Objective.—To clarify the impact of breast cancer resistance protein 1 (BCRP1)/ATP-binding cassette transporter subfamily G member 2 (ABCG2) expression on cardiac repair after myocardial infarction (MI).

Methods and Results.—The ATP-binding cassette transporter BCRP1/ABCG2 is expressed in various organs, including the heart, and may regulate several tissue defense mechanisms. BCRP1/ABCG2 was mainly expressed in endothelial cells of microvessels in the heart. MI was induced in 8- to 12-week-old wild-type (WT) and Berp1/Abcg2 knockout (KO) mice by ligating the left anterior descending artery. At 28 days after MI, the survival rate was significantly lower in KO mice than in WT mice because of cardiac rupture. Echocardiographic, hemodynamic, and histological assessments showed that ventricular remodeling was more deteriorated in KO than in WT mice. Capillary, myofibroblast, and macrophage densities in the peri-infarction area at 5 days after MI were significantly reduced in KO compared with WT mice. In vitro experiments demonstrated that inhibition of BCRP1/ABCG2 resulted in accumulation of intracellular protoporphyrin IX and impaired survival of microvascular endothelial cells under oxidative stress. Moreover, BCRP1/ABCG2 inhibition impaired migration and tube formation of endothelial cells.

Conclusion.—BCRP1/ABCG2 plays a pivotal role in cardiac repair after MI via modulation of microvascular endothelial cell survival and function. (Arterioscler Thromb Vasc Biol. 2010;30:2128-2135.)

Key Words: angiogenesis ■ BCRP1/ABCG2 ■ myocardial infarction ■ pathology ■ remodeling
out (KO) mice (FVB/N background; model 2767-F; TACONIC, Hudson, NY) were used. WT mice originated from the National Institutes of Health, Bethesda, MD; and have been bred in CLEA Japan since 1993. Thus, the genetic background of FVB/Ncl mice is the same as that of FVB/NTac mice (TACONIC). In our preliminary experiments, no potential difference was found between the 2 groups (supplemental Figure I; available online at http://atvb.ahajournals.org). KO mice were established in the laboratory of Alfred H. Schinkel, PhD, and colleagues at the Netherlands Cancer Institute and were backcrossed to FVB/N mice for 7 generations. A permanent left anterior descending artery ligation was performed as previously described. (Additional information is provided in the supplemental data).

Echocardiographic and Hemodynamic Measurements
A transthoracic echocardiographic study was performed under anesthesia with sodium pentobarbital before surgery and at 28 days after MI with a dynamically focused 15-MHz linear-array transducer (EnVisor M2540A). For hemodynamic measurement, the right carotid artery was cannulated by the micropressure transducers (supplemental data).

Histological Analysis
Mice were euthanized at baseline and at 5 and 28 days after MI. Hearts were weighed, fixed in methanol, and cut into 4 transverse sections: apical, middle, upper, and basal. Each part was embedded in paraffin and sectioned at a 5-μm thickness. Histological analysis was performed as previously described (supplemental data).

RNA Extraction and RT-PCR Analysis
Total RNA was isolated from the myocardial tissues of the peri-infarction area and cultured cells with reagent (TRizol Reagent) and an RNA isolation kit, respectively. Reverse transcription was performed with 1 μg of total RNA, random hexamer primers, and Moloney murine leukemia virus reverse transcriptase (ReverTraAce-α). For quantitative assessment of gene expression levels, quantitative real-time PCR analysis was performed (supplemental data).

Cell Cultures
Human microvascular endothelial cells from the heart (HMVEC-Cs) were purchased. Cell viability was measured by dimethylthiazol-carboxymethoxyphenyl-sulfophenyl-tetrazolium assay. The intracellular protoporphyrin IX concentration in HMVEC-Cs was measured as previously described. Migration and tube formation assays were performed using a commercially available system (BD BioCoat Angiogenesis System) according to the manufacturer’s guidelines (supplemental data).

Measurement of Protoporphyrin IX Concentration and Oxidative Stress in Murine Heart
The concentration of protoporphyrin IX, including heme in murine hearts, was measured as previously described. For assessment of oxidative stress in murine hearts, lipid peroxidation and protein oxidation were measured according to the manufacturer’s recommendation (supplemental data).

Statistical Analysis
Data are expressed as mean±SEM. The Kaplan-Meier method and the log-rank test were used for comparison of survival. Comparison between 2 groups was analyzed by the 2-tailed Student t test. The paired t test was used to compare echocardiographic parameters before and after MI. Frequencies were compared with the Fisher exact probability test. A multiple group comparison was performed by 1-way ANOVA, followed by the Bonferroni procedure, for comparison of means. P<0.05 was considered statistically significant.

Results
Expression of BCRP1/ABCG2 in the Heart
BCRP1/ABCG2 is abundantly expressed in proximal tubule cells of murine kidney. By using a kidney specimen as a positive control, we identified the expression of BCRP1/ABCG2 mRNA in murine heart by conventional RT-PCR (Figure 1A). Immunohistochemical staining of the heart from WT mice with anti-BCRP1/ABCG2 antibody demonstrated that BCRP1/ABCG2 was mainly expressed in the endothelial cells of microvessels (Figure 1B).

