Characterization of the in vitro cell models

Differentiated Caco-2 epithelium

Caco-2 cells were grown on Transwell-Clear® membranes (polyester, 0.4 µm pores, Costar). For complete differentiation into mature enterocytes they were maintained for 21 days after reaching confluence. Immunostaining of ZO-1 tight junction protein (Figure 1A) and TEM observation (Figure 1B) confirmed the presence of tight junctions; alkaline phosphatase activity (Figure 1C) and TEM observation (Figure 1D) confirmed the presence of a dense network of well-organized microvilli.

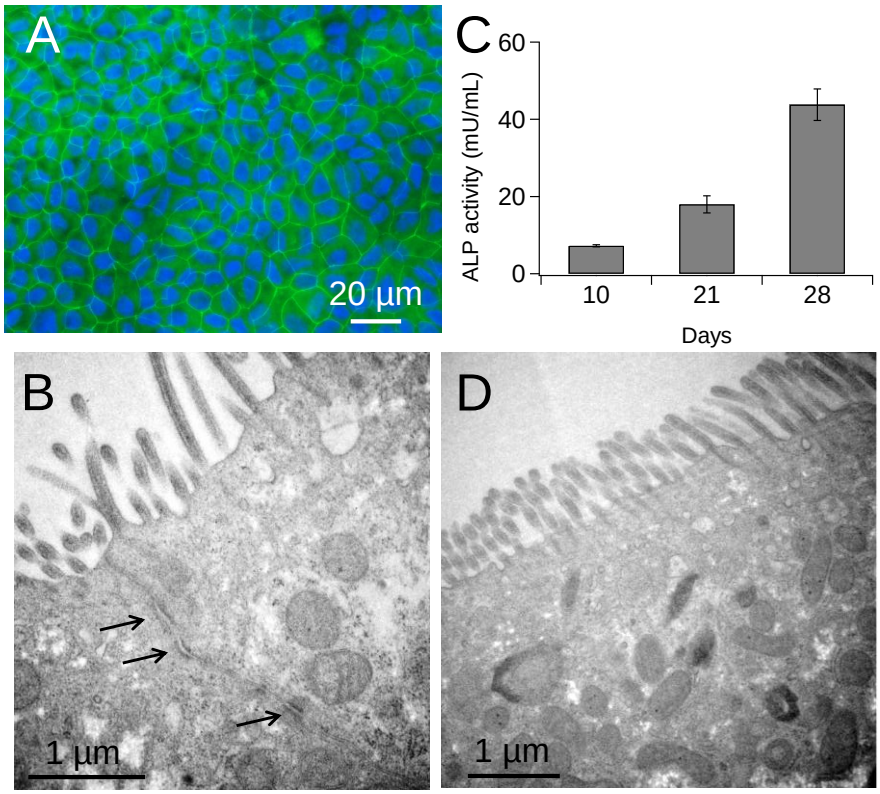

Figure 1. Differentiation of Caco-2 enterocytes. Complete differentiation was assessed by immunostaining of ZO-1 junction protein (A), TEM observation of the presence of tight junctions (B), their increased alkaline phosphatase activity (C), and TEM observation of well-organized microvilli on their apical surface (D).

Caco-2/HT29-MTX co-culture

To reproduce a mucus-secreting epithelium, Caco-2 cells were co-cultured for 4 weeks with HT29-MTX cells at the ratio of 75% Caco-2/25% HT29-MTX. Post-confluent HT29-MTX cells show a discrete brush border and secrete mucus [1]. Mucus secretion was probed by staining with 1% Alcian blue for 1 h (Figure 2A-C) [2]. HT29-MTX distribution in the co-culture was not homogeneous, and mucus production was restricted to the areas where HT29-MTX cells grew (intense blue staining in Figure 2A-C). The TEER (Figure 2D) and ALP activity (Figure 2E) were significantly lower in Caco-2/HT29-MTX co-culture than in the Caco-2 monoculture, proving that microvilli-containing cells were less abundant. Fast red staining of ALP (Figure 2F-H) showed areas in the Caco-2/HT29-MTX co-culture where ALP expression was reduced, confirming the presence of HT29-MTX cells.

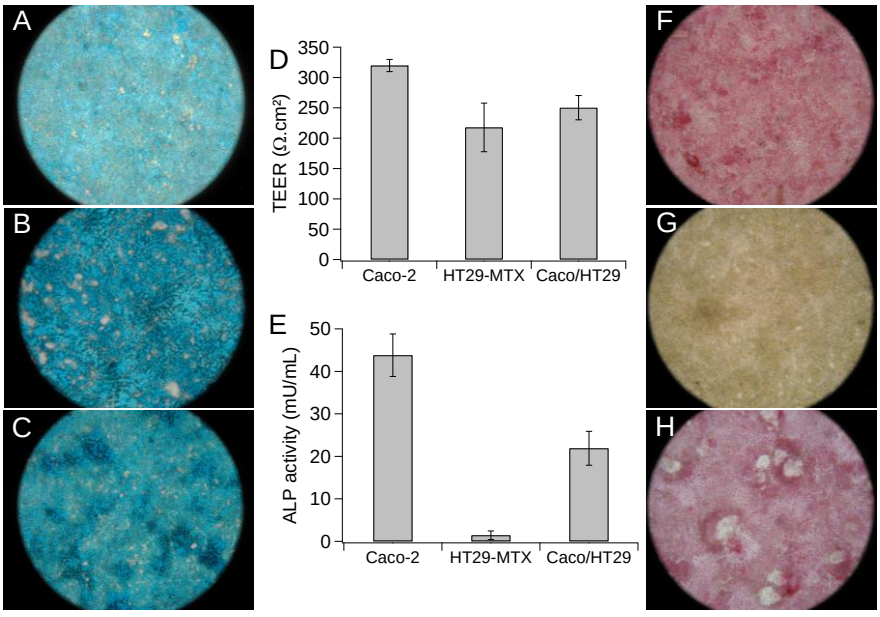

Figure 2. Characterization of the Caco-2/HT29-MTX coculture. Mucus staining in Caco-2 (A), HT29-MTX (B) and a coculture of Caco-2 and HT29-MTX (C) cells. Transepithelial resistance

