BMC Cell Biology

Research article

Development of a serum-free co-culture of human intestinal epithelium cell-lines (Caco-2/HT29-5M21)

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Abstract

Background: The absorptive and goblet cells are the main cellular types encountered in the intestine epithelium. The cell lineage Caco-2 is a model commonly used to reproduce the features of the bowel epithelium. However, there is a strong debate regarding the value of Caco-2 cell culture to mimick *in vivo* situation. Indeed, some authors report in Caco-2 a low paracellular permeability and an ease of access of highly diffusible small molecules to the microvilli, due to an almost complete lack of mucus. The HT29-5M21 intestinal cell lineage is a mucin-secreting cellular population. A co-culture system carried out in a serum-free medium and comprising both Caco-2 and HT29-5M21 cells was developed. The systematic use of a co-culture system requires the characterization of the monolayer under a given experimental procedure.

Results: In this study, we investigated the activity and localization of the alkaline phosphatase and the expression of *IAP* and *MUC5AC* genes to determine a correlation between these markers and the cellular composition of a differentiated monolayer obtained from a mixture of Caco-2 and HT29-5M21 cells. We observed that the culture conditions used (serum-free medium) did not change the phenotype of each cell type, and produced a reproducible model. The alkaline phosphatase expression characterizing Caco-2 cells was influenced by the presence of HT29-5M21 cells.

Conclusion: The culture formed by 75% Caco-2 and 25% HT29-5M21 produce a monolayer containing the two main cell types of human intestinal epithelium and characterized by a reduced permeability to macromolecules.

Background

Among human intestinal diseases, intestinal inflammatory disorders, such as food allergies and Crohn's disease, are characterized by an increase in intestinal permeability

[1-4]. The *in vitro* study of these pathologies as well as of permeability modulators requires the use of a cell model representing as closely as possible the physiological conditions.

The small intestinal epithelium is the main barrier preventing the molecules from the lumen (e.g. food, toxins) to reach the blood compartment [5]. This epithelium is composed of several cell types: enterocyte, goblet cell, paneth cell, endocrine cell and stem cell [6,7]. The absorptive and goblet cells are the two main cellular types in the intestinal epithelium. The apical side of the enterocytes is characterized by a brush border which contains several enzymes and which increases the surface for nutrient absorption. The goblet cells secrete a mucus [8,9], which covers the apical membrane of intestinal cells and limits molecule absorption [10,11]. The cellular phenotypes in the epithelium are influenced by cell-cell and cell-matrix interactions and are defined by the expression of specific genes [12].

In vitro models are increasingly developed to study drug and nutrient transport across the intestinal epithelium [13-16]. The intestinal cell lineage Caco-2 is the most commonly used cell model [17,18]. However, some authors report in Caco-2 a low paracellular permeability [14,16,19] and an ease of access of highly diffusible small molecules to the microvilli, due to an almost complete lack of mucus [16]. Caco-2 cells were obtained from human colon adenocarcinoma [20]. It differentiates spontaneously when it grows to confluence [21]. The alkaline phosphatase (EC 3.1.3.1.), coded by the *IAP* gene, is an enzyme widely used as a marker of differentiation in the Caco-2 cell type [22,23]. The parental HT29 cell line was obtained from human colorectal cancer [24]. The HT29-5M21 cell line results from the isolation of HT29 cells adapted to methotrexate (MTX, 10^{-5} M) [25,26]. The differentiation of goblet cells is characterized by the secretion of several mucins [27]. The *MUC5AC* mucin gene, usually expressed in the stomach, accounts for the major expressed mucin gene in HT29-MTX [28,29].

The aim of our study is the development of a serum-free co-culture of human intestinal epithelium cell lines. This reproducible co-culture will form an epithelial monolayer exhibiting the two main cellular types encountered in the human intestinal epithelium. The systematic use of co-culture systems requires the characterization of the monolayer under a given experimental procedure. In this study, we investigated the activity and localization of alkaline phosphatase and the expression of *IAP* and *MUC5AC* genes to determine a correlation between these markers and the cellular composition of a differentiated monol-

ayer obtained from a mixture of Caco-2 and HT29-5M21 intestinal cells.

Results

Co-culture characterization
Microscopy analysis of the Caco-2/HT29-5M21 (seeding ratio 3:1) co-cultures showed a monolayer of differentiated cells with microvilli (Fig. 1). Monolayer formed domes which have been shown to exhibit signs of enterocytic differentiation and transport properties. Viability of the Caco-2/HT29-5M21 (seeding ratio 3:1) co-cultures was assayed by fluorescent dye exclusion (Fig. 2). Living cells were observed with a green fluorescent nuclei. At the end of the culture (21 days after confluence) few cells were observed with an orange fluorescent nuclei/cytoplasm.

Permeability properties
Transepithelial electrical resistance (TEER) measurement was assayed 21 days after confluence on Caco-2/HT29-5M21 co-cultures at different seeding ratio (Fig. 3). Results showed a decrease of the TEER with the decrease of the Caco-2 proportion. Monolayer permeability to lucifer yellow and to 20 kDa fluorescent-dextran was assayed 21 days after confluence on Caco-2/HT29-5M21 co-cultures at different seeding ratio (Fig. 4). Permeability of Caco-2/HT29-5M21 co-cultures was 10-fold higher to Lucifer yellow than to 20 kDa-dextran. Results showed a decrease of the permeability to these molecules with the increase of the Caco-2 proportion in the co-culture.

