Global Tumor RNA Expression in Early Establishment of Experimental Tumor Growth and Related Angiogenesis following Cox-Inhibition Evaluated by Microarray Analysis

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Abstract: Altered expression of COX-2 and overproduction of prostaglandins, particularly prostaglandin E_2, are common in malignant tumors. Consequently, non-steroidal anti-inflammatory drugs (NSAIDs) attenuate tumor net growth, tumor related cachexia, improve appetite and prolong survival. We have also reported that COX-inhibition (indomethacin) interfered with early onset of tumor endothelial cell growth, tumor cell proliferation and apoptosis. It is however still unclear whether such effects are restricted to metabolic alterations closely related to eicosanoid pathways and corresponding regulators, or whether a whole variety of gene products are involved both up- and downstream effects of eicosanoids. Therefore, present experiments were performed by the use of an in vivo, intravital chamber technique, where micro-tumor growth and related angiogenesis were analyzed by microarray to evaluate for changes in global RNA expression caused by indomethacin treatment.

Indomethacin up-regulated 351 and down-regulated 1852 genes significantly ($p < 0.01$); 1066 of these genes had unknown biological function. Genes with altered expression occurred on all chromosomes.

Our results demonstrate that indomethacin altered expression of a large number of genes distributed among a variety of processes in the carcinogenic progression involving angiogenesis, apoptosis, cell-cycling, cell adhesion, inflammation as well as fatty acid metabolism and proteolysis. It remains a challenge to distinguish primary key alterations from secondary adaptive changes in transcription of genes altered by cyclooxygenase inhibition.

Keywords: RNA expression, microarray, angiogenesis, cyclooxygenase.

Introduction

It is well established that non-steroidal anti-inflammatory drugs (NSAIDs) attenuate tumor net growth (Peterson, 1986), where provision of unselective prostaglandin synthase inhibitors, particularly indomethacin, attenuates systemic inflammation due to reduced prostaglandin production. This leads to decreased host appearance of acute phase proteins in both tumor-bearing mice and cancer patients (Cahlin et al. 2000b; Peluffo et al. 2004; McMillan et al. 1995). Such effects are related to reduced tumor growth, improved appetite and attenuation of cachexia with subsequent prolongation of survival in experimental models (Gelin et al. 1991; Cahlin et al. 2000a; Lönnroth et al. 1995). Similar improvements have also been repeatedly observed in unselected cancer patients on systemic anti-inflammatory treatment (Lundholm et al. 1994; Lundholm et al. 2004). In a recent study we reported that unspecific COX-inhibition (indomethacin) interfered with early onset of tumor endothelial cell growth, tumor cell proliferation and apoptosis (Lönnroth et al. 1995), observations that are similar to our findings in prostaglandin receptor subtype knock out mice (Axelsson et al. 2005). It is however still unclear whether such effects are mainly restricted to metabolic alterations closely related to eicosanoid and prostanoid pathways or whether a whole variety of gene products are involved up- and downstream of effects by eicosanoids. Therefore, microarray technique was used to evaluate overall changes in global RNA expression in early onset of micro-tumor growth and related angiogenesis.

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Material and Methods

Tumor models and animal groups

Tumor model

A methylcholanthrene induced sarcoma (MCG 101) was used in present experiments. This tumor model has been continuously transplanted in vivo at our laboratory for more than 25 years. The tumor was originally induced chemically as a sarcoma, while recent histological evaluation revealed that few tumor cells, if any, have characteristics of a sarcoma. Therefore, our tumor should rather be classified as a low or undifferentiated rapidly growing epithelial-like solid tumor. It has a reproducible and exponential growth pattern with a doubling time of 55–60 hours in vivo. It leads to 100% tumor take and does not give rise to visible metastases within the time period it kills the host. The tumors comprise 15–20 % of the body weight of the tumor bearing animals at the time of spontaneous death due to anorexia and cachexia. MCG 101 cells produce increased systemic levels of prostaglandin E_2, and COX-1/COX-2 inhibition by indomethacin, with normalized systemic levels of PGE_2, reduced tumor growth, improved nutritional state and prolonged host survival (Gelin et al. 1991; Cahlin et al. 2000a). These effects by indomethacin were in part due to decreased tumor cell proliferation and increased apoptosis as well as attenuated angiogenesis (Axelsson et al. 2005).

MCG-101 tumor cells for intravital chamber inoculation were maintained in Mc Coy's 5 A medium (MP Biomedicals, Inc., Aurora, Ohio, USA) supplemented with fetal calf serum (FCS, 10%), penicillin (100 U/ml), streptomycin (100 µg/ml) and L-glutamine (292 µg/ml). Cells were split 1/5 once weekly with a medium change in between (Mc Coy´s 5A + 2% FCS, penicillin, streptomycin and L-glutamin as mentioned above). The viability of the tumor cells was >99 % evaluated by trypan blue exclusion and microscopic examination before experiment. At start of experiments, cells were trypsinized and suspended in Mc Coy´s 5A medium at a concentration of 1.15 $\times 10^5$ cells/µl, 0,5 µl was inoculated into the intravital chamber as described. Animals were sacrificed at day 5 and micro tumors were immediately frozen in liquid nitrogen and kept at –80 °C until RNA extraction.

Animal groups

Adult, weight stable, female, wild type C57 black mice (n = 24) were used. The animals were housed in a temperature controlled room (24 °C) with a 12 hour light / dark schedule. The mice were housed in separate cages during the experiments to avoid interference with the subcutaneously placed intravital chamber. All animals were allowed initial adaptation for 3 days following operation to attain stable body weight and normal food intake before experiments started with free access to ordinary rodent chow (ALAB AB, Stockholm, Sweden) and water ad libitum under all experimental conditions.

All mice were randomly assigned to treatment (n = 12) and control groups (n = 12) before implantation with tumor cell suspensions. Treatment groups received indomethacin (Confortid, 5 mg/ml, Dumex-Alpharma) provided in the drinking water for 5 days corresponding to 6 µg / ml drinking water (Gelin et al. 1991; Cahlin et al. 2000a; Lönnroth et al. 1995; Lönnroth et al. 2001). The appropriate dilution of indomethacin in the drinking water was calculated based on daily normal water consumptions of 3–4 ml water/mouse/day. This corresponds to indomethacin of 1 µg/g bw/day. Controls received ordinary drinking water. Indomethacin provision started two days before tumor cell inoculation.

Intravital chamber and surgical techniques

The dorsal skin fold chamber in the mouse was prepared as described earlier (Axelsson et al. 1997). Mice were anaesthetized with i.p. injection of 0.15 ml from a 1 ml stock-solution composed of 0.4 ml Ketalar® (50 mg/ml; Parke-Davis), 0.05 ml Rompun® vet (20 mg/ml; Bayer), and 0.55 ml physiological saline. The dorsal skin was shaved. The mice were kept at constant temperature of 36-37 °C by means of a heating pad during the procedure. A 20 mm long midline incision was made in the dorsal skin. Blunt dissection was used to free the skin from underlying tissue before introducing the chamber into the skin fold. The chamber was fixed to the skin by 4–0 sutures and the midline incision used for the installation of the chamber

was closed with two 4–0 stitches. The skin covering one side of the central hole of the chamber was removed to expose the micro vascular tree of the contralateral subcutaneous tissue. Tumor cells (5.75×10^4) were inoculated onto the upper tissue layer of the chamber preparation by means of a Hamilton needle (10 µl). Small volumes of tumor cells were applied in order to avoid disseminated growth of tumor in the chamber. The access chamber was closed by cover glass after inoculation.