Mortality in WT and KO Mice After MI
The survival rate up to 28 days after MI was significantly lower in KO than in WT mice (28.3% [n=60] versus 74.5% [n=51]; P<0.0001) (Figure 2A), whereas all of the sham-operated WT and KO mice survived until 28 days after MI (n=10 for each group). The main cause of death in KO mice was cardiac rupture in 4 to 6 days after MI, which was identified by the presence of intrathoracic hematoma. Cardiac rupture occurred more often in KO than in WT mice (67.4% versus 30.8%; P=0.02).

Echocardiography and Hemodynamics
Left ventricular dimensions and function, examined using echocardiography, were similar in WT and KO mice before
MI (Figure 2B and supplemental Table). However, at 28 days after MI, left ventricle (LV) dilatation and thickening of the noninfarcted area were worse in KO than in WT mice. In addition, ejection fraction was more decreased in KO than in WT mice. These results indicated that ventricular remodeling after MI was exaggerated in KO compared with WT mice.

There was no significant difference in aortic blood pressure, left ventricle end-diastolic diameter, maximum +dP/dt (+dP/dtmax), and maximum −dP/dt (−dP/dtmax) between WT and KO mice at baseline (Figure 2C and supplemental Table). At 28 days after MI, LVEDP, +dP/dtmax, and −dP/dtmax were more deteriorated in KO compared with WT mice. There was no significant difference between sham-operated WT and KO mice in echocardiographic and hemodynamic data.

Heart Weight:Body Weight Ratio and Infarct Size
Heart weight:body weight ratio did not differ between the 2 groups at baseline (Figure 2D). However, at 28 days after MI, heart weight:body weight ratio was greater in KO than in WT mice. In sham-operated mice, there was no significant difference in heart weight:body weight ratio between the 2 groups. Initial area at risk and initial infarct size did not differ between the 2 groups (supplemental Figure II), whereas infarct size at 28 days after MI was significantly larger in KO than in WT mice (Figure 2E).

Myocyte Cross-Sectional Area and Collagen Volume Fraction in the Noninfarcted Area
At baseline, myocyte cross-sectional area (CSA) and collagen volume fraction (CVF) did not differ between WT and KO mice (supplemental Figure III and supplemental Figure IV). At 28 days after left anterior descending artery occlusion, myocyte CSA and CVF in noninfarcted area were greater in KO than in WT mice, which indicated exacerbated ventricular remodeling in KO mice. There was no significant difference between sham-operated WT and KO mice in myocyte colony-stimulating activity and CVF.
Capillary Density and Diameter in the Peri-Infarction Area
At baseline, LV capillary density and diameter did not differ between WT and KO mice (Figure 3A). At 5 days after MI, the capillary density in the peri-infarction area was lower in KO than in WT mice. At 28 days after MI, capillary density remained significantly lower in KO than in WT mice. In addition, capillary diameter in the peri-infarction area was smaller in KO than in WT mice at 5 and 28 days after MI. Thus, mature vessel formation was impaired in KO mice.

Macrophage and Myofibroblast Infiltrations in the Peri-Infarction Area
At baseline, we rarely found macrophages and myofibroblasts in both WT and KO mice (Figure 3A). At 5 days after MI, the capillary density in the peri-infarction area was lower in KO than in WT mice. At 28 days after MI, capillary density remained significantly lower in KO than in WT mice. In addition, capillary diameter in the peri-infarction area was smaller in KO than in WT mice at 5 and 28 days after MI. Thus, mature vessel formation was impaired in KO mice.

Cytokine mRNA Expression in the Peri-Infarction Area
At baseline, cytokine mRNA expression levels were comparable between WT and KO mice (Figure 4). Gene expression levels of tumor necrosis factor α were comparable between WT and KO mice even after MI; the expression of interleukin 6 and monocyte chemotactic protein-1 (MCP-1), other proinflammatory cytokines, was more exaggerated in KO than in WT mice at 5 days after MI. The expression level of matrix metalloproteinase-9 was also higher in KO than in WT mice at 5 days after MI. When we compared angiogenesis-related genes, the expression levels of hypoxia-inducible factor (HIF)-2α and angiopoietin-1 were higher in KO than in WT mice at 5 days after MI; the expression levels of HIF-1α and vascular endothelial growth factor A were comparable between the 2 groups. At 28 days after MI, the expression level of angiopoietin-1 was still higher in KO than in WT mice, although vascular endothelial growth factor A was more expressed in WT than in KO mice. The expression levels of fibrosis-related genes, such as transforming growth factor (TGF)-β1, fibronectin, collagen type 1, alpha 1 (COL1A1), and collagen type 3, alpha 1 (COL3A1), were comparable between WT and KO mice at 28 days after MI. However, at 5 days after MI, TGF-β1, fibronectin, and COL1A1 were more expressed in KO than in WT mice.

