

Altered Gene Expression Profiles Define Pathways in Colorectal Cancer Cell Lines Affected by Celecoxib

Naheed Fatima,^{1,2} Ming Yi,³ Sadia Ajaz,⁵ Robert M. Stephens,³ Stacey Stauffer,¹ Peter Greenwald,¹ David J. Munroe,⁴ and Iqbal Unnisa Ali¹

¹Division of Cancer Prevention, National Cancer Institute, Bethesda, Maryland; ²Basic Research Program, ³Advanced Biomedical Computing Center, and ⁴Laboratory of Molecular Technology, Science Applications International Corporation, Inc., Frederick, Maryland; and ⁵Panjwani Center for Molecular Medicine, University of Karachi, Karachi, Pakistan

Abstract

It is well established that celecoxib, a selective inhibitor of cyclooxygenase-2 (COX-2) and a tested chemopreventive agent, has several COX-2-independent activities. In an attempt to better understand COX-2-independent molecular mechanisms underlying the chemopreventive activity of celecoxib, we did global transcription profiling of celecoxib-treated COX-2-positive and COX-2-deficient colorectal cancer cell lines. Celecoxib treatment resulted in significantly altered expression levels of over 1,000 to 3,000 transcripts in these cell lines, respectively. A pathway/functional analysis of celecoxib-affected transcripts, using Gene Ontology and Biocarta Pathways and exploring biological association networks, revealed that celecoxib modulates expression of numerous genes involved in a variety of cellular processes, including metabolism, cell pro-

liferation, apoptotic signaling, cell cycle check points, lymphocyte activation, and signaling pathways. Among these processes, cell proliferation and apoptotic signaling consistently ranked as the highest-scoring Gene Ontology terms and Biocarta Pathways in both COX-2 expresser and nonexpresser cell lines. Altered expression of many of the genes by celecoxib was confirmed by quantitative PCR and at the protein level by Western blotting. Many novel genes emerged from our analysis of global transcription patterns that were not previously reported to be affected by celecoxib. In the future, in-depth work on selected genes will determine if these genes may serve as potential molecular targets for more effective chemopreventive strategies. (Cancer Epidemiol Biomarkers Prev 2008; 17(11):3051–61)

Introduction

Several lines of evidence, including prevention clinical trials, epidemiologic studies, and genetic manipulation as well as pharmacologic experimentation in animal models, have provided evidence for a crucial role of the enzyme cyclooxygenase-2 (COX-2) in colorectal carcinogenesis (1-5). COX-2 is a key enzyme in generating multifunctional lipid metabolites, prostaglandins, which stimulate proliferation, increase motility and invasion, modulate immune function, and are proangiogenic and antiapoptotic. COX-2 has therefore been a logical target for the prevention and treatment of colorectal as well as a variety of other cancers. A selective COX-2 inhibitor, celecoxib, has shown efficacy in reducing polyp burden in patients with the inherited syndrome familial adeno-

matous polyposis and, more recently, with sporadic adenomas (2, 3, 5). Understandably, there has been intense interest in delineating the molecular mechanisms underlying the antiproliferative activity of celecoxib.

It has been well documented that the multifaceted nature of the anticarcinogenic activity of celecoxib cannot simply be ascribed to its ability to selectively inhibit COX-2 and that COX-2-independent mechanisms also underlie its growth-inhibitory activity (6-11). In particular, specific proteins and molecular pathways, affecting cell cycle progression and apoptosis, have been extensively studied and shown to be targeted by celecoxib (12-17).

The scope of celecoxib-modulated molecular alterations can best be studied by applying global approaches at the transcription and/or protein levels. Previously, differential proteomic and transcriptomic profiling in colorectal cancer cell lines and healthy colonic mucosa of patients with hereditary nonpolyposis colon cancer, respectively, have shown celecoxib-mediated expression changes in diverse cellular functions, including cell proliferation, apoptosis, immune function, and cell signaling (18, 19). In this investigation, we have performed transcription profiling of colorectal cancer cells to gain an unbiased global perspective of molecular networks and pathways targeted by celecoxib. The use of COX-2 expresser and nonexpresser cell lines provides an opportunity to delineate the anticarcinogenic activities of celecoxib that are independent of COX-2 inhibition and may be instructive in identifying molecular profiles relevant for celecoxib-mediated adenoma regression.

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N. Fatima and M. Yi contributed equally to this work.

Current address for I.U. Ali: Molecular Oncology Program, Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan.

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Requests for reprints: Iqbal U. Ali, Molecular Oncology Program, Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan. Phone: 92-21-763-0801; Fax: 92-21-481-9018-9. E-mail: ialiali@cyber.net.pk

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Materials and Methods

Colorectal Cancer Cell Lines. Four human colon cancer cell lines were used in this study. HCA-7 cell line, established from a primary human colonic adenocarcinoma, was purchased from the European Collection of Cell Cultures. HCT-116, HT-29, and DLD-1 cell lines were purchased from the American Type Culture Collection. HT-29 cells, with a G to A mutation in codon 273 of the *p53* gene, and DLD-1 cells, with a C to T mutation at position 241 of the *p53* gene, overexpress *p53*. HCT-116 cells carry a mutation in codon 13 of the *KRAS* proto-oncogene.

Cell Culture Conditions and Celecoxib Treatment. HCT-116 and HT-29 cells were maintained in McCoy's 5A modified medium (American Type Culture Collection), DLD-1 cells were grown in RPMI 1640, and HCA-7 cell line was maintained in DMEM (American Type Culture Collection). All cell culture media were supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin and incubated at 37°C with 5% CO₂. Celecoxib was obtained from LKT Laboratories, Inc. and dissolved in DMSO (100 mol/L stock solution stored at -20°C). Exponentially growing cells with 50% confluence were treated with DMSO alone, with 5 µmol/L celecoxib for 24, 48, and 72 h, or with 75 µmol/L celecoxib for 24 h. At the end of the incubation period, cell viability was determined as described previously (18). In parallel experiments, cells were terminated and cell pellets were stored at -20°C for RNA isolation and protein analysis.

RNA Isolation and Hybridization to Oligonucleotide Arrays. Total RNA was extracted from each cell line using RNeasy Mini kit and RNase-free DNase (Qiagen) according to the manufacturer's protocol. Human Genome U133 Plus 2.0 GeneChip oligonucleotide arrays (Affymetrix) representing 47,000 transcripts and variants, including 38,500 well-characterized human genes, were used for gene expression analysis of celecoxib-induced alterations. Biotin-labeled cRNAs from all four cell lines with or without celecoxib treatment were prepared as described by Affymetrix Expression Analysis Technical Manual. Labeling, hybridization, and subsequent washing and detection were done as recommended by the manufacturer. Array images were acquired using GeneChip scanner from Affymetrix and average intensity value for all of the arrays was adjusted to a target intensity of 500.