Microscopy

Observations on tumor growth were made by microscopy in a Nikon Eclipse E400 microscope with Nikon Plan 4X/0,10 objective and Nikon Digital Camera DXM 1200. Photographical documentations were performed immediately after implantation of tumor cells at day 0 and at day 5 following tumor cell implantation. Digital pictures were kept in a computer for subsequent analysis. Typical tumor graphs have been published elsewhere (Axelsson et al. 2005; Axelsson et al. 1997).

Image analysis of vascular beds

Image analyses were based on analysis of a given area by use of a digital photo across the tumor area. The area was composed of the tumor and its near surroundings and was the identical area in photos from day 0 and day 5. The centrum of a photo corresponded to the central part of the tumor based on visual analysis. For image analyses we used the computer program Easy Image Analysis 2000, Tekno Optik AB, and applied a technique to quantify the area (mm^2) of tumor related blood vessels and the size of the tumor area in the same plane (mm^2). Tumor related vascular area is the difference in vascular area between day 5 and day 0. This represents the appearance of net vessel formation around the tumor during five days, which was reported to be significantly reduced by indomethacin(Axelsson et al. 2005). Five days tumor growth was necessary to identify microtumors and corresponding vascular net works for isolation of RNA.

RNA extraction and amplification

Tumors from experiment 1 with 6 indomethacin-treated mice and 5 untreated control mice were pooled within groups, 3 + 3 (Indo 1A, 1B) and 3 + 2 (Ctrl 1C, 1D). Tumors from experiment 2 with 6 indomethacin-treated and 6 control mice were pooled within groups in the same way, (Indo 2A, 2B and Ctrl 2C, 2D). Total RNA Isolation Microdissected Cryosections Kit (QIAGEN Sciences, Maryland, U.S.A.) was used. Tissue disruption was done by aspiration with a syringe through 18 gauge needle 5x in lysis buffer. Quality and quantity of RNA were checked in an Agilent 2100 BioAnalyzer with RNA 6000 Nano Assay kit (Agilent Technologies, Palo Alto, CA, U.S.A.). Concentrations of RNA were measured in a NanoDrop (ND-1000A) spectrophotometer (NanoDrop Technologies, Inc.). Isolated tumor weight ranged from 8.4 to 16 mg (Indo) and 7.2 to 20.1 mg (Ctrl) wet weight and total RNA amount ranged from 4.1 to 10.1 µg (booth groups). RNA was linearly amplified with BD Smart mRNA Amplification Kit (BD Biosciences Clontech, Palo Alto, CA, USA). Unamplified total RNA used in the amplification reaction ranged from 425 ng to 946 ng with an efficiency of 160 to 240 x amplification based on the assumption that 5% of the total RNA fraction consists of polyA + mRNA. Amplified mRNA was checked for quality and quantity as mentioned above for total RNA.

cDNA Microarray profiling and data analysis

Expression array, Whole Mouse Genome Oligo Microarray (Agilent Technologies), containing 44290 features, including positive and negative control spots, was used. Four-hundred nanograms of amplified mRNA fractions from indomethacin-treated animals in experiment 1 (pool of 200 ng 1A and 200 ng 1B = test) were labeled with Cyanine 3-dCTP (Amersham Biosciences) in cDNA synthesis reaction with Agilent Fluorescent Direct Label Kit. Fourhundred nanograms of amplified mRNA fractions from untreated control mice in experiment 1 (pool of 200 ng 1C and 200 ng 1D = ctrl) were labeled with Cyanine 5-dCTP in parallel with the test-fraction. Hybridizations were performed during 18 hours with test- versus control cDNA followed by post-hybridization washes according to "in situ Hybridization Kit Plus" (Agilent Technologies) instructions. Microarrays were dried with nitrogen gas in a laminar flow bench and images were quantified on Agilent G2565 AA microarray scanner and fluorescence

intensities were extracted using the Feature Extraction software program (Agilent technologies). Dye-normalized, outlier- and background-subtracted values were further analyzed in GeneSpring software program, imported with the FE Plug-in (Agilent Technologies). Amplified mRNAs from experiment 2 were analyzed in the same way as in experiment 1 as a replicate. Technical replicates of experiment 1 and 2 were performed in a second run and the computer-based analyses were performed on all four series.

Gene expression in healthy, inbred mice was tested in muscle tissue from two individuals for assessment of the overall microarray variability. PolyA + selected RNA was extracted and 400 ng from mouse 1 were labeled with Cyanine 3-dCTP and 400 ng from mouse 2 were labeled with Cyanine 5-dCTP followed by hybridization to the same array targets with a ratio of 1.31 ± 0.03 (M ± SD) which confirms the validity of presented findings.

Statistics

Comparisons between several groups were performed by factorial analysis of variance (ANOVA). The ratio between expressed transcripts in tumor tissue of MCG 101 inoculates from study versus control animals were calculated in the GeneSpring software program. Genes with p-values outside the 99% confidence limit ($p < 0.01$) of the overall analytical variability as derived by t-testing were regarded to reflect significantly up- or down-regulated genes as defined by the software producer.

Results

24 mice were used in these experiments divided on 12 study animals and 12 controls. One mouse in the control group died initially due to the experimental procedures subsequently to implantation of the vascular chamber and was excluded from further analyses.

Indomethacin treatment reduced tumor area ($p < 0.03$), while the reduced tumor related vascular area did not reach statistical significance as observed and reported elsewhere in a larger group of animals (Axelsson et al. 2005) (Fig. 1). Tumor vascular area is positively correlated to the tumor area (not shown) as reported (Axelsson et al. 2005).

41 534 transcripts (genes) were analyzed (Fig. 2). Indomethacin treatment up-regulated 351 and down-regulated 1852 genes ($p < 0,01$). 1066 of these 2203 genes had unknown gene products and unknown biological function(s) of the corresponding protein. Such genes were therefore excluded for further consideration (Fig. 2). Genes significantly affected by indomethacin were located on all chromosomes as shown in (Fig. 3) with distribution according to functional aspects as shown in Table 1. The ten most up- and down-regulated genes according to the amounts of transcript following indomethacin treatment are shown in Table 2. Genes altered by indomethacin and strictly related to arachidonic acid metabolism are shown in Figure 4. Significantly altered genes for regulation of important but different aspects of the carcinogenic process (inflammation, angiogenesis, apoptosis, cell cycle, proliferation, cell adhesion,

