FEBS Letters 582 (2008) 3542–3546

Vitamin E dependent microRNA regulation in rat liver

Sonja Gaedicke[a], Xiangnan Zhang[b], Constance Schmelzer[a], Yijia Lou[b], Frank Doering[a], Jan Frank[a], Gerald Rimbach[a,*]

[a] *Institute of Human Nutrition and Food Science, Christian-Albrechts-University of Kiel, Hermann-Rodewald-Strasse 6, 24118 Kiel, Germany*
[b] *Institute of Pharmacology and Toxicology and Biochemical Pharmaceutics, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, China*

Received 3 September 2008; revised 11 September 2008; accepted 12 September 2008

Available online 24 September 2008

Edited by Barry Halliwell

Abstract **Dietary vitamin E (VE) is known to regulate gene expression by altering mRNA concentrations. Recently, microRNA (miRNA) have been discovered as a means of posttranscriptional gene regulation. Since the effect of VE on miRNA regulation is unknown, we fed rats for 6 months diets deficient or sufficient in VE and determined hepatic concentrations of miRNA involved in processes previously associated with VE (lipid metabolism, miRNA-122a; cancer and inflammation, miRNA-125b). VE-deficiency resulted in reduced concentrations of miRNA-122a and miRNA-125b. The findings of the present study demonstrate that differences in dietary VE may affect hepatic miRNA concentrations in vivo.**

Keywords: MicroRNA; Vitamin E; Tocopherol; Post-transcriptional gene regulation; Rat; Liver

1. Introduction

In rats, vitamin E (VE), particularly its major congener α-tocopherol (αT), has been shown to regulate gene expression by increasing or lowering mRNA concentrations in various tissues [1–6]. In mammals, mRNA levels often correlate poorly with the respective protein concentrations [7]. The recent discovery of microRNA (miRNA) revealed a possible explanation for this phenomenon. miRNA are a class of small, non-coding, double-stranded RNA that, in their mature form, are single-stranded and ~22 nucleotides long. Mature miRNA post-transcriptionally suppress gene expression by binding at the 3′ untranslated region of mRNA and inhibiting their translation into proteins [8]. It has been calculated that each miRNA binds on average 100 different target mRNA, allowing for post-transcriptional silencing of many different genes, or potentially entire pathways, by a single miRNA [9]. miRNA are encoded for in the genome and are, thus, liable to regulation. Presently, the potential regulatory effects of dietary VE on the expression of miRNA are unknown. In order to investigate the role of dietary VE on miRNA expression, we fed rats for 6 months diets deficient or sufficient in VE and analyzed miRNA concentrations in the liver. For this study, we selected miRNA that were previously shown to be involved in processes that have been associated with VE, namely lipid metabolism (miR-122a) [10,11], cancer progression and inflammation (miR-125b) [12–14].

2. Materials and methods

2.1. Experimental animals and diets

Two groups of 8 recently weaned male Fisher 344 rats (mean body weight, 51 ± 5 g; Charles River Laboratories, Sulzfeld, Germany) were randomly assigned to a VE-containing (VE^+) or a VE-deficient diet (VE^-). The composition of the semi-synthetic diets (ssniff Spezialdiaeten GmbH, Soest, Germany) was (g/kg diet): casein, 240; corn starch, modified, 480; glucose, 110; cellulose, 50; VE-free vitamin premix (E15313-2), 10; mineral premix (E15000), 60; rapeseed oil, 50. All VE in the diets originated from native or antioxidant-stripped rapeseed oils, respectively, and were as follows (mg/kg diet; analyzed by HPLC): VE^-: αT, <1; γT, <1; VE^+: αT, 12; γT, 24. Because consumption of oxidized lipids may alter gene expression [15], great care was taken to protect the used oils and diets from oxidation. Peroxide values of the oils were <0.5 mEq O_2/kg, indicating that no lipid oxidation had occurred. Butylated hydroxytoluene (Carl Roth GmbH, Karlsruhe, Germany; 200 mg/kg) was added to all oils as a preservative. Diets were vacuum-packed with oxygen absorber pads in polyethylene and stored at −20 °C. The animals had free access to tap water and the experimental diets throughout the experiment and were housed in pairs in a conditioned room (temperature, 22 ± 2 °C; relative humidity, 55%; 12 h light/dark cycle). The animal experiment was conducted in accordance with the German regulations on animal care and with permission of the responsible authority. Food consumption was recorded daily and animal weight weekly. At the end of the experiment, the rats were fasted for 12 h prior to CO_2-anaesthesia and decapitation. The liver was excized and dissected; one part was stored in RNAlater (Qiagen, Hilden, Germany) and the remainder snap-frozen in liquid nitrogen and stored at −80 °C until used.

