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Microarray Analysis of the Gene Expression Profile in Diethylnitrosamine-induced Liver Tumors in Mice

Eun-Soo Jung[1], Jung-Duck Park[2], and Doug-Young Ryu[1,*]
[1]*College of Veterinary Medicine, Seoul National University, Seoul, 151-742, Korea*
[2]*College of Medicine, Chung-Ang University, Seoul 156-756 Korea*

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ABSTRACT : Liver cancer is a leading cause of tumor-related mortality. Diethylnitrosamine (DEN) is one of the most extensively studied hepatic carcinogens to date. In this study, the mRNA expression profile in DEN-induced liver tumors in mice was analyzed using DNA microarrays. We report increased expression of genes that participate in hypoxia response, including metallothionein 1 (*Mt1*), metallothionein 2 (*Mt2*), fatty acid synthase (*Fasn*), transferrin (*Trf*), adipose differentiation-related protein (*Adfp*) and ceruloplasmin (*Cp*), as well as those involved in predisposition and development of cancers, such as cytochrome P450 2A5 (*Cyp2a5*), alpha 2-HS-glycoprotein (*Ahsg*) and Jun-B oncogene (*Junb*). The hepatic iron regulatory peptide, hepcidin (*Hamp1*), was downregulated in DEN-stimulated liver tumors. Expression of tumor suppressor genes, such as tripartite motif protein 13 (*Trim13*), was decreased under these conditions. The data collectively indicate that DEN-induced tumor development can be exploited as a possible model for liver cancer, since this process involves various genes with important functions in hepatic carcinogenesis.

Key words : **microarray, diethylnitrosamine, liver, mouse**

Introduction

Liver cancer is one of the leading causes of tumor-related death. Viral hepatitis, alcohol liver disease, and carcinogen exposure are major factors that trigger this disorder. Rat and mouse models are generally used to investigate carcinogen-induced liver tumors. While the hepatic carcinogen, diethylnitrosamine (DEN), has been extensively studied (Bai *et al.*, 2005), its molecular mechanisms of action are yet to be elucidated.

DEN is an alkylating genotoxic agent that induces liver tumors with no requirement for promoting agents, particularly in young mice. It is likely that the alterations accumulated in the genome lead to cellular transformation and tumor development. Numerous foci appear within a few months after a single DEN injection at the neonatal stage, followed by development of tumor masses by 5 to 12 months. The focus size, but not number, increases in a time-dependent manner in DEN-treated mice (Goldsworthy and Fransson-Steen, 2002).

Differential susceptibility to hepatocarcinogenesis is evident among various strains of mice, and young and adult animals (Diwan *et al.*, 1986; Drinkwater and Ginsler, 1986). The C3H/He strain displays high sensitivity to DEN-induced liver tumors, whereas C57BL/6 is comparatively resistant. Strain-specific liver tumor susceptibility is possibly controlled by intrinsic genetic differences related to growth regulation of initiated hepatocytes (Drinkwater and Ginsler, 1986).

The effects of different genes with functional importance in the predisposition and development of DEN-induced hepatocarcinogenesis have been analyzed in various genetically modified mouse strains. Mice overexpressing c-myc or TGF-alpha in the liver developed spontaneous tumors, and were more susceptible to DEN-induced hepatocarcinogenesis (Fausto, 1999). On the other hand, deletion of cyclin G1 and c-Jun decreased DEN-induced liver carcinogenesis, supporting their role in tumor development (Eferl *et al.*, 2003; Jensen *et al.*, 2003).

In the current study, we analyze gene expression profiles in DEN-induced mouse liver tumors, using DNA microarray, to extend our understanding of the molecular mechanisms of this process.

*To whom all correspondence should be addressed.

Materials and Methods

Mice

Male offsprings were obtained from mating of 8-week-old C3H/He male and female mice (Samtako, Osan, Korea). Twelve-day-old pups were intraperitoneally administered DEN dissolved in sterile phosphate-buffered saline at a dose of 20 ug/kg of body weight. Pups were weaned at 3 weeks of age, and allowed unlimited access to lab chow (Purina 5057, Purina, St Louis, MO) and sterilized water until sacrifice at 6 to 8 months. The Animal Welfare Committee of Seoul National University approved all procedures dealing with the handling of animals described in this study.