Microarray Data Analysis. GeneChip images and data sets were uploaded into the National Cancer Institute's Microarray Analysis Database (mAdb) for data normalization, extraction, and evaluation.⁶ The differentially expressed genes were defined as up-regulated or down-regulated consistently in COX-2 expresser (HCA-7 and HT-29) and nonexpresser (DLD and HCT) cell lines at least 1.5-fold after treatment with 5 or 75 µmol/L of celecoxib compared with control experiments. Genes with detection calls flagged as absent for both treated and control experiments were eliminated from the

extracted gene lists. The differentially expressed genes were extracted based on color-coded selection criteria that met the defined criteria described above using an in-house software tool, WPS (20), developed at the Advanced Biomedical Computing Center, NCI-Frederick. Pathway-level analyses using the WPS program or Ingenuity Pathway Analysis tool were performed with the differentially expressed genes. Gene Ontology (GO) and pathway enrichment analyses were also performed within WPS for all the differentially expressed genes. Comparison of pathway-level enrichment for the differentially expressed genes from different cell lines or treatment with different doses was done using enrichment scores as transformed Fisher's exact test *P* values and visualized in heat maps.⁷ The statistically enriched Biocarta Pathways⁸ and their associated genes were visualized and analyzed within gene-term association networks in the WPS program (20).

cDNA Synthesis and Quantitation by Real-time PCR. Two micrograms of RNA were reverse transcribed into first strand using the SuperScript II First-Strand cDNA Synthesis kit (Invitrogen) according to the manufacturer's protocol with hexamer primers. Quantitative real-time PCR (qPCR) reactions were carried out using the Taqman Universal PCR Master Mix (Applied Biosystems) in a total volume of 25 µL on ABI Prism 7900 Sequence Detector System (Applied Biosystems) according to the manufacturer's instructions. FAM-labeled probes and primers for all genes were purchased from Applied Biosystems. Amplification reactions were done in triplicate for each sample. The relative fold change in mRNA level of each gene of interest was calculated by the comparative C_t (2^{-ΔΔC_t}) method. *β-Actin* was used as the reference gene for normalization.

Western Blot Analysis. Celecoxib-treated cells were washed twice with cold PBS and lysed in radioimmuno-precipitation assay lysis buffer (Santa Cruz Biotechnology, Inc.). Cell lysates were sonicated for 1 min intermittently and centrifuged at 14,000 rpm for 15 min at 4°C. Protein concentration was determined using bicinchoninic acid assay (Sigma-Aldrich). Western blot analysis was done as described previously (18). Antibodies against GADD45A, SKP2, CDC2, DHFR, RAD1, BARD1, and BAX (Santa Cruz Biotechnology) and antibodies against MET and BIRC4 (Upstate Biotechnology, Inc. and Cell Signaling Technology, Inc., respectively) were used at 1:200 dilution. Antibodies against actin (Amersham Biosciences) at 1:1,000 dilution were used as a control for protein loading for all proteins analyzed.

Results

Transcription Profiling of Celecoxib Treatment. To identify genes whose expression levels respond to celecoxib treatment, four colorectal cancer cell lines, two COX-2 expressers (HCA-7 and HT-29) and two nonexpressers (HCT-116 and DLD-1), were treated with 5 or 75 µmol/L of celecoxib for 24 hours (18). The low dose of 5 µmol/L celecoxib did not affect cell viability or

⁶ <http://nciarray.nci.nih.gov/index.shtml>

⁷ M. Yi et al., in preparation.

⁸ <http://www.biocarta.com/genes/allpathways.asp>

morphology (18) and was comparable with the physiologically achievable plasma concentration in humans following the administration of highest clinical daily dose resulting in tumor inhibition after several months to years. The IC_{50} dose of celecoxib for growth inhibition was 75 $\mu\text{mol/L}$ and varies considerably depending on cell type and culture conditions (ref. 18 and references therein). Both doses of celecoxib far exceed the concentration at which celecoxib inhibits synthesis of prostaglandins (10, 21). Supplementary Table S1 provides information on the effects of 5 and 75 $\mu\text{mol/L}$ of celecoxib on key enzymes involved in the generation of prostaglandins.

Gene expression patterns of all four cell lines treated with the solvent control DMSO or with celecoxib were analyzed using Affymetrix GeneChip microarrays. We did pattern extraction with the WPS program with a minimum of 1.5-fold increase or decrease in expression level following celecoxib treatment (20). A total of 189 features were identified to be significantly up-regulated or down-regulated in the COX-2 expresser cell lines in response to celecoxib at the low dose of 5 $\mu\text{mol/L}$. In COX-2 nonexpresser cell lines, treatment with 5 $\mu\text{mol/L}$ celecoxib induced significant up-regulation or down-regulation of 245 transcripts. As expected, a much higher

number of transcripts were modulated by 75 $\mu\text{mol/L}$ celecoxib. Celecoxib-treated COX-2-positive cell lines showed significant differential expression of >1,400 transcripts, whereas over 3,000 transcripts were up-regulated or down-regulated in the COX-2 nonexpressers. The Venn diagrams displayed in Fig. 1B and C provide a measure of the extent of common and distinct celecoxib-mediated alterations in the expression levels of genes in COX-2-positive and COX-2-negative cell lines and between low and high doses of celecoxib, respectively.

Validation of Microarray Results by qPCR and Western Blotting. Initial qPCR validation of the microarray results for the expression of several genes was carried out in the COX-2 nonexpresser cell line HCT-116. Although the fold changes in the expression levels of genes detected in qPCR and microarrays were frequently not identical, the qPCR results, especially at the high dose of celecoxib, were generally comparable with the microarray analysis. Figure 2A displays two examples each of up-regulation and down-regulation of *MAP3K2* and *ATM* and *DHFR* and *BARD1*, respectively. The qPCR results confirmed celecoxib modulation of the expression of all four genes at 75 $\mu\text{mol/L}$ celecoxib as observed in microarray analysis. However, the expression levels of these genes at 5 $\mu\text{mol/L}$ celecoxib, although generally confirmed the trend for up-regulation or down-regulation, did not often reach the 1.5-fold increase or decrease in the expression level detected in the microarray analysis. Essentially similar results were obtained with the COX-2-positive HCA-7 cell line (data not shown). We reasoned that longer periods of treatment with low dose of celecoxib may affect cell viability and/or have a more pronounced effect on expression level of genes. Both HCT-116 and HCA-7 cell lines were therefore treated with 5 $\mu\text{mol/L}$ celecoxib for 24, 48, and 72 hours. As shown in Fig. 2B, extended periods of treatment with 5 $\mu\text{mol/L}$ celecoxib did not affect viability (or morphology) of both cell lines. However, the qPCR analysis showed a more pronounced trend of up-regulation or down-regulation of genes after 72 hours of celecoxib treatment in many but not all cases. Figure 2C shows an example of *CDKN1A*, the expression level of which was clearly increased following treatment with 75 $\mu\text{mol/L}$ celecoxib but was unchanged even after 72 hours of treatment with 5 $\mu\text{mol/L}$ celecoxib. The expression level of another gene, *CNB1*, was, however, markedly decreased following treatment with 5 $\mu\text{mol/L}$ celecoxib for 72 hours, whereas the up-regulation in the expression of *BIRC4* reached a level comparable with that of cells treated with 75 $\mu\text{mol/L}$ celecoxib.

