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Human Macrophage ATP7A is Localized in the *trans*-Golgi Apparatus, Controls Intracellular Copper Levels, and Mediates Macrophage Responses to Dermal Wounds

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Abstract

The copper transporter ATP7A has attracted significant attention since the discovery of its gene mutation leading to human Menkes disease. We previously reported that ATP7A is highly expressed in the human vasculature and identified a novel vascular function of ATP7A in modulation of the expression and activity of extracellular superoxide dismutase. We recently identified that ATP7A expression in THP-1 cells (a monocyte/macrophage model cell line) plays a role in the oxidation of low density lipoproteins, indicating that it is necessary to further investigate its expression and function in monocytes/macrophages. In the current study, we demonstrated the protein and mRNA expression of ATP7A in human peripheral blood mononuclear cell (PBMC)-derived macrophages and alveolar macrophages. ATP7A was strongly co-localized with the *trans*-Golgi apparatus in PBMC-derived macrophages. Intracellular copper, detected by synchrotron X-ray fluorescence microscopy, was found to be distributed to the nucleus and cytoplasm in human THP-1 cells. To confirm the role of endogenous ATP7A in macrophage copper homeostasis, we performed inductively coupled plasma mass spectrometry in murine peritoneal macrophages, which showed markedly increased intracellular copper levels in macrophages isolated from ATP7A-deficient mice versus control mice. Moreover, the role of ATP7A in regulating macrophage responses to dermal wounds was studied by introduction of control and ATP7A-downregulated THP-1 cells into dermal wounds of nude mice. Infiltration of THP-1 cells into the wounded area (detected by expression of human macrophage markers MAC2 and CD68) was reduced in response to downregulation of ATP7A, hinting decreased macrophage accumulation subsequent to dermal wounds. In summary, alongside our previous studies, these findings indicate that human macrophage ATP7A is localized in the *trans*-Golgi apparatus, regulates intracellular copper levels, and mediates macrophage responses to a dermal wound.

Keywords

macrophage; copper; ATP7A

INTRODUCTION

Vascular metallomics is an emerging field for studying the biological role of metal cations and their signaling pathways in the vasculature [1]. Among the metal cations involved in vascular homeostasis, copper is an essential redox-active metal that is required for normal cellular function [2]. During the past decade, the copper trafficking theory has been established and validated in several laboratories [3]. This theory describes a group of copper chaperones and transporters that tightly regulate copper homeostasis in cells. Among these copper chaperones and transporters, ATP7A has attracted significant attention since the discovery that its gene mutation leads to Menkes disease [4–6]. We previously reported that ATP7A is highly expressed in the human vasculature, including aortic smooth muscle cells, aortic endothelial cells, and umbilical vein endothelial cells [7]. Moreover, we identified a novel vascular function of ATP7A: modulation of the expression and activity of a copper-accepting enzyme, extracellular superoxide dismutase [7, 8]. Thus, it is compelling to investigate the expression and function of ATP7A in other vascular cell types, including macrophages.

Macrophages are a major component of innate immune defenses and are essential for tissue repair, angiogenic and inflammatory responses in vasculature. Earlier studies showed that dietary copper deficiency can influence macrophage function. For example, respiratory burst and microbicidal activities *in vitro* were reduced in peritoneal macrophages isolated from rats fed copper-deficient diets [9]; reduced macrophage microbicidal activities are postulated to contribute to increased mortality in response to the bacterial challenges in copper-deficient rodents [10]. Very recently, White *et al.* discovered a molecular mechanism to interpret this phenomenon, showing that microbicidal activity in macrophages is attenuated after inhibition of ATP7A expression in RAW264.7 cells, a murine macrophage cell line [11]; moreover, hypoxia stimulates ATP7A translocation in RAW 264.7 cells and tumor-associated macrophages in SCID mice [12]. Zheng *et al.* observed ATP7A expression in a murine cell line of microglia which is the resident macrophages of the brain [13], and IFN- γ induces ATP7A expression in microglia [13]. These studies identified a previously underappreciated role of ATP7A in regulating macrophage function. We also reported that ATP7A expression was increased in THP-1 cells, a human monocyte/macrophage cell line, after exposure to phorbol-12-myristate-13-acetate (PMA) [14], and that downregulation of ATP7A expression in THP-1 cells inhibited oxidation of low density lipoproteins [15]. Therefore, in the current study, we further characterized the expression, subcellular location, and copper regulatory function of ATP7A in freshly isolated primary macrophages. Moreover, we investigated the role of macrophage ATP7A in modulating responses to dermal wounds.