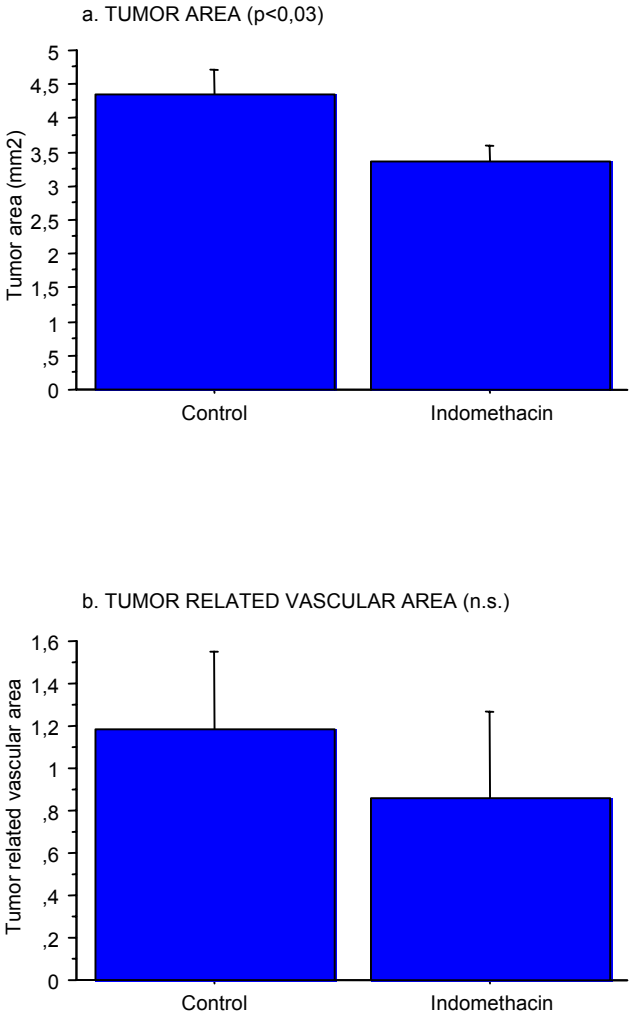

Figure 1. Effects on early tumor growth (a) ($p < 0.03$) and tumor related vascular area (b) by indomethacin provision in the drinking water to MCG 101, inoculated mice (n = 12) compare to controls (n = 12) as described in Methods.

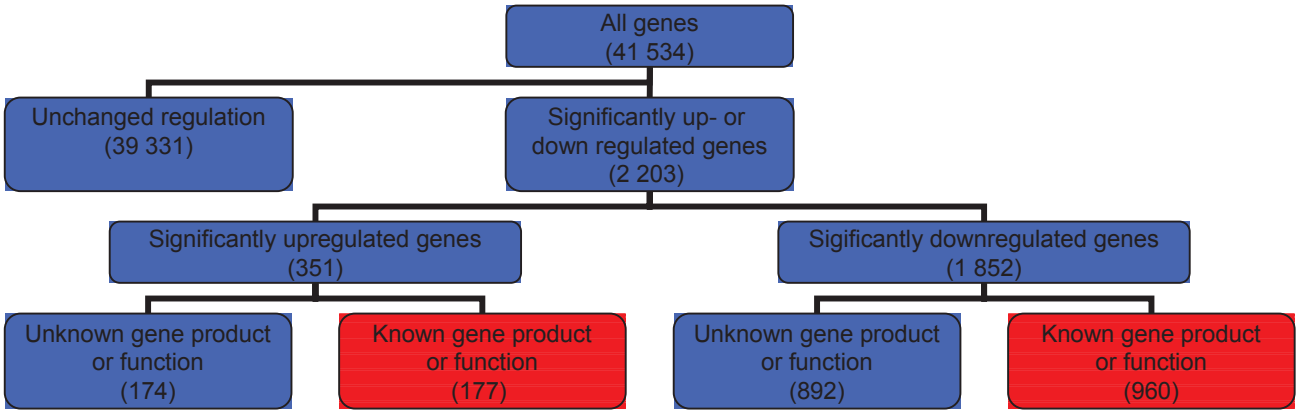

Figure 2. Stepwise analysis of global gene expression assessed by microarray technique. Genes in red boxes are subjected to functional considerations in Figure 4 and Table 4–6.

carbohydrate and fatty acid metabolism, proteolysis) are shown in the Appendix ordered by their magnitude in transcription comparing indomethacin treated tumors versus controls.

Discussion

It is well-recognized that non-steroidal anti-inflammatory drugs (NSAIDs), particularly indomethacin, attenuate tumor net growth (Peterson 1986), reduced tumor related cachexia, improved appetite and prolonged survival in tumor bearing mice (Gelin et al. 1991; Cahlin et al. 2000a; Lönnroth et al. 1995), and in part also in cancer patients (Lundholm et al. 1994; Lundholm et al. 2004). There is also evidence from population based, case control and clinical trials that regular use of NSAIDs may reduce the relative risk to develop colorectal adenomas (Baron et al. 2003; Sandler et al. 2003) and colorectal cancer (Smalley and

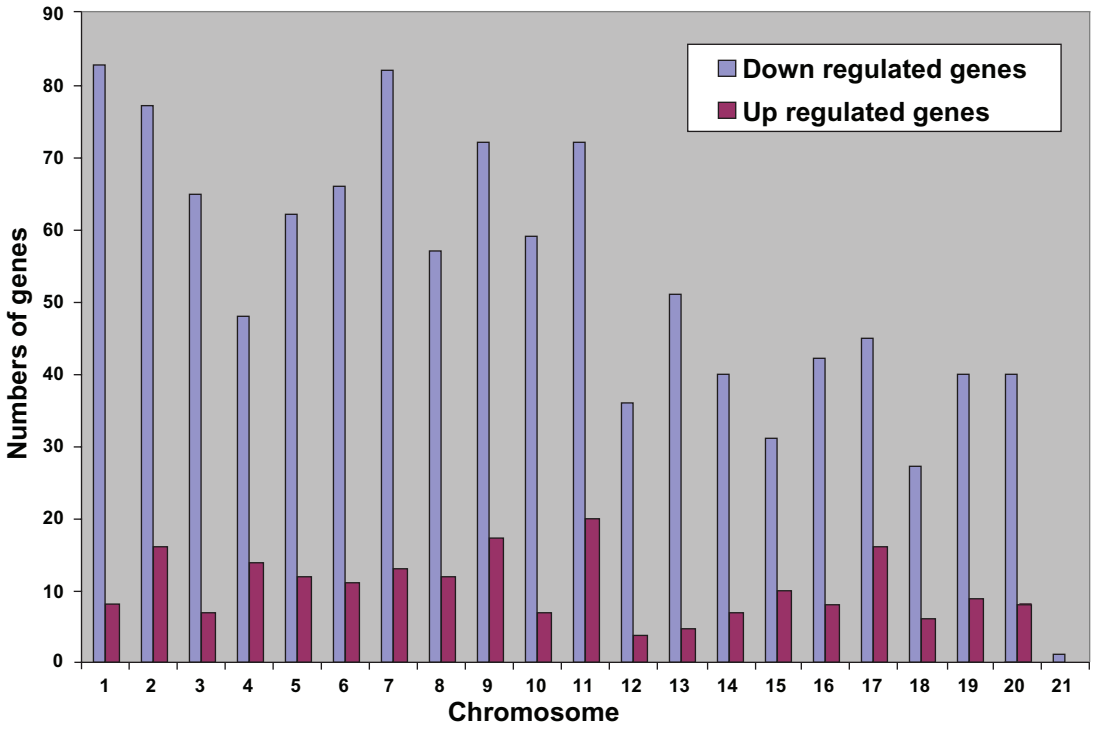

Figure 3. Distribution of tumor tissue genome wide expression of RNA transcripts (genes) significantly up- and down-regulated during indomethacin treatment of MCG 101 inoculated mice. ($p < 0.01$) (141 of the up-regulated genes and 756 of the down-regulated genes had unknown localization).

Table 1. The number of up- and down-regulated genes, regarded either stimulatory or inhibitory in function for progression of microtumors and related angiogenesis.

