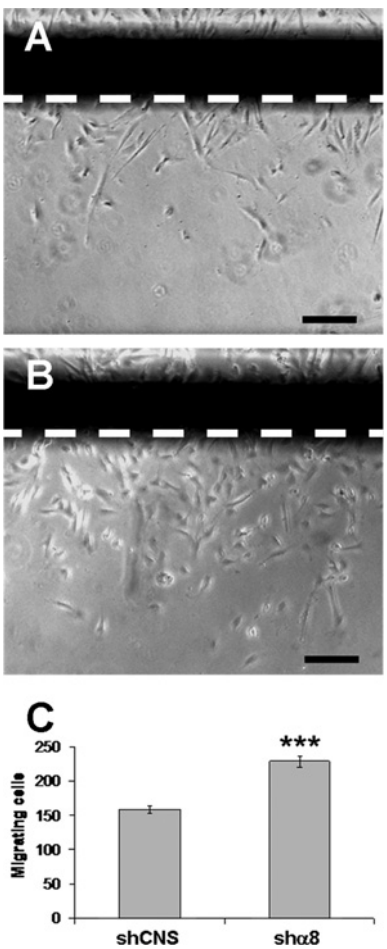
were apparently devoid of vinculin, whereas only 30% of paxillin-positive FAs were also vinculin-positive and were localized at the periphery of the cells (Figures 6B, 6D and 6F).

Reduced presence of integrin α8β1 up-regulates cell migration in the HIEC model

The functional role of RhoA in microfilament organization and its consequent impact on cell motility is well established. By performing wound assays (Figure 7), we demonstrated a 40% increase in the amount of cells that migrated across the wound edge in HIEC/shα8 cell monolayers compared

Figure 7 | Silencing of the α8 subunit increases migration in the HIEC cell line

Phase-contrast micrographs illustrating migrating HIEC/shCNS (**A**) and HIEC/shα8 cells (**B**) across the wound edge after 48 h of incubation in the presence of 2 mM hydroxyurea ($n = 5$; scale bar: 100 μm). (**C**) The histogram represents the number of cells that migrated across the wound edge ($n = 5$; $^{***}P < 0.0001$).



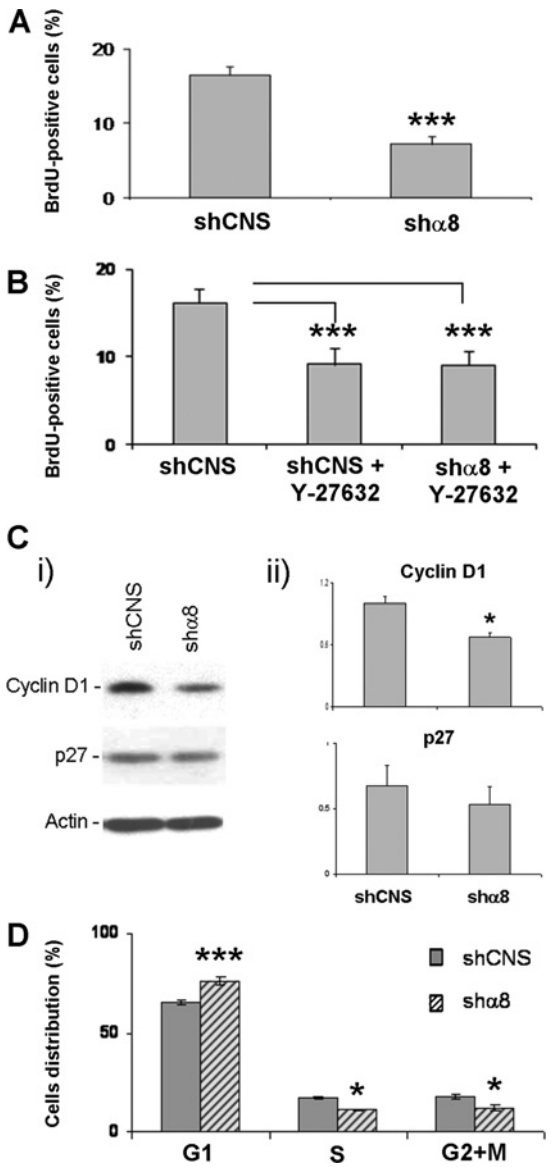
with HIEC/shCNS cells. In these experiments, cell proliferation was blocked by the addition of 2 mM hydroxyurea to the incubation medium. Thus our results confirmed that α8 silencing led to the up-regulation of intestinal epithelial HIEC cell migration.

