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Contrasting effects of ERK on tight junction integrity in differentiated and under-differentiated Caco-2 cell monolayers

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ERK (extracellular-signal-regulated kinase) activation leads to disruption of tight junctions in some epithelial monolayers, whereas it prevents disruption of tight junctions in other epithelia. The factors responsible for such contrasting influences of ERK on tight junction integrity are unknown. The present study investigated the effect of the state of cell differentiation on ERK-mediated regulation of tight junctions in Caco-2 cell monolayers. EGF (epidermal growth factor) potentiated H_2O_2-induced tight junction disruption in under-differentiated cell monolayers, which was attenuated by the MEK [MAPK (mitogen-activated protein kinase)/ERK kinase] inhibitor U0126. In contrast, EGF prevented H_2O_2-induced disruption of tight junctions in differentiated cell monolayers, which was also attenuated by U0126. Knockdown of ERK1/2 enhanced tight junction integrity and accelerated assembly of tight junctions in under-differentiated cell monolayers, whereas it had the opposite effect in differentiated cell monolayers. Regulated expression of wild-type and constitutively active MEK1 disrupted tight junctions, and the expression of dominant-negative MEK1 enhanced tight junction integrity in under-differentiated cells, whereas contrasting responses were recorded in differentiated cells. EGF prevented both H_2O_2-induced association of PP2A (protein phosphatase 2A), and loss of association of PKCζ (protein kinase Cζ), with occludin by an ERK-dependent mechanism in differentiated cell monolayers, but not in under-differentiated cell monolayers. Active ERK was distributed in the intracellular compartment in under-differentiated cell monolayers, whereas it was localized mainly in the perijunctional region in differentiated cell monolayers. Thus ERK may exhibit its contrasting influences on tight junction integrity in under-differentiated and differentiated epithelial cells by virtue of differences in its subcellular distribution and ability to regulate the association of PKCζ and PP2A with tight junction proteins.

Key words: epidermal growth factor (EGF), extracellular-signal-regulated kinase (ERK), mitogen-activated protein kinase/extracellular-signal-regulated kinase kinase (MEK), occludin, protein kinase Cζ (PKCζ), protein phosphatase 2A (PP2A).

INTRODUCTION

Epithelial tight junctions in the intestinal mucosa perform the critical function of selectively restricting the passage of macromolecules, such as toxins, allergens and pathogens from the lumen to the tissues [1]. A host of diseases like inflammatory bowel disease (ulcerative colitis and Crohn's disease), malabsorption, coeliac disease, endotoxaemia and some types of diarrhoea in humans can be attributed to disruption in the structure and function of tight junctions [2]. Tight junctions also confer a 'fence' function by segregating apical and basolateral membrane proteins, and therefore contribute to epithelial differentiation. Loss of the fence function of tight junctions has been implicated in carcinogenesis [3] and tumour metastasis [4].

Tight junctions are formed by the assembly of several transmembrane proteins like occludin, claudins, JAMs (junctional adhesion molecules), tricellulin, the intracellular scaffold proteins like ZO (Zona occludens)-1, ZO-2 and ZO-3, and plaque proteins, such as symplekin, cingulin and 7H6 [5,6]. This tight junction protein complex interacts with the actin cytoskeleton to anchor the protein complex at the apical end of the epithelium. The structural proteins of tight junctions are surrounded by a number of regulatory proteins, such as MAPK (mitogen-activated protein kinase), PKC (protein kinase C), PP2A (protein phosphatase 2A), G-proteins, Rab13, c-Src, phosphoinositide 3-kinase and phospholipase Cγ, and secondary messengers like calcium and c-AMP, that are components of several different pathways, interplay of which is vital for the maintenance and regulation of tight junction integrity [7–9]. Modulation of the activity of these signalling molecules by pharmacological methods has demonstrated their role in regulation of tight junction integrity in different epithelia. Occludin, a major component of the tight junction, is known to be highly phosphorylated on serine and threonine residues in intact junctions [10–13]. Dephosphorylation of occludin on serine and threonine residues and phosphorylation on tyrosine residues occurs during the disruption of tight junctions [14]. Thus protein kinases hold a place of primary importance in regulation of tight junction integrity.

MAPKs/ERKs (extracellular-signal-regulated kinases) are a group of serine/threonine kinases, which regulate gene expression, mitosis, differentiation, cell survival and apoptosis [15]. However, little is known about the exact role of ERK in the regulation of epithelial tight junctions. The few studies on this subject have only produced mixed results. In one study, ERK1/2 activation was found to inhibit claudin-2 expression and transiently increase tight junction integrity [16]. This process

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; dsRNA, double-stranded RNA; EGF, epidermal growth factor; ERK, extracellular-signal-regulated kinase; FBS, fetal bovine serum; GFP, green fluorescent protein; HBSS, Hank's balanced salt solution; HRP, horseradish peroxidase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; NBCS, newborn calf serum; p-Akt, phospho-Akt; p-ERK, phospho-ERK; p-GSK-3β, phospho-glycogen synthase kinase 3β; PKC, protein kinase C; PP2A, protein phosphatase 2A; RT, reverse transcription; siRNA, small interfering RNA; TER, transepithelial electrical resistance; WT-, DN- and CA-MEK, wild-type, dominant-negative and constitutively active MEK; ZO, Zona occludens.

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was blocked by the MEK (MAPK/ERK kinase) inhibitor U0126. Another study showed that activation of PKC disrupts tight junctions by a MAPK-dependent mechanism [17]. Furthermore, constitutively active Ras [18] or constitutively active Raf-1 [19] induced ERK activation and disruption of epithelial tight junctions. Down-regulation of the MAPK signalling pathway restores epithelial cell morphology and the assembly of tight junctions in Ras-transformed MDCK (Madin–Darby canine kidney) epithelial cells [20]. On the other hand, a further study showed that bile in the intestinal lumen initiates ERK1/2-dependent signalling that is essential for normal expression of key tight junction proteins and regulation of tight junction integrity [21]. In differentiated Caco-2 cell monolayers, EGF (epidermal growth factor) protects the tight junctions against oxidative stress induced by H_2O_2 [22]. This protective effect of EGF was blocked by the MEK inhibitor U0126 thus demonstrating that the action of EGF was mediated by ERK. Furthermore, activated ERK (p-ERK; phospho-ERK) was associated with the tight junction protein complex and was co-localized with occludin. These strikingly contrasting results pose a formidable challenge in defining the precise role of ERK in regulation of tight junction integrity.

In the present study, using both pharmacological and molecular techniques, we addressed some of the above-mentioned paradoxical effects of ERK in regulation of tight junctions in Caco-2 cells. The results show that ERK is involved in destabilization of tight junctions in under-differentiated Caco-2 cells, whereas it plays a role in protection of tight junctions in differentiated cells. A differential effect of ERK on PP2A and PKCζ distribution, and their association with the tight junction protein complex, may determine its contrasting effects in under-differentiated and differentiated Caco-2 cell monolayers.

EXPERIMENTAL

Chemicals

Cell culture medium (DMEM; Dulbecco's modified Eagle's medium), FBS (fetal bovine serum) and antibiotics were procured from Gibco-BRL. NBCS (newborn calf serum) was purchased from Invitrogen. FITC–inulin, Protein A–Sepharose beads, sodium vanadate, SDS, EGF and protease inhibitors were purchased from Sigma–Aldrich. The alkaline phosphatase assay kit was purchased from the Biomedical Research Service Center at SUNY Buffalo. H_2O_2 was purchased from Fisher Scientific and the MEK inhibitor U0126 was from Promega. Most of the other reagents and chemicals were purchased either from Sigma–Aldrich or Fisher Scientific.

Antibodies

Rabbit anti-ERK (phospho-Thr^{202}/phospho-Tyr^{204}) antibody was purchased from Cell Signaling. Mouse anti-ERK1/2, mouse anti-occludin, mouse anti-ZO-1, rabbit anti-occludin, rabbit anti-ZO-1 and rabbit anti-phosphothreonine antibodies were purchased from Zymed Laboratories/Invitrogen. Mouse anti-β-actin antibody, HRP (horseradish peroxidase)-conjugated anti-(rabbit Ig) and anti-(mouse Ig) secondary antibodies used in immunoblotting, and Cy3 (indocarbocyanine) or Alexa Fluor® 488-conjugated anti-(rabbit Ig) and anti-(mouse Ig) secondary antibodies used in immunostaining, were purchased from Sigma–Aldrich. Rabbit anti-PKCζ antibody was purchased from Upstate/Millipore. Mouse anti-PP2A and mouse anti-villin antibodies were purchased from BD Biosciences. Alexa Fluor® 488-conjugated anti-(mouse Ig) and anti-(rabbit Ig) antibodies and anti-GFP (green fluorescent protein) antibody were purchased from Invitrogen.

MEK constructs

A GFP sequence with cohesive SalI and EcoRV sites was amplified by PCR, using the pAcGFP1-N1 plasmid (Clontech) as template and the following primers (custom synthesized by Integrated DNA Technologies): 5′-GACGCGTCGACATGGTGAGCAAGGGCG-3′ (forward) and 5′-CCGATATCTCACTTGTACAGCTCATC-3′ (reverse). The amplified PCR product for GFP was digested with SalI and EcoRV, and ligated into the pTRE2hyg vector (digested with the same restriction endonucleases), to obtain the pTRE2hyg-pAcGFP1-N1 vector. *MEK1* cDNA sequences for wild-type MEK (WT-MEK), dominant-negative MEK1^{K97M} (DN-MEK) and constitutively active MEK1$^{S218E/S220D}$ (CA-MEK) in vector were obtained from Dr Natalie Ahn (University of Colorado, Boulder, CO, U.S.A.). *MEK* sequences with cohesive BamHI and MluI sites were amplified by PCR, using the above cDNAs as templates and the following primers (custom synthesized by Integrated DNA Technologies): 5′-GCGGATCCACCACCATGCCCAAGAAGAAG-3′ (forward) and 5′-GCACGCGTCGACGCCAGCAGCATG-3′ (reverse). The amplified PCR products for WT-MEK, DN-MEK and CA-MEK were digested with BamHI and MluI, and ligated into the pTRE2hyg-pAcGFP1-N1 vector (digested with the same restriction endonucleases) to obtain pTRE2hyg-pAcGFP1-N1-MEK constructs.

