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NON-MAMMALIAN *FAT-1* GENE PREVENTS NEOPLASIA WHEN INTRODUCED TO A MOUSE HEPATOCARCINOGENESIS MODEL

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Abstract

We investigated the effect of a non-mammalian omega-3 desaturase in a mouse hepatocarcinogenesis model. Mice containing double mutations (DM) in *c-myc* and *TGF-α* (transforming growth factor- α), leading to liver neoplasia, were crossed with mice containing omega-3 desaturase. MRI analysis of triple mutant (TM) mice showed the absence of neoplasia at all time points for 92% of mice in the study. Pathological changes of TM (*TGFα/c-myc/fat-1*) mouse liver tissue was similar to control mouse liver tissue. Magnetic resonance spectroscopy (MRS) measurements of unsaturated fatty acids found a significant difference ($p < 0.005$) between DM and TM transgenic (Tg) mice at 34 and 40 weeks of age. HPLC analysis of mouse liver tissue revealed markedly decreased levels of omega-6 fatty acids in TM mice when compared to DM (*TGFα/c-myc*) and control (CD1) mice. Mass spectrometry (MS) analysis indicated significantly decreased 16:0/20:4 and 18:1/20:4 and elevated 16:0/22:6 fatty acyl groups in both GPCho and GPEtn, and elevated 16:0/20:5, 18:0/18:2, 18:0/18:1 and 18:0/22:6 in GPCho, within TM mice compared to DM mice. Total fatty acid analysis indicated a significant decrease in 18:1n9 in TM mice compared to DM mice. Western blot analysis of liver tissue showed a significant ($p < 0.05$) decrease in NF- κ B (nuclear factor- κ B) levels at 40 weeks of age in TM mice compared to DM mice. Microarray analysis of TM versus DM mice livers at 40 weeks revealed alterations in genes involved in cell cycle regulation, cell-to-cell signaling, p53 signaling, and arachidonic acid (20:4) metabolism. Endogenous omega-3 fatty acids were found to prevent HCC development in mice.

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Keywords

Omega-3 fatty acids; liver cancer; HCC; Fat-1; TGF- α /c-myc; transgenic mouse model

1 Introduction

The World Health Organization reports that liver cancer is the third highest cause of death from cancer, with hepatocellular carcinoma (HCC) being predominantly observed in Asian and African countries [1]. Annual incidence rates of HCC for these countries are around 150 per 100,000 people [2]. HCC accounts for 80% of all primary liver cancers [1]. Epidemiologic studies show that primary liver cancer is the most visceral malignant tumor (350,000 cases per year) and in some countries is the most common cancer overall [2].

The liver is a key organ for the metabolism of free fatty acids [3]. The synthesis of fatty acids leads to products that are intrinsically involved in cellular processes. Some of the processes that fatty acids are involved in within the cell are apoptosis and cell cycle regulation [4,5]. Fatty acid metabolism results in the production of prostaglandins and leukotrienes which are important regulators of cellular growth and proliferation [4,6]. Arachidonic acid (AA) was shown to induce 11 genes that are regulated by nuclear factor- κ B (NF- κ B) including, *COX-2*, *IL-6*, and *TNF- α* , and it is also thought that AA, through PGE₂, could stimulate growth-related genes and proliferation through PI3K signaling and NF- κ B translocation to the nucleus [7].

Previous research has shown a correlation between alterations in omega-6 fatty acids and the onset and development of HCC [6,9]. Recent insight into the benefits of omega-3 fatty acids in rodent models, with some emphasis on cancer, has created a need for further study of these metabolites [8–11]. Epidemiologic studies of persons with a diet rich in omega-3 fatty acids have shown that these persons are less prone to many cancer risks [12].

The *fat-1* gene encodes an n-3 desaturase that introduces a double bond at the n-3 position of the hydrocarbon chain in n-6 fatty acids to form an n-3 fatty acid [9,10]. The *fat-1* gene is not found in mammalian cells. The gene can be found in *C. elegans* and other life forms, such as plants [13]. This gene was used to generate a transgenic mouse model to study the effects of n-3 fatty acids [9,10]. Subsequent studies utilizing this transgenic mouse model has revealed several benefits of n-3 fatty acids. One study of inflammatory responses in the colons of these transgenic mice, following dextrane sodium sulphate (DSS) administration, found a down-regulation of pro-inflammatory factors and cytokines, such as NF κ B, TNF α , iNOS and IL-1 β [8]. A study utilizing a liver acute inflammatory model investigated the benefits of the *fat-1* gene. This study found less severe inflammatory injury and histologically apparent damage as well as reduced hepatic gene expression of pro-inflammatory cytokines (TNF α , IL-1 β , IFN- γ and IL-6) and reduced apoptosis in hepatocytes [11].

MRS has been used previously as a tool to measure alterations in the degree of unsaturation of fatty acids *in vitro* that correlate with liver neoplasia. Alterations in hepatic phospholipids utilizing MRS has been extensively studied [14–20]. It has been previously established using single-voxel MRS, that changes in lipid profiles of tumor tissue during stages of development are observable with proton MRS [21]. Alterations in methyl and methylene hydrogens from lipid resonances were noted using MRS [21]. Increases in unsaturated methylene hydrogens in PUFA at 2.8 ppm and increases in unsaturated lipid olefinic hydrogens at 5.4 ppm as the disease state progressed were also noted [21].

We hypothesized that by introducing an endogenous source of omega-3 fatty acids in a mouse hepatocarcinogenesis model (TGF α /c-myc), we could reduce the occurrence or volume of tumors. In this study, we have crossed transgenic (Tg) mice that have the *fat-1* gene knocked-in, with the double mutant (DM) (TGF α /c-myc) mouse model of HCC to form a triple mutant (TM) Tg mouse model.

2 Material and Methods

2.1 Animal Models

TM mice expressing mutations in TGF- α and c-myc genes combined with a knock-in of the Fat-1 gene were the primary experimental model used in this study. The DM Tg mice develop a primary form of liver cancer in a similar fashion to what has been observed in humans [22,23]. The knock-in Fat-1 strain (C57BL6 background) has been shown to convert omega6 to omega3 fatty acids *in vivo* [9,10]. The DM Tg TGF α (CD1 background)/c-myc (C57BL/6JxCBA/J background) [22] mice were bred with Fat-1 mice to produce a TM mouse model. Genotyping was utilized in order to confirm the presence of the *fat-1* transgene. Mice toes were cut at weaning (3weeks of age), 50ul of toe lysis buffer was added, tubes were incubated at 37° C overnight, the next day tubes were heated to 100° C for 10 min to inactivate Proteinase K, vortexed, and centrifuged for 3 min at 12K rpm. Fat-1 primers: Fat1-f TGTTTCATGCCTTCTTCTTTTCC, and Fat1-r GCGACCATACTCAAACCTTGA. TGF- α primers: TGF α -f AGTTCTGCTTCCATGCAACC, and TGF α -r TGATGATAAGGACAGCCAGG. c-myc primers: cmyc-f ACAGCAGCTCGCCCAAATCC, and cmyc-r GGGCTGGAGCACTTGCGG.