Impact of BCRP1/ABCG2 on Survival of Microvascular Endothelial Cells Under Hypoxia and Oxidative Stress
Survival of microvascular endothelial cells under hypoxia or oxidative stress is important for angiogenesis after MI. We assessed the impact of BCRP1/ABCG2 expression in microvascular endothelial cells of the heart on their survival by using HMVEC-Cs. The expression of BCRP1/ABCG2 in HMVEC-Cs was identified by conventional RT-PCR (Figure 5A). The inhibition of BCRP1/ABCG2 by fumitremorgin c (FTC) did not alter the cell viability of HMVEC-Cs under normal conditions. However, under oxidative stress with 200-μmol/L hydrogen peroxide, the cell viability of HMVEC-Cs with FTC was more impaired than that without FTC (Figure 5B). Under hypoxia, no significant difference was found in the cell viability between HMVEC-Cs with and without FTC.

Impact of BCRP1/ABCG2 Inhibition on Intracellular Protoporphyrin IX Concentration in HMVEC-Cs
The regulation of porphyrins and heme within a cell is important for cellular defense against oxidative stress.21–23 Recent data1 suggested that BCRP1/ABCG2 plays an important role in intracellular protoporphyrin IX concentration by active efflux. Flow cytometry analysis demonstrated that protoporphyrin IX concentration was higher in HMVEC-Cs with FTC than in those without FTC (Figure 5C), which indicated that BCRP1/ABCG2 plays an important role in the efflux of intracellular protoporphyrin IX in microvascular endothelial cells of the heart in normal expression level.

Protoporphyrin IX Level and Oxidative Stress In Vivo
The levels of protoporphyrin IX, including heme in the heart, were higher in KO than in WT mice (Figure 5D), although serum bilirubin levels did not differ between the 2 groups in our breeding environment (0.79±0.07 versus 0.75±0.09 mol/L; P=0.77). Lipid peroxidation assay and oxyblot analysis demonstrated that oxidative stress in the peri-infarction...
area at 5 days after MI was exaggerated in KO compared with WT mice, although there was no significant difference between the 2 groups at baseline and at 28 days after MI (Figure 5E and F).

Impact of BCRP1/ABCG2 on Function of Microvascular Endothelial Cells
For assessment of the function of HMVEC-Cs, gene expressions of intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and endothelial NO synthase under inflammatory conditions and migration and tube formation properties were examined. Real-time PCR showed that inhibition of BCRP1/ABCG2 did not alter the expression pattern of intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and endothelial NO synthase even under oxidative stress in HMVEC-Cs (Figure 6A). However, pharmacological inhibition of BCRP1/ABCG2 impaired migration and tube formation of HMVEC-Cs under both normal and oxidative stress conditions (Figure 6B-D).

Discussion
In the present study, we found that BCRP1/ABCG2 is expressed mainly in the endothelial cells of microvessels in the heart. We also demonstrated that genetic disruption of BCRP1/ABCG2 deteriorated mortality and cardiac remodeling after MI. In KO mice, angiogenesis and recruitment of macrophages and myofibroblasts were impaired. In vitro experiments showed that BCRP1/ABCG2 impaired migration and tube formation of HMVEC-Cs under both normal and oxidative stress conditions (Figure 6B-D).

The cardiac healing process after MI was divided into 3 overlapping phases: inflammatory, proliferative, and maturation.24 Because most deaths in KO mice occurred in 4 to 6 days after MI, we assessed the healing process at 5 days after MI, which is under the proliferative phase. During the proliferative phase of healing, fibroblasts and endothelial cells activated by cytokines and growth factors proliferate, leading to the formation of highly vascularized granulation tissue. In addition, activated myofibroblasts produce extracellular matrix proteins and an extensive microvascular network is formed.

Because BCRP1/ABCG2 is expressed mainly in endothelial cells of microvessels, we first assessed angiogenesis. At 5 days after MI, mature vessel formation was impaired in KO compared with WT mice, although the expression levels of angiogenesis-related cytokines were comparable or higher in KO than in WT mice. Previous studies3,15 showed that BCRP1/ABCG2 played an important role in cell survival under hypoxia or oxidative stress, which is induced in ischemic conditions and exaggerates cardiac injury.25 In our in vitro experiments, we found that BCRP1/ABCG2 is essential for survival of microvascular endothelial cells of the heart under oxidative stress, which is important for angiogenesis in damaged tissues.

Previous studies3,15 suggested the mechanisms by which BCRP1/ABCG2 expression protect cell death. BCRP1/ABCG2 may cause effluxion of protoporphyrin IX. The regulation of porphyrins and heme within a cell is important because the accumulation of heme within a cell can ultimately lead to the accumulation of iron and the production of...
cell-damaging reactive oxygen species by the Fenton reaction. In our in vitro experiments, we found that BCRP1/ABCG2 inhibition leads to accumulation of protoporphyrin IX in microvascular endothelial cells of the heart. This result may explain why inhibition of BCRP1/ABCG2 led to impaired survival of microvascular endothelial cells under oxidative stress but not under normal conditions. In fact, we demonstrated that the protoporphyrin IX level in the heart was higher in KO than in WT mice. In addition, oxidative stress in the peri-infarction area was exaggerated in KO versus WT mice at 5 days after MI, although there was no significant difference at baseline between the 2 groups. These results support our hypothesis that elevated protoporphyrin IX levels in microvascular endothelial cells may result in exaggeration of oxidative stress and, thus, lead to impaired angiogenesis under oxidative stress in KO mice.