Evaluation of tumor growth

Livers were removed, and lobes separated after sacrificing mice. Tumor nodules more than 2 mm in diameter were counted by digital enlargement of captured images. Lumps smaller than 2 mm were excluded, since they could not be identified with certainty as nodules. Liver lesions were scored according to the following scheme: 0, no detectable gross lesion (no nodule); 1, several nodules (1~10 nodules); 2, large number of small nodules (more than 10); 3, multiple small and large nodules; 4, multiple large nodules and grossly necrotic liver. All liver lobes were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin (H&E).

Microarray analysis

Total RNA was extracted from frozen liver tissues using TRIzol reagent, following the manufacturer's protocol (Sigma, St. Louis, MO). RNA extracted from control mouse liver was used as the reference, and labeled with Cyanine-3-dUTP. DEN-induced liver tumor RNA was labeled with Cyanine-5-dUTP (DeRisi *et al.*, 1997). Labeled cDNA samples were mixed and purified using Microcon YM-30 columns (Amicon, Bedford, MA), and prepared for hybridization by adding buffer (final 50% formamide, 4.1× Denhardts, 4.4X SSC, 100 ug/ml herring sperm DNA). Hybridization procedures were performed in a total volume of 50 ul under hybrid slips (Grace Biolabs, Bend, OR) at 42°C for 16 h in the HybChamber (Gene Machines, San Carlos, CA). Hybrid slips were gently removed in 4× SSC washing buffer, and arrays washed by immersion into 1× SSC, 0.1% SDS for 2 min, 0.1× SSC, 0.1% SDS twice for 2 min, and 0.1× SSC twice for 1 min. Fluorescence signals on the array were scanned with GenePix 4000B (Molecular Devices, Sunnyvale, CA), and analyzed using GenePix Pro 5.0 array analysis software (Molecular Devices, Sunnyvale, CA) and BRB-Array Tools (http://linus.nci.nih.gov/).

Real-time RT-PCR

PCR oligonucleotides were designed using Primer Express (ver 2.0, Applied Biosystems, Foster City, CA), based on mRNA sequences from GenBank (Table 1). Reverse transcription for cDNA synthesis was performed in a volume of 25 ul with 2 ug of total RNA, MuLV reverse transcriptase, and random hexamers (Promega, Madison, WI). Real-time PCR was performed using 0.4 ul cDNA, gene-specific primer pairs, and the ABI SYBR Green PCR Master Mix (Applied Biosystems) in an ABI Prism 7000 sequence detection system. The following conditions were employed: (i) denaturation (10 min at 95°C); (ii) 40 cycles of amplification (15 s at 95°C, 1 min at 60°C); (iii) melting curve (60-99°C with a heating rate of 0.1°C per s, and a continuous fluorescence measurement). Serial dilutions of cDNA were prepared to generate a standard curve from cycle thresholds (CTs), allowing for comparisons between samples. A standard curve of CTs for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control.

Statistical Analysis

The significance of data was assessed using the Student *t*-test (two-tailed). Values of $p < 0.05$ and $p <$

Table 1. Real-time RT-PCR oligonucleotides

Gene Abbreviation	Accession Number	Primer Sequence (Forward/Reverse)
Hamp	NM_032541	5'-GGCAGACATTGCGATACCAA-3' / 5'-TGGCTCTAGGCTATGTTTTGCA-3'
Mt1	NM_013602	5'-GCTGTGCCTGATGTGACGAA-3' / 5'-AGGAAGACGCTGGGTTGGT-3'
Cp	NM_007752	5'-AGGAGTATGAGGGAGCCGTCTA-3' / 5'-CCGGGAAGCACTTTGTCATC-3'
Trf	NM_133977	5'-TCACTGCCATTCGGAATCAG-3' / 5'-CCACTTCACTGGCGAGTTGTC-3'