2.2. Quantification of tocopherols

Tocopherols were quantified by HPLC with fluorescence detection as described by Augustin et al. [16].

2.3. Total RNA isolation

Total RNA including miRNA were extracted by using the miRNeasy® Isolation Kit (Qiagen). DNA digestion was performed with the RNase-Free DNase Set (Qiagen). The concentration of isolated RNA was determined spectrophotometrically by measuring the absorbance at 260 nm; purity was determined using the ratio of 260/280 nm and a ratio of 1.6–1.9 was considered acceptable. Total RNA aliquots were stored at −80 °C until PCR or TaqMan analysis.

2.4. Real-time qRT-PCR

Primer sequences for real-time quantitative RT-PCR experiments were designed with Primer3 software (version 0.4.0; http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primer pairs (Table 1) were obtained from MWG Biotech AG (Ebersberg, Germany). One-step qRT-PCR was carried out with the QuantiTec® SYBR®

*Corresponding author. Fax: +49 431 880 2628.
E-mail address: rimbach@foodsci.uni-kiel.de (G. Rimbach).

0014-5793/$34.00
doi:10.1016/j.febslet.2008.09.032

Table 1
Nucleotide sequences of primers used for the real-time qRT-PCR experiments

Gene	Primer sequence *forward* (5′–3′)	Primer sequence *reverse* (5′–3′)
β-Actin	GGG GTG TTG AAG GTC TCA AA	TGT CAC CAA CTG GGA CGA TA
CD36	AAG CAA GGT TGC CAT AAT TG	CCA AAC ACA GCA TAG ATG GA
5α-R1	TTA TGC TGA AGA CTG GGT GA	AAA TAG TTG GCT GCA GAT ACG
γ-GCS_m	TGT GTG ATG CCA CCA GAT TT	GCT TTT CAC GAT GAC CGA GT
αTTP	GCT TTT CAA ATT ACC CCA TC	GAT CCC ACG AAC TTT CAA TG
HO-1	GGG TGA CAG AAG AGG CTA AG	GCT GAT CTG GGA TTT TCC TC

Green RT-PCR kit (Qiagen). RT-PCR amplification was performed in a Rotor-Gene 3000 thermocycler (Corbett Research, Sydney, Australia). Relative mRNA concentrations are given as the ratios between the amount of the target gene and the housekeeping gene β-actin.

2.5. TaqMan analyses

TaqMan® MicroRNA Assays were obtained from Applied Biosystems (ABI, Foster City, CA, USA). miRNA quantification was performed as two-step RT-PCR. Reverse transcription was performed in a thermocylcer (Biometra, Goettingen, Germany) with specific miRNA primers from the TaqMan® MicroRNA Assays and reagents from the TaqMan® MicroRNA Reverse Transcription Kit. During the PCR step, PCR products were amplified from cDNA samples using the TaqMan MicroRNA Assay together with the TaqMan® Universal PCR Master Mix (ABI). PCR was performed in an Applied Biosystems 7300 Real-Time PCR System. Target sequences were as follows: hsa-miR-122a, UGGAGUGUGACAAUGGUGUUUGU; hsa-miR-125b, UCCCUGAGACCCUAACUUGUGA; hsa-miR-146a, UGAGAACUGAAUUCCAUGGGUU. Relative miRNA concentrations are given as the ratios between the amount of the target gene and the endogenous control snoRNA (Rat).

2.6. Western blot experiments in liver homogenates

Liver tissue (100 mg) was homogenized in RIPA buffer, lysates were purified by centrifugation (4 °C, 14000 × *g*, 30 min) and total protein concentrations in each lysate were quantified using a BCA Protein Assay Kit (Pierce). Total proteins of the lysate (40 μg per lane) were separated by SDS gel electrophoresis followed by transferring the proteins to a PVDF membrane. Samples were then incubated with the primary antibodies (diluted in 5% non-fat milk (1:1000)) and the secondary antibodies (Santa Cruz Biotechnology; anti-mouse horseradish peroxidase) in blocking buffer (anti-HO-1, 1:4000; anti-β-actin, 1:1000). The blots were exposed to Immun-Star Western Chemiluminescent Kit (Bio-Rad Laboratories, Hercules, CA, USA) and scanned with a Chemidoc (Bio-Rad). Digital images were captured and quantified using the Quantity-One system (Bio-Rad). Relative concentrations of proteins were quantified as the ratio between the amount of target protein and the amount of the housekeeping protein β-actin.

