Research Article

Pathway and single gene analyses of inhibited Caco-2 differentiation by ascorbate-stabilized quercetin suggest enhancement of cellular processes associated with development of colon cancer

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The aim was to investigate mechanisms contributing to quercetin's previously described effects on cell-proliferation and -differentiation, which contradicted its proposed anticarcinogenic potency. In a 10-day experiment, 40 μM quercetin stabilized by 1 mM ascorbate reduced Caco-2 differentiation up to 50% ($p < 0.001$). Caco-2 RNA from days 5 and 10, hybridized on HG-U133A2.0 Affymetrix GeneChips®, showed 1743 affected genes on both days ($p < 0.01$). All 14 Caco-2 differentiation-associated genes showed decreased expression ($p < 0.01$), including intestinal alkaline phosphatase, that was confirmed technically (qRT-PCR) and functionally (enzyme-activity). The 1743 genes contributed to 27 pathways ($p < 0.05$) categorized under six gene ontology (GO) processes, including apoptosis and cell-cycle. Genes within these GO-processes showed fold changes that suggest increased cell-survival and -proliferation. Furthermore, quercetin down-regulated expression of genes involved in tumor-suppression and phase II metabolism, and up-regulated oncogenes. Gene expression changes mediated by ascorbate-stabilized quercetin were concordant with those occurring in human colorectal carcinogenesis (≈80–90%), but were opposite to those previously described for Caco-2 cells exposed to quercetin without ascorbate (≈75–90%). In conclusion, gene expression among Caco-2 cells exposed to ascorbate-stabilized quercetin showed mechanisms contrary to what is expected for a cancer-preventive agent. Whether this unexpected *in vitro* effect is relevant *in vivo*, remains to be elucidated.

Keywords: Caco-2 / Colorectal cancer / Differentiation / Microarray / Quercetin

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1 Introduction

Quercetin is a phytochemical that belongs to the polyphenol group of antioxidants. This dietary compound is part of the flavonol subclass of flavonoids and can be found in glycosylated forms in fruits and vegetables, including curly kale, broccoli, blueberries, and onions [1]. Following intake, conjugated quercetin can be hydrolyzed by β-glycosidases present in the cytoplasm or on the membrane of small intestinal cells [2]. When absorbed from the small intestine, quercetin can be metabolized by phase II enzymes present in small intestinal cells and the liver. On the other hand, conjugated quercetin that reaches the colon can be hydro-

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Abbreviations: ALPI, alkaline phosphatase, intestinal; **APC,** anaphase promoting complex; **CDC,** cell division cycle; **CDK,** cyclin-dependent kinase 1; **COX-2,** cyclo-oxygenase 2; **CRC,** colorectal cancer; **GAPDH,** glyceraldehyde-3-phosphate dehydrogenase; **GO,** gene ontology; **IAP,** inhibitor of apoptosis proteins; **MMP7,** matrix metalloprotease 7; **qRT-PCR,** quantitative reverse transcriptase PCR; **ROS,** reactive oxygen species; **STK,** serine/threonine kinase; **SULT,** sulfotransferase; **TEER,** transepithelial electrical resistance; **UGT,** UDP-glucuronosyltransferase

lyzed by bacterial β-glycosidases [3], leading to colonic release and absorption of quercetin [4].

In the healthy colon, uptake of nutrients from the colon lumen is facilitated by foldings in the intestinal mucosa that enlarge the absorption area. The base of the colonic crypts contains a limited number of stem cells which after proliferation migrate towards the lumen and can differentiate into absorptive cells (enterocytes), goblet cells (responsible for secretion of protective mucins), or enteroendocrine cells (secretion of hormones, including serotonin) [5]. Within 4 – 8 days after migration from the base of the crypt, these different cell types reach the top of the villus, become apoptotic and are shedded in the gut lumen [6]. The continuous process of proliferation, differentiation, and apoptosis that occurs along the crypt axis is strictly regulated. Once this equilibrium is perturbed, for example as a result of underlying gene mutations, colorectal tumors may develop through a gradual series of well-characterized histopathological changes, also known as the adenoma – carcinoma sequence [7].

The human-derived colon cancer cell line Caco-2 is a well-accepted model to study cell proliferation, differentiation, and apoptosis as a function of time [8]. When these cells proliferate toward a monolayer, contact inhibition occurs that leads to cell cycle arrest and spontaneous differentiation into absorptive cells [5]. This time-dependent process mimicks phenotypic changes that normal colonic epithelial cells undergo during migration along the crypt-villus axis *in vivo* [5]. A typical feature of differentiated Caco-2 cells is the brush border with a high density of microvilli. Brush border enzymes that are known to be positively correlated with the differentiation grade of enterocytes and therefore extensively used as Caco-2 differentiation markers, include intestinal alkaline phosphatase (ALPI) [6] and sucrase-isomaltase [8, 9].

Quercetin is generally recognized as a compound that inhibits mechanisms involved in development of (colorectal) cancer, as demonstrated by *in vitro* [10, 11] and *in vivo* [12, 13] studies. However, data regarding its mechanisms of action are relatively scarce and the beneficial effect of quercetin on colorectal cancer (CRC) is under debate [13, 14]. In a previous study performed with Caco-2 cells, quercetin-treatment resulted in effects opposite to what would be expected for a phytochemical with anticarcinogenic potency, including increased proliferation and inhibited differentiation [15]. The aim of the present study was to further investigate this unexpected effect in Caco-2 cells by characterising quercetin-induced changes in cellular physiology and gene expression in the context of biological pathways and gene ontology (GO) processes.

2 Materials and methods

2.1 Cell culture

The human colon cancer cell line, Caco-2, was obtained from the American Type Culture Collection (Manassas, VA, USA). For subculturing, near-confluent Caco-2 monolayers of passage 35 and 36 were seeded in a 1:10 split ratio in T75 flasks (Costar, Cambridge, UK) with DMEM culture medium, as described previously [15]. For differentiation experiments, Caco-2 cells of passage 37 were seeded (1:10 split ratio) in triplicate on polycarbonate membrane Transwell® inserts (Corning Life Sciences, Cambridge, UK), with a membrane diameter of 75 mm (growth area 44 cm^2, 0.4 μm pore size) at a density of ± 40 000 cells/cm^2. After 2 days, cell cultures reached confluency, and this time point was considered experimental day 0.

2.2 Quercetin exposure

To prevent quercetin's instability in culture medium [11], 40 μM quercetin (Sigma – Aldrich, Zwijndrecht, The Netherlands) out of a 200 × stock solution in DMSO (Sigma – Aldrich) was prepared in DMEM culture medium shortly before cell exposure and stabilized by 1 mM (final concentration) sodium ascorbate (Boom B. V., Meppel, The Netherlands) [15]. This culturing condition is further referred to as "40 μM quercetin". From days 0 – 9, Caco-2 monolayers were exposed to 8 mL of 40 μM quercetin in the apical compartment only, in order to mimic exposure as it would occur in the gut. As controls, cells were exposed to 8 mL of 1 mM sodium ascorbate, including the quercetin solvent (0.5% DMSO), a condition previously shown not to interfere with Caco-2 differentiation [15]. The latter culture condition is further referred to as "control". Basolateral compartments of both experimental conditions were filled with 12 mL DMEM culture medium. In the course of the experiment, culture medium in both compartments was refreshed every 24 h.

2.3 Transepithelial electrical resistance (TEER)

TEER was measured as a marker for Caco-2 cell differentiation [15]. As temperature fluctuations may influence the outcome of the TEER measurement, Transwell® inserts were cooled down to room temperature (20 ± 1°C) prior to TEER measurement. On days 0, 3, 5, 7, and 10 postconfluency TEER was measured in triplicate, using a Millicel-ERS Volt Ohm meter (Millipore, Amsterdam, The Netherlands). TEER values were calculated according to the following equation: TEER = $R \times$ filter area ($\Omega \cdot cm^2$).

