Research Article

Infection of cultured intestinal epithelial cells with severe acute respiratory syndrome coronavirus

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Abstract. To identify a model for the study of intestinal pathogenesis of severe acute respiratory syndrome (SARS) we tested the sensitivity of six human intestinal epithelial cell lines to infection with SARS coronavirus (SARS-CoV). In permissive cell lines, effects of SARS-CoV on cellular gene expression were analysed using high-density oligonucleotide arrays. Caco-2 and CL-14 cell lines were found to be highly permissive to SARS-CoV, due to the presence of angiotensin-converting enzyme 2 as a functional receptor. In both cell lines, SARS-CoV infection deregulated expression of cellular genes which may be important for the intestinal pathogenesis of SARS.

Key words. SARS, SARS-CoV; coronavirus; intestine; Caco-2; CL-14, microarray.

Severe acute respiratory syndrome (SARS) is caused by infection with the SARS coronavirus (SARS-CoV) [1]. Approximately 25% of patients with SARS are likely to progress to severe respiratory failure with characteristics of acute respiratory distress syndrome [2]. Although the pathogenesis of SARS is still unclear, it is believed that after binding to its functional receptor, i.e. angiotensin-converting enzyme 2 (ACE2), SARS-CoV replicates in permissive cells resulting in their lysis [3]. Therefore, damage of infected tissue may result directly from virus replication (cytolytic activity). On the other hand, the progression of SARS to respiratory failure in the later phase of the disease (weeks 2 and 3) which occurs in a notable proportion of patients despite lowering of virus load suggests an immunopathological mechanism [4].

Gastrointestinal symptoms are frequently observed in patients with SARS. In the Hong Kong and Toronto outbreaks, a considerable proportion of patients had watery diarrhoea, sometimes preceding the onset of respiratory symptoms [5, 6]. Peiris et al. [4] reported that up to 70% of their patients in the community outbreak in Hong Kong developed watery diarrhea. Patients with diarrhea had higher rates of intensive care unit admission and intubation; however, there was no association with oxygen requirement and overall mortality [5, 7]. Despite a relatively normal endoscopic and microscopic appearance of the gut in SARS patients with gastrointestinal involvement, SARS-CoV was found in the large and small bowel by both electron microscopy and viral culture [5]. We previously demonstrated that the intestinal Caco-2 cell line is highly permissive to SARS-CoV infection [8]. In the present study, we compared the sensitivity of Caco-2 cells to SARS-CoV infection with that of five other human intestinal cell lines. Only the CL-14 cell line was...
highly sensitive to SARS-CoV. Both Caco-2 and CL-14 were used to define the effects of SARS-CoV infection on the cellular gene expression profile.

Materials and methods

Cell cultures
Human cell lines derived from colon carcinoma including Caco-2, CL-14, HT-29, SW-480, DLD-1 and HCT-15 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany). The cells were grown at 37°C in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 10% fetal bovine serum (FBS) and containing 100 IU/ml of penicillin and 100 µg/ml of streptomycin. All culture reagents were purchased from Biochrom (Berlin, Germany).

Virus preparation
SARS-CoV strain FFM-1 was isolated from respiratory specimens of a SARS patient admitted to the Infectious Diseases Department of Frankfurt am Main University Hospital, Germany, on Vero (African green monkey kidney; ATCC CCL81) cells [9]. SARS-CoV strain 6109 (courtesy of Prof. W. Lim, Government Virus Unit, Hong Kong) was obtained from a Hong Kong patient early in the outbreak there and is thus one transmission generation earlier than the FFM-1 strain. SARS-CoV stocks used in the experiments had undergone five passages on Vero cells and were stored at –80°C. Virus titers were determined as TCID50/ml in confluent cells in 96-well microtitre plates [9].

Immune staining of viral antigens
Cells infected with SARS-CoV at multiplicity of infection (MOI) 1 and MOI 10 were collected at different times post infection (p.i.) by trypsinization of adherent cells. Non-adherent cells (the numbers of which increased with time after infection) were collected by centrifugation of culture supernatants. Both adherent and non-adherent cells were fixed on glass slides with 60/40 methanol/acetone for 15 min. Immune peroxidase staining was performed using human immune serum obtained from a SARS patient as described previously [9].

SARS-CoV receptor blocking experiments
To investigate whether ACE2 is a functional receptor for SARS-CoV in intestinal epithelial cell cultures, the cells were pre-treated for 60 min at 37°C with goat antibody directed against the human ACE2 ectodomain (R&D Systems; Wiesbaden-Nordenstadt, Germany). After treatment, the cells were washed three times with phosphate-buffered saline (PBS) and infected with one of the SARS-CoV strains at MOI 1. Twenty-four hours p.i. the cells were fixed and stained for viral antigens as described above. Goat anti-ACE1 antibody (R&D Systems) was used as control. Both antibodies were added at a concentration of 50 µg/ml.

Flow cytometry
To investigate expression of cell surface ACE2, intestinal cell lines were washed twice with PBS and incubated for 30 min with goat anti-ACE2 antibody (R&D Systems). After washing with PBS, the cells were incubated with FITC-conjugated anti-goat IgG (Becton Dickinson, Heidelberg, Germany) for 30 min. As controls, cells were stained with irrelevant primary antibody (goat anti-mouse IgG; Sigma Biochemicals, Seelze, Germany) or without a primary antibody to determine unspecific and background fluorescence, respectively. Instrument settings of the flow cytometer (FACSscan; Becton Dickinson) were adjusted to obtain background mean fluorescence in the histogram mode between 1 and 10 on the logarithmic scale.

Electron microscopy
Caco-2 cells were infected 2–3 days after reaching confluence with SARS-CoV at MOI 1. One day p.i., the cells were processed for ultrastructural analysis as described previously [10]. Briefly, cells were pelleted and fixed with 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in ethanol and embedded in Durpan-Epon. Thin sections were contrasted with uranyl acetate and lead citrate and viewed with a Jeol JEM, 2000 CX electron microscope (Arishima, Japan).