2.7. Statistical analyses

Statistical comparisons were made by means of a one-tailed unpaired *t*-test (normal distribution, homogeneity of variances), Welch-corrected *t*-test (heterogeneity of variances), or a Mann–Whitney *U*-test (skewed data) as appropriate using the software Instat 3 for Macintosh (version 3.0b; GraphPad Software, Inc., San Diego, CA).

3. Results and discussion

Neither symptoms of ataxia, a classical sign of severe VE-deficiency, nor differences in feed intake and life weight gain were observed in the animals fed VE-deficient (VE^-) or VE-sufficient (VE^+) diets for 24 weeks (data not shown), which is in accordance with previous findings [6]. As expected, hepatic αT concentrations were reduced in the VE^- rats (Fig. 1A), in agreement with the literature [17]. Differences in hepatic αT were previously shown to affect relative mRNA concentrations in the liver of rats [6]. In order to verify the differential expression of genes regulated by αT, we quantified relative mRNA

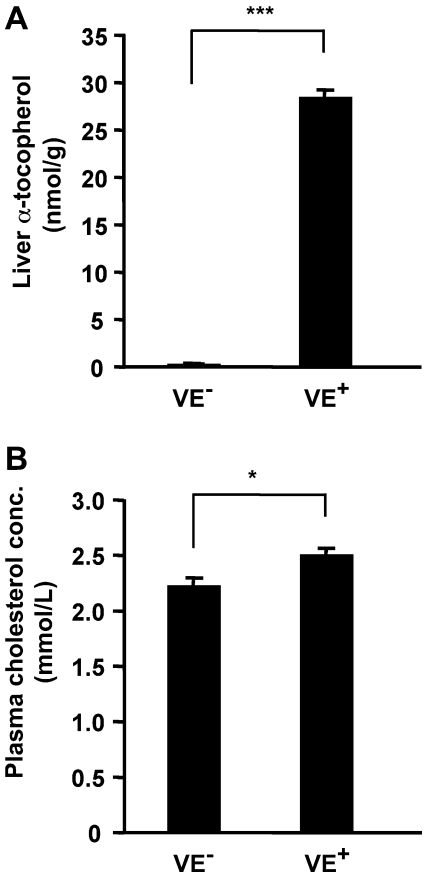

Fig. 1. Effect of dietary VE on αT concentrations in the liver (A) and plasma cholesterol concentrations (B) of rats fed VE-deficient (VE^-) or -sufficient (VE^+) diets for 6 months. Values are expressed as mean ± S.E.M., $n = 8$. $^*P < 0.05$, $^{***}P < 0.0001$.

concentrations of known VE sensitive genes in the livers of our rats. As expected, mRNA concentrations of CD36 and 5α-R1 were significantly increased, and mRNA concentrations of αTTP and γ-GCS_m were significantly decreased in the VE^- animals (Fig. 2).

Recently, miRNA have been discovered as molecules that effect post-transcriptional gene-silencing by a process termed RNA interference [18]. To date, the impact of dietary VE on hepatic miRNA concentrations is unknown. VE has long been investigated for its potential cardiovascular disease (CVD) and cancer preventive properties [19,20]. Increased circulating concentrations of blood lipids, especially cholesterol, and pro-inflammatory cytokines have been associated with increased risk for CVD and cancer [21,22]. For the current investigations, we selected two miRNAs (122a, 125b) that were shown to regulate genes associated with lipid metabolism and cancer.