The resulting DNA plasmids were then transformed into *Escherichia coli* DH5αFIQ competent cells and purified by using Qiagen maxi- or mini-prep kits. The plasmids were sequenced at the Molecular Resources Center facility of the University of Tennessee. The expression of various mutants of MEK upon their transfection into the cells was also confirmed by RT (reverse transcription)–PCR.

Synthesis of long double-stranded ERK siRNA (small interfering RNA)

ERK1 cDNA (GenBank® accession number NM_002745) was amplified by RT–PCR and cloned into the TopoII vector (Invitrogen). The identity of the cloned product was verified by sequencing. The template was then digested with SpeI or XbaI to linearize it, and sense and anti-sense strands were obtained using T7 or SP6 polymerases respectively. β-Globin cDNA was used to make control dsRNA (double-stranded RNA). Sense and antisense RNAs were synthesized from cDNA inserts cloned into plasmid vectors using the MEGAscript RNA kit (Ambion). Equimolar concentrations of sense and antisense RNA were mixed, extracted with phenol/chloroform (1:1), precipitated and resuspended in annealing buffer (10 mM Tris/HCl, pH 7.5, and 1 mM EDTA). The sense and antisense RNA mixture was boiled for 1 min in a boiling water bath and the temperature of the bath was allowed to cool to room temperature (25 °C) over 12–16 h, resulting in the formation of long dsRNA as described previously [23]. Integrity of the long dsRNA was checked by gel electrophoresis. This long dsRNA was subsequently digested with RNase III to produce an enzymatically cleaved pool of siRNA.

Cell culture

Caco-2 cells purchased from A.T.C.C. were grown under standard cell culture conditions [24] in DMEM containing 10 % (v/v) FBS or NBCS, high-glucose, L-glutamine, pyruvate and fortified with penicillin, streptomycin and gentamicin. The cells were grown as monolayers in 100-mm-diameter petri dishes or T75 flasks. Experiments were conducted on cells grown in polycarbonate membrane transwell inserts (Costar) of various diameters, i.e. 6.5 mm, 12 mm and 24 mm. Studies were performed on days 3 or

4 (under-differentiated cells) and 7–14 (differentiated cells) post-seeding. Analysis for alkaline phosphatase and villin indicated that these cells switch to the differentiated state on approximately day 5 post-seeding.

Transfection of siRNA into Caco-2 cells

Caco-2 cells (125 000 cells/well) were seeded in six-well cluster plates, and allowed to grow and attach for 24–48 h. Upon reaching 60–70 % confluence, cells were washed twice with HBSS (Hank's balanced salt solution) containing calcium to remove all traces of antibiotics from the cells. Transfection was performed using 0.9 ml of antibiotic-free DMEM, 15 μg of the ERK-specific siRNA or non-specific siRNA per ml of medium (13.5 μg per well), 3.5 μl of Oligofectamine™ and 1 μl of Plus reagent per μg of siRNA for each well. After 24 h, the cell monolayers were trypsinized and seeded on to transwell inserts. The TER (transepithelial electrical resistance) and cell morphology were monitored.

Generation of the Tet-on Caco-2 cell line

Caco-2 cells at 60–70 % confluence were transfected with commercially available pTet-on vector (Clontech), using Lipofectamine™ LTX and Plus reagent. The cells were then selected with G0418 sulfate (700 μg/ml). After 14 days, the cell colonies were cloned, cultured and maintained in a lower concentration (350 μg/ml) of G0418 sulfate. These clones were characterized by immunoblotting the whole-cell lysate for Tet-R (Tet repressor) protein, and by luciferase assay for doxycycline-induced expression.

Transfection of Tet-on Caco-2 cells with MEK constructs

Tet-on Caco-2 cells (125 000 cells/well) were seeded in six-well cluster plates, and allowed to grow and attach for 24–48 h. Upon reaching 60–70 % confluence, cells were washed twice with HBSS containing calcium to remove all traces of antibiotics from the cells. Transfection was performed using 0.9 ml of antibiotic-free DMEM, 1 μg of MEK expression vector or vector control, 3 μl of Lipofectamine™ and 1 μl of Plus reagent per μg of plasmid DNA for each well. After 24 h, the cell monolayers were trypsinized and seeded on to transwell inserts. The TER and cell morphology were monitored.

H_2O_2 and EGF treatments

On days 3 or 4 (under-differentiated cells) and days 7–14 (differentiated cells), the cells in transwell inserts were washed and bathed in PBS (Dulbecco's saline containing 10 mM glucose, 0.6 % BSA, 1 mM $MgCl_2$ and 1.2 mM $CaCl_2$). The cells were pre-incubated for 50 min with or without 10 μM U0126 added to both the apical and basal medium. Human EGF (30 nM) was administered into both the apical and basal medium 10 min before the administration of H_2O_2. H_2O_2 (20 μM) was administered to both the apical and basal medium as described previously [25]. Control cell monolayers were incubated in PBS without EGF and/or H_2O_2.

Tight junction assembly by calcium switch

Caco-2 cells were grown to confluence in the transwell inserts in regular medium. Calcium was chelated from the medium by administering 4 mM EGTA into both the apical and basal medium for approx. 10 min. Disruption of tight junctions was monitored by measuring the TER. Cells were washed three times with the regular medium to remove EGTA, and cell monolayers were incubated in regular calcium-containing medium for 3–6 h. Reassembly of tight junctions and restoration of barrier function was monitored by measuring the TER and unidirectional flux of FITC–inulin.

Measurement of TER

TER was measured according to the method described by Hidalgo et al. [26] using the Millicell electrical resistance system (Millipore). The resistance was calculated as Ω/cm^2, by dividing the observed value by the surface area of the membrane. The background resistance of the transwell membrane ($\sim$30 Ω/cm^2) was subtracted from the observed resistance values.

Paracellular inulin flux measurement

Cell monolayers in the transwell inserts were incubated under various experimental conditions in the presence of 500 μg/ml FITC–inulin administered to the basal medium. At various time intervals, 100 μl aliquots were taken from the apical as well as the basal buffers. An equal volume of the medium or the buffer was used for controls. The fluorescence was measured (excitation at 485 nm and emission at 538 nm) using an FLx800 Microplate fluorescence reader (BioTEK Instruments) and the KCjunior™ software. The unidirectional flux of inulin into the apical compartment was calculated as a percentage of inulin administered into the basal compartment per cm^2 surface area of the cell monolayer.

Immunofluorescence imaging

Cell monolayers washed with PBS were fixed in an acetone/methanol (1:1) mixture at 0 °C for 5 min. Fixed cell monolayers were permeabilized by incubating with 0.2 % Triton X-100 in PBS, and blocked in 4 % (w/v) dried milk powder in TBST [Tris-buffered saline (20 mM Tris/HCl, pH 7.2, and 150 mM NaCl) with 0.05 % Tween 20] for 30 min at room temperature, and double-labelled by incubating with primary antibodies for different proteins, followed by a 1 h incubation with the secondary antibodies [goat anti-(rabbit IgG) and goat anti-(mouse IgG) conjugated with either Alexa Fluor® 488 or Cy-3]. Fluorescence was visualized using a confocal laser-scanning microscope (Zeiss LSM510 PASCAL). Images from serial *XY* sections (1 μm thick) were collected. The images were stacked using the Image J software and processed in the Adobe Photoshop software.

Preparation of detergent-insoluble fraction

Cell monolayers were washed with ice-cold PBS, and lysed in lysis buffer CS (20 mM Tris/HCl, pH 7.2, containing 1.0 % Triton X-100, 2 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml bestatin, 10 μg/ml pepstatin-A, 2 mM vanadate and 1 mM PMSF). The lysate was centrifuged at 15 600 ***g*** for 4 min at 4 °C, to sediment the highly dense actin-cytoskeleton-rich Triton-insoluble fraction. The sedimented actin cytoskeleton pellet was suspended in lysis buffer CS, and the sediment was homogenized by sonication in an ice bath using three sets of five bursts 5 s apart. Aliquots were taken for protein estimation by the BCA (bicinchoninic acid) method (Pierce). The sonicated extract of Triton-insoluble fraction was used for immunoprecipitation of occludin.

Immunoprecipitation

The extracts from the Triton-insoluble fraction (400 μg of protein) were incubated with 3 μg of rabbit polyclonal anti-occludin antibody at 4 °C on the rocker for 16 h. Immune complexes were isolated by conjugating with Protein A–Sepharose beads at 4 °C for 1 h. Proteins in the washed beads were extracted in 20 μl of Laemmli's sample buffer by heating at 100 °C for 10 min. Non-specific binding levels were determined by conducting the immunoprecipitation assay using rabbit pre-immune IgG instead of anti-occludin antibody.

Immunoblot analysis

Cell monolayers were washed twice with ice-cold PBS and lysed with heated lysis buffer D (10 mM Tris/HCl, pH 7.4, 0.3 % SDS, 10 μM sodium vanadate, 100 μM sodium fluoride and 10 μl/ml protease inhibitor cocktail). The lysate was sonicated and heated at 100 °C for 10 min. An aliquot was taken for protein estimation, and the lysate was mixed with an equal volume of 2× Laemmli's sample buffer and heated at 100 °C for 10 min. Proteins were separated by SDS/PAGE (7 % gel) and transferred on to a PVDF membrane. The membranes were probed for p-ERK, ERK1/2, PKCζ, PP2A, ZO-1, occludin or β-actin by using a combination of the specific primary antibodies with corresponding HRP-conjugated anti-(mouse IgG) or HRP-conjugated anti-(rabbit IgG) secondary antibodies. The blot was developed using the ECL (enhanced chemiluminescence) system (Amersham/GE Healthcare). Densitometric analysis of specific bands was performed using the Image J software.