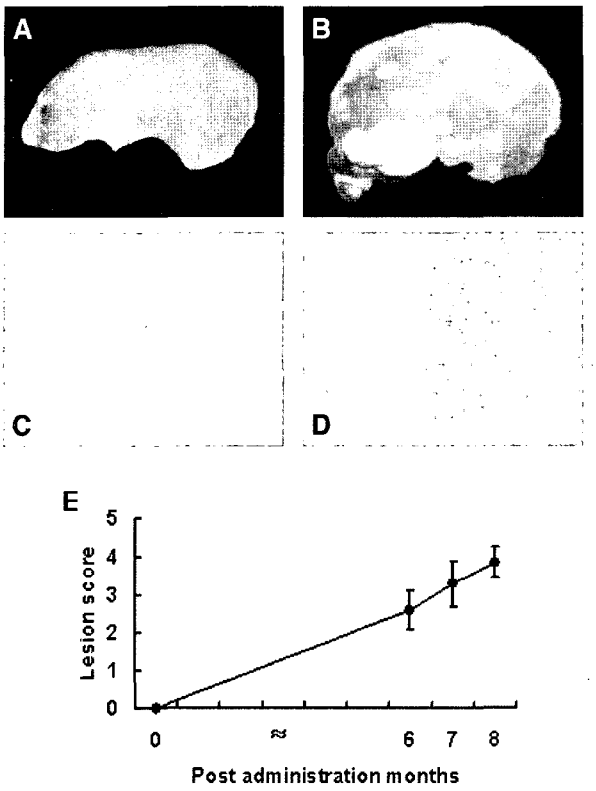

Fig. 1. Evaluation of liver tumor growth 6 to 8 months after DEN treatment of 12-day-old male mice.
Macroscopic view of livers from (A) control and (B) DEN-treated mice, and histological examination of liver sections in (C) control (100×, H&E) and (D) DEN-treated mice (100×, H&E). (E) Macroscopic lesion score of liver tumors in mice treated with DEN.

0.01 were considered statistically significant. All statistical analyses were performed using SigmaPlot (Systat, Richmond, CA).

Results

The incidence of neoplastic nodules was evaluated in DEN-treated mouse livers. Macroscopic lumps more than 2 mm in diameter on the surface of each lobe were counted (Fig. 1). Histological examination of DEN-induced liver tumors revealed loss of the normal architecture of liver parenchyma, as well as typical changes of hepatocyte adenoma and carcinoma, analogous to previous reports (Goldsworthy and Fransson-Steen, 2002; Boissan *et al.*, 2005).

Among the 30 annotated mRNA species downregulated more than two-fold in DEN-induced liver tumors compared to control liver (Table 2), 15 were expressed preferentially, but not exclusively, in liver. These include CYP7B1 (Schwarz *et al.*, 1997), Slco1a1 (Oatp1 and Slc21a1) (Hagenbuch *et al.*, 2000), Slco1b2 (OATP2, OATP-C, or LST-1)(Ogura *et al.*, 2000), Aox1 (Kurosaki *et al.*, 1999), Es-31 (Aida *et al.*, 1993), CYP1A2, Mup3 (Shahan *et al.*, 1987), C4 (Miyagoe *et al.*, 1994), regucalin (Murata and Yamaguchi, 1997), Serpina3k (Salier *et al.*, 1993), CYP2C50 (Wang *et al.*, 2004a), Mgst1 (Kelner *et al.*, 2004), carboxylesterase 3 (Dolinsky *et al.*, 2001), CYP2D10 (Wong *et al.*, 1989), and Ugt1a6a (Vasiliou *et al.*, 1997). Among the transcripts upregulated more than 2-fold in DEN-induced tumors (Table 3), Apoa4 (Reue *et al.*, 1993), Fasn (Paulauskis and Sul, 1988), CYP2A5 (Ulvila *et al.*, 2004), Trf (Chen and Bissell, 1987), Ahsg (Schinke *et al.*, 1996), ATF5 (Hansen *et al.*, 2002), and Cp (Klomp *et al.*, 1996) were expressed preferentially in the liver.

Several genes, including *Mt1, Mt2, Fasn, Trf, Adfp* and *Cp*, encoding proteins that have roles against hypoxia, were transcriptionally induced in DEN-induced liver tumors, compared to control livers. These results suggest that DEN-stimulated liver tumor tissues undergo hypoxia, and thus adapt accordingly, as observed for various other diseases, including human cancers. Mt1 and Mt2 are induced in response to hypoxia and oxidative stress (Wang *et al.*, 2004b). Fasn provides a cellular proliferative advantage under conditions of hypoxia, since the FAS pathway supplies oxidizing power for oxidative steps (Hochachka *et al.*, 2002; Baron *et al.*, 2004; Menendez *et al.*, 2005). Cells adapt to hypoxia-related conditions, such as heart disease and tumorigenesis, by stimulating the expression of genes involved in iron homeostasis, including *Trf* (Rolfs *et al.*, 1997) and *Cp* (Mukhopadhyay *et al.*, 2000). Additionally, *Adfp* plays a potential role in adaptation to hypoxia and tumorigenesis (Saarikoski *et al.*, 2002). On the other hand, regucalcin, a protein that has a protective role in conditions of cell injury, such as apoptosis and hypoxia (Ishigami *et al.*, 2002), is downregulated in DEN-induced liver tumors.

