Introduction

Copper is an essential trace element and required for survival by a wide range of species, from yeast to mammals [1]. It functions as a cofactor and is required for structural and catalytic properties of a variety of enzymes because of its capacity to act as an intermediary in the transfer of electrons that makes it central to a variety of enzymes because of its capacity to act as an intermediary in the transfer of electrons that makes it central to many vital biological processes, such as cellular respiration and iron transport, required for growth and development [4]. Thus, Cu supplements were used to treat anemia in animals in the 1960s, and later in chicks [5], pigs [6], infants [7], and adult humans with good success [8].

Feeds provide most of the copper as an essential micronutrient consumed by animals, and drinking water contributes about 6–13% of average daily intake of copper [9,10,11,12]. Most of ingested copper is absorbed in the small intestine, and very small amounts in the stomach [10]. The absorption of copper in the body depends on a variety of factors including its chemical form [10]. Chelated copper has been proven to improve the utilization of copper, which is absorbed more efficiently through an amino acid transport system [13] by increasing intestinal absorption and renal tubular reabsorption of copper, and the chelated form displays increased retention in the body compared with its inorganic form [14,15,16], as has been demonstrated in many compounds, such as copper-lysine [17], organic copper chelates [18], copper carbonate [19] and copper-metallothionein (copper-MT) complex [15].

If inorganic coppers are transformed to copper-MT through binding by the MT produced endogenously by animals, such organic copper could be utilized effectively, and then lower doses of inorganic copper could be added in feed, which would, in turn, lead to reduced fecal copper contents. To investigate the feasibility of this hypothesis, we developed transgenic mice that secrete MT specifically as copper chelatin in yeast. For specific expression in the salivary glands by a promoter of gene coding pig parotid secretory protein, Transgenic CUP1 was highly expressed in the parotid and submandibular salivary glands and secreted in saliva as a 9-kDa copper-chelating protein. Expression of salivary copper-chelating proteins reduced fecal copper contents by 21.61% and increased body-weight by 12.97%, suggesting that chelating proteins improve the utilization and absorbed efficacy of copper. No negative effects on the health of the transgenic mice were found by blood biochemistry and histology analysis. These results demonstrate that the introduction of the salivary CUP1 transgene into animals offers a possible approach to increase the utilization efficiency of copper and decrease the fecal copper contents.

Abstract

Copper is required for structural and catalytic properties of a variety of enzymes participating in many vital biological processes for growth and development. Feeds provide most of the copper as an essential micronutrient consumed by animals, but inorganic copper could not be utilized effectively. In the present study, we aimed to develop transgenic mouse models to test if copper utilization will be increased by providing the animals with an exogenous gene for generation of copper chelatin in salvia. Considering that the S. cerevisiae CUP1 gene encodes a Cys-rich protein that can bind copper as specifically as copper chelatin in yeast, we therefore constructed a transgene plasmid containing the CUP1 gene regulated for specific expression in the salivary glands by a promoter of gene coding pig parotid secretory protein. Transgenic CUP1 was highly expressed in the parotid and submandibular salivary glands and secreted in saliva as a 9-kDa copper-chelating protein. Expression of salivary copper-chelating proteins reduced fecal copper contents by 21.61% and increased body-weight by 12.97%, suggesting that chelating proteins improve the utilization and absorbed efficacy of copper. No negative effects on the health of the transgenic mice were found by blood biochemistry and histology analysis. These results demonstrate that the introduction of the salivary CUP1 transgene into animals offers a possible approach to increase the utilization efficiency of copper and decrease the fecal copper contents.

Citation: Xie X, Ma Y, Chen Z, Liao R, Zhang X, et al. (2014) Transgenic Mice Expressing Yeast CUP1 Exhibit Increased Copper Utilization from Feeds. PLoS ONE 9(9): e107810. doi:10.1371/journal.pone.0107810

Editor: Vladimir V. Kalinichenko, Cincinnati Children’s Hospital Medical Center, United States of America

Received March 2, 2014; Accepted August 21, 2014; Published September 29, 2014

Copyright: © 2014 Xie et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by the National Transgenic Breeding Program (grant no.: 2014ZX08006-004; 2014ZX08009-003-006). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* Email: panyuchun1963@aliyun.com

† These authors contributed equally to this work.