Enzyme activity and localization
In order to study the relationship between enzyme activity and the cellular composition of the monolayer, experiments concerning the specific activity of alkaline phosphatase and enzyme localization were conducted in cell cultures. We used Caco-2 cells, HT29-5M21 cells and co-cultures of these two cell types (seeding ratios 1:3, 2:2, 3:1). The alkaline phosphatase specific activity (SA) was detected in the Caco-2 cultures, whereas no activity was detected in the HT29-5M21 cultures. In the co-cultures and in the cell homogenate mixtures, the SA of alkaline phosphatase increased with the proportion of Caco-2 ($p < 0.001$ in both cases). The alkaline phosphatase SA was significantly ($p < 0.001$) greater in the co-cultures than in the mixtures of Caco-2 and HT29-5M21 homogenates (Fig. 5). Comparisons of the pairs showed that this general effect was the result of the 2:2 and 3:1 ratios, where the alkaline phosphatase SA was significantly (respectively $p < 0.01$ and $p < 0.001$) greater in the co-cultures than in the mixtures of Caco-2 and HT29-5M21 homogenates. The specific detection of alkaline phosphatase activity was further investigated by cytochemistry. Results showed a significant linear correlation ($r = 0.960$, $p < 0.001$) between alkaline phosphatase positive-cells and Caco-2 proportion in the culture. However, Caco-2 culture did not show
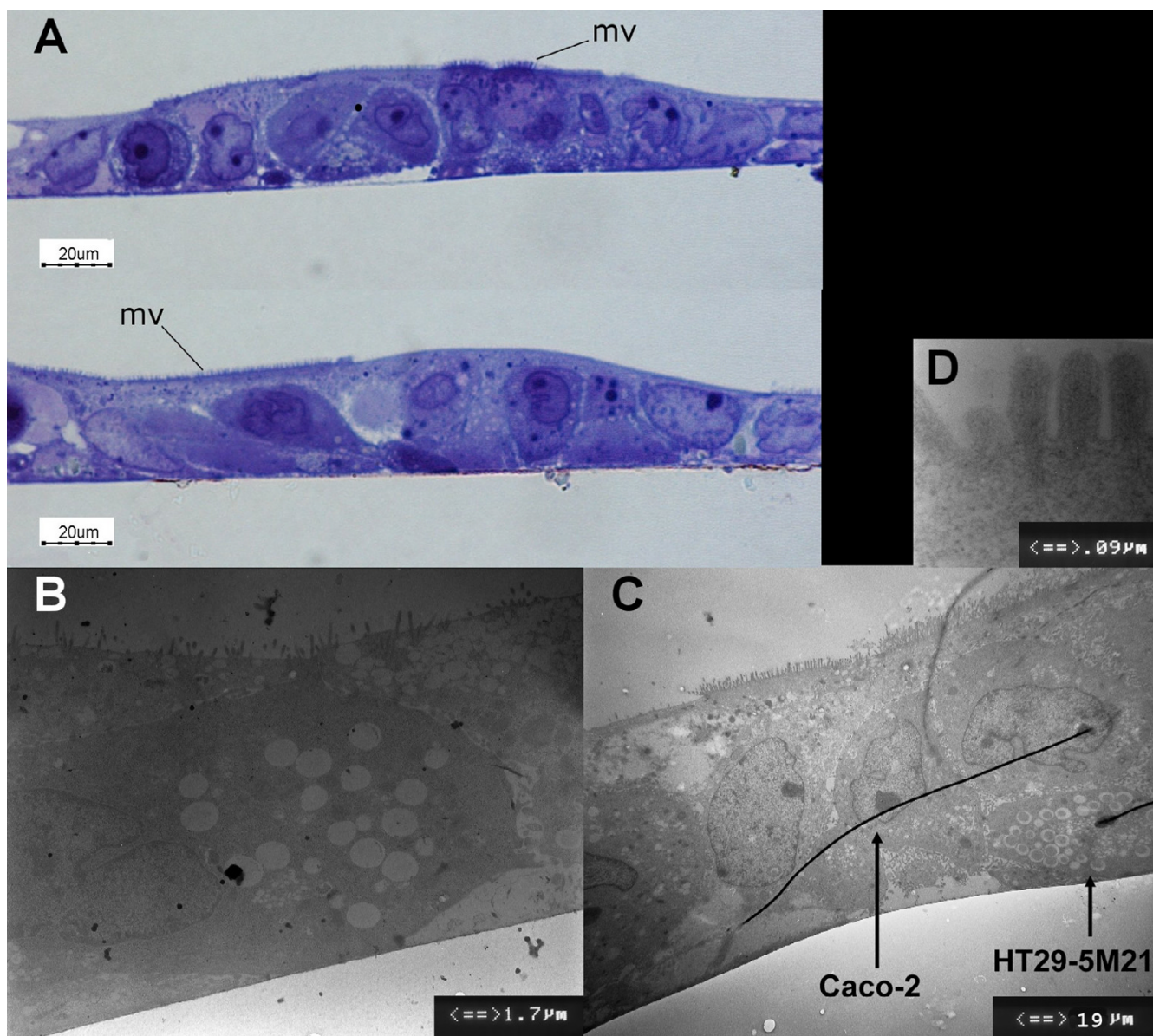
Figure 1
Semi-thin cross sections (toluidine blue staining) (A) and electronic microscopy ultra-thin sections (B-D) of Caco-2/HT29-5M21 co-culture monolayer (seeding ratio 3:1). Mv: microvilli.

an alkaline phosphatase localization on the whole culture surface (Fig. 6). A progressive bias towards the Caco-2 proportion and the alkaline phosphatase-positive area resulted from a 2:2 Caco-2/HT29-5M21 seeding ratio.