We subsequently selected a subset of 45 differentially expressed genes identified in microarrays of cells treated with 75 $\mu\text{mol/L}$ celecoxib for quantitative analysis by real-time PCR (Table 1). The selection of this subset of genes was based on the extent of differential expression detected by WPS as well as their known cellular functions. Furthermore, eight of these genes, *BIRC4*, *FOXP1*, *ATM*, *JMJD2B*, *Ninein*, *TRIM14*, *RIPK1*, and *MET*, were among the common genes affected by both low and high dose of celecoxib (Fig. 1C). For these 45 genes, there was ~70% concordance between the microarray and qPCR results. Examples of the qPCR-confirmed expression levels of celecoxib-responsive

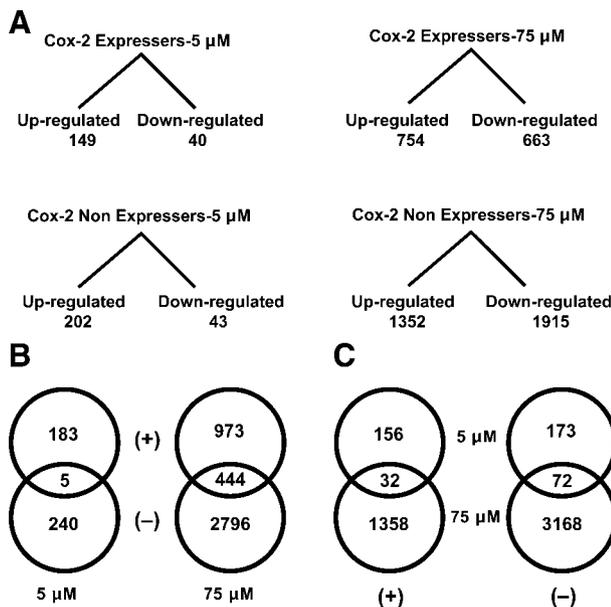


Figure 1. Differential transcription profiling of celecoxib-treated colorectal cancer cell lines. **A.** Numbers indicate up-regulated or down-regulated transcripts at 5 or 75 $\mu\text{mol/L}$ of celecoxib compared with DMSO-treated controls in COX-2-positive HCA-7 and HT-29 cell lines and COX-2-negative HCT-116 and DLD-1 cell lines. **B.** Venn diagrams showing numbers of differentially expressed unique and common transcripts between 5 and 75 $\mu\text{mol/L}$ of celecoxib treatment in COX-2-positive HCA-7 and HT-29 cell lines and COX-2-negative HCT-116 and DLD-1 cell lines. **C.** Venn diagrams showing numbers of differentially expressed unique and common transcripts between COX-2-positive HCA-7 and HT-29 cell lines and COX-2-negative HCT-116 and DLD-1 cell lines at 5 or 75 $\mu\text{mol/L}$ of celecoxib treatment.

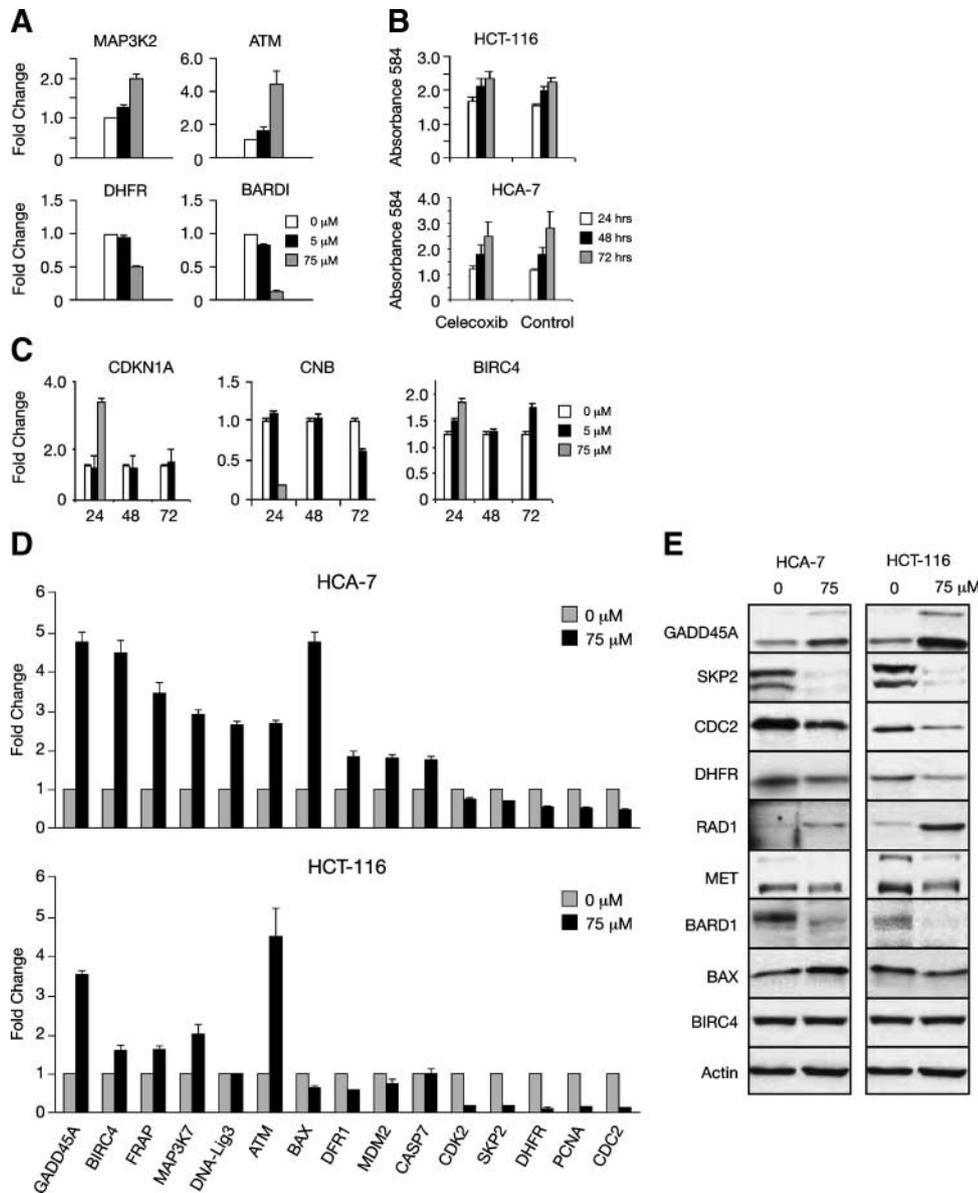


Figure 2. Validation of differential expression identified by microarray analysis at transcriptional and translational levels. COX-2-positive HCA-7 and COX-2-negative HCT-116 cells were treated with DMSO or 75 μ mol/L celecoxib. **A**, **C**, and **D**. qPCR of selected transcripts. A relative up-regulation or down-regulation was calculated by setting the value of DMSO-treated controls as 1. Each bar represents a mean of three qPCRs from independent experiments. **B**. Effect of 5 μ mol/L celecoxib on the growth of HCT-116 and HCA-7 cell lines. Cell viability was measured at indicated times and represents three experiments with a mean of six wells in each experiment. **E**. Western blots of nine differentially expressed proteins in HCA-7 and HCT-116 cells probed against the respective antibodies. β -Actin, used as the loading control, is shown for one protein but was done for all nine proteins. The data are representative of at least two, in most cases three, separate experiments.

genes in the COX-2 expresser HCA-7 and the non-expresser HCT-116 cell lines show similarities as well as differences in the celecoxib-mediated transcriptional regulation (Fig. 2D). Expression of about a third of the genes was deregulated in the same direction by celecoxib in both cell lines. *GADD45A*, *mTOR/FRAP*, *ATM*, and *MAP3K2* are examples of the up-regulated genes and *SKP2*, *CDC2*, *DHFR*, and *PCNA* represent the down-regulated genes by celecoxib in COX-2 plus and minus cell lines. Some genes, such as *DNL3*, *CDK2*, and *CASP7*, were modulated in one but not the other cell line. In other instances, the expression change was in opposite direction in COX-2 plus versus minus cell lines as was the case with the *Ninein*, *MDM2*, *DRF1*, and *JMJD2B* genes.