Transgenic Mice Expressing Yeast CUP1 Exhibit Increased Copper Utilization from Feeds

Xiaoxian Xie*, Yufang Ma*, Zhenliang Chen, Rongrong Liao, Xiangzhe Zhang, Qishan Wang, Yuchun Pan*

School of Agriculture and Biology, Department of Animal Sciences, Shanghai Jiao Tong University, Shanghai, PR China, Shanghai Key Laboratory of Veterinary Biotechnology, Shanghai, PR China
these motifs occurs through the Cys residues [21,24]. In this study, we took advantage of the yeast CUP1 gene to establish a transgenic mouse model to determine whether endogenous expression of CUP1 can increase copper utilization by mice.

Materials and Methods

Ethics Statement
The FVB and ICR mouse varieties were used in this research. All animal procedures received approval from the Institutional Animal Care and Use Committee (IACUC) of Shanghai city, China. The mice were housed in the Animal Care Facility at Shanghai Jiao Tong University (IACUC permit numbers: SYXK (Shanghai) 2013-0052).

Construction of the recombinant plasmids expressing the CUP1 gene
A fragment, which contained the complete open reading frame (ORF) of the CUP1 gene, was synthesized based on the published sequence in GenBank (NM_001179185). The vector pPSP (pig parotid secretory protein) was a gift from Ning Li (College of Biological Sciences, China Agricultural University, Beijing, China). The recombinant plasmid pPSP-CUP1 was constructed by insertion of the fragment containing the ORF of the CUP1 gene, which was digested with As I (TaKaRa, Japan), into the same endonuclease-digested pPSP vector. The recombinant plasmid was confirmed by restriction analysis and DNA sequencing.

Transgene purification, quantification and pronuclear microinjection
The linear DNA fragment containing the pPSP promoter, signal peptide and CUP1 gene was obtained by digestion with Xho I and Not I and subsequently purified by agarose gel electrophoresis as described by Yin et al. [25]. DNA was resuspended in microinjection buffer, which consisted of 0.1 mmol/L ethylenediaminetetraacetic acid and 10 mmol/L Tris Cl (pH 7.4) at a concentration of 20 ng/μL, and stored at -20°C. The injection of transgene DNA was performed according to Hogan et al. [26].

Transgenic examination by PCR and southern blot
The presence of the CUP1 transgene in the transgenic founders and offspring was confirmed by PCR analysis of genomic DNA derived from tail biopsies and DNA sequencing. PCR was performed with specific primers (forward primer 5’TGTGTAAGCGTGGTAGGTGCTCATC 3’, reverse primer 5’GACACCTACTCAGACAATGCGATGC 3’), and the transgene length was 337 bp. The transgenic founders (G0) were confirmed by Southern blot analysis. Genomic DNA was isolated from the mouse tails using a ZR Genomic DNA-Tissue MiniPrep Kit (Zymo Research, USA). Twenty micrograms of DNA was confirmed by restriction analysis and DNA sequencing by insertion of the fragment containing the ORF of the CUP1 gene, which was digested with As I (TaKaRa, Japan), into the same endonuclease-digested pPSP vector. The recombinant plasmid was confirmed by restriction analysis and DNA sequencing.