	Up-regulated	Down-regulated	P-value (sign test)
Angiogenesis	1	4	n.s.
Stimulatory genes	1	3	n.s.
Inhibitory genes	0	1	n.s.
Apoptosis	9	22	0.05
Stimulatory genes	9	19	n.s.
Inhibitory genes	0	3	n.s.
Carbohydrate metabolism	4	5	n.s.
Stimulatory genes	4	5	n.s.
Inhibitory genes	0	0	n.s.
Cell cycle & cell proliferation	8	39	<0.01
Stimulatory genes	7	31	<0.01
Inhibitory genes	1	6	n.s.
Cell adhesion	3	19	<0.01
Stimulatory genes	3	19	<0.01
Inhibitory genes	0	0	n.s.
Fatty acid metabolism	3	16	<0.01
Stimulatory genes	3	16	0.01
Inhibitory genes	0	0	n.s.
Inflammation	9	14	n.s.
Stimulatory genes	8	13	n.s.
Inhibitory genes	1	1	n.s.
Proteolysis	5	26	<0.01
Stimulatory genes	5	26	<0.01
Inhibitory genes	0	0	n.s

DuBois 1997; Thun et al. 1991; Williams et al. 1999; Giovannucci et al. 1994; Giovannucci et al. 1995; Smalley et al. 1999). Altered expression of COX-2 and overproduction of prostaglandins are common in colorectal cancers (Eberhart et al. 1994; Marnett and DuBois 2002; Williams et al. 1999; Joo et al. 2002), breast (Rolland et al. 1980), gastric (Uefuji et al. 2001), esophagus (Shamma et al. 2000), pancreatic (Juuti et al. 2006), bile duct (Hayashi et al. 2001), papillary thyroid (Siironen et al. 2006), malignant pheochromocytomas (Salmenkivi et al. 2001), urinary tract (Tuna et al. 2004), prostate (Tkacz et al. 2005), cervical (Kulkarni et al. 2001), retinoblastoma (Karim et al. 2000) and lung cancer (Wolff et al. 1998). Elevated levels of COX-2 and PGE_2 are also seen in premalignant conditions such as Barrett´s esophagus (Kandil et al. 2001) and colorectal adenomas (Khan et al. 2001). Thus, there are strong evidence that both COX-1 and COX-2 are of importance in several cancer forms in man. (Gupta et al. 2003; Huang et al. 2006).

We have earlier reported that indomethacin increased tumor cell apoptosis and reduced tumor cell proliferation and endothelial cell growth in animal studies (Axelsson et al. 2005), where unselective prostaglandin synthase inhibitors, (indomethacin), attenuate systemic inflammation reflected by reduction in plasma concentrations of prostaglandin E_2 and acute phase proteins (Cahlin et al. 2000b; Peluffo et al. 2004; McMillan et al. 1995). It is assumed that tumor reducing effects by indomethacin are caused by blocking prostaglandin production (Vane 1971), with competitive inhibition of substrate binding at COX- isoenzymes. Highly selective COX-2 inhibitors have retained anti-tumor effects, despite a lack of COX-1 inhibition, suggesting that COX-2 mediators are as well important for tumor development (Sheng et al. 1997; Evans 2003; Peluffo et al. 2004; Huang et al. 2006). The present study was based on intravital chamber (Axelsson et al. 1997) and microarray technique in order to evaluate overall changes in global RNA synthesis caused by indomethacin treatment on initial tumor growth to further map out important genetic areas behind tumor reducing effects by cyclooxygenase inhibition.

Indomethacin reduced tumor size as in earlier studies (Axelsson et al. 2005) and altered expres-

Table 2. Ten most up- and down-regulated tumor tissue genes during indomethacin treatment of MCG 101 inoculated mice.

Gene name	Accession number	Ratio	t-test p-value	Product	Biological process
Gpr2	NM_007721	8,20	0,0008	G protein-coupled receptor 2	G-protein coupled receptor protein signaling pathway; chemotaxis; defense response; positive regulation of cytosolic calcium ion concentration
Cdh2	NM_007664	3,27	0,0083	cadherin 2	calcium-dependent cell-cell adhesion; cell migration; homophilic cell adhesion
AK054131	AK054131	3,20	0,0002	RIKEN cDNA E230022H04 gene	
1500031H04Rik	NM_025893	3,20	0,0035	SMAD-interacting zinc finger protein 2	
Mal	NM_010762	3,11	0,0044	T-cell differentiation protein	differentiation of T-cells
Qscn6	NM_023268	2,98	0,0092	quiescin Q6	
M13540	M13540	2,96	0,0054	histocompatibility 2, class II antigen A, beta 1	immune response; antigen presentation & processing (exogenous peptide antigen via MHC class II)
AF053980	AF053980	2,95	0,0044	adenylyl cyclase type I	adenylate cyclase activation; cAMP biosynthesis; cyclic nucleotide biosynthesis; intracellular signaling cascade
AF464943	AF464943	2,95	0,0065	glutathione S-transferase	nitrobenzene metabolism; xenobiotic catabolism
AK038526	AK038526	2,90	0,0071	cytochrome P450, family 4	biological process unknown
BC030460	BC030460	0,41	0,0065	sprouty-related protein	development; inactivation of MAPK; regulation of signal transduction
V2r14	NM_009489	0,40	0,0035	vomeronasal 2	G-protein coupled receptor protein signaling pathway
C330027C09Rik	NM_172616	0,40	0,0047	RIKEN cDNA C330027C09	
Il1r1	NM_008362	0,40	0,0023	interleukin 1 receptor, type I	cell surface receptor linked signal transduction; cytokine and hemokine mediated signaling pathway
Crtap	NM_019922	0,40	0,0052	cartilage associated protein	
AK002825	AK002825	0,39	0,0033	synaptobrevin like 1	intracellular protein transport
AK078146	AK078146	0,38	0,0015	RIKEN cDNA 1700013G20	
Kcnn3	NM_080466	0,37	0,0085	calcium-activated potassium channel protein 3	ion transport; potassium ion transport
AK033404	AK033404	0,37	0,0022	transmembrane protein 34	
BC006023	BC006023	0,36	0,0070	cytochrome b	electron transport

The 99% confidence interval for the relative expression ratio was 1.31±0.03 (2.3%) based on 4 arrays.

