ATP-Binding Cassette B10 Regulates Early Steps of Heme Synthesis

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Rationale: Heme plays a critical role in gas exchange, mitochondrial energy production, and antioxidant defense in cardiovascular system. The mitochondrial transporter ATP-binding cassette (ABC) B10 has been suggested to export heme out of the mitochondria and is required for normal hemoglobinization of erythropoietic cells and protection against ischemia–reperfusion injury in the heart; however, its primary function has not been established.

Objective: The aim of this study was to identify the function of ABCB10 in heme synthesis in cardiac cells.

Methods and Results: Knockdown of ABCB10 in cardiac myoblasts significantly reduced heme levels and the activities of heme-containing proteins, whereas supplementation with δ-aminolevulinic acid reversed these defects. Overexpression of mitochondrial δ-aminolevulinic acid synthase 2, the rate-limiting enzyme upstream of δ-aminolevulinic acid export, failed to restore heme levels in cells with ABCB10 downregulation. ABCB10 and heme levels were increased by hypoxia, and reversal of ABCB10 upregulation caused oxidative stress and cell death. Furthermore, ABCB10 knockdown in neonatal rat cardiomyocytes resulted in a significant delay of calcium removal from the cytoplasm, suggesting a relaxation defect. Finally, ABCB10 expression and heme levels were altered in failing human hearts and mice with ischemic cardiomyopathy.

Conclusions: ABCB10 plays a critical role in heme synthesis pathway by facilitating δ-aminolevulinic acid production or export from the mitochondria. In contrast to previous reports, we show that ABCB10 is not a heme exporter and instead is required for the early mitochondrial steps of heme biosynthesis. (Circ Res 2013;113:279-287.)

Key Words: δ-aminolevulinic acid ■ ATP-binding cassette transporters ■ cardiomyopathies ■ heme ■ mitochondria
organs from single-cell bacteria to humans.\textsuperscript{10,11} They use the energy of ATP hydrolysis to transport diverse substrates across cellular membranes.\textsuperscript{11} Although >50 ABC proteins have been identified in the human genome, only 4 of them localize to the mitochondria, and their functions in the heart remain understudied.\textsuperscript{12} ABCB6 is an outer membrane protein and has been suggested to import coproporphyrinogen III into the mitochondria for the synthesis of heme,\textsuperscript{13,14} although a controversy about its mitochondrial localization and role in heme synthesis exists.\textsuperscript{15} ABCB7 regulates mitochon- 
drial iron homeostasis and was shown to 
interact with ferrochelatase.\textsuperscript{16} A mutation of this protein was 
found in patients with X-linked sideroblastic anemia with 
atalia, suggesting that ABCB7 may participate in heme bio-
synthesis.\textsuperscript{17} ABCB8 (also known as mABC1) was recently 
shown to protect against oxidative stress,\textsuperscript{18} facilitate iron 
export out of the mitochondria, and play a role in the matu-
rature of cytosolic iron–sulfur cluster proteins.\textsuperscript{19} However, 
heme levels were not altered in the hearts of ABCB8 knock-
out mice, suggesting that this transporter may not be 
involved in the regulation of heme homeostasis.\textsuperscript{19} Finally, 
several reports suggested that ABCB10 (also known as 
ABC-me and mABC2) plays a role in hemoglobinization of 
erythropoietic cells and could function to export heme out 
of the mitochondria,\textsuperscript{20,21} although the biological substrate 
for this protein has not been identified yet.

ABCB10 is a mitochondrial inner membrane half-protein 
that homodimerizes to form a functional transporter.\textsuperscript{22} Tissues 
with the highest levels of ABCB10 expression include hematopoietic lineages, heart, liver, and kidney.\textsuperscript{20,22} and ABCB10 was 
shown to be required for normal red blood cell development.\textsuperscript{24} 
Overexpression of ABCB10 enhances heme synthesis 
during maturation of murine erythroleukemia (MEL) cells 
but does not initiate hemoglobin synthesis in undifferentiated cells.\textsuperscript{25} ABCB10 interacts with and stabilizes Mfrn1, but not 
Mfrn2, in developing hematopoietic cells. This interaction is 
associated with enhanced iron uptake into the mitochondria.\textsuperscript{25} Furthermore, ferrochelatase was found to transiently interact 
with ABCB10–Mfrn1 complex,\textsuperscript{23} suggesting coupling of mitochon-
drial iron import and integration of iron into the PPIX 
ing ring for heme synthesis. Finally, ABCB10 knockout mice die 
in utero from severe anemia,\textsuperscript{26} and ABCB10 heterozygous mice display impaired recovery from ischemia–reperfusion 

injury in the heart as a result of increased production of reactive oxygen species (ROS).\textsuperscript{27} 

In this study, we show that ABCB10 plays a role in the early steps of heme synthesis and ALA export into the cytoplasm in 
cardiac myoblasts, providing evidence contrary to the commonly held hypothesis that ABCB10 may function as a heme 
exporter. We report that downregulation of ABCB10 impairs 
heme synthesis and the activities of heme-containing proteins 
in cardiac cells and that addition of ALA, but not overexpression 
of ALAS2, reverses these defects.

### Methods

Methods for H9c2 cell culturing, lentiviral and adenoviral transduction, heme and total iron content determination, enzyme 
activity analyses, microscopy and ROS quantification, mRNA and 
protein level measurements, \textsuperscript{14}C-glycine incorporation, Ca\textsuperscript{2+} 
transient analysis, cell death quantification, neonatal rat cardio-
myocyte (NRCM) isolation, as well as myocardial infarction 
(MI) surgery and tissue harvesting in mice, are described in the 
detailed methods provided in the online supplement. Data are 
expressed as mean±SEM. Statistical significance was assessed with 
the unpaired Student \textit{t} test; \( P<0.05 \) was considered statistically 
significant.