Alkaline phosphatase assay

The alkaline phosphatase assay was performed using a SUNY Buffalo kit, according to the vendor's instructions. Briefly, 3-day- or 14-day-old Caco-2 cells were lysed using 100 μl of cell lysis solution, and 10 μl of each cell lysate was transferred into a clear 96-well assay plate and mixed with 100 μl of the alkaline phosphatase assay solution. After incubation at 37 °C for 30 min, the reaction was terminated by addition of 20 μl of 1 M sodium hydroxide. Absorbance was read at 410 nm using a Spectramax spectrophotometer. Alkaline phosphatase activity was calculated as units (nmoles of *p*-nitrophenol)/μg of protein per h.

Statistical analysis

The observed results in the two different groups were compared using Student's *t* tests for grouped data. Significance in all tests was set at a 95 % or greater confidence level.

RESULTS

Contrasting effects of EGF on H_2O_2-mediated disruption of barrier function in under-differentiated and differentiated cell monolayers

Extensive studies indicate that EGF protects the epithelial barrier function from a variety of insults [28,29]. However, EGFR (EGF receptor) activation can also disrupt cell–cell adhesion and promote cell migration [30]. Therefore it is crucial to define the conditions that render differential EGF responses. We evaluated the effect of EGF on H_2O_2-induced barrier disruption in under-differentiated and differentiated cell monolayers. Identity of cell state (under-differentiated compared with differentiated) was confirmed by measuring alkaline phosphatase activity and villin expression. Expression of alkaline phosphatase, an enzyme present on the brush border of the intestinal epithelial cells, increases as the cells mature and differentiate [31,32]. Expression of villin, a cytoskeletal protein, also increases as the cells grow and differentiate [33,34]. Our results show that alkaline phosphatase activity in 14-day-old differentiated cells was 6-fold higher than that in the 4-day-old under-differentiated cells (Figure 1A). The differentiated state of cells was further confirmed by high expression of villin in 14-day-old differentiated cells (Figure 1B). We performed a more rigorous analysis of cells at days 4, 7 and 14 to distinguish the state of differentiation in these cells. Besides alkaline phosphatase and villin, we also analysed p-GSK-3β (phospho-glycogen synthase kinase 3β), p-Akt (phospho-Akt), the TER and the unidirectional flux of FITC–inulin on different days post-seeding. Several-fold higher levels of alkaline phosphatase activity were detected in 7- and 14-day-old cells (Supplementary Figure S1 at http://www.BiochemJ.org/bj/433/bj4330051add.htm). The levels of villin, p-GSK-3β and p-Akt were also high in 7- and 14-day-old cells. Cells on day 7 and day 14 were similar except for differences in the TER and p-GSK-3β levels. The rate of inulin permeability was significantly lower in 7- and 14-day-old cell monolayers compared with 3- and 4-day-old cell monolayers. Thus 14-day-old Caco-2 cells are mature and differentiated, whereas 4-day-old cells can be defined as under-differentiated.

We next evaluated the effects of EGF on H_2O_2-mediated disruption of barrier function and the role of ERK in EGF-mediated protection of tight junctions in day 4 under-differentiated and day 14 differentiated cell monolayers. In both 4- and 14-day-old cells, H_2O_2 treatment led to disruption of tight junctions as was evident from decreased TER (Figures 1C and 1E) and increased inulin flux (Figures 1D and 1F). Similar to our previous observation [22], on day 14, EGF significantly attenuated the H_2O_2-induced decrease in the TER (Figure 1C) and increase in inulin flux (Figure 1D). Pretreatment of cells with MEK inhibitor (U0126) significantly inhibited the effect of EGF on H_2O_2-induced changes in the TER (Figure 1C) and inulin permeability (Figure 1D). In contrast with 14-day-old cells, in 4-day-old cells EGF exacerbated the effect of H_2O_2 on TER (Figure 1E) and inulin flux (Figure 1F). EGF-mediated potentiation of the H_2O_2 effect on inulin permeability was prevented by U0126 (Figure 1F).

Immunostaining of fixed cell monolayers for tight junction proteins demonstrated that H_2O_2 treatment disrupts the junctional organization of occludin and ZO-1 in both 4-day-old under-differentiated and 14-day-old differentiated cell monolayers (Figure 2). In 14-day-old cells, EGF attenuated the H_2O_2-induced redistribution of occludin and ZO-1 from the tight junctions, and U0126 treatment inhibited this protective effect of EGF (Figure 2A) indicating that this effect is mediated by ERK. In 4-day-old cell monolayers, EGF enhanced the H_2O_2-induced redistribution of occludin and ZO-1 (Figure 2B).

Reduced expression of ERK1/2 promotes assembly of tight junctions in under-differentiated cells, whereas it delays tight junction assembly in differentiated cells

To confirm the role of ERK in regulation of tight junction integrity at different stages of differentiation, we knocked down ERK1/2 by using siRNA. Immunoblot analysis revealed that transfection with ERK1/2 siRNA, but not control RNA, resulted in a significant decrease in ERK levels in the cells on day 3 and day 7 (Figures 3A and 3B). The levels of p38-MAPK, a closely related kinase, remained unaffected at all times, thus confirming the specificity of the ERK1/2 siRNA used. Transfection with siRNA increased the basal TER in 3-day-old under-differentiated cell monolayers, whereas it decreased the basal TER in 7-day-old differentiated cell monolayers (Figure 3C) compared with corresponding cells transfected with control RNA. In support of the change in the TER values, the basal inulin permeability in cell monolayers transfected with siRNA was lower in 3-day-old under-differentiated cell monolayers and higher in 7-day-old differentiated cell monolayers, as compared with inulin

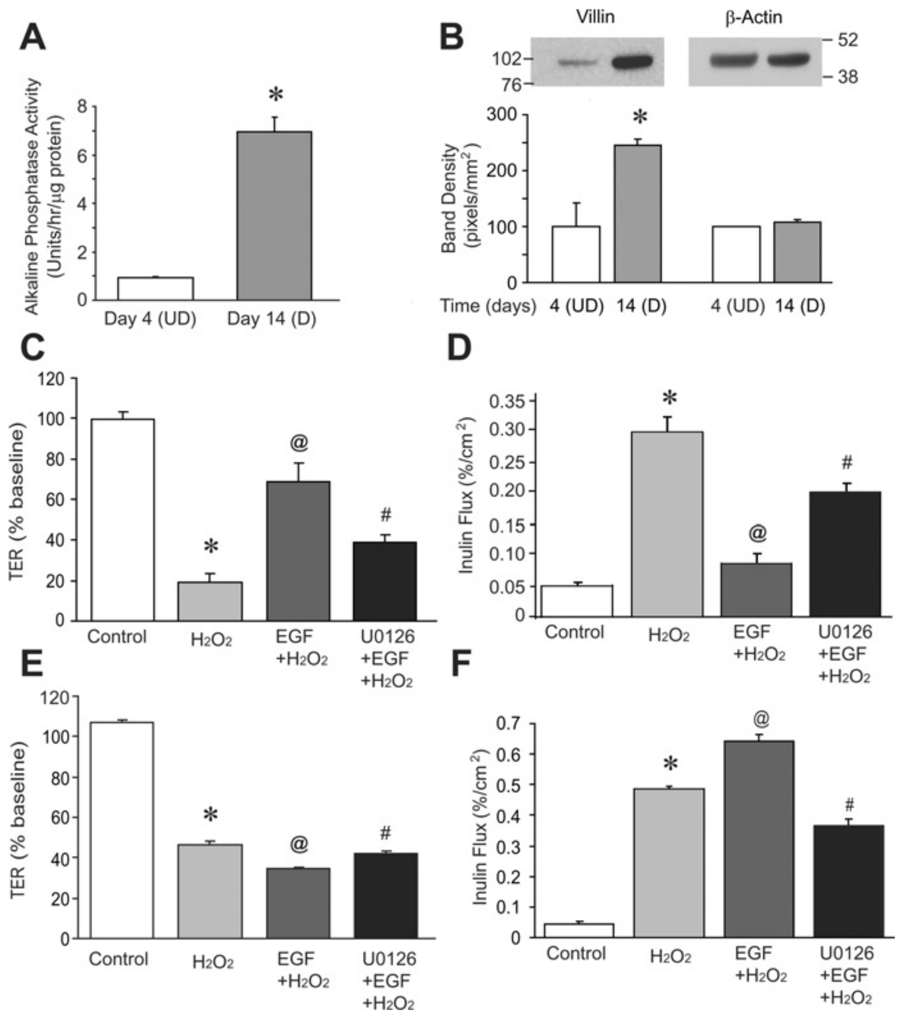

Figure 1 Contrasting effects of EGF on H_2O_2-induced epithelial barrier disruption in under-differentiated and differentiated cell monolayers

(**A**) Alkaline phosphatase activity was measured in extracts from under-differentiated (UD) 4-day-old and differentiated (D) 14-day-old Caco-2 cell monolayers. Values are means ± S.E.M. ($n = 4$). $^*P < 0.05$. (**B**) Extracts from Caco-2 cell monolayers on day 4 or 14 after seeding were immunoblotted for villin and β-actin. The molecular mass in kDa is indicated. Band density was quantified by densitometric analysis. Values are means ± S.E.M. ($n = 4$). $^*P < 0.05$. (**C** and **D**) 14-day-old or (**E** and **F**) 4-day-old Caco-2 cell monolayers were pretreated with 10 μM U0126 for 50 min prior to administration of 30 nM EGF. H_2O_2 (20 μM) was administered 10 min after EGF. Control cells without treatments, as indicated, are also shown. (**C** and **E**) TER and (**D** and **F**) inulin permeability were measured at various times. Values are means ± S.E.M. ($n = 6$). $^*P < 0.05$ compared with corresponding control values, @$P < 0.05$ compared with H_2O_2-treated values, #$P < 0.05$ compared with EGF+H_2O_2 values.

permeability in corresponding cells transfected with non-specific or control RNA (Figure 3D). Immunostaining and confocal microscopy showed that the reduction of ERK expression, by siRNA, resulted in an enhanced localization of occludin and ZO-1 at the intercellular junctions on day 3 (Figure 3E). In contrast, on day 7, the reduced ERK expression resulted in reduced localization of occludin and ZO-1 at the junctions (Figure 3F).