Specificity of the primers

The specificity of the primer pairs (IAP hu Fo/Ba, MUC5AC Fo/Ba, bACT hu Fo/Ba – see table 1) was tested on Caco-2 and HT29-5M21 cDNA. Caco-2 cDNA gave rise to a 452 bp amplicon with IAP hu Fo/Ba primers and was

not amplified with MUC5AC Fo/Ba primers (Fig. 7). HT29-5M21 cDNA gave rise to a 408 bp amplicon with MUC5AC Fo/Ba primers and was not amplified with IAP hu Fo/Ba primers (Fig. 7). In the Caco-2/HT29-5M21 co-culture a 452 or a 408 bp amplicon was detected with, respectively, IAP hu Fo/Ba and MUC5AC Fo/Ba primer pairs (Figs. 7). The bACT hu Fo/Ba primer pairs amplified a 485 bp fragment in Caco-2 and HT29-5M21 cDNA (Fig. 7).
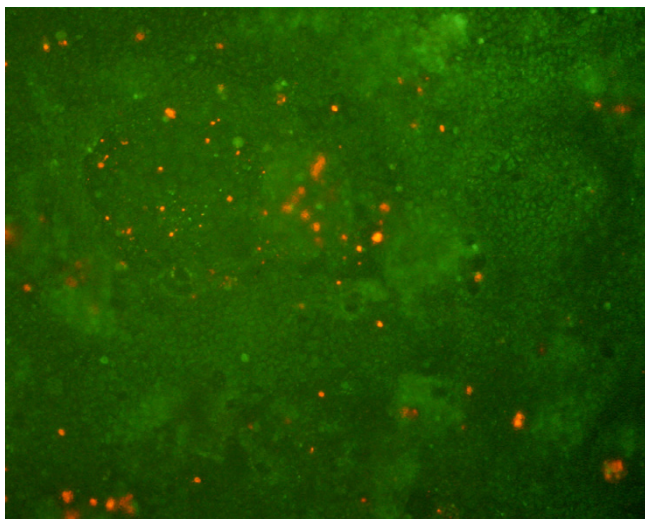
Figure 2
Fluorescent dye exclusion in Caco-2/HT29-5M21 co-culture monolayer (seeding ratio 3:1) 21 days after the confluence.

Expression of IAP and MUC5AC genes

The expression level of *IAP* and *MUC5AC* genes was determined by real-time PCR in the Caco-2 and HT29-5M21 co-cultures. After 40 PCR cycles, the fluorescence, corresponding to SYBER Green® binding, was recorded and plotted against the cycle number (data not shown).

Our results showed that the threshold cycle number (Ct) value, corresponding to the amount of *IAP* cDNA, is the same in the Caco-2 culture and in co-cultures at different
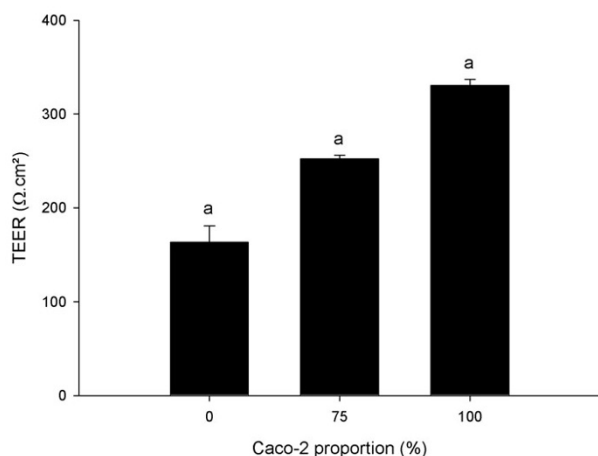

Figure 3
Transepithelial Electrical resistance of Caco-2/HT29-5M21 co-culture monolayer 21 days after the confluence. Results are expressed as $\Omega.cm^2$. Groups sharing the same letter are significantly different (^{a} p < 0.001, N ≥ 4).
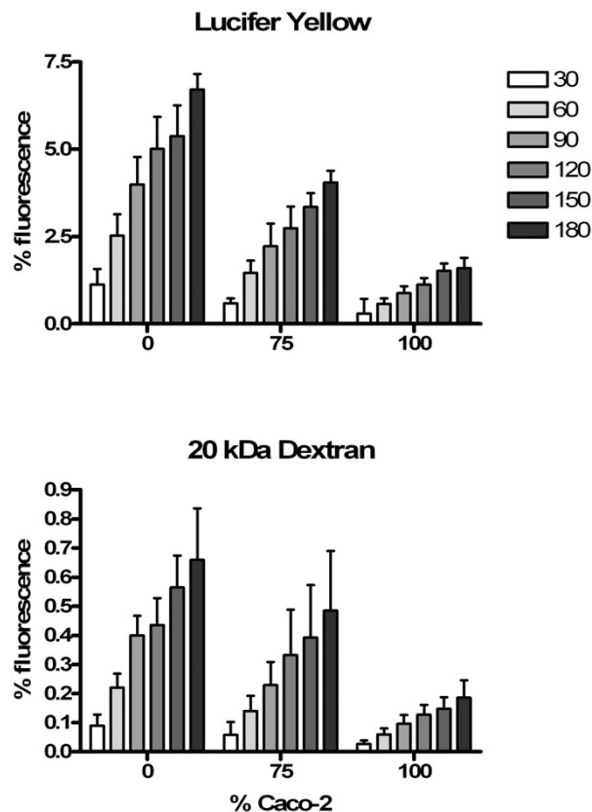