### Results

**ABCB10 Regulates Heme Levels in Cardiac Cells**

Studies have shown that ABCB10 is required for normal 
hematopoiesis possibly via heme export from the mitochon-
dria.\textsuperscript{20,21,24,26} To test this hypothesis, we measured heme levels 
and total mitochondrial iron content with ABCB10 modula-
tion in H9c2 cardiac myoblasts. We effectively downregul-
at ABCB10 by lentiviral transduction of short hairpin RNA (shRNA) and overexpressed this protein using adenovirus, as 
evidenced by Western blot (Figure 1). Furthermore, our 
isoation protocol yielded highly enriched mitochondrial and 
cytoplasmic fractions (Online Figure IIA).

Overexpression of ABCB10 had no effect on mitochon-
drial or total cellular heme levels in H9c2 cells (Figure 1A 
and 1B). However, cellular and mitochondrial heme con-
tent was significantly reduced by ABCB10 knockdown 
in H9c2 cells (Figure 1C and 1D) and in NRCM (Online 
Figure IIB–IID). The reduction in heme was not caused by 
a decrease in nonheme mitochondrial iron levels, which were 
unaffected by ABCB10 downregulation (Online Figure IIE).

It is important to note that mitochondrial heme levels were 
reduced, not increased, by ABCB10 knockdown, in contrast 
to the commonly stated hypothesis that ABCB10 functions 
in heme export.

Consistent with a reduction in heme content, the activities 
of several heme-containing enzymes were decreased with 
ABCB10 knockdown (Figure 2A–2C), whereas their mRNA 
and protein levels remained unchanged (Online Figure IIIA 
and IIIB). However, we observed no change in the activities 
or mRNA levels of heme-containing enzymes with ABCB10 
overexpression (Figure 2D–2F and Online Figure IIC), which 
is in agreement with unaltered heme content in these cells.

Thus, our results suggest that ABCB10 regulates the heme 
biosynthetic pathway, but does not seem to be involved in 
heme export out of the mitochondria.

### Nonstandard Abbreviations and Acronyms

<table>
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<th>Description</th>
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<tr>
<td>ALA</td>
<td>δ-aminolevulinic acid</td>
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<tr>
<td>ALAS1/2</td>
<td>ALA synthase 1/2</td>
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<tr>
<td>ABCB10</td>
<td>ATP-binding cassette B10</td>
</tr>
<tr>
<td>CoA</td>
<td>coenzyme A</td>
</tr>
<tr>
<td>Mfrn1/2</td>
<td>mitoferrin 1 and 2</td>
</tr>
<tr>
<td>MEL</td>
<td>murine erythroleukemia</td>
</tr>
<tr>
<td>MI</td>
<td>myocardial infarction</td>
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<tr>
<td>NRCM</td>
<td>neonatal rat cardiomyocytes</td>
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<tr>
<td>PPIX</td>
<td>protoporphyrin IX</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
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<td>siRNA</td>
<td>small interfering RNA</td>
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ABCBl0 Does Not Alter Levels of Heme Synthetic or Degrading Enzymes

To determine how knockdown of ABCBl0 reduces heme content, we measured the levels of enzymes catalyzing each of the 8 steps in the heme biosynthesis pathway. The mRNA and protein levels of ALAS1, the rate-limiting enzyme in heme synthesis, were not altered with ABCBl0 knockdown (Figure 3A and 3B; Online Figure IVA). In addition, there was a small increase in mRNA levels of uroporphyrinogen decarboxylase and protoporphyrinogen oxidase and no difference in mRNA levels of other heme synthetic enzymes (Online Figure IVA). Similarly, there was no change in the expression of ALAS1 and most of the other heme synthetic enzymes with ABCBl0 overexpression (Online Figure IVB). Finally, we detected no difference in the levels of heme oxygenase 1 and 2 with ABCBl0 expression (Online Figure IVB). Finally, we detected no difference in the levels of heme oxygenase 1 and 2 with ABCBl0 expression (Online Figure IVB). Finally, we detected no difference in the levels of heme oxygenase 1 and 2 with ABCBl0 expression (Online Figure IVB). Finally, we detected no difference in the levels of heme oxygenase 1 and 2 with ABCBl0 expression (Online Figure IVB). Finally, we detected no difference in the levels of heme oxygenase 1 and 2 with ABCBl0 expression (Online Figure IVB).

A recent study showed that ABCBl0 transiently interacts with ferrochelatase, the last enzyme in the heme biosynthetic pathway. The mRNA and protein levels of ferrochelatase in heme content was not because of enhanced degradation. Thus, we hypothesized that ABCBl0 may play a role in the incorporation of iron into the PPIX ring to form heme. To test this, we assessed the effects of ABCBl0 knockdown on incorporation of 55Fe into PPIX by supplementing cells with ALA (Figure 3C). Incorporation of 55Fe into the PPIX ring was unaffected by ABCBl0 knockdown. Consistently, the reduction in total cellular and mitochondrial heme levels with ABCBl0 knockdown (Figure 3C) was completely reversed this defect (Figure 3D). However, incorporation of 55Fe into the PPIX ring was unaffected by ABCBl0 knockdown (Figure 3E), arguing against the hypothesis that ABCBl0 is involved in the last steps of heme synthesis.

ABCBl0 Facilitates ALA Production or Export From the Mitochondria

We next hypothesized that ABCBl0 may play a role in the early mitochondrial steps of heme biosynthetic pathway, namely the production of ALA by ALAS1 or export of ALA from the mitochondria. This hypothesis predicts that addition of exogenous ALA would rescue the heme defect observed with ABCBl0 knockdown. Consistently, the reduction in total cellular and mitochondrial heme levels with ABCBl0 knockdown (Figure 1C) is partially reversed by ALA (Figure 3D). However, incorporation of 55Fe into the PPIX ring was unaffected by ABCBl0 knockdown (Figure 3E), arguing against the hypothesis that ABCBl0 is involved in the last steps of heme synthesis.

Figure 1. ATP-binding cassette (ABC) B10 regulates heme levels in cardiac myoblasts. Cellular (A) and mitochondrial (B) heme content with ABCBl0 overexpression in H9c2 cells (n=6). Cellular (C) and mitochondrial (D) heme levels with ABCBl0 knockdown in H9c2 cells (n=6). Data are presented as mean±SEM. *P<0.05 vs control. Ad-ABCBl0 indicates adenovirus encoding ABCBl0 protein; Ad-GFP, adenovirus encoding green fluorescent protein; and shRNA, short hairpin RNA.