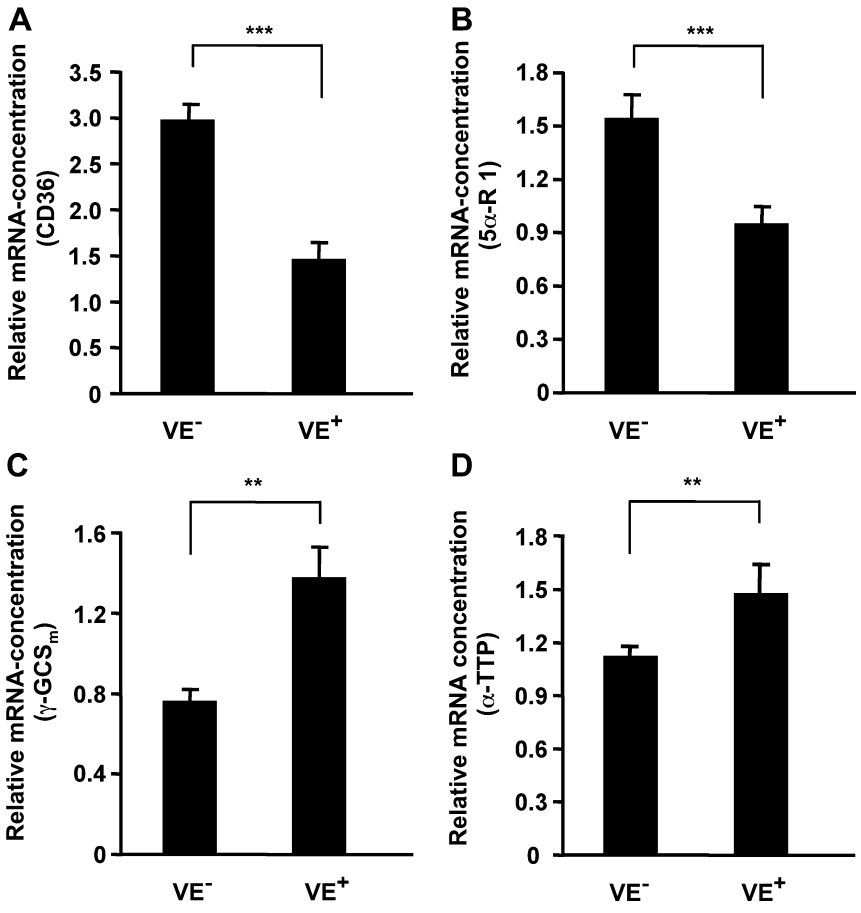

Fig. 2. Effects of dietary VE on scavenger receptor CD36, 5-α-steroid reductase type 1 (5α-R1), regulatory subunit gamma-glutamylcysteinyl synthetase (γ-GCS_m), and αT transfer protein (αTTP) mRNA concentrations in rat liver. Values are mean ± S.E.M., $n = 8$. $^{**}P < 0.001$, $^{***}P < 0.0001$.

Because miRNA are evolutionary conserved across species, the chosen miRNA are found in rat as well as in human tissue [23].

miR-122 is liver-specific and the most abundant miRNA in the liver [24]. In our rats, concentrations of miR-122a in the liver (Fig. 3) and cholesterol in plasma (Fig. 1B) were significantly lower in the VE^- than in the VE^+group. Esau and co-workers [10] used antisense oligonucleotide inhibition to specifically inhibit miR-122 in mice, which resulted in significantly increased hepatic concentrations of 108 mRNA related to lipid metabolism and significantly reduced plasma cholesterol and triacylglycerol concentrations. A comparable experiment recently supported the finding that cholesterol concentrations are only weakly reduced upon targeted inhibition of miR-122, although a large number of genes related to lipid metabolism were differentially regulated [25]. Apart from its role in lipid metabolism, miR-122 was down-regulated in rodent and human hepatocellular carcinomas [11] and is thought to play a critical role in liver development [26]. Shan and colleagues demonstrated that inactivation of miR-122 by antagomir 122, a chemically synthesized oligonucleotide with sequence complimentary to miR-122, resulted in decreased Bach-1 and increased HO-1 mRNA concentrations [27]. Bach-1 is a repressor of the redox-sensitive transcription factor nuclear factor erythroid 2-like 2 (Nrf2), which controls HO-1 expression [28]. In agreement, HO-1 mRNA as well as protein concentrations were increased in our VE-deficient rats (Fig. 4). Because oxidative stress is known to cause Nrf2 activation [29], the induction of HO-1 expression in our rats might also have occurred by a miR-122 independent mechanism.

