Abcg2-Labeled Cells Contribute to Different Cell Populations in the Embryonic and Adult Heart

Michelle J. Doyle,^{1,*} Travis J. Maher,^{1,*} Qinglu Li,¹ Mary G. Garry,¹
Brian P. Sorrentino,² and Cindy M. Martin¹

ATP-binding cassette transporter subfamily G member 2 (*Abcg2*)-expressing cardiac-side population cells have been identified in the developing and adult heart, although the role they play in mammalian heart growth and regeneration remains unclear. In this study, we use genetic lineage tracing to follow the cell fate of *Abcg2*-expressing cells in the embryonic and adult heart. During cardiac embryogenesis, the *Abcg2* lineage gives rise to multiple cardiovascular cell types, including cardiomyocytes, endothelial cells, and vascular smooth muscle cells. This capacity for *Abcg2*-expressing cells to contribute to cardiomyocytes decreases rapidly during the postnatal period. We further tested the role of the *Abcg2* lineage following myocardial injury. One month following ischemia reperfusion injury, *Abcg2*-expressing cells contributed significantly to the endothelial cell lineage, however, there was no contribution to regenerated cardiomyocytes. Furthermore, consistent with previous results showing that *Abcg2* plays an important cytoprotective role during oxidative stress, we show an increase in *Abcg2* labeling of the vasculature, a decrease in the scar area, and a moderate improvement in cardiac function following myocardial injury. We have uncovered a difference in the capacity of *Abcg2*-expressing cells to generate the cardiovascular lineages during embryogenesis, postnatal growth, and cardiac regeneration.

Introduction

ATP-BINDING CASSETTE transporter subfamily G member 2 (*Abcg2*), a member of the ABC (ATP-binding cassette) transporter family, is expressed by stem cells isolated from a wide range of tissues, including the heart. *Abcg2* is an energy-dependent transporter located in the plasma membrane and is capable of effluxing a variety of substances, including cytotoxic drugs [1]. Side population (SP) cells, defined by their ability to efflux the DNA-binding dye Hoechst 33342, rely principally on *Abcg2* for the efflux of Hoechst 33342 dye [2]. SP cells have been shown to possess stem cell activity in numerous tissues, including bone marrow and the heart [2,3]. When isolated by flow cytometry and cultured with the proper stimulation, cardiac SP cells have the ability to differentiate into all cardiac cell lineages, including cardiomyocytes, smooth muscle cells, and endothelial cells [3–7]. When delivered to animals following cardiac injury, isolated cardiac SP cells can migrate to the area of injury and differentiate into the cardiac lineages [6,7].

In the adult heart, *Abcg2* expression is predominantly localized to the interstitial spaces located between muscle fibers. The majority of *Abcg2*-positive cells express microvasculature markers, including coexpression with endothelial cell markers [8–10]. Following myocardial injury,

cardiac expression of *Abcg2* increases [11]. In addition to its well-described expression in adult tissue-specific stem cell populations, *Abcg2* is expressed throughout embryonic development [12]. At embryonic day (e)8.5 in mouse development, *Abcg2* is expressed in the yolk sac and the heart tube. Expression in the heart decreases significantly between e11.5 and e13.5 [3]. Although *Abcg2* is widely expressed in the developing embryo, its role during embryonic development has not been previously investigated.

Recent genetic lineage-tracing studies have expanded the stem cell characterization of *Abcg2* by allowing for cell fate analysis of *Abcg2*-expressing cells and their progeny [13]. Using this lineage-tracing technique, Fatima et al. demonstrated that *Abcg2* labels tissue-specific progenitor cell populations in multiple tissues, including marking of hematopoietic stem cells, intestinal stem cells, and interstitial cells in cardiac and skeletal muscle [13]. Labeled cells in cardiac and skeletal muscle are predominantly vascular and coexpress the endothelial gene CD31 [13–15]. Upon introduction of systemic oxidative stress, the contribution of the *Abcg2* lineage to vascular cells increased [15]. Following either chemical injury in skeletal muscle or response to cardiac oxidative stress, *Abcg2*-labeled cells contribute to microvascular cells but contribute only minimally to regenerated skeletal muscle fibers and do not contribute to

¹Department of Medicine, Lillehei Heart Institute, University of Minnesota, Minneapolis, Minnesota.