Figure 2. Heme-containing enzyme activities are reduced by ATP-binding cassette (ABC) B10 knockdown. Catalase (A), peroxidase (B), and mitochondrial complex IV (C) activities in H9c2 with ABCBl0 knockdown (n=3–6). D–F, Heme-containing enzyme activities with ABCBl0 overexpression in H9c2 cells (n=3–6). Data are presented as mean±SEM. *P<0.05 vs control. Ad-ABCBl0 indicates adenovirus encoding ABCBl0 protein; Ad-GFP, adenovirus encoding GFP; and shRNA, short hairpin RNA.
Figure 3. ATP-binding cassette (ABC) B10 does not regulate heme synthesis. 
A, Protein levels of δ-aminolevulinic acid synthase 1 (ALAS1), the rate-limiting enzyme in heme synthesis. B, Densitometry analysis of ALAS1 Western blots (n=6). Total porphyrin levels with ABCB10 knockdown alone (C) or with ABCB10 knockdown and ALA supplementation (D; n=6). E, Incorporation of 55Fe into protoporphyrin IX (PPIX), with ABCB10 knockdown in H9c2 cells. Cells are treated with ALA to promote porphyrin synthesis and incubated with 55Fe, followed by quantification of 55Fe saturation of PPIX in the organic fraction containing porphyrins (n=6). Data are presented as mean±SEM. *P<0.05 vs control, #P<0.05 vs ABCB10 shRNA.

and 1D) was completely abolished by ALA supplementation (Figure 4A and 4B), and the difference in heme content between ABCB10 shRNA and control shRNA groups was no longer significant. Similar results were obtained in ABCB10 shRNA-treated NRCM supplemented with ALA (Online Figure IVD). Although ABCB10 knockdown alone was associated with a significant decrease in the activities of heme-containing enzymes (Figure 2A–2C), this difference was no longer present in ALA-treated cells transduced with ABCB10 shRNA lentivirus (Figure 4C–4E). To confirm these findings, we measured incorporation of 14C-labeled glycine into heme with ABCB10 knockdown in H9c2 cells. Glycine is one of the first 2 substrates for ALA synthesis inside the mitochondria, and a reduction in ALA synthesis or export is expected to reduce radioactivity in the heme-containing organic fraction isolated from these cells. Accordingly, ABCB10 knockdown resulted in a reduced
inclusion of radioactive glycine into heme that was measured in the organic phase (Online Figure IVE).

One potential explanation for the defect in heme synthesis with ABCB10 shRNA and its reversal by the addition of exogenous ALA is a reduction in mitochondrial bioenergetic function as a result of increased ROS levels and subsequent deficiency in succinyl CoA, a necessary substrate for the synthesis of ALA and heme. To address this possibility, we supplemented cells with membrane-permeable methyl succinate and measured the effects of ABCB10 knockdown on heme production. The addition of methyl succinate led to a small but significant increase in heme levels in the control cells, but failed to restore heme content in ABCB10 shRNA cells (Figure 4F), suggesting that the defect observed with ABCB10 knockdown is not because of succinyl CoA deficiency.

As ABCB10 knockdown is associated with a defect in hemo-globinization and differentiation of erythropoietic lineage both in vitro and in vivo, we assessed the effects of ALA supplementation on these parameters in MEL cells with ABCB10 knockdown. We used small interfering RNA (siRNA) approach to knock down ABCB10 in MEL cells and achieved 50% reduction in its mRNA and protein levels (Online Figure VA and VB). Consistent with our hypothesis, ABCB10 knockdown reduced both heme content and differentiation of MEL cells (Online Figure VC and VE), whereas addition of exogenous ALA reversed these defects (Online Figure VD and VE). These findings suggest that ABCB10 also facilitates mitochondrial ALA synthesis or export in erythroid progenitor cells, and this function of ABCB10 is necessary for erythrocytic differentiation.

**ALAS2 Overexpression Does Not Restore Heme Levels With ABCB10 Knockdown**

Finally, to show that ABCB10 functions downstream of ALA synthesis step, we measured heme levels with simultaneous knockdown of ABCB10 and overexpression of ALAS2, the mitochondrial rate-limiting enzyme that combines glycine and succinyl-CoA to form ALA. We successfully overexpressed zebrafish ALAS2 using a lentiviral vector (Online Figure VIA and VIB), which resulted in a significant increase in total cellular heme content (Online Figure VIC). However, we observed no increase in heme levels with zebrafish ALAS2 overexpression in cells treated with ABCB10 shRNA (Figure 4G). Overexpression of zebrafish ALAS2 in NRHM similarly increased cellular heme levels in control shRNA group, but failed to rescue the heme defect in ABCB10 shRNA group (Online Figure VID–VIF). These data, together with our finding that ABCB10 knockdown or overexpression does not alter the mRNA and protein levels of ALAS1, suggest that ABCB10 functions to facilitate ALA export, rather than to regulate production of ALA by ALAS1 enzyme.

**ABCB10 Is Important for Cardiomyocyte’s Response to Hypoxia**

We recently showed that hypoxia increases heme production in the heart via upregulation of the rate-limiting enzyme, ALAS2, catalyzing mitochondrial synthesis of ALA. The increased rate of ALA synthesis would require higher export capacity to efficiently deliver this intermediate into the cytosol. Consistently, mRNA and protein levels of ABCB10 were higher in H9c2 cells subjected to 48 hours of 1% O2 than in cells grown in normoxia (Figure 5A). Treatment of cells with ABCB10 siRNA completely reversed hypoxic induction of this response.