Knockdown of the integrin α8 subunit impairs cell-cycle progression

Considering that the RhoA/ROCK pathway affects cell-cycle progression, we assessed the proliferation rate of HIEC/shCNS and HIEC/shα8 cells

Figure 8 | Integrin α8β1 and cell-cycle progression in the HIEC cell lines

Histograms illustrating the percentage of BrdU-labelled cells for (**A**) HIEC/shCNS compared with HIEC/shα8 and for (**B**) non-treated HIEC/shCNS compared with Y-27632-treated HIEC/shCNS and HIEC/shα8 (***$P < 0.0005$). (**C**) Quantification of the amounts of cyclin D1 and $p27^{Kip1}$ was performed in HIEC/shCNS and HIEC/shα8. (i) Western-blot analysis; (ii) the relative amounts of cyclin D1 and $p27^{Kip1}$ detected for each group according to the loading control actin ($n = 3$; *$P = 0.012$). (**D**) Histogram illustrating the percentage of cells distributed in each of G_1, S and G_2+M cell-cycle phases for HIEC/shCNS and HIEC/shα8 according to iCys LSC analysis ($n = 3$; *$P = 0.015$, ***$P = 0.0002$).



by BrdU (bromodeoxyuridine) incorporation experiments. These experiments demonstrated a decrease of ~50% in the number of BrdU-positive cells in HIEC/shα8 cultures compared with control HIEC cells (Figure 8A). Moreover, HIEC/shCNS cells treated with Y-27632 confirmed that ROCK inhibition in these control cultures resulted in a significant decrease in cell growth, comparable with that observed for α8-depleted cells (Figure 8B). Importantly, the treatment of HIEC/shα8 cells with Y-27632 had no additive effect on proliferation inhibition, suggesting that the integrin α8β1 stimulated proliferation via ROCK (Figure 8B). HIEC α8-depleted cells also exhibited a corresponding significant decrease in cyclin D1 expression levels, but no significant changes were noted for $p27^{Kip1}$ (Figure 8C), two cell-cycle regulators that have been reported to be potential downstream targets of the RhoA/ROCK pathway in various cell models (Croft and Olson, 2006). LSC (laser scanning cytometry) analysis of HIEC/shα8 and HIEC/shCNS populations showed that the decrease in cell growth found in HIEC/shα8 cells was associated with a significantly higher percentage of cells in G_1 phase (Figure 8D).

RNAi (RNA interference)-resistant α8 cDNA restores the wild-type phenotype in HIEC/shα8 cells

To confirm that the observed cell adhesion and proliferation decreases, as well as the cell migration increase in HIEC, were due specifically to the depletion of α8β1 and not because of non-specific off-target effects of the RNAi pathway, we generated an α8 subunit 'rescue' vector bearing three silent point mutations in the α8 cDNA that was resistant to RNAi-mediated knockdown. When HIEC/shα8 cells were infected with the α8 subunit rescue lentivirus, the expression levels of the α8 subunit were restored (Supplementary Figure S3A at http://www.biolcell.org/boc/101/boc1010695add.htm) and the cellular adhesion-, migration- and proliferation-related phenotypes were totally abolished and comparable with wild-type levels (Supplementary Figures S3B–S3D). Taken together, these results show that the phenotypes induced by α8 shRNA are the result of specific depletion of the integrin α8 subunit.

Discussion

In the present study, we report for the first time that the integrin α8β1 is expressed by epithelial cells. In the human small intestine, α8β1 was found to be restricted to the crypt cell population in the intact fetal intestine. Expression of α8β1 increases dramatically during development of the smooth musculature from 16 weeks onwards (Beaulieu et al., 1993), including adult tissue (results not shown). Indeed, smooth muscle cells express high levels of α8β1 (Schnapp et al., 1995a). Nevertheless, detection of the α8 transcript in purified epithelial fractions at mid-gestation, as well as in the crypt-like HIEC cells, confirmed the expression of α8β1 in epithelial crypt cells in the functional small intestine.

Additional experiments using HIEC cells revealed that the integrin α8β1 interacted in an RGD-dependent manner and promoted accurate FA and stress fibre assembly through RhoA activation, to enhance cell adhesion and proliferation, accompanied by a decrease in cell migration. Taken together, these findings suggest a role for α8β1 in the stimulation of proliferation while simultaneously restraining migration of these undifferentiated, progenitor epithelial cells towards the terminal differentiation compartment.