To determine the role of ERK in the assembly of tight junctions, we evaluated the effect of ERK siRNA on calcium-induced assembly of tight junctions. Transfection of ERK siRNA markedly accelerated the calcium-induced restoration of the TER (Figure 4A) and development of the barrier to inulin (Figure 4B) in 3-day-old under-differentiated cell monolayers, compared with control-RNA-transfected cells. On the other hand, in 7-day-old differentiated cell monolayers, siRNA transfection resulted in a significant delay in the recovery of the TER (Figure 4C) and restoration of barrier function (Figure 4D) compared with cells transfected with control RNA.

To determine the effect of ERK siRNA on the state of differentiation we evaluated the effect of control non-specific RNA and ERK siRNA on alkaline phosphatase and villin levels. Our results show that transfection with the ERK siRNA produced no significant change in alkaline phosphatase or villin levels in 3- and 7-day-old cells (Supplementary Figures S2A and S2B at http://www.BiochemJ.org/bj/433/bj4330051add.htm).

Regulated expression of MEK demonstrates differentiation-dependent influence on tight junction integrity

In order to demonstrate further the role of ERK in the regulation of epithelial tight junctions in Caco-2 cells, we transiently expressed WT-MEK, DN-MEK or CA-MEK in under-differentiated (day 3) and differentiated (day 7) cells by doxycycline-mediated regulation of expression. The expression of MEK (WT and mutants) was confirmed by monitoring the expression of GFP in the cells at 6 h after addition of doxycycline in 3-day-old (Figure 5A) as well as 7-day-old cell monolayers (Figure 5B).

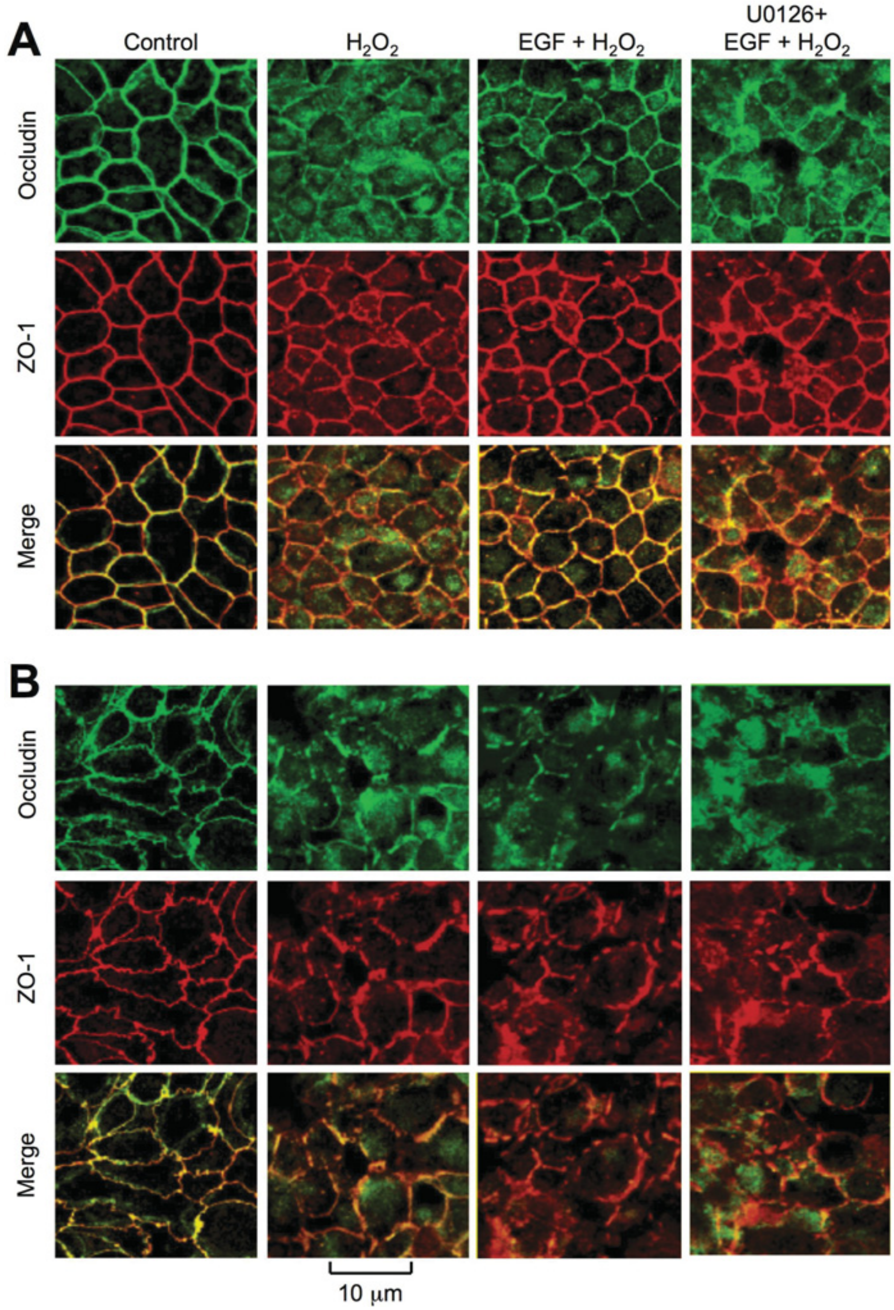

Figure 2 Contrasting effects of EGF on H_2O_2-induced disruption of tight junctions in under-differentiated and differentiated cell monolayers

(**A**) Differentiated 14-day-old or (**B**) under-differentiated 4-day-old Caco-2 cell monolayers were pretreated with 10 μM U0126 for 50 min prior to administration of 30 nM EGF. H_2O_2 (20 μM) was administered 10 min after EGF. Control cells without treatments, as indicated, are also shown. Cell monolayers were fixed and immunostained for occludin and ZO-1.

Confocal microscopy revealed that on day 3, expression of WT-MEK and CA-MEK led to a decreased localization of occludin and ZO-1 at the intercellular junctions. The redistribution of junctional proteins was more pronounced with the expression of CA-MEK compared with WT-MEK (Figure 5C). In contrast, expression of DN-MEK produced increased junctional localization of occludin and ZO-1. On the other hand, on day 7, expression of WT-MEK and CA-MEK resulted in an enhanced junctional localization of occludin and ZO-1, as compared with cells expressing vector alone (Figure 5D), whereas the expression of DN-MEK produced a decreased junctional localization of occludin and ZO-1.

We consistently observed the doxycycline-regulated expression of GFP–WT-MEK, GFP–DN-MEK and GFP–CA-MEK when examined by an immunofluorescence method and by RT–PCR for GFP. However, only weak bands of GFP–MEK were detected by immunoblotting analysis. We believe that MEK, being a tightly regulated signalling molecule in mammalian cells, is rapidly degraded when expressed at high levels. This degradation is probably enhanced during the extraction procedure during sample preparation for immunoblotting analysis. On the other hand, quick fixation of cell monolayers for immunostaining prevents such degradation.

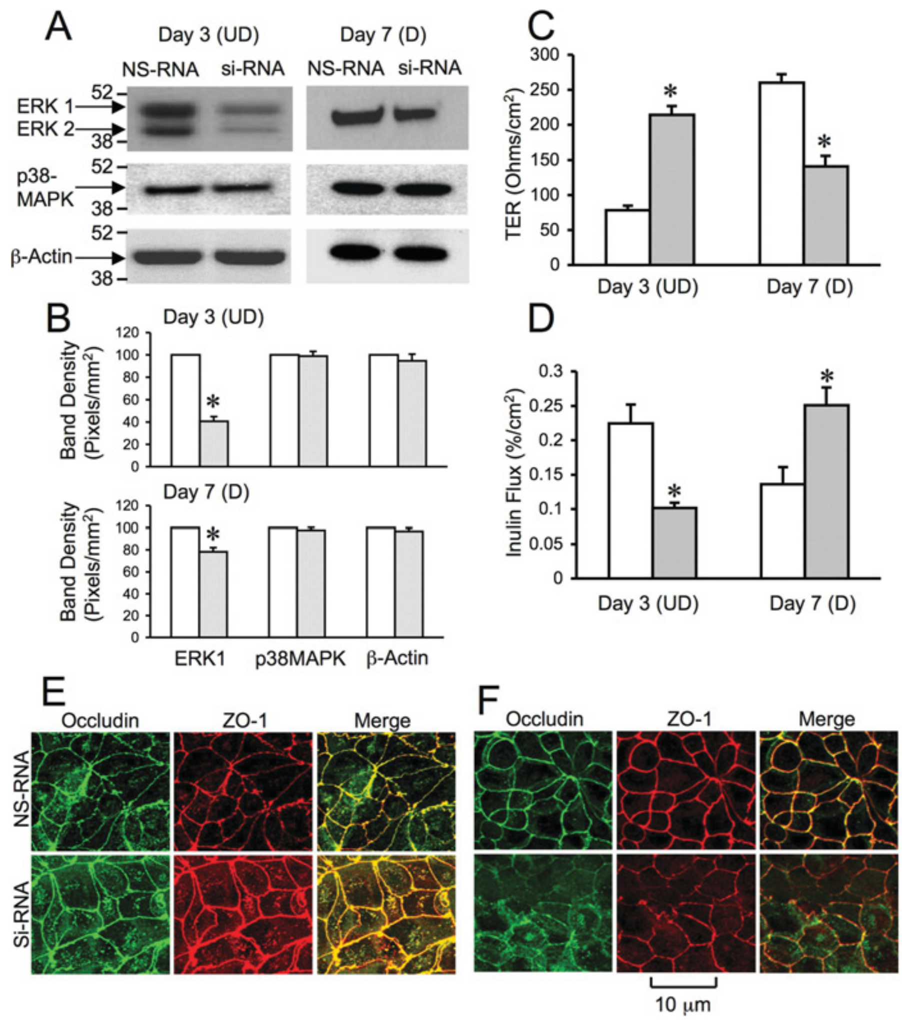

Figure 3 Contrasting effects of knockdown of ERK on epithelial barrier disruption in under-differentiated and differentiated cell monolayers