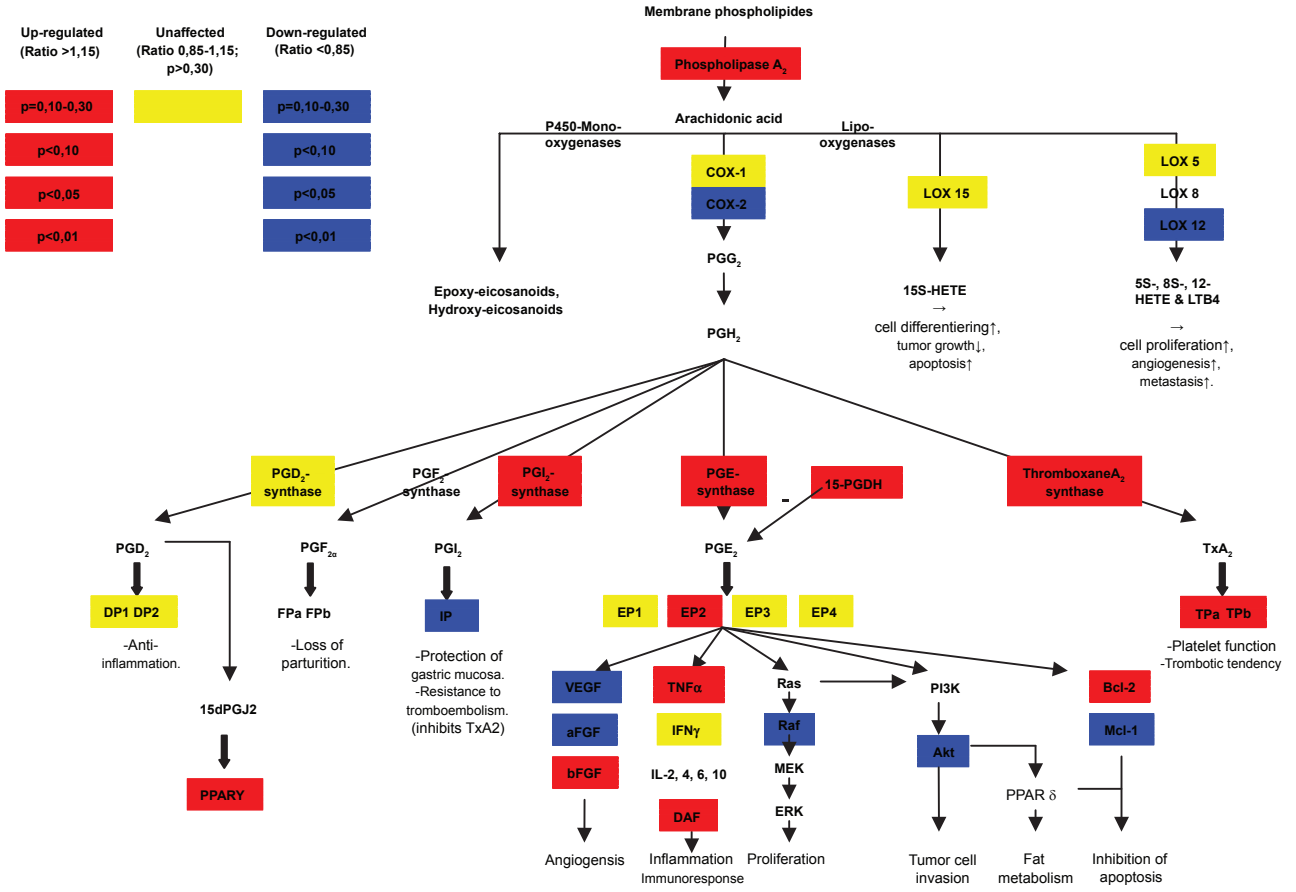

Figure 4. Alterations in overall tumor tissue levels of transcript expression in arachidonic acid metabolism during indomethacin treatment of MCG 101 inoculated mice. Blue boxes indicate down-regulated genes and colors turning towards red indicate increasing grades of up-regulation of transcripts.

sion of 2 203 genes out of 41 534 (5,3%). These genes were widely and relatively uniformly spread over the entire genome on all chromosomes. Indomethacin down-regulated five times as many genes being up-regulated. Affected genes were predominantly stimulatory in function. Thus, effects by indomethacin were overall down-regulating stimulatory genes. However, indomethacin influenced on the expression of a large number of genes responsible for important steps in the carcinogenic process. Genes with mainly reduced expression involved apoptosis, cell cycle and proliferation, cell adhesion, fatty acid metabolism and proteolysis, while effects on angiogenesis, carbohydrate metabolism and inflammation displayed both up- and down-regulation.

We have previously reported that tumor effects by indomethacin involve apoptosis and cell proliferation in vivo (Axelsson et al. 2005), as confirmed by others in vitro (Huang et al. 2006). Earlier and resent studies indicate that COX-2 derived PGE_2 are involved in the control of programmed cell death (Sheng et al. 1998; Huang et al. 2006), although exact mechanisms are unknown. PGE_2 may reduce apoptotic rates by increasing levels of antiapoptotic proteins like Bcl-2 (Sheng et al. 1998), Mcl-1 or other key mediators as NF-κB (Poligone and Baldwin 2001) and by activation of two main apoptotic pathways: the death receptor and mitochondrial pathways (Huang et al. 2006). Such results agree with our present in vivo findings, which indicate that indomethacin down-regulated Mcl-1, while Bcl-2 and TNF-receptor associated factor 5 were significantly increased. Genes involved in the NF-κB cascade were both up- and down-regulated by indomethacin. Prior studies have shown that PGE_2 stimulates cell proliferation in colorectal cancer (Sheng et al. 2001), although downstream pathways are still unknown. There are indications that cell proliferation is stimulated by activation of the epidermal growth factor receptor (EGFR) (Yoshimoto et al. 2002). Tumor growth is

also stimulated by the oncogene Ras, which induces cell proliferation, cell transformation and cell survival by activation of Raf-MEK-ERK and PI3K-Akt pathways. Indomethacin treatment in present experiments down-regulated Raf and Akt genes, which would reduce tumor cell proliferation. Genes responsible for the production of EGF and EGF-receptor were not significantly changed, while other genes involving cell proliferation mainly showed reduced expression as reported elsewhere (Lönnroth et al. 2001).

Angiogenesis is though to play a central role in cancer progression (Folkman 1971). Main proangiogenic factors are vascular endothelial growth factor (VEGF) and fibroblast growth factor 1 & 2 (acidFGF & basicFGF). There is also evidence that COX-2 plays a role in tumor-associated angiogenesis (Williams et al. 2000; Oshima et al. 1996) by modulation of proangiogenic factors with correlations between COX-2 and VEGF expression in tumor tissue (Joo et al. 2003). PGE_2 is then thought to be the mediator behind COX-2 activities in tumor angiogenesis (Hernandez et al. 2001). Both selective and nonselective COX-inhibitors can however reduce tumor angiogenesis, by inhibiting production of proangiogenic factors and subsequent proliferation, migration and tube formation of endothelial cells (Axelsson et al. 2005; Peterson 1986; Tsujii et al. 1998; Masferrer et al. 2000; Skopinska-Rozewska et al. 1998; Sawaoka et al. 1999). In the present analysis the gene coding for one of three forms of VEGF (VEGF-A) was down-regulated by indomethacin whereas the other two (VEGF-B & C) were unaffected. AcidFGF showed a trend towards down-regulation, while basicFGF displayed a trend to up-regulation. Other genes in angiogenesis were mainly down-regulated. Our results therefore support that indomethacin affects tumor angiogenesis in addition to other processes more related to tumor cell proliferation (Axelsson et al. 2005).

Malignant disease is also characterized by attenuation of cell mediated anti-tumor immune response. Probably directed in part by PGE_2, based on reduced production of anti-tumor Th1 cytokines (TNFα, IFNγ and IL-2) (Harris et al. 2002) and increased production of Th2 cytokines (IL-4, IL-10 and IL-6) (Shreedhar et al. 1998; Huang et al. 1998; Della Bella et al. 1997). In present experiments the gene coding for TNFα was up-regulated, while genes coding for mentioned cytokines were not changed by indomethacin. Our previous results imply that main tumor reducing effects by indomethacin are caused by blockade of prostaglandin production (Vane 1971). Accordingly, genes coding for these enzymes in prostaglandin biosynthesis were decreased by indomethacin including the COX-2 enzyme. This suggests that indomethacin also acts on transcription of cyclooxygenases.

Genes in control of fatty acid and protein metabolism were significantly and highly down-regulated by indomethacin, while genes for carbohydrate metabolism seemed to be both up- and down-regulated. These observations may contribute to reported beneficial overall host-metabolic effects by indomethacin attenuating catabolism caused by a growing tumor (Gelin et al. 1991; Lundholm et al. 2004). Distant metastases are the major cause of death in cancer. Over-expression of COX-2 and increased production of PGE_2 may promote metastatic potential and tumor cell invasiveness (Tsujii et al. 1997), while treatment with NSAIDs reduced this imbalance (Lundholm et al. 1994; Yamauchi et al. 2003; Yao et al. 2003). The PI3K-Akt pathway is highly important in regulating tumor cell migration and invasion where PGE_2 is known to stimulate this pathway (Sheng et al. 2001). In present experiments the gene coding for Akt was significantly down-regulated and genes coding for proteins regulating cell adhesion were likewise mainly down-regulated by indomethacin.