Integrin-dependent adhesion involves heterogeneous molecular complexes with the typical composition of anchor proteins such as vinculin and paxillin. The integrin–matrix interactions are followed by the sequential formation of FXs (focal complexes), FAs and FBs (fibrillar adhesions), which leads to linkage of the actin network to the cell membrane (Zaidel-Bar et al., 2004; Zimerman et al., 2004). In adherent and spread cultured cells, stress fibres are associated with FAs and maintain an isometric actomyosin-based tension applied to the ECM (Geiger and Bershadsky, 2001). The regulation of this phenomenon is primarily mediated through the small G-protein RhoA. The positive effect of the integrin α8β1 on the promotion of adhesion is not without precedent since other groups have reported that this RGD-dependent integrin is important for the spreading and generation of strong adhesive forces in mesangial, neuroendocrine, myofibroblastic cells and VSMCs, where interaction of α8β1 with the ECM led to cytoskeleton/FA assembly (Muller et al., 1995; Lu et al., 2002; Bieritz et al., 2003; Zargham et al., 2007b). In HIEC, reduction of adhesion in cells lacking α8 was accompanied by the decreased presence of vinculin in FAs and cortical rearrangement of the actin cytoskeleton. As a plausible explanation, it has been well documented that RhoA activity exerts a control on actin stress fibre assembly through the action of ROCK, the principal effector of this GTPase. This action contributes to decreased cell migration by enhancing adhesion on the ECM (Zaidel-Bar et al., 2003; Cetin et al., 2004; Russo et al., 2005). As reported previously, α8β1 interacts either directly or indirectly with the GTPase RhoA, and facilitates RhoA recruitment to the plasma membrane, allowing its activation by membrane-bound associated proteins (Zargham et al., 2007a). In further investigating this feature, we observed that HIEC α8-knockdown cells displayed a lower amount of RhoA in membrane protein extracts than in control HIEC, while membrane-associated levels of other GTPases such as Rac1 and Cdc42 remained constant. Moreover, pull-down experiments of the RhoA active form revealed a decrease of this activated GTPase in α8-knockdown HIEC cells. These findings suggest that the absence of integrin α8β1 in intestinal epithelial cells impairs the recruitment of RhoA to the membrane and, consequently, its activation.

As stated above, we observed that HIEC α8-knockdown cells displayed a reduced presence of vinculin at the FA, compared with wild-type HIEC, without affecting the expression level of this protein. In contrast, we observed no substantial change in the localization of paxillin, another well-characterized FA component (Turner, 2000), suggesting that α8β1 contribution to FA assembly was specific to vinculin recruitment. Similar observations were made when the control HIEC were grown on the non-RGD substratum collagen I. These observations indicated that vinculin recruitment, but not paxillin, depends on the RGD cell–matrix interaction system through integrin α8β1. Based on the proposed scheme of temporal stages in the formation and organization of cell–matrix adhesions (Zaidel-Bar et al., 2004), vinculin recruitment occurs during the late FX stage, while paxillin is already present in early FX. Thus our results suggest that the knockdown of α8 in HIEC impaired the maturation of focal contacts at the late FX stage.

Other groups have proposed that RhoA activity is involved in the recruitment of some FA components including vinculin (Barry and Critchley, 1994;

Zaidel-Bar et al., 2003). Thus our findings strongly suggest that α8β1 is involved, through RhoA activity, in the recruitment of vinculin specifically to the developing focal contacts in intestinal epithelial cells. To emphasize the association between integrin α8β1 and RhoA GTPase in intestinal cells, we have shown that the inhibition of ROCK signalling led to a phenotype similar to that of the α8 knockdown in terms of both actin stress fibre organization and vinculin/paxillin distribution. This supports the hypothesis that the cytoskeleton rearrangement and FA aberrations observed in α8-knockdown cells are mediated largely by the RhoA/ROCK pathway. We also show that loss of α8 is associated with a decrease in cell proliferation, via a RhoA/ROCK mechanism. This signalling pathway has been identified as a modulator of cell-cycle progression through molecular events by which RhoA/ROCK/LIMK (LIM domain kinase) inhibition led to stress fibre disassembly, a decrease in ERK1/2 (extracellular-signal-regulated kinase 1/2) phosphorylation and subsequent cyclin D inhibition (Walker and Assoian, 2005).

Furthermore, Croft and Olson showed that RhoA/ROCK activation led to down-regulation of $p27^{Kip1}$ and cyclin D1 up-regulation via independent mechanisms (Croft and Olson, 2006). In HIEC cells, the observed antiproliferative effect of α8 knockdown was accompanied by a decrease in cyclin D1 expression, whereas the expression of the CDK (cyclin-dependent kinase) inhibitor $p27^{Kip1}$ was not affected. In agreement with these findings, the analysis of cell-cycle progression in α8-depleted cells has shown that a significantly higher percentage of the population accumulated in G_1 phase when compared with control HIEC. Thus cell-cycle progression analysis indicated that integrin α8β1 facilitated the progression from G_1 to S-phase in epithelial progenitor cells of the human intestine. Moreover, inhibition of ROCK in HIEC cells also resulted in a reduction in the amount of proliferative cells compared with α8-knockdown HIEC, suggesting that the α8β1-dependent mechanism of proliferation control acted mainly via RhoA/ROCK signalling.