Figure 4
Lucifer yellow (452 Da) and dextran (20 kDa) fluorescence in the basolateral pole compartment of Caco-2/HT29-5M21 co-culture monolayer 21 days after the confluence. Basolateral pole compartment samples were analysed 30 to 180 minutes after permeability tracer addition into the apical compartment. Results are expressed as % of total fluorescence.

seeding Caco-2/HT29-5M21 proportions (1:3, 2:2 and 3:1; Fig. 8). With regard to the HT29-5M21 cDNA, no amplification product was detected until a Ct value corresponding to the value of the negative control was obtained without template. The Ct value did not differ significantly with the proportion of Caco-2 cells in the culture. The same results (Ct ~ 27) were obtained for *MUC5AC* (data not shown).

Discussion

To understand the cellular events leading to a leaky gut in pathologies such as inflammatory bowel diseases, an *in vitro* approach using cell models is required. The intestinal cell lineage Caco-2 is the cell model most commonly used to reproduce the features of the human small intestine epithelium [17,18]. Nevertheless, the Caco-2 cell model is different in some respects to the human epithelium. For example, it has a low paracellular permeability and allows
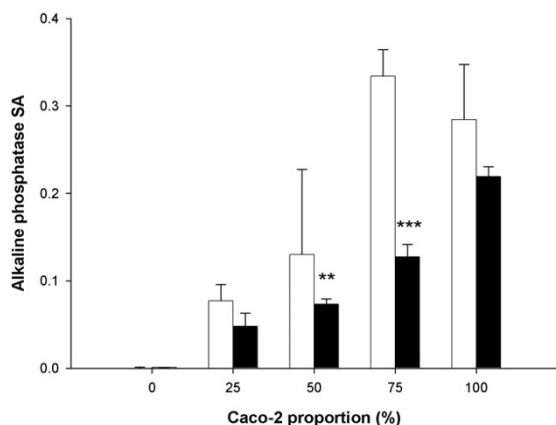
Figure 5
Alkaline phosphatase specific activity measured in Caco-2/
HT29-5M21 co-cultures (open bars) and in mixtures (black
bars) of these cells in the proportions 3:1, 2:2, 1:3. Results
are expressed as specific activity (SA, μmol/min.mg protein).
**p < 0.01, ***p < 0.001 (N = 3).

access of highly diffusible small molecules to the micro-
villi too easily [14,17,19,30,31], owing to the lack of an
adequate mucus layer. As an alternative, therefore, we
analyzed the properties of a co-culture of Caco-2 cells and
HT29-5M21 cells differentiating spontaneously into
mucus-secreting cells. This co-culture system exhibits the
two main important cellular types encountered in the
human intestinal epithelium. In order to make this proce-
dure reliable and reproducible, we replaced the usual
serum-containing culture medium with a serum-free
medium supplemented with hormones and amino acids.
Although the proportions of both cellular types are deter-
mined at culture seeding, they can rapidly modify during
culture owing to the ability of each cell line to adhere to
and to proliferate on the culture substrate. More, the het-
erogeneity of Caco-2 cells between 0 and 30 days of con-
fluence [32] can deeply influence the co-culture
composition.

A brief characterization of the Caco-2/HT29-5M21 co-cul-
ture, initially seeded with a 3:1 ratio, was performed.
Results showed a monolayer containing few cells with
condensed DNA (ethidium bromide accumulation). The
ethidium bromide accumulation may reflect an early pro-
liferative/apoptotic activity and modified nucleic acid
expression. Indeed, an increase in DNA content is a fea-
ture of actively-proliferating cells that would therefore
exhibit higher fluorescence peaks when labeled by ethid-
ium bromide [33]. Moreover, previous studies reported
the appearance of an increase in DNA staining by ethid-
ium bromide during the early phases of apoptosis, known
as the hyperdiploid peak, [34]. On the other hand, intrac-

ellular ethidium bromide fluorescence could also be
enhanced by an increase in DNA base pair accessibility
associated with the differentiation state [35]. Then, a
recent study indeed suggests that cell proliferation and
apoptosis can co-exist [36]. In our case we can not specu-
late between these possibilities. Whatever the solution
may be, our results show that our co-culture has a high
proportion of living cells.