2.4 Alkaline phosphatase

Activity of intestinal alkaline phosphatase, a Caco-2 differentiation marker, was determined on a BM/Hitachi 911 analyzer according to a colorimetric assay as described previously [6]. In brief, ALP converts the substrate *p*-nitrophenol phosphate into *p*-nitrophenol. Concomitantly, the time-dependent release of *p*-nitrophenol is proportional to the ALP activity. The activity of this enzyme was determined in the apical culture medium, since in previous studies intracellular ALP was shown to be correlated with its presence and activity in the apical culture medium [16, 17]. ALP activity among quercetin-treated and control cells was first corrected for baseline ALP activity in DMEM culture medium. Subsequently, corrected ALP activity was normalized for the number of cells in culture, by measurement of total protein in the apical culture medium.

2.5 RNA extraction, cleanup, and quality control

On days 5 and 10 postconfluency, cells were first rinsed with ice cold PBS without calcium or magnesium (Invitrogen® Life Technologies, Breda, The Netherlands). Subsequently, cells were harvested in 2×0.75 mL of ice-cold TRIzol® (Life Technologies, Paisley, UK), immediately frozen in liquid nitrogen and stored at $-80^{\circ}C$ for up to 2 months, until RNA isolation according to the TRIzol protocol. Following isolation, total RNA was purified with RNeasy Midi columns (QIAGEN, Westburg, Leusden, The Netherlands), including a DNAse incubation step. Quality of purified total RNA was determined on a UV-VIS spectrophotometer (Shimadzu Benelux, 's Hertogenbosch, The Netherlands) by calculation of the A_{260}/A_{280} ratio (1.7–2.0) and confirmed on a Lab-on-a-Chip on the Agilent 2100 bioanalyser (Agilent Technologies, Palo Alto, California, USA) by determining the 28S/18S ribosomal RNA ratio (1.7–2.0).

2.6 RNA preparation for Affymetrix GeneChip® arrays and hybridization

RNA originating from quercetin treated and control Caco-2 cells harvested on days 5 and 10, was used as an input for hybridization to HG-U133A 2.0 Affymetrix GeneChip® arrays that comprise 22 215 genes, of which 14 500 are well-characterized human genes.

For hybridization, the GeneChip® One-Cycle Eukaryotic Target Labeling Assay for expression analysis was performed, as described in the Affymetrix GeneChip® Expression Analysis Technical Manual (GeneChip® Expression Analysis Technical Manual, 2004, http://www.affymetrix.com/support/technical/manual/expression_manual.affx). In brief, 2 μg of purified total RNA was reverse transcribed using a T7-Oligo(dT) Promoter Primer for synthesis of the first-strand complementary DNA (cDNA). Subsequently, double stranded cDNA was produced to serve as a template for the *in vitro* transcription. After cRNA fragmentation, meant for optimal hybridization efficiency, 10 μg of fragmented cRNA was hybridized to Affymetrix GeneChip® HG-U133A 2.0 arrays and finally detected with the GeneChip® Scanner 3000 7G (Affymetrix, Santa Clara, California, USA).

2.7 Analysis of Affymetrix GeneChip® array data

CEL files containing raw signal intensities, were imported in Rosetta Resolver 5.0 (Rosetta Inpharmatics LLC, Seattle, USA), applying data preprocessing (to estimate and reduce systematic errors) and error modeling (for improvement of accuracy of the present and absent calls for low replicate numbers). Genes with an "absent call" ($p \geq 0.065$ for HG-U133A 2.0 GeneChip® arrays) across all eight arrays ($n = 7285$) were considered not to be affected by quercetin treatment and/or incubation time and were therefore excluded from further analyses (see Fig. 1 of Supporting Information). Subsequently, normalized intensities originating from replicate Affymetrix GeneChip® arrays ($n = 2$/group) were combined and fold changes (quercetin *vs.* control) were calculated for days 5 and 10. For selection of genes that changed upon quercetin-treatment in time, the remaining genes ($n = 14\,930$) were analyzed by a combination of the two-way ANOVA ($p < 0.01$), including error-weighting (to increase degrees of freedom) and the false discovery rate (FDR < 0.05) [18]. This analysis retrieved 1743 significantly changed genes (≈8% of the initial number of genes). To interpret these differentially expressed genes at a higher biological level rather than the level of single genes only, data were loaded in the pathway mapping tool MetaCore™ (GeneGo, St. Joseph, MI, USA). Significantly changed pathways were subsequently used to determine the relative contribution of significantly changed

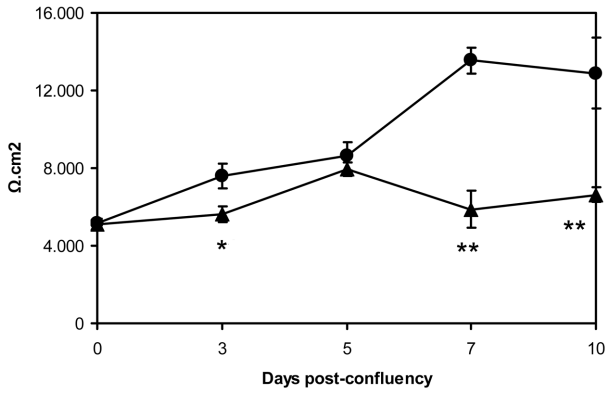

Figure 1. Time-dependent TEER values in 40 μM quercetin (triangles) and control cells (dots). Values are given as mean ± SD ($n = 3$/group). * $p < 0.01$ and ** $p < 0.001$ when compared to control cells.

genes to pathway-associated GO processes. To this end, different pathways categorized under identical GO processes were first grouped. Subsequently, within each set of grouped pathways, *i. e.*, within each GO process, the number of significantly changed genes was expressed as percentage of the total number of genes.

MIAME (minimum information about a microarray experiment) compliant data described in this publication have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE7259.

2.8 Confirmation of Affymetrix GeneChip® array expression data

To support the biological relevance of the statistically filtered dataset, a search was performed for the presence of previously described Caco-2 differentiation markers, regardless of the direction and/or magnitude of the fold change [8].

To confirm Affymetrix GeneChip® array expression data and the biological relevance of the dataset obtained by statistical analyses, a subset of relevant genes, including the Caco-2 differentiation marker ALPI [6], and the human CRC-associated genes encoding for cyclo-oxygenase 2 (COX-2) [19] and matrix metalloprotease 7 (MMP7) [20], was confirmed by means of real-time quantitative reverse transcriptase PCR (qRT-PCR). Primers for real-time qRT-PCR were developed in Beacon Designer 4.02 (Premier Biosoft, Palo Alto, California, USA). Furthermore, the ALPI enzyme was confirmed functionally by measurement of its activity corrected for total protein in the apical culture medium [6].

2.9 qRT-PCR

Per sample (n = 3/group) 200 ng of total RNA was reverse transcribed into complementary DNA (cDNA) in an iCycler iQ™ Real-Time PCR Detection System (BioRad, Veenendaal, The Netherlands), using the iScript™ cDNA synthesis kit (BioRad, Veenendaal, The Netherlands). This reaction was performed in a final volume of 20 μL containing 5% v/v of a Moloney murine leukaemia virus (MMLV)-derived reverse transcriptase with RNAse inhibitor and 20% v/v of a 5x iScript Reaction Mix. Subsequent real-time qRT-PCR was performed either with the iQ™ SYBR® Green Supermix or the TaqMan® assay (see Table 1 of Supporting Information). The SYBR® Green assay was performed in a final volume of 25 μL containing 50% v/v of the 2x iQ™ SYBR® Green Supermix, 0.3 μM primermix with both forward and reverse primers (Biolegio, Nijmegen, The Netherlands) and 5 ng of cDNA. An initial denaturation step of 3 min at 95°C was followed by 45 cycles, each consisting of 94°C for 15 s, followed by annealing (temperatures given in see Table 1 of Supporting Information) for 30 s and elongation at 72°C for 20 s. Subsequently, a melting curve was generated to ensure amplification of only the proper PCR product. This was performed by 80 cycles of 10 s each, starting at 54°C, increasing with 0.5°C per cycle up to 94°C and measuring the fluorescence.