MATERIALS AND METHODS

Cells

The human THP-1 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in culture using RPMI 1640 medium (Hyclone, Logan, UT) in the presence of 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μ g/ml). Cultures were maintained in 5% CO₂ tissue culture incubator maintained at 37°C until ready for use. Human alveolar macrophages were prepared from bronchoalveolar lavage fluids obtained from non-smoking volunteers (kindly provided by Dr. Dennis

McGraw at the University of Cincinnati with an approved IRB protocol). Human peripheral blood mono-nuclear cells (PBMCs) were isolated from the blood of health volunteers (Hoxworth Blood Center, University of Cincinnati Medical Center) by a Ficoll–Hypaque method. Macrophages were then generated by an adherence-based method in RPMI 1640 medium with 10% heat-inactivated human serum for 6–8 days before use. To prepare peritoneal macrophages, cells were harvested from the peritoneal cavity of mice 3 days after intraperitoneal administration of 3% thioglycollate broth (Sigma-Aldrich, St. Louis, MO). Cells were then plated onto tissue culture plastic overnight, and adherent cells were collected for further study.

Animals

The heterozygous blotchy ATP7A-deficient mouse females and wild-type males were obtained from the Jackson Laboratory (Bar Harbor, ME) to establish colonies. In the current study, male hemizygous mice carrying the X-linked blotchy ATP7A mutation and control littermates were used and designated as ATP7A-deficient mice (ATP7A^{-y}) and control mice (ATP7A^{+y}), respectively. Nude mouse males (Nu/J, 8–10 weeks) were also purchased from the Jackson Laboratory for the dermal wound study. All animal protocols were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Synchrotron X-ray Fluorescence Microscopy (SXRF)

THP-1 cells were cultured under the same conditions as described above, except that an autoclave-sterilized silicon nitride window (500-nm thickness, 200-μm thick silicon frame, Silson Ltd., Northampton, UK) was added to each well prior to cell seeding. THP-1 cells were first incubated with 100 nM PMA to differentiate to the macrophage phenotype. Adherent cells were treated with 200 μM CuSO₄ for 4 h, washed in phosphate-buffered saline (PBS), and fixed by 4% paraformaldehyde in PBS for 10 min followed by air drying. This method of cell fixation and preparation for SXRF is comparable to plunge freezing method with the minimized disruption of metal topography and the limited alteration of typical cellular copper content [16]. SXRF was performed at beamline 2-ID-D of the Advanced Photon Source at the Argonne National Laboratory (Argonne, IL). Incident X-rays of 10 keV energy were chosen to excite elements Cu and Zn, accompanied by monochromatization with a monochromator. A Fresnel zone plate focused the X-ray beam to a spot size of 0.3×0.2 μm² on the specimen, which was raster-scanned in steps of 0.5 μm. Fluorescence spectrum from the specimen was captured for 1 s of dwell time. Spectral analysis of the fluorescence spectrum of each raster pixel then provided spatial images for Cu and Zn.

Western Blotting

Cell preparation and Western blotting to detect ATP7A protein levels was performed as described previously [7].

RT-PCR

Total RNA preparation and RT-PCR were performed as described previously [15]. The sequences of RT-PCR primers for the target genes were produced using Primer 3 (<http://frodo.wi.mit.edu/primer3/>).

Immunofluorescence and Confocal Microscopy

Cells were plated at 1×10⁵ per 35 mm dish onto coverslips for 24 h. Cells were washed in 100 mM of PBS, pH 7.4, and fixed in 4% paraformaldehyde, pH 7.4 (20 min, 4°C). After washing with PBS again, proteins were localized using the primary antibodies: ATP7A (1:100) or TGN46 (1:100) (overnight, 4°C). Cells were then washed and incubated with

secondary antibodies coupled to rhodamine red-X or FITC (1:200, 1 h, room temperature), respectively. The stained cells were mounted on slides in Vectashield antifade medium (Vector Laboratories, Burlingame, CA). Cells were observed with a Zeiss Axioplan Imaging 2 microscope coupled to a cooled, charge-coupled device camera. Images were captured with Metamorph software and merged with Photoshop software. Cells were also observed with a Zeiss laser-scanning confocal microscope (LSM Meta 510) using argon laser excitation parameters of 488 and 514 nm with emission filters of 540/30 and 580/32 nm. Images were captured with Zeiss software and merged with Photoshop software.