Cell viability assay
To assess effects of SARS-CoV infection on Caco-2 cell viability, confluent cell layers in 96-well plates were infected at MOI 1 and MOI 10. The viability was measured at different times p.i. using the MTT assay performed as described previously [10].

Gene expression analysis by oligonucleotide microarrays and RT-PCR
Gene array analysis was done according to the principles of MiamCe [11] We used the Affymetrix HG-U133A chip (Affymetrix, Santa Clara, Calif.). This oligonucleotide microarray targets 22,000 genes. Sample preparation was done by the RNeasy Mini Kit (Qiagen, Hilden, Germany) standard protocol. Generation of biotin-labelled cRNA, hybridization and staining were done according to standard protocols available from Affymetrix. Data analysis was performed using Microarray Analysis Suite (Affymetrix) and GeneSpring software version 4.0 (Silicon Genetics, San Carlos, Calif.) as published previously [12]. In brief, the lowest raw data value was arbitrarily defined as ‘11’ in order not to eliminate genes which are expressed only in one sample. To eliminate false
‘fold-change’ calls, genes that were classified as ‘up-regulated’ had to be flagged as ‘present’ in the infected samples, while genes that were classified as ‘down-regulated’ had to be flagged as ‘present’ in the mock-infected samples. Within those parameters, genes were selected if they were either up- or down-regulated at least threefold in duplicate. Following microarray analysis, genes related to apoptosis, cytokines, chemokines or interferons were confirmed by RT-PCR, according to standard protocols [10]. PCR primer and amplification conditions were determined by the software Primer3 (Whitehead Institute for Biomedical Research, Cambridge, Mass.) [13].

Results

Virus growth

Previously, we demonstrated that Caco-2 cells are highly permissive to infection with SARS-CoV strain FFM-1 [8]. In the present study we extended our observations by comparing the sensitivity of Caco-2 cells to SARS-CoV with that of five other intestinal cell lines including CL-14, HT-29, SW-480, DLD-1 and HCT-15. In addition to strain FFM-1, the sensitivity of intestinal cell lines to infection with SARS-CoV strain 6109 was tested. Caco-2 and CL-14 cells were the only cell lines permissive to infection with both SARS-CoV strains. In Caco-2 cells infected with the FFM-1 strain at MOI 1, infectious virus titers increased from none at 1 h p.i. to a maximum of $5.4 \times 10^7$ TCID$_{50}$/ml at 48 h p.i. (fig. 1A). Infection of Caco-2 cells at MOI 10 resulted in 10-fold ($1.5 \times 10^6$ vs $1.1 \times 10^5$ TCID$_{50}$/ml) and 4-fold ($8.3 \times 10^7$ vs $2.1 \times 10^7$ TCID$_{50}$/ml) higher infectious titers 12 and 24 h p.i. relative to cultures infected at MOI 1. The maximum virus titers in cultures infected at MOI 10 were similar to those of cultures infected at MOI 1 ($8.3 \times 10^7$ vs $5.4 \times 10^7$ TCID$_{50}$/ml); however, the maximum was already achieved at 24 h p.i. (fig. 1A).

In CL-14 cells infected at MOI 1 with the FFM-1 strain, a maximum virus titer of $3.7 \times 10^7$ TCID$_{50}$/ml was measured 72 h p.i., i.e. 1 day later than in Caco-2 cells (fig. 1B). In CL-14 cultures infected at MOI 10, a maximum virus titer of $4.5 \times 10^7$ TCID$_{50}$/ml had already developed 48 h p.i. (fig. 1B).

In both Caco-2 and CL-14 cells infected at MOI 1, a cytopathic effect (CPE) did not appear before 48 h p.i. The CPE at 72 h p.i. was very marked, with numerous rounded and enlarged cells, some of which became detached (fig. 2A). Although most of the cells stained positive for viral antigens 72 h p.i., the exact numbers of infected cells could not be determined due to the extensive cellular lysis. Some cells which were not destroyed continued to adhere to the culture vessel surface and did not stain with immune serum (fig. 2A). For both cell lines, the CPE developed 1 day earlier in cultures infected at MOI 10 than in those infected at MOI 1.

Infection of Caco-2 and CL-14 cells with the SARS-CoV 6109 strain resulted in a similar viral titer and similar CPE when compared with cells infected with the FFM-1 strain (data not shown).

Effects on cell viability

Using the MTT assay, the cell viability of both Caco-2 and CL-14 cultures infected at MOI 1 or 10 measured 24 h p.i. was similar to mock-infected cultures (fig. 2B). The viability was significantly lowered 48 and 72 h p.i. in cultures infected at MOI 10 and 1, respectively. The maximum decrease in cell viability was achieved 96 h p.i. However, at least 35% cell viability was recorded even in cultures infected at MOI 10, and this did not decrease any further (fig. 2B).

Figure 1. Production of infectious virus titers in Caco-2 cells (A) and CL-14 cells (B) infected with SARS-CoV FFM-1 strain (MOI 1 and 10) at different time points p.i. Values represent the mean (± SD) from three independent experiments.
SARS-CoV receptor studies
Since ACE2 was identified as a functional SARS-CoV receptor in different cell types [3] we measured whether its expression may correlate with the sensitivity of intestinal cell lines to SARS-CoV infection. Caco-2 and CL-14 expressed ACE2 mRNA and protein which were not detectable in the other cell lines tested (fig. 3A, B). Pre-treatment of both Caco-2 and CL-14 cells with anti-ACE2 antibody reduced at least 20-fold the numbers of SARS-CoV-infected cells, while ACE1 antibody used as control had no effects on numbers of infected cells (fig. 3C).

Ultrastructural study
Caco-2 cell cultures 24 h p.i. consisted of mostly poorly differentiated cells (fig. 4A, B) but also some well-differentiated (villus) enterocytes (fig. 4D). Coronavirus particles were found in both, with more than 30% of cells found to be infected. Viral particles were observed intracellularly within dilated cytoplasmic vesicles (fig. 4C) consistent with dilated endoplasmic reticulum but not in nuclei, and extracellularly both attached to the cytoplasmic membrane and detached from the cells (fig. 4B, E).