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**Figure 5.** Hypoxic upregulation of ATP-binding cassette (ABC) B10 is required for cell survival. A, Western blot analysis showing that ABCB10 is upregulated by hypoxia in H9c2 cells, and this increase is prevented by treatment of hypoxic cells with ABCB10 small interfering RNA (siRNA). B, Heme levels in H9c2 subjected to 48 hours of hypoxia (1% O2) or normoxia, with or without suppression of hypoxic ABCB10 upregulation by siRNA (n=6). C, Mitochondrial ALA synthesis or export in erythroid progenitor cells, and this function of ABCB10 is necessary for erythrocytic differentiation. D, Overexpression of zebrafish ALAS2 in NRHM similarly increased cellular heme levels in control shRNA group, but failed to rescue the heme defect in ABCB10 shRNA group (Online Figure VID–VIF). These data, together with our finding that ABCB10 knockdown or overexpression does not alter the mRNA and protein levels of ALAS1, suggest that ABCB10 functions to facilitate ALA export, rather than to regulate production of ALA by ALAS1 enzyme.

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protein (Figure 5A) and abolished the hypoxia-mediated increase in heme (Figure 5B). These findings suggest that upregulation of ABCB10 is needed to support the increase in heme synthesis in hypoxia, and inhibition of ABCB10 upregulation is sufficient to halt heme production under hypoxic conditions.

To determine whether hypoxia-driven upregulation of ABCB10 bears physiological consequences, we assessed ROS levels and cell death in cardiac cells subjected to 48 hours of hypoxia, followed by 30 minutes of reoxygenation with and without knockdown of ABCB10. Failure to upregulate ABCB10 in hypoxia as a result of siRNA treatment was associated with increased oxidative stress, as determined by the superoxide-sensitive dye MitoSox (Figure 5C). The accumulation of MitoSox in the mitochondria was not caused by changes in mitochondrial membrane potential, as tetramethylrhodamine, ethyl ester staining did not differ between the groups (Online Figure VI). Furthermore, cell death was significantly increased in hypoxic cells in which ABCB10 upregulation was suppressed (Figure 5D) and further augmented by addition of hydrogen peroxide as a source of oxidative stress (Figure 5E). Thus, in contrast to forced overexpression of ABCB10 in normoxia which does not alter heme synthesis, physiological induction of ABCB10 in hypoxia is necessary to support increased rates of heme synthesis, and failure to maintain adequate ABCB10 levels compromises cell survival.

**ABCB10 Levels Are Increased in Cardiomyopathy**

To determine whether ABCB10 expression is altered in ischemic heart disease, we measured the levels of this protein in the hearts of mice subjected to MI. We observed a progressive increase in ABCB10 expression in the heart after MI compared with sham-operated controls, with >2-fold elevation in ABCB10 protein levels 1 month after MI (Figure 6A). The increase in ABCB10 protein was associated with higher cellular and mitochondrial heme levels in mouse hearts 1 month after MI (Figure 6B and 6C). Furthermore, there was also an increase in ABCB10 content in the explanted hearts from human patients with ischemic cardiomyopathy compared with the nonmyopathic human hearts (Figure 6D), which was paralleled by increased heme levels in myopathic hearts (Figure 6E).

Finally, to determine whether ABCB10 has an effect on cardiomyocyte physiology, we assessed Ca²⁺ handling in NRCM with ABCB10 knockdown. In the absence of electric pacing, fewer cells in the ABCB10 siRNA group were beating spontaneously compared with the control group (17/27 versus 9/24 spontaneously beating/total cell count for control and ABCB10 siRNA, respectively). Analysis of Ca²⁺ transients revealed no difference in the magnitude of transient (F/F₀) and rise time, but a significant prolongation of the decay time (Figure 6F–6H), indicative of impaired Ca²⁺ removal from the cytoplasm after systolic influx. Consistent with a calcium removal defect, we

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**Figure 6. ATP-binding cassette (ABC) B10 is upregulated in the ischemic heart and modulates Ca²⁺ transients.** A, ABCB10 protein levels in sham-operated mice and mice subjected to myocardial infarction (MI) injury 1 week and 1 month after the surgery. Densitometric analysis is presented below the Western blot (n=3–4). Total cellular (B) and mitochondrial (C) heme levels in mouse hearts 1 month after MI or sham operation (n=3). D, ABCB10 protein levels in explanted human hearts with ischemic cardiomyopathy or in noncardiomyopathic control human hearts. Densitometric analysis is shown on the right. E, Heme levels in myopathic and nonmyopathic human hearts (n=10). Analysis of Ca²⁺ transients in neonatal rat cardiomyocytes with ABCB10 and control small interfering RNA (siRNA); representative images (F), F/F₀ as a measure of transient magnitude (n=10–11 cells from 3 coverslips; G), and rise and decay times (n=15 cells from 4 coverslips; H). Data are presented as mean±SEM. *P<0.05 vs control. ICM indicates ischemic cardiomyopathy; and NM, nonmyopathic.
observed a significant reduction in protein levels of sodium–calcium exchanger in NRCM treated with ABCB10 siRNA, compared with control (Online Figure VII).

In summary, we showed that ABCB10 facilitates ALA export from the mitochondria into the cytosol and is required for heme synthesis. ABCB10 is positively regulated by hypoxia, and failure to increase the levels of this protein results in increased oxidative stress and loss of cell viability after hypoxia–reperfusion injury. Finally, low ABCB10 levels are associated with slower Ca^{2+} removal from the cell, and upregulation of ABCB10 in ischemic myocardium may help to protect cardiac function.

Discussion

The production of heme requires efficient shuttling of synthetic intermediates between mitochondrial and cytosolic compartments, but the transporters mediating these events are not known. In this report, we show for the first time that ABCB10 plays a role in early mitochondrial steps of heme synthesis, likely by facilitating ALA export out of the mitochondria, and is important for maintenance of cellular heme homeostasis in cardiac cells. Knockdown of ABCB10 in cardiac myoblasts reduces heme levels and the activities of heme-containing proteins, whereas addition of exogenous ALA completely reverses these defects. However, increased production of ALA in the mitochondria by overexpression of the rate-limiting enzyme ALAS2 or supplementation with methyl succinate, a precursor of succinyl-CoA, fails to restore heme levels. These results suggest that ABCB10 may be involved in ALA export out of the mitochondria (Figure 7).

