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Copper elevated embryonic hemoglobin through reactive oxygen species during zebrafish erythrogenesis

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ABSTRACT

Copper, as an essential trace mineral, can cause diseases such as childhood leukemia at excess levels, but has been applied in anemia therapy for a long time. However, few reports have studied its role during hematopoiesis at the molecular level in an animal model. In this study, by microarray, qRT-PCR, whole-mount *in situ* hybridization and *O*-dianisidine staining detections, we revealed the increased expression of hemoglobin in copper-exposed embryos. Secondly, we found that copper-exposed embryos exhibited high levels of reactive oxygen species (ROS), and genes in oxygen binding and oxygen transporting were up-regulated in the embryos. Finally, we found that ROS scavengers NAC, GSH, and DMTU not only inhibited *in vivo* ROS levels induced by copper, but also significantly decreased high expression of hemoglobin back to almost normal levels in copper exposed embryos, and also helped with copper elimination from the embryos. Our data first demonstrated that ROS mediated copper induced hemoglobin expression in vertebrates, partly revealing the underlying molecular mechanism of copper therapy for anemia. Moreover, we revealed that copper homeostasis was broken by its induced ROS and ROS helped with copper overloading in the body, which could be applied as a novel therapy target for copper-caused diseases.

1. Introduction

As a catalytic and structural cofactor in many enzymes, copper plays essential and critical roles in biological processes. It is critical for cells to maintain homeostatic concentrated copper in the body, and the unbalance of copper in the body, *i.e.*, excess or deficiency, will lead to pathological conditions (Bremner, 1998; Halfdanarson et al., 2008; Maria and Hazegh-Azam, 1996). Different concentrated copper, from 1 mM to 90 mM, was found in the surface water over the world, and high concentrated copper usually distributed in the water around the mining area or other copper related factories (Mattie et al., 2008). Copper in excess is very toxic to aquatic and invertebrate species (Mattie et al., 2008). Studies have revealed that copper in excess affected development and damaged the gill, liver, blood and nervous system (Johnson et al., 2007), and abnormally high levels of copper in the body were associated with gastrointestinal disorders, liver cirrhosis, pancreatic cancer and acute leukemia in mammals and humans (Demir et al., 2011; Ishida et al., 2013; Mattie et al., 2008). On the contrary, copper deficiency is associated with anemia, neutropenia and bone marrow hypocellularity (Bustos et al., 2013; Halfdanarson et al., 2008), and copper has been applied as anemia therapy for a long time (Harada et al., 2011; Keil and Nelson, 1931; Schultze et al., 1936). However, few reports have discussed the underlying mechanisms of copper in hematopoiesis process.

Many studies have reported that environmental toxicants induce reactive oxygen species (ROS) in vertebrates. Copper, via Fenton-like reactions, was found to catalyze the formation of ROS (Pham et al., 2013). To balance the redox homeostasis, cells in the body will express a variety of genes involved in the detoxification of ROS or in repair of ROS-induced protein, lipid and nucleic acid damage under oxidative stress (Fedeli et al., 2010; Guo et al., 2010; Hodges et al., 2008; Liu et al., 2015; Mugoni et al., 2014; Valko et al., 2006). Nuclear factor erythroid-related factor 2 (Nrf2), as a critical transcriptional factor, is very sensitive to cellular oxidative stress and positively regulates an array of antioxidant cytoprotective genes including heme oxygenase 1 (*hmos1*), glutathione peroxidase (*gpx*) and others (Bhagi-Damodaran et al., 2014; Liu et al., 2015). In addition, via the antioxidant/electrophile response element (ARE/EpER), ROS induce the expression of antioxidant genes

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by the JNK/SAPK, AP-1 and p38 signal transduction pathways (Guo et al., 2010; Mattie et al., 2008).

ROS can induce DNA damage (Hodges et al., 2008; Yahata et al., 2011), contribute to cellular aging and the senescence process (Yahata et al., 2011), alter signaling pathways and influence genetic and epigenetic changes in the cells (Ghaffari, 2008; Maryanovich and Gross, 2013). Abnormal elevation of intracellular ROS levels has been implicated in the pathogenesis of various diseases, such as ataxia telangiectasia, bone marrow failure and leukemia (Craig et al., 2007; Du et al., 2008; Halliwell, 1991; Uziel et al., 2008). In hematopoiesis process, ROS not only regulated hematopoietic stem cell self-renewal, differentiation and apoptosis (Ghaffari, 2008; Ito et al., 2006), but also mediated several compounds in elevating hemoglobin expression (Aerbajinai et al., 2007; Hsiao et al., 2006).

Hemoglobin consists of two α- and two β-type chains and delivers oxygen and ion in the blood for metabolism in active tissues (Tiedke et al., 2011). The embryonic and fetal hemoglobin possesses more high oxygen affinity than adult hemoglobin (Tiedke et al., 2011). The genes *scf4* (solute carrier family 4, member 1a) and *scf25* (solute carrier family 25, member 37) are specifically expressed in erythrocytes, belonging to ion transport proteins in the solute carrier family (Alan and Leonard, 2004), and *alas* (aminolevulinate, delta-, synthase2), a gene encoding the key enzyme for heme biosynthesis, also specifically labels erythrocytes during zebrafish erythrocyte development (Alan and Leonard, 2004). Hemoglobin was reported to act in cells to reduce oxygen and oxidative stress (Baines and Ho, 2003; Bhagi-Damodaran et al., 2014; Fedeli et al., 2010), and hypoxia causes a significantly reduced expression of α and β mRNA of hemoglobin (Roesner et al., 2006).