VE-depletion in our rats caused significant, but relatively weak changes in miR-122a when compared to targeted inhibition [10,25]. In fact, dietary factors are not expected to exert drastic effects on miRNA expression. Nevertheless, even small changes in hepatic miRNA expression over a prolonged period may be of biological significance.

In our study, miR-125b was significantly down-regulated in the VE^- as compared to the VE^+ animals (Fig. 3). A role for miR-125b in cancer development has been suggested because miR-125b was down-regulated in human prostate cancer tissues [12,13], lung cancer cell lines [30], breast cancer [31], and in squamous cell carcinoma of the tongue [32]. Shi and colleagues [33], on the other hand, found over-expression of miR-125b in prostate cancer cell lines and prostate cancer samples. Retroviral infection of the breast cancer cell line SKBR3 with constructs expressing 125b resulted in ca. 35% suppression of the oncogenic proteins ERBB2 and ERBB3 at the transcript as well as protein levels, and the cells exhibited reduced migration and invasion capacities [34]. A role of miR-125b in inflammation is supported by a study that identified TNFα as a direct target of miR-125b. A decrease of miR-125b resulted in increased TNFα production and inflammation in LPS stimulated macrophages [35]. Thus, the reduced miR-125b levels ob-

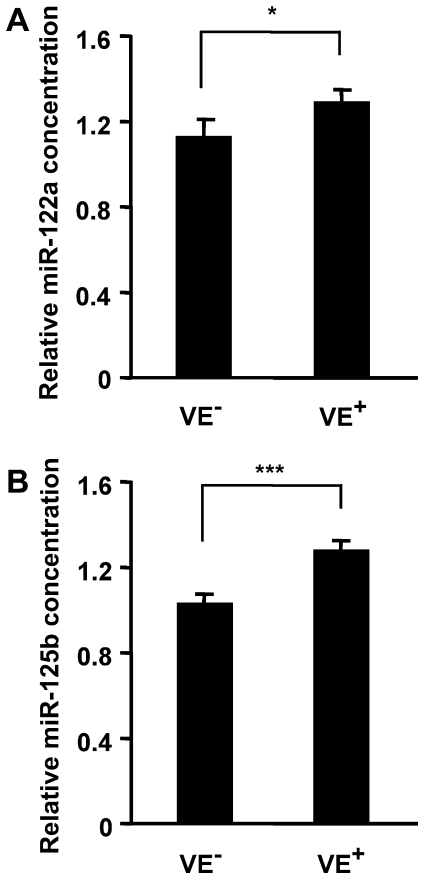

Fig. 3. Effects of dietary VE on miR-122a and miR-125b concentrations in rat livers. Values are mean ± S.E.M., $n = 8$. $^{*}P < 0.05$, $^{***}P < 0.0001$.

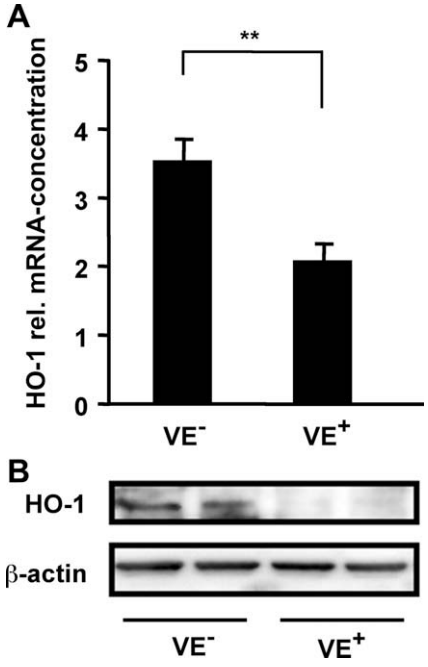

Fig. 4. Effects of dietary VE on (A) HO-1 mRNA concentration (values are mean ± S.E.M., $n = 8$, $^{**}P < 0.001$) and (B) protein levels of HO-1 and β-actin (loading control). Western blots of two representative animals per group are shown.

served in our VE-deficient rats may be associated with an enhanced inflammatory response due to VE-deficiency, as previously described [36,37].

In conclusion, in rats fed a VE-depleted diet for 6 months, VE-deficiency resulted in reduced concentrations of miR-122a and miR-125b, which may play an important role in lipid metabolism, carcinogenesis, and inflammation. Thus, the gene regulating properties of αT may be, at least partly, mediated by changes in miRNA levels.