Our results demonstrate that ABCB10 knockdown reduces heme synthesis in 3 distinct cell lines, nonerythropoietic H9c2, NRCM, and erythropoietic MEL cells, providing first evidence that hemoglobinization defect observed in ABCB10 knockout mice may be because of reduced cytosolic ALA availability and impaired heme synthesis. However, the exact function of ABCB10 in hematopoiesis and the ability of ALA supplementation to reverse embryonic lethality of ABCB10 knockout mice warrant in-depth investigation. In addition, the finding of elevated heme content in mouse MI hearts indicates a novel role of ABCB10 in hematopoiesis and the ability of ALA supplementation to increase in oxidative stress triggered by ischemia–reperfusion,27 although the source of ROS has not been identified yet. We also show that knockdown of ABCB10 in cardiac myoblasts leads to increased ROS production and is associated with reduced activities of heme-containing antioxidant enzymes, peroxidases and catalases. Thus, it is tempting to speculate that the potential mechanism for the increase in ROS observed with ABCB10 deletion/knockdown may be through a decrease in the antioxidant capacity of the cells caused by reduced availability of heme, although mitochondrial accumulation of prooxidant ALA may contribute to and further exacerbate the damage. In addition to increases in oxidative stress and cell death, we show that ABCB10 knockdown impairs Ca^{2+} handling by prolonging transient decay time. Although the exact mechanism for this finding is yet to be elucidated, we show that sodium–calcium exchanger protein levels are reduced by ABCB10 knockdown, consistent with the observed reduction in protein levels of sodium–calcium exchanger in NRCM treated with ABCB10 siRNA, compared with control (Online Figure VII).
with previous findings of sodium–calcium exchanger being sensitive to oxidative stress.\textsuperscript{34,35} Furthermore, sarcoplasmic reticulum Ca\textsuperscript{2+} ATPase 2 is thought to play an important role in Ca\textsuperscript{2+} removal during the diastolic phase. Sarcoplasmic reticulum Ca\textsuperscript{2+} ATPase 2 is exquisitely sensitive to oxidative damage, undergoing multiple posttranslational modifications, including oxidation and sulfenylation of cystines, which impair its activity and cause prolongation of decay phase of the transient.\textsuperscript{36,37} Interestingly, Liesa et al\textsuperscript{27} found sarcoplasmic reticulum Ca\textsuperscript{2+} ATPase 2 activity to be significantly reduced in the hearts of ABCB10 heterozygous mice after ischemia–reperfusion injury, which is supportive of our data. Disruption of Ca\textsuperscript{2+} transients by ABCB10 knockdown suggests that changes in the levels of this protein may indirectly influence cardiac contractility.

Overexpression of ABCB10 in cardiac myoblasts did not increase heme content, consistent with previous reports and with the rate-limiting role of ALAS1 in heme synthesis,\textsuperscript{8} whereas knockdown of ABCB10 significantly reduced heme levels. This raises a question about physiological significance of ABCB10 upregulation in the mouse MI model and in human hearts with ischemic cardiomyopathy. Important to note, however, that in the former set of experiments ABCB10 levels were increased artificially through forced adenoviral overexpression. As shown in Online Figure IVB, ABCB10 overexpression did not alter the levels of heme synthetic enzymes. Although ALA export capacity was increased in this setting, the levels of ALAS1, the enzyme required for ALA production in the mitochondria, remained unchanged. Thus, one would not expect heme levels to be altered by forced overexpression of ABCB10. However, upregulation of ABCB10 in hypoxia and ischemic hearts parallels overall induction of heme synthetic pathway and heightened requirements for ALA export. We reported earlier that heme synthesis is increased in ischemic human failing hearts and in cardiac cells subjected to hypoxia through upregulation of ALAS2.\textsuperscript{20} In this setting, upregulation of ABCB10 is required to support increased demand for ALA transport. Consistently, we show that suppression of ABCB10 upregulation by hypoxia is sufficient to repress heme production and leads to increased cell death. Our conclusions are supported by Shirihai et al,\textsuperscript{20} who also show that forced overexpression of ABCB10 does not increase hemoglobinization of undifferentiated MEL cells in which heme synthesis pathway is inactive. However, ABCB10 overexpression, together with induction of differentiation (which is a strong stimulus for increased heme production), promotes hemoglobinization.\textsuperscript{20} Thus, overexpression of ABCB10 facilitates heme synthesis when other steps in heme synthesis (particularly those needed for ALA production) are also active.

A recent study showed that ABCB10 binds to and stabilizes Mfrn1 in MEL cells,\textsuperscript{25} although this interaction has not yet been confirmed in the heart. Thus, ABCB10 knockdown is expected to reduce mitochondrial iron levels through destabilization of Mfrn1, which we failed to observe in our studies. Mfrn1 is expressed in low levels in the heart, whereas Mfrn2 is the predominant isoform responsible for the majority of iron imported into the mitochondria.\textsuperscript{7,9} Given that ABCB10 does not regulate expression or stability of Mfrn2,\textsuperscript{25} reduction in Mfrn1 with ABCB10 loss is unlikely to alter mitochondrial iron uptake in cardiac myoblasts. Finally, a weak interaction between ABCB10 and ferrochelatase has been reported in hematopoietic cells\textsuperscript{21} but has not been studied in other cell types. We show that ABCB10 knockdown in cardiac cells does not affect ferrochelatase levels or activity, as we observed no change in iron incorporation into PPIX. Consistent with our findings, Chen et al\textsuperscript{22} also report no difference in iron incorporation into PPIX, with coexpression of Mfrn1 and ABCB10 in MEL cells. Thus, the relevance of ferrochelatase–ABCB10 interaction remains to be determined.

In summary, we have shown that knockdown of ABCB10 causes a reduction in cytosolic and mitochondrial heme levels, possibly as a result of reduced cytosolic ALA levels. Importantly, our findings are contrary to the commonly held hypothesis that ABCB10 functions in mitochondrial heme export, which would predict increased mitochondrial and reduced cytosolic heme levels because of mitochondrial trapping of heme with ABCB10 knockdown.

**Acknowledgments**

We are grateful to all members of Feinberg Cardiovascular Research Institute and Northwestern University Medical Scientists Training Program for insightful comments and support.