Inductively Coupled Plasma Mass Spectrometry

Cell sample preparation was performed as described in our previous publication [14]. For tissue sample preparation, after harvesting, the tissue was placed in a Petri dish and cut into fine pieces before being stored at -80°C for future assay. After transferring to a liquid nitrogen-cooled mortar, the tissue was ground into powder with a pestle. Then the tissue was transferred to a 1 mL glass tissue grinder for a second homogenization. The homogenized tissue was then digested in nitric acid (final concentration 5%). An Agilent 7500ce system (Agilent Technologies, Santa Clara, CA) was used to detect the copper concentration in cells and tissues. Instrumental parameters were as follows: forward power, 1,500 W; plasma gas flow rate, 15.0 L min^{-1} ; auxiliary gas flow rate, 1.0 L min^{-1} ; carrier gas flow rate, 0.99 L min^{-1} ; makeup gas flow rate, 0.14 L min^{-1} ; and monitored isotope, ^{63}Cu . Samples were read five times for cell samples and three times for tissue samples and then averaged for reproducibility. The sample values were normalized to total protein content, as determined by a bicinchoninic acid protein assay.

Dermal Wound Experiment

Using a sterile 3-mm biopsy punch, full-thickness skin wounds were created on the dorsorostral back skin without injuring the underlying muscle. THP-1 cells (1×10^6) were grown on polyethylene-coated silica microcarrier beads and incubated with either 5 nM of ATP7A-specific or control siRNA oligonucleotides for 24 h. The efficacy of ATP7A siRNA in THP-1 cells is demonstrated in our previous studies [14, 15]. The beads were then loaded into the skin on day 4 after wounding. Following 7 h of incubation, the microcarrier beads were removed and the wound was carefully washed with PBS before a collection for further assays. Wound tissue biopsies containing THP-1 cells labeled with or without a fluorescence marker, calcein acetoxymethyl ester (calcein AM, $1.5\text{ }\mu\text{M}$; Anaspec) were imaged with a Zeiss LSM510 META confocal microscope (argon laser excitation wavelength of 488 nm with emission filters of 505/550 nm).

Statistical Analyses

Data are presented as mean $\pm$ SEM. Data were compared between groups by Student's *t* test when one comparison was performed or by ANOVA for multiple comparisons. When significance was indicated by ANOVA, the Tukey–Kramer post hoc test was used to specify between-group differences. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Subcellular Copper Topography in Human Macrophages

Using SXRF, we detected intracellular copper in THP-1 cells after incubation with copper (Fig. 1). Because as a co-factor of transcription factor, zinc mainly accumulates in the nucleus, we compared the topography of copper (Fig. 1a) and zinc (Fig. 1b). This study showed that although both metals co-localized within the nucleus, a significant amount of

copper was also detected in the cytoplasm in THP-1 cells, presumably is localized in mitochondrion and *trans*-Golgi apparatus of cytoplasm [17].

Human Macrophage Expressing ATP7A

Previously, we reported that ATP7A is expressed in the human THP-1 cell line and that PMA robustly increases ATP7A protein levels in a dose- and time-dependent fashion [18]. To assess ATP7A expression in freshly isolated human macrophages, we performed Western blotting and RT-PCR to examine the protein and mRNA expression, respectively, in human PBMC-derived macrophages and alveolar macrophages. As demonstrated in Fig. 2, protein and mRNA expression of ATP7A were detected in both types of human macrophages.

Subcellular Location of ATP7A in Human Macrophages

To assess the subcellular distribution of ATP7A in macrophages, immunofluorescence and confocal microscopy were performed. We detected a partial co-localization between ATP7A and the *trans*-Golgi apparatus (labeled by TGN46) in human PBMC-derived macrophages (Figs. 3e and i) around one side of perinuclear space (Fig. 3f).

Increased Intracellular Copper Level in ATP7A-Deficient Macrophages

Our previous study showed that the intracellular copper level was significantly higher in human THP-1 cells treated with ATP7A siRNA as compared to control siRNA [18]. Moreover, PMA treatment led to increased ATP7A expression and decreased cellular copper levels, which was blocked by downregulation of ATP7A [18]. To further confirm the role of endogenous ATP7A in regulating copper in freshly isolated primary macrophages, we assayed cellular copper levels in peritoneal macrophages isolated from ATP7A-deficient mice versus control mice. Consistent with our findings in ATP7A siRNA treated THP-1 cells, we observed that murine peritoneal macrophages lacking ATP7A have higher cellular copper levels as compared with wild-type macrophages (Fig. 4). These findings confirm that endogenous ATP7A regulates copper levels in macrophages.