A number of other genes with functional importance in the predisposition and development of human cancers and animal models of cancer were additionally up- or downregulated in DEN-induced liver tumors. *Trim13* (also known as RFP2 and LEU5), a tumor suppressor candidate in human and mouse (Kapanadze

Table 2. List of genes downregulated 8 months after DEN treatment of 12-day-old male mice. Genes downregulated at least 2.0-fold are included. The results represent normalized average ratios of three separate and independent experiments.

Fold Change ± S.E.	Accession Number	Gene Identity	Abbreviation	Chromosome Location
- 7.31 ± 1.59	NM_008649	major urinary protein 5	Mup5	4
- 4.44 ± 0.38	NM_007825	cytochrome P450, family 7, subfamily b, polypeptide 1	Cyp7b1	3
- 4.3 ± 0.5	NM_032541	hepcidin antimicrobial peptide 1	Hamp1	7
- 4.06 ± 1.03	NM_013797	solute carrier organic anion transporter family, member 1a1	Slco1a1	6
- 3.17 ± 1.25	NM_008648	major urinary protein 4	Mup4	4
- 2.94 ± 1.05	NM_020495	solute carrier organic anion transporter family, member 1b2	Slco1b2	6
- 2.91 ± 0.31	NM_201239	ribonuclease, RNase A family 4	Rnase4	14
- 2.86 ± 0.51	NM_013855	ATP-binding cassette, sub-family A (ABC1), member 3	Abca3	17
- 2.84 ± 0.48	NM_011675	uridine-cytidine kinase 1	Uck1	2
- 2.77 ± 0.52	NM_024264	cytochrome P450, family 27, subfamily a, polypeptide 1	Cyp27a1	1
- 2.7 ± 0.34	NM_009676	aldehyde oxidase 1	Aox1	1
- 2.69 ± 0.42	NM_010001	cytochrome P450, family 2. subfamily c, polypeptide 37	Cyp2c37	19
- 2.68 ± 0.42	NM_144511	esterase 31-like	Es31	8
- 2.65 ± 0.35	NM_009993	cytochrome P450, family 1, subfamily a, polypeptide 2	Cyp1a2	9
- 2.57 ± 0.35	NM_011413	sex-limited protein	Slp	17
- 2.51 ± 0.52	NM_053200	carboxylesterase 3	Ces3	8
- 2.45 ± 0.28	NM_010845	major urinary protein 3	Mup3	4
- 2.44 ± 0.41	NM_009780	complement component 4 (within H-2S)	C4	17
- 2.39 ± 0.2	AK004666	potassium voltage-gated channel, shaker-related subfamily, b1	Kcnab1	3
- 2.36 ± 0.39	NM_198927	submandibular gland protein C	Smgc	15
- 2.34 ± 0.3	NM_023233	tripartite motif protein 13	Trim13	14
- 2.32 ± 0.24	NM_009060	Regucalcin	Rgn	X
- 2.31 ± 0.26	NM_145079	UDP glucuronosyltransferase 1 family, polypeptide A6A	Ugt1a6a	1
- 2.3 ± 0.28	NM_011458	serine (or cysteine) peptidase inhibitor, clade A, member 3K	Serpina3k	12
- 2.29 ± 0.21	NM_017382	RAB11a, member RAS oncogene family	Rab11a	9
- 2.28 ± 0.19	NM_134144	cytochrome P450, family 2, subfamily c, polypeptide 50	Cyp2c50	19
- 2.16 ± 0.14	NM_013541	glutathione S-transferase, pi 1	Gstp1	19
- 2.16 ± 0.13	NM_019946	microsomal glutathione S-transferase 1	Mgst1	6
- 2.12 ± 0.11	XM_283480	RIKEN cDNA A230048G03 gene		
- 2.12 ± 0.15	NM_019688	Rap guanine nucleotide exchange factor (GEF) 4	Rapgef4	2
- 2.1 ± 0.11	NM_010005	cytochrome P450, family 2, subfamily d, polypeptide 10	Cyp2d10	15

et al., 1998; Baranova *et al.*, 2003; van Everdink *et al.*, 2003) was downregulated in DEN-induced tumors. *Trim13* is conserved in human and mouse at the genomic sequence level. Specifically, in humans, TRIM13 is located at the 13q14.3 locus that is frequently deleted in various malignancies (Kapanadze *et al.*, 2000; Corcoran *et al.*, 2004). Transcripts for the antioxidant enzymes, GSTP1 and MGST1, were also downregulated. On the other hand, high levels of Apoa4 with antioxidant-like activity (Ferretti *et al.*, 2002) were detected in DEN-induced tumors.