(**A**) 3-Day-old under-differentiated (UD) and 7-day-old differentiated (D) Caco-2 cells transfected with control siRNA (NS-RNA) or ERK1/2-specific siRNA (si-RNA) were immunoblotted for ERK, p38-MAPK and β-actin. The molecular mass in kDa is indicated. (**B**) Densitometric analysis of immunoblots shown in (**A**). Values are means ± S.E.M. ($n = 3$). *$P < 0.05$ compared with NS-RNA. (**C**) The TER and (**D**) inulin permeability were measured in cell monolayers transfected with NS-RNA (white bars) or ERK-specific siRNA (grey bars) on day 3 or 7 after seeding on transwell inserts. Values are means ± S.E.M. ($n = 6$). *$P < 0.05$ compared with NS-RNA values. Extracts from 3-day-old (**E**) or 7-day-old (**F**) cell monolayers transfected with NS-RNA or ERK-specific siRNA (Si-RNA) were fixed and immunostained for occludin and ZO-1.

Expression of WT-MEK and its mutants for a short time (18 h) did not have any effect on alkaline phosphatase or villin levels in under-differentiated (Supplementary Figurse S2C and S2E) or differentiated cells (Supplementary Figures S2D and S2E), thus indicating that ERK regulates the tight junction integrity without significantly altering the differentiation state of the cells. The alkaline phosphatase and villin levels were slightly higher in the absence of doxycycline in 7-day-old cells (Figure S2D), but not in 3-day-old cells (Figure S2C).

EGF modulates PP2A and PKCζ distribution

The studies mentioned above have demonstrated that ERK has contrasting effects on regulation of tight junctions in under-differentiated and differentiated cells. Evidence indicates that PP2A [14,35–38] and PKCζ [39] are involved in the regulation of threonine residue phosphorylation of occludin and that EGF prevents H_2O_2-induced threonine residue dephosphorylation of occludin in Caco-2 cell monolayers [22,40]. To determine whether the differentiation-dependent effects of ERK on tight junctions involved modulation of PKCζ and/or PP2A, we evaluated the effect of EGF and H_2O_2 in 4- and 14-day-old cell monolayers. The results showed that levels of PP2A, PKCζ and tight junction proteins, such as claudin-4, are not different in 4-day-old under-differentiated and 14-day-old differentiated cell monolayers (Figures 6A and 6B). Previous studies have demonstrated that PP2A directly interacts with occludin and regulates its phosphorylation on threonine residues [38]. Therefore we evaluated the effect of EGF and H_2O_2 on co-immunoprecipitation of occludin with PP2A or PKCζ in 4-day-old under-differentiated and 14-day-old differentiated cell monolayers. H_2O_2 treatment caused a significant increase in co-immunoprecipitation of PP2A with occludin (Figures 6C and 6D) in 14-day-old differentiated cell monolayers, but not in 4-day-old under-differentiated cell monolayers. EGF effectively decreased PP2A association with occludin and this effect of EGF was attenuated by U0126 (Figures 6C and 6D). Neither EGF nor U0126 had a significant influence on PP2A co-immunoprecipitation with occludin in 4-day-old cell monolayers. In contrast with PP2A, PKCζ co-immunoprecipitation with occludin was significantly

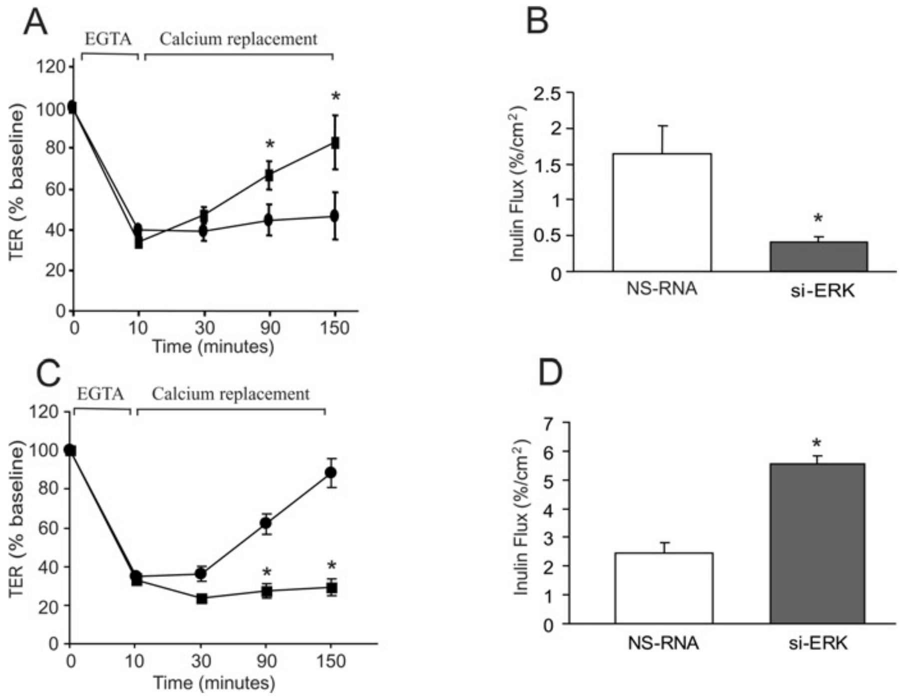

Figure 4 Contrasting effects of knockdown of ERK on the assembly of tight junctions in under-differentiated and differentiated cell monolayers

(**A** and **B**) Under-differentiated 3-day-old Caco-2 cells transfected with NS-RNA (●, white bars) or ERK-specific siRNA (■, grey bars) were subjected to calcium-switch-mediated tight junction assembly. (**A**) The TER was measured during EGTA treatment and calcium replacement. (**B**) Inulin permeability measured at 150 min after calcium replacement. (**C** and **D**) Tight junction assembly was also studied in differentiated 7-day-old cells transfected with control siRNA (NS-RNA; ●, white bars) or ERK-specific siRNA (si-ERK; ■, grey bars). (**C**) The TER and (**D**) inulin permeability were measured. Values are means $\pm$ S.E.M. ($n = 6$). $^*P < 0.05$ compared with NS-RNA values.

reduced by H_2O_2 treatment in 14-day-old differentiated cell monolayers (Figures 6C and 6E). EGF pretreatment abrogated the H_2O_2 effect on co-immunoprecipitation of PKCζ with occludin and this effect of EGF was attenuated by U0126 (Figure 6E). In 4-day-old under-differentiated cell monolayers, however, H_2O_2 and EGF failed to influence PKCζ association with occludin. The immunoprecipitation procedure using rabbit pre-immune IgG showed no precipitation of occludin or PP2A in control cell extracts (Supplementary Figure S3 at http://www.BiochemJ.org/bj/433/bj4330051add.htm).

Immunostaining and confocal microscopy showed that H_2O_2 did not affect the distribution of PKCζ in both 4-day-old under-differentiated and 14-day-old differentiated cell monolayers, whereas EGF enhanced junctional distribution of PKCζ in 14-day-old differentiated cell monolayers, but not in 3-day-old under-differentiated cell monolayers (Figure 7). This effect of EGF on PKCζ redistribution in 14-day-old cell monolayers was attenuated by U0126. EGF induced redistribution of PKCζ even in the absence of H_2O_2, whereas U0126 alone had no influence on PKCζ distribution.

Differential distribution of ERK in under-differentiated and differentiated cells

In order to determine whether the level and distribution of ERK are different in under-differentiated and differentiated cells, we analysed active ERK (p-ERK) and total ERK in these cells by immunoblotting and immunofluorescence methods. The level of total ERK in 14-day-old differentiated cells was higher than that in 4-day-old under-differentiated cells (Figures 8A and 8B). In contrast, the level of p-ERK was greater in 4-day-old under-differentiated cells compared with that in 14-day-old differentiated cells. EGF treatment rapidly and robustly increased p-ERK in 14-day-old differentiated cells. EGF-mediated activation of ERK was much more pronounced in 14-day-old differentiated cells compared with that in 4-day-old under-differentiated cells (Figure 8C). Immunofluorescence localization indicated that p-ERK was predominantly distributed in the intracellular compartment in 3-day-old under-differentiated cell monolayers, whereas in EGF-treated 14-day-old differentiated cell monolayers p-ERK was predominantly distributed at the peri-junctional region (Figure 8D).

A review of all of the ERK blots from the present study revealed that the levels of ERK2 were low in 7–14-day-old cells compared with in 3-day-old cells. However, when blotted for p-ERK, both bands appeared in equal amounts, indicating that active forms of ERK1 and ERK2 are present in both under-differentiated and differentiated cells, although the total ERK protein levels vary. The significance of this observation is unclear at this time.