The TaqMan® assay was carried out in a final volume of 25 μL with 57% v/v TaqMan® universal mastermix (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands), ≈0.9 μM primer mix with both forward and reverse primers, ≈0.2 μM of the corresponding TaqMan® probe (Applied Biosystems) and 50 pg of cDNA. After an initial denaturation step of 10 min at 95°C, 45 cycles were run, each of which consisted of 95°C for 15 s and 60°C for 60 s.

Real-time qRT-PCR reactions for both TaqMan® and SYBR® Green assays were performed in the iCycler iQ™ Real-Time PCR Detection System. Absolute copy numbers of the genes of interest were determined by linear regression from cDNA calibration curves of each gene. Expression data obtained by real-time qRT-PCR were normalized against the amount of total RNA used for each sample (200 ng), expressed as copies per microgram of RNA [21] and against the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin (ACTB) that were both unchanged upon quercetin treatment, according to the microarray data.

2.10 Comparison with human colorectal carcinogenesis

Quercetin-induced fold changes in gene expression among Caco-2 cells were compared to fold changes previously described for human colorectal carcinogenesis. To this end, genes contributing to significantly changed pathways were selected based on an identical direction of fold change on both days 5 and 10 and a minimum up- or downregulation of 1.5 on both days (see Fig. 1 of Supporting Information). Gene expression data for human colorectal carcinogenesis were retrieved from PubMed articles, including microarray based studies, or from the Cancer Profiling Database Oncomine™ (http://www.oncomine.org/main/index.jsp).

In addition to MetaCore™ based analyses, the total set of 1743 differentially expressed genes was evaluated for the presence of genes contributing to functional groups that are likely to be involved in nutritional modulation of CRC, *i. e.* tumor suppressor genes, oncogenes, and genes encoding for enzymes involved in xenobiotic metabolism (see Fig. 1 of Supporting Information). These *in vitro* data were retrieved and compared with previously published data for human colorectal carcinogenesis, as described above.

2.11 Comparison to microarray data of Caco-2 cells exposed to quercetin without ascorbate

To determine whether effects on Caco-2 cells are caused by interference of ascorbate that is required for quercetin-stabilization, two comparisons were made with data from our

Table 1. Expression data of genes that are positively correlated with Caco-2 cell differentiation and their behavior upon quercetin treatment, either with or without ascorbate stabilization

Gene title (synonym)	Gene symbol	Accession number	Function	References	Fold change quercetin *versus* control + ascorbate, +quercetin			Fold change quercetin *versus* control − ascorbate, +quercetin	
					Day 5	Day 10	$p <$	5 μM ($p <$)	50 μM ($p <$)
Alkaline phosphatase, intestinal	*ALPI*	NM_001631	Cleavage of phosphate groups on DNA, RNA, (deoxy)ribonucleosides and proteins	[5, 6, 22]	−2.1	−2.1	0.00609	1.5 (0.05)	1.5 (0.1)
Sucrase-isomaltase	*SI*	NM_001041	Digestion of sucrose and isomaltose in the intestine	[9]	−38.0	−3.8	2.3E−06	–	–
Calbindin 3 (Calbindin-D9K)	*S100G (CALB3)*	NM_004057	Vitamin D-dependent calcium binding	[8]	−4.6	−2.3	6.9E−12	–	–
Dipeptidylpeptidase IV (CD26)	*DPP4*	NM_001935	Proteolysis, immune response	[22]	−2.2	−1.4	0.00125	–	–
Glutaminase	*GLS*	NM_014905	Glutamine catabolism	[8]	−1.8	−1.9	0.00042	–	–
Hephaestin	*HEPH*	NM_014799	Copper and iron ion transport	[8]	−2.0	−1.2	9.6E−07	–	–
Transferrin	*TF*	AI073407	Secreted Fe^{3+} transport protein	[9]	−7.2	−1.9	0.00007	–	–
Aquaporin 3	*AQP3*	N74607	H_2O transport	[8]	−6.4	−2.6	4.0E−13	–	–
Fatty acid binding protein 6, ileal (gastrotropin)	*FABP6*	U19869	Lipid metabolism and negative regulation of cell proliferation	[38]	−2.2	−2.5	0.00697	–	–
Alanyl (membrane) aminopeptidase (aminopeptidase N, aminopeptidase M, Microsomal aminopeptidase, CD13, p150)	*ANPEP*	NM_001150	Cell differentiation and development	[22]	−4.4	−1.4	1.9E−08	–	–
Membrane-bound aminopeptidase P (XNPEP2)	*XPNPEP2*	AF195953	Proteolysis by metalloexopeptidase activity	[22]	−6.9	−3.0	0.00002	–	–
Angiotensin I converting enzyme peptidyl-dipeptidase A) 2	*ACE2*	AK026461	Proteolysis by carboxypeptidase activity and zinc ion binding	[22]	−3.5	−1.6	0.00972	–	–
Apolipoprotein B	*APOB*	NM_000384	Lipid metabolism	[8]	−3.6	−1.6	1.1E−16	–	–
Apolipoprotein M (G3A)	*APOM*	NM_019101	Membrane lipid metabolism, lipid transport	[8]	−3.7	−2.0	0.00116	−1.4 (0.5)	−1.4 (0.01)

All 14 Caco-2 cell differentiation related genes were significantly down-regulated by 40 μM quercetin stabilized by 1 mM ascorbate. References indicate articles in which these genes have been reported as being positively correlated with Caco-2 cell differentiation. For comparison of quercetin-mediated effects in the absence of ascorbate, data are shown from Caco-2 cells exposed to 5 or 50 μM quercetin only, as described in our previous report [10].

previous report in which postconfluent Caco-2 cells were exposed to 5 and 50 μM quercetin for 48 h, without ascorbate [10]. First, significantly affected genes as presented in Table 1 (differentiation genes), Table 2 (genes contributing to significantly changed pathways) and Table 3 (single gene analysis) were compared to genes found in our previous study performed with quercetin only [10]. For the latter study, genes were included that were found to be present in at least three out of four microarrays per treatment group, showing an identical direction of fold change for both quercetin concentrations and at least one significant p-value of < 0.05, or in the case of data available for only one of two quercetin concentrations, showing a p-value of < 0.05.

Secondly, the list of 281 significantly changed genes from our previous study performed with quercetin only [10] was first filtered as mentioned above, and subsequently compared to the subset of 1743 differentially expressed genes in the present study performed with ascorbate-stabilized quercetin, retrieved after filtering on genes showing an identical direction of fold change on both days.