(E) and alkaline phosphatase activity (F). Alkaline phosphatase staining using FastRed on Caco-2 (G), HT29-MTX (H) and a coculture of Caco-2 and HT29-MTX (I) cells.

Caco-2/RajiB co-culture

In the absence of specific markers of human M-cells, differentiation of Caco-2 cells into M-cells was confirmed by TEM observation (Figure 3). The epithelium was dense (Figure 3A), presented cell junctions and irregular microvilli (Figure 3B-C). ALP activity in Caco-2 clone 1 cells was 142 ± 13 mU/mL.min while it dropped to 71 ± 11 mU/mL.min in the Caco-2/Raji B co-culture.

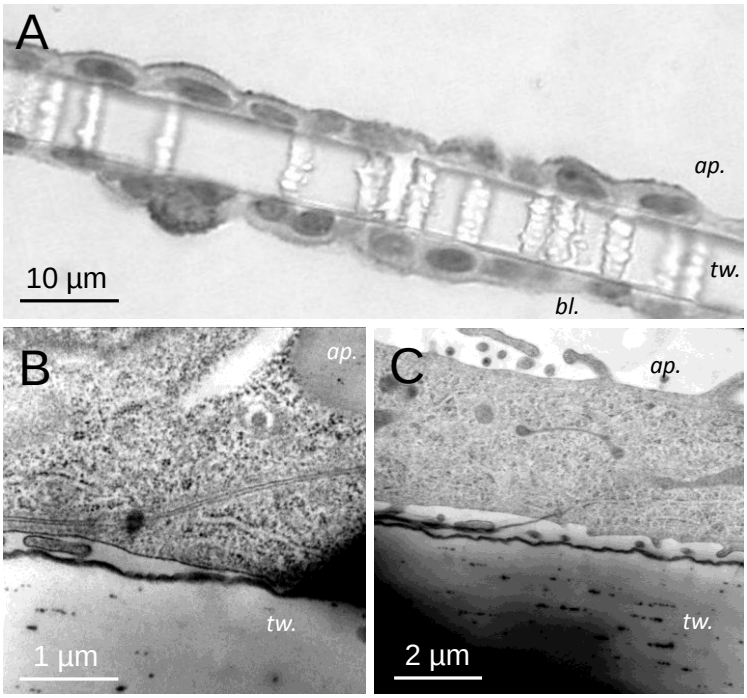

Figure 3. Microscopic observation of M-cells. A: optical microscopy image of the Caco-2/RajiB coculture, forming a dense and homogeneous epithelium. B, C: TEM images of Caco-2/RajiB coculture, presenting several features of M-cells: cell-cell junctions (B), disorganized and rare microvilli (C). ap.: apical compartment, bl. Basolateral compartment, tw.: transwell insert.

Reduced expression of ALP and sucrase isomaltase (SI), involved in tight junction structure, as well as claudin 5 and 8 (CLDN5, CLDN8), occludin (OCCL) and myosin light chain kinase long isoform (MLCK), involved in microvilli development, were observed (Table 1).

Table 1. Differential expression of genes involved in microvilli and tight junction structure[a]

Gene	**ALP**	**SI**	**CLDN2**	**CLDN5**	**CLDN8**	**OCLN**	**TJP1**	**MLCK**
Caco-2	1.02±0.44	1.16±0.21	0.92±0.13	1.03±0.15	1.05±0.09	1.02±0.12	1.03±0.14	1.05±0.33
Caco-2/RajiB	0.13±0.05*	0.38±0.21	0.91±0.32	0.54±0.12*	0.62±0.24*	0.59±0.19*	0.95±0.12	1.91±0.60*

[a]Differential expression was measured by RT-qPCR in Caco-2 monoculture and Caco-2/RajiB coculture. Expression is expressed as fold increase ($2^{-\Delta\Delta Ct}$) where Ct is the cycle threshold. Markers of microvilli: ALP: alkaline phosphatase, SI: sucrase isomaltase. Markers of tight junctions: CLDN: claudin, OCLN: occludin, TJP1: tight junction protein 1 (ZO-1), MLCK: myosin light chain kinase. Statistical significance was tested by randomization tests using REST2009.

References

1. Lesuffleur T, Barbat A, Dussaulx E, Zweibaum A: **Growth adaptation to methotrexate of HT-29 human colon carcinoma cells is associated with their ability to differentiate into columnar absorptive and mucus-secreting cells.** *Cancer Res* 1990, **50:**6334-6343.
2. Walter E, Janich S, Roessler BJ, Hilfinger JM, Amidon GL: **HT29-MTX/Caco-2 cocultures as an in vitro model for the intestinal epithelium: in vitro-in vivo correlation with permeability data from rats and humans.** *J Pharm Sci* 1996, **85:**1070-1076.