In addition, we have shown that forced expression of GATA-4, a key transcription factor involved in enterocyte differentiation (Escaffit et al., 2005a; Bosse et al., 2007), led to markedly reduced α8 expression in the HIEC cell model. Mapping of the *ITGA8* (integrin α8) human promoter led to the identification of six putative GATA-binding sites in the 2800 bp upstream of the transcription initiation site (results not shown), which could mediate integrin α8 subunit transcriptional repression. These findings are in accordance with the fact that differentiated epithelial cells lack integrin α8β1 (the present study) and that RhoA activity is down-regulated during the intestinal epithelial differentiation process (Gout et al., 2001). Also, our observations regarding α8β1 localization in the crypt–villus axis and the regulation of cell-cycle progression via α8β1/RhoA are concordant with the inhibition of RhoA activity that normally occurs in the differentiating intestinal epithelium, where proliferative cells have to stop cycling prior to terminal differentiation (Pageot et al., 2000). Thus the striking adhesion decrease, due to the loss of α8 expression, could be indicative of an induction of cell migration that occurs normally in the crypt–villus axis after the triggering of the differentiation process in intestinal epithelial cells (Beaulieu, 1999; Jobin et al., 1999; Lussier et al., 2000). As expected, wound assays revealed that the absence of α8β1 led to an increase in cell migration. Considering the antimigratory phenotype resulting from activation of RhoA GTPase in intestinal epithelial cells (Cetin et al., 2004, 2007), our results reinforce RhoA's status as a key element of the intracellular mechanism underlying integrin α8β1 activation.

Materials and methods

Tissues

Specimens of small intestine (jejunum) from fetuses ranging in age from 14 to 20 weeks (post-fertilization) were obtained after legal abortion. The project was performed in accordance with protocols approved by the Institutional Human Research Review Committee of the Université de Sherbrooke for the use of human material. In some experiments, preparations of pure epithelial and mesenchymal fractions were prepared and analysed as previously described (Perreault et al., 1998).

Cell culture

The HIEC cell line was generated and grown as previously described (Perreault and Beaulieu, 1996). These cells express a number of crypt markers and are thus considered to be non-differentiated crypt-like progenitor cells (Quaroni and Beaulieu, 1997; Pageot et al., 2000; Escaffit et al., 2005b; Tremblay et al., 2006). The Caco-2/15 cell line has been generated and characterized previously for its ability to morphologically and functionally differentiate like villus small intestinal cells (Beaulieu and Quaroni, 1991; Vachon and Beaulieu, 1992; Tremblay et al., 2006).

Establishment of α8-knockdown HIEC cells and GATA-4-expressing HIEC cells

Stable shRNA α8 subunit knockdown and CNS (shCNS) HIEC cell populations were established by lentiviral infection. The same strategy was used to generate a stable GATA-4-expressing HIEC cell population. The oligonucleotides used to generate shα8 (5′-GATCCGATCAGAGTTAATGGAACCTTCAAAGAGAGGTTCCATTAACTCTGATCTTTTTTGGAAC-3′ and complement 5′-TCGAGTTCCAAAAAAGATCAGAGTTAATGGAACCTCTCTTGAAGGTTCCATTAACTCTGATCG-3′), shCNS (5′-GATCGTTCTCCGAACGTGTCACGTTTCAAGAGAAACGTGACACGTT-CGGAGAATTTTTTGGAAC-3′ and complement 5′-TCGAGTTCCAAAAAATTCTCCGAACGTGTCACGTTCTCTTGAAACGTGACACGTTCGGAGAAC-3′) and full-length α8 (5′-CGATAAGCTTCTCCTTCCAG-3′and complement 5′-AACATCTGATACGACAACGG-3′) were synthesized by IDT (Coralville, IA, U.S.A.). Silent mutations resistant to α8 shRNA were introduced into full-length human α8 subunit cDNA by PCR mutagenesis. Specifically, nucleotide residues 357 (C→T), 358 (A→C) and 360 (A→T) from the *ITGA8* coding sequence (GenBank® accession number NM_003638) were substituted to generate the ^{355}atTCgTgttaatggaacc^{372} mutant. The oligonucleotide annealed products were subcloned into pLVX-puro (Clontech, CA, U.S.A.). pLPCX/hGATA-4, pLPCX/EV, pLVX/α8-rescue, pLENTIU6/shα8 and pLENTIU6/shCNS vectors (where EV is empty vector) were used to produce viruses in HEK-293T cells [human embryonic kidney cells expressing the large T-antigen of SV40 (simian virus 40)] as previously described (Boudreau et al., 2007). Subconfluent HIEC cells were infected with viral suspensions containing 4 μg/ml polybrene (Sigma–Aldrich) for 48 h at 37°C. The shα8- and shCNS-infected cells were then selected for 10 days with 5 μg/μl blasticidin, while pLPCX/EV- and pLPCX/GATA-4-infected cells were selected for 10 days with 1 μg/μl puromycin to generate stable cell populations. GATA-4- and EV-infected cells were grown as described above for HIEC for 30 days and RNA was extracted for subsequent RT–PCR analysis.