Table 2. Selection of genes contributing to GO processes affected by 27 significantly changed pathways

GO process	Gene title	Gene symbol (synonym)	Accession number	Fold change quercetin *versus* control + ascorbate, +quercetin		Fold change in human colorectal carcinogenesis		In agreement	Fold change quercetin *versus* control − ascorbate, +quercetin		Opposite effect?
				Day 5	Day 10	Direction	Reference	5 μM ($p <$)	50 μM ($p <$)		
Cell cycle	BUB1 budding uninhibited by benzimidazoles 1 homolog (yeast)	*BUB1*	AL137654	1.8	2.2	↑	[39] S	Yes	–	−1.5 (0.05)	Yes
	Citron (rho-interacting, serine/threonine kinase 21)	*CIT*	AI861788	1.5	1.9				–	–	
	Antigen identified by mAb Ki-67	*MKI67*	BF001806	1.9	2.2	↑	[39] S	Yes	–	–	
	HRAS-like suppressor 3	*HRASLS3 (HREV107)*	BC001387	1.8	1.5	↑	[39] S	Yes	1.3 (0.02)	1.3 (0.05)	No
	Aurora kinase B	*AURKB (AIM-1, STK12, ARK2)*	AB011446	2.1	1.7	↑	[40]	Yes	–	–	
	Kinesin family member 23	*KIF23*	NM_004856	1.5	1.9				–	–	
	CDC6 cell division cycle 6 homolog (*S. cerevisiae*)	*CDC6 (CDC18)*	NM_001254	1.6	2.9	↑	[39] S	Yes	−4.1 (0.01)	-	Yes
	Discs, large homolog 7 (Drosophila)	*DLG7 (DLG1)*	NM_014750	1.6	2.2	↑	[41]	Yes	–	–	
	Polo-like kinase 1 (Drosophila)	*PLK1*	NM_005030	1.8	2.1	↑	[42] S	Yes	−1.5 (0.02)	−1.7 (0.01)	Yes
	Annexin A1	*ANXA1*	NM_000700	4.9	5.8	↑	[27]	Yes	−1.3 (0.1)	−0.3 (0.05)	Yes
	Cyclin D2	*CCND2*	NM_001759	2.9	7.4	↑	[39] S	Yes	–	–	
	G0/G1switch 2	*G0S2*	NM_015714	−2.7	−2.5	↑	[39] S	No	–	–	
Apoptosis, cell death	Egl nine homolog 3 (C. Elegans)	*EGLN3*	NM_022073	2.9	4.2				–	–	
	Pleckstrin homology-like domain, family A, member 1	*PHLDA1*	AA576961	2.0	1.6	↑	[43]	Yes	–	–	
	Baculoviral IAP repeat-containing 3	*BIRC3*	U37546	2.8	1.5	↑	[44]	Yes	–	–	
	Pleckstrin homology-like domain, family A, member 2	*PHLDA2*	AF001294	1.7	3.9				–	–	
	Immediate early response 3	*IER3*	NM_003897	2.6	5.3				–	–	
	Lectin, galactoside-binding, soluble, 1 (galectin 1)	*LGALS1*	NM_002305	3.9	10.8	↑	[45] P	Yes	–	–	
	Epithelial membrane protein 3	*EMP3*	NM_001425	8.0	3.4				−1.2 (0.5)	−1.2 (0.05)	Yes
	Transforming growth factor, beta 1 (Camurati–Engelmann disease)	*TGFB1*	BC000125	4.1	2.8	↑	[46]	Yes	−1.4 (0.01)	−1.4 (0.5)	Yes
	Epithelial membrane protein 1	*EMP1*	NM_001423	2.3	5.2	↓	[42] S	No	–	–	
	BCL2-like 14 (apoptosis facilitator)	*BCL2L14*	NM_030766	−3.4	−1.7				–	–	
	Deoxyribonuclease I	*DNASE1*	M55983	−4.1	−2.3	↑	[47] O	No	–	–	
	Tumor necrosis factor (ligand) superfamily, member 10	*TNFSF10 (TRAIL)*	NM_003810	−4.2	−2.0	↓	[42] S	Yes	–	–	
Transcription	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	*MAFF*	AL021977	1.7	2.7	↑	[39] S	Yes	–	–	
	RAD54 homolog B (*S. cerevisiae*)	*RAD54B*	NM_012415	1.7	2.2				–	–	
	Tribbles homolog 3 (Drosophila)	*TRIB3*	NM_021158	1.9	2.2				–	–	
	MCM4 minichromosome maintenance deficient 4 (*S. cerevisiae*)	*MCM4 (CDC21, CDC54)*	AA604621	1.7	2.2	↑	[48] O	Yes	–	–	

Table 2. Continued

GO process	Gene title	Gene symbol (synonym)	Accession number	Fold change quercetin *versus* control + ascorbate, +quercetin		Fold change in human colorectal carcinogenesis		In agreement	Fold change quercetin *versus* control − ascorbate, +quercetin		Opposite effect?
				Day 5	Day 10	Direction	Reference	5 μM ($p <$)	50 μM ($p <$)		
	Zinc finger protein 165	*ZNF165*	NM_003447	1.6	2.3	↑	[49]	Yes	−1.6 (0.05)	−1.5 (0.01)	Yes
	Activating transcription factor 3	*ATF3*	NM_001674	2.0	3.9	↑	[39] S	Yes	–	–	
	v-myb myeloblastosis viral oncogene homolog (avian)-like 2	*MYBL2 (BMYB)*	NM_002466	1.8	1.8	↑	[39] S	Yes	–	–	
	Heat shock 70 kDa protein 1A, heat shock 70 kDa protein 1B	*HSPA1A, HSPA1B*	NM_005345	2.6	1.6	↑	[25]	Yes	–	–	
	General transcription factor IIH, polypeptide 1, 62 kDa	*GTF2H1*	BC000365	−2.7	−1.5	↑	[48]	No	–	–	
	WW domain-containing transcription regulator 1	*WWTR1*	BF674349	−2.5	−1.6				–	–	
Protein kinase cascade	Serine/threonine kinase 17b (apoptosis-inducing)	*STK17B*	NM_004226	2.6	2.2				–	–	
Proteolysis	Mucosa-associated lymphoid tissue lymphoma translocation gene 1	*MALT1*	AB026118	2.1	2.2	↑	[39] S	Yes	–	–	
	Oxidized low density lipoprotein (lectin-like) receptor 1	*OLR1*	AF035776	2.3	2.5				1.1 (0.5)	1.7 (0.05)	No
	Matrix metallopeptidase 7 (matrilysin, uterine)	*MMP7*	NM_002423	23.6	5.9	↑	[20] P	Yes	–	–	
	Angiotensin I converting enzyme (peptidyl-di-peptidase A) 2	*ACE2*	NM_021804	−3.5	−1.6				–	–	
	X-prolyl aminopeptidase (aminopeptidase P) 2, membrane-bound	*XPNPEP2*	AF195953	−6.9	−3.0				–	–	
	Macrophage stimulating 1 (hepatocyte growth factor-like)	*MST1*	U37055	−2.6	−1.5				–	–	
	Transmembrane protease, serine 6	*TMPRSS6*	AI912086	−4.4	−2.5				–	–	
	Macrophage stimulating, pseudogene 9	*MSTP9*	AA911235	−2.6	−1.5				–	–	
	Myosin IA	*MYO1A*	AF009961	−4.0	−1.6				–	–	
	Meprin A, alpha (PABA peptide hydrolase)	*MEP1A*	NM_005588	−4.7	−1.5	↑	[50] P	No	−1.3 (0.5)	−1.2 (0.05)	No
	Cathepsin O	*CTSO*	AV729484	−5.0	−2.0				−1.1 (1)	−1.7 (0.05)	No
	B-factor, properdin	*BF (CFB)*	NM_001710	−2.6	−2.1	↑	[43]	No	1.4 (0.01)	1.1 (0.5)	Yes
Small GTPase-mediated signal transduction	RAB3B, member RAS oncogene family	*RAB3B*	BC005035	3.1	3.4						

Genes with an identical direction of fold change on both days 5 and 10 and a minimum of 1.5 up- or downregulation on both days ($n = 48$), contributing to significantly changed pathways. *O*, gene expression data are only accessible via the Cancer Profiling Database Oncomine (http://www.oncomine.org/main/index.jsp); *P*, differential expression determined at the protein level; *S*, gene expression data are only accessible via the online supplementary data of the corresponding articles; The column "In agreement" indicates whether the direction of gene expression changes in Caco-2 cells exposed to ascorbate-stabilized quercetin is in agreement with the direction of gene expression changes occurring in human colorectal carcinogenesis.; For comparison of quercetin-mediated effects in the absence of ascorbate, data are shown from Caco-2 cells exposed to 5 or 50 μM quercetin only, as described in our previous report [10]. The column "Opposite effect?" indicates whether the direction of fold change as found by ascorbate-stabilized quercetin is reversed when the Caco-2 cells are exposed to quercetin only, without ascorbate.