For this study, we used alkaline phosphatase as an indica-
tor of the cell proportion in co-culture models. The alka-
line phosphatase is a membrane-bound enzyme localized
in the brush border of enterocytes in the human intestinal
epithelium and was used previously by Matsumoto *et al*.
[23] to monitor the differentiation of enterocyte-like cells.
In order to analyze the correlation between the propor-
tion of the enterocyte-like cells and the alkaline phos-
phatase in the Caco-2/HT29-5M21 co-cultures, we
estimated the enzyme SA. This enzyme activity is
increased with the Caco-2 proportion. However, the alka-
line phosphatase SA in co-cultures seems to be higher
than in cell mixtures with the same proportions (1:3, 2:2
and 3:1). This result suggests a positive interaction
between Caco-2 and HT29-5M21 cells. This interaction
could lead to an increase of the gene expression, an
increase of the translation rate or a decrease of enzyme
degradation. This last possibility was already proposed by
Matsumoto *et al*. [23] during cell differentiation.

The alkaline phosphatase activity was detected in co-cul-
tures by the cytochemistry method. Our results show that
the alkaline phosphatase was restricted to the enterocyte-
like cell and could then be used as a potential marker for
the monitoring of these cells under several co-culture con-
ditions. The discrepancy between the alkaline phos-
phatase-positive area and the Caco-2 seeding proportion
in culture could be explained by the heterogeneity of the
cell lineage [32]. Indeed, this cell line differentiates spon-
taneously in enterocyte-like cells, but also in other cell
types which do not express alkaline phosphatase. Our
results underline this heterogeneity, as we observed, by
cytochemistry at cellular level, several red-scales (meaning
alkaline phosphatase activity) in the same culture. This
differentiation seems to be influenced by the Caco-2/
HT29-5M21 co-culture and thus by a cell-cell interaction.

In order to collect more data on the proportionate compo-
sition of the monolayer, we investigated the expression
level of the alkaline phosphatase coding gene and of the
MUC5AC gene. Though the two cell lineages were both of
an adenocarcinoma origin [16,21,24], we showed that the
IAP and *MUC5AC* gene expressions are restricted, respec-
tively, to Caco-2 cells and HT29-5M21 cells. We showed
by real-time PCR that the expression level of the *IAP* and
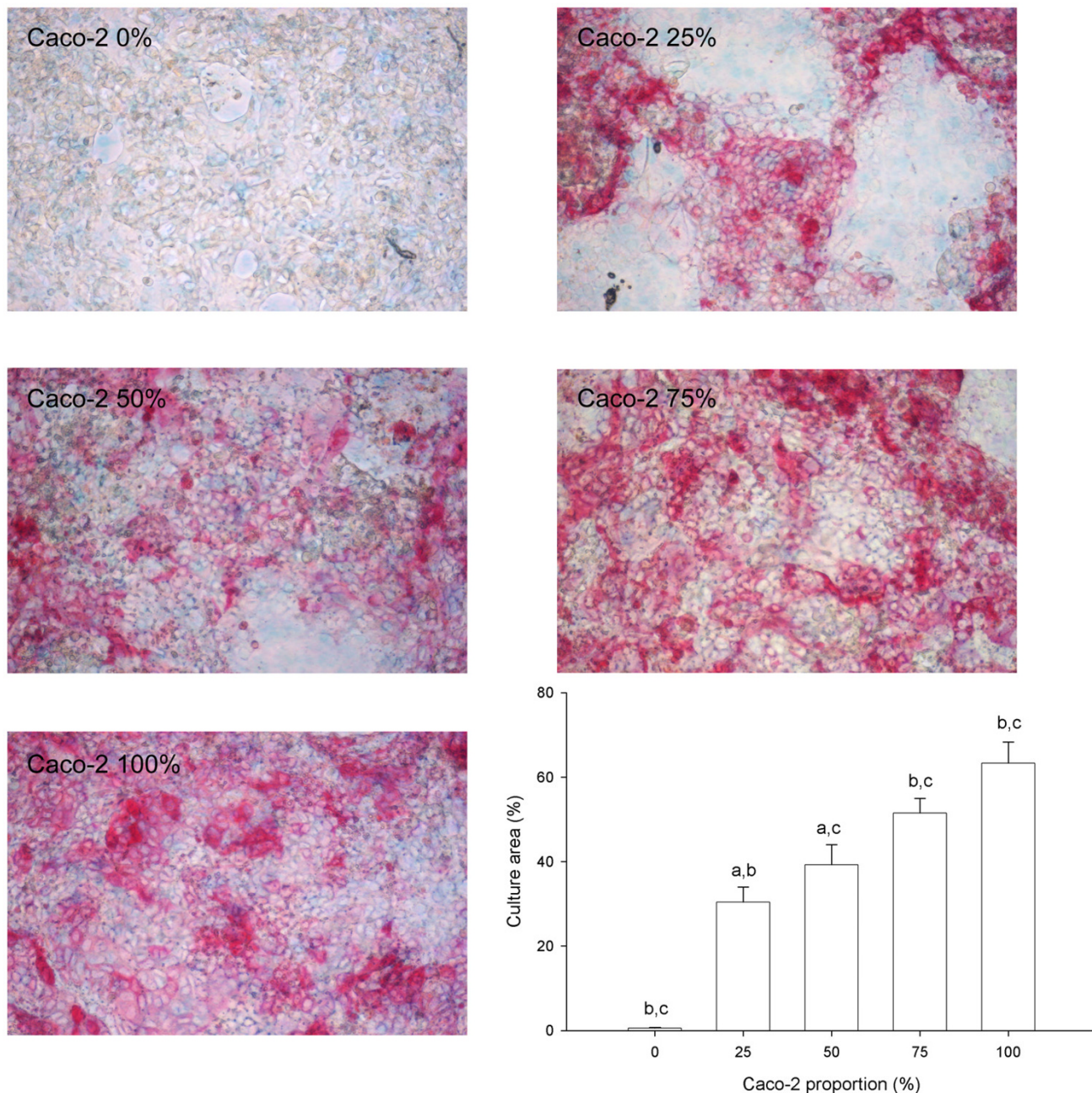*MUC5AC* genes in the monolayer is independent of the