²Department of Experimental Hematology, St. Jude Children's Research Hospital, Memphis, Tennessee.

*These two authors contributed equally to this work.

cardiomyocytes [14,15]. Furthermore, when cardiac injury is performed on *Abcg2*-null mice, they exhibit decreased cardiac function resulting in increased mortality contributing to impaired angiogenesis and antioxidant responses [9,10]. Despite the importance of *Abcg2* in cardiac regeneration, the fate of *Abcg2*-expressing cells following cardiac injury has not been assessed.

We have undertaken studies using an inducible lineage-tracing technique to further investigate the cell fate of cardiac *Abcg2*-expressing cells in two settings. First, we examined the cell fate of *Abcg2*-labeled cells during cardiovascular development. We show significant *Abcg2* labeling of cardiomyocytes throughout the developing heart, as well as labeling of endothelial cells and smooth muscle cells. The ability of *Abcg2*-labeled cells to contribute to cardiomyocytes decreases in the early postnatal period. Second, we analyzed the *Abcg2* lineage contribution in the adult heart following ischemia reperfusion (IR) injury. In combination with preconditioning using the oxidative stress agent paraquat dichloride (PQ), we show that the *Abcg2* lineage contributes to endothelial cells but not to newly formed cardiomyocytes following IR injury.

For the first time, we have analyzed the cardiac role of *Abcg2* during embryonic development and we observed a striking difference in the cell lineages that *Abcg2* contributes to in the embryonic and adult heart. *Abcg2*-expressing cells contribute to multiple cardiovascular lineages during embryonic heart development and also primarily to vascular cells in the adult heart.

Materials and Methods

Experimental animals

All experiments were performed in accordance with the Guide for the Care and Use Laboratory Animals, and the experimental protocol was submitted and approved by the University of Minnesota Institutional Animal Care and Use Committee. All studies used adult mice aged 2–6 months. *Abcg2*:CreER^{T2} mice were obtained from Dr. B.P.S. (St. Jude Children's Research Hospital Memphis, TN). C57Bl/6J wild-type (000664) and RosaCAGZsGreen (007906) mice were purchased from The Jackson Laboratories.

Tamoxifen administration

Tamoxifen was administered by intraperitoneal injection. For the determination of embryonic ages, noon on the day of the postcoital plug was considered e0.5. For the cell fate analysis of the embryonic heart, timed pregnant females (RosaCAGZsGreen heterozygous mated to *Abcg2*:CreER^{T2} heterozygous males) received one to three doses of 60 mg/kg body weight tamoxifen (Sigma) dissolved in corn oil beginning at embryonic day 7.5. To reduce the abortion rate associated with tamoxifen, 30 mg/kg body weight progesterone (Sigma) was included for intraperitoneal injections as previously described [16]. For cell fate analysis in the postnatal heart, postnatal day 1 to day 21 pups received a single dose of 1 mg tamoxifen subcutaneously. Animals were sacrificed at 8 weeks of age to determine the *Abcg2*-labeled cells in the heart. For adult heart injury studies, each mouse received 60 mg/kg body weight of tamoxifen (Sigma) dissolved in corn oil or vehicle control (corn oil) for 12 consecutive days, 24 h apart.

Immunofluorescence staining

Adult hearts and embryos (various ages) were harvested, fixed with 4% paraformaldehyde, cryoembedded, and processed for tissue staining. Ten-microgram sections were stained overnight at 4°C with the following primary antibodies: desmin (1:500; Novus Biologicals), CD31 (1:500; R&D Systems), c-kit (1:200; R&D Systems), dystrophin (1:500; Abcam), endomucin (1:500; Abcam), Sca-1 (Ly6a, 1:200; BD Biosciences), Sox10 (1:500; Santa Cruz), α -smooth muscle actin (α -SMA: 1:500; Sigma, clone 1A4), Wt1 (1:200; Abcam), and OSF2 (periostin, 1:500; BioVendor). Secondary staining was performed for 1 h at room temperature using the appropriate AF555- or AF594-conjugated secondary antibody (Life Technologies). Images were taken using a Zeiss LSM 510 Meta Confocal Microscope.