DISCUSSION

ERK has a variable effect on the integrity of epithelial tight junctions; ERK can disrupt tight junction integrity in some cases [8,16,17,41], whereas it can also protect tight junction integrity in some other cases [21,22]. The mechanistic basis for this paradoxical ERK activity in tight junction regulation remained unclear. In the present study, we hypothesized that the state of cell differentiation is pivotal for ERK-induced differential regulation of tight junction integrity in intestinal epithelial cell monolayers. Towards this end, we have shown that ERK destabilizes tight junctions in under-differentiated epithelial cells, whereas it protects tight junction integrity in

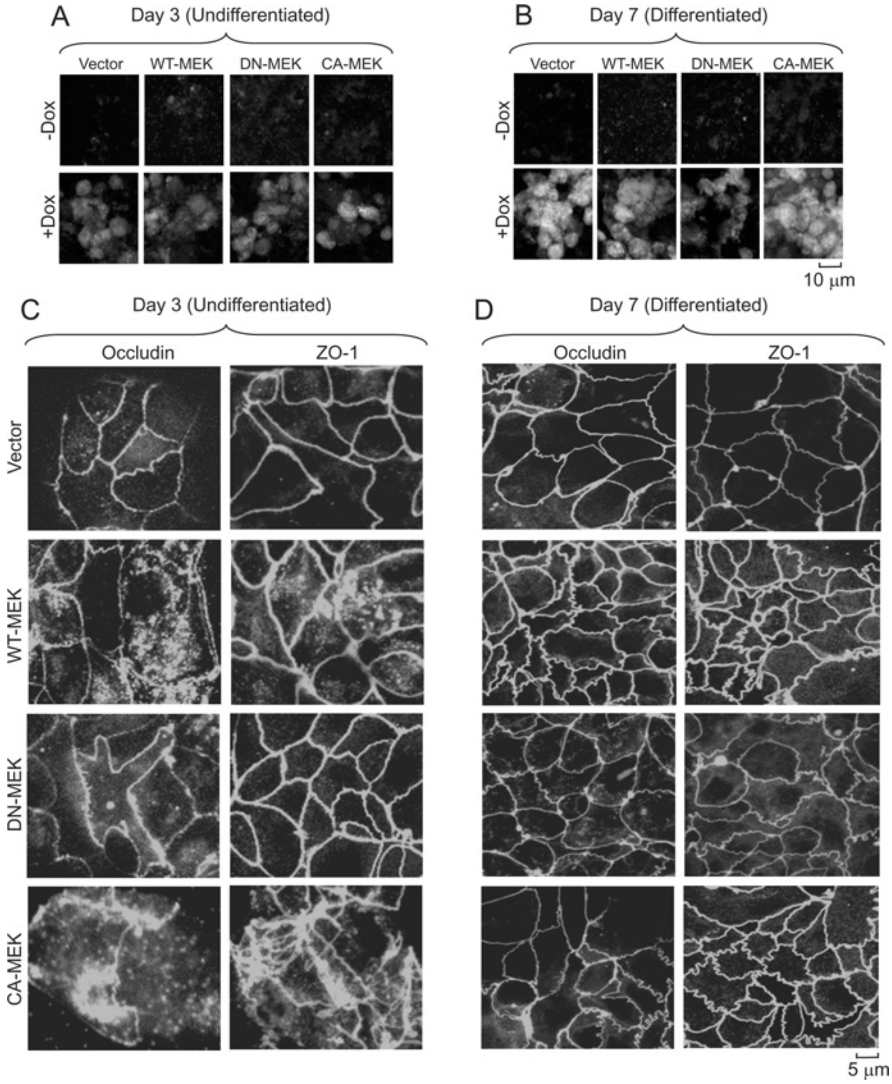

Figure 5 Effects of expression of active and inactive MEK1 on tight junction integrity at different stages of differentiation

(**A** and **B**) Caco-2 cells expressing Tet-on regulator were transfected with GFP or GFP-tagged WT-MEK1, DN-MEK1 or CA-MEK in pTRE2hyg-GFP-N vector, and the expression of GFP and MEK was induced by administering doxycycline on (**A**) day 3 or (**B**) day 7. Expression of GFP before and after doxycycline (Dox) administration was visualized by immunofluorescence staining and confocal microscopy. (**C**) Under-differentiated 3-day-old and (**D**) differentiated 7-day-old cell monolayers, in which MEK expression was induced by doxycycline, were fixed and stained for occludin and ZO-1.

differentiated epithelial cells. We conclude that differences in the spatial distribution of active ERK and its influence on distribution of PP2A and PKCζ contribute to the differential response to ERK in under-differentiated and differentiated Caco-2 cell monolayers.

Caco-2 cell monolayers at 3–4 days or 7–14 days post-seeding were used as models of under-differentiated and differentiated cell monolayers respectively. High alkaline phosphatase activity and villin expression confirmed the differentiation status of 7–14-day-old cells, as compared with 3–4-day-old cells. A rapid decrease in the TER and increase in inulin permeability upon treatment with H_2O_2 indicated that H_2O_2 disrupts the epithelial barrier function in both under-differentiated and differentiated Caco-2 cell monolayers. Additionally, H_2O_2 treatment resulted in redistribution of occludin and ZO-1 from the intercellular junctions in both under-differentiated and differentiated cell monolayers. Previous studies have shown that oxidative stress induced by H_2O_2 disrupts tight junctions in differentiated epithelial cells [14,22,36–38,42]. Furthermore, EGF, functioning as a gastrointestinal mucosal protective factor, is known to attenuate H_2O_2-induced disruption of tight junctions and barrier dysfunction [21,22]. EGF is also known to activate ERK1/2 [43–45] and prevent H_2O_2-induced barrier disruption by an ERK-dependent mechanism in Caco-2 cells [22]. In agreement with these previous studies, we have shown in the present study that in differentiated Caco-2 cell monolayers, EGF prevents H_2O_2-induced disruption of tight junctions by an ERK-dependent mechanism. In contrast to the protective effect of EGF in 14-day-old differentiated cell monolayers, in 4-day-old under-differentiated cell monolayers, EGF potentiates H_2O_2-induced barrier disruption. This effect was supported by an enhancement of the H_2O_2-induced redistribution of occludin and ZO-1 from the intercellular junctions. The inhibition of the EGF-mediated potentiation of the H_2O_2 effect upon U0126 treatment indicates that the effect of EGF in under-differentiated cell monolayers was also mediated by ERK.

The contrasting response of ERK in under-differentiated and differentiated cell monolayers was investigated further by

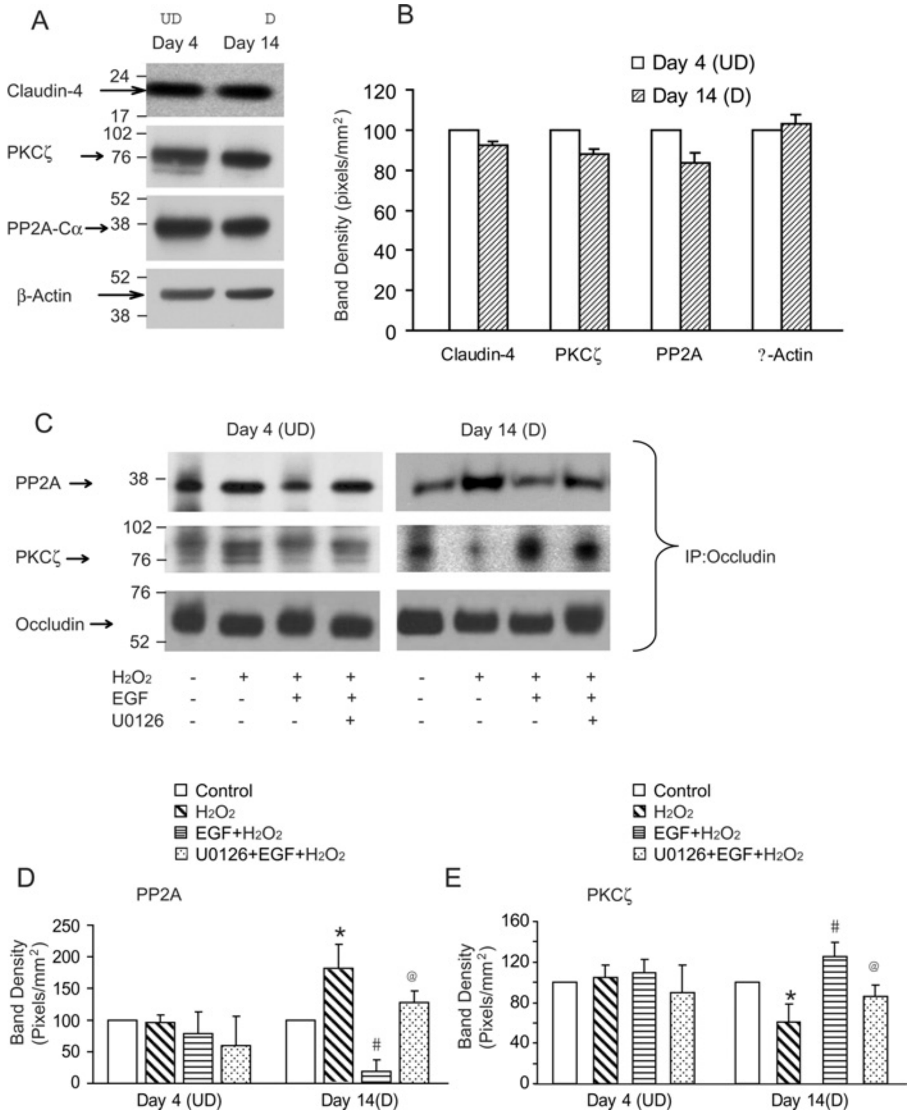

Figure 6 Differential effects of EGF on cellular distribution of PP2A and PKCζ in under-differentiated and differentiated cell monolayers

(**A** and **B**) Extracts from 4-day-old under-differentiated (UD) and 14-day-old differentiated (D) Caco-2 cells were (**A**) immunoblotted for claudin-4, PKCζ and PP2A-Cα and (**B**) the bands were quantified by densitometric analysis. The molecular mass in kDa is indicated. Values are means $\pm$ S.E.M. ($n = 3$). (**C**) Caco-2 cell monolayers were pretreated with 10 μM U0126 for 50 min prior to administration of 30 nM EGF. H_2O_2 (20 μM) was administered 10 min after EGF. Control cells without treatments, as indicated, are also shown. Occludin was immunoprecipitated (IP) from detergent-insoluble fractions of 4-day-old or 14-day-old old Caco-2 cells and immunoblotted for PKCζ and PP2A-Cα. The molecular mass in kDa is indicated. Densitometric analysis of (**D**) PP2A-Cα and (**E**) PKCζ bands in experiments described in (**C**). Values are means $\pm$ S.E.M. ($n = 3$–5). $^*P < 0.05$ compared with control values, $\#P < 0.05$ compared with the corresponding H_2O_2 values, @$P < 0.05$ compared with the corresponding EGF+H_2O_2 values.

knockdown of ERK1/2 using siRNA. By using siRNA, we were able to show a significant reduction in ERK levels at both 3 and 7 days post-transfection. The present study shows that knockdown of ERK increases the TER and decreases inulin permeability in 3-day-old under-differentiated cell monolayers, indicating an enhancement of tight junction barrier function. In contrast, the TER was reduced and inulin permeability was elevated in 7-day-old differentiated cell monolayers transfected with siRNA against ERK. The junctional distribution of occludin and ZO-1 was enhanced by ERK siRNA, but not control RNA in 3-day-old cell monolayers, whereas, it was reduced in 7-day-old cell monolayers. These results demonstrate further that ERK exerts disruptive influence on tight junctions in under-differentiated cells, whereas it enhances tight junction integrity in differentiated cell monolayers.