ATP7A Downregulation Modulates THP-1 Cell Responses to a Dermal Wound

In our previous study, we validated siRNA-mediated downregulation of ATP7A expression in THP-1 cells [18]. Thus, we used this approach to investigate whether endogenous ATP7A regulates macrophage responses to a dermal wound. First, in order to verify that THP-1 cells can accumulate in wounded tissue, the cells were labeled with a fluorescence marker, calcein AM. Using a confocal microscopy, we readily detected the fluorescence signal in the tissue (Fig. 5a), indicating the accumulation of THP-1 cells in the wound. The signal was specific for THP-1 cells because no fluorescence signal was detected in a wound loaded with unlabeled THP-1 cells (Fig. 5b). Moreover, compared with identically labeled cells adherent to glass slides, THP-1 cells present in wounded tissues were smaller in size and inhomogeneous in appearance (compare Figs. 5a with c), consistent with phenotypic alterations during migration and tissue adherence.

Next, we examined the impact of ATP7A down-regulation on THP-1 cell accumulation in dermal wounds. We assayed the expression of macrophage markers (MAC2 and CD68) to quantify THP-1 cell accumulation because downregulation of ATP7A only does not change the expression of these markers (data not shown). Figure 5d shows that the expression of MAC2 was reduced in wounds loaded with ATP7A-downregulated THP-1 cells at 4 days. Additional studies to detect the expression of CD68 (another macrophage marker) led to the same tendency ($n=4$; $p<0.05$). Note that the PCR primers used here are specific for human

and do not amplify mouse sequences. These results indicate that endogenous ATP7A mediates macrophage responses to dermal wounds *in vivo*.

Alteration of Copper Concentration During the Progression of Dermal Wounds

Finally, we examined the kinetics of copper accumulation in tissues during wound healing. We found that 11 days are required for complete healing of the wounds in this model (Fig. 6, top panel). In the early stages of wound healing, the copper concentrations remained unchanged. And the copper concentration showed a tendency to increase at day 11 postwounding (Fig. 6, bottom panel), accompanied by regenerated epidermis (data not shown).

DISCUSSION

Trace metals are thought to be inhomogeneously distributed in biological cells and tissues. Thus, it is necessary to apply novel bioimaging techniques with high spatial resolution to investigate the physiology of biometals [19, 20]. SXRF is a new generation of X-ray technique to map the metal topography in biological samples [21]. The copper topography in nucleus and cytoplasm can be readily observed by this technique in THP-1 cells after copper treatment (Fig. 1), and the copper concentration in nucleus is higher than cytoplasm, which is also observed in several other studies using the cultured cells [21]. However, these *in vitro* findings are inconsistent with a recent *in vivo* study by Ralle *et al.* [22]. In the latter study using *in situ* hepatocytes, although significant amount of copper was detected in the nucleus (20–69 μM), copper was highest in the cytoplasm [22]. The reasons for this discrepancy are unknown; note that extracellular environments and intracellular signaling pathways between cultured cells and intact tissues may be distinct. In addition, previous study indicated that estimated free copper is extraordinarily limited and is less than 10^{-18} M in a cell [3]. Therefore, intracellular copper content is tightly controlled in cells by a group of copper chaperones and transporters [3], such as ATP7A.

Consistent with the previous study, our data indicate that ATP7A mainly localizes to *trans*-Golgi apparatus and egresses copper in macrophages studied herein. Recently, Collins *et al.* [23] reported that ATP7A is strongly expressed in the nucleus of IEC-6 and Caco-2 cells and in rat intestine. Although we did not replay this finding in our cells, it is intriguing to examine whether ATP7A can translocate into nucleus under certain stimulations in macrophages in the future study. In addition to *trans*-Golgi apparatus and nucleus, other reported subcellular locations of ATP7A include lipid rafts [24] and phagosome [11].

We chose nude mice to study the function of ATP7A in macrophages *in vivo* because these T-cell-deficient mice are widely used to investigate the function of transplanted human cells, particularly genetically modified cells, in dermal wounds [25] and cancer. Moreover, macrophage distribution and density are not significantly altered between nude and control mice after a wound [26], indicating that nude mice can replicate the physiological behavior of macrophages. Because macrophage accumulation occurs in the early stages of wound healing, we chose day 4 after wounding to load THP-1 cells into the wound.

