Up-Regulation of miR-34a by Zinc Deficiency
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Abstract
Mild-to-moderate zinc deficiency is common throughout the world. The biochemical changes associated with zinc deficiency have been extensively studied. However, the study of the regulation of microRNAs expression by zinc has just begun. MicroRNAs play a significant role in the regulation of gene function by binding to complementary regions of specific target mRNAs. MicroRNAs whose expression is altered in response to low zinc may play a role in adaptive responses to low zinc. Herein, the effect of low zinc intake on the expression of miR-34a in intestine, liver and thymus was analyzed in mice. In addition, the effect of low and excess zinc on the expression of miR-34a was analyzed in human hepatoma cells HepG2. Mice fed a low zinc diet exhibited low zinc content in tibia and increased expression of zinc transporter Slc39a4 in intestine. Moreover, the expression of miR-34a was increased in intestine and thymus of zinc deficient mice. The expression of miR-34a was also increased in HepG2 cells grown in low zinc medium. However, the expression of this microRNA was not affected by excess zinc. The up-regulation of miR-34a associated with zinc deficiency could be part of an adaptive response to cues generated in cells under low zinc conditions such as oxidative stress and inflammation.

Keywords: microRNAs; DMSO; RT-PCR

Introduction
Zinc deficiency is largely related to low zinc intake or poor absorption of zinc from the diet [1]. Severe or clinical zinc deficiency was defined as a condition characterized by short stature, hypogonadism, impaired immune function, skin disorders, cognitive dysfunction and anorexia [2]. Although severe zinc deficiency is rare, mild-to-moderate zinc deficiency is common throughout the world [3]. Recent estimates indicate that 31% of the world’s population is at risk of zinc deficiency [4]. The regulation of microRNAs expression by zinc has begun to be studied recently. MiR-31 and miR-21 were found to be upregulated in esophagus of zinc deficient mice [4]. In humans, mild zinc depletion caused decreased serum levels of miR-200b, miR-204, miR-296-5p, miR-145 and miR-375 [5]. MicroRNAs regulated by zinc levels in cells may act as mediators of the regulation of cellular processes by zinc. Moreover, microRNAs could also be involved in the regulation of zinc homeostasis [6]. We analyzed global microRNA expression profile in small intestine of young CD-1 mice fed both a zinc-sufficient or zinc-deficient diet and identified miR-34a (also known as miR-34a-5p) as the most up-regulated microRNA [7]. MiR-34a is considered as a tumor suppressor since its upregulation causes apoptosis and growth arrests in some cell lines [8]. In mammals, the family of miR-34 comprises 3 microRNAs; miR34a, miR-34b and miR-34c [8]. MiR-34a has its own transcript whereas miR-34b/c shares the same transcript. MiR-34a is expressed ubiquitous while miR-34b/c is prevalently expressed in the lungs [8]. Herein, we studied the regulation of miR-34a by zinc in mice and in human hepatoma cells HepG2 cells.

Methods
Cell culture and treatments
HepG2 cells were purchased from ATCC (American Type Culture Collection, Manassas, VA). Cells were grown in a 5% CO2-enriched atmosphere in DMEN medium containing 22 mM glucose supplemented with 10% fetal bovine serum, non-essential amino acids, 2 mM L-glutamine, 100 µg/ml streptomycin, and amphotericin B (Sigma, St. Louis, MO). Cells were seeded in 12 well plates (1 × 105 cells per well) and allowed to grow for 24h before treatment. In some experiments, cells were incubated with either the zinc chelator N,N,N’,N’-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) (4 µM) (Sigma) or vehicle Dimethyl sulfoxide (DMSO). Intracellular zinc depletion was also caused by incubating the cells in medium with fetal bovine serum treated with Chelex-100 (10% w/v) (BioRad, Hercules, CA) [9]. Chelex treatment did not affect the levels of iron in medium. Excess zinc condition was produced by treating cells with ZnCl2.

mRNA and microRNA quantitation by quantitative RT-PCR
Total RNA was isolated using TRizol reagent (Life Technologies, Grand Island, NY). Isolated RNA was treated with Turbo DNase (Life Technologies). MiR-34a levels were measured using TaqMan® MicroRNA Reverse Transcription Kit (Life Technologies) and predesigned primers for miR-34a and RNU6b (Life Technologies). Mouse Slc39a4 mRNA levels were measured as reported previously [10]. TaqMan® Gene Expression Master Mix (Life Technologies) was used to perform one-step PCR. MiR-34a and Slc39a4 values were normalized to levels of RNU6b and 18S rRNA respectively.