Cell expression quantification

All immunohistochemical images were quantified using the Image Pro Plus 6.0 or the FIJI Software analysis. For all analyses following IR injury, five random fields of view were chosen per mouse from at least three mice per group. For postnatal *Abcg2* lineage analysis, three whole heart sections were analyzed from at least three mice per group. Dystrophin membrane staining was used to identify and count the total number of cardiomyocytes, and then the number of ZsGreen-positive cardiomyocytes was counted. The total number of *Abcg2* lineage-positive cells was determined by counting ZsGreen-positive cells and normalized to section area.

Myocardial injury

Following tamoxifen treatment, *Abcg2*:CreER^{T2}, RosaCAGZsGreen double heterozygous mice then received two doses of 20 mg/kg PQ [Sigma; dissolved in sterile 1× phosphate-buffered saline (PBS)] or vehicle control (sterile 1× PBS) 1 week apart beginning 2 weeks before cardiac injury. Sex-matched mice aged 3–4 months underwent temporary ligation of the left anterior coronary artery. Briefly, mice were anesthetized with 1.5% isoflurane and intubated to the level of the carina, placed on a heating pad, and put on a ventilator to aid respiration. Thoracotomy was performed, the heart exposed, and the proximal left anterior coronary artery was ligated below the atrial appendage using a 9-0 silk suture for 30 min. Ligature was then released, and blood flow was confirmed by visualization. Thoracic wall was approximated using 3-0 silk sutures and extubation was achieved. Mice were kept on a heating pad and observed until fully recovered.

Echocardiography

Echocardiography was obtained under 2% isoflurane anesthesia using a Vevo 770 (VisualSonics) high-frequency ultrasound biomicroscope using a 30-Mhz probe operating at a range of 100–120 frames per second. Echocardiography was performed at baseline, 1 week, and 1 month following myocardial injury. Ejection fraction (EF%) and shortening fraction (FS%) values were calculated from M- and B-mode images of the left ventricle in standard long- and short-axis views, respectively. EF and FS were calculated using the following formulas:

$$EF\% = [(LVEDV - LVESV)/LVEDV] \times 100$$

$$FS\% = [(LVIDd - LVIDs)/LVIDd] \times 100$$

Scar size assessment

Hearts were perfused with cold PBS and cut transversely into three 3-mm rings (base, middle, and apex). Ten-micrometer sections at the landmark level of the papillary muscles were stained with Sirius Red–Fast Green. Mosaic images were taken on a Zeiss AxioObserver Z1 Inverted Microscope with the Zeiss AxioVision software and scar size, indicated by red-stained collagen, was calculated as a percent of the entire left ventricle using the ImageJ software.

Statistical analysis

All results are mean $\pm$ SEM. Significance was established by two-tailed Student's *t*-test, *P* < 0.05.

Results

Cell fate of *Abcg2*-positive progenitor cells in the embryonic heart

Abcg2 is expressed by stem cells in the adult heart and it is expressed during heart development. To explore the role of

Abcg2 during cardiac development, we used the previously described *Abcg2*:CreER^{T2} mouse [13] crossed to Rosa-CAGZsGreen reporter mouse. In double transgenic animals, the reporter allele is expressed following inducible activation of Cre recombinase resulting in reporter expression in *Abcg2*-positive cells and their progeny allowing us to track the cell fate of *Abcg2*-expressing cells throughout heart development. We initially delivered a single dose of tamoxifen to timed pregnant females at e8.5 and analyzed the reporter expression 2 days later at e10.5. A small number of *Abcg2*-labeled cells were present in the heart and they coexpress the muscle interfilament protein desmin (Supplementary Fig. S1A; Supplementary Data are available online at www.liebertpub.com/scd).