Generation of recombinant tenascin-C peptides: TNfn3 RGD and RAA

cDNA encoding recombinant tenascin-C fragments containing the third fibronectin type-III repeat with the intact RGD motif (TNfn3 RGD) and the mutated site (TNfn3 RAA) were generated by the method of Yokosaki et al (1998).

Antibodies and reagents

The rabbit polyclonal antibody targeting the human α8 cytoplasmic domain (Schnapp et al., 1995b) was used at a 1:500 dilution for indirect immunofluorescence experiments. A rabbit anti-(chick α8) serum (a gift from Dr Louis F. Reichardt, Department of Physiology, University of California San Francisco, San Francisco, CA, U.S.A.) was also used at a 1:100 dilution in initial immunofluorescence experiments (data not shown). Antiserum directed against the α8 integrin subunit was kindly provided by Dr Gaétan Thibault (IRCM, Montreal, QC, Canada) (Zargham and Thibault, 2005), and used at 1:3000 for Western-blot experiments. Other antibodies used for Western blot were polyclonal anti-αv (AV1; 1:1000 dilution; Chemicon), anti-cyclin D1/D2 (1:5000; Santa Cruz Biotechnology), anti-p27^{Kip1} (1:1000; Santa Cruz Biotechnology), anti-Cdc42 (1:500; Santa Cruz Biotechnology), monoclonal anti-cytokeratin 18 (CY-90; 1:10000; Sigma), anti-vinculin (7F9; 1:10000; Chemicon), anti-RhoA (26C4; 1:200; Santa Cruz Biotechnology), anti-Rac1 (1:1000; BD Transduction Laboratories), anti-β1 integrin [mAb13; 1:1000; a gift from Dr Kenneth M. Yamada, NIH (National Institutes of Health)/NIDCR (National Institute of Dental and Craniofacial Research), Bethesda, MD, U.S.A. (Fogerty et al., 1990)], and anti-actin (C4; 1:75000; Chemicon) antibodies. For immunolocalization, we used the monoclonal antibodies anti-vinculin (7F9; 1:500; Chemicon) and anti-paxillin (1:500; BD Transduction Laboratories). For vinculin/paxillin co-immunolocalization, TRITC (tetramethylrhodamine β-isothiocyanate)-conjugated anti-paxillin (1:500; BD Transduction Laboratories) was used, after vinculin immunodetection. The actin cytoskeleton was stained using TRITC-conjugated phalloidin (1:1000; Chemicon). Blocking anti-integrin antibodies used were anti-β1 (mAb13; 10 μg/ml) and anti-α5 (mAb16; 25 μg/ml) both obtained from Dr K.M. Yamada (Fogerty et al., 1990), anti-α9 [Y9A2; 10 μg/ml; originally obtained as a gift from Dr Dean Sheppard, Lung Biology Center, University of California San Francisco, San Francisco, CA, U.S.A. (Wang et al., 1996) and then purchased from Abcam], anti-αv (mAb2021Z; 25 μg/ml; Chemicon), anti-αvβ3 (LM609; 10 μg/ml; Biogenesis) and anti-αvβ5 (P1F6; 5 μg/ml; Gibco BRL) antibodies. Mouse IgG (Sigma) was used as a negative control at a 10 μg/ml concentration. Y-27632 (Calbiochem), the specific inhibitor of ROCK, was used at a concentration of 20 μM.

Membrane fractionation

For the preparation of fractions enriched in plasma membrane proteins, HIEC cells were treated with ice-cold lysis buffer containing 250 mM sucrose, 10 mM Tris (pH 7.5), 1 mM PMSF and 1% protease inhibitor cocktail (Sigma). After three cycles of freeze-thaw, protein samples were centrifuged at 100 000 *g* at 4°C for 1 h. The supernatant was removed and the pellet was washed twice with lysis buffer and resuspended in 100 μl of lysis buffer containing 0.1% SDS and 1% Triton X-100. The sample was centrifuged to clear cell debris (10 000 *g* at 4°C for 20 min) and the supernatant was harvested and stored at –20°C until used for Western-blot analyses.

RNA extraction and RT–PCR

Total RNA, mRNA and cDNA were prepared as previously described (Dydensborg et al., 2006). Single-stranded cDNA was amplified (Taq polymerase; New England Biolabs) for 30 cycles of denaturation (45 s at 94°C), annealing (45 s at 60°C) and extension (1 min at 72°C). For human α8 subunit detection, we used the forward primer 5′-CAGTTTGGACGAATCCACCT-3′ and the reverse primer 5′-TGCTGTCTGGATTGTCCTTG-3′. Primer sequences for sucrase-isomaltase (oligo-1,6-glucosidase; EC 3.2.1.10), DPPIV and RPLP0 (ribosomal protein, large, P0) detection and the specific conditions used have been previously described (Dydensborg et al., 2006; Escaffit et al., 2006).