Copper has been postulated as a dynamic signal molecule [1]. In response to hypoxia [12] or PMA induction [14], intracellular copper contents and the corresponding expression of copper transporters start to change, although the biological consequence remains unknown. Moreover, our current data hints that as compared with control macrophages, ATP7A-deficient macrophages accumulate to a lesser extent in response to dermal wounds. Note that copper has been used to treat dermal wounds and copper deficiency has been proposed to contribute to the delayed healing of human chronic wounds [27, 28]. In order to better understand the mechanism related to the role of copper in dermal wounds, we sought to

determine whether copper concentrations change during the process of wound healing. However, we did not detect a significant change in the copper concentration of wound tissues in the earlier stage of dermal wound healing.

Macrophage accumulation includes adhesion and migration. Chavakis *et al.* observed that treatment of Cu^{2+} in a concentration range between 5 and 100 $\mu\text{mol/L}$ has no effect on adhesion of U937 cells [29]. We have not yet examined the more detailed signaling pathways related to the reduction of macrophage accumulation within the dermal wound in response to ATP7A downregulation. However, recent studies hint that the following machineries could involve into. First, Rac proteins induce lamellipodia extension that push the plasma membrane outward through actin polymerization and play an important role in cell migration [30]. Ashino *et al.* [24] recently reported that downregulation of ATP7A expression using siRNA significantly decreases vascular smooth muscle cell (VSMC) migration in response to platelet-derived growth factor (PDGF) and wound scratch [24]. To gain the mechanistic insight, the authors discovered that downregulation of ATP7A reduces PDGF-induced Rac1 translocation and lamellipodia formation [24]. Macrophages, such as THP-1 cells, form long pseudopodia and lamellipodia once they attach to the endothelium [31] or are under the stimulation of LPS [32] and cytokine [33]. Therefore, the finding by Ashino *et al.* in VSMC could be replayed in macrophages, downregulation. Second, vascular endothelial growth factor (VEGF) receptor 1, an important member of the VEGF receptor family, binds VEGF and mediates macrophage recruitment and cancer metastasis [34–36]. Zhou *et al.* reported that a copper-induced cardiovascular event is associated with VEGF receptor 1 signaling pathway *in vivo* [37]. Moreover, the studies by other laboratory and ours showed that PMA markedly induces VEGF receptor 1 expression in THP-1 cells [14, 38–40], which is reversed by ATP7A downregulation [14]. Therefore, downregulation of ATP7A in THP-1 cells might reduce their migration in response to dermal wounds via the regulation of the VEGF receptor 1 expression. Third, cytosolic phospholipase A_2 (cPLA $_2$) is a critical regulatory enzyme in integrin adhesion, and downregulation of cPLA $_2$ decreases cell migration [41, 42]. Jak-2/STAT-3/cPLA $_2$ pathway is involved into PDGF-induced VSMC motility [43]. Moreover, ATP7A downregulation leads to reduced cPLA $_2$ expression and activity in THP-1 cells [15]. Therefore, cPLA $_2$ could lead another candidate pathway to regulate ATP7A dependent macrophage migration. Fourth, SOD3, a key regulator of reactive oxygen species (ROS) in the extracellular space, and ROS have been proposed as signaling molecules and are critical for neovascularization in the ischemic heart [44], hindlimb [45], and wound healing [46] *in vivo*. Recruitment of inflammatory cells into ischemic tissues is significantly reduced in SOD3 knockout mice as compared with controls [47]. In the previous reports, we also have identified that vascular ATP7A is required for the full activation of SOD3, because ATP7A deficiency leads to impaired activity of SOD3 [7, 8]. Overall, we posit that a dynamic network related to the regulation of ROS, integrin, and actin polymerization may contribute to the reduced macrophage accumulation in response to ATP7A downregulation.

In summary, we report that copper is distributed to the nucleus and cytoplasm in human THP-1 cells. ATP7A is primarily distributed to the *trans*-Golgi apparatus in macrophages. Peritoneal macrophages harvested from ATP7A-deficient mice exhibited increased cellular copper levels, indicating a critical role for endogenous ATP7A in regulating copper homeostasis in these cells. Finally, downregulation of ATP7A in THP-1 cells led to reduced THP-1 cell accumulation in a dermal wound model, indicating that ATP7A mediates responses of macrophages to wounds. Together, these findings provide new insight into copper regulation in macrophages and hint that ATP7A may modulate aspects of macrophage function pertinent to wound healing, inflammation, and perhaps, atherosclerosis.