Blood biochemistry and histology analysis
Blood samples of approximately 1 mL per mouse were obtained from the retro-orbital venous plexus of the transgenic and control mice using heparinized capillary tubes. Five mice at 6 wk of age and ten mice at 1 yr of age in each group were used for the blood biochemistry analysis. The blood samples were centrifuged at 3000 rpm for 10 min for the sera. The sera were stored at -80°C prior to blood biochemistry analysis. Nineteen blood biochemical parameters, including Ca (Calcium ion), Fe (ferrum ion), GLU (glucose), CRE (creatinine), CHO (cholesterol), BUN (blood urea nitrogen), AMY (amylase), ALT (alanine aminotransferase), AST (aspartate aminotransferase), and ALP (alkaline phosphatase), were detected using an auto-analyzer (Hitachi 7180, Hitachi, Japan). After blood drawing, the mice were sacrificed for histopathology analysis. Tissues (heart, liver, spleen, stomach, kidney, intestine, brain, parotid gland and submandibular gland) were collected and fixed in PBS buffered 10% formalin. The specimens, after paraffin
embedding, were sectioned horizontally at 5 μm thickness, stained with hematoxylin and eosin according to standard protocol, and observed using a microscope (Nikon, Japan) at an excitation wavelength of 559 nm.

**Statistical analysis**

The phenotypic data (the fecal ash copper contents and the body-weight increases of transgenic and control mice) were analyzed separately based on a general linear model (SAS 9.3):

\[
y_{ijk} = \mu + s_i + d_j + g_k + e_{ijk}
\]

Where

- \( \mu \): an overall mean
- \( s_i \): a fixed paternal effect
- \( d_j \): a fixed maternal effect
- \( g_k \): a fixed CUP1 gene effect
- \( e_{ijk} \): a residual error effect with a normal distribution N \((0, \sigma^2)\)

**Results**

**Generation of transgenic mice**

The 12.5-kb linear transgene pPSP-CUP1 (construction shown in Fig. S1) was generated by digestion with \(Xho\) I and \(Nol\) I and introduced into fertilized mouse oocytes through pronuclear injection.

Four male (No. 5, 6; FVB mice; No. 20, 22; ICR mice) and two female (No. 15: FVB mouse; No. 26: ICR mouse) transgenic founder (G0) mice obtained from 29 mice were confirmed by PCR screening and DNA sequencing among 46 G1 transgenic mice were confirmed by PCR amplification from genomic DNA and sequencing among 77 offspring (Table S1).

**Expression of yeast CUP1 transgene in the salivary glands**

The CUP1 transgene mRNA expression in the salivary glands of transgenic founder was analyzed by reverse transcription PCR. The results revealed that CUP1 was expressed in the parotid and submandibular glands and was barely expressed in the heart, liver, spleen, stomach, kidney, intestine, and brain tissue of the transgenic founders. However, the CUP1 gene was not expressed in all tissues of the control mice (Fig. S2).

The CUP1 protein was detected in the parotid and submandibular glands using anti-CUP1 antibodies to probe western blot analysis. The level of β-actin in each sample was determined as the control for protein loading. The results indicated the presence of CUP1 in both detected tissues and indicated relatively high expression in the submandibular glands after normalization against β-actin. In contrast, relatively low expression was observed in the parotid glands (Fig. 1B). CUP1 protein was also detected in the salivary fluid of the transgenic mice by western blot analysis (Fig. 1B), and no CUP1 protein was detected in the saliva of the control mice. The molecular mass of the protein containing CUP1 was 9 kDa as identified in the salivary glands and the secreted saliva.