CD1 mice were used as non-Tg controls. During MRI analysis, animals were anesthetized (2–3% Isoflurane, 100% O₂). Respiration was monitored while animals were being scanned using a respiration pillow and SAM PC gating software (SAI Instruments, Manchester, England). Mice were administered a standard laboratory rodent diet which contained 1.22% linoleic acid, 0.1% linolenic acid, <0.01% arachidonic acid, 0.19% omega-3 fatty acids, and 1.60% total monounsaturated fatty acids (LabDiet, Hiland, TX). Mice were euthanized via CO₂ gas asphyxiation. Liver tissue was immediately removed from mice upon euthanasia. Liver tissue sections were taken at time of sacrifice and sent for histologic preparation (H&E stains). Experiments were carried out in conformity with the PHS policy and with Oklahoma Medical Research Foundation's Institutional Animal Care and Use Committee (IACUC) approval.

2.2 MRI

MRI and MRS data were collected on a Bruker 300MHz (7T) small animal horizontal bore spectrometer (Ettlingen, Germany). Animals (n=20 DM, n=12 TM, n=6 controls) were placed at the magnet isocenter, using the base of the sternum for anatomical positioning. A gradient echo fast low angle shot (FLASH) scan was utilized to obtain multi-slice images, as previously described [24]. Tumor volumes were measured utilizing *ImageJ* and means $\pm$ S.D. were obtained. Briefly, images were loaded into *ImageJ* software by defining the pixel range as 256 $\times$ 256. Once the MRI images were loaded into *ImageJ*, they were rescaled to the FOV (field-of-view) used in the study (38.4 $\times$ 38.4 mm). Then, utilizing a freehand selection tool, the tumor volumes were outlined and the area of the tumor was measured for every slice taken that contained a nodular mass. The tumor volumes from each slice were summed for each animal at each time point. A non-biased blind study of tumor volumes was also performed on DM Tg mouse MRI data sets in order to confirm tumor volume growth in DM mice over time. Animals were assessed *in vivo* beginning at week 20 of age, then following at weeks 26, 30, 34 and 40 weeks before they were sacrificed.

2.3 MRS

MRS studies were conducted at the same time as MRI data acquisition on animals at all time points. Image-guided ^1H -MRS data were obtained as previously described [24]. Briefly, a spin echo chemical shift imaging (CSI) scan with a TE of 3.5 ms, a TR of 1500 ms, CHESS (chemical shift selective imaging) water suppression, a $38.4\text{ mm} \times 38.4\text{ mm}$ field of view, and an acquisition time of 30 minutes, was used. A Gaussian excitation pulse of 1.021 ms and a refocusing pulse with twice the duration were applied. A total of 1024 scans were acquired. The spatial matrix was 32×32 voxels, reconstructed to 64×64 voxels containing spectra with 2048 complex points. The slice was 1.5 mm thick and located over the mid liver region. Animals were anesthetized using 2–3% Isoflurane and 100% oxygen during collection of all MR images and spectra. Respiration was constantly monitored using a respiration pillow and SAM PC gating software (SAI Instruments, Manchester, England) while the animals were being scanned. Respiratory gating (SAI Instruments) was used for all MR data acquisition.

2.4 Processing of MRS data

Spatially encoded chemical shift spectra were produced by 3D Fourier transform of the acquired free induction decays with *Paravision* software (Bruker, Ettlingen, Germany). These were saved as magnitude spectra (i.e. the square root of sum of squares of the real and imaginary components of the Fourier transformed spectra). Prior to Fourier transform the spatial dimension were zero-filled once to twice their collected value. Thus the CSI data used for analysis was composed of 64×64 spatial voxels totaling to 4096 individual spectra, from a mid-liver slice predominantly containing liver tissue, for each animal at each time point. Each spectrum contains 2048 data points, 4006 Hz (13.3 ppm) leading to a total of 8.3×10^6 points of data per liver dataset. This large data set would be difficult to process without the ability to filter through the data and isolate individual spectra of interest. A *Mathematica* notebook was written, which can upload, process, filter, and isolate single spectra from the MRS data.

The procedures below have been described previously for single high resolution spectra [25]. Briefly the plot of voxels in the CSI data set were parameterized from a discrete set of points to the more commonly used ppm scale, (i.e. (x, y) pairs of ppm, magnitude). The central point was assigned 4.78 ppm. A Savitzky-Golay filter [25,26] was applied to denoise the spectra. An interpolating polynomial of the spectra was constructed (built in *Mathematica* function) and the area under the bis-allyl and vinyl peaks at 2.8 and 5.3 ppm were determined by analytical integration of the interpolating polynomial with appropriate integration bounds as enunciated in [25]. The ratio R , which is the ratio of areas determined as described above of the bis-allyl ($-\text{CH}_2-\text{CH}_2-\text{CH}=\text{}$) and vinyl ($-\text{CH}=\text{CH}-$) hydrogen atoms of fatty acids was determined. Batch processing of the data is feasible using the set of procedures described above, and allows a hands-free operation of the data. The ratio R may also be converted to number of double bonds, $n_{\text{bonds}} = -1/(R - 1)$. In our case, R values were correlated with corresponding MRI anatomical images, i.e. the spectra used to produce the R values were taken from a similar MR image ROI at each time point. This allowed the plotting of low R values that corresponded with neoplastic or tumor formations as a function of time. The ROIs were determined by using MRI images of the DM mouse livers at stages of visually apparent neoplastic or tumor growth, and then backtracking through the time points, selecting ROIs in the animals at each time until we were selecting similar ROIs at earlier time points with no visual indication of neoplastic formations in the MRI images. Data values are displayed as mean $\pm$ S.D. One-way ANOVA (Graph Pad Prism version 5, GraphPad Software, Inc. La Jolla, CA) using a Kruskal-Wallis test (non-parametric) was used to evaluate differences ($p < 0.05$) between control and DM and TM Tg animal groups, or between DM and TM animal groups, for individual R values.

2.5 Lipid Extraction and Thin Layer Chromatography (TLC)

The whole liver was removed at the time of sacrifice and tumorous regions were dissected away from non tumorous regions, using MRI spatial coordinates, and stored separately for analysis. Lipids were extracted from 200 mg of liver tissue from TM ($n \geq 3$ /time point) and DM ($n \geq 3$ /time point) mice at various time points, following a modified Folch extraction method [27], as previously described [24]. One-dimensional TLC, utilizing a mobile phase of 50:25:8:4 chloroform: methanol: glacial acetic acid: water v/v, was used to separate lipid classes in lipid extracts and GPCho fractions were collected.