2.12 Statistics on "non-omics" parameters

Quercetin-induced effects on TEER, real-time qRT-PCR, and ALPI activity were tested for significance with the Student's *t*-test and considered significantly changed when $p < 0.05$.

3 Results

3.1 TEER

TEER was measured as an indicator of Caco-2 cell differentiation. In the course of the experiment, quercetin-treated cells showed a significantly lower increase in TEER values when compared to control cells (Fig. 1). On day 10 post-confluency, TEER values for quercetin-treated cells amounted to ≈50% of control cells. These results indicate that exposure of Caco-2 cells to 40 μM quercetin resulted in decreased cell differentiation.

3.2 Confirmation of decreased Caco-2 differentiation

To further investigate whether decreased Caco-2 cell differentiation could be supported by Affymetrix GeneChip® array data, fold changes of Caco-2 differentiation related genes among the 1743 significantly changed genes were compared with available Caco-2 literature. As can be seen in Table 1, mRNA expression of all 14 Caco-2 differentiation markers found among the significantly changed genes, including *ALPI*, sucrase-isomaltase, dipeptidylpeptidase IV (*DPP4* = *CD26*), aminopeptidase N and P that are expressed in the enterocyte brush border [5, 8, 22], were found to be decreased by quercetin. In addition, the main regulator of intestinal cell differentiation, *CDX2* [23], was near-significantly down-regulated (fold change day 5: −2.0 and day 10: −1.2, $p < 0.03$).

Decreased Caco-2 cell differentiation upon exposure to ascorbate-stabilized quercetin, as measured by the physiological parameter TEER, was thus confirmed by decreased expression of differentiation related genes among the statistically filtered dataset.

3.3 Confirmation of Affymetrix GeneChip® array expression data

In addition to the above mentioned literature-based confirmation, decreased mRNA expression of the Caco-2 differentiation marker *ALPI* as indicated by Affymetrix GeneChip® arrays was confirmed both technically – by means of real-time qRT-PCR – and functionally by measurement of ALPI enzyme activity. As can be seen in Fig. 2A, ALPI expression was significantly decreased on days 5 and 10 as determined with Affymetrix GeneChip® arrays, as quantified by real-time qRT-PCR corrected for both total RNA concentration and the housekeeping genes *GAPDH* and β-actin, and as determined with ALPI enzyme activity corrected for the number of cells in culture.

In addition, since decreased cell differentiation is a hallmark of (colon) cancer [24], the possibility of differential expression of the colon cancer-associated genes encoding for COX-2 [19] and MMP7 [20] was also investigated by means of real-time qRT-PCR. Fold changes of these genes as obtained with Affymetrix GeneChip® arrays compared to fold changes obtained with real-time qRT-PCR after correction for *GAPDH*, β-actin, and total RNA, were also in agreement with one another (Figs. 2B and C).

3.4 Biological pathways and corresponding GO processes

Since Affymetrix GeneChip® array expression data were confirmed with additional techniques, this reliable gene expression dataset was subsequently used for further analyses. In order to visualize and to interpret the statistically filtered set of 1743 significantly changed genes at a higher biological level, this dataset was loaded in the MetaCore™ software, in order to find affected biological pathways and their corresponding GO processes. Table 2 of Supporting Information shows an overview of significantly changed pathways ($n = 27$, $p < 0.05$) and GO processes. When the number of significantly changed genes within each GO process was expressed as percentage of the total number of genes within the very same GO process, the following percentages were found: cell cycle: 31%, apoptosis and cell death: 31%, transcription: 29%, protein kinase cascade: 26%, proteolysis: 25%, small GTPase-mediated signal transduction: 24%, and unknown: 30%. Within the specified GO processes showing the highest percentage of differentially expressed genes, *i.e.*, "cell cycle", and "apoptosis and cell death", the most significantly changed pathways are "Role of anaphase promoting complex (APC) in cell cycle regulation" ($p = 0.0004$) and "Role of inhibitor of apoptosis proteins (IAP) in apoptosis" ($p = 0.035$), respectively (see Table 2 of Supporting Information). In the Role of APC in cell cycle regulation pathway, fold changes in gene expression suggest increased cell proliferation, as depicted in Fig. 3. Among the six above mentioned GO processes (GO process "unknown" was excluded) affected by significantly changed pathways, 48 genes showed a combination of an identical direction of fold change and a minimum up- or downregulation of 1.5 on both days (Table 2). As can be seen in the GO processes apoptosis and cell death, quercetin-treatment probably resulted in inhibition of apoptosis, as suggested by upregulation of apoptosis inhibitors, *e.g.*, *BIRC3* and downregulation of apoptosis inducers, *e.g.*, *TNFSF10*.