During angiogenesis, not only cell survival, but also migration and tube formation of endothelial cells are essential. In the present study, we found that pharmacological inhibition of BCRP1/ABCG2 resulted in impaired migration and tube formation of HMVEC-Cs, even under normal conditions. These results suggest that BCRP1/ABCG2 may also play an important role in chemotaxis of microvascular endothelial cells. Therefore, impaired survival under oxidative stress and impaired chemotaxis of microvascular endothelial cells might lead to impaired mature vessel formation in KO mice. Impaired angiogenesis might result in delayed cardiac repair after MI and then exaggerated expression of proinflammatory signals in KO mice, which was supported by exacerbated expression of interleukin 6.

In the present study, macrophage recruitment was also impaired in KO mice at 5 days after MI, although the expression level of MCP-1 was higher in KO than in WT mice. The previous study showed that loss of BCRP1/ABCG2 does not affect hematopoiesis. In addition, our in vitro experiments showed that BCRP1/ABCG2 had no effect on expression of adhesion molecules, such as intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 or endothelial NO synthase in microvascular endothelial cells under inflammatory conditions, even with oxidative stress. Therefore, impaired angiogenesis might lead to impaired macrophage recruitment from the bloodstream. Because macrophages play an important role in clearance of necrotic cardiomyocytes, impaired macrophage recruitment might contribute to impaired cardiac healing and fragility of myocardium.

In this study, the number of myofibroblasts was reduced in KO compared with WT mice at 5 days after MI. Myofibroblasts have been thought to derive from resident fibroblasts. However, our data showed that TGF-β1, a cytokine that induces differentiation of fibroblasts into myofibroblasts, was highly expressed in KO mice. In addition, COL1A1 and
fibronectin expressions were higher in KO than in WT mice. These results appear discrepant between fibroblast activity and myofibroblast recruitment. However, TGF-β is a pleiotropic and multifunctional cytokine, known to exert diverse and often contradictory cellular effects on all cell types. TGF-β-mediated actions are not only dependent on cell type but also on its stage of differentiation and on the cytokine milieu. Thus, impaired cytokine balance by impaired angiogenesis may explain this discrepancy.

Because myofibroblasts play a pivotal role in wound strengthening in the healing process, impaired recruitment of myofibroblasts, together with exaggerated expression of matrix metalloproteinases and impaired clearance of necrotic cardiomyocytes by macrophages, may explain why cardiac rupture was more often observed in KO mice in 4 to 6 days after MI. In fact, when we compared specimens of mice that died of cardiac rupture at 5 days after MI, granulation tissue formation was impaired in KO compared with WT mice (supplemental Figure V). In addition, the augmented fragility of myocardium in the early stage of cardiac healing might lead to excessive infarct expansion and then to exaggerated infarct size and ventricular remodeling at 28 days after MI in KO compared with WT mice, together with exaggerated expression of proinflammatory signals and fibrosis-related cytokines.

In previous studies, BCRP1/ABCG2-expressing cardiac progenitor cells had the potential to differentiate into cardiomyocyte-like cells and endothelial-like cells in vivo after cardiac injury. However, the number of these cells is extremely small. In addition, the contribution of these cells to cardiac repair after MI remains controversial. The significance of these cells and impact of BCRP1/ABCG2 expression through regulating function of these cells in cardiac healing after MI must be elucidated in further studies.

Our findings suggest that upregulation of BCRP1/ABCG2 expression may improve the cardiac healing process after MI and post-MI outcomes. In the previous study, a peroxisome proliferator–activated receptor γ agonist reg-
ulated Bcrp1/Abcg2 expression positively. Shiomi et al. demonstrated that pioglitazone ameliorates ventricular remodeling after MI in mice. Upregulation of BCRP1/ABCG2 might, at least in part, contribute to this result. The development of drugs that upregulate BCRP1/ABCG2 effectively appears to be promising.

In conclusion, we demonstrated that BCRP1/ABCG2 plays a pivotal role in cardiac repair after MI via modulation of microvascular endothelial cell survival and function. Our results suggest that BCRP1/ABCG2 may be of interest for a therapeutic target to improve clinical outcomes after MI.

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Disclosures

None.