2.6 HPLC

Phenacyl-ester extracts of GPCho lipid fractions were analyzed for alterations in fatty acid profiles using a gradient HPLC method previously described [24]. *LabView* software was used to record voltage readings from the detector, and voltage readings were processed utilizing *Mathematica* software. Data values are displayed as mean $\pm$ S.E. A two-way ANOVA (Graph Pad Prism version 5, GraphPad Software, Inc. La Jolla, CA) test (parametric) was used to evaluate differences ($p < 0.05$) between control and DM and TM animal groups, or between DM and TM animal groups, for individual fatty acyl species. Solvents were all filtered HPLC grade (Honeywell Burdick and Jackson, Morristown, NJ). Standards for docosahexaenoic, eicosapentaenoic, oleic, linoleic, γ -linolenic and arachidonic acids had a purity of $\geq 98\%$ (Sigma-Aldrich, St. Louis, MO).

2.7 Tandem Mass Spectrometry Analysis of GPCho and GPETn Lipids

Dried liver tissue extracts were suspended in HPLC grade methanol/chloroform (1:1 v/v; 500 μ L). Based on results from total phosphorus [28] and preliminary mass spectrometry analyses of each lipid extract at a range of different dilutions (in isopropanol/methanol/chloroform (4:2:1 v/v) solution containing 20 mM NH_4Ac), a dilution range was established at which linearity in response of specific lipids was observed, and ensuring that ratios of specific lipid ion abundances compared to other lipids within the mixture remained constant [29,30]. Aliquots (10–40 μ L) from each sample were diluted to 1600 μ L with isopropanol/methanol/chloroform (4:2:1 v/v) solution containing 20 mM NH_4Ac . Samples were then centrifuged and loaded into Whatman Multichem 96-well plates (Fisher Scientific, Pittsburgh, PA), and sealed with Teflon Ultra-Thin Sealing Tape (Analytical Sales and Services, Pompton Plains, NJ). Samples were then introduced to a Thermo Fisher model TSQ Quantum Ultra triple quadrupole mass spectrometer (San Jose, CA) via a chip-based nano-electrospray ionization (nESI) source (Advion NanoMate, Ithaca, NY) operating in infusion mode using an ESI HD chip A, as previously described [24,31]. All MS and CID-MS/MS spectra were acquired automatically for 1–20 minutes at a rate of 500 m/z sec^{-1} by methods created using Xcalibur software (Thermo, San Jose, CA). In MS mode, the Q1 peak width was maintained at 0.5 Da. For precursor ion (PI) scan mode CID-MS/MS experiments, Q1 was operated at a peak width of 0.5 Da while Q3 was maintained at 1 Da. For neutral loss (NL) CID-MS/MS scans, Q1 and Q3 were both operated at a peak width of 0.5 Da. For product ion scan mode CID-MS/MS experiments, Q1 and Q3 were operated with peak widths of 1.0 Da. The Q2 collision gas pressure was set at 0.5 mtorr. Collision energies were 45V, 35V and 45 V for precursor ion, neutral loss and product ion scan mode MS/MS experiments, respectively. Identification of GPCho lipids was achieved using a lipid class specific precursor ion (PI) scan mode experiment to monitor the characteristic phosphocholine product ion at m/z 184 (PI 184). Identification of GPETn lipids was achieved using a lipid class specific neutral loss (NL) scan mode experiment to monitor the characteristic loss of phosphoethanolamine of 141 Da (NL 141). Assignment of acyl substituents present in each of the identified GPCho and GPETn lipids was then achieved by correlation with the information provided by the use of product ion scan mode MS/MS experiments in negative ion mode at the specific m/z values at which individual $[\text{M}+\text{Ac}]^-$

GPCho lipids or $[M-H]^-$ GPEtn lipids were observed. Note that characterization of the fatty acyl chains in the lipids identified here was restricted to determination of the total number of carbons and double bonds, and that information regarding their relative sites of attachment to the glycerol backbone, and the specific position of the double bonds, was not obtained. A five point Gaussian smooth was applied to all spectra prior to data analysis. All solvents used were filtered HPLC grade (methanol (MeOH) (J.T. Baker, Phillipsburg, NJ), ammonium acetate (NH_4Ac) and chloroform ($CHCl_3$) (EMD Chemicals, Gibbstown, NJ).

Quantitative lipid analysis was achieved by determining peak areas from GPCho and GPEtn specific PI or NL scan mode MS/MS experiments using a Lipid Mass Spectrum Analysis (LIMSA) v.1.0 software peak model fit algorithm [32], in conjunction with a user-defined database of hypothetical lipid compounds for automated peak finding and for the correction of ^{13}C isotope effects. No attempts were made to quantitatively correct for different ESI responses of individual lipids due to concentration, acyl chain length or degree of unsaturation, or different CID-MS/MS fragmentation efficiencies as a function of precursor ion mass. Thus, the abundance of individual molecular species within each lipid class are reported here as % total lipid area for each class. One-way ANOVA (Graph Pad Prism version 5, GraphPad Software, Inc. La Jolla, CA) followed by Tukey as a post-test (incorporating a Bonferroni type correction) was used to evaluate significant differences ($p < 0.05$) between control and DM and TM animal groups, or between DM and TM animal groups, for individual lipid molecular species.

2.8 Total Fatty Acid Analysis

Ten to forty μL aliquots from resuspended liver extracts were dried then saponified by reaction in 0.4 N KOH in 80% methanol at 50° C for 1 hr. Saponified fatty acids were then acidified and extracted with diethyl ether, as previously described [33], and then stored in methanol. Saponified fatty acids were fractionated and quantified by RP-HPLC using a Shimadzu Prominence System. A linear gradient of 75–100% acetonitrile + 0.1% acetic acid was employed over 65 minutes with a YMC J'sphere H80 column with a flow rate of 1.0 mL/min. Fatty acids were detected using UV absorbance at 192 nm and evaporative light scattering (ELS), as previously described [33]. Authentic fatty acid standards (16:0, 16:1n7, 18:0, 18:1n9, 18:2n6, 18:3n3, 18:3n6, 20:3n6, 20:4n6, 20:5n3, 22:5n3 and 22:6n3) (Nu-Chek Prep) were used to generate calibration curves for fatty acid verification and quantification. Statistical significance ($p < 0.05$) in the differences in fatty acid concentrations between control and DM and TM groups, or between DM and TM groups was determined by 1-way ANOVA as described above.

