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Research Article

Differential gene expression between wild-type and *Gulo*-deficient mice supplied with vitamin C

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Abstract

The aim of this study was to test the hypothesis that hepatic vitamin C (VC) levels in VC deficient mice rescued with high doses of VC supplements still do not reach the optimal levels present in wild-type mice. For this, we used a mouse scurvy model (*sfx)* in which the L-gulonolactone oxidase gene (*Gulo)* is deleted. Six age- (6 weeks old) and gender- (female) matched wild-type (WT) and *sfx* mice (rescued by administering 500 mg of VC/L) were used as the control (WT) and treatment (MT) groups (n = 3 for each group), respectively. Total hepatic RNA was used in triplicate microarray assays for each group. EDGE software was used to identify differentially expressed genes and transcriptomic analysis was used to assess the potential genetic regulation of *Gulo* gene expression. Hepatic VC concentrations in MT mice were significantly lower than in WT mice, even though there were no morphological differences between the two groups. In MT mice, 269 differentially expressed transcripts were detected (≥ twice the difference between MT and WT mice), including 107 up-regulated and 162 down-regulated genes. These differentially expressed genes included stress-related and exclusively/predominantly hepatocyte genes. Transcriptomic analysis identified a major locus on chromosome 18 that regulates *Gulo* expression. Since three relevant oxidative genes are located within the critical region of this locus we suspect that they are involved in the down-regulation of oxidative activity in *sfx* mice.

Key words: gene expression, L-gulonolactone oxidase, liver, oxidative stress, vitamin C.

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Introduction

Oxidative stress is a major pathogenic event associated with nearly all chronic liver disorders ranging from hepatitis to cancer (Padayatty *et al.*, 2003; Briley *et al.*, 2006; Chen *et al.*, 2007). Consequently, antioxidants, such as vitamin C (VC), are widely used as therapeutic agents. However, the precise mechanism of antioxidant action is still unclear and the role of VC in oxidative stress is still controversial (Bader *et al.*, 2007; Besaratinia *et al.*, 2007). Humans lack the ability to synthesize VC and obtain this vitamin primarily from food. VC deficiency in humans leads to scurvy, which is still occasionally encountered in some parts of the world (Burk and Molodov, 2007; Larralde *et al.*, 2007). A study of the long-term effects of VC deficiency in the liver using these rare scurvy patients is practically impossible, especially at the molecular level, since it would require liver tissue. The alternative is to use animal models for such studies.

Traditionally, rodents have been used to investigate the effects of VC, particularly through VC-deficient models. The spontaneous bone fracture (*sfx)* mouse (Beamer *et al.*, 2000) is a useful model because it lacks the gene for L-gulonolactone oxidase (*Gulo*), a key enzyme in the ascorbic acid (AA) synthesis pathway (Jiao *et al.*, 2005). *Sfx* mice are derived from an inbred strain of Balb/c mice and therefore have a Balb/c genomic background, with the key difference being that they possess the mutated gene for Gulo. The mutation for Gulo is inherited as a recessive gene

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and produces spontaneous bone fracture(s) 6-8 weeks after birth. *Sfx* mice were initially considered as a model for stage-specific bone growth failure and fracture (Beamer *et al.*, 2000). Based on rough mapping data obtained from 100 *sfx* F2 mice we found that chromosome 14 contains an ~38 kb deletion of genomic DNA that includes the entire *Gulo* gene (Jiao *et al.*, 2005). Further studies confirmed that this deletion is responsible for the bone and tissue disease phenotypes in these mice (Beamer *et al.*, 2000; Jiao *et al.*, 2005; Yan *et al.*, 2007), which suggests that this deletion has a wide range of effects in different mouse organs.

The administration of VC has been used to rescue VC-deficient mice in various studies, although the amount required varies considerably. For example, Yazama *et al.* (2006) used a VC concentration of 330 mg/L in drinking water and suggested that this ameliorated the VC deficiency in *Gulo*-/- mutant mice. Wang *et al.* (2007), in a study of the effects of VC on liver damage in lead-exposed mice, used VC doses of 140, 420 and 1260 mg kg^{-1} body weight together with vitamin B1 (10, 30 and 90 mg kg^{-1}) in a 3 x 3 factorial design. These authors concluded that the most effective combination was 420 mg of VC kg^{-1} and 10 mg of vitamin B1 kg^{-1}. However, since the mice used in this study were wild type, they were already producing VC. In a study in which the gastric lesions and Th1 immune responses to *H. pylori* were compared in VC-deficient B6.129P2-*Gulo*(-/-) mice supplemented with a low (33 mg/L) or high (3,300 mg/L) concentration of VC in the drinking water, Lee *et al.* (2008) observed that VC did not protect *Gulo*(-/-) mice from developing *H. pylori*-induced premalignant gastric lesions, despite the high concentrations of VC achieved in plasma and gastric tissue.