**The contents of copper in mouse manure ash and changes of body weight**

Forty-four G1 offspring were selected from the total G1 mice considering similar weights and used for further experiments, of which 28 were transgenic mice, and 16 were control mice. To determine the effect of the expressed CUP1 on the transgenic mice, the usage efficiency of copper in the prepared feed was investigated by detecting the contents of copper in mouse manure from the transgenic and control mice at a dietary level of 10 mg/kg copper. For the first week, the transgenic mice exhibited manure ash copper contents of 168.285 ± 18.849 mg/kg, a reduction of 18.41% \((P = 0.0022<0.01)\) compared with the control mice (206.263 ± 42.307 mg/kg) raised under the same conditions. At the second week, the manure ash copper contents of 171.449 ± 10.767 mg/kg were significantly lower \((P = 0.0003<0.01)\) compared with that (218.713 ± 49.831 mg/kg) of the control mice. This represents a reduction of 21.61% in the ash copper contents under the same conditions (Fig. 2A). The effect of the expressed CUP1 on body weight of the transgenic mice was also analyzed. At day 0, the body weight of the control group was 20.531 ± 1.099 g and that of the transgenic group was 20.835 ± 1.214 g. After 1 wk, the body-weight increases of the transgenic mice (6.906 ± 0.998 g) were significantly greater \((P = 0.025<0.05)\) compared with those (5.063 ± 1.214 g) of the control mice (Fig. 2B). On average, the transgenic mice were 7.2% heavier than the control mice raised under the same conditions.
After 2 wk, the body-weight increases of the transgenic mice (17.884 ± 0.728 g) were significantly greater (P = 0.019, 0.05) compared with those (13.475 ± 1.556 g) of the control mice (Fig. 2B). On average, the transgenic mice were 12.97% heavier than the control mice raised under the same conditions.

Analysis of blood biochemistry and histology

The blood biochemistry results revealed that the levels of GLU, BUN, AMY, ALT, AST, and ALP in serum were slightly elevated in the transgenic mice at 6 wk of age compared with the control mice. In contrast, the blood concentration of CRE was slightly decreased, and all differences were not significant (P > 0.05; Table 1). The similar results were observed in the mice at 1 yr of age, but the level of ALP was slightly decreased in the transgenic mice (Table S2). Differences between the transgenic and control mice were hardly observed for other serum biochemical parameters.

At the ages of 6 wk and 1 yr, the transgenic mice were in good health and did not exhibit any gross pathological abnormalities or illness. Histological analysis was performed in the tissues (heart, liver, spleen, stomach, kidney, intestine, brain, parotid gland, and submandibular gland) of the mice, and no obvious changes were observed in the tissues of the transgenic mice compared with those of the control mice, and the results were shown in Fig. 3 and Fig. S3, respectively.

Discussion

Metallothionein is a highly conserved family of closely related proteins.

Table 1. Blood biochemistry results in the transgenic and control mice at 6 wk of age.

| Parameter | Transgenic | Control |
|-----------|------------|---------|
| ALB (g/L) | 18.167 ± 1.002 | 18.400 ± 1.365 |
| GLOB (g/L) | 24.333 ± 0.577 | 24.5 ± 2.082 |
| A/G | 0.757 ± 0.038 | 0.7 ± 0.026 |
| TP (g/L) | 42.2 ± 1.323 | 42.967 ± 3.362 |
| GLU (mmol/L) | 5.165 ± 0.323 | 4.335 ± 0.458 |
| CHO (mmol/L) | 2.133 ± 0.153 | 1.717 ± 0.097 |
| TG (mmol/L) | 1.3 ± 0.386 | 1.347 ± 0.375 |
| Ca (mmol/L) | 1.58 ± 0.115 | 1.43 ± 0.076 |
| Fe (mmol/L) | 36.833 ± 1.501 | 33.767 ± 3.465 |
| HDL (mmol/L) | 1.513 ± 0.101 | 1.533 ± 0.163 |
| LDL (mmol/L) | 0.463 ± 0.148 | 0.427 ± 0.154 |
| UA (μmol/L) | 187.5 ± 25.03 | 176.867 ± 15.689 |
| BUN (mmol/L) | 6.6 ± 0.917 | 5.567 ± 0.586 |
| CRE (μmol/L) | 3.145 ± 0.518 | 6.76 ± 0.679 |
| LDH (U/L) | 1165.867 ± 1173.167 | 1155.867 ± 1161.645 |
| AMY (U/L) | 240.333 ± 195.493 | 230.433 ± 190.493 |
| ALT (U/L) | 180.333 ± 153.047 | 170.433 ± 149.047 |
| AST (U/L) | 130.633 ± 15.314 | 120.633 ± 14.314 |
| ALP (U/L) | 64.5 ± 8.839 | 62.6 ± 7.839 |

The differences between the transgenic (n = 5) and control mice (n = 5) were not significant (P > 0.05) for all examined serum biochemical parameters.