Diets and animals
CD-1 male mice (6 wk) were purchased from Charles River Laboratories (Wilmington, MA). Mice were housed individually in metabolic cages in a temperature- and humidity-controlled environment, and randomly assigned to either low zinc (<0.5 mg/kg) or zinc-adequate diet (30 mg/kg) (3 mice each group). According to power calculation, a sample size of 3 is sufficient to detect a 2 fold increase 80% of the time (http://www.statisticalsolutions.net/pss_calc.php).

Purified diets were purchased from Test Diets (Diets# 55VP and 55VQ, Purina, St. Louis, MO), and were custom prepared using a modified AIN-93G diet. Mice were fed the assigned diets for 21d. Food and water were provided ad libitum. The dietary intakes and body weights of all mice were monitored every 2 days throughout the entire study. At the termination of the experiments, mice were killed in the

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morning of day 22 by anesthesia overdose (Isoflurane (2-chloro-2- (difluoromethoxy)-1,1,1-trifluoro-ethane)) and tissues were harvested and stored in RNAlater (Life Technologies). The animal protocol was approved by the Institutional Laboratory Animal Care and Use Committee of Florida International University.

**Analysis of zinc content in tibia bone**

Bones were cleaned of all soft tissue, dried for 12 h at 105°C, and then extracted in a Soxhlet apparatus with petroleum ether for 48h, and ashed with muffle furnace at 450°C - 490°C for 12 hours. Ashed samples were digested by refluxing with HNO₃ over a 3-day period; digests were reconstituted with 2% HNO₃. Samples were analyzed by inductively coupled argon plasma emission spectrometry using a Thermo 6500 dual view ICP instrument (Thermo Fisher Scientific Inc. Waltham, MA 02454).

**Statistical analysis**

Data are presented as means ± S.D. One-way ANOVA and Student Newman-Keuls post-test were used for comparison of three groups. For comparison between two groups, Student’s t tests were used. Statistical significance was set at p<0.05.

**Results**

Low zinc intake did not affect food intake or growth (data not shown). However, tibia zinc content was decreased in mice fed low zinc diet (Figure 1). The expression of Slc39a4 mRNA in intestine, a gene known to be up-regulated by zinc depletion, was significantly increased in zinc deficient mice (Figure 2).

Low zinc intake increased expression of miR-34a in thymus and small intestine; however, the expression of miR-34a in liver was not significantly affected by zinc intake (Figure 3). In addition, the levels of miR-34a were increased in human hepatoma cells HepG2 cells grown in medium treated with the zinc chelators TPEN or Chelex (Figure 4A and 4B). Nevertheless, excess zinc did not influence the levels of miR-34a in cells (Figure 4B).

**Discussion**

The results from this study indicate that miR-34a expression is induced by intracellular zinc depletion. Marginal zinc deficiency in mice was confirmed by significantly lower tibia zinc and elevated expression of Slc39a4 in intestine, a zinc transporter that is known to be upregulated during zinc deficiency [10]. The stimulative effect of zinc deficiency on miR-34a expression was observed in mice and in two “in vitro” models of zinc deficiency. Currently, there is no ideal cell model to simulate zinc deficiency. Thus, the combination of experiments with Chelex-100 and TPEN is the best approach to obtain reliable results. To test the effect of zinc depletion on miR-34a expression, human hepatocyte cells HepG2 cells were employed. The HepG2 cell line has been used extensively and is a well recognized model of liver cells [11].

Interestingly, miR-34a expression was induced zinc depletion in HepG2 cells but not in liver in mice. It is possible that the zinc depletion caused by intake of low zinc diet was not as severe as the zinc depletion induced by growing the cells in medium treated with zinc chelators. The fact that mice did not become anorexic is an indication that they had a mild zinc deficiency rather than a severe zinc deficiency. Inasmuch as in humans mild zinc deficiency is more common than severe zinc deficiency, the model of zinc deficiency used in this study...
could be more relevant.

Of note, our results indicate that miR-34a expression was not affected by excess zinc in cells. This suggests that the overexpression of miR-34a caused by zinc depletion may not be directly mediated by zinc, but instead by cues generated in cells under low zinc conditions such as oxidative stress and inflammation [9,12]. These cues are known to create an environment that promotes cancer [13-14]. More studies are needed to confirm this assumption. If this hypothesis is correct, it is conceivable that the induction of miR-34a by zinc depletion is a mechanism to promote apoptosis and growth arrest in order to prevent carcinogenesis. Further research will determine whether miR-34a plays an important role in the prevention of tumor formation under low zinc conditions.