References

The ATP-Binding Cassette Transporter BCRP1/ABCG2 Plays a Pivotal Role in Cardiac Repair After Myocardial Infarction Via Modulation of Microvascular Endothelial Cell Survival and Function

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Methods

Experimental Model of Myocardial Infarction

This study was approved by the Animal Care and Use Committee of the University of Tokyo. All experiments were performed according to the “Guide for the Care and Use of Laboratory Animals”.1 Eight- to 12-week-old female wild-type FVB/NJcl (WT) mice (CLEA Japan, Hamamatsu, Japan) and Bcrp1/Abcg2 knock-out (KO) mice (TACONIC, Hudson, NY, FVB/N background; model number, 2767-F) were used.2 WT mice originated from the National Institute of Health, and have been bred in CLEA Japan since 1993. Thus, the genetic background of FVB/NJcl mice is the same as that of FVB/NTac mice (TACONIC, Hudson, NY, USA). KO mice were established in the laboratory of Alfred H. Schinkel and colleagues at the Netherlands Cancer Institute,2 and were backcrossed to FVB/N mice for 7 generations. A permanent left anterior descending artery (LAD) ligation was performed as described previously.3 Briefly, mice were anesthetized by intra-peritoneal injection of sodium pentobarbital (50mg/kg of body weight). A 24-gauge polyethylene tube was inserted into the trachea and mechanical ventilation was provided by a rodent ventilator (Model SAR-830; CWE Inc., Ardmore, PA, USA). The chest was opened by the second left intercostal incision and the heart was exposed. The LAD was ligated by 7-0 nylon suture approximately 2mm below the tip of the left atrial appendage. Some mice were randomly chosen and sacrificed to be analyzed for initial area at risk and initial infarct size. Sham animals were prepared identically without undergoing ligation of the LAD.
Comparison of FVB/NTac and FVB/NJcl mice

FVB/NTac mice were purchased from TACONIC (Hudson, NY, USA). The expression levels of BCRP1/ABCG2 mRNA in the heart and the kidney, survival rate after myocardial infarction (MI) and echocardiographic data at baseline and at 28 days after MI were assessed in FVB/NTac and FVB/NJcl mice. MI was induced also in KO mice to assess whether the results of our experiments changes if FVB/NTac mice are used as WT mice.

Echocardiographic and Hemodynamic Measurements

Transthoracic echocardiographic study was performed under anesthesia with sodium pentobarbital before surgery and at 28 days after MI with a dynamically focused 15MHz linear-array transducer (EnVisor M2540A; Philips Medical System, Best, The Netherlands) with a depth setting of 15mm. Two-dimensional images and M-mode tracings were recorded from the short axis view at the papillary muscle level of the left ventricle (LV). For hemodynamic measurement, the right carotid artery was cannulated by the micro pressure transducers with an outer diameter of 0.42mm (Samba 201 and Samba Preclin 420 LP; Samba Sensors AB, Vastra Frolunda, Sweden), which was then advanced into the LV. Pressure signals were recorded using a MacLab data acquisition system (Model 400 with chart v4.2 software; AD Instruments, Colorado Springs, CO, USA) with a sampling rate of 5000Hz. Heart rate was kept at approximately 320-370 beats per minute to minimize data deviation.

Histological Analysis

Mice were sacrificed at baseline, and 5 days and 28 days after MI. Hearts were weighed and fixed in methanol and cut into 4 transverse sections: apical, middle, upper and basal sections. Each part was
embedded in paraffin, and sectioned at 5 μm thickness. Specimens were stained with hematoxylin and eosin (H&E) or sirius red dyes as described previously. Myocyte cross-sectional area (CSA) and collagen volume fraction (CVF) were determined by quantitative morphometry of tissue sections from the middle section of the LV in H&E and sirius red staining, respectively. Twenty cardiomyocytes per slide were measured for myocyte CSA calculation. Five random fields were examined for CVF analysis (magnification, ×200). Immunohistochemical staining was performed with the following primary antibodies: anti-BCRP1/ABCG2 antibody (BXP-53; Alexis Biochemicals, Farmingdale, NY, USA), anti-CD31 antibody (MEC13.3; BD Pharmingen, San Diego, CA, USA), anti-macrophage antibody Mac-3 (M3184; BD Pharmingen, San Diego, CA, USA), and anti-α-smooth muscle actin (α-SMA) antibody (1A4; Sigma-Aldrich, St. Louis, MO, USA). For BCRP1/ABCG2 staining, antigen retrieval was performed with 0.1% sodium azide solution (pH 9.0) and heat. Antibody distribution was visualized by the avidin-biotin complex technique and Vector Red substrate (Vector Laboratories, Youngstown, OH, USA). Quantitative assessment of capillary, macrophage and myofibroblast density was performed in the peri-infarction area of the middle section of the heart, which was identified by anti-CD31, anti-Mac3 and anti-α-SMA staining, respectively. Five random fields were examined, and density was expressed as the number/square millimeter (magnification, ×400). The diameters of capillaries in the peri-infarction area were calculated as the average lengths of a major and a minor axis of capillaries in specimens stained by anti-CD31 antibody. Five random fields were examined (magnification, ×400).