The differences between the transgenic (n = 5) and control mice (n = 5) were not significant (P > 0.05) for all examined serum biochemical parameters.

ALB: albumin; GLOB: globulin; A/G: ALB/GLOB; TP: total protein; GLU: glucose; CHO: cholesterol; TG: triglyceride; Ca: calcium ion; Fe: ferrum ion; HDL: high density lipoprotein; LDL: low-lipid lipoprotein; UA: uric acid; BUN: blood urea nitrogen; CRE: creatinine; LDH: lactate dehydrogenase; AMY: amylase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase.

doi:10.1371/journal.pone.0107810.t001
Yeast CUP1 is a member of the MT family and accounts for copper-binding in *S. cerevisiae* [31]. Mammals have the MT gene, and it possesses multiple isoforms [32]. In this study, we took advantage of yeast CUP1 for transgene as it shares functional sequence identity to mammalian MT. Thus, yeast CUP1 binds to copper through Cys residues by the formation of metal-thiolate linkages, as well as the mammalian MT proteins [33], suggesting that this gene may be effective in copper-binding in mammals. This gene has been used for transgene in some organisms. Yeast CUP1 was introduced to tobacco plants, and its expression contributed to copper content because of its role in copper-binding [32]. In *Drosophila*, this gene was selected as the transgene instead of the endogenous Mtn gene, which has a similar structure and function with yeast CUP1, to determine its role in binding copper [34].

Many researchers have used the promoter of the mouse salivary gland-specific PSP gene to express the exogenous genes such as phytase gene in the saliva of transgenic mice, which has been confirmed to be feasible [25,35,36]. A similar phenomenon was examined in our results, and constitutive expression in the PSP/CUP1 mice was notably specific for the parotid and submandibular glands. A three-fold higher expression was detected in submandibular glands compared with the parotid glands, and a similar phenomenon was detected by Mikkelsen et al. (1992) [37], and the opposite observation was reported by Golavan et al. (2001) [36].

Copper as a feed additive is effective in growth enhancement and disease prevention in weanling pigs, and it is widely used in pork production around the world, especially in China [38,39]. However, copper in pig diets heavily exceeds the minimum requirements for normal performance (5–25 mg/kg copper for different classes of pigs) [40], and most of the ingested copper, acting as promotants, by pigs is excreted in the manure (>90%) [41]. The concentrations of copper in pig manure are 5–12 times those in pig feeds with additives [42], which are higher than for other agricultural animals, such as cattle and sheep [43]. The application of pig manure directly onto agricultural land as fertilizer is common practice in China [39]. Because of copper’s low mobility and non-degradation, copper can accumulate in soils [44], which leads to environmental consequences. When manure is repeatedly applied as fertilizer, copper can cause surface pollution with severe biological consequences, e.g., causing toxicity to plants, elevating bacterial resistance to toxic metals and increasing human exposure to copper via the food chain [39,40,45]. Still, the present inputs of copper are too high and reducing the contents of copper in the diet should reduce concentrations in the pig manure [46]. In the present study, we validated a method to increase copper utilization efficiency and to decrease the fecal copper content by providing the animals with an exogenous gene for generation of copper chelatin in the saliva. We determined that this approach can reduce mouse fecal copper content by 21.61%. Therefore, this might provide an important clue for preventing the pollution caused by the fecal copper in the pig production.

In addition, we reduced the dietary supply of copper with a 10 mg/kg concentration and demonstrated its feasibility for decreasing fecal copper content. Similar results were observed by Jondreville et al. (2003) [47]. The CUP1 transgene mice, at a dietary level of 10 mg/kg copper, displayed a body weight-increase response, with the transgene mice 12.97% heavier.