Figure 6
Localization of alkaline phosphatase activity (in red) by cytochemistry in a co-culture of Caco-2 and HT29-5M21. Groups sharing the same letter are significantly different (^{a} p < 0.01; ^{b,c} p < 0.001, N = 4).

cell proportion and thus of the Caco-2 or HT29-5M21 abundancy. However, the specific activity of the alkaline phosphatase was dependant on the Caco-2 proportion. Consequently, there is a complex control of the gene expression, enzyme translation and degradation regarding the cell-cell interaction.

Monolayer permeability is influenced by the presence of HT29-5M21 cells in the system. This influence is in accordance with an increase of the permeability. Indeed, we showed a decrease of the TEER and an increase of the Lucifer yellow and 20 kDa dextran permeability with the increase of HT29-5M21 proportion in the monolayer at

Table 1: List of primers used in this study

Primer sets	Sequences 5'→3'	Predicted PCR product size (bp)	Tm (°C)	References
IAP hu Fo	CCG-CTT-TAA-CCA-GTG-CAA-CA		60	
IAP hu Ba	CCC-ATG-AGA-TGG-GTC-ACA-GA	452	62	-
MUC5AC Fo	TGA-TCA-TCC-AGC-AGC-AGG-GT		62	
MUC5AC Ba	CCG-AGC-TCA-GAG-GAC-ATA-TGG-G	408	70	[25]
bACT hu Fo	AGA-AAA-TCT-GGC-ACC-ACA-CC		60	
bACT hu Ba	GTC-AGG-CAG-CTC-GTA-GCT-CT	485	64	-

seeding. The cell ratio is established at seeding and is probably not indicative of the situation when permeability is measured. However, HT29-5M21 cells seem to remain in sufficient proportion in the culture to increase cell permeability. The range of molecular masses (Lucifer yellow 452 Da & dextran 20 kDa) used in this study to explore monolayer permeability allow to characterize para-cellular permeability [37,38]. Lucifer yellow is not absorbed by epithelial cell but is able to pass through cell junctions as mannitol does. Dextrans are largely used as macromolecule permeability tracer.

Conclusion

The phosphatase alkaline, a well-established marker for enterocyte-like cells, could be proposed, in combination with *MUC5AC*, as a new marker of great interest for monitoring the cellular composition of the co-culture. We are currently developing an immuno-labelling system to quantify the level of this protein.

On the basis of the enterocyte/goblet cell ratio observed *in vivo* [39,40], the cell proportion 3:1 allows us to decrease the permeability to macromolecules who becomes closer to the situation observed in a human intestinal epithelium.

Permeability studies, using this co-culture, indicate that Caco-2/HT29-5M21 co-culture is more permissive than Caco-2 cell culture regarding to para-cellular permeability. This characteristic is probably in accordance with the *in vivo* permeability. We can expect to use the model to study some human diseases involving permeability alterations as coeliac disease.

Methods
Chemicals

All chemicals were purchased from Sigma-Aldrich (Poole, Dorset, UK) and from VWR (Brussels, Belgium). Cell culture media were obtained from Gibco BRL (Merelbeke, Belgium) and from Sigma-Aldrich. The molecular biology reagents were obtained from Promega (Madison, USA). Lucifer yellow and 20 kDa-dextran were obtained from Sigma-Aldrich.

Cell lines and culture conditions

The HT29-5M21 cell line, derived from the HT29-MTX 10-5M cell line [27], was obtained at passage 9 from Dr. T. Lesuffleur (INSERM U505, Villejuif, France). Caco-2 cells were obtained at passage 22 from Cambrex Bio Science (Verviers, Belgium). These cells were progressively adapted (until passage 30 for HT29-5M21 and passage 40 for Caco-2) to a serum-free medium: the basal defined medium (BDM) [41] supplemented with a mixture of non-essential amino acids and growth factors [42]. Initially, Caco-2 and HT29-5M21 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing glucose (4.15 mg/l) buffered with HEPES (25 mM) containing fetal calf serum (10%) and antibiotics (1%: penicillin [1 U/ml], streptomycin [1 μg/ml] and amphotericin as fungizone® [2.5 ng/ml]). Then, these cells were progressively sub-cultured in BDM containing glucose (4.15 mg/l) supplemented with non-essential amino acids and growth factors [42]. Cells were used at passages 30 (HT29-5M21) and 40 (Caco-2) to avoid dedifferentiation [43]. The cells were seeded at 4×10^4 cells/cm^2 in plastic flasks (Greiner labortechnik SA, Wemmel, Belgium) and cultured at 37°C in a CO_2-air (5:95) atmosphere (Heraeus EK incubator). The culture medium was changed every day.