The influence of ERK on assembly of tight junctions in under-differentiated and differentiated cells was determined by the calcium switch method. The calcium-mediated restoration of barrier function was significantly faster in ERK siRNA-transfected 3-day-old under-differentiated cell monolayers compared with that in control siRNA-transfected cells. In contrast, reassembly of tight junctions by calcium was delayed by knockdown of ERK in 7-day-old differentiated cell monolayers. These observations suggest that ERK activity directly influences the mechanisms involved in tight junction assembly.

Most importantly, our studies show that the switch in the ERK effect on tight junction integrity occurs at approx. 5 days post-seeding. This observation is complementary to previous reports by Nelson and co-workers that Caco-2 cells become differentiated on day 4 or 5 [46,47]. We evaluated the effect of reduced expression

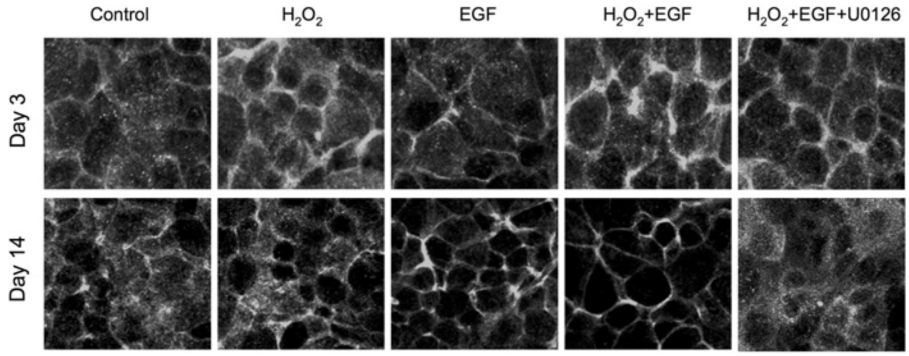

Figure 7 Differential effects of EGF on redistribution of PKCζ in under-differentiated and differentiated cell monolayers

Under-differentiated 3-day-old and differentiated 14-day-old cell monolayers were pretreated with 10 μM U0126 for 50 min prior to administration of 30 nM EGF. H_2O_2 (20 μM) was administered 10 min after EGF. Control cells without treatments, as indicated, are also shown. Cell monolayers were fixed and immunostained for PKCζ.

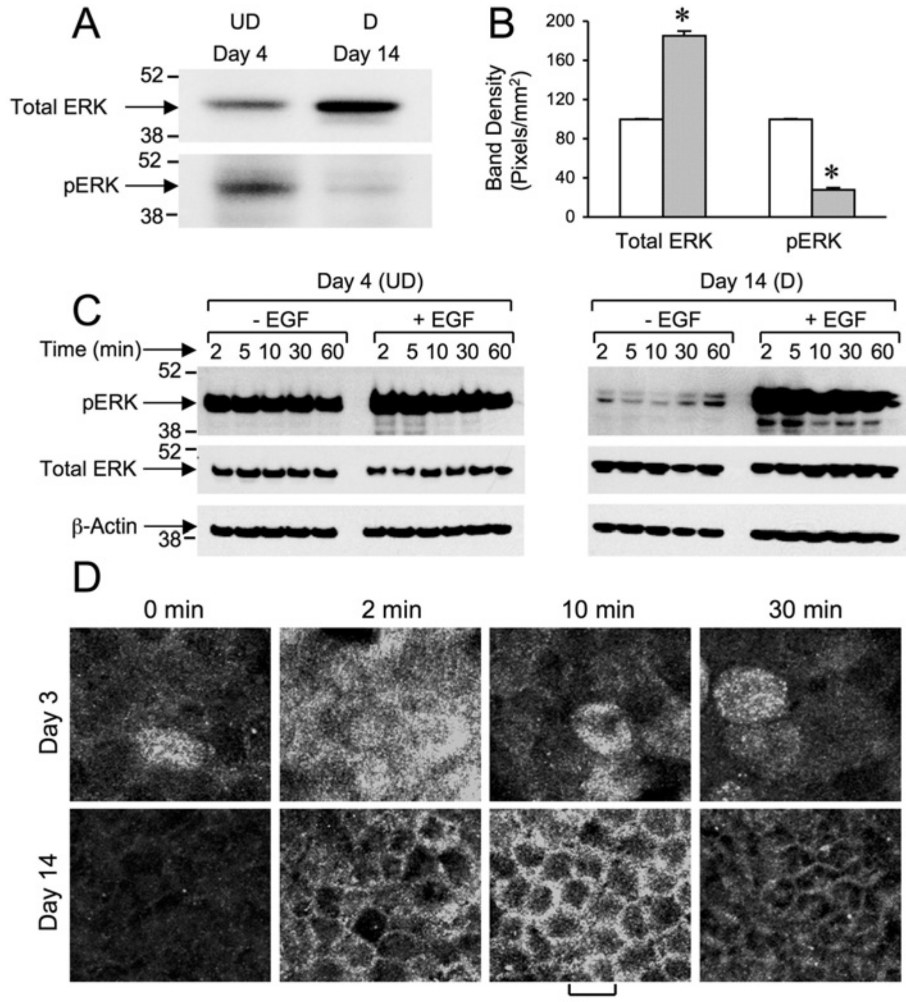

Figure 8 Differential distribution of active ERK in EGF-treated cell monolayers

(**A** and **B**) Extracts from under-differentiated (UD) 4-day-old and differentiated (D) 14-day-old Caco-2 cell monolayers were immunoblotted for total ERK and p-ERK (active), and the bands were quantified by densitometric analysis. Values are means $\pm$ S.E.M. ($n = 6$). $^*P < 0.05$ compared with corresponding 3-day-old values. (**C**) Caco-2 cell monolayers were incubated with or without EGF for various times and the cell extracts were immunoblotted for total and p-ERK. The molecular mass in kDa is indicated. (**D**) Caco-2 cell monolayers were incubated with EGF for varying times and the cell monolayers fixed and immunostained for p-ERK.

of ERK using siRNA on days 3, 4, 5 and 6 and revealed that tight junctions in ERK-knockdown cells were stronger than in the control cells on days 3 and 4, whereas the tight junction was weaker than in the control cells on days 5 and 6. Therefore Caco-2 cells appear to switch into the differentiated state at approx. day 4 in cell culture. Owing to the low stability of siRNA and slight leakiness of the pTRE vector, we used day 7 to represent differentiated cells in the present study.

Expression of WT-MEK and CA-MEK on day 3 caused disrupted junctional organization of occludin and ZO-1 compared with that in vector-transfected cells, whereas the expression of DN-MEK enhanced the organization of occludin and ZO-1 at the intercellular junctions. In contrast, on day 7, expression of WT-MEK and CA-MEK enhanced the junctional organization of occludin and ZO-1, and the expression of DN-MEK showed a small, but significant, disruption of junctional occludin and ZO-1. These studies demonstrate further that ERK has contrasting influences on the tight junction integrity in under-differentiated and differentiated cell monolayers. Therefore the state of differentiation of epithelial cells may determine the type of influence on tight junction integrity by ERK. Expression of siRNA or MEK did not alter the levels of alkaline phosphatase or villin, indicating that the differentiation state of the cells was unaffected. This observation indicated that ERK plays a direct role in the regulation of tight junction integrity. Our study also raises the point that the weaker tight junction integrity in under-differentiated cells is likely to be caused by higher ERK activity, as shown by the increase in tight junction integrity in these cells upon knockdown of ERK.

The mechanism involved in the differential response to ERK in under-differentiated and differentiated cell monolayers is unclear. It is likely that downstream signalling events in response to ERK activation may be different in these cells. Our previous study showed that EGF prevented H_2O_2-induced dephosphorylation of occludin by an ERK-dependent mechanism [22]. Occludin is known to be highly phosphorylated on threonine residues in epithelium with intact tight junctions [10–13], whereas it is dephosphorylated during the disruption of tight junctions by calcium depletion [12–14,38], phorbol esters [48] or acetaldehyde [14]. Occludin phosphorylation appears to be required for the assembly of tight junctions [14]. Evidence indicates that PP2A [14,35–38] and PKCζ [39] play roles in the regulation of threonine residue phosphorylation of occludin. A previous study demonstrated that H_2O_2-induced occludin dephosphorylation is mediated by PP2A translocation to tight junctions [40]. The effect of H_2O_2 on PKCζ association with tight junctions was hitherto unknown. Therefore we investigated the effect of H_2O_2 and EGF on the association of PP2A and PKCζ with occludin, and their distribution in 4-day-old under-differentiated and 14-day-old differentiated cell monolayers.