In conclusion, this study has evaluated the magnitude of net overall changes on whole genome transcription in micro-tumors during a period of unspecific cyclooxygenase blockade by indomethacin. The results, derived in vivo by application of microarray, demonstrate an overwhelming number of influences by indomethacin of which inhibitory effects (down-regulation) appeared to dominate. It remains a challenge to distinguish primary key effects from secondary adaptive alterations in transcription of all these affected genes by NSAID treatment.

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Appendix

Significantly up- and down-regulated tumor tissue genes controlling important steps of tumor progression during indomethacin treatment of MCG 101 inoculated mice.

ANGIOGENESIS

Gene name	Accession number	Ratio	t-test p-value	Product	Biological process
Nte	NM_015801	1,19	0,0097	neuropathy target esterase	angiogenesis
Hif1a	NM_010431	0,73	0,0047	hypoxia inducible factor 1, alpha subunit	angiogenesis; response to hypoxia
AK004663	AK004663	0,71	0,0004	caveolin 2	negative regulation of endothelial cell proliferation
Enpep	NM_007934	0,70	0,0021	glutamyl aminopeptidase	angiogenesis
Robo4	NM_028783	0,68	0,0083	roundabout homolog 4	angiogenesis

APOPTOSIS

Gene name	Accession number	Ratio	t-test p-value	Product	Biological process
Cidea	NM_007702	2,70	0,0080	cell death-inducing DNA fragmentation factor	apoptosis
Bcl2a1c	NM_007535	1,97	0,0074	B-cell phospholi/lymphoma 2 related protein A1c	apoptosis
Traf5	NM_011633	1,96	0,0009	Tnf receptor-associated factor 5	apoptosis; positive regulation of cell proliferation
Mnt	NM_010813	1,82	0,0087	max binding protein	cell aging; regulation of apoptosis & cell cycle
BC012955	BC012955	1,76	0,0097	tribbles homolog 3	apoptosis
Ppp1r13b	NM_011625	1,73	0,0011	protein phosphatase 1, regulatory subunit 13B	apoptosis
Ddit3	NM_007837	1,68	0,0080	DNA-damage inducible transcript 3	cell cycle arrest; apoptosis
Clca2	NM_030601	1,24	0,0010	chloride channel calcium activated 2	apoptosis
Gadd45g	NM_183358	1,16	0,0013	growth arrest and DNA-damage-inducible 45 gamma	apoptosis; cell cycle
Mcl1	NM_008562	0,86	0,0013	myeloid cell leukemia sequence 1	apoptosis
AK020218	AK020218	0,82	0,0096	Tial1 cytotoxic granule-associated RNA binding protein	apoptosis
AK129340	AK129340	0,77	0,0073	programmed cell death 6 interacting protein	apoptosis
AK079110	AK079110	0,74	0,0034	Bcl-2-associated transcription factor	positive regulation of apoptosis
Hif1a	NM_010431	0,73	0,0047	hypoxia inducible factor 1, alpha subunit	apoptosis
AK012111	AK012111	0,72	0,0003	cell division cycle and apoptosis regulator 1	apoptosis
Casp2	NM_007610	0,71	0,0037	caspase 2	apoptosis
Casp6	NM_009811	0,69	0,0038	caspase 6	apoptosis
Cflar	NM_009805	0,68	0,0089	CASP8 & FADD-like apoptosis regulator isoform 1 & 2	apoptosis
2810413N20Rik	NM_134141	0,67	0,0045	cytokine induced apoptosis inhibitor 1	anti-apoptosis
AF121215	AF121215	0,67	0,0096	muc2	apoptosis; negative regulation of cell proliferation
Unc5d	NM_153135	0,66	0,0028	netrin receptor Unc5h4	apoptosis
Pdcd8	NM_012019	0,62	0,0061	programmed cell death 8	DNA fragmentation & mitochondrial changes of apoptosis
Pten	NM_008960	0,61	0,0050	phosphatase and tensin homolog	apoptosis; negative regulation of cell cycle
Casp12	NM_009808	0,57	0,0055	caspase 12	apoptosis
Thoc1	NM_153552	0,57	0,0099	THO complex 1	apoptosis
Cycs	NM_007808	0,52	0,0092	cytochrome c	apoptosis
Lgals7	NM_008496	0,52	0,0054	galactose binding lectin	apoptosis
Api5	NM_007466	0,51	0,0073	apoptosis inhibitor 5	anti-apoptosis
Bad	NM_007522	0,51	0,0090	Bcl-associated death promoter	apoptosis
Ube1c	NM_011666	0,50	0,0061	ubiquitin-activating enzyme E1C	apoptosis; cell cycle
Birc6	NM_007566	0,47	0,0082	baculoviral IAP repeat-containing 6	anti-apoptosis

CARBONHYDRATE METABOLISM

Gene name	Accession number	Ratio	t-test p-value	Product	Biological process
Chst4	NM_011998	2,21	0,0082	carbohydrate sulfotransferase 4	expression of 6-sulfo sialyl Lewis X (L-selectin ligand displayed by CD34)
Pmm1	NM_013872	1,98	0,0031	phosphomannomutase 1	mannose biosynthesis & metabolism
Ppp1r1a	NM_021391	1,87	0,0056	protein phosphatase 1, subunit 1A	glycogen metabolism

Ins1	NM_008386	1,83	0,0072	insulin I	glucose metabolism & transport
Man2b1	NM_010764	0,80	0,0088	mannosidase 2, alpha B1	carbohydrate metabolism
Ppara	NM_011144	0,70	0,0082	peroxisome phospholipids activated receptor alpha	glucose metabolism
Large	NM_010687	0,68	0,0069	like-glycosyltransferase	carbohydrate biosynthesis
BC005552	BC005552	0,67	0,0017	asparagine synthetase	asparagine biosynthesis; glutamine metabolism
Bad	NM_007522	0,51	0,0090	Bcl-associated death promoter	glucose catabolism; glucose homeostasis
Man2a1	NM_008549	0,45	0,0078	mannosidase 2, alpha 1	carbohydrate metabolism