In view of the widespread use of VC as an antioxidant (Al-Shamsi and Amin, 2006; Bader *et al.*, 2007; Besaratinia *et al.*, 2007; Montecinos *et al.*, 2007; Plantinga *et al.*, 2007; Siriwardena *et al.*, 2007), it is important to understand the molecular mechanisms of this molecule in liver. Since, in rodents, VC is synthesized in the liver and transported to other parts of the body, the hepatic content of VC is critical in assessing whether the body has sufficient VC. In the present work, we hypothesized that although VC supplements in previous studies successfully rescued mutant mice with a seemingly normal phenotype, the hepatic VC content of these mice was still below the optimal level found in wild type mice. Indeed, as shown here, even with 500 mg of VC/L in the drinking water, the hepatic content of VC in *sfx* mice was still not normal. As a result of insufficient hepatic VC, *sfx* mice had a different gene expression profile compared to wild type mice.

Materials and Methods

Animals

Heterozygous *sfx*/+ mice were obtained from Jackson Laboratories. Homozygous *sfx*/*sfx* mice were produced by intercrossing the heterozygous *sfx*/+ mice at the University of Tennessee Health Science Center (UTHSC). Experimental procedures for this study were approved by the Institutional Animal Care and Use Committee at UTHSC. Normal and *sfx* littermates produced from the same heterozygous parental pair were used in the mutation screening. Three six-week-old female *sfx* mice and three age- and gender- matched WT mice were used for the microarray assays and RT-PCR. Additional WT and *sfx* mice (n = 20) were used to measure VC levels. The mice were handled according to a protocol described elsewhere (Jiao *et al.*, 2005). When six weeks old, the mice were sacrificed and tissue samples were immediately obtained and preserved in liquid nitrogen.

Vitamin C test

Prior to the vitamin C test, the mice were genotyped using a pair of primers (forward: TGAGGCAAACATT GGAGATG, reverse: CCCCATACCATAACCAGCAG) flanking exon 2 of the *Gulo* gene. Subsequently, three homozygous *sfx/sfx* mice were housed in the same animal room on the same cartridge with three +/+ BALB/cBy mice. All of the mice were fed the same food except that the *sfx/sfx* mice received 500 mg of VC/L in their drinking water. Plasma and hepatic VC levels were measured by the α, α'-dipyridyl method, as described by Kutnink *et al.* (1987). Statistical analysis was done using an Excel *t*-test, with $p < 0.05$ indicating significance.

DNA and RNA extraction

Genomic DNA was extracted from the livers of WT and *sfx* mice using a commercial kit (Qiagen, CA) according to the manufacturer's instructions. DNA quality and quantity were assessed spectrophotometrically (Eppendorf photometer, Eppendorf Scientific Inc., Westbury, NY) after which the DNA was used for large scale mutational screening. RNA was extracted from livers using Trizol reagent (Invitrogen, CA) (Yan *et al.*, 2007). Total RNA was purified using the RNeasy MinElute cleanup kit (Qiagen). RNA quality and integrity were checked with an Agilent Bioanalyzer.

Microarray analysis

Total RNA was isolated from the livers of three WT mice and three *sfx* mice treated with VC. Subsequently, 200 ng of high-quality RNA with a RIN (RNA Integrity Score) number of more than 7 was used to generate cDNA and cRNA using an Illumina® TotalPrep RNA amplification kit (Ambion). From each of the six samples, 1.5 μg of cRNA was hybridized overnight to the Mouse-6 v1B BeadChip in a multiple step procedure, according to the manufacturer's instructions. The chips were washed, dried and scanned on a BeadArray Reader (Illumina, CA) and the raw data were generated using BeadStudio 2.3.41 software (Illumina).

The raw data were normalized with the quantile method using BeadStudio software. After p value assessment ($p < 0.05$) (Lee *et al.*, 2009) and filtering for fold changes = 2, statistical analysis was done using EDGE software (Vollrath *et al.*, 2009) to identify differentially expressed genes that were then used for hierarchical and functional clustering with Cluster/TreeView and bioinformatics DAVID tools, respectively. Genes were considered to be differentially expressed when the difference in expression between the two groups was greater than two fold (Schena *et al.*, 1995; Gu *et al.*, 2003).

Quantitative RT-PCR

The expression levels of 9 genes were assessed by end-point RT-PCR. These genes were selected based on their fold increase in expression and possible relevance to the *Gulo* pathway. Total RNA was isolated from age- and sex-matched WT and *sfx* mice. Reverse transcription and PCR were done using a one-step RT-PCR kit (Invitrogen) as recommended by the manufacturer. The specific primers for the selected genes are shown in Table 1. The reactions were done in 50 μL containing 4 ng of total RNA/μL and 0.2 μM of sense and anti-sense primers (final concentrations). cDNA synthesis and pre-denaturation were done using one cycle of 50 °C for 40 min and 94 °C for 2 min, followed by PCR amplification with 33 cycles of 94 °C for 30 s, 54-58 °C for 36 s and 72 °C for 2 min. The PCR products were analyzed by electrophoresis in 2.0% agarose gels. Quantitative analysis of the PCR products was done using Scion Image software.

Tanscriptome mapping

Transcriptome mapping done with Genenetwork software was used to identify the chromosomal regions containing genes that affect the expression of *Gulo*. Gene expression data from the livers of 46 recombinant inbred (RI) mice derived from strains C57BL/6J and DBA/2 (BXD) were used in this analysis, which involved three major steps. First, *Gulo* was identified from the probes used to detect liver-specific gene expression in BXD RI strains. Second, interval mapping was done to establish *Gulo* linkage maps for the entire genome. Permutations of 1000 tests were used to assess the strength and consistency of the linkages. Third, the maps determined in the previous step were reassessed by focusing on the major locus identified by whole genome mapping.