Effects on cellular gene expression
The relative abundance of specific mRNA in SARS-CoV-infected cells was compared to mock-infected confluent Caco-2 cell cultures (same passage and identical culture conditions) 24 h p.i. when cell viabilities were similar (fig. 2B). All gene expression experiments were done in duplicate and only genes which were up- or down-regulated in both samples underwent further evaluation. After applying strong restrictions as described in Materials and Methods, resulting genes were grouped according to their function (table 1).

We focussed on genes related to apoptosis, chemokines, interferon-induced genes and transcription factors, since these gene groups may play an important role in the pathogenesis of SARS. Expression of the selected genes was confirmed by RT-PCR (fig. 5). In the infected cells, we found an up-regulation of some anti-apoptotic genes including Bcl-2 (only in Caco-2 but not in CL-14 cells) and A20, while several pro-apoptotic genes including Bid, Bad, caspase-2 and caspase-6 were down-regulated. On the other hand, the anti-apoptotic programmed cell death 4 gene (PDCD4) was down-regulated in infected cells. Increased levels of mRNA of members of the AP-1 family of cellular transcription factors including c-jun.
Figure 3. Expression of ACE2 mRNA (A) and surface protein (B) on Caco-2 and CL-14 cells as well as blocking of SARS-CoV infection by anti-ACE2 antibody (C). mRNA was measured by means of RT-PCR and amplification products of ACE2 and GAPDH were visualized with ethidium bromide on an agarose gel. ACE2 surface expression was measured by flow cytometry. Blocking experiments were performed by treatment of cells 60 min before infection with the FFM-1 strain (MOI 1) using goat antibody directed against ACE2 or control antibody directed against ACE1. The data are means (± SD) from three independent experiments.

Figure 4. Ultrastructural appearance of mock-infected (A) and SARS-CoV-infected (B–E) Caco-2 cells. A poorly differentiated cell shows viral particles in dilated cytoplasmic vesicles (arrow; B and C) as well as extracellularly (arrowheads; B). A well-differentiated (villus) enterocyte shows viral particles in cytoplasmic vesicles (arrows) and on the cell surface (arrowheads; D). Some viral particles attach onto the microvilli, whereas some detach from the cell surface (E). c, cytoplasm; n, nucleus.
Table 1. Genes differentially expressed at least threefold in SARS-infected CaCo-2 cells 24 h p.i. compared to mock-infected cells.