**Sources of Funding**

M. Bayeva is supported by the American Heart Association (AHA) Midwest Affiliate Predoctoral Fellowship (10PRE4430021). R. Wu is supported by the AHA grant (0920130G). M. Liesa is supported by Fundación Ramón Areces postdoctoral fellowship. B. Pav is supported by the March of Dimes Foundation (6-FY09-289) and the National Institutes of Health (R01 DK070838 and P01 HL032262). H. Ardehali is supported by the National Institutes of Health grants (K02 HL107448, R01 HL104181, and 1P01 HL108795).

**Disclosures**

None.

**References**

complete. Production of heme requires coordinated movement of synthetic intermediates between mitochondria and cytosol. ABCB10 is a mitochondrial transporter that was previously suggested to participate in heme export out of this organelle. In contrast to this view, our data indicate that knockdown of ABCB10 does not result in heme accumulation inside the mitochondria. Instead, we show that ABCB10 is required for early mitochondrial steps of the pathway and may facilitate transport of the first synthetic intermediate, δ-aminolevulinic acid, from mitochondria to the cytosol. We found that supplementation with exogenous δ-aminolevulinic acid is sufficient to reverse the defect in heme production because of ABCB10 knockdown, whereas no rescue is observed when δ-aminolevulinic acid production is stimulated inside the mitochondria. Finally, our studies show that ABCB10 expression and heme levels are elevated in human hearts with ischemic cardiomyopathy and that knockdown of ABCB10 in cardiomyocytes is associated with increased oxidative stress and a defect in calcium transient decay time. These findings suggest that ABCB10 may play an important role in cardiac response to ischemic insults.
ATP-Binding Cassette B10 Regulates Early Steps of Heme Synthesis
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_Circ Res._ 2013;113:279-287; originally published online May 29, 2013; doi: 10.1161/CIRCRESAHA.113.301552

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/113/3/279

Data Supplement (unedited) at:
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SUPPLEMENTAL METHODS

Cell culture and reagents
H9c2 cardiac myoblasts were purchased from ATCC and kept in complete DMEM medium (ATCC, VA) supplemented with 10% FBS (Invitrogen, CA) and 1% penicillin-streptomycin (P/S). MEL cells were grown in suspension in complete RPMI-1640 (Cellgro, VA) medium supplemented with L-glutamine, 10% FBS and 1% P/S. Isolation and culture of neonatal rat cardiomyocytes (NRCM) was performed as described previously.\(^1\)

Adenoviral and Lentiviral Transduction of Cells
A recombinant adenoviral vector encoding GFP and human ABCB10 cDNA, separated by the polio virus internal ribosome entry site (IRES) element and under the transcriptional control of CMV promoter, was constructed. Briefly, ABCB10 cDNA was cloned into the adenovirus shuttle vector pAdCGI\(^2\) to make the vector designed pAdCGI-ABCB10. A 25 cm\(^2\) flask (T25; Sarstedt, Newton, NC) of CRE8 cells was cotransfected with 2.1 μg of γ5 viral packaging plasmid and 2.1 μg of shuttle vector plasmid using Lipofectamine Plus (Invitrogen). Cells were incubated 5–9 days until cytopathic effects were observed. Cells and supernatant were collected and subjected to three cycles of freeze-thaw. Following centrifugation, 2 ml of the vector-containing supernatant was added to a 90% confluent T25 and returned to the incubator until cytopathic effects were observed. This procedure was repeated three to four times after which the vector was plaque purified and expanded. Large-scale vector preparations were purified on a cesium chloride gradient, as described.\(^3\) Virus titers were determined by plaque assays. The insertion of ABCB10 cDNA was confirmed by sequencing the vector genome, and the vector was functionally validated by the expression of GFP and ABCB10. NRCMs were transduced on day 6 after isolation, and were imaged 2 days following transduction by a laser scanning confocal microscope (UltraVIEW; PerkinElmer), using a 403 water-immersion lens and
X40 optical zoom. Western blot analysis was also performed to assess gene expression.
Lentivirus for shRNA-mediated knockdown of ABCB10 was purchased from Open Biosystems (cat #: V2LMM_5302) and non-silencing shRNA was used for control experiments. For ALAS2 overexpression experiments, zebrafish ALAS2 cDNA (GenBank #NM_131682) were cloned into pMMPA-HA retroviral vectors as described.\(^4\) Viral transduction of H9c2 cells was carried out in complete medium for 48 hours.

**MEL Cell Differentiation and siRNA treatment**
On the day of differentiation induction 1 x 10\(^5\) to 1 x 10\(^6\) cells/mL were collected by centrifugation and transfected with siGENOME SMARTpool siRNA against mouse ABCB10 (Dharmacon, CO) using DharmaFECT 1 siRNA transfection reagent (Dharmacon, CO) according to the manufacturer’s protocol. Cells were incubated with the transfection mix for 6 hours and differentiation was initiated by addition of complete medium containing 2% DMSO and 5mM hexamethylene bisacetamide (HMBA, Sigma-Aldrich, USA). To maintain efficient ABCB10 knockdown, the transfection was repeated on Day 3 of differentiation, and the differentiation medium was replaced. Cells were collected five days after induction of differentiation and knockdown of ABCB10 was confirmed by qRT-PCR and Western blot analyses. Differentiation was assessed by staining cells with benzidine and counting blue (differentiated) and unstained (undifferentiated) cells.

**Western Blot**
Fifteen-30 µg of protein were resolved on SDS-PAGE gels and transferred to nitrocellulose membranes (Invitrogen, CA). The membranes were probed with antibodies against ABCB10, ALAS1/2 (Abcam, MA), ferrochelatase (Proteintech,IL), GAPDH (Santa Cruz, CA), NCX (Swant, Switzerland) and tubulin (Abcam, MA). HRP-conjugated donkey anti-rabbit and donkey anti-
mouse were used as secondary antibodies (Santa Cruz, CA) and visualized by Pierce SuperSignal Chemiluminescent Substrates.