Figure 2E displays expression levels of 10 genes at the protein level in HCA-7 and HCT-116 cells before and after treatment with 75 μ mol/L celecoxib. The expression levels of seven of these genes were concordant at both

transcription and protein levels. In the case of three genes, *BIRC4*, *BAX*, and *ATM*, both microarray and qPCR analyses detected celecoxib-mediated up-regulation at the transcriptional level. However, at the translational level, there was no change in the amount of the BIRC4 protein in both cell lines, whereas the expression of BAX protein was decreased in the COX-2 expresser HCT-116 cell line. The expression of ATM protein has previously shown to be down-regulated in both COX-2-positive and COX-2-negative cell lines.⁹

Pathway/Function Annotation of Celecoxib-Modulated Transcripts. Similarities and differences at pathway-level or GO term-level within the celecoxib-modulated transcripts in all four colorectal cancer cell lines treated

⁹ Submitted for publication.

with 75 $\mu\text{mol/L}$ celecoxib were evaluated using enrichment scores and then visualized and clustered as heat maps for Biocarta Pathways and GO terms. As is evident from the Biocarta Pathways annotation displayed in Fig. 3, expression levels of transcripts involved in numerous cellular processes were affected by celecoxib in all four cell lines. In general, it was difficult to discern a clear pattern of celecoxib modulation in COX-2 expressers versus non-expressers based on changes in expression levels of transcripts involved in most cellular processes (Fig. 3A). The heterogeneity of response to celecoxib, also evident in GO Biological Processes (GOBP) annotations (data not shown), may be a general reflection of the genetic background of the four colorectal cancer cell lines. However, as shown in Fig. 3B, there were similarities in the expression patterns of genes involved in specific pathways and cellular processes. In particular, in all four cell lines, there were enrichments of differentially expressed genes within

Biocarta Pathways, such as apoptotic signaling in response to DNA damage, G₁-S checkpoint of the cell cycle, and mitogen-activated protein kinase signaling (Fig. 3B), suggesting that such enrichment is independent of their genetic background as well as COX-2 expression status. On the other hand, several pathways were selectively enriched for celecoxib-responsive genes in either COX-2 expressers or nonexpressers (Fig. 3C), implying that celecoxib modulation of these transcripts may be dictated, among other factors, by the presence or absence of the enzyme COX-2.

The consistently up-regulated or down-regulated genes for all four cell lines, treated with high dose of celecoxib, were analyzed together (Supplementary Tables S1A and S2A) or separately as COX-2 expressers and nonexpressers (Supplementary Tables S1B and S2B and S1C and S2C, respectively) using GO or Biocarta functional annotations. The Fisher's exact test shows cell

Table 1. Differential expression of celecoxib-induced genes in HCA-7 and HCT-116 cell lines validated by qPCR

Gene symbol	Gene name	Fold change	
		HCA-7	HCT-116
<i>GADD45A</i>	Growth arrest and DNA damage inducible, α	4.7	3.5
<i>BIRC4</i>	Baculoviral IAP repeat-containing 4	4.4	1.5
<i>FRAP</i>	FK506 binding protein 12-rapamycin associated protein	3.4	1.6
<i>FOXP1</i>	Forkhead box P1	3.3	0.9
<i>ATM</i>	Ataxia-telangiectasia mutated	2.6	3.9
<i>JMJD2B</i>	Jumonji domain containing 2B	3.1	0.6
<i>MAP3K2</i>	Mitogen-activated protein kinase kinase kinase 2	2.9	2
<i>DNA Lig 3</i>	Ligase III, DNA	2.6	0.9
<i>NQO1</i>	NAD(P)H dehydrogenase, quinone 1	2.4	1.2
<i>CDKN1A</i>	Cyclin-dependent kinase inhibitor 1A	2.2	2.4
<i>RAB2B</i>	Member RAS oncogene family	2.2	1.2
<i>AGPAT3</i>	1-Acylglycerol-3-phosphate <i>O</i> -acyltransferase 3	2.1	0.9
<i>BAX</i>	BCL2-associated X protein	2	0.6
<i>DDR1</i>	Discoidin domain receptor family, member 1	2.1	0.4
<i>NINEIN</i>	Ninein (GSK3B interacting protein)	2	0.6
<i>CASP7</i>	Caspase-7	1.7	1
<i>MDM2</i>	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein	1.7	0.7
<i>EP300</i>	E1A binding protein p300	1.9	1.2
<i>DRF1</i>	DBF4 homologue B	1.8	0.5
<i>RAD1</i>	RAD1 homologue	1.6	0.4
<i>CCNB1</i>	Cyclin B1	1.6	0.3
<i>TREX1</i>	Three prime repair exonuclease 1	1.5	0.2
<i>GATA2</i>	GATA binding protein 2	1.4	0.3
<i>INPP5A</i>	Inositol polyphosphate-5-phosphatase	1.3	0.5
<i>TNFAIP3</i>	Tumor necrosis factor, α -induced protein 3	1.3	1.7
<i>BCL2L1</i>	BCL2-like 1	1.2	0.8
<i>GPR116</i>	G protein-coupled receptor 116	1.2	1
<i>MET</i>	Met proto-oncogene	1.2	0.5
<i>TRIM14</i>	Tripartite motif-containing 14	1.2	0.4
<i>MYD88</i>	Myeloid differentiation primary response gene (88)	1.1	0.2
<i>RIPK1</i>	Receptor (TNFRSF)-interacting serine-threonine kinase 1	1.1	1.1
<i>CYC1</i>	Cytochrome <i>c</i> -1	1	0.3
<i>DNAJB1</i>	DnaJ (Hsp40) homologue, subfamily B, member 1	1	0.5
<i>WEE1</i>	WEE1 homologue (<i>S. pombe</i>)	0.9	0.3
<i>RPA1</i>	Replication protein A1	0.8	0.4
<i>CDK2</i>	Cyclin-dependent kinase 2	0.7	0.1
<i>CDC25A</i>	Cell division cycle 25 homologue A	0.6	0.09
<i>BARD1</i>	BRCA1-associated RING domain 1	0.6	0.1
<i>SKP2</i>	S-phase kinase-associated protein 2 (p45)	0.6	0.1
<i>CDC2</i>	Cell division cycle 2	0.4	0.1
<i>RRM1</i>	Ribonucleotide reductase M1 polypeptide	0.5	0.1
<i>DHFR</i>	Dihydrofolate reductase	0.5	0.1
<i>PCNA</i>	Proliferating cell nuclear antigen	0.5	0.1
<i>MYT1</i>	Myelin transcription factor 1	ND	0.3

NOTE: Numbers >1 = up-regulation, <1 = down-regulation, and 1 = no change. Abbreviation: ND, not determined.