| Accession No. | Gene     | Map      | Description                                      |
|---------------|----------|----------|--------------------------------------------------|
| **Up-regulation** |          |          |                                                  |
| Chemokines/cytokines/receptors |          |          |                                                  |
| NM_001146     | ANGPT1   | 8q22.3-q23| angiopoietin 1                                   |
| NM_001200.1   | BMP2     | 20p12    | bone morphogenetic protein 2                     |
| NM_001201.1   | BMP3     | 4p14-q21 | bone morphogenetic protein 3                     |
| NM_004591.1   | CCL20    | 2q33-q37 | chemokine (C-C motif) ligand 20                   |
| NM_002090.1   | CCL3     | 17q11-q21| chemokine (C-C motif) ligand 3                    |
| NM_001511.1   | CXCL1    | 4q21     | chemokine (C-X-C motif) ligand 1                  |
| M57731.1      | CXCL2    | 4q21     | chemokine (C-X-C motif) ligand 2                  |
| NM_002620.1   | CXCL3    | 4q21     | chemokine (C-X-C motif) ligand 3                  |
| NM_000584.1   | CXCL8    | 4q13-q21 | chemokine (C-X-C motif) ligand 8                  |
| NM_001565.1   | CXCL10   | 4q21     | chemokine (C-X-C motif) ligand 10                 |
| AF030514.1    | CXCL11   | 4q21.2   | chemokine (C-X-C motif) ligand 11                 |
| NM_002009.1   | FGF7     | 15q15-q21.1| fibroblast growth factor 7 (keratinocyte growth factor) |
| BG389073      | GDF11    | 12q13.13 | growth differentiation factor 11                 |
| NM_014440.1   | IL1F6    | 2q12-q14.1| interleukin 1 family, member 6 (epsilon)         |
| NM_000418.1   | IL4R     | 16p11.2-12.1| interleukin 4 receptor                           |
| NM_002608.1   | PDGFB    | 22q13.1  | platelet-derived growth factor beta polypeptide  |
| NM_002620.1   | PF4V1    | 4q12-q21 | platelet factor 4 variant                        |
| M19154.1      | TGF2B    | 1q41     | transforming growth factor, beta 2               |
| **Cell cycle** |          |          |                                                  |
| S67788.1      | APC      | 5q21-q22 | adenomatosis polyposis coli                      |
| AI421559      | RALGDS   | 9q34.3   | ral guanine nucleotide dissociation stimulator   |
| NM_002923     | RGS2     | 1q31     | regulator of G-protein signalling 2, 24 kDa      |
| **Apoptosis** |          |          |                                                  |
| AF086790.1    | ADCA     |          | aconitase precursor                              |
| NM_006633.1   | BCL2     | 18q21.3  | B cell CLL/lymphoma 2                            |
| NM_006561.1   | CUGBP2   | 10p13    | CUG triplet repeat, RNA-binding protein 2        |
| NM_017523.1   | HSXIAPF1 | 17p13.2  | XIAP-associated factor-1                         |
| AI078167      | NFKBIA   | 14q13    | NfkB light polypeptide gene enhancer in B cells inhibitor α |
| NM_000315.1   | PTH      | 11p15.3-p15.1| parathyroid hormone                            |
| NM_006290     | A20(TNFAIP3) | 6q23 | tumour necrosis factor, alpha-induced protein 3 |
| **Differentiation** |          |          |                                                  |
| NM_000348.1   | SRD5A2   | 2p23     | steroid-5-alpha-reductase                        |
| NM_005725     | TSPAN-2  | 1p12     | tetraspan 2                                     |
| **Signal transduction** |          |          |                                                  |
| D32201        | ADRA1A   | 8p21-p11.2| adrenergic, alpha-1A-, receptor                  |
| NM_004041.2   | ARRB1    | 11q13    | arrestin, beta 1                                |
| L24959        | CAMK4    | 5q21.3   | calcium/calmodulin-dependent protein kinase IV   |
| NM_014421.1   | DKK2     | 4q25     | dickkopf homologue 2 (Xenopus laevis)            |
| NM_003165.1   | DLG4     | 17p13.1  | discs, large (Drosophila) homologue 4            |
| NM_00417.2    | DUSP1    | 5q34     | dual specificity phosphatase 1                   |
| BC005047.1    | DUSP6    | 12q22-q23| dual specificity phosphatase 6                   |
| NM_004431     | EPHA2    | 1p36     | EphA2                                           |
| U35398.1      | GPR68    | 14q31    | G protein-coupled receptor 68                    |
| NM_014920     | ICK      | 6p12.3-p11.2| intestinal cell kinase                          |
| AW338791      | IGFL5    | 16p13.3  | IGF-binding protein, acid-labile subunit        |
| NM_003604     | IRS4     | Xq22.3   | insulin receptor substrate 4                    |
| BC002844      | NFKB2    | 10q24    | NfkB light polypeptide gene enhancer in B cells 2|
| AA149639      | QKI      | 6q26-27  | homologue of mouse quaking QKI                  |
| NM_004841     | RASA2    | 1p24     | RAS protein activator-like 2                    |
| SS9049        | RGS1     | 1q31     | regulator of G-protein signalling 1              |
| AI332407      | SFRP1    | 8p12-p11.1| secreted frizzled-related protein 1              |
| U91903.1      | SFRP3    | 2pter    | frizzled-related protein                        |
| AF036269      | SH3GL3   | 15q24    | SH3-domain GRB2-like 3                         |
| XS2075        | SPN      | 16p11.2  | sialophorin (gpL115, leukosialin, CD43)          |
| **Transcription** |          |          |                                                  |
| NM_021570.2   | BARX1    | 9q12     | BarH-like homeobox 1                            |
| BE675435      | COPEB    | 10p15    | core promoter element-binding protein            |
| NM_001964.1   | EGR1     | 5q31.1   | early growth response 1                         |
| N25429        | ELF3S6   | 8q22-q23 | eukaryotic translation initiation factor 3, subunit 6, 48 kDa |
| BF060791      | ETV5     | 3q28     | ets variant gene 5 (ets-related molecule)       |
| NM_002017.2   | FLI1     | 11q24.1-q24.3| Friend leukaemia virus integration 1            |
| Accession No. | Gene   | Map          | Description                                                                 |
|--------------|--------|--------------|------------------------------------------------------------------------------|
| V01512       | FOS    | 14q24.3      | c-fos                                                                        |
| NM_001546.1  | ID4    | 6p22-p21     | inhibitor of DNA-binding 4,                                                  |
| J04111       | JUN    | 1p32-p31     | c-jun                                                                        |
| NM_000475.2  | NR0B1  | Xp21.3-p21.2 | nuclear receptor subfamily 0, group B, member 1                              |
| NM_002616.1  | PER1   | 17p13.1-17p12| period homologue 1 (Drosophila)                                              |
| BC005325.1   | SSX4   | Xp11.23      | synovial sarcoma, X breakpoint 4                                             |
| NM_030751.1  | TCF8   | 10p11.2      | transcription factor 8 (represses interleukin 2 expression)                  |
| BC004145.1   | TNRC4  | 1q21         | trinucleotide repeat-containing 4                                            |
| NM_014112.1  | TRPS1  | 8q24.12      | trichorhinophalangeal syndrome 1                                             |
| NM_000551.1  | VHL    | 3p26-p25     | von Hippel-Lindau syndrome                                                   |
| NM_013256.1  | ZNF180 | 19q13.2      | zinc finger protein 180 (IHZ168)                                             |
| NM_006448.1  | ZNF348 |             | zinc finger protein 384                                                     |

**Protein folding**
- AF007162 CR Y AB 11q22.3-q23.1 crystallin, alpha B
- AF052173 TBCD 17q25.3 tubulin-specific chaperone d

**DNA recombination**
- NM_004507 HUS1 7p13-p12 HUS1 checkpoint homologue

**Metabolism**
- AF182276.1 CYP2E1 10q24.3-qter cytochrome P450, family 2, subfamily E, polypeptide 1
ectonucleotide pyrophosphatase/phosphodiesterase 3 glutamine-fructose-6-phosphate transaminase 2
- AL527430 GSTM3 1p13.3 glutathione S-transferase M3 (brain)
- AU121975 PAICS 4pter-q21 phosphoribosylaminoimidazole carboxylase
- NM_000329.1 RPE65 1p31 retinal pigment epithelium-specific protein 65 kDa

**Interferon-induced genes**
- NM_005101.1 G1P2 1p36.33 interferon, alpha-inducible protein (clone IFI-15K)
- NM_022873.1 G1P3 1p35 interferon, alpha-inducible protein (clone IFI-6-16)
- NM_006417.1 IFI44 1p31.1 interferon-induced protein 44
- NM_001548.1 IFIT1 10q25-q26 interferon-induced protein with tetraciclopeptide repeats 1
- NM_001549.1 IFIT4 204747_at interferon-induced protein with tetraciclopeptide repeats 4
- NM_003641.1 IFITM1 11p15.5 interferon induced transmembrane protein 1 (9-27)
- NM_006435.1 IFITM2 11p15.5 interferon induced transmembrane protein 2 (1-8D)
- NM_004030.1 IRF7 11p15.5 interferon regulatory factor 7
- NM_005101 ISG15 1p36.33 interferon-stimulated protein, 15 kDa
- NM_002462.1 MxA 21q22.3 interferon-ducible protein p78
- NM_016817.1 OAS2 12q24.2 2'-5'-oligoadenylate synthetase 2, 69/71 kDa
- NM_003733.1 OASL 12q24.2 2'-5'-oligoadenylate synthetase-like