Zebrafish embryos and larvae are sensitive to many environmental pollutants, and they have been widely used in environmental studies, pharmaceutical screening, and physiological analysis in recent years (Zhang et al., 2015a, 2015b). We have recently found that copper inhibited the specification of axons and Schwann cell myelination during neurogenesis (Zhang et al., 2015a), and by in-depth gene profiling characterization in copper-exposed embryos, we found that hemoglobin genes were elevated by copper in embryos. In this study, we verified the elevated hemoglobin in copper-exposed embryos by RT-PCR detection, whole-mount *in situ* hybridization and *O*-dianisidine staining. Then, we revealed that ROS mediated the copper-induced increased expression of hemoglobin. Finally, we revealed that modulation of ROS levels in copper-exposed embryos by the oxygen scavengers NAC (*N*-acetylcysteine, Beyotime, Suzhou, China), GSH (Reduced Glutathione, GBCBIO Tec. Inc., Guangzhou, China) and DMTU (1,3-Dimethylthiourea, TCI, Japan), not only recovered the elevated hemoglobin to normal levels, but also accelerated copper excretion in the copper-exposed embryos.

2. Materials and methods

2.1. Maintenance of fish stocks and embryo collection

According to standard procedures, adult zebrafish (AB strain) were maintained in a circulating filtration system (28 ± 0.5 °C,

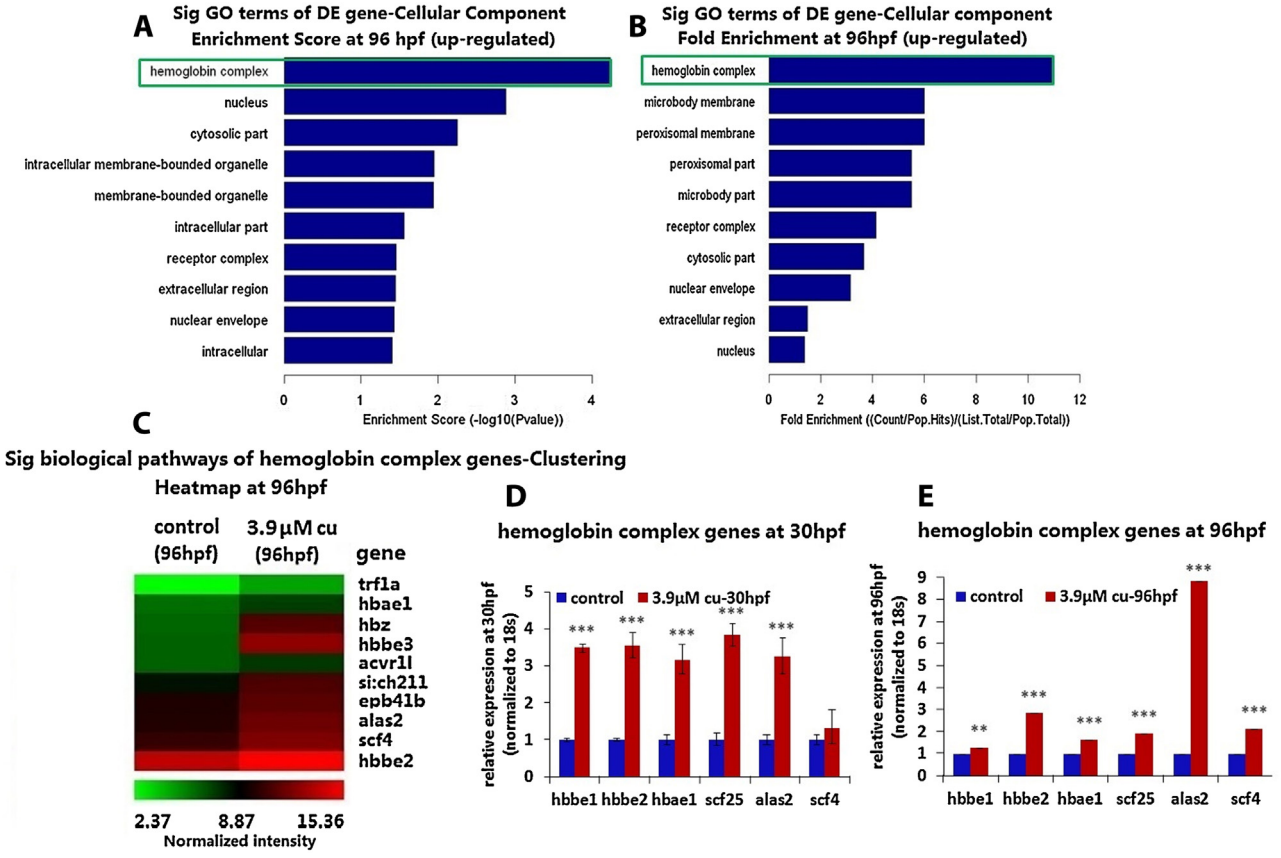

Fig. 1. Copper induced increased transcripts of hemoglobin complex genes. (A, B) Cellular Component GO (Gene ontology) Enrichment Score and Fold Enrichment both showed that hemoglobin complex genes exhibited the most significant increase in copper-exposed embryos. (C) Clustering Analysis showed that hemoglobin complex genes exhibited increased expression in copper-exposed embryos. (D, E) qRT-PCR detection verified that copper induced increased expression of hemoglobin complex genes at 30 hpf (D) and at 96 hpf (E). *hbbe1*: hemoglobin *beta* embryonic-1; *hbbe2*: hemoglobin *beta* embryonic-2; *hbae1*: hemoglobin *alpha* embryonic-1; *Scf25*: solute carrier family 25; *Scf4*: solute carrier family 4, *band3*. The rate and percentage calculated method is mentioned in Section 2. *** indicates $P < 0.01$; ** indicates $P < 0.05$; * indicates $P < 0.1$. Data were performed with 3 biological replicates and analyzed by one-way ANOVA.