2.9 Microarray

RNA was isolated from liver tissue, further purified by using an RNeasy Mini Kit (Qiagen, Valencia, CA), and RNA concentration was determined as previously described [24]. The Illumina TotalPrepTM RNA Amplification Kit was used for labeling cRNA (Ambion, Austin, TX), as previously described [24]. Illumina BeadStudio software (as previously described [24]) was used to quantitate the signal from BeadChips, and MATLAB normalized values were imported into BRB ArrayTools (Biometric Research Branch, National Cancer Institute). The data were log transformed, and differentially expressed genes were identified between any two classes by using a multivariate permutation test [34,35] to provide a median false discovery rate (FDR) of 5% (90% confidence). Random variance *t*-statistics for each gene was used [36]. Genes differentially expressed ($< 5\%$ FDR) and simultaneously having a ratio 2-fold or larger were used in further analyses. Pathways were explored by placing the only genes that passed our significance/ratio threshold into Ingenuity Pathways Analysis (IPA) (Ingenuity[®] Systems, www.ingenuity.com). The steps in the Network Generation Algorithm used by IPA involves (1) Identifying which molecules of interest

interact with other molecules in the Ingenuity Knowledge Base that are then classified as Network Eligible molecules. (2) These Network Eligible molecules are used to generate networks, by maximizing specific connectivity. (3) Networks are scored based on the number of Network Eligible molecules they contain. The higher the score, the lower the probability of finding the observed number of Network Eligible molecules in a given network by random chance (Ingenuity® Systems, www.ingenuity.com).

2.10 Western Blots

A Western blot assay of NFκB expression in mouse liver tissues was also conducted. Frozen tissue was weighed and sliced into thin pieces using a razor blade and then subsequently thawed in lysis buffer containing proteases and phosphatase inhibitors. Tissues were disrupted and homogenized at 4°C, incubated on ice for 30 min, transferred to microcentrifuge tubes and centrifuged (10000xg, 10 min, 4°C). The supernatant fluid was the total cell lysate. After determining total protein concentrations, lysates were separated on SDS-PAGE (Bio-Rad, Emeryville, CA), and transferred to nitrocellulose membranes. Western analysis was performed using antibodies against NFκB. The NFκB antibody was a rabbit polyclonal (Abcam Inc., Cambridge, MA). Secondary antibodies were labeled with horseradish peroxidase. The ECL Advance Western Blotting Detection Kit (Amersham Biosci., Piscataway, NJ) was used to detect immunoreactive proteins. NIH Image J was used to calculate density regions in Western blot bands.

3 Theory

It is known that cancer can be reduced with exogenous omega-3 fatty acids. With the use of a transgenic mouse model, the *fat-1* gene (not found naturally in mammals) was used to allow mice to convert omega-6 fatty acids to omega-3 fatty acids. Our thought was that HCC in a TGF-κ/c-myc transgenic mouse model could be reduced if the *fat-1* gene was also incorporated. This concept can also be applied to other cancer models, and this study further supports the benefit of omega-3 fatty acids as a means of reducing various cancers.

4 Results

We investigated the effect of including the *fat-1* gene in the mouse HCC model by several methods. Histological analysis was undertaken, comparing the TM to DM mouse liver tissue. MRI analysis of nodule and tumor incidence and tumor volumes in TM mice (n=12) compared to DM and control mice was performed. HPLC studies of the levels of specific fatty acids from the liver tissue from 30 to 40 weeks of age (corresponding with observed neoplasia in DM mice) were done, in addition to MS and total lipid analyses and implementation of microarray analysis comparing TM and DM liver tissue at 40 weeks of age.

4.1 Histology

H&E stained histological sections taken from livers of control (i), TM (ii), and DM (iii) mice were histologically analyzed (Fig. 1(i-iii)). Upon histological analysis, TM mice were found to exhibit similar liver histology to that of CD1 control mice, whereas DM mouse liver tissue exhibited nuclear pleomorphism and vacuolation. Pathology reports stated that there was moderate diffuse hepatocellular cytoplasmic vacuolation with no evidence of acute tissue necrosis or inflammation in both TM and control mouse models at 40 weeks of age. Pathology reports for DM mice, from 30 to 40 weeks of age, identified marked diffuse hepatocellular vacuolation with multiple small foci of telangiectasis and the presence of cysts. In DM mice, sinusoidal spaces and central veins were moderately to markedly congested. Neoplastic formations become visible at 30 and 36 weeks of age, and the

presence of hepatocellular adenomas and HCCs become more prominent in the livers at 40 weeks of age.

4.2 MRI

MRI analysis of TM mice found, in all cases but one, that there are no nodular developments that occur over the life span of the animals (Fig. 1(iv–ix)). In contrast, DM mice develop neoplastic formations around 30 weeks of age, and tumorous formations (consisting of both adenomas and HCCs) are noted in most male DM mice at 40 weeks of age. The tumor masses were identified as hypointense regions (darkened) with a loss of organization in the liver architecture in the MRI images as compared to surrounding liver tissue. Only one of the TM mice developed some neoplastic formations, and those were delayed in forming when compared to DM mice. This single male TM with verifiable neoplasia had nodules forming at 34 weeks, as opposed to 30 weeks in male DM, and tumor formations by 40 weeks of age, as opposed to some of the male DM which exhibited tumors at 34 weeks of age. None of the CD1 mice developed neoplastic formations. A histogram of the tumor volumes for DM and TM mice shows significant differences in tumor volumes between TGF- κ /c-myc mice and CD1 ($p < 0.01$), as well as between TGF- κ /c-myc mice and Fat-1/TGF- κ /c-myc mice ($p < 0.05$) (Fig. 2). Tumor volumes are shown as averages $\pm$ S.D.

4.3 MRS

Ratio (R) values for fatty acyl species (2.8 ppm peak ($-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-$): 5.3 ppm peak ($-\text{CH}=\text{CH}-$)) (Fig. 3A) in livers of TM mice were determined. It was found that R values for TM mice were similar to that of control mice, while the R value for fatty acids in DM mice was markedly less (Fig. 3B). MRS analysis showed that the lowest R value for TM mice was at 34 weeks, with a value of 0.377. The corresponding DM R value at the same time point was 0.189. One-way ANOVA analysis was used to show a significant difference in R values between DM and TM mice ($p < 0.005$) at 34 and 40 weeks of age. In contrast, there were significant differences (one-way ANOVA) in R values between DM and controls at 26 and 40 weeks ($p < 0.05$), but not at 34 weeks. An overlay of a 40 week old Tg mouse CSI data set, highlighting regions where ratio values between 0.05 and 0.15 appear, coincided with MRI visible neoplastic formations at 40 weeks in the same animal (Fig. 3C). A plot of the CSI data combined with an MRI image of the same Tg mouse at 40 weeks showed a relatively low amount of R values between 0.35 and 0.45 when compared to the lower R values between 0.05 and 0.15 (Fig. 3D). CD1 mouse data shows R values between 0.35 and 0.45 that are located throughout the liver (Fig. 3E), as the MRS data indicates (Fig. 3B). TM mice had similar R values to CD1 mice. Subcutaneous fat in Tg and CD1 mice contains both lower and higher R values (as seen in Figs. 3C-E).