**Miscellaneous genes**
- NM_000443.2 ABCB4 7q21.1 ATP-binding cassette, sub-family B (MDR/TAP), member 4
- NM_000706.2 AVPR1A 12q14-q15 arginine vasopressin receptor 1A
- NM_016279.1 CDH9 5p14 cadherin 9, type 2 (T1-cadherin)
- NM_004397.2 DDX6 11q23.3 DEAD (Asp-Glu-Ala-Asp) box polypeptide 6
- AK022087.1 DES 2q35 desmin
- U56236.1 FCAR 19q13.2-q13.4 Fc fragment of IgA
- NM_006682.1 FG1L2 7q11.23 fibrinogen-like 2
- NM_005204.1 MAP3K8 10p12.1 mitogen-activated protein kinase kinase kinase 8
- NM_005952.1 MT1X 16q13 metallothionein 1X
- NM_014314.1 RIG-I 9p12 DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide
- NM_014139.1 SCN11A 3p24-p21 sodium channel, voltage-gated, type XI, alpha
- NM_003095.1 SNRF1 12q23.1 small nuclear ribonucleoprotein polypeptide F
- NM_000593.2 TAPI 6q12 tissue factor pathway inhibitor 2
- (MDR/TAP)AL574096 TFP2 15q13 tight junction protein 1 (zona occludens 1)
- AAM13018 TJP1 15q13 tight junction protein 1 (zona occludens 1)

**Down-regulation**

**Chemokines/cytokines/receptors**
- M60459 EPOR 19p13.3-p13.2 erythropoietin receptor
erythropoietin receptor precursor
- NM_001562.1 IL18 11q22.2-q22.3 interleukin 18 (interferon gamma-inducing factor)
- NM_002415.1 MIF 22q11.23 macrophage migration inhibitory factor