Co-cultures

Five types of culture cells were produced: Caco-2 cells alone, HT29-5M21 cells alone and co-cultures of these cells in various seeding ratios (1:3, 2:2 and 3:1). These cultures (4×10^4 cells/cm^2 per flask) were maintained in BDM supplemented with non-essential amino acids, a mixture of growth factors [42] and antibiotics (see above). The medium was changed every two days. Co-cultures were confluent 7 days after the seeding. The Caco-2 and HT29-5M21 cells were maintained in culture during 21
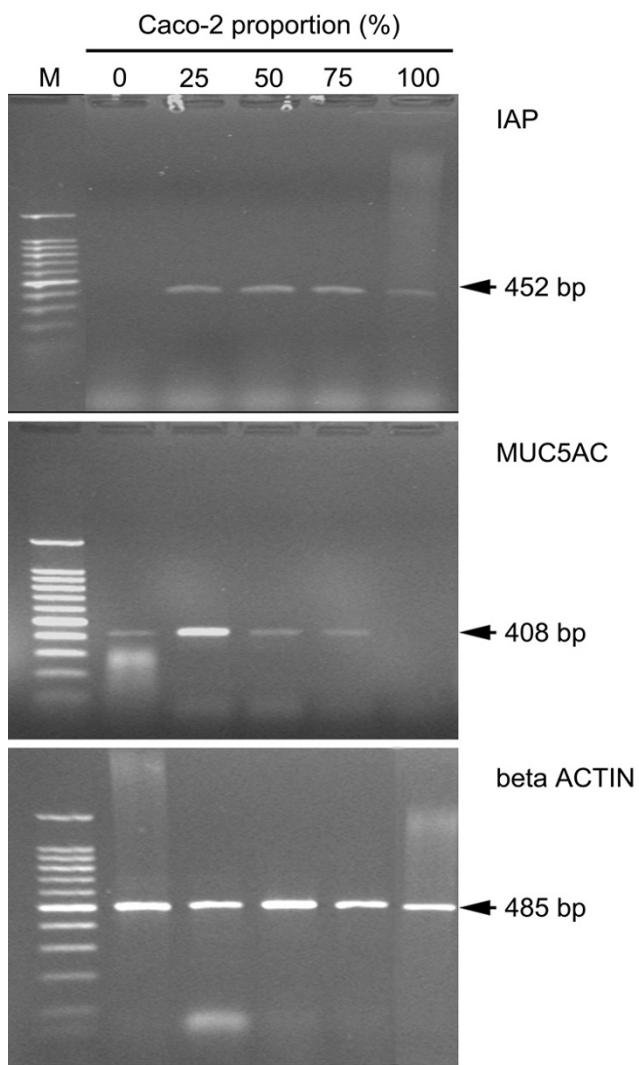
Figure 7
IAP, *MUC5AC* and *beta ACTIN* PCR on mRNA isolated from cell cultures. Lanes: M: 100 bp DNA ladder. Analysis was repeated three times.

days of differentiation after confluence. Under these conditions, the maximum expression of intestinal differentiation-specific markers was achieved. For the enterocytes, the differentiation marker was the alkaline phosphatase activity associated with the microvilli [17,30,31]. With regard to the goblet cells, the differentiation marker was the mucin synthesis [44]. Cells were used for mRNA extraction, enzyme assays and localization. Cells in co-culture were briefly characterized by phase contrast microscopy, light microscopy (semi-thin cross sections stained with toluidine blue), electronic microscopy and vital dye (ethidium bromide/acridine orange) exclusion [45].

Permeability assays
Permeability analyses were performed on cells cultured on 0.4 µm polyethylene terephtalate membrane coated with collagen as described above. Transepithelial electrical resistance measurement was measured in Hank's buffer [46,47]. Lucifer yellow and fluorescent dextran were measured in the baso-lateral pole compartment with a fluorimeter (excitation 430 nm – emission 540 nm).

Enzyme assays
Enzyme activity assays were performed on cell homogenates. Just before use, the monolayer was washed with PBS buffer (pH 7.2) and 1 ml of water was added. Cells were collected by scraping and were disrupted by ultrasonication (3 times for 20 sec each; ultrasonicer MSE PE818). Enzyme activity was assayed on co-culture homogenates and on Caco-2 and HT29-5M21 homogenate mixtures in 1:3, 2:2 and 3:1 ratios. Alkaline phosphatase activity was assayed according to the method described by Garen and Levinthal [48], with *p*-nitrophenolphosphate as substrate. Results were expressed as milliunit mg^{-1} of protein (specific activity = SA). One unit was defined as the activity equal to one µmol of substrate hydrolyzed per min at 37°C. Proteins were assayed by the method described by Bradford [49]. Phosphatase alkaline activity was localized on cell culture by cytochemistry using the fast red substrate (naphtol combined to fast red; Roche Diagnostics GmbH, Penzberg, Germany). Labelled cell-culture pictures were analyzed using ImageJ 1.33u software (Wayne Rasband, NIH, USA). Images were converted in 256 greyscale files, then a threshold was defined in order to include the red channel in the selection. The area of the selection was measured as a percentage of the picture surface.