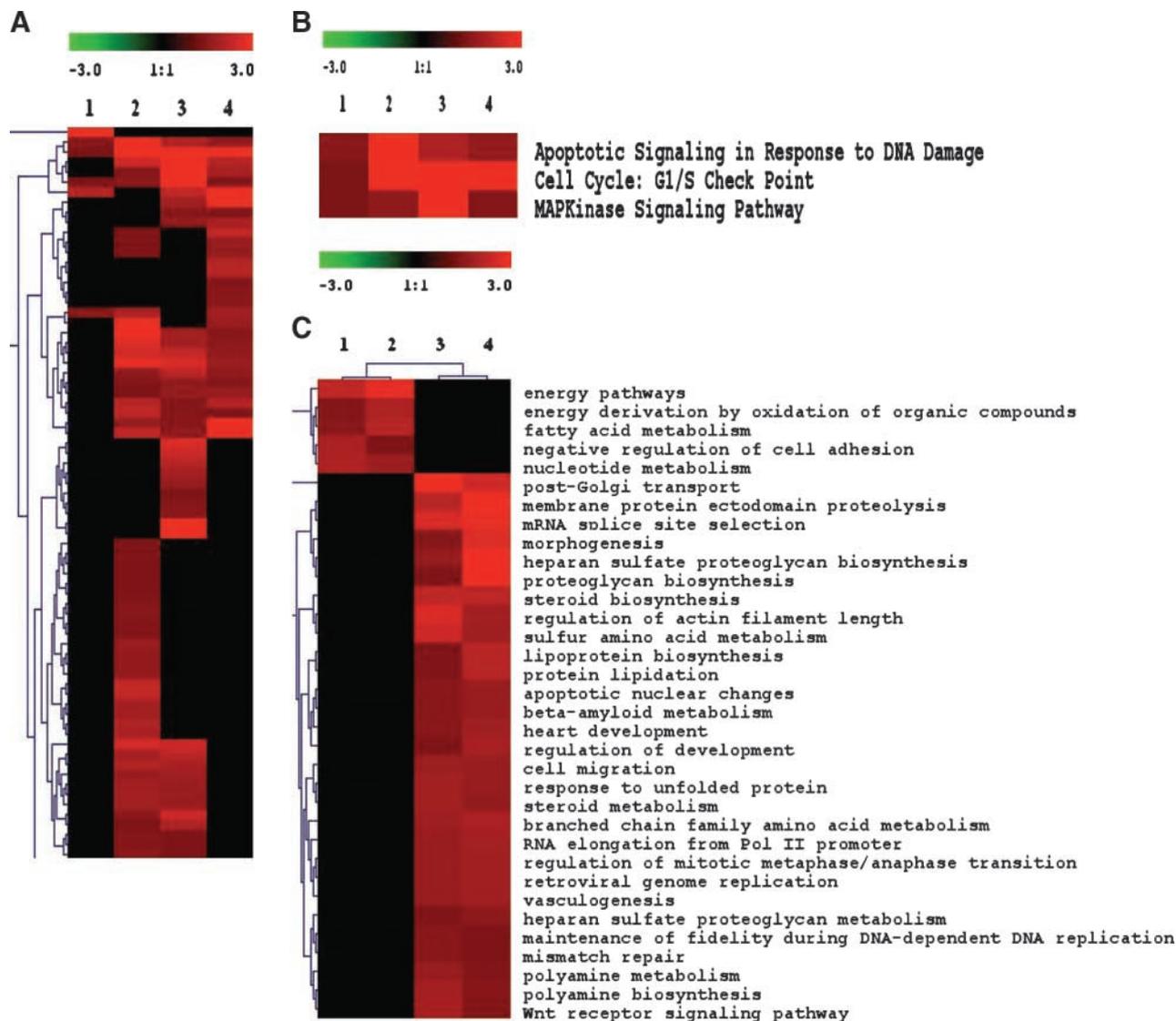


Figure 3. Cluster dendrograms showing Biocarta Pathways annotations of celecoxib-modulated transcripts in colorectal cancer cell lines. Heat maps of celecoxib-affected transcripts are based on enrichment scores displaying heterogeneous expression patterns (A), similarities in expression patterns of genes from specific cellular processes (B), and selective enrichment of genes from various cellular processes (C). The bars with the color spectrum for the range of expression are shown. *Lanes 1 and 2*, COX-2 expressers, HCA-7 and HT-29; *lanes 3 and 4*, COX-2 nonexpressers, DLD-1 and HCT-116.

cycle to be the top-ranked enriched term. This is consistent with the Biocarta Pathways findings displayed in Fig. 3B. Interestingly, analysis of GOBP and KEGG functional annotations of celecoxib-responsive genes at 5 and 75 $\mu\text{mol/L}$ celecoxib treatments in all four COX-2 expresser and nonexpresser cell lines identified cell cycle and cell proliferation to be among the prominent categories common between the two doses (Supplementary Table S3A and B). In addition, celecoxib, at both low and high concentrations, seems to affect DNA replication and various metabolic processes.

We next examined the functional annotation of those genes that were found to be commonly modulated in microarray profiling by celecoxib both at 5 and 75 $\mu\text{mol/L}$ concentrations and are shown in Fig. 1C. The pooled list

of 32 transcripts in COX-2 expressers and 72 in nonexpressers (Supplementary Table S4A and B, respectively) that were commonly modulated by treatment with both low and high concentrations of celecoxib was subjected to enrichment analysis in GOBP annotations, Biocarta, and KEGG pathways. Despite the small numbers, Fisher's exact test identified cell cycle, G₁-S check-point, apoptosis, metabolism, and specific signaling pathways to be among the top-ranked processes affected by celecoxib independent of low or high concentration (Supplementary Tables S5 and S6).

Finally, we subjected only the qPCR-validated, celecoxib-responsive genes from HCT-116 and HCA-7 cell lines to Ingenuity Pathway Analysis to identify the functional networks and physiologic processes

preferentially targeted by celecoxib. This computational tool of curated database integrates genomic data with various mining techniques to predict functional linkages. Essentially, the highly significant overrepresented functions determined by the mechanism-proposing software tool were related to cell proliferation, cell cycle, cell death, and immune function modulation (Fig. 4A and B), which is consistent with the findings using GO and Biocarta Pathways (Fig. 3; Supplementary Tables S1 and S2).

Biological Association Networks Affected by Celecoxib. The interactions among the celecoxib-modulated genes can be gleaned by incorporating the differentially expressed probe sets into molecular interactions and biological association networks. The differentially expressed genes identified in the microarray analysis and those validated by qPCR were compared in parallel. Figure 4C shows analysis of enriched Biocarta Pathways of celecoxib-modulated genes from the HCA-7 cell line within the context of gene-term association networks along with their associated genes. Very similar results were obtained when the biological network analysis was done with celecoxib-responsive genes from the HCT-116 cell line (Supplementary Fig. S1). Consistent with the results revealed by multiple bioinformatics tools (Figs. 3 and 4A and B; Supplementary Tables S1 and S2), several celecoxib-modulated genes play a crucial role in cell cycle regulation and apoptosis and serve as important hubs or interaction points between many cellular processes or pathways. These genes included *DHFR*, *SKP2*, *CDC2*, *CDC25A*, *ATM*, *mTOR/FRAP*, *GADD45A*, *BAX*, *CASP7*, and *BIRC4*.