4.4 HPLC

HPLC analysis of several fatty acids from GPCho lipids revealed marked differences between TM and DM mice (Fig. 4). Those mice containing the additional *fat-1* gene had lower levels of oleic acid (Fig. 4A), arachidonic acid (Fig. 4D), and combined omega-6 FA (Fig. 4F) in TM mice at 40 weeks of age, compared to mice with DM ($p < 0.05$). All of the fatty acid levels in TM mice were significantly lower ($p < 0.05$) than control mouse levels (Fig. 4A-D), indicating that there was an omega-6 to omega-3 change in lipid composition.

4.5 Mass Spectrometry

MS results indicated significantly ($p < 0.05$) elevated 16:0/22:6 and decreased 16:0/20:4 and 18:1/20:4 fatty acyl groups in both GPCho and GPEtn, and increased 16:0/20:5, 18:0/18:2, 18:0/18:1 and 18:0/22:6 in GPCho, within TM mice compared to DM mice (Fig. 5A and B). In many cases (except for 16:0/16:0, 16:0/20:5 and 18:2/22:6 GPCho) fatty acyl

compositions of TM mice were similar (i.e., not significantly different) to the wild-type non-tumor-bearing mice. Comparisons between wild-type controls to DM mice indicated increased 16:0/16:0 GPCho, 16:0/18:1 GPCho, 16:0/20:4 GPCho and GPEtn and 18:1/20:4 GPCho and GPEtn, and decreased 16:0/18:2 GPCho and GPEtn, 18:0/18:2 GPCho, 16:0/22:6 GPCho and GPEtn and 18:0/22:6 GPEtn in DM mice. Total fatty acid analysis indicated a significant decrease in 18:1n9 in TM mice compared to DM mice, a decrease in 16:0 and 18:0 and an increase in 18:1n9 and 20:3n6 in DM mice compared to controls (Fig. 5C).

4.6 Microarray

Microarray analysis of TM versus DM mouse liver tissue at 40 weeks revealed alterations in several genes of interest with a 10% FDR and >2 fold difference. The major networks that were involved included mainly cell cycle alterations (networks 1–3), cell-to-cell signaling and interaction (network 4), and inflammatory response (network 5) (see Table 1a). In comparison, the major networks associated with gene alterations when comparing control to TM, were lipid metabolism (networks 1–3), cell morphology (network 4), and gene expression, and hepatic system disease (network 5) (see Table 1b). A total listing of the major genes affected when comparing DM vs. TM are shown in Table 2. Most genes associated with cell cycle alterations, cell-to-cell signaling and interactions, and inflammatory responses are down-regulated in the TM mice. In contrast the genes most affected when comparing controls to TM are mainly associated with lipid metabolism (see Table 3). There are also differences in gene expressions associated with cell morphology that is cancer-related, and hepatic system disease, which is likely due to the mutations in *c-myc* and *TGF- α* . The most significantly affected canonical pathway in TM mice, when compared to DM mice, was ATM signaling ($p=1.17\times10^{-4}$), VDR/RXR activation ($p=1.66\times10^{-4}$), cell cycle: G2/M DNA damage checkpoint regulation ($p=2.93\times10^{-4}$), p53 signaling ($p=4.37\times10^{-4}$), and hepatic fibrosis/hepatic stellate cell activation ($p=7.53\times10^{-4}$). Of interest, an exploration into the microarray data utilizing the Ingenuity Pathway Analysis (IPA) software showed that multiple genes, involved in cell cycle and cell death either down- or upstream of NF- κ B, were altered in the TM to DM mice (see Fig. 6A). Western blot analysis of NF- κ B protein levels in control, DM and TM mouse liver samples is shown in Figure 6B. NF- κ B levels in the TM mouse livers are significantly lower than those found in the DM livers ($p<0.01$) and control livers ($p<0.001$) (Fig. 6B).

5 Discussion

We have found that the inclusion of the non-mammalian omega-3 desaturase gene, *fat-1*, is able to prevent the development of neoplasia in a Tg mouse HCC model through the conversion of omega-6 to omega-3 fatty acids. The ratio of omega-6:omega-3 fatty acids was altered, leading to a more healthy state of the liver in these mice as has been previously reported [9,10]. Additionally, the presence of endogenous omega-3 fatty acids led to alterations in the inflammatory process and acute phase response to inflammation, both known contributors to cancer development [9,10,37]. For instance, there was a reduction in systemic inflammation in *fat-1* transgenic mice with acute pancreatitis, as well as tissue fibrosis in chronic pancreatitis by increasing the tissue content of omega-3 polyunsaturated fatty acids [38]. It has also been reported that there is reduced colitis-associated colon cancer in *fat-1* transgenic mice, which may be mediated, in part, by the anti-inflammatory properties of n-3 PUFA [39,40]. Of interest, the reduced incidence and growth rate of colon tumors induced by inflammation (dextran sodium sulfate) plus treatment with a carcinogen (azoxymethane) was accompanied by lower activity of nuclear factor kappa B (NF- κ B) [39].

Although the possibility cannot be eliminated that the anti-cancer effect of the *fat-1* gene may be due to non-enzymatic roles, there are a few reasons to suggest that the anti-tumor

effect is due to the enzymatic function of *fat-1*. The function of the *fat-1* gene is unique in that it produces an enzyme that converts n-6 to n-3. This gene does not encode for or act as a transcriptional factor, thereby decreasing the likelihood that the *fat-1* protein directly interacts with other proteins and triggers other effects [9,10]. The *fat-1* mice have shown no adverse effects or abnormalities [9,10]. Therefore, it is unlikely that the *fat-1* gene directly interacts with other proteins or affects their functions. Furthermore, parallel feeding studies with n-3 have exhibited the same anti-cancer effect as the *fat-1* gene in previous studies [39,40]. This suggests that it is the level of n-3 that causes the anti-cancer effect, rather than a protein interaction caused by the *fat-1* gene.

Histological analysis of liver tissue removed from TM mice was nearly identical to that of control liver tissue at the same time points. There were no readily observable alterations in the structure of the liver, nor were there any observable changes to the hepatocytes themselves. MRI analysis yielded only one mouse out of twelve that showed any signs of neoplasia. This mouse however, was delayed in its formation of neoplastic lesions and tumors, as compared to the DM mice, by four to six weeks.