Western-blot analysis

Proteins were extracted, separated by SDS/PAGE and transferred on to nitrocellulose membranes as previously described (Perreault and Beaulieu, 1996; Escaffit et al., 2005a). For RhoA–GTP pull-down assays, active RhoA was extracted using the Rho Activation Assay Biochem kit (Cytoskeleton) as previously described (Cetin et al., 2007). Membranes were blocked in 5% (w/v) non-fat dried skimmed milk powder/PBS and 0.1% Tween 20 (Bio-Rad) and then incubated with primary antibodies overnight at 4°C. Primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies (anti-mouse and anti-rabbit; Amersham) and developed using the Immobilon Western® kit (Millipore).

Indirect immunofluorescence staining

The preparation and OCT (optimal cutting temperature; Canemco-Marivac, Lakefield, QC, Canada) embedding of specimens, cryosectioning as well as staining procedures were performed as previously described (Beaulieu et al., 1992). HIEC cell immunofluorescence was done as previously described (Escaffit et al., 2005b). Cells were seeded on to type I collagen (BD Biosciences) or serum-pretreated coverslips (Fisher) 24 h before fixation. The secondary antibodies used were anti-mouse and anti-rabbit conjugated with FITC (Chemicon). For vinculin/paxillin co-immunolocalization, mouse IgG (Sigma) supplementary blocking and 2% (w/v) PFA (paraformaldehyde) post-fixation steps were found to be sufficient to block potential cross-reactions with FITC-conjugated anti-mouse. Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole), whereas tissue sections were counterstained with 0.01% Evan Blue. Vinculin/paxillin-stained FXs were counted using the MetaMorph Imaging System (Universal Imaging, West Chester, PA, U.S.A.).

Cell adhesion assays

Adherent HIEC cells were harvested using PBS/0.5 mM EDTA (incubation for 10 min at 37°C) and centrifuged at 100 *g* for 5 min at 4°C. Cell pellets were resuspended in OptiMEM (Invitrogen) culture medium supplemented with 0.2% FBS (fetal bovine serum) and 0.3 mM $MnCl_2$. For 24 h adhesion assays, cycloheximide was added to the medium at a concentration of 10 μg/ml throughout the assay to avoid endogenous ECM secretion (Vainionpaa et al., 2006). However, for short-term adhesion assays, cycloheximide treatment was omitted since endogenous secretion was considered to be negligible. For assays involving blocking antibodies (short-term assays), suspensions of cells were incubated for 30 min at 37°C with the blocking antibody. Then, 48-well plates were treated with the appropriate coating (40 μg/ml of GST, GST–TNfn3 RGD and GST–TNfn3 RAA in PBS) and blocked with 2% (w/v) BSA. Cells were seeded (5×10^4 cells per well) and incubated for 1 h for short-term assays (with blocking antibodies) and 24 h for long-term assays (shRNA cells). Non-adherent cells were gently washed away with PBS and the remaining cells were fixed with 2% PFA (pH 7.4) for 1 h at 4°C and permeabilized with 0.1% Triton X-100. Cell nuclei were stained with DAPI and adherent cells were counted manually.

Cell migration assay

HIEC cells were seeded on to 100 mm^2 plates and cultured until 2 days post-confluence. Cell monolayers were wounded with a 19 mm plastic cell-scraper (Costar, Corning, NY, U.S.A.). Wounded cell monolayers were incubated for 48 h in OptiMEM with 2 mM hydroxyurea (Sigma) to block cell proliferation (Tetreault et al., 2008). After incubation, DAPI staining was performed and the number of cells that had migrated across the wound edge was manually counted by immunofluorescent microscopy.

BrdU incorporation experiments

Each cell line was seeded on to serum-pretreated coverslips in a 24-well plate (Falcon) at 5×10^4 cells per well (and treated with Y-27632, where indicated), 24 h before the addition of BrdU labelling reagent (Roche). The procedure and reagents used for detection of S-phase positive HIEC cells were in accordance with the FLUOS® In Situ Cell Proliferation kit protocol (Roche).

Cell-cycle progression analysis by LSC

Each cell line was seeded on to a 12-well plate (Falcon) at 5×10^4 cells per well, 48 h before methanol fixation and DAPI staining. DAPI-stained cells were scanned with an iCys imaging cytometer (Compucyte, Cambridge, MA, U.S.A.) to measure DNA content using violet diode laser excitation (405 nm) and appropriate filters (463/39 nm) for fluorescence detection. DNA content was measured for at least 3000 isolated nuclei per sample in three separate experiments to assess cell-cycle distribution.