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ABBREVIATIONS

calcein AM	Calcein acetoxymethyl ester
cPLA₂	Cytosolic phospholipase A ₂
DAPI	4 ,6-Diamidino-2-phenylindole
DIC	Differential interference contrast
ICP-MS	Inductively coupled plasma mass spectrometry
PBMC	Peripheral blood mono-nuclear cell
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PMA	Phorbol-12-myristate-13-acetate
SXRF	Synchrotron X-ray fluorescence
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell

References

1. Easter RN, et al. Vascular metallomics: copper in the vasculature. *Vascular Medicine*. 2010; 15(1): 61–69. [PubMed: 19808712]
2. Mertz W. The essential trace elements. *Science*. 1981; 213(4514):1332–1338. [PubMed: 7022654]
3. Rae TD, et al. Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase. *Science*. 1999; 284(5415):805–808. [PubMed: 10221913]
4. Chelly J, et al. Isolation of a candidate gene for Menkes disease that encodes a potential heavy metal binding protein. *Nature Genetics*. 1993; 3(1):14–19. [PubMed: 8490646]
5. Levinson B, et al. The mottled gene is the mouse homologue of the Menkes disease gene. *Nature Genetics*. 1994; 6(4):369–373. [PubMed: 8054976]
6. Mercer JF, et al. Isolation of a partial candidate gene for Menkes disease by positional cloning. *Nature Genetics*. 1993; 3(1):20–25. [PubMed: 8490647]
7. Qin Z, et al. Essential role for the Menkes ATPase in activation of extracellular superoxide dismutase: implication for vascular oxidative stress. *The FASEB Journal*. 2006; 20(2):334–336.
8. Qin Z, et al. Role of Menkes ATPase in angiotensin ii-induced hypertension. A key modulator for extracellular super-oxide dismutase function. *Hypertension*. 2008; 52:945. [PubMed: 18768397]
9. Babu U, Failla ML. Respiratory burst and candidacidal activity of peritoneal macrophages are impaired in copper-deficient rats. *The Journal of Nutrition*. 1990; 120(12):1692–1699. [PubMed: 2175781]
10. Jones DG, Suttle NF. The effect of copper deficiency on the resistance of mice to infection with *Pasteurella haemolytica*. *Journal of Comparative Pathology*. 1983; 93(1):143–149. [PubMed: 6841690]
11. White C, et al. A role for the ATP7A copper-transporting ATPase in macrophage bactericidal activity. *The Journal of Biological Chemistry*. 2009; 284(49):33949–33956. [PubMed: 19808669]