CELL CYCLE & CELL PROLIFERATION

Gene name	Accession number	Ratio	t-test p-value	Product	Biological process
Cdc37	NM_016742	2,10	0,0048	cell division cycle 37 homolog	cell cycle
Traf5	NM_011633	1,96	0,0009	Tnf receptor-associated factor 5	positive regulation of cell proliferation; apoptosis
Mnt	NM_010813	1,82	0,0087	max binding protein	regulation of cell cycle; cell aging; regulation of apoptosis
Pard3	NM_033620	1,68	0,0038	partitioning-defective protein 3 homolog	cell cycle
Chc1	NM_133878	1,62	0,0053	chromosome condensation 1	cell cycle; mitosis
Myg1	NM_021713	1,55	0,0033	melanocyte proliferating gene 1	proliferation
Mapk3	NM_011952	1,41	0,0098	mitogen activated protein kinase 3	cell cycle
Gadd45g	NM_011817	1,16	0,0013	growth arrest & DNA-damage-inducible 45 gamma	cell cycle
Ccng1	NM_009831	0,84	0,0077	cyclin G1	cell cycle; mitosis
Ccnt1	NM_009833	0,84	0,0045	cyclin T1	cell cycle
AW209059	NM_023058	0,78	0,0048	tyrosine- & threonine specific cdc2-inhibitory kinase	cell cycle
U27177	U27177	0,77	0,0089	retinoblastoma-like protein 1	negative regulation of cell cycle
Orc3l	NM_015824	0,76	0,0090	origin recognition complex, subunit 3-like	DNA replication
Cul4b	NM_028288	0,76	0,0041	cullin 4B	cell cycle
X61753	X61753	0,75	0,0038	heat shock factor 1	negative regulation of cell proliferation
Prim2	NM_008922	0,74	0,0075	DNA primase, p58 subunit	DNA replication
AB003502	AB003502	0,74	0,0023	G1 to S phase transition 1	G1/S transition of mitotic cell cycle; cell proliferation; cell cycle
Cspg6	NM_007790	0,72	0,0010	chondroitin sulfate proteoglycan 6	DNA repair; cell cycle; meiosis; mitosis
Prim1	AK089063	0,71	0,0047	DNA primase small subunit	DNA replication
Mina	NM_025910	0,71	0,0009	myc induced nuclear antigen	cell proliferation
Pdgfb	NM_011057	0,71	0,0072	platelet derived growth factor, B polypeptide	cell proliferation; cell cycle
Ppara	NM_011144	0,70	0,0082	peroxisome hospholipids activated receptor alpha	regulation of transcription
Gsg2	NM_010353	0,67	0,0096	germ cell-specific gene 2	cell cycle
Mcm8	NM_025676	0,67	0,0028	minichromosome maintenance deficient 8	DNA replication; cell cycle
Chek1	NM_007691	0,66	0,0055	checkpoint kinase 1 homolog	G2/M transition of mitotic cell cycle
Cdk2	NM_016756	0,66	0,0011	cyclin-dependent kinase 2 isoform 1&2	cell cycle
03-sep	NM_011889	0,65	0,0017	septin 3	cell cycle
Orc2l	NM_008765	0,63	0,0000	origin recognition complex, subunit 2	DNA replication
AF129738	AF129738	0,63	0,0059	cullin 3	cell cycle
AK052506	AK052506	0,63	0,0002	cyclin J	cell cycle
AK044216	AK044216	0,62	0,0074	nucleolar protein 5	cell growth
Pten	NM_008960	0,61	0,0050	phosphatase and tensin homolog	negative regulation of cell cycle; induction of apoptosis
Tax1bp3	NM_029564	0,60	0,0031	Tax1 binding protein 3	negative regulation of cell proliferation
Mcm7	NM_008568	0,58	0,0042	minichromosome maintenance protein 7	cell cycle; cell proliferation
Mafk	NM_010757	0,59	0,0068	v-maf	cell cycle
Igfbp7	NM_008048	0,56	0,0084	insulin-like growth factor binding protein 7	cell growth
BC031460	BC031460	0,54	0,0066	anaphase-promoting complex subunit 10	cell cycle; mitosis
Cks2	NM_025415	0,53	0,0072	CDC28 protein kinase regulatory subunit 2	cell cycle
Stag1	NM_009282	0,53	0,0028	stromal antigen 1	cell cycle; chromosome segregation; mitosis
BC053028	BC053028	0,52	0,0004	large tumor suppressor 2	negative regulation of cell cycle
Ube1c	NM_011666	0,50	0,0061	ubiquitin-activating enzyme E1C	cell cycle; apoptosis
BC029198	BC029198	0,50	0,0015	M-phase phosphoprotein 10	
Ccnh	NM_023243	0,49	0,0009	cyclin H	cell cycle
Ect2	NM_007900	0,45	0,0038	ect2 oncogene	cell cycle
AY259532	AY259532	0,44	0,0051	M-phase phosphoprotein	
Top1	NM_009408	0,44	0,0031	topoisomerase I	DNA replication

CELL ADHESION

Gene name	Accession number	Ratio	t-test p-value	Product	Biological process

Pard3	NM_033620	1,68	0,0038	partitioning-defective protein 3 homolog	cell-cell adhesion; cytokinesis
Selpl	NM_009151	1,33	0,0044	selectin	cell adhesion
BC039767	BC039767	1,26	0,0080	protein tyrosine phosphatase, receptor type F	cell adhesion
Hapln1	NM_013500	1,17	0,0046	hyaluronan and proteoglycan link protein 1	cell adhesion
Rgmb	AK047390	0,79	0,0067	repulsive guidance molecule B	cell adhesion
AK077250	AK077250	0,79	0,0043	plakophilin 4	cell adhesion
AK039018	AK039018	0,78	0,0021	neuroligin 3	cell adhesion
Ptpru	NM_011214	0,77	0,0066	protein tyrosine phosphatase, receptor type L	cell adhesion
AK016518	AK016518	0,76	0,0095	Down syndrome cell adhesion molecule	cell adhesion
Catnd2	NM_008729	0,74	0,0051	catenin delta 2	cell adhesion
Col6a1	NM_009933	0,72	0,0018	procollagen, type VI, alpha 1	cell adhesion
Itgb2l	NM_008405	0,70	0,0076	integrin beta 2-like	cell-matrix adhesion
AF169388	AF169388	0,68	0,0094	procollagen, type IV, alpha 4	cell adhesion
AK034300	AK034300	0,67	0,0058	brain link protein 2	cell adhesion
Omd	NM_012050	0,67	0,0017	osteomodulin	cell adhesion
Lims1	NM_026148	0,61	0,0076	LIM & senescent cell antigen-like domains 1	cell-matrix adhesion
NM_017383	NM_017383	0,61	0,0094	contactin 6	cell adhesion
Col5a1	NM_015734	0,59	0,0049	procollagen, type V, alpha 1	cell adhesion
X15050	X15050	0,59	0,0040	neural cell adhesion molecule 1	cell adhesion
AK032156	AK032156	0,58	0,0096	protocadherin beta 14	cell adhesion
Fbln5	NM_011812	0,54	0,0082	fibulin 5	cell adhesion
Pscdbp	NM_139200	0,52	0,0011	pleckstrin homology binding protein	cell adhesion
Pcdhb22	NM_053147	0,47	0,0088	protocadherin beta 22	cell adhesion

FATTY ACID METABOLISM

Gene name	Accession number	Ratio	t-test p-value	Product	Biological process
Cidea	NM_007702	2,70	0,0080	cell death-inducing DNA fragmentation factor	lipid metabolism
BC026669	BC026669	2,58	0,0037	acetyl-Coenzyme A acyltransferase 1	fatty acid metabolism
Galgt1	NM_008080	1,29	0,0049	beta-1,4-N-acetylgalactosaminyltransferase	lipid glycosylation
AY259499	AY259499	0,74	0,0072	nephrocystin 3	lipid metabolism
Ppara	NM_011144	0,70	0,0082	peroxisome phospholipids activated receptor alpha	fatty acid metabolism
Crot	NM_023733	0,69	0,0093	carnitine O-octanoyltransferase	fatty acid metabolism & transport
AK083617	AK083617	0,67	0,0049	RIKEN cDNA 4933425A18	fatty acid biosynthesis
1600020H07Rik	AK041686	0,63	0,0089	2-hydroxyphytanoyl-CoA lyase	lipid metabolism
Pla1a	NM_134102	0,62	0,0081	phospholipase A1 member A	lipid metabolism
Ch25h	NM_009890	0,61	0,0024	cholesterol 25-hydroxylase	cholesterol metabolism
BC026817	BC026817	0,59	0,0058	acetoacetyl-CoA synthetase	fatty acid metabolism
Sftpb	NM_147779	0,58	0,0054	surfactant associated protein B	lipid metabolism
Hsd17b7	NM_010476	0,57	0,0039	hydroxysteroid dehydrogenase 7	steroid biosynthesis
Gnpat	NM_010322	0,53	0,0037	glyceronephosphate O-acyltransferase	ether lipid biosynthesis; phospholipids biosynthesis
AF017175	AF017175	0,52	0,0056	carnitine palmitoyltransferase 1a	fatty acid metabolism & transport
3632410F03Rik	NM_028721	0,51	0,0042	nephrocystin 3	lipid metabolism
Hadhb	AK033462	0,64	0,0070	hydroxyacyl-Coenzyme A dehydrogenase / 3-ketoacyl-Coenzyme A thiolase / enoyl-Coenzyme A hydratase	fatty acid beta-oxidation
Acox2	NM_053115	0,59	0,0086	acyl-Coenzyme A oxidase 2	fatty acid beta-oxidation
Elovl6	NM_130450	0,54	0,0055	ELOVL family member 6	fatty acid elongation