Statistical analysis

Results were expressed as means ± S.E.M. Each experiment was repeated at least three times and representative results are shown. Values from adhesion assays and BrdU labelling experiments were subjected to the mixed linear model. All other values were subjected to the two-tailed Student's t test. $P < 0.05$ was considered to be statistically significant.

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online data

Supplementary online data

Integrin $\alpha 8\beta 1$ regulates adhesion, migration and proliferation of human intestinal crypt cells via a predominant RhoA/ROCK-dependent mechanism

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Figure S1 | Cellular localization of small GTPases in α8-depleted HIEC

Relative amounts of total and membrane-associated RhoA (**A**), Cdc42 (**B**) and Rac1 (**C**) detected by Western blotting for shCNS and shα8 according to the integrin β1 (Int β1) subunit used as a loading control ($n = 3$, $^*P = 0.05$).

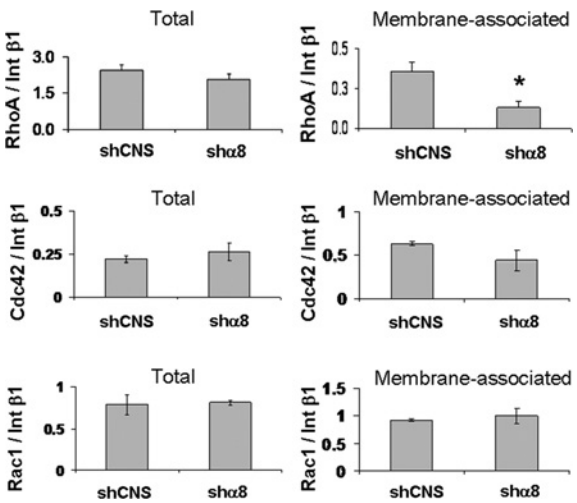

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Figure S2 | Impact of integrin α8β1 on vinculin and paxillin cellular localization in HIEC

Representative immunofluorescence micrographs of vinculin (i) and paxillin (ii) localization in normal HIEC (**A**), HIEC/shCNS (**B**), HIEC/shα8 (**C**) and Y-27632-treated (20 μM) HIEC (**D**) cultured on serum coating at 24 h after seeding. (**E**) Vinculin (i) and paxillin (ii) localization respectively in HIEC/shCNS cultured on type I collagen coating at 24 h after seeding. In this case, 10 μg/ml of cycloheximide was added to the adhesion medium to avoid endogenous fibronectin secretion/deposition ($n = 3$, scale bar: 50 μm). Amounts of vinculin- and paxillin-stained FA per cell were counted for each experimental conditions (iii) ($n = 8$, $^{***}P < 0.0001$).