In differentiated cell monolayers, H_2O_2 enhanced co-immunoprecipitation of PP2A with occludin, whereas it decreased the association of PKCζ with occludin. This increased association of PP2A and reduced association of PKCζ may explain the previous observation that H_2O_2 rapidly dephosphorylated occludin on threonine residues. In differentiated cells, only EGF attenuated the H_2O_2-induced increase in PP2A association with occludin, and decreased the PKCζ association with occludin, via an ERK-dependent mechanism. Such a regulation of PP2A and PKCζ association with occludin by H_2O_2 and EGF was absent in under-differentiated cell monolayers. The effect of EGF on PKCζ redistribution was further confirmed by confocal immunofluorescence localization. EGF induced an enhanced localization of PKCζ at the intercellular junctions via an ERK-dependent mechanism in 14-day-old differentiated cell monolayers, whereas such an effect was not seen in 4-day-old under-differentiated cell monolayers. These observations suggest that the signalling events downstream of ERK activation are distinct and different in under-differentiated and differentiated cells.

Another probable difference in ERK signalling in under-differentiated and differentiated cells may involve differential distribution of active p-ERK in the cells. Analysis of ERK and p-ERK indicated that much higher levels of active ERK are present in under-differentiated cells compared with those in differentiated cell monolayers, although the level of total ERK is greater in differentiated cells. EGF induced a rapid and robust increase in active ERK in differentiated cells. On the other hand, in under-differentiated cells, EGF induced only a slight increase in active ERK levels, which were already high prior to EGF stimulation. Immunofluorescence microscopy indicated that active ERK in under-differentiated cells was distributed in the intracellular compartment, whereas significant amounts of active ERK were localized at the perijunctional region in differentiated cell monolayers. Therefore the differences in the subcellular distribution of activated ERK may contribute to different downstream signalling events in under-differentiated and differentiated cells. The differential distribution of p-ERK may be responsible for its distinct effects on PP2A and PKCζ in differentiated and under-differentiated cells.

In conclusion, the present study demonstrates that ERK has a disruptive influence on tight junctions in under-differentiated Caco-2 cells, whereas it has a protective role on tight junctions in differentiated cell monolayers. This difference in response may be caused by differences in ERK distribution and its downstream signalling leading to a change in its influence on PP2A and PKCζ association with tight junction proteins.

AUTHOR CONTRIBUTION

Sudhir Aggarwal, Takuya Suzuki and William Taylor contributed by conducting the experiments and processing the data. Radhakrishna Rao was responsible for designing the experimental setup and directing co-authors in the execution of the experiments. Sudhir Aggarwal and Radhakrishna Rao also contributed by participating in manuscript writing. Aditi Bhargava contributed by generating specific reagents for some of the studies.

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SUPPLEMENTARY ONLINE DATA

Contrasting effects of ERK on tight junction integrity in differentiated and under-differentiated Caco-2 cell monolayers

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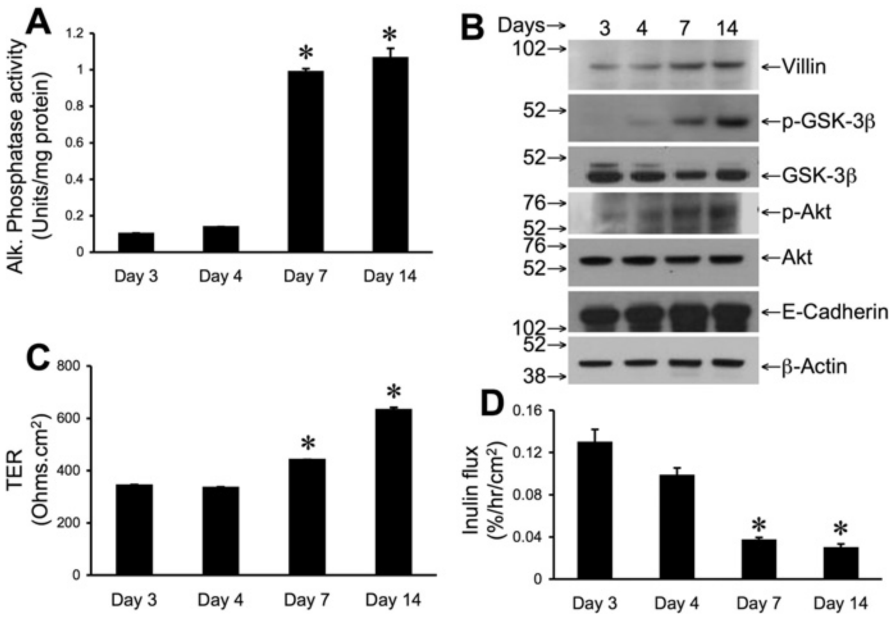

Figure S1 Differentiation markers in under-differentiated and differentiated cells

Proteins extracted from Caco-2 cell monolayers on different days post-seeding were analysed for (**A**) alkaline (Alk.) phosphatase activity and (**B**) immunoblotted for different proteins. The molecular mass in kDa is indicated. Cell monolayers were also analysed for the barrier function by evaluating (**C**) the TER and (**D**) inulin permeability. Values are means ± S.E.M. ($n = 6$). *$P < 0.05$ compared with corresponding values for 3- or 4-day-old cells.

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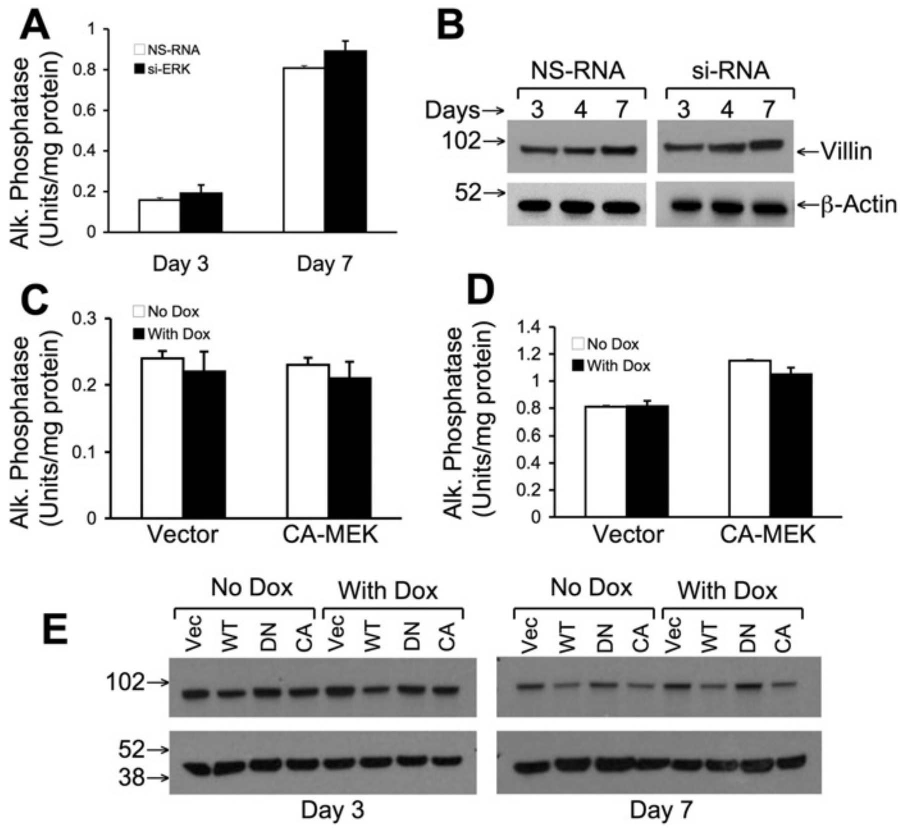

Figure S2 Effect of MAPK expression on differentiation markers

(**A** and **B**) Caco-2 cells were transfected with control (NS-RNA) or ERK-specific siRNA (si-RNA). At various days post-transfection protein extracts were analysed for (**A**) alkaline (Alk.) phosphatase activity and (**B**) villin. Alkaline phosphatase activity was also measured in vector- or CA-MEK-transfected cells on (**C**) day 3 or (**D**) day 7. (**E**) Extracts from cells transfected with vector (Vec), WT-MEK (WT), DN-MEK (DN) or CA-MEK (CA) were immunoblotted for villin (upper panel) and actin (lower panel). The molecular mass in kDa is indicated. Dox, doxycycline.

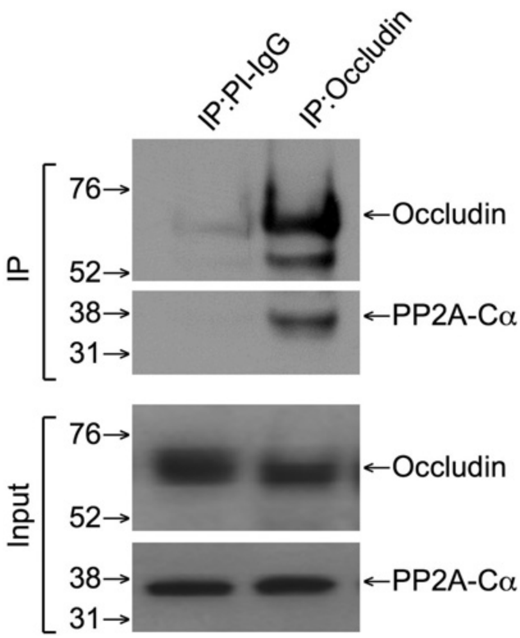

Figure S3 Specificity of occludin immunoprecipitation

Protein extracts from Caco-2 cells were subjected to immunoprecipitation (IP) using anti-occludin antibody (2 μg) or rabbit pre-immune IgG (5 μg) and the isolated immunocomplexes were immunoblotted for occludin and PP2A-Cα. The input shows the total protein extracts (10 μg of protein) also immunoblotted for occludin and PP2A-Cα.

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