Discussion

Our study of whole genome expression microarrays of celecoxib-treated colorectal cancer cell lines represents an unbiased global perspective of celecoxib-responsive genes. The use of various bioinformatics tools has shown that a broad range of celecoxib-mediated expression changes affects crucial processes of cellular growth and apoptosis both in COX-2-positive and COX-2-deficient cell lines; genes affecting cell cycle, energy metabolism, and nucleotide metabolism also ranked at the top in many of these annotations. These findings are consistent with our earlier investigation of diverse proteomic changes in colorectal cancer cells treated with celecoxib (18). In that investigation, we had also shown that when colorectal cancer cells were treated with physiologically comparable 5 $\mu\text{mol/L}$ or an IC_{50} dose of 75 $\mu\text{mol/L}$ celecoxib, there was ~30% overlap in celecoxib-responsive proteomic markers between the two doses. That the potentials of using a higher concentration of celecoxib (e.g., maximizing the effects and avoiding false negatives) may outweigh the perils (e.g., toxicity) prompted us to use both low and high doses for examining the transcriptional signatures of celecoxib in colorectal cancer cell lines in the present investigation.

The relevance of using a high dose of celecoxib was strengthened by the following observations in our present study. First, in the list of randomly selected 45 genes (Table 1), 8 were modulated at both low and high doses of celecoxib. Second, an extended period of

treatment with the same low dose of celecoxib resulted in a more pronounced effect on the expression levels of some celecoxib-responsive genes comparable with those observed at the high dose (Fig. 2C). Third, application of various bioinformatics tools has provided evidence that, in the context of biological pathways, by and large the same cellular processes are affected by low and high doses of celecoxib (Supplementary Tables S3A and B, S5, and S6). Finally, the 75 $\mu\text{mol/L}$ celecoxib-mediated transcriptional alterations were comparable with those observed in patients of a clinical trial treated with celecoxib (see section on hereditary nonpolyposis colorectal cancer patients below; Fig. 5). Based on these considerations, we relied on 75 $\mu\text{mol/L}$ celecoxib-mediated transcriptional profiles to obtain a deeper insight into the COX-2-dependent and COX-2-independent mechanisms underlying the antiproliferative effect of celecoxib.

Transcriptional Deregulation and Biological Pathways Affected by Celecoxib in COX-2 Expresser and COX-2 Nonexpresser Colorectal Cancer Cell Lines. To discern the gene sets/cellular processes affected, uniquely or commonly, by celecoxib in COX-2-positive versus COX-2-negative cell lines, we subjected the sets of genes, which were commonly affected (444), unique to COX-2 expressers (973), or unique to COX-2 nonexpressers (2,796; Fig. 1B), to Biocarta Pathways and GOBP annotations enrichment analysis (Supplementary Tables S1, S2, S7, and S8). In all four cell lines, cell cycle/cell proliferation, DNA replication, DNA metabolism, DNA repair, and response to DNA damage were the most common categories. When the genes affected by celecoxib in COX-2 expressers but not in nonexpressers and vice versa were analyzed for GOBP terms with a total of 16,762 annotated genes in the GOBP database, the top-ranking categories of these two distinct classes were again cell cycle, cell proliferation, and metabolism (Supplementary Table S8B and C). On the other hand, genes in the cell death category were enriched in COX-2 nonexpressers (Supplementary Table S8C). Of note is the fact that Biocarta annotation of celecoxib-modulated genes in COX-2-deficient cell lines showed significant enrichment of genes in ATM and mTOR signaling pathways and in the regulation of the downstream effectors of mTOR, eIF4e, and p70 S6 kinase (Supplementary Table S7C). Although the levels of the ATM and mTOR transcripts as well as proteins seem to be modulated in the COX-2 expresser HCA-7 and non-expresser HCT-116, a combination of lower numbers of celecoxib-modulated genes in the COX-2-positive cell lines and the annotated genes in the Biocarta Pathways made it difficult to assess the significance of ATM and mTOR signaling in the presence of COX-2.

Comparison of Celecoxib-Induced Transcriptional Changes in Colorectal Cancer Cell Lines and Normal Colonic Mucosa. The vast range of transcriptional alterations observed in colorectal cancer cell lines in response to celecoxib was also evident in specimens of the normal colonic mucosa from a clinical trial of hereditary nonpolyposis colorectal cancer patients following long-term treatment with a high dose of celecoxib (19). A comparative analysis of the two data sets showed a large number of common genes