Initial area at risk and initial infarct size were assessed by injection of Evans blue dye and
triphenyltetrazolium chloride (TTC) staining 24 hours after producing MI (n=5 for each group). The LV was sliced into 4 sections, and each section was weighed. Area at risk and infarct size were calculated by the following equations: area at risk (%) = (weight of the LV - the weight of the LV stained blue)/weight of the LV; infarct size (%) = (weight of the LV - weight of the LV stained red)/weight of the LV. For infarct size analysis at 28 days after MI, the boundary lengths of the infarcted and noninfarcted epicardial and endocardial surfaces were traced with a planimeter digital image analyzing software (ImageJ; Bethesda, MD, USA) after sirius red staining. Infarct size was calculated as the sum of the endocardial and epicardial scar length divided by the sum of the LV endocardial and epicardial circumferences of all the sections.

**RNA Extraction and RT-PCR Analysis**

Total RNA was isolated from the myocardial tissues of the peri-infarction area and cultured cells with TRIzol Reagent (Invitrogen Corp., Carlsbad, CA, USA) and RNA Isolation Kit (Agilent Technologies, Santa Clara, CA, USA), respectively. Reverse transcription was performed with 1 μg of total RNA, random hexamer primers and MMLV reverse transcriptase (ReverTraAce-α; TOYOBO, Osaka, Japan). For confirmation of the expression of Bcrp1/Abcg2 in murine organs and cultured cells, conventional reverse transcription polymerase chain reaction (RT-PCR) was performed. PCR for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also performed to confirm equality of amounts of loaded cDNA. PCR reactions were carried out for 25 cycles for murine and human GAPDH, 30 cycles for murine Bcrp1/Abcg2 and 35 cycles for human Bcrp1/Abcg2 with 30 seconds of denaturation at 367.15K (94°C), 30 seconds of annealing at 338.15K (65°C) and 60 seconds of
extension at 345.15K (72°C), followed by 3 minutes of final extension at 345.15K (72°C). PCR products were detected on a 3% agarose gel by electrophoresis in 1×TAE buffer. The primers for conventional RT-PCR were as follows: murine Bcrp1/Abcg2 (523bp), 5'-GTGGCATCTCTGGAGGAGGA-3' and 5'-TCCTGAGCTCCTGGGAAGTTG-3'; murine GAPDH (452bp), 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCTGTGTTGCTGTA-3'; human Bcrp1/Abcg2 (379bp), 5'-AGTTCCATGGCACTGGCCATA-3' and 5'-TCAGGTTAGGCAATTGTGAGG-3'; human GAPDH (532bp), 5'-AGGTCGGAGTCAACCGGATTTG-3' and 5'-GTGATGGCATGGACTGTGGT-3'. For quantitative assessment of gene expression levels, quantitative real time PCR analysis was performed. Specific mRNAs were quantified by SYBR Green real-time PCR Master Mix (TOYOBO, Osaka) in an ABI PRISM 7000 thermocycler (Applied Biosystems, Foster City, CA) under standard manufacturer’s conditions. Data are expressed in arbitrary units that were normalized by correction for the signal obtained in the same cDNA preparation for GAPDH mRNA. The primers for real time PCR were as follows: murine Bcrp1/Abcg2, 5'-GCCTTGGAGTACTTTGCATCA-3' and 5'-AAATCCGCAGGGTTGTTGTA-3'; murine tumor necrosis factor-α (TNF-α), 5'-TCCCAGGGTTCTCTTCAA-3' and 5'-GGTGAGGAGCACGTAGTCGG-3'; murine interleukin-6 (IL-6), 5'-ACACACCAGGCGCTCTCCCTACTT-3' and 5'-CACGATTTCGAGAGACATGTG-3'; murine monocyte chemotactic protein-1 (MCP-1), 5'-CCACTCACCCTGTGACTCATC-3' and 5'-TGTTGATCTCTTTATGCTCTCC-3'; murine matrix metalloproteinase-9 (MMP-9), 5'-AGACAAAGGTACAGCCTGTTC-3' and 5'-GGCACGCTGGAATGATCTAAG-3'; murine
hypoxia-inducible factor-1α (HIF-1α), 5’-CCCATTCTCATCCGTCAA-3’ and 5’-CCGGTCATAACCCCATCA-3’; murine hypoxia-inducible factor-2α (HIF-2α), 5’-CAGGTTAGAACTAACAGGACACAGCA-3’ and 5’-AGAAAGTCACGCTCGGTGGA-3’; murine vascular endothelial growth factor A (VEGFA), 5’-AAAAACGAAAGCGCAAGAAA-3’ and 5’-TTTCTCCGCTCTGAAACAAGG-3’; murine angiopoietin-1, 5’-CGGATTTTCTTCCAGAAAC-3’ and 5’-TCCGACTTCATATTTTCCACAA-3’; murine transforming growth factor-β (TGF-β1), 5’-GCCTGAGTGCTGCTTCTTTTG-3’ and 5’-CGTGGAGTTTTGGATCTTGCTGT-3’; murine fibronectin, 5’-TGTGAAAGGGAACCAGCAGA-3’ and 5’-CTCGGTGTGTTAAGGGAGAATG-3’; murine collagen type I, alpha 1 (COL1A1), 5’-CCGAACCCCAAGGAAAAGA-3’ and 5’-GTGGACATTAGGCGCAGGA-3’; murine collagen type III, alpha 1 (COL3A1), 5’-TCCCCTGGAATCTGTGAATC-3’ and 5’-TGAGTCGAATTGGGAGAAT-3’; murine GAPDH, 5’-ATGACAACTTTGTCAAGCTCATTT-3’ and 5’-GGTCCACCACCCTGTTGCT-3’; human inter-cellular adhesion molecule-1 (ICAM-1), 5’-CCTTCCTCACCCTGCTTGCTGT-3’ and 5’-AGCGTAGGGTAAGGTTCTTGC-3’; human vascular cell adhesion molecule-1 (VCAM-1), 5’-GGAAAAGGGAATCCAGGTG-3’ and 5’-ACAGTGACAGAGCTCACCATT-3’; human endothelial nitric oxide synthase (eNOS), 5’-AGGAACCTGTGTTGACCCCTCA-3’ and 5’-TATCCAGGTCCCATGACAGA-3’; human GAPDH, 5’-CGCTCTCTGCTCTCCTGTT-3’ and 5’-AAATCCGTTGACTCCGACCTT-3’.