PCR primers
All the PCR primers used in this study were obtained from Eurogentec (Seraing, Belgium). Three pairs of primers were chosen: IAP hu Fo/Ba was a specific of an alkaline phosphatase gene; MUC5AC Fo/Ba was a specific of the *MUC5AC* mucin gene [29]; and bACT hu Fo/Ba was a specific of the cytosolic *beta actin* coding-gene. The primer3 program [50] was used to design the IAP hu Fo/Ba and bACT hu Fo/Ba primers. The sequences of the primers used in this study are presented in Table I.

RNA isolation
Total mRNA was isolated using a kit manufactured by Promega (RNagents® total RNA isolation system) from confluent monolayer cells. RNA integrity was checked by electrophoresis in agarose gel (1%) containing ethidium bromide (0.5 µg/ml). RNA concentration was estimated by spectrophotometry at 260 nm.
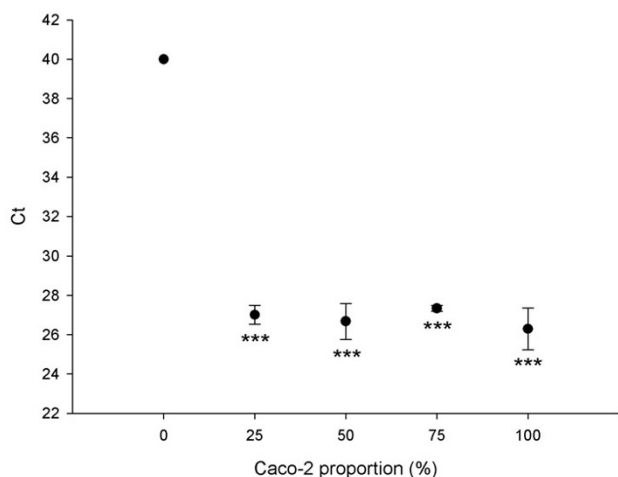
Figure 8
Alkaline phosphatase real-time PCR Ct values against the
Caco-2/HT29-5M21 seeding ratio. *** p < 0.001 in compari-
son with the 0% Caco-2 condition (N ≥ 3).

Reverse transcription
cDNA was synthesized using 5 µg of DNase-treated mRNA
in a reaction mixture containing RT buffer (1x), dNTP (0.6
mM), RNase inhibitor (1 U/µl), OligodT primers (0.2
µM) and RT Mo-MuLV (0.008 U/µl). The mix was incu-
bated for 1 h at 42°C.

PCR and real-time PCR
The PCR was performed on the cDNA in a final volume of
50 µl. A sample (2 µl) of this cDNA (0.04 µg) was added
to the PCR mixture containing Mg^{2+} free buffer (1x),
$MgCl_2$ (2.5 mM for IAP hu Fo/Ba, 3 mM for MUC5AC Fo/
Ba and 1.5 mM for bACT hu Fo/Ba), dNTP (200 µM),
primers (1 µM each) and Taq polymerase (0.1 U). The
PCR was carried out with a thermal cycler (Thermolyne,
Amplitron® II).

The real-time PCR has been described elsewhere [51]. In
our conditions, we used the SYBER®Green, which is a dou-
ble-strand DNA dye that gives rise to a fluorescence when
it is intercalated between DNA bases. Real-time PCR was
carried out in a final volume of 50 µl containing 25 µl of
SYBER®Green PCR Master Mix (Applied Biosystems), 2 µl
of cDNA (0.04 µg) and 1µM of each primer.

In both procedures, the reaction was performed in dupli-
cate for each cDNA. The PCR conditions were as follows:
initial denaturation step at 95°C for 3 min, followed by
40 cycles at 95°C for 1 min, annealing temperature for 1
min (60°C for IAP hu Fo/Ba, 62°C for MUC5AC Fo/Ba
and 60°C for bACT hu Fo/Ba) and 72°C for 1 min. A final
elongation step was performed for 5 min at 72°C.

The PCR products (10 µl) were separated by electrophore-
sis in agarose gels (1%) containing ethidium bromide
(0.5 µg/ml). The nucleolytic DNA polymerase overlapped
the hybridization probe, and the resulting relative
increase in the reporter fluorescent dye emission was
monitored in real time using a sequence detector (Gene-
Amp 5700 System, Applied Biosystems). The fluorescence
intensity was a function of the cycle number and was
determined using the sequence detector software (Applied
Biosystems), giving the Ct at which PCR amplification
reached the exponential phase. The Ct was plotted against
the culture types. The value of the Ct was inversely linearly
correlated with the logarithmic value of cDNA quantity.

Statistics
Results are reported as mean ± standard deviation (SD).
With regard to heteroscedasticity, statistical analysis was
performed using Newman-Student-Keuls or Kruskal-Wal-
lis ANOVA. A p-value below 0.05 was considered statisti-
cally significant.

Authors' contributions
GN carried out cell culture, molecular biology, biochem-
istry analysis and experimental design. She was involved
in manuscript drafting and revising. CD participated in
cell culture and in experimental design. BEM participated
in real-time PCR studies and in drafting the manuscript.
WZ participated in manuscript revision. PD participated
in statistical analysis. OP was involved in morphometry
and statistical analysis as well as in drafting and revising
manuscript. GD and YJS were the coordinators of the
study, participated in the drafting and gave the final
approval of the version to be published. All authors read
and approved the final manuscript. The authors declare
that they have no competing interests.

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