12. White C, et al. Copper transport into the secretory pathway is regulated by oxygen in macrophages. *Journal of Cell Science*. 2009; 122(Pt 9):1315–1321. [PubMed: 19351718]
13. Zheng Z, et al. Altered microglial copper homeostasis in a mouse model of Alzheimer's disease. *Journal of Neurochemistry*. 2010; 114(6):1630–1638. [PubMed: 20626553]
14. Afton S, et al. Copper egress is induced by PMA in human THP-1 monocytic cell line. *Biomaterials*. 2009; 22(3):531–539. [PubMed: 19205902]
15. Qin Z, et al. Participation of ATP7A in macrophage mediated oxidation of LDL. *Journal of Lipid Research*. 2010; 51(6):1471–1477. [PubMed: 19965596]
16. Finney L, et al. X-ray fluorescence microscopy reveals large-scale relocalization and extracellular translocation of cellular copper during angiogenesis. *Proceedings of the National Academy of Sciences of the United States of America*. 2007; 104(7):2247–2252. [PubMed: 17283338]
17. Yang L, et al. Imaging of the intracellular topography of copper with a fluorescent sensor and by synchrotron X-ray fluorescence microscopy. *Proceedings of the National Academy of Sciences of the United States of America*. 2005; 102(32):11179–11184. [PubMed: 16061820]
18. Afton S, et al. Copper egress is induced by PMA in human THP-1 cells: a potential regulator of VEGFR1. *Biomaterials*. 2009; 22(3):9.
19. Qin Z, et al. Quantitative trace metal imaging with high spatial resolution: applications in biomedicine. *Metallomics*. 2011; 3(1):28–37. [PubMed: 21140012]
20. Becker JS, et al. Bioimaging of metals and biomolecules in mouse heart by laser ablation inductively coupled plasma mass spectrometry and secondary ion mass spectrometry. *Anal Chem*. 2010; 82:9528–33. [PubMed: 20977196]
21. Kemner KM, et al. Elemental and redox analysis of single bacterial cells by X-ray microbeam analysis. *Science*. 2004; 306(5696):686–687. [PubMed: 15499017]
22. Ralle M, et al. Wilson disease at a single cell level: intracellular copper trafficking activates compartment-specific responses in hepatocytes. *The Journal of Biological Chemistry*. 2010; 285(40):30875–30883. [PubMed: 20647314]
23. Collins JF, et al. Alternative splicing of the Menkes copper ATPase (Atp7a) transcript in the rat intestinal epithelium. *American Journal of Physiology—Gastrointestinal and Liver Physiology*. 2009; 297:G695–707. [PubMed: 19679821]
24. Ashino T, et al. Unexpected role of the copper transporter ATP7A in PDGF-induced vascular smooth muscle cell migration. *Circ Res*. 2010; 107:787–799. [PubMed: 20671235]
25. Rees RS, Adamson BF, Lindblad WJ. Use of a cell-based interactive wound dressing to enhance healing of excisional wounds in nude mice. *Wound Repair and Regeneration*. 2001; 9(4):297–304. [PubMed: 11679138]
26. Manuel JA, Gawronska-Kozak B. Matrix metalloproteinase 9 (MMP-9) is upregulated during scarless wound healing in athymic nude mice. *Matrix Biology*. 2006; 25(8):505–514. [PubMed: 17010584]
27. Borkow G, Gabbay J, Zatcoff RC. Could chronic wounds not heal due to too low local copper levels? *Medical Hypotheses*. 2008; 70(3):610–613. [PubMed: 17689198]
28. Liusuwan RA, et al. Impaired healing because of copper deficiency in a pediatric burn patient: a case report. *The Journal of Trauma*. 2008; 65(2):464–466. [PubMed: 18695485]
29. Chavakis T, et al. Molecular mechanisms of zinc-dependent leukocyte adhesion involving the urokinase receptor and beta2-integrins. *Blood*. 1999; 93(9):2976–2983. [PubMed: 10216093]
30. Wells CM, et al. Rac1-deficient macrophages exhibit defects in cell spreading and membrane ruffling but not migration. *Journal of Cell Science*. 2004; 117(Pt 7):1259–1268. [PubMed: 14996945]
31. Ronald JA, et al. Differential regulation of transendothelial migration of THP-1 cells by ICAM-1/ LFA-1 and VCAM-1/ VLA-4. *Journal of Leukocyte Biology*. 2001; 70(4):601–609. [PubMed: 11590197]
32. Patel TR, Corbett SA. Simvastatin suppresses LPS-induced Akt phosphorylation in the human monocyte cell line THP-1. *The Journal of Surgical Research*. 2004; 116(1):116–120. [PubMed: 14732356]

33. Capo C, et al. Effect of cytotoxic necrotizing factor-1 on actin cytoskeleton in human monocytes: role in the regulation of integrin-dependent phagocytosis. *Journal of Immunology*. 1998; 161(8): 4301–4308.
34. Wey JS, et al. Vascular endothelial growth factor receptor-1 promotes migration and invasion in pancreatic carcinoma cell lines. *Cancer*. 2005; 104(2):427–438. [PubMed: 15952180]
35. Fan F, et al. Expression and function of vascular endothelial growth factor receptor-1 on human colorectal cancer cells. *Oncogene*. 2005; 24(16):2647–2653. [PubMed: 15735759]
36. Yang AD, et al. Vascular endothelial growth factor receptor-1 activation mediates epithelial to mesenchymal transition in human pancreatic carcinoma cells. *Cancer Research*. 2006; 66(1):46–51. [PubMed: 16397214]
37. Zhou Y, Bourcy K, Kang YJ. Copper-induced regression of cardiomyocyte hypertrophy is associated with enhanced vascular endothelial growth factor receptor-1 signalling pathway. *Cardiovascular Research*. 2009; 84(1):54–63. [PubMed: 19542178]
38. Akuzawa N, et al. Zinc finger transcription factor Egr-1 activates Flt-1 gene expression in THP-1 cells on induction for macrophage differentiation. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2000; 20(2):377–384.
39. Worley JR, et al. Metalloproteinase expression in PMA-stimulated THP-1 cells. Effects of peroxisome proliferator-activated receptor-gamma (PPAR gamma) agonists and 9-cis-retinoic acid. *The Journal of Biological Chemistry*. 2003; 278(51):51340–51346. [PubMed: 14534304]
40. Tuomisto TT, et al. Analysis of gene and protein expression during monocyte-macrophage differentiation and cholesterol loading—cDNA and protein array study. *Atherosclerosis*. 2005; 180(2):283–291. [PubMed: 15910854]
41. Zhu X, et al. Cytosolic phospholipase A2 activation is essential for beta 1 and beta 2 integrin-dependent adhesion of human eosinophils. *Journal of Immunology*. 1999; 163(6):3423–3429.
42. Myou S, et al. Blockade of eosinophil migration and airway hyperresponsiveness by cPLA2-inhibition. *Nature Immunology*. 2001; 2(2):145–149. [PubMed: 11175812]
43. Neeli I, et al. An essential role of the Jak-2/STAT-3/ cytosolic phospholipase A(2) axis in platelet-derived growth factor BB-induced vascular smooth muscle cell motility. *The Journal of Biological Chemistry*. 2004; 279(44):46122–46128. [PubMed: 15322111]
44. Maulik N. Redox signaling of angiogenesis. *Antioxidants Redox Signaling*. 2002; 4(5):805–815. [PubMed: 12470509]
45. Tojo T, et al. Role of gp91phox (Nox2)-containing NAD (P)H oxidase in angiogenesis in response to hindlimb ischemia. *Circulation*. 2005; 111(18):2347–2355. [PubMed: 15867174]
46. Sen CK. The general case for redox control of wound repair. *Wound Repair and Regeneration*. 2003; 11(6):431–438. [PubMed: 14617282]
47. Kim HW, et al. Essential role of extracellular SOD in reparative neovascularization induced by hindlimb ischemia. *Circulation Research*. 2007; 101(4):409–419. [PubMed: 17601801]