**Cell cycle**
- NM_002012.1 FHIT 3p14.2 fragile histidine triad gene
- NM_005256.1 GAS2 11p14.3-15.2 growth arrest-specific 2
| Accession No. | Gene | Map | Description |
|---------------|------|-----|-------------|
| NM_005862.1   | STAG1| 3q22.2 | stromal antigen 1 |
| BG034328      | TFD2 | 3q23  | transcription factor Dp-2 (E2F dimerization partner 2) |
| **Apoptosis** |      |      |             |
| U68879        | BAD  | 11q12.3 | BCL2-antagonist of cell death |
| NM_001196.1   | BID  | 22q11.1 | BH3 interacting domain death agonist |
| NM_001224.1   | CASP2| 7q34-q35 | caspase 2, apoptosis-related cysteine protease |
| U20537.1      | CASP6| 4q25  | caspase 6, apoptosis-related cysteine protease |
| NM_004430.1   | CIDEB| 14q11.2 | cell death-inducing DFFA-like effector |
| BC005299.1    | CYCS | 7p15.2 | cytochrome c, somatic |
| AA485440      | SPHK2| 19q13.3 | sphingosine kinase 2 |
| M55983.1      | DNASE1| 16p13.3 | deoxyribonuclease |
| AK024029.1    | MOAP1| 14q2  | modulator of apoptosis 1 |
| NM_004456.1   | PDCD4| 10q24 | programmed cell death 4 (neoplastic transformation inhibitor) |
| NM_006281.1   | STK3 | 8q22.1 | serine/threonine kinase 3 (STE20 homologue, yeast) |
| **Signal transduction** | | | |
| NM_001177.2   | ARL1 | 12q23.3 | ADP-ribosylation factor-like 1 |
| NM_005308.1   | GPRK5| 10q24-pter | G protein-coupled receptor kinase 5 |
| AL570294      | HRMT1L1| 21q22.3 | HMT1 hmRNP methyltransferase-like |
| US0748.1      | LEPR | 1p31  | leptin receptor |
| NM_021183.1   | LOC57826| Xq25 | hypothetical protein similar to small G proteins, especially RAP-2A |
| NM_002757.1   | MAP2K5| 15q22.2 | mitogen-activated protein kinase kinase 5 |
| AW195581      | MCLC | 1p13.2 | Mid-1-related chloride channel 1 |
| BF001665      | OGT | Xq13  | O-linked N-acetylgalactosamine (GlcNAc) transferase |
| NM_008848.2   | PAK4 | 19q13.13 | p21(CDKN1A)-activated kinase 4 |
| AL574319      | PDK2 | 17q21.32 | pyruvate dehydrogenase kinase, isoenzyme 2 |
| AI198609      | RAB2 | 8q12.1 | RAB2, member RAS oncogene family |
| AF125393.1    | RAB7A| 15q15-q21.1 | RAB7A, member RAS oncogene family |
| BG338251      | RAB7L1| 1q2 | RAB7, member RAS oncogene family-like 1 |
| AB018283.2    | RHOBTB1| 10q21.2 | Rho-related BTB domain-containing 1 |
| AB014486.1    | SCAP2| 7p21-p15 | src family-associated phosphoprotein 2 |
| NM_003025.1   | SH3GL1| 19p13.3 | SH3-domain GRB2-like 1 |
| NM_013381.1   | TRHDE| 12q15-q21 | thyrotropin-releasing hormone-degrading ectoenzyme |
| **Protein folding** | | | |
| NM_003796     | C19orf2| 19q12 | chromosome 19 open reading frame 2 |
| NM_016074.1   | CGI-143| 1p36.13-q31.3 | CGI-143 protein |
| AF19061.1     | CITED2| 6q23.3 | Cbp/p300-interacting transactivator |
| BC005250.1    | CRSP9 | 5q33.3 | cofactor required for Sp1 transcriptional activation |
| NM_017946     | FKB14 | 7p15.1 | FK506 binding protein 14, 22 kDa |
| AI935162      | HP1P | 1q21.3 | haematopoietic PBX-interacting protein |
| NM_028317.1   | L2TFL1| 3p21.3 | leucine zipper transcription factor-like 1 |
| NM_005360.2   | MAF | 16q22-q23 | v-maf musculoaponeurotic fibrosarcoma oncogene homologue |
| AA421957      | MED8 | 1p34.1 | mediator of RNA polymerase II transcription |
| AV756536      | MLLT3| 9p22  | myeloid/lymphoid or mixed-lineage leukaemia |
| AB028973.1    | MYT1 | 20q13.33 | myelin transcription factor 1 |
| L20433.1      | POU4F1| 13q21.1-q22 | POU domain, class 4, transcription factor 1 |
| AW027312      | RXF5 | 1q21  | regulatory factor X, 5 (influences HLA class II expression) |
| BC000519.1    | RUVBL1| 3q21 | RuvB-like 1 |
| NM_003113.1   | SP100 | 2q37.1 | nuclear antigen Sp100 |
| BC002802.1    | SUP34H1| 17q21-q23 | suppressor of Ty 4 homologue 1 |
| AA081084      | TAZ | 3q23-q24 | transcriptional coactivator with PDZ-binding motif (TAZ) |
| NM_003447.1   | ZNF165| 6p21.3 | zinc finger protein 165 |
| **Metabolism** | | | |
| NM_001086.1   | AADAC| 3q21.3-q25.2 | arylylacetamide deacetylase (esterase) |
| A1653169      | AK3 | 1p31.3 | adenylyl kinase 3 |
| U05598.1      | AKR1C2| 10p15-p14 | aldo-keto reductase family 1, member C2 |
| NM_002108.2   | AKR1D1| 7q32-q33 | aldo-keto reductase family 1, member D1 |
| BC000977.1    | ALAD | 9q34  | aminolevulinate, delta-, dehydratase |
| NM_000694.1   | ALDH3B1| 11q13 | aldehyde dehydrogenase 3 family, member B1 |
| AU149534      | ALDH7A1| 5q31 | aldehyde dehydrogenase 7 family, member A1 |
| NM_005165.1   | ALDOC| 17cen-q12 | aldolase C, fructose-bisphosphate |
| NM_001150.1   | ANPEP| 15q25-q26 | alanyl (membrane) aminopeptidase |
| AF161454.1    | APOM| 6p21.3 | apolipoprotein M |
| Accession No. | Gene      | Map        | Description                                      |
|--------------|-----------|------------|--------------------------------------------------|
| NM_000049.1  | ASPA      | 17pter-p13 | aspartoacylase (aminoacylase 2, Canavan disease) |
| NM_000709.1  | BCKDHA    | 19q13.1-q13.2 | branched-chain keto acid dehydrogenase E1     |
| AY028632.1   | CAT       | 11p13      | catalase                                         |
| AL568982     | CDS2      | 20p13      | CDP-diacylglycerol synthase                      |
| AA723370     | CGI-105   | 2p24.3-p11.2 | CGI-105 protein                                 |
| AF154830.1   | CPS1      | 2q35       | carnitine palmitoyltransferase 1, mitochondrial |
| BF001714     | CPT1A     | 11q13.1-q13.2 | carnitine palmitoyltransferase 1A (liver)      |
| NM_000998.1  | CPT2      | 1p32       | carnitine palmitoyltransferase II               |
| AL534872     | CTH       | 1p31.1     | cystathionase (cystathionine gamma-lyase)       |
| AJ222967     | CTNS      | 17p13      | cystosis, nephropathic                          |
| NM_018973.1  | DPM3      | 1q21.3     | dolichyl-phosphate mannosyltransferase polypeptide 3 |
| NM_000120.2  | EPHX1     | 1q42.1     | epoxide hydrolase 1, microsomal (xenobiotic)   |
| AF233336.1   | EPHX2     | 8p21-p12   | epoxide hydrolase 2, (cytoplasmic)             |
| NM_020973.1  | GBA3      | 4p15.31    | glucosidase, beta, acid 3 (cytosolic)          |
| AW299507     | GGPS1     | 1q43       | geranylgeranyl diphasate synthase 1             |
| X62078       | GM2A      | 5q31.3-q33.1 | GM2 ganglioside activator protein              |
| NM_015761.1  | GMPR2     | 14q11.2    | guanosine monophosphate reductase 2            |
| NM_000862.1  | GSTM1     | 1p13.3     | glutathione S-transferase M1                    |
| BC001453.1   | GSTZ1     | 14q24.3    | glutathione transferase zeta 1 (maleylacetoacetate isomerase) |
| NM_005326.1  | HAGH      | 16p13.3    | hydroxyacyl glutathione hydrolase              |
| NM_002108.2  | HAL       | 12q22-q24.1 | histidine ammonia-lyase                        |
| NM_001911.1  | HMGCL     | 1p36.1-p35 | 3-hydroxy-2-methylglutaric-3-carboxymethyltransferase |
| NM_000862.1  | HSD3B1    | 1p13.1     | hydroxy-delta-5-steroid dehydrogenase          |
| D55639.1     | KYNU      | 2q22.1     | kynureninase (L-kynurenine hydrolyase)          |
| NM_020379.1  | MAN1C1    | 1p35       | mannosidase, alpha, class 1C, member 1         |
| NM_000898.1  | MAOB      | Xp11.4-p11.3 | monooamine oxidase B                           |
| AI039874     | NQO1      | 16q22.1    | NAD(P)H dehydrogenase, quinone 1                |
| AL574319     | PDK2      | 17q21.32   | pyruvate dehydrogenase kinase, isoenzyme 2     |
| NM_016134.1  | PGCP      | 8q22.2     | plasma glutamate carboxypeptidase               |
| AK021676.1   | PGM3      | 6q14.1-q15 | phosphoglucomutase 3                           |
| NM_002676.1  | PMM1      | 22q13.2    | phosphomannomutase 1                           |
| BC059897.1   | PNLPRT8   | 10q26.11   | pancreatic lipase-related protein 2            |
| NM_022128.1  | RBSP      | 2p23.3     | ribokinase                                      |
| AW190316     | SHMT2     | 12q12-q14  | serine hydroxymethyltransferase 2 (mitochondrial) |
| NM_000340.1  | SLC2A2    | 3q26.1-q26.2 | solute carrier family 2 (facilitated glucose transporter), member 2 |
| AF059203.1   | SOAT2     | 12q13.13   | sterol O-acetyltransferase 2                   |
| NM_003167.1  | SULT2A1   | 19q13.3    | sulphotransferase family, cytosolic, 2A member 1 |
| BE895437     | TK2       | 16q22-q23.1 | thymidine kinase 2, mitochondrial               |
| NM_001076.1  | UGT2B15   | 4q13       | UDP glycosyltransferase 2 family, polypeptide B15 |
| NM_001077.1  | UGT2B28   | 4q13       | UDP glycosyltransferase 2 family, polypeptide B17 |
| AF177227.1   | UGT2B28   | 4q13.3     | UDP glycosyltransferase 2 family, polypeptide B28 |
| NM_016327.1  | UPB1      | 22q11.2    | ureidopropionase, beta                         |
| M14016.1     | UROD      | 1p34       | uroporphyrinogen decarboxylase                  |