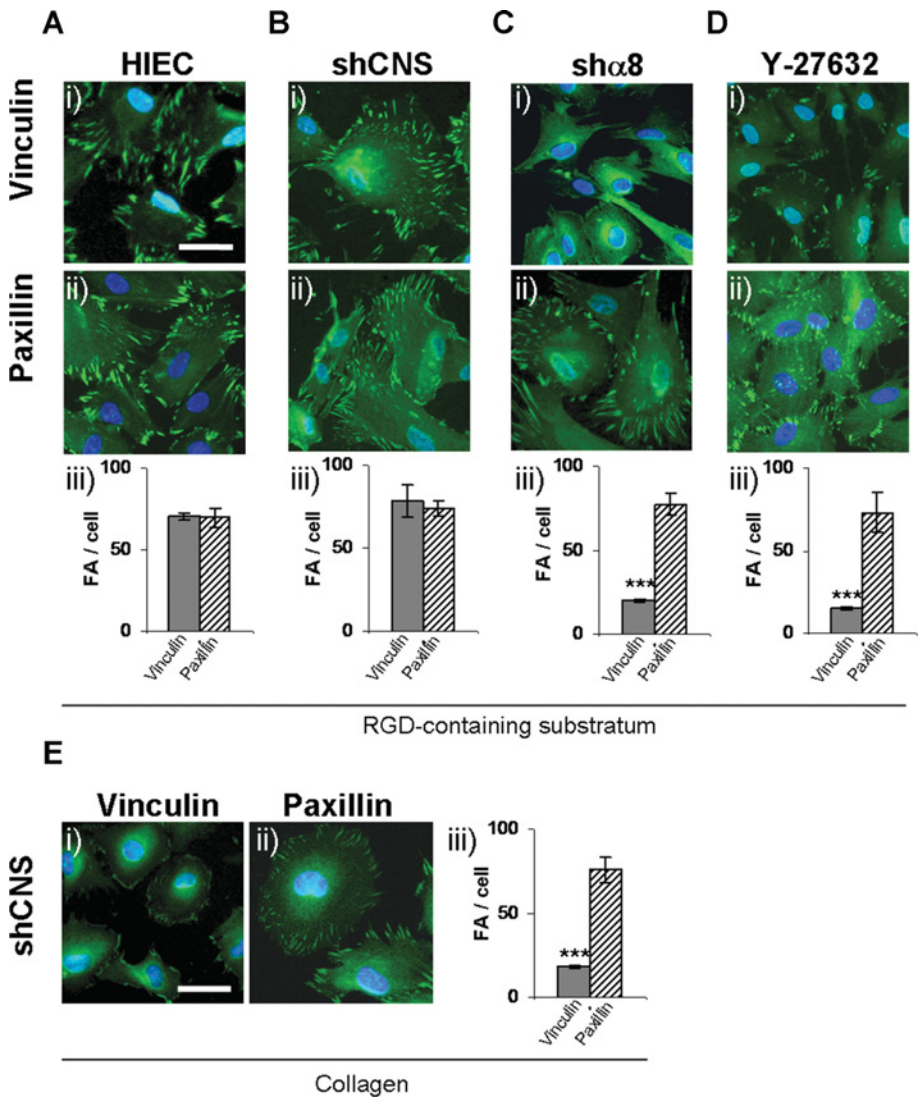
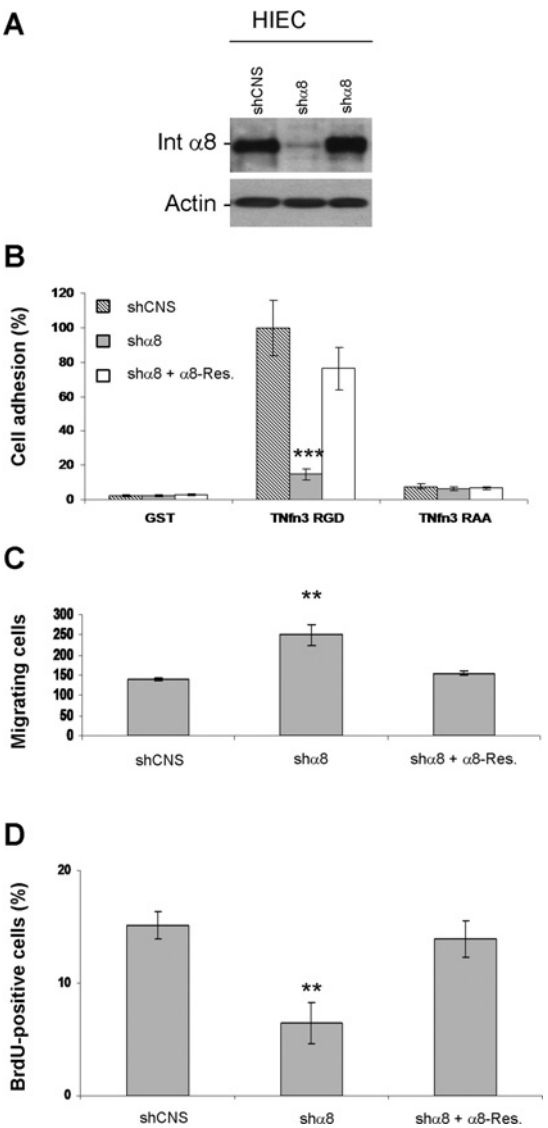


Figure S3 | RNAi-resistant α8 cDNA restores wild-type phenotype in HIEC shα8 cells

(**A**) Representative Western-blot analysis indicating a re-expression of the α8 subunit (Int α8) in HIEC/shα8 cells after the insertion of RNAi-resistant α8 cDNA (HIEC/shα8/α8-rescue) compared with HIEC/shCNS and HIEC/shα8. (**B**) Long-term (24 h) adhesion assays with HIEC/shCNS, HIEC/shα8 and HIEC/shα8/α8-rescue (shα8 + α8-Res.) cells plated on GST, RGD-containing GST–TNfn3 or RAA-mutated GST–TNfn3. Cycloheximide (10 μg/ml; long-term assays only) was added to the adhesion medium to avoid endogenous fibronectin secretion/deposition ($n = 3$, $^{***}P = 0.0004$). (**C**) Histogram showing the amount of HIEC/shCNS, HIEC/shα8 and HIEC/shα8/α8-rescue cells that migrated across the wound edge after 48 h of incubation in the presence of 2 mM hydroxyurea ($n = 3$; $^{**}P = 0.002$). (**D**) Histogram illustrating the percentage of BrdU-labelled cells for HIEC/shCNS compared with HIEC/shα8 and HIEC/shα8/α8-rescue ($n = 3$; $^{**}P = 0.005$).



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