Cell Cultures
Human microvascular endothelial cells from the heart (HMVEC-Cs) were purchased from Lonza (Basel, Switzerland). HMVEC-Cs were cultured in EGM-2 medium (Lonza, Basel, Switzerland) at 310.15K (37°C) in a mixture of 95% air and 5% CO2. For cell viability assay, HMVEC-Cs were cultured in 96 well dish for 5000 cells per well in the presence of vehicle or 10 µmol/L fumitremorgin C (FTC) (Calbiochem, Madison, WI, USA), an inhibitor of BCRP1/ABCG2, under normal condition, oxidative stress with H2O2 (200 µmol/L), or hypoxia (<1% O2 and 5% CO2; Aneropack Kenki 5%; Mitsubishi Gas Chemical Co., Tokyo, Japan) for 24 hours (n=7 for oxidative stress and n=4 for hypoxia). Cell viability was measured by means of MTS (dimethylthiazol-carboxymethoxyphenyl-sulfophenyl-tetrazolium) assay (Promega, Madison, WI), and calculated as the percentage of the absorbance to the well with cells under normal condition with the vehicle. For assessment of gene expression under inflammatory condition, HMVEC-Cs were cultured in 6 well dish at 80% confluency, and were exposed to human recombinant TNF-α (20ng/ml; R&D Systems Inc., Minneapolis, MN, USA) for 24 hours under normal condition or oxidative stress (H2O2, 200 µmol/L) with presence or absence of FTC (10 µmol/L) (n=3 for each group). From cultured cells, mRNA was extracted, and the expression of ICAM-1, VCAM-1 and eNOS was assessed by real-time PCR. Migration assay was performed using the BD BioCoat™ Angiogenesis System: Endothelial Cell Migration (BD Biosciences, Bedford, MA, USA) according to the manufacturer’s guidelines. In brief, HMVEC-Cs were suspended in EBM-2 medium (Lonza, Basel, Switzerland) including 0.1% fetal bovine serum with vehicle or 10µmol/L FTC, and were seeded onto 3µm inserts coated with a thin-layer of human fibronectin (1.0×105 cells per insert) with or without
oxidative stress (H$_2$O$_2$, 200μmol/L). EGM-2 medium was added to the lower chambers of a 24-well plate as chemoattractant solution. Following 22 hour incubation at 310.15K (37°C), the cells were labeled with 4μg/ml of Calcein AM (BD Biosciences, Bedford, MA, USA) for 90 minutes at 310.15K (37°C). The fluorescence of the cells was measured using a fluorescence microplate reader (POWERSCAN HT, DS Pharma Biomedical, Osaka, Japan) at excitation/emission wavelengths of 485/528 nm. Data were expressed as the fold migration derived from the fluorescent units of inserts with chemoattractant solution divided by those without chemoattractant solution. Tube formation was evaluated using the BD BioCoat™ Angiogenesis System: Endothelial Cell Tube Formation (BD Biosciences, Bedford, MA, USA) according to the manufacturer’s guidelines. Briefly, HMVEC-Cs were seeded at 2×10$^4$ cells per well onto a BD Matrigel Matrix coated 96-well plate with vehicle or 10μmol/L FTC under normal condition or oxidative stress (H$_2$O$_2$, 200μmol/L). Following 18 hour incubation at 310.15K (37°C), cells were labeled with 8μg/ml Calcein AM. The tube area and length, and the number of joints and paths were measured using the KURABO Angiogenesis Image Analyzer Ver.2 (KURABO, Osaka, Japan).