Table 3. Overview of differentially expressed genes grouped by function

Functional group	Gene title	Gene symbol (synonym)	Accession number	Fold change quercetin *versus* control + ascorbate, +quercetin		Fold change in human colorectal carcinogenesis		In agreement	Fold change quercetin *versus* control − ascorbate, + quercetin		Opposite effect?
				Day 5	Day 10	Direction	Reference		5 μM ($p <$)	50 μM ($p <$)	
Xenobiotic metabolism	Cytochrome P450, family 1, subfamily A, polypeptide 1	*CYP1A1*	NM_000499	12.5	5.6	↑	[51] P	Yes	1.2	11.4	No
	Cytochrome P450, family 2, subfamily B, polypeptide 7 pseudogene 1	*CYP2B7P1*	M29873	−3.0	−2.4				–	–	
	Glutathione S-transferase A1	*GSTA1*	NM_000846	−2.3	−1.5				–	–	
	UDP glucuronosyltransferase 1 family, polypeptide A9	*UGT1A9*	AV691323	1.5	1.9				–	–	
	UDP glucuronosyltransferase 1 family, polypeptide A10	*UGT1A10*	NM_021027	1.5	1.9				–	–	
	UDP glucuronosyltransferase 2 family, polypeptide B15	*UGT2B15*	NM_001076	−1.6	−2.1	↓	[47]	Yes	–	–	
	Epoxide hydrolase 1, microsomal (xenobiotic)	*EPHX1*	NM_000120	−1.3	−1.9	↓	[52] P	Yes	–	1.3 (0.02)	Yes
	Epoxide hydrolase 2, cytoplasmic	*EPHX2*	AF233336	−3.0	−1.9	↓	[48] O	Yes	–	–	
	Sulfotransferase family, cytosolic, 1C, member 1	*SULT1C1*	NM_001056	−2.6	−2.5				–	–	
	Sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone (DHEA)-preferring, member 1	*SULT2A1*	U08024	−4.2	−2.6				–	1.3 (0.01)	Yes
	Flavin-containing monooxygenase 1	*FMO1*	NM_002021	−2.4	−5.6	↓	[39] S	Yes	–	1.9 (0.05)	Yes
	Flavin-containing monooxygenase 5	*FMO5*	NM_001461	−4.6	−1.8	↓	[42]	Yes	–	–	
	Glutathione peroxidase 2 (gastrointestinal)	*GPX2*	NM_002083	4.4	2.7	↓	[48] O	No	1.7 (0.02)	1.3 (0.05)	No
	ATP-binding cassette, sub-family A (ABC1), member 1	*ABCA1*	NM_005502	−4.2	−2.0				–	–	
umor suppressor genes and oncogenes	TP53 activated protein 1	*TP53AP1*	BC002709	−2.9	−2.9	↓	[53] O	Yes	–	–	
	RAB3B, member RAS oncogene family	*RAB3B*	BC005035	3.1	3.4				–	–	
	v-myb myeloblastosis viral oncogene homolog (avian)-like 2	*MYBL2*	NM_002466	1.8	1.8	↑	[39] S	Yes	–	–	
	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	*MAFF*	AL021977	1.7	2.7	↑	[39] S	Yes	–	–	
	Epithelial membrane protein 1	*EMP1*	NM_001423	2.3	5.2				–	–	
	Transmembrane 4 L six family member 1	*TM4SF1 (TAAL6)*	AI346835	1.5	2.6	↑	[54]	Yes	−1.4 (0.02)	1.4 (0.5)	Yes
	Rho GDP dissociation inhibitor (GDI) beta	*ARHGDIB (LY-GDI)*	NM_001175	2.8	2.5	↑	[55]	Yes	–	–	
Miscellaneous	Heat shock 27 kDa protein 1	*HSPB1*	NM_001540	2.9	2.8				–	–	
	Heat shock 70 kDa protein 1A, heat shock 70 kDa protein 1B	*HSPA1A, HSPA1B*	NM_005345	2.6	1.6	↑	[25]	Yes	–	–	
	Trefoil factor 2 (spasmolytic protein 1)	*TFF2 (SP)*	NM_005423	−7.4	−3.1				–	–	
	CD24 antigen (small cell lung carcinoma cluster 4 antigen)	*CD24*	BG327863	3.1	3.0	↑	[39] S	Yes	–	–	
	Frizzled homolog 2 (Drosophila)	*FZD2*	L37882	2.7	1.5	↑	[56] P	Yes	–	–	

Genes with an identical direction of fold change on both days 5 and 10 and a minimum of 1.5 up- or downregulation on both days, that are part of the 1743 significantly changed genes. *O*, gene expression data are only accessible *via* the Cancer Profiling Database Oncomine (http://www.oncomine.org/main/index.jsp); *P*, differential expression determined at the protein level; *S*, gene expression data are only accessible *via* the online supplementary data; The column In agreement indicates whether the direction of gene expression changes in Caco-2 cells exposed to ascorbate-stabilized quercetin is in agreement with the direction of gene expression changes occurring in human colorectal carcinogenesis. For comparison of quercetin-mediated effects in the absence of ascorbate, in the last two columns data are shown from Caco-2 cells exposed to 5 or 50 μM quercetin only, as described in our previous report [10]. The column "Opposite effect?" indicates whether the direction of fold change as found by ascorbate-stabilized quercetin is reversed when the Caco-2 cells are exposed to quercetin only, without ascorbate.

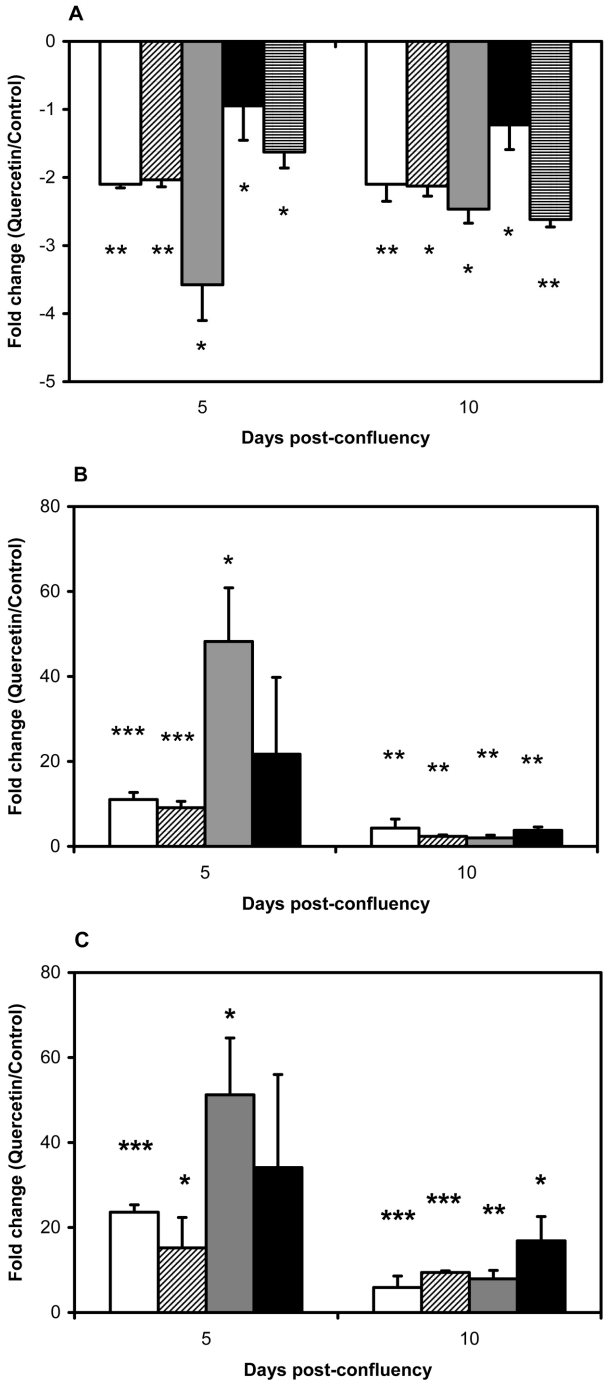

Figure 2. Fold changes of *ALPI* (A), *PTGS2*, which encodes the *COX-2* enzyme (B) and *MMP7* (C) on days 5 and 10, determined by measurement with Affymetrix GeneChip® arrays (white bars), by real-time qRT-PCR corrected for the house keeping genes *GAPDH* (diagonally lined bars), β-actin (grey bars) or for total RNA (black bars), and measurement of *ALPI* activity in the apical culture medium corrected for the number of cells (Panel A, horizontally lined bars, expressed as μmol nitrophenol/min/mg protein). Values are presented as mean ± SD, $n = 3$/group for real-time qRT-PCR and $n = 2$/group for Affymetrix GeneChip® arrays. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.5 Gene analysis by functional groups

In addition to analyses based on the MetaCore™ software, the total set of 1743 significantly changed genes was evaluated for functional groups that are involved in nutritional modulation of CRC, *i.e.*, tumor suppressor genes, oncogenes, and xenobiotic metabolism. Genes involved in xenobiotic metabolism showed a tendency towards decreased expression of phase II metabolism genes (Table 3).

Differentially expressed genes in the functional group "tumor suppressor genes and oncogenes" show a decreased expression of tumor suppressor genes and increased expression of oncogenes. As can be seen in the miscellaneous functional group, quercetin up-regulated expression of heat shock proteins, which is a phenomenon also observed in human colorectal carcinogenesis [25].

3.6 Comparison with human colorectal carcinogenesis

Gene expression changes categorized by GO processes and functional groups were compared with changes reported to occur in human colorectal carcinogenesis.

Comparison by GO processes showed that in total, 28 out of 48 genes (≈58%) could be compared to available literature on human colorectal carcinogenesis (Table 2). Within these 28 genes, 22 (≈79%) showed a direction of fold change that is concordant with fold changes reported for human colorectal carcinogenesis.