To maximize the number of labeled cells, we administered multiple doses of tamoxifen 24 h apart to timed pregnant females beginning at e7.5 for analysis at later embryonic and fetal time points. We found that for embryos to survive to fetal time points, we could deliver a maximum of two tamoxifen doses to pregnant females, even with the addition of progesterone. In double transgenic embryos at e13.5, we saw scattered endogenous ZsGreen-positive cells throughout the myocardium and epicardium in both the atria and ventricles (Fig. 1A). At e19.5, there was extensive ZsGreen expression throughout the ventricular myocardium and in the atrium (Fig. 1B). We continued to see ZsGreen-positive cells in the epicardium and we now also saw

FIG. 1. The *Abcg2* lineage contributes to multiple cardiovascular cell types during embryonic heart development. Embryos were analyzed at e13.5 (A, C, D, G, H) or e19.5 (B, E, F, I, J) following induction with tamoxifen at e7.5–e9.5 or e7.5–e8.5, respectively. *Abcg2* lineage-labeled cells (ZsGreen-positive) are green, lineage markers are red, and nuclei are labeled with DAPI (blue) in all images. ZsGreen is coexpressed in desmin-positive cardiomyocytes (C, E) and in Wt1 (G), endomucin (F), CD31 (I), α -actin, smooth muscle isoform (α -SMA) (J) positive cells. ZsGreen is not coexpressed with endomucin (D) or Sox10 (H) at e13.5. Arrowheads indicate cells that coexpress ZsGreen with the appropriate lineage marker. Scale bars = 200 μ m (A, B) and 50 μ m (C–J). *Abcg2*, ATP-binding cassette transporter subfamily G member 2.

ZsGreen-positive cells in the endocardium. No reporter expression was observed with the delivery of tamoxifen in embryos lacking either the *Abcg2*:CreER^{T2} or RosaCAGZsGreen allele, and it was also not observed with the vehicle control delivered heterozygous to embryos, indicating low recombination leakiness (data not shown).

At both e13.5 and e19.5, some ZsGreen-positive cells resemble adjacent cardiomyocytes in size and shape and coexpress the muscle protein desmin (Fig. 1C, E, Supplementary Figs. S2A and S3A). ZsGreen-positive cells did not coexpress endomucin at e13.5 (Fig. 1D and Supplementary Fig. S2B), however, by e19.5 there was coexpression of ZsGreen and endomucin in the endocardial cell layer (Fig. 1F and Supplementary Fig. S3B). Together these observations indicate that the *Abcg2*-positive progenitor cells contribute to cardiomyocytes early in heart development and subsequently contribute to endocardial cells as well. To confirm the presence of reporter-positive cells in the epicardium, we assessed the coexpression of ZsGreen with the epicardial cell marker Wt1 (Wilms' Tumor 1) [17] and observed coexpression of Wt1 in some ZsGreen-positive cells (Fig. 1G and Supplementary Fig. S2C).

Additionally, because a subset of cardiac SP cells express neural precursor markers and cardiac SP cells may derive from neural crest cells in the heart [18], we evaluated the coexpression of ZsGreen with the neural crest marker Sox10. Although we observed Sox10-positive and ZsGreen-positive cells in the outflow tract (OFT) region, we did not see any cells that coexpressed ZsGreen and Sox10 (Fig. 1H and Supplementary Fig. S2D), suggesting that it is unlikely *Abcg2*-labeled cells in the heart are derived from neural crest cells.

In addition to labeling cardiomyocytes, we also saw a high expression of ZsGreen in interstitial cells located between the cardiomyocytes, so we looked at coexpression with interstitial cell population markers. At e19.5, we saw considerable coexpression of ZsGreen with the endothelial cell marker CD31 (Fig. 1I and Supplementary Fig. S3C) in both small and large blood vessels and some coexpression with the smooth muscle isoform of α -actin (α -SMA) (Fig. 1J and Supplementary Fig. S3D). We did not observe ZsGreen coexpression with periostin (OSF2)-expressing cardiac fibroblasts [19] (Supplementary Fig. S3E). Therefore, during embryonic heart development, the *Abcg2* lineage contributes to multiple cardiovascular lineages, including cardiomyocytes, endothelial cells, and vascular smooth muscle cells (VSMCs).