Results

Body weight gain in *sfx* mice supplemented with dietary VC

sfx mice given supplementary VC (500 mg/L) in the drinking water did not develop spontaneous fractures. Body weight gain in *sfx* mice was not significantly different from that of WT mice (Figure 1A). However, the VC concentration in *sfx* mice was significantly lower than in normal WT mice (Figure 1B). Since previous work has shown that various organs of *sfx* mice develop abnormally (Beamer *et al.*, 2000; Jiao *et al.*, 2005) we also examined the influence of VC on the weight of selected organs in WT and *sfx* mice. Table 2 shows that there were no significant differences in the organ weights of WT and *sfx* mice.

Hepatic VC level in treated *sfx* mice

Figure 2 shows that the hepatic VC level in treated *sfx* mice was significantly lower than in normal WT mice. This finding indicates that the concentration of VC (500 mg/L) in the drinking water was not sufficient to restore hepatic VC levels to normal in *sfx* mice.

Differential gene expression in livers from VC-treated mice

After normalizing the data using the Cubic spline algorithm with BeadStudio, the data from the three normal and VC-treated mice were coherent for each group (Figure 2). In general, the expression profile in VC-treated *sfx*

Table 1 - Primers for the different genes studied by real-time PCR.

Gene	PCR product size (bp)	Forward primer	Reverse primer
Fgf21	368	TTCAAATCCTGGGTGTCAAAG	CAGGAAGAGTCAGGACGCATA
Jun	319	TCCTGCCCGTGTTTGTAAAT	CAGTCTGGACTTGTGTGTTGC
Birc5	321	CAGATCTGGCAGCTGTACCTC	TGCAATTTTGTTCTTGGCTCT
Mdm2	340	CCAGCATTTTCAGCTTTTTGT	CAAAGCTATCCTTCGCTTCCT
Trp53	305	CGTAAACGCTTCGAGATGTTC	GCTGAGCCCTAGCTACAAGGT
Gulo	329	TTCTTCTTCTGGCTGCTGTTC	GTCTCATAGGCCAGCCAGTAA
Cth	366	GCACCAACAGGTACTTCAGGA	AACGAAGCCGACTATTGAGGT
Cfhl1/Cfhr1	358	TCTGTCGCCTGTTGCTCTTAG	CCTTGATTGCAGACCACTTGT
Cyp3a41	337	TTTGATGGTCAAATGCCTCTC	TGCTGGTGATCACATCTATGC
Map2k6	330	CTACCTGGTCGACTCTGTTGC	GGATGTTGCATAAGCTCTGGA
Gapdh	533	GGGTGGAGCCAAACGGGTC	GGAGTTGCTGTTGAAGTCGCA

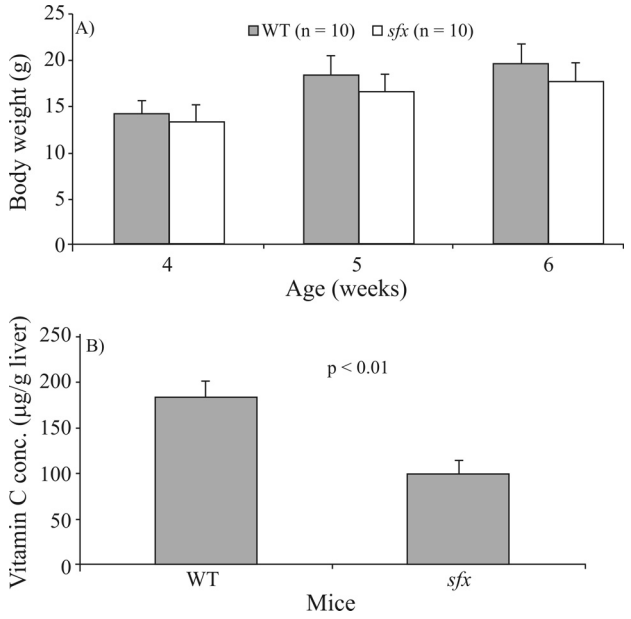

Figure 1 - Body weight (A) and hepatic VC concentration (B) in WT and VC-treated *sfx* mice. There were no significant differences in body weight between the two groups, but vitamin C concentration was lower in *sfx* mice. The columns represent the mean ± SD of 10 mice per group.

mice was greatly improved in relation to none VC-treated mice. In our previous comparison between VC- deficient and WT mice (Jiao *et al.*, 2007), we detected 398 differentially expressed genes in a total of 12,000 mouse transcripts. In this study, we only detected 269 differentially expressed genes in a total of 47,000 oligonucleotide probes, the latter corresponding to a total of 23,000 well characterized genes. The ratio of differentially expressed genes was 30 *vs.* 86 per gene (or 174.7 per probe) for the current study *vs.* the previous study. Furthermore, the degree of change in these genes in the current study was much smaller than in our previous investigation, *i.e.*, 1.7-fold change *vs.* 4.1-fold change, respectively. The altered expressions levels of major genes in VC deficiency *sfx* mice have been corrected into normal levels in VC-treated *sfx* mice. For example, the expression levels of major urinary protein (*Mup*) genes in VC deficiency *sfx* mice were significantly lower than that of the wild type in our previous study (Yan *et al.*, 2007); however in our current study we did not find significant difference between those genes in VC-treated *sfx* mice and the wild type. After detecting significant p values (< 0.05) and filtering to remove genes with = 2-fold change in expression (this cutoff value was chosen based on published studies: Shahan *et al.*, 1987; Schena *et al.*, 1995; Gu *et al.*, 2003), EDGE software detected 269 differentially expressed genes out of a total of 47,000 oligonucleotide probes, corresponding to > 23,000 well-characterized genes and > 23,000 ESTs or predicted genes. Of these 269 genes, 107 were up-regulated and 162 were down-regulated (Supplementary material, Table S1). Hierarchical and functional clustering of the changes in gene expression using Cluster/TreeView identified genes that were differentially expressed in the two groups. Overall, there was a decrease in the expression of genes associated with signaling and protein binding, but an increase in those associated with biosynthesis activity (Table 3).