**DNA repair**
| NM_007195.1  | POLJ      | 18q21.1 | polymerase (DNA directed) iota |
| NM_005053    | RAD23A    | 19p13.2 | RAD23 homolog A                |
| NM_002907.1  | RECQL     | 12q12   | RecQ protein-like (DNA helicase Q1-like) |
| NM_014311.1  | SMUG1     | 12q13.11 | single-strand selective monofunctional uracil DNA glycosylase |
| NM_003380.1  | XPA       | 9q22.3  | xeroderma pigmentosum, complementation group A |

**RNA processing**
| NM_030934    | C1orf25   | 1q25.2  | chromosome 1 open reading frame 25 |
| NM_014953    | DIS3      | 13q21.32 | mitotic control protein dis3 homologue |
| NM_006567    | FARSI     | 6p25.1  | phenylalanine-tRNA synthetase |
| NM_006867    | RBPMS     | 8p12-p11 | RNA-binding protein with multiple splicing |

**Proliferation**
| NM_013407.1  | DHPS      | 19p13.11 | deoxyhypusine synthase |
| NM_00508.2   | FGA       | 4q28     | fibrinogen, A alpha polypeptide |
| NM_01481.1   | GAS8      | 16q24.3  | growth arrest-specific 8 |
| NM_005537.1  | ING51     | 13q34    | inhibitor of growth family, member 1 |
| NM_002888.1  | RARRES1   | 3q25.32  | retinoic acid receptor responder (tazarotene induced) 1 |
| NM_006443.1  | RCL       | 6p21.1   | putative c-Myc-responsive |
| NM_005981.1  | SAS       | 12q13.3  | sarcoma-amplified sequence |

**Miscellaneous genes**
| NM_004306    | ANXA13    | 8q24.1-q24.2 | annexin A13 |
Concerning cytokine/chemokine-related genes, our results showed an up-regulation of several CXC chemokines; among the down-regulated genes we found interleukin (IL)-18 and macrophage migration inhibitory factor (MIF). Several interferon-induced genes were up-regulated, including the human 2'-5' oligoadenylate synthetase 2 gene (OAS2) and human myxovirus resistance-1 gene (MxA). SARS-CoV strains FFM-1 and 6109 influenced similarly the expression of the selected genes (fig. 5). Neither UV-inactivated virus nor virus-free filtered cell culture supernatants caused any changes in gene expression pattern compared to mock-infected cells (data not shown).

**Discussion**

To provide an experimental model for the study of SARS gastrointestinal pathology, we tested the sensitivity of six intestinal cell lines to SARS-CoV infection. In addition to Caco-2 cells which were previously shown to be permissive to SARS-CoV [8], only CL-14 cells promoted SARS-CoV replication. CL-14 cells show features of well-differentiated enterocytes [14] while Caco-2 cells show an undifferentiated phenotype with the ability to undergo spontaneous enterocytic differentiation after reaching confluence [15]. We infected Caco-2 cells 2–3 days after confluence, i.e. when electron microscopy identified mostly poorly differentiated enterocytes and only few well-differentiated (villus) enterocytes. Both poorly and well-differentiated enterocytes supported SARS-CoV replication suggesting that the sensitivity of intestinal epithelial cells does not depend on a particular stage of cellular differentiation.

ACE2 has recently been shown to be a functional receptor for SARS-CoV [3] and surface ACE2 is abundantly present on enterocytes of the small intestine [16]. The present study indicates that cell surface expression of ACE2 appears to be essential for infection of intestinal cells. Depending on the MOI used, CL-14 and Caco-2 cells produced maximum infectious virus titres 48–72 h p.i., at times when few effects on cell viability and CPE were observed. Later, i.e. 72 and 96 h p.i., cell viability was markedly decreased without a concomitant further increase of virus titres. Although some cells that survived virus infection even in cultures infected at MOI 10 for 96 h did not stain with immune serum, we did not succeed in sub-cultivation of surviving cells. Whether or not these cells are infected and whether expression of viral antigens is below the detection limit of the method used is not clear at present.

Gene expression analysis performed 24 h p.i. demonstrated that SARS-CoV infection influences expression of several cellular genes which may be important for SARS intestinal pathogenesis. For example, SARS-CoV up-regulated anti-apoptotic genes such as Bcl-2 and A20 while it down-regulated pro-apoptotic genes such as Bid, Bad, caspase-2 and caspase-6. In a murine model, Bcl-2 overexpression in gut epithelial cells, decreased the apoptosis [17] and protected against intestinal injury [18]. Although Bcl-2 was detectable only in Caco-2 cells, other regulators of the apoptotic mitochondrial pathway including pro-apoptotic Bcl-2 homologues Bid and Bad were down-regulated by SARS-CoV in both Caco-2 and CL-14 cells. Since Bid and Bad are involved in the regulation of intestinal epithelial cell survival [19], their role in SARS-CoV intestinal infection should be studied further. In both cell lines, SARS-CoV up-regulated A20 which may protect different cell types against tumour necrosis factor (TNF)-mediated programmed cell death and is critical for limiting inflammation by terminating TNF-induced nuclear factor (NF)-kB responses in the intestine and other organs [20]. In addition, down-regulation of caspase-2 and caspase-6 in infected intestinal cell lines may be of interest as these caspases were shown to be important mediators of apoptosis in gastrointestinal

![Figure 5. Validation of microarray data with semiquantitative RT-PCR. Transcripts from one housekeeping gene (GAPDH) and 19 other cellular genes were assessed for their relative abundance in mock-infected and SARS-infected (strains FFM-1 and 6109) colon carcinoma cell lines (Caco-1 and CL-14). All genes showed a pattern of transcript abundance consistent with the microarray data.](image-url)
epithelium [21, 22]. The results show that SARS-CoV-infected epithelial cells develop an anti-apoptotic response which may be important to inhibit or delay destruction of infected enterocytes. These findings are consistent with clinical observations demonstrating a relatively normal endoscopic and microscopic appearance of the intestine in patients with SARS [5].