INFLAMMATION

Gene name	Accession number	Ratio	t-test p-value	Product	Biological process
M13540	M13540	2,96	0,0054	histocompatibility 2, class II antigen A, beta 1	antigen presentation & processing (exogenous peptide antigen via MHC class II)
Rmcs1	NM_207105	2,34	0,0010	response to metastatic cancers 1	antigen presentation & processing (exogenous peptide antigen via MHC class II)
AK008094	AK008094	1,96	0,0097		humoral immune response
Gab3	NM_181584	1,84	0,0078	growth factor receptor bound protein 2	macrophage differentiation
BC037477	BC037477	1,64	0,0017	adaptor-related protein complex 3	antigen presentation & processing (exogenous lipid antigen); positive regulation of NK T-cell differentiation

AJ298147	AJ298147	1,45	0,0006	defensin beta 7	defense response to bacteria
H2-M10.2	NM_177923	1,44	0,0010	histocompatibility 2, M region locus 10.2	antigen presentation & processing (endogenous antigen via MHC class I)
BC021779	BC021779	1,33	0,0030	Fms interacting protein	negative regulation of macrophage differentiation
Ccl19	NM_011888	1,24	0,0051	chemokine ligand 19	chemotaxis; immune response; inflammatory response
Ly6d	NM_010742	1,19	0,0028	lymphocyte antigen 6 complex, locus D	defense response
Gadd45g	NM_011817	1,16	0,0013	growth arrest & DNA-damage-inducible 45 gamma	T-helper 1 cell differentiation; interferon-gamma biosynthesis
AK080873	AK080873	0,91	0,0023	toll-like receptor adaptor molecule 2	regulation of I-kappaB kinase / NF-kappaB cascade; regulation of cytokine production
BC030872	BC030872	0,83	0,0088	attractin-like 1	inflammatory response
Tlr4	NM_021297	0,82	0,0041	toll-like receptor 4	immune response; inflammatory response
Fcgr2b	NM_010187	0,77	0,0000	Fc receptor	negative regulation of B-cell proliferation; negative regulation of immune response; positive regulation of phagocytosis; antigen presentation (exogenous peptide antigen via MHC class II)
Ifi47	NM_008330	0,69	0,0044	interferon gamma inducible protein 47	defense response
Ap3b1	NM_009680	0,67	0,0088	adaptor-related protein complex 3, beta 1 subunit	antigen presentation & processing (exogenous lipid antigen); positive regulation of NK T-cell differentiation
Il18	NM_008360	0,66	0,0087	interleukin 18	immune response
X97991	X97991	0,64	0,0010	calcitonin / calcitonin-related polypeptide	inflammatory response
Ifi203	NM_008328	0,63	0,0049	interferon activated gene 203	immune response
Bad	NM_007522	0,51	0,0090	Bcl-associated death promoter	positive regulation of B- & T-cell differentiation
AB022307	AB022307	0,49	0,0055	interleukin 15	NK T-cell proliferation & differentiation; lymph gland development ; regulation of antiviral response
9230106L14Rik	NM_138685	0,48	0,0058	elafin-like protein I	defense response to bacteria
Cxcl10	NM_021274	0,47	0,0009	chemokine ligand 10	chemotaxis; immune response; inflammatory response
Irf2	NM_008391	0,44	0,0083	interferon regulatory factor 2	immune response

PROTEOLYSIS

Gene name	Accession number	Ratio	t-test p-value	Product	Biological process
Mst1	NM_008243	2,08	0,0019	macrophage stimulating 1	proteolysis and peptidolysis
Zfp61	NM_009561	2,03	0,0075	zinc finger protein 61	proteolysis and peptidolysis
AK081629	AK081629	1,76	0,0089	dual specificity phosphatase 11	protein amino acid dephosphorylation
F12	NM_021489	1,42	0,0038	coagulation factor XII	proteolysis and peptidolysis
BC027403	BC027403	1,17	0,0014	bleomycin hydrolase	proteolysis and peptidolysis
AK036071	AK036071	0,75	0,0050	membrane-bound transcription factor protease	proteolysis and peptidolysis
Col6a1	NM_009933	0,72	0,0018	procollagen, type VI, alpha 1	proteolysis and peptidolysis
Casp2	NM_007610	0,71	0,0037	caspase 2	proteolysis and peptidolysis
Enpep	NM_007934	0,70	0,0021	glutamyl aminopeptidase	proteolysis and peptidolysis
AK041155	AK041155	0,70	0,0015	CDC14 cell division cycle 14 homolog B	protein amino acid dephosphorylation
BC050834	BC050834	0,70	0,0095	metalloprotease	proteolysis and peptidolysis
Casp6	NM_009811	0,69	0,0038	caspase 6	proteolysis and peptidolysis
Hspa14	NM_015765	0,68	0,0062	heat shock protein 14	proteolysis and peptidolysis
Cflar	NM_009805	0,68	0,0089	CASP8 & FADD-like apoptosis regulator isoform 1 & 2	proteolysis and peptidolysis
Ndst1	NM_008306	0,67	0,0079	N-deacetylase / N-sulfotransferase 1	proteolysis and peptidolysis
Ube3a	NM_011668	0,65	0,0043	ubiquitin protein ligase E3A isoform 1 & 2	protein modification; ubiquitin-dependent protein catabolism
Pep4	NM_008820	0,65	0,0101	peptidase 4	collagen catabolism; proteolysis and peptidolysis
Spc18	NM_019951	0,63	0,0090	Sec11-like 1	proteolysis and peptidolysis
Pten	NM_008960	0,61	0,0050	phosphatase and tensin homolog	protein amino acid dephosphorylation
Ubl3	NM_011908	0,61	0,0081	ubiquitin-like 3	protein modification
AK083534	AK083534	0,57	0,0080	metalloprotease	proteolysis and peptidolysis
Casp12	NM_009808	0,57	0,0055	caspase 12	proteolysis and peptidolysis
Proz	NM_025834	0,56	0,0077	protein Z	proteolysis and peptidolysis
Ptpn8	NM_008979	0,54	0,0024	protein tyrosine phosphatase	protein amino acid dephosphorylation
Tpp2	NM_009418	0,54	0,0086	tripeptidyl peptidase II	proteolysis and peptidolysis
BC057997	BC057997	0,53	0,0048	proteasome 26S ATPase subunit 6	protein catabolism
AK082233	AK082233	0,51	0,0055		protein modification
Ube1c	NM_011666	0,50	0,0061	ubiquitin-activating enzyme E1C	protein modification
Cpa3	NM_007753	0,44	0,0015	carboxypeptidase A3	proteolysis and peptidolysis
AK078301	AK078301	0,44	0,0096	dipeptidylpeptidase 9	proteolysis and peptidolysis
Yme1l1	NM_013771	0,43	0,0044	YME1-like 1	protein catabolism; proteolysis and peptidolysis