We observed upregulation of CYP2A5 in DEN-induced tumors. CYP2A5 and its human ortholog, CYP2A6, are elevated in liver tumors in mice (Wastl *et al.*, 1998; Tetri *et al.*, 2002) and humans (Raunio *et al.*, 1998). Ahsg (also known as fetuin-A) is a growth promoter in serum that influences tumor establishment and growth (Leite-Browning *et al.*, 2004; Kundranda *et al.*, 2005). Ahsg mRNA was highly expressed in DEN-induced tumors. On the other hand, the protein suppresses

Table 3. List of genes upregulated 8 months after DEN treatment of 12-day-old male mice. Genes upregulated at least 2.0-fold are included. The results represent normalized average ratios of three separate and independent experiments.

Fold Change ± S.E.	Accession Number	Gene Identity	Abbreviation	Chromosome Location
7.85 ± 0.75	NM_009242	secreted acidic cysteine rich glycoprotein	Sparc	11
6.59 ± 3.03	NM_007468	apolipoprotein A-IV	Apoa4	9
4.36 ± 1.17	NM_008630	metallothionein 2	Mt2	8
4.27 ± 2.03	NM_013602	metallothionein 1	Mt1	8
3.75 ± 0.15	NM_007988	fatty acid synthase	Fasn	11
3.61 ± 0.78	NM_007812	cytochrome P450, family 2, subfamily a, polypeptide 5	Cyp2a5	7
3.59 ± 1.1	XM_205565	RIKEN cDNA 4930526H21 gene		5
3.26 ± 1.09	AK028479	poliovirus receptor-related 4	Pvrl4	1
3.17 ± 0.65	NM_027733	RIKEN cDNA 5133400G04 gene		18
2.95 ± 0.68	XM_130789	RIKEN cDNA 1700003N22 gene		
2.87 ± 0.89	NM_026065	DNA segment, Chr 10, ERATO Doi 322, expressed		10
2.68 ± 0.64	NM_007752	Ceruloplasmin	Cp	3
2.63 ± 0.46	NM_013465	alpha-2-HS-glycoprotein	Ahsg	16
2.58 ± 0.56	XM_486083	kelch repeat and BTB (POZ) domain containing 11	Kbtbd11	8
2.54 ± 0.71	NM_008583	multiple endocrine neoplasia 1	Men1	19
2.52 ± 0.29		RIKEN cDNA 1200015M12 gene		
2.49 ± 0.51	NM_008416	Jun-B oncogene	Junb	8
2.48 ± 0.54	NM_007712	CDC-like kinase 2	Clk2	3
2.46 ± 0.28	NM_011767	zinc finger RNA binding protein	Zfr	15
2.46 ± 0.44		RIKEN cDNA E430024C06 gene		
2.38 ± 0.41	NM_009565	Vzinc finger and BTB domain containing 7B	Zbtb7b	3
2.33 ± 0.35	XM_358398	RIKEN cDNA A230066D03 gene		
2.32 ± 0.18	NM_053086	nucleolar and coiled-body phosphoprotein 1	Nolc1	19
2.32 ± 0.21	NM_133977	Transferrin	Trf	9
2.28 ± 0.29	NM_030693	activating transcription factor 5	Atf5	7
2.24 ± 0.18	NM_007408	adipose differentiation related protein	Adfp	4
2.04 ± 0.06	NM_175511	RIKEN cDNA A130092J06 gene		2
2.03 ± 0.06	NM_177640	RIKEN cDNA D030056L22 gene		19

Table 4. Real-time RT-PCR analysis on selected genes. Selected genes displaying 2.0-fold or greater expression at 8 months in livers of DEN-treated mice, compared to control mice, were independently examined by real-time RT-PCR, and compared with microarray data shown in Tables 1 and 2.

Gene Abbreviation	Microarray (Fold Change ± S.E., n = 3)	Real time RT-PCR (Fold Change ± S.E., n = 5)
Hamp	- 4.30 ± 0.50	- 4.56 ± 1.18
Mt1	4.27 ± 2.03	4.92 ± 2.67
Cp	2.68 ± 0.64	2.05 ± 0.06
Trf	2.32 ± 0.21	2.57 ± 0.18

TGF-beta-dependent signaling, and thus inhibits tumor progression driven by this growth factor (Swallow *et al.*, 2004). An activating protein-1 transcription factor, Jun-B, important in the control of cell growth, differentiation and neoplastic transformation, is upregulated in DEN-induced liver tumors. Mouse models of under- or

overexpression support the tumor suppressor effect of Men1, which is also significantly elevated in these tumors (Agarwal *et al.*, 2005).