For gene expression changes categorized by functional groups, in total 15 out of 26 (≈58%) genes could be compared to available literature on human colorectal carcinogenesis (Table 3). Overall, 14 out of the 15 genes (≈93%), showed quercetin-mediated fold changes that were concordant with those occurring in human colorectal carcinogenesis.

3.7 Comparison with Caco-2 cells exposed to quercetin without ascorbate

To determine whether the above mentioned adverse effects in Caco-2 cells are caused by possible interference of the antioxidant ascorbate that is required for stabilization of quercetin in the culture medium, a comparison was made with expression data from our previous report in which postconfluent Caco-2 cells were exposed to 5 and 50 μM quercetin for 48 h without ascorbate [10]. First, significantly changed genes presented in the Tables 1–3 were compared to data from our previous study [10]. Only two significantly changed differentiation related genes in the present study were also found in the previous study. *ALPI* expression was up-regulated by quercetin in the absence of ascorbate, which was opposite to expression data obtained in the presence of ascorbate in the present study, where this gene was down-regulated; *APOM* was down-regulated in

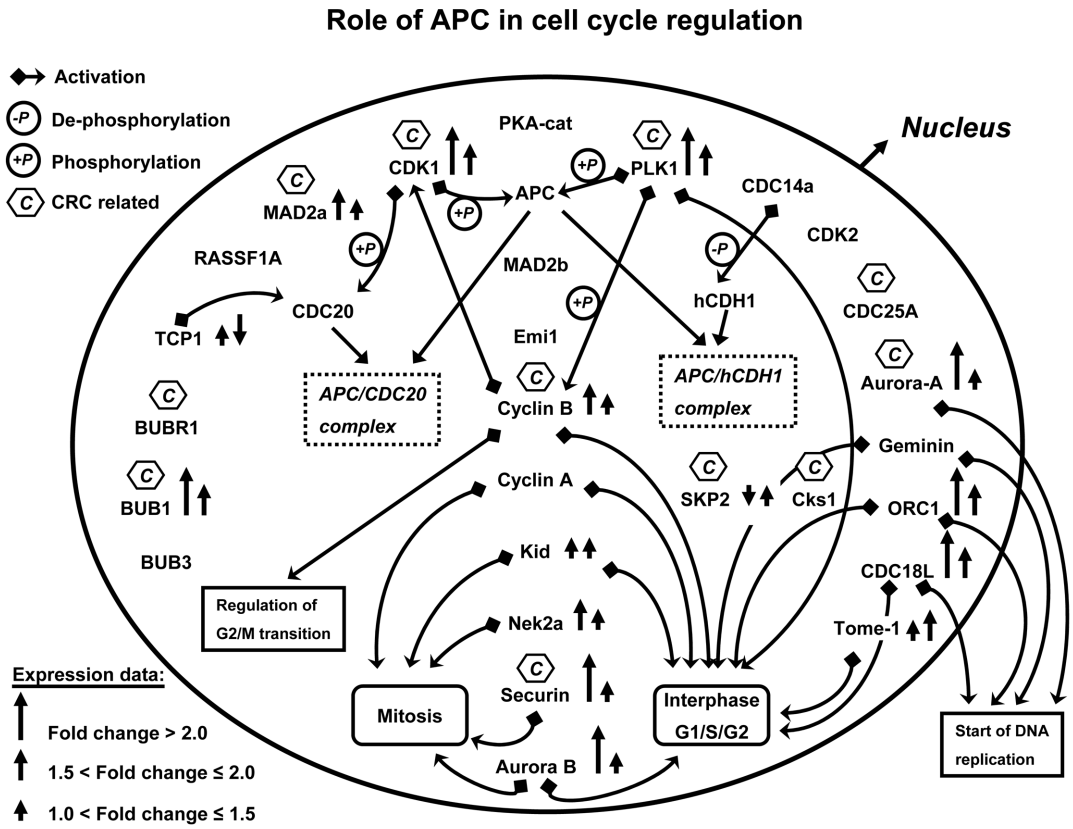

Figure 3. Pathway entitled Role of APC in cell cycle Regulation, including visualization of fold changes and CRC related genes expressed in the nucleus (adapted from MetaCore™). Per gene, the two neighbouring arrows indicate fold changes on day 5 (left arrow) and day 10 (right arrow). Abbreviations used and synonyms: APC: anaphase promoting complex; Aurora-A (=*STK6* in mice, *STK15* in humans): serine/threonine kinase 6; *BUB1*: budding uninhibited by benzimidazoles 1; *PLK1*: polo-like kinase 1; *CDC*: cell division cycle; *CDC18L = CDC6*; *CDK1 = CDC2*; *Kid = KIFF22*: kinesin family member 22; *MAD2a = MAD2L1*: mitotic arrest-deficient 2; *ORC1*: origin recognition complex subunit 1; Securin = *PTTG1*: pituitary tumor-transforming 1 (= tumor transforming protein 1); Tome-1 = *CDCA3*: cell division cycle associated 3.

both conditions (Table 1). Similar analyses among genes contributing to the GO processes affected by the 27 significantly changed pathways showed that 12 genes could be compared among both datasets. In total, 8 out of these 12 genes (≈67%) showed an opposite direction of fold change when data were compared with and without addition of ascorbate (Table 2). In the absence of ascorbate, seven of these eight genes (≈88%) showed a direction of fold change that is contrary to that occurring in development of human CRC, and includes inhibition of cell cycle genes.

Among the genes grouped by function, four out of the six genes in the present study that were also found in the previous study (≈67%) showed an opposite direction of fold change when data were compared with and without addition of ascorbate (Table 3). In the absence of ascorbate, three of these four genes (=75%) showed a direction of fold change that is contrary to that occurring in development of human CRC, and includes increased expression of genes involved in xenobiotic metabolism.

Overall, 13 out of the total number of 20 matching genes as described in Tables 1–3 (=65%) showed an opposite direction of fold change when expression data obtained with ascorbate-stabilized quercetin were compared to those obtained with quercetin only. Furthermore, in the absence of ascorbate, 10 out of these 13 genes (≈77%) showed a direction of fold change that is contrary to that occurring in development of human CRC.

In the second type of analysis, the significantly affected genes from our previous study with quercetin only were compared to a subset within the 1743 significantly changed genes (by ascorbate-stabilized quercetin) showing an identical direction of fold change on both days. This comparison learned that 19 genes matched between the two datasets, of which 13 (≈68%) showed an opposite direction of fold change (see Table 3 of Supporting Information).

4 Discussion

Epidemiological studies suggest that regular dietary intake of quercetin occurs at 16.3 mg/day, when expressed as aglycone [26]. Based on these data, the concentration of querce-

tin aglycone in the colonic lumen has been estimated at 40 μM [15], and as such provided an input to perform the present *in vitro* experiments at this physiologically relevant concentration. In the present paper, quercetin at 40 μM reduced Caco-2 differentiation, which confirms our previous study conducted under identical experimental conditions [15]. Caco-2 differentiation was characterized using the TEER and ALPI, the latter determined by Affymetrix GeneChip® arrays, real-time qRT-PCR and enzyme activity. Furthermore, reduced Caco-2 differentiation was in accordance with the decreased expression of all differentiation related genes present in the statistically filtered dataset. In addition, ascorbate-stabilized quercetin near-significanlty down-regulated the intestine-specific caudal type homeobox transcription factor 2 (*CDX2*) which targets sucrase isomaltase, and is considered to be the main regulator of intestinal cell differentiation [23] and also down-regulated in human CRC [27]. Importantly, the quercetin-mediated decrease in Caco-2 differentiation has been found with multiple techniques, which were all concordant. In addition, Affymetrix GeneChip® array data indicated quercetin-induced upregulation of the human CRC-associated genes *PTGS2*, which encodes the *COX-2* enzyme [19], and *MMP7* [20], both of which were confirmed by real-time qRT-PCR. Based on these findings, it is concluded that statistical filtering retrieved a reliable gene expression dataset that was therefore used for further analyses of quercetin-mediated biological effects in Caco-2 cells.