MRS has been used in previous research to show that there is a correlation between alterations in 2.8 ppm and 5.3 ppm proton resonances and the onset of liver neoplasia [21,24,25]. We implemented this method in this study to compare unsaturated fatty acyl resonances between TM, DM and control mice. We found that the ratio of 2.8 ppm to 5.3 ppm protons (*R* value) remains relatively constant in TM mice over time, indicating a balanced amount of PUFA: MUFA. MRS analyses produced *R* values for TM mice at all time points that were similar to controls. However, DM mice had a drastic decrease in *R* values (2.8 to 5.3 ppm protons), indicating either a dramatic loss of bis-allyl protons, a dramatic increase in methylene protons, or the combination of both. This significant decrease in the *R* value (2.8 to 5.3 ppm proton resonances) has been shown to coincide with liver neoplasia onset [24]. We have previously shown that DM mice have increased levels of stearoyl-CoA desaturase-1 (SCD1) [24]. High levels of n-3 fatty acids, and corresponding low levels of n-6 fatty acids, have been shown to decrease SCD1 levels [24], which would lead to low 18:1n-9 levels.

We note that while magnitude spectra are less than ideal for quantitative measurements of peak areas, for the low resolution spectra acquired here, measurement errors are negligible. Phased spectra from these data may also be used, but we have found whilst scrutinizing a small subset of cases that the constant and linear phase terms are different for all spectra from this subset in each voxel and in the absence of any robust automated method for determining such we present magnitude spectra only as these can be computed much faster. This is partly because of the random sign of the acquired signal in the vicinity of the suppressed water peak. The detailed treatment of analyses of these spectra guaranteeing the smallest variability are outside the context of this article, suffice it to say magnitude spectra provide the best precision thus far, however there is room for improvement.

HPLC analysis of target fatty acids from GPCho lipids was conducted from liver tissues. We found that all of the omega-6 fatty acids had decreased levels for TM mice, compared to DM and control mice. This helps to support the point that n-3 desaturase, capable of converting omega-6 fatty acids to omega-3 fatty acids, is highly beneficial to normal as well as diseased animals by lowering the ratio of omega-6 to omega-3 fatty acids. We attempted to measure eicosapentaenoic acid (20:5(n-3)) and docosahexaenoic acid (DHA) (22:6(n-3)) levels with HPLC, however, we did not find a significant alteration in these fatty acids in TM mice, as compared to DM or control mice.

MS analysis of 16:0/20:4 and 18:1/20:4 from GPCho and GPEtn also indicated significant decreases in the n-6 fatty acyl groups in the TM compared to the DM, with concurrent significant increases in n3 fatty acyl groups (e.g., 16:0/22:6 GPCho and GPEtn, and 18:0/22:6 GPCho lipids). Total fatty acid analysis also indicates that de-novo lipogenesis and fatty acid synthesis (FAS) are differentially regulated in DM vs. TM knock-outs.

A comparison of gene expression from microarray analysis between DM and TM indicated a down-regulation in the TM livers in several cancer-associated genes involved in cell cycle pathways, such as cell-to-cell signaling and interaction, and cell death. Exploration of the microarray data utilizing the Ingenuity Pathway Analysis (IPA) software indicated that there were multiple genes involved in cell cycle and cell death either down- or upstream of NF- κ B that were altered in the TM to DM mice (Fig. 6A). Western blot data on NF- κ B indicated that endogenous omega-3 fatty acids in the TM mice significantly decreased NF- κ B levels ($p < 0.01$) (Fig. 6B). It is known that NF- κ B plays an important role during liver neoplastic development via transcriptional regulation of pro-survival genes, which then affect death-inducing signals elicited by the host immune response [41]. It was found that transforming growth factor- α inhibits the intrinsic pathway of c-myc induced apoptosis via the activation of NF- κ B in hepatocellular carcinomas generated in TGF- α /c-myc mice [42]. Oxidative stress driven by the inflammatory response plays an important role in hepatocellular cancer [43]. In the DM transgenic mouse model, co-expression of the c-myc proto-oncogene and TGF- α results in the early generation of an oxidative stress environment and predisposes mice to 100% incidence of liver cancer by 8 months of age [22,23]. Omega-3 fatty acids have also been found to inhibit hepatocellular carcinoma cell growth by blocking β -catenin and cyclooxygenase-2 [44].

6 Conclusion

We have shown in this study, both visually and metabolically, that the presence of endogenous omega-3 desaturase fatty acids (decrease in omega-6 or n-6/n-3 ratio) is able to prevent the formation of neoplastic tumors in almost all cases within the TGF- α /c-myc mouse HCC model. It would be of interest to study the effects of an exogenous source of omega-3 fatty acids in this same HCC model, in order to determine whether the commonly used means of administering omega-3's would be as successful as the endogenous expression of omega-3 desaturase. Overall, out of 22 total animals, we only had two male TM mice (one being from the twelve mice used in the MRI/MRS study) that developed neoplasia at later time points than that observed for the DM mice. These results reveal the importance of maintaining a healthy amount of omega-3 fatty acids in the diet, and that omega-3 fatty acids may play a role in the chemoprevention and treatment of HCC.

Research Highlights

It is well known that exogenous omega-3 fatty acids can have an effect in reducing cancer. The *fat-1* gene (not found naturally in mammals) was used to allow mice to convert omega-6 fatty acids to omega-3 fatty acids, in a transgenic mouse model. Our hypothesis was that hepatocellular carcinomas (HCC), that develop within a TGF- α /c-myc transgenic mouse model, could be reduced if the *fat-1* gene was also incorporated in a triple mutation transgenic mouse. Liver fatty acyl groups, within *fat-1*/TGF- α /c-myc (triple mutant or TM) mice compared to HCC-bearing TGF- α /c-myc (double mutant or DM) transgenic mice, were assessed *in vivo* with the use of the magnetic resonance spectroscopy (MRS) method, chemical shift imaging (CSI), as well as in *ex vivo* liver extracts with the use of high performance liquid chromatography (HPLC) and mass spectrometry (MS) analyses. Fatty acyl groups in phospholipids were found to be substantially altered in TM mice compared to DM mice, with marked decreases in

omega-6 fatty acids. Microarray analysis indicated genes associated with cell cycle, cell-to-cell signaling and interactions, and inflammatory responses were down-regulated in TM mice compared to DM mice. Of interest there were a number of genes associated with nuclear factor κ B (NK- κ B), which were down-regulated in TM mice (compared to DM mice) in the microarray data, which correlated with decreased protein levels of NK- κ B in Western blot analysis. The concept of introducing the fat-1 gene can also be applied to other cancer models, and this study further supports the benefit of omega-3 fatty acids as a means of reducing various cancers.