Acknowledgements: SG is an associated member of the Ph.D. programme "Natural Antioxidants" (GRK 820) supported by the German Research Foundation (DFG). GR is supported by a grant (No. 528/051) from the Union zur Foerderung von Oel- und Proteinpflanzen (UFOP) e.V.

References

[1] Ricciarelli, R., Zingg, J.M. and Azzi, A. (2000) Vitamin E reduces the uptake of oxidized LDL by inhibiting CD36 scavenger receptor expression in cultured aortic smooth muscle cells. Circulation 102, 82–87.
[2] Vasu, V.T., Hobson, B., Gohil, K. and Cross, C.E. (2007) Genome-wide screening of alpha-tocopherol sensitive genes in heart tissue from alpha-tocopherol transfer protein null mice (ATTP(−/−)). FEBS Lett. 581, 1572–1578.
[3] Rota, C., Barella, L., Minihane, A.M., Stöcklin, E. and Rimbach, G. (2004) Dietary alpha-tocopherol affects differential gene expression in rat testes. IUBMB Life 56, 277–280.
[4] Rimbach, G., Fischer, A., Stoecklin, E. and Barella, L. (2004) Modulation of hepatic gene expression by alpha-tocopherol in cultured cells and in vivo. Ann. NY. Acad. Sci. 1031, 102–108.
[5] Hundhausen, C., Frank, J., Rimbach, G., Stoecklin, E., Muller, P.Y. and Barella, L. (2006) Effect of vitamin E on cytochrome P450 mRNA levels in cultured hepatocytes (HepG2) and in rat liver. Cancer Genom. Proteom. 3, 183–190.
[6] Barella, L. et al. (2004) Identification of hepatic molecular mechanisms of action of alpha-tocopherol using global gene expression profile analysis in rats. Biochim. Biophys. Acta: Mol. Basis Dis. 1689, 66–74.
[7] Nelson, P.T. and Keller, J.N. (2007) RNA in brain disease: no longer just "the messenger in the middle". J. Neuropathol. Exp. Neurol. 66, 461–468.
[8] Boyd, S.D. (2008) Everything you wanted to know about small RNA but were afraid to ask. Lab. Invest. 88, 569–578.
[9] Lewis, B.P., Burge, C.B. and Bartel, D.P. (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120, 15–20.
[10] Esau, C. et al. (2006) miR-122 Regulation of lipid metabolism revealed by in vivo antisense targeting. Cell Metab. 3, 87–98.
[11] Kutay, H., Bai, S., Datta, J., Motiwala, T., Pogribny, I., Frankel, W., Jacob, S.T. and Ghoshal, K. (2006) Down regulation of miR-122 in the rodent and human hepatocellular carcinomas. J. Cell Biochem. 99, 671–678.
[12] Ozen, M., Creighton, C.J., Ozdemir, M. and Ittmann, M. (2008) Widespread deregulation of microRNA expression in human prostate cancer. Oncogene 27, 1788–1793.
[13] Porkka, K.P., Pfeiffer, M.J., Waltering, K.K., Vessella, R.L., Tammela, T.L. and Visakorpi, T. (2007) MicroRNA expression profiling in prostate cancer. Cancer Res. 67, 6130–6135.
[14] Sonkoly, E. et al. (2007) MicroRNAs: novel regulators involved in the pathogenesis of Psoriasis? PLoS ONE 2, e610.
[15] Koch, A., Konig, B., Spielmann, J., Leitner, A., Stangl, G.I. and Eder, K. (2007) Thermally oxidized oil increases the expression of insulin-induced genes and inhibits activation of sterol regulatory element-binding protein-2 in rat liver. J. Nutr. 137, 2018–2023.
[16] Augustin, K., Blank, R., Boesch-Saadatmandi, C., Frank, J., Wolffram, S. and Rimbach, G. (2008) Dietary green tea polyphenols do not affect vitamin E status, antioxidant capacity and meat quality of growing pigs. J. Anim. Physiol. Anim. Nutr., doi:10.1111/j.1439-0396.2007.00768.