Table 2 - Organ weights (in grams) in seven-week-old WT Balb/c mice and VC-treated *sfx* mice.

Organs	Balb/c mice	*Sfx* mice with VC (500 mg/L)	N	p
Thymus	0.082 ± 0.011	0.081 ± 0.017	5	0.4578
Spleen	0.106 ± 0.008	0.086 ± 0.005	5	0.0008
Liver	1.013 ± 0.116	0.989 ± 0.083	5	0.3581
Heart	0.133 ± 0.024	0.129 ± 0.021	5	0.4142
Lung	0.130 ± 0.013	0.128 ± 0.005	5	0.3509
Kidney	0.274 ± 0.032	0.250 ± 0.025	5	0.1159

Values (in grams) are the mean ± SD of the number of mice indicated. P values are for *sfx* mice compared to WT Balb/c mice.

Down-regulation of target genes involved in the regulation of MAPK signaling

Since VC is a well-known antioxidant, we examined the gene expression levels of mitogen-activated protein kinase (MAPK) signaling in the liver. We found that a group of genes related to a stress-activated protein kinase (*Sapks*) pathway was down-regulated (Figure 3A). These genes included dual-specificity phosphatase (*Dusp*) 3, 8 and 10, growth arrest- and DNA damage-inducible gene gadd45, beta (*Gadd45b*), fibroblast growth factor 21 (*Fgf21*) and oncogene jun (*Jun*). *Dusp* 3 suppresses T-cell receptor (TCR)-induced activation of *Erk2* and *Jnk* in the MAPK signaling pathway. Consequently, expression of *Jun* is also decreased by *Dusp* 3. *Gadd45b* may mediate activation of the *p38/Jnk* pathway, via *Mtk1*, in response to environmental stress (Takekawa *et al.*, 2002; Chi *et al.*, 2004). *Fgf21* treatment of mouse adipocytes is associated with phosphorylation of *Frs2*, a docking protein linking *Fgf* receptors to the MAPK pathway (Kharitonenkov *et al.*, 2005; Wente *et al.*, 2006). Surprisingly, there were no down-regulated liver-specific genes in VC-treated *sfx* mice.

Up-regulation of liver-specific genes in VC-treated *sfx* mice

Among the genes with altered expression, a group of genes with important functions in several diseases was found to be up-regulated (Figure 3B). Coagulation factor VIII (F8) was up-regulated in VC-treated *sfx* mice. An *F8* deficiency causes the bleeding disorder known as hemophilia A. Individuals develop a variable phenotype consisting of hemorrhaging in joints and muscles, easy bruising, and prolonged bleeding from wounds. Bleeding is one of the consequences of scurvy caused by a VC deficiency. The factor h-related gene 1 (*Cfhl1)* has recently been associated with a decreased risk of age-related macular degeneration