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Abbreviations

HCC	Hepatocellular carcinoma
GPCCho	glycerophosphatidylcholine
GPEtn	glycerophosphatidylethanolamine
IACUC	Institutional Animal Care and Use Committee
FLASH	fast low angle shot
TE	echo time
TR	relaxation time
DM	double mutant
TM	triple mutant
Tg	transgenic

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Figure 1. Liver histology (H&E staining) of different mice studied

H&E stained slides of liver tissue taken from control (i), TM (ii) and DM (iii) mice. Pathological analysis of control and TM tissue revealed nearly identical pathologies, whereas DM mice had noted nuclear pleomorphism and vacuolation. T1-weighted MRI images of TM mice at 30 (iv), 34 (v) and 40 (vi) weeks of age. Tumor masses were identified as hypointense regions (darkened) with a loss of organization in the liver architecture in the MRI images as compared to surrounding liver tissue. There are no noticeable alterations to the appearance of the liver in TM mice at any time point. The appearance of TM livers is similar to control mouse at 40 weeks of age (vii). There was only one TM mouse (viii) that developed neoplasia similar to DM mice (ix) at 40 weeks of age.

Figure 2. Tumor volumes measured from morphological MR images

Tumor volume comparisons between DM (TGF- α /c-myc) (n=5), TM (Fat-1/TGF- α /c-myc) (n=5), and control (CD1) (n=6) at 40 weeks of age. Results are shown as averages $\pm$ S.D. There is a significant difference between tumor volumes for DM and control mice (p<0.01), and between DM and TM mice (p<0.05).

Figure 3. MR spectroscopy assessment of lipids in mice livers

(A) Representative spectrum from a control mouse at 40 weeks of age. The vinyl (5.3 ppm), bis-allyl allyl (2.8 ppm), water (H_2O , 4.7 ppm), and methyl (0.5–1.0 ppm) peaks are indicated on the spectrum.

(B) MRS analysis of the alteration in unsaturated fatty acid protons revealed the ratio (R) of 2.8 ppm bis-allyl protons (found only in polyunsaturated fatty acids) to the 5.3 ppm methylene protons (found in all unsaturated fatty acids, but in MUFA the only present resonance) is similar between the Tg and control mice. DM mice meanwhile had a significantly altered R value from that of TM mice beginning at 26 weeks of age ($p=0.003$). For all time points, $n \geq 5$ animals/time point. Results are shown as means $\pm$ S.D.

(C) An overlay of a 40 week old Tg mouse CSI data set on top of an MR image of the same mouse, highlighting regions where ratio (R) values between 0.05 (red on the color scale) and 0.15 (purple on the color scale) appear, coincided with MRI visible neoplastic formations at 40 weeks in the same animal (highlighted in Fig. 3D). Some low R values are also present in subcutaneous fat located in the skin (thin layer around periphery of the image surrounding the liver).

(D) A plot of the CSI data overlaid on an MRI image of the same Tg mouse at 40 weeks showed a relatively low amount of R values between 0.35 and 0.45 when compared to the lower R values between 0.05 and 0.15 as was seen in Fig. 3C.

(E) R values between 0.35 and 0.45 appear throughout the liver of this control mouse at 40 weeks of age. This is in agreement with the R values obtained in Fig. 3B. Some high R values are also present in subcutaneous fat located in the skin.

Figure 4. HPLC analysis of lipids in mice livers

HPLC analysis of fatty acyl species oleic (A), linoleic (B), γ -linolenic (C), and arachidonic (D) from GPCho lipids showed a difference in all of the species between TM (X; X with dashed line) mice and DM (■; closed square with solid line) mice. Indeed, TM fatty acid levels for the omega-6 fatty acids were even less than controls (◇; open grey diamond with dotted line) (B-D). Levels of the omega-3 fatty acid eicosapentaenoic acid (E) between DM (■) and TM (X) mice as well as control (◇) mice revealed a relatively equal distribution of the fatty acid among all groups with differing trends. By averaging all omega-6 fatty acids analyzed (F), we found that TM (X) mice possessed much less of the fatty acids than DM (■) or controls (◇). Results are shown as averages of each fatty acid $\pm$

S.E. of $n=5$ animals for DM per time point and $n\geq 3$ animals for TM and controls per time point. At 40 weeks there was a significant difference ($p<0.05$) in oleic acid (A), arachidonic acid (D), and combined omega-6 FA (F), when comparing DM to TM mouse liver extracts. At 40 weeks there was also a significant difference ($p<0.05$) in all fatty acids assessed, when comparing Controls to TM mouse liver extracts.

Figure 5. MS and total fatty acid analyses of mice livers

Quantitative ESI-MS/MS analysis of (A) GPCho and (B) GPEtn lipids from crude liver tissue extracts of 40 week control (n=5), DM (n=5) and TM (n=5) mice. Only those lipid species whose fatty acid ion relative abundances were observed to be greater than 10% were assigned. *The GPCho 18:1/18:2 lipid was also found to contain an abundant GPCho 16:0/20:3 lipid species, while the GPCho 18:1/20:4 lipid also contained an abundant GPCho 16:0/22:5 lipid. The GPEtn 16:0/20:5 lipid was also found to contain an abundant GPEtn 16:1/20:4 lipid species. (C) Total fatty acid analysis of crude liver tissue extracts from 40 week control (n=5), DM (n=5), and TM (n=5) mice. The key used to indicate significant ($p < 0.05$) differences between sample groups is: #, control and DM groups; φ, control and TM groups; and ψ, DM and TM groups.

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Figure 6. Microarray analysis of mice livers

(A) A network map showing NF- κ B related pathways of genes down-regulated (green) and up-regulated (red) following microarray analysis when a comparison is made between mouse liver mRNA obtained in DM versus TM mice at 40 weeks of age. Genes differentially expressed ($< 5\%$ FDR) and simultaneously having a ratio 2-fold or larger were used to generate a network pathway with genes that passed the significance/ratio threshold in Ingenuity Pathways Analysis (IPA) (Ingenuity® Systems, www.ingenuity.com). This network was ranked third by the IPA software and reflected a score of 28, meaning there were 28 network eligible molecules. (B) Western blot data on NF- κ B protein levels in TM, DM and control mouse livers at 34 and 40 weeks of age. NF- κ B levels, measured as

optical density (ImageJ), were significantly decreased in TM mice compared to DM mice (** $p < 0.01$) and controls (** $p < 0.001$) at 40 weeks of age, but not at 34 weeks of age. Insert depicts a representative Western blot of NF- κ B and β -actin protein levels at 40 weeks of age. β -Actin is used as a loading control.