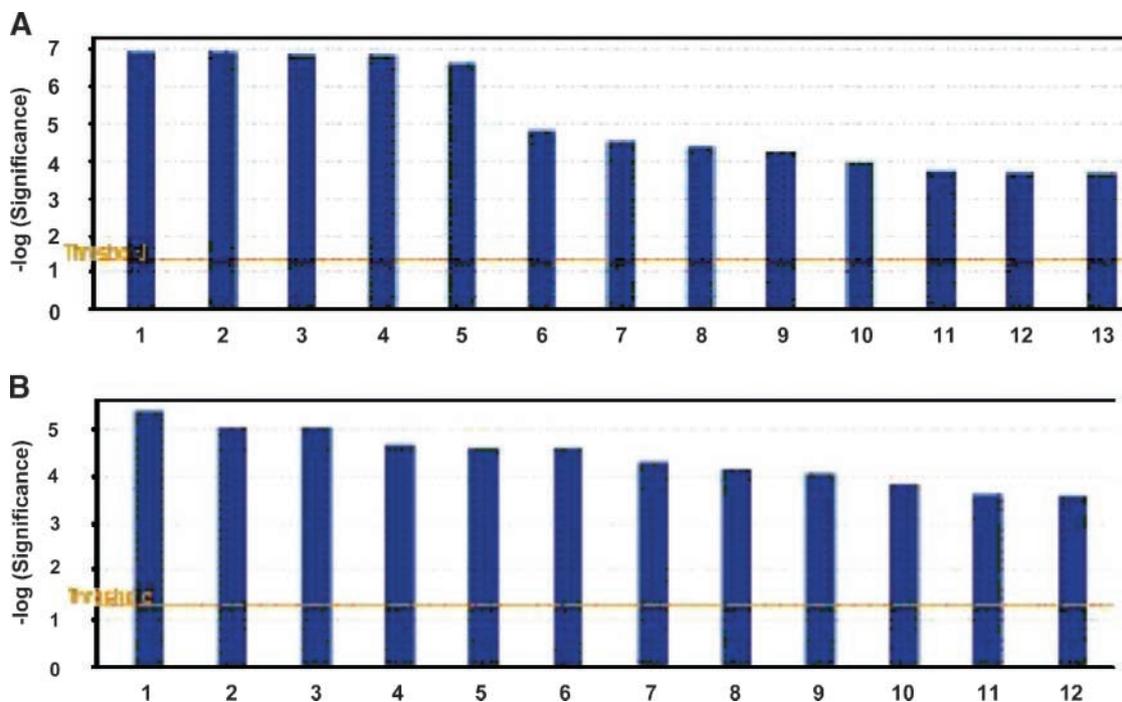


Figure 4. Enriched functional categories and pathways in colorectal cancer cell lines following treatment with celecoxib. The celecoxib-modulated genes that were validated by qPCR were subjected to Ingenuity Pathway Analysis computational tool. **A.** HCA-7. 1, cancer; 2, cell cycle; 3, hematologic disease; 4, cell death; 5, immunologic disease; 6, connective tissue development and function; 7, reproductive system disease; 8, DNA replication, recombination, and repair; 9, gastrointestinal disease; 10, neurologic disease; 11, respiratory disease; 12, cellular growth and proliferation; 13, genetic disorder. **B.** HCT-116. 1, cell cycle; 2, cell death; 3, immunologic disease; 4, connective tissue development and function; 5, cancer; 6, cell growth and proliferation; 7, DNA replication, recombination, and repair; 8, neurologic disease; 9, cellular assembly and organization; 10, reproductive system disease; 11, hematologic disease; 12, genetic disorder. The numbered functional terms represented by bars were identified for their significant enrichment levels using P values calculated with right-tailed Fisher's exact test. The threshold line in the graph corresponds to a P value of 0.05 as the conventional cutoff. The functional terms above the threshold line would be considered as significantly modulated by celecoxib. The Y axis shows the negative log of P value.

despite the heterogeneity of transcriptional alterations in 75 $\mu\text{mol/L}$ celecoxib-treated COX-2 expresser and nonexpresser cell lines (Fig. 5). It is noteworthy that the level of expression of genes altered by celecoxib in colonic mucosa was subtle and much less than that observed in colorectal cancer cell lines. Functional annotation of the commonly altered transcripts by terms defined in GOBP annotation database again showed cell cycle, cell proliferation, DNA replication, and intracellular transport to be the major categories affected by celecoxib in human colonic mucosa and colorectal cancer cell lines (data not shown).

Examples of Novel Celecoxib-Modulated Genes. The investigation of global gene expression changes induced by celecoxib by the powerful DNA microarrays technology provided an extensive list of potentially novel targets for celecoxib. A few previously reported genes affected by celecoxib and involved in the regulation of cell cycle and apoptosis, such as *BAX*, *SKP2*, *BCL2L1*, *CDK2*, *CDKN1A*, and *TP53*, were among the celecoxib-modulated and PCR-validated genes in our study (Table 1). However, most of the validated genes in Table 1 have not been reported previously in the vast body of celecoxib literature to be the potential targets of celecoxib.

Examples of these novel celecoxib-modulated genes identified in our study include genes such as protein kinases, *ATM* and *mTOR/FRAP*, that are centrally involved in cell survival, growth, and metabolism (22, 23). *ATM* is a critical signaling molecule and plays a crucial role in numerous DNA damage response pathways in cells (Supplementary Fig. S4; refs. 22, 24), and *mTOR* occupies a central position in a complex signaling network regulating protein synthesis, cell growth and proliferation, and autophagy (Supplementary Fig. S5; ref. 25). The *mTOR* pathway is the target of many clinical trials (23, 26). Interestingly, *ATM*, *mTOR/FRAP*, and *DNAL3* were among the celecoxib-modulated proteins identified by a global proteomic profiling approach in the serum samples of FAP patients¹⁰ and were also up-regulated in celecoxib-treated colorectal cancer cell lines (18).

Consistent with the effect of celecoxib on cell cycle regulation and cell death (13, 15), many other celecoxib-responsive genes, such as *CDC2*, *CDC25A*, *GADD45A*,

¹⁰ Submitted for publication.

C

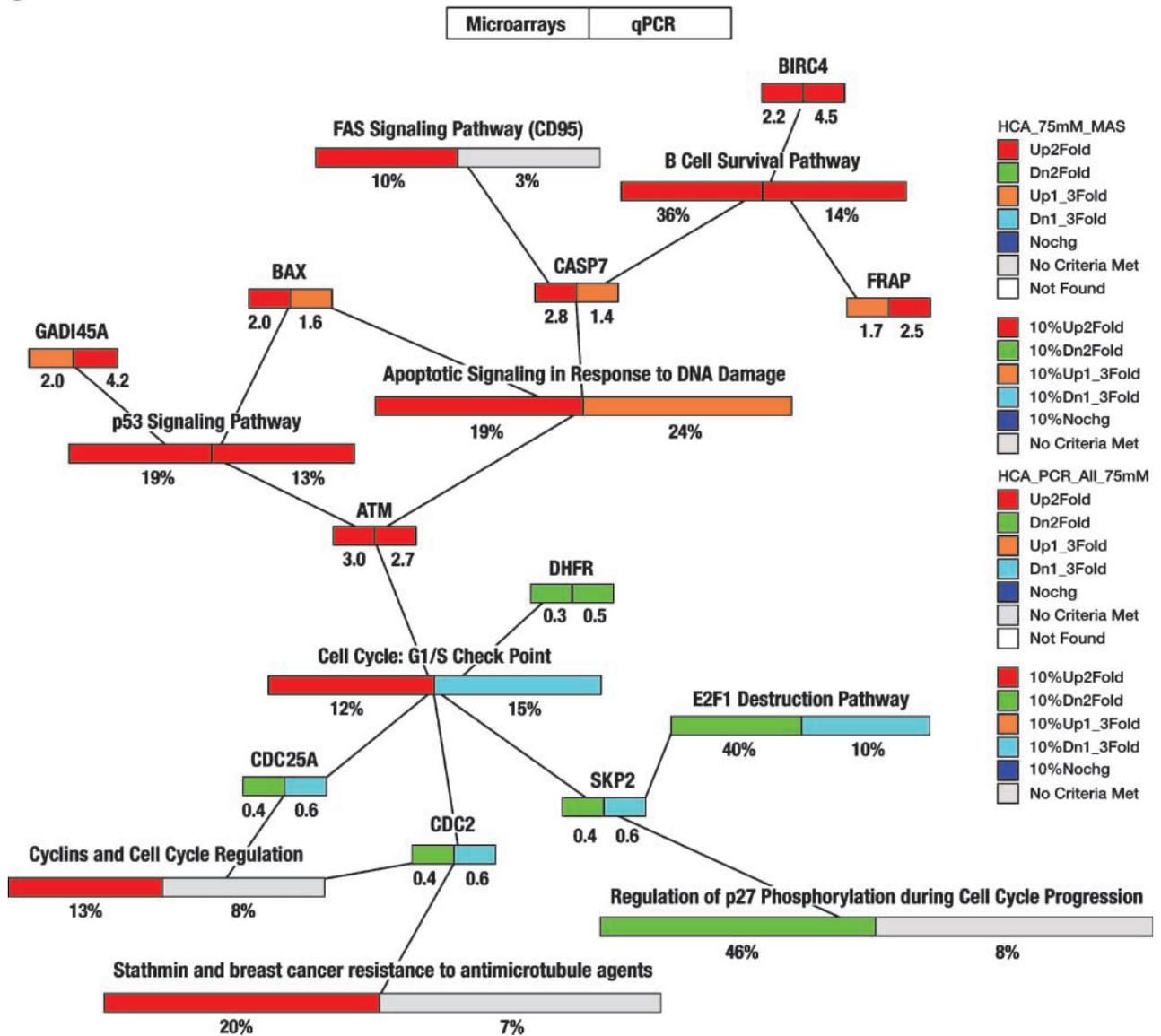


Figure 4 Continued. C. Celecoxib-modulated genes from the HCA-7 cell line were analyzed as gene-term association networks and are shown in the context of biological pathways. Numbers under the gene names indicate the extent of up-regulation or down-regulation and under the pathways provide an estimate of the genes in that particular pathway affected by celecoxib. The colors of the bars represent fold change as indicated by the blocks on the right.