Real-time RT-PCR was employed as an independent method to confirm the expression changes of a selected set of genes identified by microarray. We selected several genes associated with iron homeostasis for confirmation analysis. The trend (increase or decrease) of expression changes detected with microarray analysis was confirmed by real-time RT-PCR (Table 4). The magnitude of the changes obtained with microarrays was consistent with real-time RT-PCR data for Hamp, Mt1, Cp and Trf.

Discussion

DEN is a DNA-alkylating mutagen and potential liver carcinogen. In this study, liver tumors were observed in C3H male mice after a single neonatal dose of DEN, consistent with earlier analyses (Goldsworthy and Fransson-Steen, 2002; Boissan *et al.*, 2005), indicating that damage by the carcinogen is sufficiently severe to induce cellular transformation of hepatocytes.

Several researchers have investigated whether the differences in mouse susceptibility to hepatocarcinogens are at the level of initiation, promotion or progression of liver tumor development (Drinkwater and Ginsler, 1986; Hanigan *et al.*, 1988; Lee *et al.*, 1989; Hanigan *et al.*, 1990; Lee *et al.*, 1991; Pugh and Goldfarb, 1992; Goldsworthy and Fransson-Steen, 2002). Strain-specific liver tumor susceptibility is genetically controlled by intrinsic differences related to growth regulation of initiated hepatocytes. Multiple lines of evidence suggest that this susceptibility is dependent on distinct genes. In support of this theory, a time-dependent increase in focus size, but not number, was evident in liver tissues of DEN-treated mice (Goldsworthy and Fransson-Steen, 2002). Strain-dependent increases in incidence, number, volume fraction and size of foci and masses were additionally observed (Goldsworthy and Fransson-Steen, 2002).

Oncogene activation and tumor suppressor inactivation result in deregulated cellular proliferation. A variety of genes are regulated in DEN-induced liver tumors, but not in control liver samples (Tables 2 and 3), reflecting the direct effects of this carcinogenic compound. It is assumed that neonatal treatment with DEN causes unknown genetic and epigenetic changes in rapidly proliferating neonatal hepatocytes. Other groups of genes regulated in DEN-induced liver cancers include those influenced by the local cellular environment during tumor development. Regulation of genes in this group is not specific for DEN-induced liver tumors, but may be common to most types of solid tumors. Most tumors larger than 1 mm^3 contain regions of hypoxia due to an imbalance between oxygen supply and consumption (Michiels, 2004). In DEN-induced liver tumors, genes that function in hypoxia response, such as *Mt1, Mt2, Fasn, Trf, Adfp* and *Cp*, are upregulated, consistent with findings in various human and animal models of cancer (Rolfs *et al.*, 1997; Hochachka *et al.*, 2002; Saarikoski *et al.*, 2002; Baron *et al.*, 2004; Wang *et al.*, 2004b; Menendez *et al.*, 2005).

As a central organ in iron distribution and homeostasis, the liver responds to iron deficiency anemia and hypoxia, by increasing both iron release from reticuloendothelial cells and intestinal iron absorption (Pietrangelo and Trautwein, 2004). An iron-deficient diet triggers a considerable decrease in the level of mouse hepcidin mRNA. In addition, hypoxia reduces hepcidin mRNA *ex vivo* in human hepatoma cells and *in vivo* in mice housed in hypobaric hypoxia chambers. Hepcidin acts by inhibiting the efflux of iron through MTP1, the sole known iron exporter of enterocytes, macrophages and hepatocytes (Ganz, 2005). Hepcidin expression was downregulated in DEN-induced liver tumors (Tables 2 and 4), indicative of anemia and/or hypoxia. Since transformed hepatocytes undergo hypoxia due to high oxygen demand caused by local tumor development, it is possible that Hamp1 downregulation is not specific for cancer induced by DEN, but common in all liver tumors.

While gene expression studies yield data on modifications in gene levels that may not mean a net biological change, profiles obtained with this method provide information on a section of overall cellular response to an altered environment. The profile of DEN-induced liver tumors discloses that various genes of functional importance in liver cancers are regulated in this process. Accordingly, DEN-induced tumors may be employed as a model for liver cancers.

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