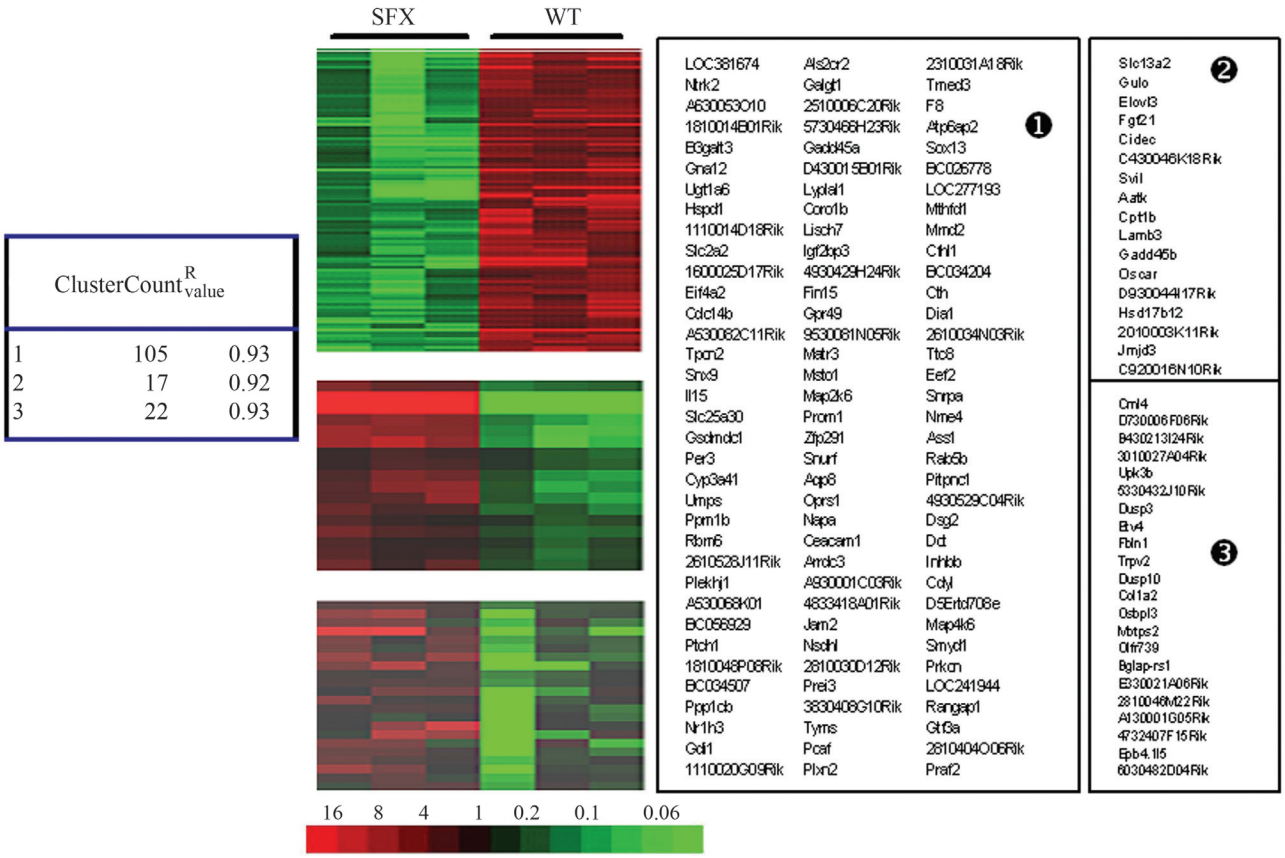

Figure 2 - Major clusters of differentially expressed genes in livers of VC-treated *sfx* mice. Three major clusters with a correlation coefficient (r) ≥ 0.9 and more than 10 transcripts were identified. The probe-to-transcript hybridization signal intensity is color-coded from low (green) to high (red). Genes with the same or similar color have the same or similar expression levels. The relative fold change for a transcript can be estimated from the color scale at the bottom of the figure.

Table 3 - Genes with altered expression in VC-treated *sfx* mice based on microarray analysis.

Change	Category	Gene number	p value	Genes affected*
Down-regulated	Signaling	19	1.40E-04	Upk3b, 17rb, Smpd1, Ly6d, Edn1, Gp5, Col1a2, Gdf15, fbln1, Lamb3, Htra3, **Fgf21**, Inhbe, Lrfn3, Chrna2, Gfra3, Cxcl13, Gpr172b, Bglap-rs1
	Extracellular region	23	5.50E-03	Mesdc2, Il17rb, Ly6d, Edn1, 9430028L06Rik, Gp5, Col1a2, Fbln1, Gdf15, Oscar, Cldn9, Adamts9, Fgf21, Htra3, Inhbe, Lamb3, Chrna2, Gfra3, 1110028A07Rik, Cxcl13, Gpr172b, Bglap-rs1, Slc13a2
	Protein binding	27	5.40E-03	Gadd45b, Cidec, Il17rb, Edn1, Trim6, Cldn9, Inhbe, Zfp295, Gfra3, Synpo, Meig1, **Birc5**, Plscr2, **Jun**, Svil, Gp5, 2610301F02Rik, Gdf15, Fbln1, Dtnb, Lamb3, Fgf21, Htra3, Myom1, Nolc1, Cxcl13, **Mdm2**
	Development	17	2.40E-03	Gadd45b, Mesdc2, Gtf2ird1, Edn1, Jun, 2610301F02Rik, Epb4.1l5, Htra3, Lor, Cbfa2t3h, Adra1a, Cxcl13, Birc5, Foxo1, Bglap-rs1
	Membrane	21	8.60E-04	Upk3b, Il17rb, Ly6d, 9430028L06Rik, Plscr2, Gp5, Svil, Evc2, Mas1, Cpt1b, Cldn9, Gulo, Trpv2, lrfn3, Chrna2, Myom1, Gfra1a, Elovl3, Gpr172b, Slc132
Up-regulated	Nucleotide binding	17	6.30E-04	Hspd1, Gna12, 1810048P08Rik, Als2cr2, BC034204, **Map2k6**, Igf2bp3, Rab5b, Ntrk2, Snrpa, Mthfd1, Nme4, Eef2, Eif4a2, Matr3, Prkcn, BC034507
	Biosynthesis	12	1.90E-03	Inhbb, **Cth**, B3galt3, Smyd1,Tyms, Dct, Umps, Mthfd1, Nme4, Eif4a2, Eef2, Nsdhl
	Membrane	19	1.20E-03	Gna12, Nsdhl, Slc2a2, Prss8, Aqp8, Mmd2, Ptch1, Dsg2, Prei3, Jam2, Praf2, Tmed3, B3galt3, Ntrk2, Prom1, Ceacam1, Atp6ap2, Dct, **Cyp3a41**
	Purine nucleotide binding	14	4.50E-03	Hspd1, Gna12, 1810048P08Rik, Als2cr2, BC034204, **Map2k6**, Rab5b, Ntrk2, Mthfd1, Nme4, Eef2, Eif4a2, Prkcn, BC034507

*The expression of genes marked in bold was also analyzed by quantitative RT-PCR.