[17] Vatassery, G.T., Brin, M.F., Fahn, S., Kayden, H.J. and Traber, M.G. (1988) Effect of high doses of dietary vitamin E on the concentrations of vitamin E in several brain regions, plasma, liver, and adipose tissue of rats. J. Neurochem. 51, 621–623.
[18] Rana, T.M. (2007) Illuminating the silence: understanding the structure and function of small RNAs. Nat. Rev. Mol. Cell Biol. 8, 23–36.
[19] Jialal, I., Traber, M. and Devaraj, S. (2001) Is there a vitamin E paradox? Curr. Opin. Lipidol. 12, 49–53.
[20] Tucker, J.M. and Townsend, D.M. (2005) Alpha-tocopherol: roles in prevention and therapy of human disease. Biomed. Pharmacother. 59, 380–387.
[21] Belluco, C., Nitti, D., Frantz, M., Toppan, P., Basso, D., Plebani, M., Lise, M. and Jessup, J.M. (2000) Interleukin-6 blood level is associated with circulating carcinoembryonic antigen and prognosis in patients with colorectal cancer. Ann. Surg. Oncol. 7, 133–138.
[22] Scott, J. (2004) Pathophysiology and biochemistry of cardiovascular disease. Curr. Opin. Genet. Dev. 14, 271–279.
[23] Bartel, D.P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116, 281–297.
[24] Girard, M., Jacquemin, E., Munnich, A., Lyonnet, S. and Henrion-Caude, A. (2008) miR-122, A paradigm for the role of microRNAs in the liver. J Hepatol 48, 648–656.
[25] Elmen, J. et al. (2008) Antagonism of microRNA-122 in mice by systemically administered LNA-antimiR leads to up-regulation of a large set of predicted target mRNAs in the liver. Nucl. Acid Res. 36, 1153–1162.
[26] Chang, J. et al. (2004) miR-122, A mammalian liver-specific microRNA, is processed from hcr mRNA and may down regulate the high affinity cationic amino acid transporter CAT-1. RNA Biol. 1, 106–113.
[27] Shan, Y., Zheng, J., Lambrecht, R.W. and Bonkovsky, H.L. (2007) Reciprocal effects of micro-RNA-122 on expression of heme oxygenase-1 and hepatitis C virus genes in human hepatocytes. Gastroenterology 133, 1166–1174.
[28] Reichard, J.F., Motz, G.T. and Puga, A. (2007) Heme oxygenase-1 induction by NRF2 requires inactivation of the transcriptional repressor BACH1. Nucl. Acid Res. 35, 7074–7086.
[29] Itoh, K., Tong, K.I. and Yamamoto, M. (2004) Molecular mechanism activating Nrf2-Keap1 pathway in regulation of adaptive response to electrophiles. Free Radic. Biol. Med. 36, 1208–1213.
[30] Nagayama, K., Kohno, T., Sato, M., Arai, Y., Minna, J.D. and Yokota, J. (2007) Homozygous deletion scanning of the lung cancer genome at a 100-kb resolution. Genes Chromosome Cancer 46, 1000–1010.
[31] Iorio, M.V. et al. (2005) MicroRNA gene expression deregulation in human breast cancer. Cancer Res. 65, 7065–7070.
[32] Wong, T.S., Liu, X.B., Wong, B.Y., Ng, R.W., Yuen, A.P. and Wei, W.I. (2008) Mature miR-184 as potential oncogenic microRNA of squamous cell carcinoma of tongue. Clin. Cancer Res. 14, 2588–2592.
[33] Shi, X.B. et al. (2007) An androgen-regulated miRNA suppresses Bak1 expression and induces androgen-independent growth of prostate cancer cells. Proc. Nat. Acad. Sci. USA 104, 19983–19988.
[34] Scott, G.K., Goga, A., Bhaumik, D., Berger, C.E., Sullivan, C.S. and Benz, C.C. (2007) Coordinate suppression of ERBB2 and ERBB3 by enforced expression of micro-RNA miR-125a or miR-125b. J. Biol. Chem. 282, 1479–1486.
[35] Tili, E. et al. (2007) Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. J. Immunol. 179, 5082–5089.
[36] Lim, Y. et al. (2008) Severe vitamin E deficiency modulates airway allergic inflammatory responses in the murine asthma model. Free Radic. Res. 42, 387–396.
[37] Yamaoka, S., Kim, H.S., Ogihara, T., Oue, S., Takitani, K., Yoshida, Y. and Tamai, H. (2008) Severe vitamin E deficiency exacerbates acute hyperoxic lung injury associated with increased oxidative stress and inflammation. Free Radic. Res. 42, 602–612.