At the cellular level, (colorectal) carcinogenesis encompasses a decrease in cell differentiation, apoptosis, and metabolism, and an increase in cell proliferation [24], as was also observed in the present study with quercetin exposed Caco-2 cells. The most significantly changed pathway in relation to cell proliferation is the Role of APC in cell cycle Regulation, comprising proliferation related genes that are mentioned hereafter. Proliferating cells express both cyclin B1 (*CCNB1*) and cyclin B2 (*CCNB2*) that complex with and activate cyclin-dependent kinase 1 (*CDK1* = p34 = *CDC2*), which is required for cells to undergo mitosis [28]. Cell division cycle 18 (*CDC18L* = *CDC6*) is involved in DNA replication during the cell cycle during the S-phase [10] suggesting that upregulation of this gene may contribute to increased cell proliferation. Serine/threonine kinase 6 (*STK6* = Aurora-A) is a cell cycle-regulated kinase that is involved in microtubule formation and increased in human colon carcinoma cell lines [29]. *PTTG1* (Securin) prevents segregation of chromosomes, is overexpressed in human colonic polyps and carcinomas at both the gene and protein level, and related to tumor invasion [30].

The most significantly changed pathway in relation to apoptosis is the Role of IAP-proteins in apoptosis. Genes expressed in this pathway suggest decreased apoptosis and include baculoviral IAP repeat-containing 3 (*BIRC3* = *c-IAP2*), baculoviral IAP repeat-containing 5 (*BIRC5* = Survivin), cyclin B1 (*CCNB1*), and cyclin-dependent kinase 1 (*CDK1* = *CDC2*). BIRC5 is known as an inhibitor of apoptosis and hypothesized to be lowly expressed in differentiated adult tissues, but overexpressed in the human colon cancer cell line HCT116 [31].

Moreover, ascorbate-stabilized quercetin caused upregulation of oncogenes and downregulation of tumor suppressor genes, which is a typical disequilibrium in (colorectal) carcinogenesis [7], and in contrast with quercetin's anticarcinogenic potency. Quercetin also up-regulated expression of heat shock proteins that prevent apoptosis, thus enabling cell survival, which is a mechanism also involved in development of human CRC [25].

Comparisons between fold changes in mRNA expression among quercetin exposed Caco-2 cells and fold changes reported for human colorectal carcinogenesis showed high similarity (≈80 – 90%), suggesting that ascorbate-stabilized quercetin in this *in vitro* model promotes processes involved in the development of human CRC. Obviously, not all Caco-2 gene data could be compared to human CRC data, because of a lack of available literature. When assuming that fold changes for these genes are not restricted to quercetin-exposed Caco-2 cells, it can be hypothesized that these genes might be novel biomarkers for human colorectal carcinogenesis.

Quercetin down-regulated expression of the majority of phase II enzymes, which is in contrast with its proposed anticarcinogenic potency [32]. Proposed mechanisms involved in the anticarcinogenic activity of dietary components *in vivo* comprise induction of detoxifying phase II enzymes that protect the colon mucosa against dietary carcinogens. Glutathione S-transferases (GSTs), UDP-glucuronosyltransferases (UGTs), and sulfotransferases (SULTs) are phase II detoxifying enzymes that metabolize xenobiotics via conjugation to glutathione, glucuronic acid, or sulphate, respectively, thereby generating less active polar and water-soluble metabolites suitable for rapid excretion. Our *in vitro* data suggest that ascorbate-stabilized quercetin diminished this protective effect by downregulating gene expression of these enzymes.

Strikingly, in the current model quercetin stabilized by 1 mM ascorbate evoked effects that are contrary to what would be expected for a compound with anticarcinogenic potency. A possible explanation is that quercetin-induced oxygen radicals that have the ability to eradicate tumor cells, might have been annihilated by ascorbate, which is a hypothesis based on previous studies [33 – 35]. The dietary flavonoid flavone and the anticancer drug camptothecin are reported to evoke mitochondrial $O_2^{-\bullet}$ that induced apoptosis of the human colon cancer cell line HT-29 [33]. When these two agents were coadministered with 1 mM ascorbate, HT-29 cells demonstrated a reduction in apoptosis, caused by a decrease in both $O_2^{-\bullet}$ induction and caspase 3-like activity. Additional evidence is provided by a study showing that intake of ascorbate supplements by patients with colorectal

adenomas is associated with decreased apoptosis in the healthy rectal mucosa [34]. The authors hypothesized that intake of ascorbate supplements by patients with colorectal adenomas may be contra-indicated as ascorbate might scavenge reactive oxygen species (ROS) that are meant to induce apoptosis among aberrant cells. Supporting evidence for beneficial effects of ROS is also described for the potentially anticarcinogenic polyunsaturated fatty acids (PUFA) that reduced Caco-2 cell proliferation, whereas coadministration of the very same PUFA with the antioxidants ascorbate (vitamin C) or α-tocopherol (vitamin E) resulted in increased Caco-2 cell proliferation [35]. Accordingly, the hypothesis of ROS scavenging by vitamin C was supported by comparison of the data from the present study to data from our previous study, in which Caco-2 cells were exposed to quercetin without ascorbate, demonstrating the anticarcinogenic potency of this flavonoid [10]. In the absence of ascorbate, ≈80% of the genes showed a quercetin-mediated direction of fold change that is contrary to that occurring in the development of human CRC, thus supporting this flavonoid's anticarcinogenic potency. Furthermore, Caco-2 cells exposed to quercetin, in the absence of ascorbate, showed a dose-dependent increase of the TEER as a marker of cell differentiation [36], which provides additional evidence for quercetin's anticarcinogenic potency. The above mentioned mechanisms found by quercetin-exposure in the absence of ascorbate, are in line with what would be expected for an anticarcinogenic compound, but are opposite to mechanisms found when quercetin was coadministered with ascorbate. Flavonoids, including quercetin, induce expression of phase II enzymes in case of a high oxidative cellular state, *e.g.*, in the presence of ROS and/or electrophiles [37]. A mechanistic explanation for the opposite effects found in the present study might be scavenging of quercetin-induced ROS by ascorbate, which probably resulted in a lower cellular oxidative state and consequently in reduced expression of genes encoding for phase II metabolism enzymes. Together with the present data, it can be hypothesized that oxidative stress induced by dietary quercetin is a mechanism contributing to its anticarcinogenic potency. Most likely, both induction and maintenance of ROS are required for eradication of tumor cells by quercetin, pointing to a beneficial effect of supposed adverse ROS.

In conclusion, exposure of Caco-2 cells to ascorbate-stabilized quercetin at the physiologically relevant concentration of 40 μM resulted in decreased expression of genes involved in cell differentiation, apoptosis, tumor suppression and phase II metabolism, and up-regulated expression of oncogenes and genes involved in cell proliferation. These findings indicate that treatment of Caco-2 cells with ascorbate-stabilized quercetin leads to stimulation instead of inhibition of mechanisms also involved in (human) colorectal carcinogenesis. Under which conditions this unexpected *in vitro* effect mediated by a supposed cancer preventive food ingredient would also be relevant for the *in vivo* situation, and to what extent the observation is due to the possibility that in the *in vitro* model quercetin acts by mechanisms dissimilar from its actual physiological mechanism, remain to be elucidated.

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