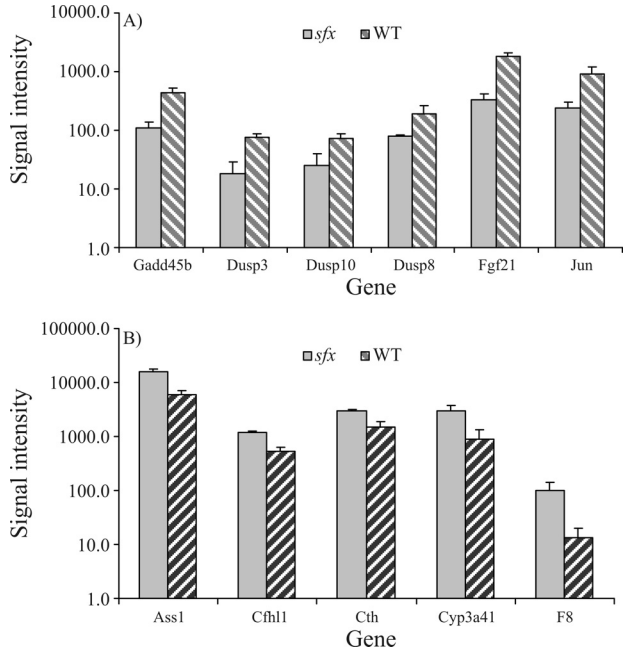

Figure 3 - Gene expression in WT Balb/c mice and VC-treated *sfx* mice based on microarray analysis. Down-regulation of genes involved in MAPK signaling (A) and up-regulation of liver-specific genes (B) in VC-treated *sfx* mice compared to WT mice. In both cases, the fold change is shown in each pair of columns for the given genes.

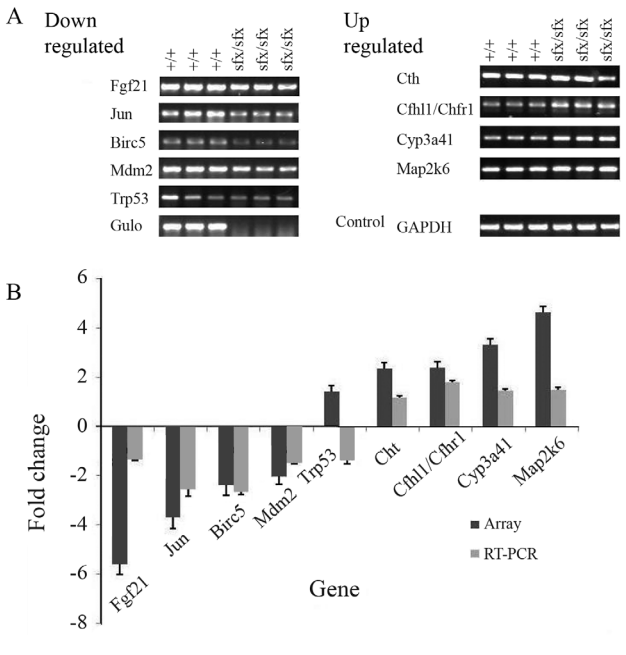

Figure 4 - Confirmation of selected gene expression levels by semi-quantitative RT-PCR. Nine genes with different expression patterns identified by microarray analysis were selected for validation of the microarray assay. A. PCR products from quantitative RT-PCR. B. Changes in expression levels measured by microarray and RT-PCR. The Y-axis shows the ratio (expressed as fold change) of gene expression between VC-treated *sfx* mice and normal (WT) mice for each gene and technique used.

(Venables *et al.*, 2006) whereas the cystathionine gammalyase (*Cth*) gene is important for transforming cystathionine into cysteine in the liver. Cytochrome p450, subfamily IIIa, polypeptide 41 (Cyp3a41) is a female-specific isoform that is predominantly expressed in the liver (Sakuma *et al.*, 2002). Estrogen signaling is not responsible for the female specificity of *Cyp3a41*, but its expression level is regulated by growth hormone (GH) signaling (Jarukamjorn *et al.*, 2006, 2007). However, female *sfx* mice supplied with small amounts of VC have an increased level of *Cyp3a41*. Argininosuccinate synthetase 1 (Ass1) is the major lipid A-interacting protein in the liver (Satoh *et al.*, 2006).

Semi-quantitative RT-PCR confirmation of microarray data

Although three WT and VC-treated *sfx* mice were used for the microarray experiments systematic errors may have biased the overall results. To confirm the validity of our findings, RT-PCR was done for nine genes: three liver-specific genes (*Cth*, *Cth11* and *Cyp3a41*) and mitogen-activated protein kinase 6 (*Map2k6*) were used as up-regulated genes, whereas two MAPK pathway genes (*Fgf21* and *Jun*) and three apoptosis related genes (*Birc5*, *Mdm 2* and *Trp53*) were used as down-regulated genes. *GAPDH* and *Gulo* were used as positive and negative controls, respectively. Figure 4 shows the electrophoretic profile of the RT-PCR products. Eight of the nine genes showed PCR amplification and confirmed the microarray results. The only exception was *Trp53*, which the microrarray data showed as being up-regulated 1.4-fold, while the RT-PCR showed that it was down-regulated (Chen *et al.*, 2009). *Cth*, *Cth11* and *Cyp3a41* were all up-regulated in the microarray analysis (Figure 3) and this was confirmed by RT-PCR (Figure 4). *Fgf21* and *Jun* were down-regulated in RT-PCR (Figure 4), in agreement with the microarray data (Figure 3). *Map2k6* was up-regulated 4.6-fold in the microarray assay, whereas RT-PCR revealed a lower amplification than in WT mice. *Birc5* and *Mdm 2* were down-regulated 2.4- and 2.1-fold, respectively, in the microarray assay, and their amplification by RT-PCR was also lower than in WT mice.