**Quantitative RT-PCR**

RNA was isolated with RNA STAT-60 (TEL-TEST, Inc, TX), reverse-transcribed with a Random Hexamer (Applied Biosystems, CA), and amplified on a 7500 Fast Real-Time PCR system with SYBR Green PCR Master Mix (Applied Biosystems, CA). Primers were designed using Primer3 (v. 0.4.0) software to target sequences spanning an exon-intron-exon boundary and their specificity was confirmed by running a dissociation curve. mRNA levels were calculated by the comparative threshold cycle method and normalized to β-actin gene.

**Heme Content Determination**

For determination of cellular heme levels, cells were lysed in 1% Triton-X100 in TBS, followed by centrifugation at 5,000 x g for 10 minutes to remove debris. For heme content in mouse hearts, ~5 mg of frozen tissue was homogenized in 1% Triton-X100 in TBS and centrifuged at 5,000 x g for 10 minutes to remove debris. For determination of mitochondrial heme levels, mitochondrial fraction was isolated using Mitochondrial Isolation Kit for Cultured Cells or Tissue (Pierce) according to the manufacturer’s protocol. Protein concentration of cellular or mitochondrial lysate was quantified by BCA assay (Pierce, IL) and heme was quantified as described. Briefly, equal amounts of protein were mixed with 2M oxalic acid, heated to 95°C for 30 minutes to release iron from heme and generate protoporphyrin IX. Samples were then centrifuged for 10 min at 1,000 x g at 4°C to remove debris, the fluorescence of the supernatant was assessed at 405nm / 600nm on Spectra Max Gemini fluorescence microplate reader and normalized to protein concentration of each sample.

**Mitochondrial ^{55}Fe Analysis**
$^{55}\text{Fe}$ (Perkin-Elmer, MA) was conjugated to nitriloacetic acid (NTA, Sigma-Aldrich, USA) and dissolved to the final concentration of 150-250 nM in complete, serum-containing medium. H9c2 cells were grown until 80% confluent, followed by modulation of ABCB10 levels and incubation in $^{55}\text{Fe}$ containing-medium for 48 hours. Cells were then washed once with cold PBS and twice with 500 μM bathophenanthrolinedisulfonate (BPS, Sigma, USA) in PBS to remove membrane-associated radioactivity. Mitochondrial fraction was isolated using the Mitochondrial Isolation Kit for Cultured Cells (Pierce), washed once with 500 μM BPS in PBS to remove residual $^{55}\text{Fe}$, and resuspended in 1% Triton-X100 in TBS. The radioactivity of mitochondrial fraction was analyzed on Beckman scintillation counter and normalized to the protein concentration of each sample determined by BCA assay.

**Total Porphyrin Content Quantification**

The assay was modified from Sorte et. Al. Briefly, cells were resuspended in 1% Triton X-100 in TBS and centrifuged for 5 min at 5,000 x g to remove debris. Supernatant was collected, mixed with one part of Ehrlich’s reagent and two parts of saturated sodium acetate solution. The fluorescence was measured at 405 nm on Spectra Max Gemini fluorescence microplate reader and normalized to protein concentration of each sample. Porphobilinogen (Sigma-Aldrich, USA) was used to generate the standard curve.

**Assessment of $^{55}\text{Fe}$ and $^{14}\text{C}$-glycine Incorporation into Heme**

Following lentiviral treatment, cells were incubated with 1.2 mM δ-aminolevulinic acid (ALA) and 200 nM $^{55}\text{Fe}$-NTA for 8 hours, washed once with PBS and twice with 500 μM BPS in PBS to remove membrane-associated $^{55}\text{Fe}$, and lysed. For $^{14}\text{C}$-glycine incorporation into heme, complete growth medium was supplemented with 10 μCi/mL $^{14}\text{C}$-glycine followed by 3-hour incubation, three washes with PBS to remove radioactivity, and cell lysis. Protein content of each sample was determined by BCA assay. Next, one part of 0.1M HCl was added to 5 parts
of cell lysate, followed by mixing with an equal volume of ethylacetate: acetic acid (3:1) and vortexing. Fractions were separated by centrifugation at 15,000 x g for 5 min and the radioactivity in the organic fraction containing heme was determined by scintillation counting and normalized to protein concentration of each sample.

**Enzyme Activities**

Complex IV activity was measured using the Sandwich ELISA Kits – Dipsticks assay (MitoSciences) according to the manufacturer’s protocol. Peroxidase activity was assessed with the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, CA) as absorbance at 560nm and normalized to protein concentration of each sample. Catalase activity was determined using the Catalase Assay Kit (Abcam) in accordance with manufacturer’s guidelines as absorbance at 570nm, and normalized to protein content.

**Hypoxia-Reoxygenation Treatment**

H9c2 cells were transfected with ABCB10 or control siRNA as described above, followed by incubation in a humidified hypoxic chamber set at 1%O₂, 5%CO₂, at 37˚C for 48 hours, and 30 minutes of reoxygenation at 21%O₂ and 5%CO₂ at 37˚C. To induce additional oxidative stress, cells were treated with 200μM H₂O₂ for 12 hours prior to reoxygenation, where indicated.

**Mitochondrial ROS and Membrane Potential Quantification**

MitoSox Red (Invitrogen) was used to assess mitochondrial O₂⁻-production and TMRE staining was employed as a marker of mitochondrial membrane potential. Cells were visualized by confocal microscopy and ROS levels were quantified by ImageJ software. Four fields per each sample were obtained and averaged. Nuclei were counterstained with Hoescht and subtracted from the total Mitosox fluorescence to exclude the dye which localized to the nucleus.
Assessment of Cell Death

Following hypoxia-reoxygenation, cells were collected by trypsinization and labeled with propidium iodine (Sigma-Aldrich, USA) and Alexa Fluor® 350-conjugated Annexin V (Molecular Probes, NY). Cell death was analyzed by flow cytometry in a FacsCanto flow cytometer (BD Bioscience).