On the other hand, SARS-CoV suppressed expression of the anti-apoptotic gene PDCD4 which is constitutively expressed in most normal tissues including lung and intestine [23]. Apart from its effects in the regulation of apoptosis, PDCD4 was shown to play a role in inhibition of translation by direct interaction with eukaryotic translation initiation factor 4A (eIF4A) [23, 24]. This finding is of interest since activity of eIF4E (together with eIF4A and eIF4G forming the translation initiation factor complex eIF4F) was shown to be important for replication of murine coronavirus [25]. Moreover, PDCD4 has the ability to suppress transactivation of activator protein (AP)-1 [23]. Since the SARS-CoV nucleocapsid was shown to activate the AP-1 transduction pathway [26], it will be of interest to show whether there may be a mechanistic link between PDCD4 suppression and enhancement of AP-1 transactivation in SARS-CoV-infected cells. The present observations demonstrate that SARS-CoV infection elevated mRNA levels of AP-1 subunits c-Fos and c-Jun in intestinal cells which could also increase AP-1 transactivation.

Infection with most viruses up-regulates different interferon (IFN)-induced genes which may establish an anti-viral state within cells. Its major effectors and indicators include double-stranded RNA-dependent protein kinase (PKR), OAS and MX proteins [27]. SARS-CoV infection of Caco-2 cells up-regulated OAS2 and MXA but not PKR genes. The discrepancy between transcriptional activation of IFN-induced genes and the ability of SARS-CoV to replicate in Caco-2 cells could be explained by the existence of a specific viral mechanism for escaping IFN-induced anti-viral effects common to most viruses [28]. The enteropathogenic potential of HCoV-OC43 (strain Paris) has been suggested to be due to its inability to induce PKR which was not stimulated by IFN-α [29]. Recently, we showed that IFN-α and IFN-β (type I IFN) inhibited SARS-CoV replication in Caco-2 cells while IFN-γ (type II IFN) was not effective [8]. Moreover, IFN-β was 50–90 times more potent than IFN-α against different SARS-CoV strains. The differences in anti-viral activity of different types of IFN could result from their ability to differentially influence expression of cellular genes important for anti-viral activity. For example, treatment of the human fibrosarcoma cell line HT1080 (expressing both type I and type II IFN receptors) with IFN-β stimulated PKR which was not stimulated by IFN-α or IFN-γ [30]. Since PKR was not up-regulated in the infected Caco-2 cells and virus replication progressed despite up-regulation of OAS2 and MXA, a role for PKR in SARS-CoV replication must be elucidated.

Although enteric pathogens such as viruses, protozoans, multicellular helmiths and enteroinvasive bacteria vary in their mode of infection, enterocytes display a common chemokine/cytokine profile in response to infection. Several ELR+CXC chemokines (containing a conserved glutamate-leucine-arginine sequence) including CXCL1 (groα), CXCL2 (groβ), CXCL3 (groγ) and CXCL8 (IL-8) were up-regulated in SARS-CoV infected Caco-2 cells. These chemokines mainly regulate neutrophil trafficking [31]. In addition, SARS-CoV induced in Caco-2 cells non-ELR CXC chemokines CXCL10/IFN-γ inducible protein-10 (IP-10) and CXCL11/IFN-inducible T cell alpha chemoattractant (I-TAC) which are potent CD4+ T cell chemoattractants [32, 33]. Rotavirus infection was shown to induce CXCL1, CXCL8 and CXCL10 in intestinal cell lines [34]. Mucosal inflammation associated with rotavirus infection is predominantly mononuclear, i.e. consists of monocytes and T lymphocytes [35], although neutrophil infiltration is found in some cases [34]. In contrast, biopsy specimens taken from the colon and terminal ileum of patients with SARS failed to demonstrate any inflammatory infiltrates [5]. Neutrophil infiltration in the intestine of SARS patients may be limited despite neutrophilia due to changes of cytokine/chemokine levels in the intestinal environment. We observed that SARS-CoV infection of Caco-2 cells inhibited expression of IL-18 which is constitutively expressed in intestinal epithelial cells [36]. Suppression of IL-18 levels reduces neutrophil accumulation in liver and lungs [37]. The absence of T lymphocyte infiltration of the intestine in SARS may be a consequence of the profound decline of both CD4+ and CD8+ lymphocytes in the blood [38], possibly resulting from lymphocyte apoptosis [39]. Although macrophage counts were increased in lungs [40], macrophage infiltration was absent from the gut of SARS patients [5]. In Caco-2 cells, SARS-CoV down-regulated MIF. Recently, MIF was identified as a major factor produced by intestinal cells in response to microbial infection regulating macrophage emigration, inflammation and cell metabolism [41]. Some of the chemokines we found up- or down-regulated in vitro were also changed in serum samples from SARS patients. For example, serum levels of CXCL10 and IL-8 were increased whereas IL-18 was decreased [42, 43]. This justifies the use of intestinal cell lines as a model to study the direct effects of SARS-CoV infection on gene expression in permissive human cells. Given the intestinal tropism of SARS-CoV, the results presented here provide several important hints at possible mechanisms of intestinal pathogenesis and potential novel therapeutic targets in SARS.

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