Transcriptomic loci and genes for oxidative proteins that regulate *Gulo*

Transcriptome mapping using data for gene expression in the livers of 46 RI mice led to the identification of two loci involved in regulating *Gulo* gene expression (Figure 5A). The major transcriptome locus was located on chromosome 18 (Figure 5A). Examination of the genes in the peak region between 13 Mbp and 23 Mbp identified 20 transcripts (Figure 5B). Investigation of the potential function of these genes identified three important oxidative-related genes, namely, *transthyretin*, *cadherin 2* and *aquaporin 4*. These three genes were located in the critical region of the locus (Figure 5B). This finding suggests that the down-regulation of any or all of these three oxidative

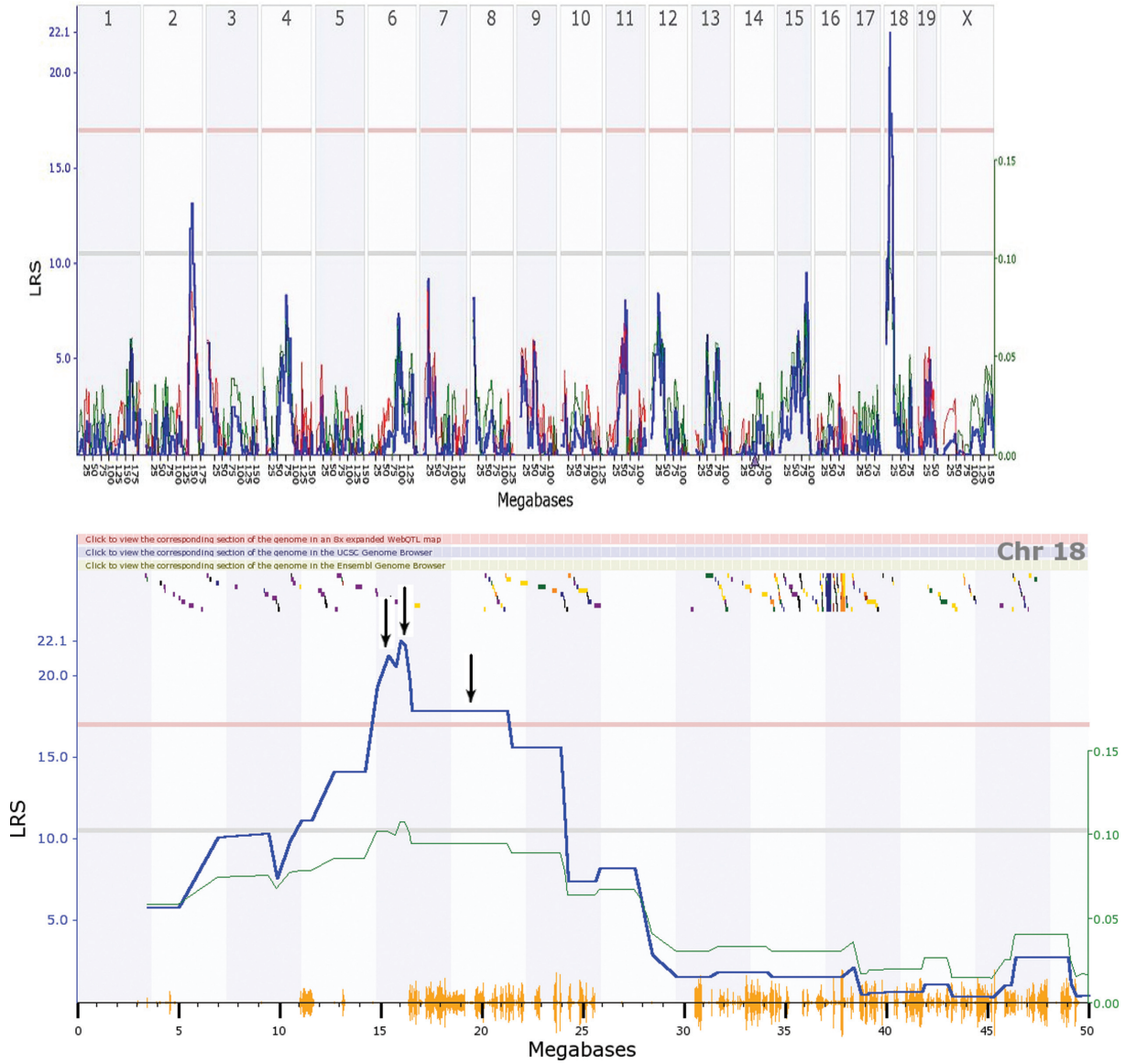

Figure 5 - Transcriptomic mapping of loci that regulate *Gulo* expression. A. Whole genome mapping revealed loci on chromosomes 2 and 18. B. Critical regions of chromosome 18 that regulate *Gulo* gene expression.

genes in the liver of VC-treated *sfx* mice results in increased oxidative stress.