Analysis of Calcium Transients

NRCM were cultured on glass cover slips followed by transfection with ABCB10 or control siRNA. Cells were loaded with 10μM fluo-4AM for 20 minutes. Intracellular Ca\(^{2+}\) transients were measured at 36°C via linescan imaging using a Zeiss LSM510 confocal microscope. Fluorescence emission was measured through a 40x water immersion objective (Apochromat, NA 1.2) at wavelengths >520nm during excitation at 488nm with a 25mW argon laser (<10% laser power). Linescan imaging was recorded in spontaneously contracting cells and in cells paced at BCL=2000msec at equilibrium. Calcium transients were recorded from 10-15 cells on 4 separate coverslips in each group and F/F₀, transient rise and decay times were analyzed using Zeiss LSM Examiner and ClampFit (Molecular Devices) software.

Human Samples

Tissue samples were obtained from the tissue bank at Feinberg Cardiovascular Research Institute (Northwestern University) and consisted of samples from non-failing (n=10) and failing ischemic (n=10) human hearts. Failing ischemic tissues were obtained from the explanted hearts of cardiac transplant recipients. Non-failing heart tissue samples were obtained from unmatched organ donors whose hearts were unsuitable for transplantation but had no known cardiac disease. Explanted hearts were immediately placed in cold cardioplegic solution and subsequently frozen in liquid nitrogen. Protocols for tissue procurement were approved by the Institutional Review Board of the Northwestern University. Informed consent
was obtained from all transplant patients and from the families of organ donors before tissue collection.

**Myocardial Infarction and Tissue Harvesting**

Myocardial infarction without reperfusion was induced in 10-12 week-old wild type C57 black mice by permanent ligation of the left coronary descending artery with a silk suture as previously described. Anesthesia was induced with 2% isoflurane and maintained with 1.5% isoflurane throughout the procedure. Disruption of blood flow was confirmed by the pallor of left ventricle, as well as ST segment elevation and QRS complex widening on ECG, followed by chest closure in layers. In sham-operated animals the chest wall was surgically opened and closed without coronary ligation. Mice were allowed to recover for one week or one month for the MI group, and one month for the sham group. After that, mouse hearts were excised under anesthesia, washed in cold PBS and flash-frozen in liquid nitrogen. All animal studies were performed in accordance with the guidelines established by Northwestern University.
REFERENCES


Online Figure I. Overexpression and Downregulation of ABCB10 in H9c2 cells

A, ABCB10 protein in H9c2 cells transduced with control non-silencing shRNA or ABCB10 shRNA lentivirus. Densitometry analysis of the Western blot is presented on the right. B, Adenoviral overexpression of GFP and ABCB10 using various multiplicities of infection (MOI). Densitometry analysis of the Western blot is presented on the right. 5MOI dose of each virus was used in all subsequent overexpression studies. Data are presented as mean ± SEM. * p<0.05 vs. control.
Online Figure II. ABCB10 knockdown reduces heme levels in NRCM

A, Enrichment of mitochondrial and cytosolic fractions isolated from H9c2 cells was evaluated by Western blot analysis of mitochondria- and cytosol-specific proteins, cytochrome c and actin, respectively. B, Western blot analysis of ABCB10 knockdown in NRCM with using shRNA-encoding lentivirus (n=3). C,D, Cellular (C) and mitochondrial (D) heme levels in NRCM with shRNA-mediated knockdown of ABCB10 (n=6). E, Mitochondrial $^{55}$Fe content in H9c2 with ABCB10 knockdown (n=6). Mito, mitochondrial. Data are presented as mean ± SEM. * p≤0.05 vs. control.
Online Figure III. Levels of heme containing enzymes are unaffected by ABCB10 modulation

**A, B** qRT-PCR analysis of mRNA levels (n=6) (**A**) and Western blot analysis of protein levels (n=3) (**B**) of heme-containing enzymes with ABCB10 knockdown. **C**, mRNA levels of heme-containing enzymes with ABCB10 overexpression (n=6). Data are presented as mean ± SEM.
Online Figure IV. ABCB10 does not regulate heme synthesis or degradation

A,B, qRT-PCR analysis of mRNA levels of enzymes involved in heme synthesis in H9c2 with ABCB10 downregulation (A) and ABCB10 overexpression (B) (n=5-6). C, mRNA levels of heme oxygenases (HOX), enzymes involved in heme degradation, with ABCB10 shRNA (n=6). D, Cellular heme levels in NRCM supplemented with ALA with and without ABCB10 knockdown (n=6). E, Incorporation of C14-glycine into heme in H9c2 cells with and without ABCB10 knockdown (n=6). Data are presented as mean ± SEM. * p<0.05 vs. control.
Online Figure V. ABCB10 knockdown and ALA supplementation in MEL cells

A,B, mRNA (A) and protein (B) levels of ABCB10 in MEL cells following ABCB10 knockdown at day 5 of differentiation. Densitometry analysis is shown on the right. C, Cellular heme levels with ABCB10 knockdown in MEL cells (n=6). D, Cellular heme levels in MEL cells with ABCB10 knockdown following ALA supplementation (n=6). E, Effects of ABCB10 knockdown with and without ALA supplementation of MEL cell differentiation (n=6). Data are presented as mean ± SEM. * p<0.05 vs. control.
Online Figure VI. zALAS2 overexpression increases cellular heme levels at baseline, but not with ABCB10 knockdown

A,B, mRNA (A) and protein (B) levels of zALAS2 in H9c2 cells transduced with zALAS2 or GFP lentivirus. Densitometry analysis is shown below the Western blot. C, Cellular heme levels with zALAS2 overexpression (n=6). D,E, mRNA (D) and protein (E) levels of zALAS2 in NRCM (n=3-6). F, Cellular heme levels with zALAS2 overexpression in NRCM with and without ABCB10 knockdown (n=6). G, Flow cytometry analysis of TMRE intensity as an indicator of mitochondrial membrane potential in H9c2 with ABCB10 knockdown (n=6). Data are presented as mean ± SEM. * p<0.05 vs. control.
Online Figure VII. ABCB10 knockdown alters expression of a cellular calcium transporter

A, Western blot analysis of NCX expression in NRCM treated with ABCB10 or control siRNA. B, Densitometry analysis of the blot in A (n=3). Data are presented as mean ± SEM. * p<0.05 vs. control.