Figure 3. Histological analysis of the tissues of the transgenic and control mice. The heart, liver, spleen, stomach, kidney, small intestine, large intestine, brain, parotid gland, and submandibular gland tissue samples from the transgenic mice (transgenic; n = 5) and control mice (n = 5) at 6 wk of age were analyzed by histology observation. In the above pictures, α and γ are whole tissues, and β and δ are amplified regions of the tissues. The length of the scale bar is 100 μm in all micrographs. The profiles of the tissues of the transgenic and control mice were determined. No obvious changes were observed in the tissues of the transgenic mice compared with those of the control mice.

doi:10.1371/journal.pone.0107810.g003
compared with the control mice after 2 wk. These results are
similar to those obtained when animals were fed 250 mg/kg of
dietary copper [40]. The decreased fecal copper contents and
increased body-weight increases caused by the transgenic yeast
CUP1 suggest that the CUP1 transgene most likely enhanced the
usage of copper in the diet. Copper is able to stimulate the
secretion of several neuropeptides and growth hormones [49], in
addition to being a component of the growth factor IGF [48].
Therefore, copper could influence the growth regulatory system in
many ways and might be the main reason for the growth
stimulation.

No significant difference was found in a range of markers and
the histology of tissues of the transgenic mice compared with the
controls. These results suggest that the CUP1 transgene did not
affect the blood composition and histology of the mice. The
transgenic mice were confirmed to be in good health and did not
exhibit any gross pathological abnormalities or illness.

In summary, we have demonstrated that the repertoire of
copper-chelating proteins produced by a model animal can be
modified by introduction of CUP1 transgene into its genome. The
salivary copper-chelating proteins in these mice lead to a
significant reduction of fecal copper levels and a significant
increase of body weight, suggesting the enhancement of the
utilization efficiency of the dietary copper by transgenic mice. Our
findings provide the essential data toward elucidating the
physiological functions of MT gene on copper metabolism.

Supporting Information

Figure S1 Construction of the recombinant plasmids expressing CUP1 gene and confirmation by PCR and restriction. [A] The recombinant plasmid pPSP-CUP1 was constructed by insertion of the fragment containing the ORF of the CUP1 gene into the same endonuclease-digested pPSP vector.

(B) The recombinant plasmid was confirmed by restriction analysis, DNA sequencing, and by PCR.

Figure S2 RT-PCR analysis of yeast CUP1 transgene expression. (A, B) The CUP1 transgene mRNA expression was analyzed by RT-PCR in the salivary glands of the transgenic founders and the control mice, respectively.

Figure S3 Histological analysis of the tissues of the transgenic and control mice at 1 yr of age. The heart, liver, spleen, stomach, kidney, small intestine, brain, parotid gland, and submandibular gland tissue samples from the transgenic mice (transgenic; n = 10) and control mice (n = 10) at 1 yr of age were analyzed by histology observation. In the above pictures, α and γ are whole tissues, and β and δ are amplified regions of the tissues. The length of the scale bar is 100 μm in all micrographs. The profiles of the tissues of the transgenic and control mice were determined. No obvious changes were observed in the tissues of the transgenic mice compared with those of the control mice.

Table S1 Generation of G1 transgenic mice.

Table S2 Blood biochemistry results in the transgenic and control mice at 1 yr of age.

Author Contributions

Conceived and designed the experiments: YP XX. Performed the experiments: XX YM ZC RL. Analyzed the data: QW XX XZ. Contributed reagents/materials/analysis tools: QW XX. Wrote the paper: YP XX.