Fig. 1.

Copper and zinc topography in human THP-1 cells. THP-1 cells were cultured and prepared as described in the “MATERIALS AND METHODS” section. Subcellular topography of **a** copper and **b** zinc were determined using SXRF, and co-localization in nucleus between copper and zinc is revealed in **c**.

Fig. 2. ATP7A expression in human macrophages. After isolation of the protein and total RNA from human alveolar macrophages (from two health volunteers) and human PBMC-derived macrophages (from three health volunteers), Western blot and RT-PCR were performed as described in the “MATERIALS AND METHODS” section, respectively. A representative blot (*top panel*) and gel picture (*bottom panel*) are presented here.

Fig. 3. Subcellular location of ATP7A in human PBMC-derived macrophages using **a–f** immunofluorescence microscopy and **g–i** confocal microscopy. The optical image was taken with differential interference contrast (DIC, **a**). The nucleus was counterstained with 4 ,6-diamidino-2-phenyli-ndole (DAPI; *blue*, **b**, **f**). ATP7A expression is shown in *red* (**c**, **e–g**, **i**), and TGN46 expression in *green* (**d**, **e**, **h**, and **i**).

Fig. 4. Cellular copper levels in peritoneal macrophages isolated from ATP7A deficient (ATP7A^{-/y}) versus control mice (ATP7A^{+/y}). Cell lysates were prepared, and the supernatants were subjected to copper measurement as described in the “MATERIALS AND METHODS” section. The sample values were normalized to the total protein content and are presented as relative copper concentration. Values are the mean ± SE for three independent experiments, and each experiment was repeated five times; $p < 0.05$ versus ATP7A^{+/y} cells.

Fig. 5.

THP-1 cell accumulation in the dermal wound of nude mice. Wound tissue biopsies containing THP-1 cells labeled **a** with or **b** without calcein AM were observed by a Zeiss LSM510 META confocal microscope, as described in the “MATERIALS AND METHODS” section. **c** THP-1 cells labeled with calcein AM on a glass slide were observed by a confocal microscope under the same conditions. **d** After incubation with either 5 nM of ATP7A-specific or control siRNA oligonucleotides for 24 h, human THP-1 cells were loaded into wounds as described in the “MATERIALS AND METHODS” section. After that, the wound tissues were homogenized, and total RNA was isolated to detect expression of human MAC-2 as an index of THP-1 gene expression; * $p < 0.01$ versus control, $n = 3$ wounds per group.

Fig. 6. Wound reduction (*top panel*) and copper concentration (*bottom panel*) during wound healing. Four 3-mm punch wounds were cut into each mouse and left to heal by secondary intention. *Top panel* Morphometric evaluation of wound reduction was performed by measuring the diameter of wounds at days 0, 3, 9, and 11 postwounding. The percentage of wound reduction at each time point was calculated by following formula: $[1 - (\text{current wound size} / \text{initial wound size})] \times 100$; $n=3$ wounds per group. *Bottom panel* Wound tissue biopsies were collected for copper analysis as described in the “MATERIALS AND METHODS” section and expressed as described in Fig. 4; $n=3$ wounds per group.