Table 1

Major networks from microarray analysis following comparison of gene expression in (A) DM versus TM, and (B) control versus TM, from mouse liver mRNAs at 40 weeks of age.

Comparisons	Network	Associated Network Functions	Score
A	1	Cell Cycle, Cell-to-Cell Signaling and Interaction, Cancer	38
	2	Cell Cycle, Cell Death, Embryonic Development	31
	3	Cell Cycle, Cell Death, Reproductive System Development and Function	28
	4	Cell-to-Cell Signaling and Interaction, Hematological System Development and Function, Immune Cell Trafficking	28
	5	Inflammatory Response, Cellular Movement, Cell-to-Cell Signaling and Interaction	28
B	1	Lipid Metabolism, Small Molecule Biochemistry, Vitamin and Mineral Metabolism	29
	2	Lipid Metabolism, Small Molecule Biochemistry, Drug Metabolism	28
	3	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry	22
	4	Cell Morphology, Cancer, Developmental Disorder	22
	5	Gene Expression, Genetic Disorder, Hepatic System Disease	15

Table 2

Total listing of major affected down-regulated gene expressions in networks 1-5, with a 3-fold or higher fold change, when comparing DM versus TM mouse liver mRNAs at 40 weeks.

Symbol	ENTREZ Gene Name	Fold Change	Networks
ANLN	Aniline, actin binding protein	-4.298	1
APOA4	Apolipoprotein A-IV	-6.161	1
CD63	CD63 molecule	-13.834	1
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	-5.973	1
CIDEA	Cell death-inducing DFFA-like effector <i>a</i>	-3.035	1
IER3	Immediate early response 3	-3.407	1
LPL	Lipoprotein lipase	-5.072	1
PLSCR1	Phospholipid scramblase 1	-3.509	1
RHOC	Ras homolog gene family, member C	-3.252	1
TES	Testis derived transcript (3 LIM domains)	-3.631	1
AURKA	Aurora kinase A	-3.838	2
BIRC5	Baculoviral IAP repeat-containing 5	-6.365	2
CCNB1	Cyclin B1	-4.658	2
CDC20	Cell division cycle 20 homolog (<i>S. cerevisiae</i>)	-6.094	2
CENPA	Centromere protein A	-3.733	2
COL4A1	Collagen, type IV, alpha 1	-3.297	2
CTGF	Connective tissue growth factor	-3.223	2
CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	-5.696	2
CYR61	Cysteine-rich, angiogenic inducer, 61	-3.284	2
IGFBP1	Insulin-like growth factor binding protein 1	-12.494	2
NID1	Nidogen 1	-4.838	2
NUPR1	Nuclear protein, transcriptional regulator, 1	-4.080	2
SMOC2	SPARC related modular calcium binding 2	-6.691	2
TFF3	Trefoil factor 3 (intestinal)	-16.122	2
BEX2	Brain expressed X-linked 2	-4.900	3
DUSP1	Dual specificity phosphatase 1	-6.314	3
DUSP6	Dual specificity phosphatase 6	-3.197	3
GADD45A	Growth arrest and DNA-damage-inducible, alpha	-6.236	3
GDF15	Growth differentiation factor 15	-7.744	3
PIM3	Pim-3 oncogene	-4.791	3
PLK3	Polo-like kinase 3 (<i>Drosophila</i>)	-5.150	3
RBP1	Retinol binding protein 1, cellular	-5.780	3
SLPI	Secretory leukocyte peptidase inhibitor	-4.589	3
TNFRSF12A	Tumor necrosis factor receptor superfamily, member 12A	-7.670	3
BHLHE40	Basic helix-loop-helix family, member e40	-3.766	4
FGF21	Fibroblast growth factor 21	-10.846	4

Symbol	ENTREZ Gene Name	Fold Change	Networks
LCN2	Lipocalin 2	-7.572	4
MT2A	Metallothionein 2A	-12.927	4
NEDD9	Neural precursor cell expressed, developmentally down-regulated 9	-3.375	4
PNPLA3	Patan-like phospholipase domain containing 3	-5.293	4
SAA3P	Serum amyloid A3 pseudogene	-3.828	4
SOCS3	Suppressor of cytokine signaling 3	-3.131	4
ATF3	Activating transcription factor 3	-12.838	5
CD14	CD14 molecule	-3.895	5
ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	-4.100	5
JUNB	Jun B proto-oncogene	-7.585	5
OMD	Osteomodulin	-3.744	5
RGS16	Regulator of G-protein signaling 16	-50.680	5
RHOB	Ras homolog gene family, member B	-6.594	5

Table 3

Total listing of major affected gene expressions in networks 1–5, with a 3-fold or higher fold change, when comparing Control versus TM mouse liver mRNAs at 40 weeks. Down-regulated (clear), and up-regulated (5% grey-shading).

Symbol	ENTREZ Gene Name	Fold Change	Networks
CSAD	Cysteine sulfinic acid decarboxylase	−3.214	1
CYP7A1	Cytochrome P450, family 7, subfamily A, polypeptide 1	−4.572	1,3
ERCC5	Excision repair cross-complementing rodent repair deficiency, complementation group 5	4.750	1
NDUFB10	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10, 22kDa	7.115	1
NUDT6	Nudix (nucleoside diphosphate linked moiety X)-type motif 6	5.440	1
TOMM22	Translocase of outer mitochondrial membrane 22 homolog (yeast)	5.185	1
TRIM33	Tripartite motif-containing 33	5.345	1
BCKDHB	Branched chain keto acid dehydrogenase E1, beta polypeptide	4.694	2
CCNG2	Cyclin G2	4.530	2
CTSC	Cathepsin C	4.750	2
DBP	D site of albumin promoter (albumin D-box) binding protein	−11.660	2,3
NNMT	Nicotinamide N-methyltransferase	17.497	2
RNASE4	Ribonuclease, RNase A family, 4	−3.030	2
VNN3	Vanin 3	5.204	2
ACPP	Acid phosphatase, prostate	3.862	3
HSD3B2	Hydroxyl-delta-5-steroid dehydrogenase, 3 beta- and steroid delta- isomerase 2	−3.530	3
PRDX2	Peroxiredoxin 2	3.026	3
ARL3	ADP-ribosylation factor-like 3	3.659	4
CAPN8	Calpain 8	−3.121	4
IL6ST	Interleukin 6 signal transducer (gp130, oncostatin M receptor)	3.085	4
SRP9	Signal recognition particle 9kDa	3.111	4
ADH7	Alcohol dehydrogenase 7 (class IV), mu or sigma polypeptide	6.774	4
RPGRIP1	Retinitis pigmentosa GTPase regulator interacting protein	3.704	5
UAP1	UDP-N-acetylglucosamine pyrophosphorylase 1	4.468	5