References

1. Pena MM, Lee J, Thiele DJ (1999) A delicate balance: homeostatic control of copper uptake and distribution. J Nutr 129: 1251–1260.
2. Shiels ME, Shike M (2006) Modern nutrition in health and disease. Lippincott Williams & Wilkins.
3. Gambling L, Kennedy C, McArdle HJ (2011) Iron and copper in fetal development. Semin Cell Dev Biol 22: 637–644.
4. Gaetke LM, Chow CK (2003) Copper toxicity, oxidative stress, and antioxidant nutrients. Toxicology 190: 147–163.
5. Gehrke G, Hart E (1929) The relation of iron and copper to hemoglobin synthesis in the chick. J Biol Chem 84: 131–141.
6. Gehrke G, Hart E (1932) The necessity of copper as a supplement to iron for hemoglobin formation in the pig. J Biol Chem 95: 365–370.
7. Gehrke G, Guckes D, Mendelssohn DR (1937) Iron versus iron and copper in the treatment of anemia in infants. Am J Dis Child 53: 785–793.
8. Harris ED (2003) Basic and clinical aspects of copper. Crit Rev Clin Lab Sci 40: 547–586.
9. Sandstead HH (1995) Requirements and toxicity of essential trace elements, illustrated by zinc and copper. Am J Clin Nutr 61: 621S–624S.
10. Turnlund JR, Scott KC, Pfeiffer GL, Jamal AM, Keyes WR, et al. (1997) Copper status of young men consuming a low-copper diet. Am J Clin Nutr 65: 72–78.
11. Fitzerfeldt HJ (1989) Safety guidelines for copper in water. Am J Clin Nutr 67: 1098S–1102S.
12. Potykja J, Ballou ER, Childers DS, Brown AJ (2014) Conflicting Interests in the Pathogen-Host Tug of War: Fungal Micronutrient Scavenging Versus Mammalian Nutritional Immunity. PLoS Pathog 10: e1003910.
13. Jacob RA, Skala JA, Omary ST, Turnlund JR (1987) Effect of varying ascorbic acid intakes on copper absorption and ceruloplasmin levels of young men. J Nutr 117: 2109–2115.
14. Coffey R, Cromwell G, Moneyer H (1994) Efficacy of a copper-lysine complex as a growth promoter for weanling pigs. J Anim Sci 72: 2680–2686.
15. Bunch R, McColl J, Speer V, Hays V (1963) Copper supplementation for weanling pigs. J Anim Sci 24: 995–1000.
16. Bremner I (1987) Involvement of metallothionein in the hepatic metabolism of copper. J Anim Sci 117: 19–29.