**Measurement of Protoporphyrin IX Concentration In Vitro and In Vivo**

Intra-cellular protoporphyrin IX concentration in HMVEC-Cs was measured as described previously.\(^8\)

Briefly, HMVEC-Cs were cultured in 100mm dish at 80% confluency with or without FTC (10 μmol/L) for 24 hours. Then, cells were harvested and washed once with PBS, and protoporphyrin IX concentration was measured by flow cytometry. To induce protoporphyrin IX fluorescence, the excitation wavelength was set at 405 nm, and the emission filter was set at 695nm. The concentration
of protoporphyrin IX including heme in murine hearts was measured as described previously.\textsuperscript{9}

Briefly, the murine hearts were homogenized thoroughly in distilled water (20\% w/v). After centrifugation, 500 $\mu\text{l}$ of 2M oxalic acid solution was added to 50 $\mu\text{l}$ of the supernatant. Following incubation at 373.15K (100\textdegree C) for 30 minutes, the fluorescence was measured using a fluorescence microplate reader (POWERSCAN HT, DS Pharma Biomedical, Osaka, Japan) at excitation/emission wavelengths of 360/645 nm. Protoporphyrin IX concentration was determined according to the standard curve made by the fluorescence of known levels of protoporphyrin IX solutions.

**Assessment of Oxidative Stress in Murine Heart**

For assessment of oxidative stress in murine hearts, lipid peroxidation (Lipid Peroxidation Assay Kit, Oxford Biomedical Research, Oxford, MI, USA) and protein oxidation (Oxyblot Protein Oxidation Detection Kit, Millipore Corp., Billerica, MA, USA) in the myocardial tissues of the peri-infarction area were measured according to the manufacturer’s recommendation.\textsuperscript{10} In lipid peroxidation assay, we measured concentrations of malondialdehyde (MDA) and 4-hydroxyalkenals (HAE). For Oxyblot analysis, the total densitometric values of detectable bands were measured using the densitometry software (Multi Gauge Ver3.0, FUJIFILM, Tokyo, Japan). Data were expressed as the ratio to the mean densitometric values at baseline in WT mice.

**Statistical Analysis**

Data are expressed as mean±SEM. Kaplan-Meier method and the log-rank test were used for comparison of survival. Comparison between two groups was analyzed by the two-tailed Student’s t-test. Paired t test was used to compare echocardiographic parameters before and 28 days after MI.
Frequencies were compared with Fisher’s exact probability test. Multiple group comparison was performed by one-way analysis of variance (ANOVA) followed by the Bonferroni procedure for comparison of means. Values of p<0.05 were considered statistically significant.

References


9. Sassa S. Sequential induction of heme pathway enzymes during erythroid differentiation of
Supplemental Figure I. Comparison of FVB/NTac and FVB/NJcl mice. (A) The expression level of BCRP1/ABCG2 mRNA in the heart and kidney did not differ between the 2 groups (n=3). (B) Survival curve of FVB/NTac, FVB/NJcl and KO mice after MI. Continuous line, FVB/NTac mice (n=20); pecked line, FVB/NJcl mice (n=15); dotted line, knock-out mice (n=16). (C) Echocardiographic data at baseline and at 28 days after MI (FVB/NTac mice, n=14; FVB/NJcl mice, n=11; Bcrp1/Abcg2 knock-out mice, n=5). KO, knock-out mice. *P<0.05 versus FVB/NTac mice; †P<0.05 versus FVB/NJcl mice.
Supplemental Figure II. Initial area at risk and initial infarct size were assessed by injection of Evans blue dye and triphenyltetrazolium chloride staining 24 hours after producing MI (n=5 for each group). No significant difference was found between the 2 groups.
Supplemental Figure III. Myocyte cross-sectional area in the non-infarcted area at 28 days after MI or sham operation. Control, n=6 for WT and n=7 for KO; sham, n=5 for each group; MI, n=15 for WT and n=10 for KO. Open columns, WT mice; filled columns, KO mice. Scale bars=20 μm. *P<0.01 versus WT mice.
Supplemental Figure IV. Collagen volume fraction in the non-infarcted area at 28 days after MI or sham operation. Control, n=5 for WT and n=7 for KO; sham, n=5 for each group; MI, n=7 for WT and n=9 for KO. Open columns, WT mice; filled columns, KO mice. Scale bars=50 μm. *P<0.01 versus WT mice.
Supplemental Figure V. Peri-infarction area of ruptured mice at 5 days after MI in specimens with H&E staining. MI, myocardial infarcton area; NMI, non-myocardial infarction area. Scale bars=100 μm. Granulation tissue formation was impaired in KO mice compared with WT mice.
### Supplemental Table I. Echocardiographic and Hemodynamic Data

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EF, ejection fraction; IVS, interventricular septum; KO, knock-out; LVEDD, left ventricle end-diastolic diameter; LVEDP, left ventricle end-diastolic pressure; MI, myocardial infarction; PW, posterior wall; sSBP, systolic blood pressure; WT, wild-type. *P<0.0001 versus baseline; †P<0.0001 versus sham; ‡P<0.0001 versus WT mice; §P<0.004 versus WT mice; ‖P<0.01 versus baseline; ¶P<0.01 versus sham; #P<0.01 versus WT mice.