Discussion

The results described here suggest that attention should be paid to how much VC is supplied in studies using *Gulo*-deleted mice. Our findings also raise questions about the influence that insufficient supplementary VC in *Gulo*-deleted mice has on studies using these mice. The growth of *sfx* mice given 500 mg of VC/mL in their drinking water was essentially normal. Of the various organs examined, only the spleen of *sfx* mice showed a significant difference in weight compared to WT. Another relevant question that this phenotype raises is whether lower than normal VC levels compromise the immune system. In a study using chickens, Wu *et al.* (2000) discovered that dietary supplementation with ascorbic acid ameliorated the immunosuppression caused by IBDV vaccination and improved humoral and cellular immune responses. We have previously shown that *sfx* mice have a much lower number of white blood cells compared to normal controls (0.83 x 10^9 *vs.* 2.16 x 10^9) (Plantinga *et al.*, 2007). These studies indicate the need for a careful investigation of the influence of a VC deficiency on the immune system.

The down-regulation of several genes in the *MAPK* pathway pointed to a potential molecular mechanism for the antioxidant activity of VC. The up-regulation of several liver- specific and/or highly expressed genes was alarming in view of the serious concern that a lack of VC in early

childhood may result in liver damage because of impairment of the intrinsic defense system.

Our data also raise the question of whether VC metabolism is similar in humans and mice, *i.e.*, are the pathways involved in the metabolism of endogenous and exogenous VC different? The major pathways of VC metabolism are probably similar in these two species, although there may be minor differences. Several of the 269 differentially expressed genes identified in this study encode for important proteins in oxidative pathways and liver metabolism and were expressed at different levels in WT and VC-treated mice. These findings support the notion of differences in VC metabolism. Previously, in a study using VC deficient mice, Lee *et al.* (2008) found that VC supplementation does not protect VC-deficient mice from *Helicobacter pylori*-induced gastritis and gastric premalignancy, at either low or high levels of VC supplementation. In their study, the high level of VC supplementation is 3,300 mg/L vitamin C in drinking water. As shown here (Figure 1), VC-treated sfx mice at 500 mg/L had a lower hepatic concentration of VC than WT mice, indicating that VC supplementation does not correct all of the changes in gene expression caused by a lack of endogenous VC. An obvious explanation for this could be that endogenous VC production in wild type mice is stimulated when required whereas in humans, VC availability is dependent on the food intake and may not reach tissues as rapidly as in mice. Together, these considerations suggest that VC-deficient mice provide a better model of the human condition than wild type mice.

The identification of a major locus and of three oxidative genes within this locus further emphasizes the importance of VC and *Gulo* in attenuating oxidative stress. In particular, transthyretin is a soluble human plasma protein. Inflammatory and oxidative stress pathways are triggered by fibrillar and non-fibrillar Transthyretin (TTR) deposits (Saraiya 2003; Sousa *et al.*, 2004; Tiahou *et al.*, 2004; Maleknia *et al.*, 2006). Aquaporin 4 has been implicated in astrocyte swelling during hepatic encephalopathy in response to oxidative stress and changes in the mitochondrial permeability transition (Bienert *et al.*, 2007; Rama and Norenberg, 2007). Further studies of the relationship between *Gulo*, *Ttr*, and *Aquaporin 4* may shed light on the mechanism by which VC regulates oxidative stress.

Animals lacking the *Gulo* gene would appear to be good models for understanding the molecular mechanisms of VC in humans since the latter also lack this gene. Selman *et al.* (2006) reported that life-long supplementation with VC does not affect the extent of oxidative damage or lifespan in C57BL/6 mice. However, it is unclear to what extent these findings with C57BL/6 mice can be extended to humans. If humans lack *Gulo* and cannot synthesize VC then how can true comparisons be made between humans and mice since WT mice have *Gulo* and consequently naturally produce VC? For this reason, comparative studies involving humans and rodents should use *Gulo*-deficient rodents in order to mimic the human condition as closely as possible.

The *sfx* mouse is not the first mouse model that lacks the *Gulo* gene, but it is the first such model to be used in a microarray analysis of the effects of VC deficiency on hepatic antioxidant function. Earlier mouse *Gulo* mutation models were used to examine other aspects of the biology of VC (Horio *et al.*, 2001; Hasan *et al.*, 2004; Telang *et al.*, 2007), partly because of the investigators different research interests and partly because of the unavailability of high throughput gene expression technologies at the time of these studies. With the introduction of new technologies and of genomic and bioinformatics tools many previously accepted aspects of the biology of VC may need to be re-examined in order to confirm their validity and introduce modifications where necessary.

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Internet Resources

Genenetwork (http://www.genenetwork.org/).
Scion Image software (http://rsb.info.nih.gov/nih-image).

Supplementary Material

The following online material is available for this article:

Table S1 - Differential gene expression in wild-type mice versus VC-treated Gulo-deficient mice.

This material is available as part of the online article from http://www.scielo.br/gmb

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