18. Apgar G, Kornegay E, Lindemann M, Notter D (1995) Evaluation of copper sulfate and a copper lysine complex as growth promoters for weanling swine. J Anim Sci 73: 2680–2686.
19. Stanbury W, Trumble I, Orr D (1990) Effect of chelated copper sources on performance of nursery and growing pigs. J Anim Sci 68: 1318–1322.
20. Armstrong T, Cook D, Ward M, Williams C, Spears J (2006) Effect of dietary copper source (cupric citrate and cupric sulfate) and concentration on growth performance and fecal copper excretion in weanling pigs. J Anim Sci 82: 1234–1244.
21. Kaigs JH, Vallee BL (1960) Metallothionein: a cadmium-and zinc-containing protein from equine renal cortex. J Biol Chem 235: 3346–3365.
22. Ecker DJ, Butt T, Sternberg E, Neeper M, Debouck C, et al. (1986) Yeast metallothionein function in metal ion detoxification. J Biol Chem 261: 16055–16060.
23. Jensen LT, Howard WR, Strain JJ, Winges DR, Caluotta VC (1996) Enhanced effectiveness of copper ion buffering by CUP1 metallothionein compared with CRNS metallothionein in Saccharomyces cerevisiae. J Biol Chem 271: 18514–18519.
24. Yin H, Fan B, Yang B, Liu Y, Luo J, et al. (2006) Cloning of pig parotid secretory protein gene upstream promoter and the establishment of a transgenic CRS5 metallothionein in Saccharomyces cerevisiae. J Biol Chem 271: 18514–18519.
29. Hu Y, Nakagawa Y, Purushotham KR, Humphreys-Beber MG (1992) Functional changes in salivary glands of autoimmune disease-prone NOD mice. Am J Physiol- Endocrinol Metab 263: E607–E614.
30. Le J, Nie Z-K, Zhang J-L, Lau F-Y, Wang Z-Z, et al. (2013) Corn Peptides Protect Against Thioacetamide-Induced Hepatic Fibrosis in Rats. J Med Food 16: 912–919.
31. Richards MP (1989) Recent developments in trace element metabolism and function: role of metallothionein in copper and zinc metabolism. J Nutr 119: 1062–1070.
32. Thomas JC, Davies EC, Malick FK, Endreszl C, Williams GR, et al. (2003) Yeast metallothionein in transgenic tobacco promotes copper uptake from contaminated soils. Biotechnol Prog 19: 273–280.
33. Kaege JH, Schaeffer A (1988) Biochemistry of metallothionein. Biochemistry 27: 8509–8515.
34. Meyer JL, Hoy MA, Jeyaprakash A (2006) Insertion of a yeast metallothionein gene into the model insect Drosophila melanogaster (Diptera: Drosophilidae) to assess the potential for its use in genetic improvement programs with natural enemies. Biol Control 36: 129–138.
35. Madken HO, Hjorth JF (1985) Molecular cloning of mouse PSP mRNA. Nucleic Acids Res 13: 1–13.
36. Golovan SP, Hayes MA, Phillips JP, Forsberg CW (2001) Transgenic mice expressing bacterial phytase as a model for phosphorous pollution control. Nat Biotechnol 19: 429–433.
37. Mikkelesen TR, Brandt J, Larsen HJ, Larsen BB, Poulsen K, et al. (1992) Tissue-specific expression in the salivary glands of transgenic mice. Nucleic Acids Res 20: 2249–2255.
38. Cromwell GL, Stahly TS, Monerue HJ (1989) Effects of source and level of copper on performance and liver copper stores in weanling pigs. J Anim Sci 67: 2996–3002.
39. Xiong X, Xuxia L, Wei L, Chunye L, Wei H, et al. (2010) Copper content in animal manures and potential risk of soil copper pollution with animal manure use in agriculture. Resour Conserv Recy 54: 985–990.
40. de Lange K, Nyachoti M, Birker S (1999) Manipulation of diets to minimize the contribution to environmental pollution. Adv Pork Prod 10: 173–186.
41. Delahaye R, Fong P, Van Ezendt M, Van der Hoek K, Olthoorn C (2003) Emissie van zevent metalen naar landbouwgrond. CBS, Voorburg/Heerlen.
42. Eohe H, Sekimoto H (1999) A survey of the contents of heavy metals in blended feeds, feces and composts of swine. Jpn. J Soil Sci Plant Nutr 70: 39–44.
43. Ogita M, Sakamoto K, Suzuki H, Ushio S, Anzai T, et al. (2005) Accumulation of zinc and copper in an arable field after animal manure application. Soil Sci Plant Nutr 51: 801–808.
44. Graber I, Hansen JF, Olsen SE, Petersen J, Ostergaard H, et al. (2005) Accumulation of copper and zinc in Danish agricultural soils in intensive pig production areas. Geografisk Filskri-Danish J 105: 15.
45. Poulsen HD (1998) Zinc and copper as feed additives, growth factors or unwanted environmental factors. J Anim Feed Sci 7: 133–142.
46. Aarnink A, Verstegen M (2007) Nutrition, key factor to reduce environmental load from pig production. Livest Sci 109: 194–203.
47. Jondreville C, Revy P, Dourmad J (2003) Dietary means to better control the environmental impact of copper and zinc by pigs from weaning to slaughter. Livest Prod Sci 84: 147–156.
48. Zhou W, Kornegay ET, Lindemann MD, Swincke JW, Welten MK, et al. (1994) Stimulation of growth by intravenous injection of copper in weanling pigs. J Anim Sci 72: 2393–2403.
49. Tsou R, Daley R, McLanahan C, Parent A, Tindall G, et al. (1977) Luteinizing hormone releasing hormone (LHRH) levels in pituitary stalk plasma during the preovulatory gonadotropin surge of rabbits. Endocrinology 104: 534–539.