RAD1, and *RRM1*, were identified that are directly or indirectly involved in cell proliferation and survival. *CDC2* is a cyclin-dependent kinase that drives cells into mitosis and therefore is targeted to arrest the cells in G_2 in response to DNA damage (27). *CDC25A* is a dual specificity phosphatase that regulates cell cycle transitions at the G_1 -S and G_2 -M entry points (28-31). *GADD45A*, a p53- and BRCA1-inducible gene, has multiple roles in the cell cycle checkpoint, signal transduction, DNA repair, and maintenance of genomic integrity (32), and *RAD1* is an integral part of a multifunctional complex involved in checkpoint control and DNA repair (33). Transcription of another DNA

repair enzyme, *RRM1*, which is involved in carcinogenesis and tumor progression (34), seems to be modulated by celecoxib.

Other novel potential targets of celecoxib identified in our study are genes involved in cellular adhesion and migration, such as *DDR1*, whose interaction with collagen facilitates adhesion, migration, and intracellular signaling (35), and *Ninein*, which is a centrosomal protein with a central role in microtubule nucleation and cell division (36, 37). Genes for two transcription factors, *GATA2* and *FOXP1*, were among those modulated by celecoxib (38, 39), so was the gene for an executioner caspase, *CASP7* (40). Celecoxib also altered the expression levels of *MDM2*

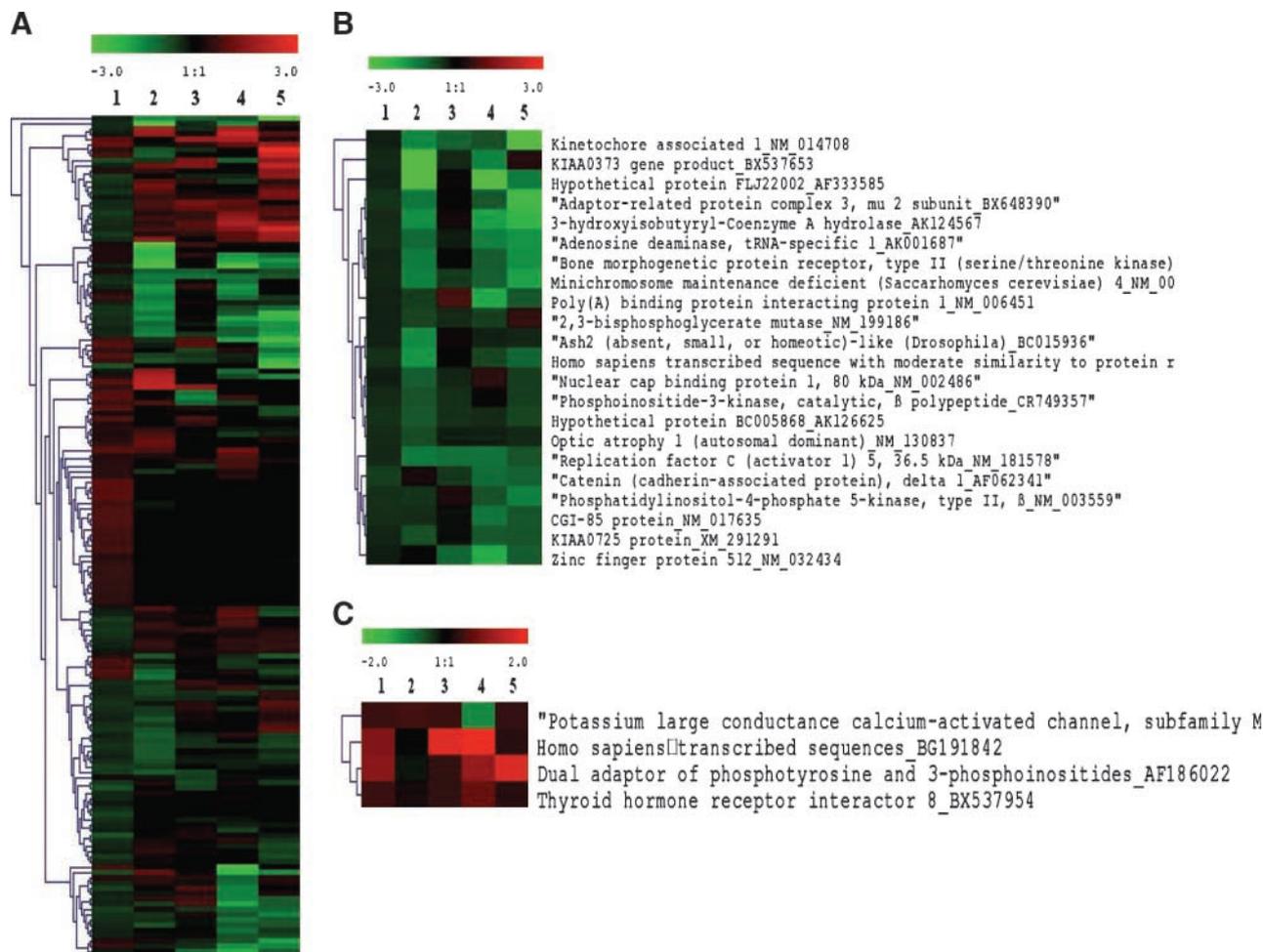


Figure 5. Comparative celecoxib-induced transcriptional profiles from normal colonic mucosa and colorectal cancer cell lines. Heat maps of the transcripts are based on *z* scores. **A**, Heat map of celecoxib-modulated transcripts from normal colonic mucosa (15) and colorectal cancer cell lines. Commonly down-regulated and up-regulated genes between colonic mucosa and at least three of the four colorectal cell lines are displayed in **B** and **C**, respectively. 1, colonic mucosa; 2, HCA-7; 3, HT-29; 4, DLD-1; 5, HCT-116. The bars with the color spectrum show the range of expression.

and BARD1, regulators of p53 and BRCA1, respectively (41, 42).

In summary, our data have furnished a rich catalogue of potential targets of celecoxib. It seems that COX-2, if not a collateral target, is certainly not the main or central target of celecoxib. Future studies focused on the similarities and differences in the patterns of celecoxib-modulated gene expression in COX-2 expresser and nonexpresser cells identified here will provide a clearer understanding of the molecular details of the events that culminate in growth inhibition and induction of apoptosis. Furthermore, the extensive list of celecoxib-responsive genes identified in our study represents a source of potentially novel targets that may be better suited for the rational design of more effective and less toxic chemopreventive strategies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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