The ability of Abcg2-labeled cells to contribute to cardiomyocytes decreases rapidly postnatally

Because we observed *Abcg2* lineage contribution to cardiomyocytes during heart development, we evaluated the potential of *Abcg2*-labeled progenitor cells to contribute to cardiomyocytes postnatally. To do so, we delivered a single dose of tamoxifen to postnatal pups at four time points, postnatal day 1 (p1), day 6 (p6), day 14 (p14), and day 21 (p21) and analyzed the contribution of *Abcg2*-derived cells in the heart at 8 weeks of age. When tamoxifen is delivered at p1, p6, or p14, a similar number of ZsGreen-positive cells were detected throughout the heart. In animals treated with tamoxifen at p21, we observed approximately a 50% decrease in the total number of ZsGreen-positive cells (Fig. 2A; $P < 0.05$). We assessed the coexpression of ZsGreen with dystrophin-positive

cardiomyocytes. Interestingly, we observed $\sim 116 \pm 33.7$ *Abcg2*-labeled cardiomyocytes in a whole heart cross section when lineage labeling is done at p1 (Fig. 2B, C; $n = 3$). This represented about 1% of total cardiomyocytes. When tamoxifen was delivered at p6, the number of ZsGreen-positive cardiomyocytes decreased significantly to 87 ± 29.2 (Fig. 2B, D; $P < 0.05$, $n = 3$). The ability of *Abcg2*-labeled cells to contribute to cardiomyocytes continued to decrease significantly and they were on average 6 ± 5.8 and 3 ± 2.5 ZsGreen-positive cardiomyocytes when tamoxifen was delivered at p14 and p21, respectively (Figs. 2B, E, F; $P < 0.05$, $n = 3$). The observed decrease in *Abcg2*-derived cardiomyocytes was greater than the change in the expression of total ZsGreen-positive cells throughout the studied postnatal period, thus supporting the conclusion that the ability of the *Abcg2* lineage to contribute to cardiomyocytes decreases with age.

We also assessed the ability of the *Abcg2* lineage to contribute to endothelial cells postnatally and observed a significant expression of ZsGreen in cells expressing CD31 beginning with postnatal day 1 tamoxifen delivery (Fig. 2G–J). In contrast to our findings with the cardiac lineage, we did not observe a difference in *Abcg2* lineage-labeled endothelial cells when tamoxifen was delivered at different time points. This observation is consistent with prior observations that the *Abcg2* lineage contributes significantly to endothelial cells [15].

Abcg2 labeled cells express the stem cell markers, Sca1, and c-kit

Cardiac SP cells are heterogenous and express the cell surface markers Sca1 and c-kit in addition to expressing *Abcg2* [3–5]. We assessed the coexpression of these stem cell markers within the *Abcg2* lineage-labeled cells in the adult heart following the induction of Cre recombination at postnatal day 1. ZsGreen-positive cells coexpress Sca1 (Fig. 3A) or c-kit (Fig. 3B) throughout the adult heart, indicating that some of the *Abcg2* lineage-marked cells likely retain stem cell characteristics.

Abcg2-labeled cells contribute to endothelial cells but not to cardiomyocytes following ischemia-reperfusion injury

To evaluate the ability of *Abcg2*-labeled cells to contribute to the cardiovascular lineages following a significant cardiac injury, we performed an IR injury. We have previously shown that *Abcg2* plays a protective role in response to oxidative stress in vivo, and therefore mice were pretreated with PQ, a systemic oxidative stress agent. For injury analysis, *Abcg2*:CreER^{T2}, RosaCAGZsGreen adult mice were placed in two groups 1 month before cardiac injury. Following 12 consecutive days of tamoxifen administration, one group received two doses of PQ 1 week apart, whereas the control group received two doses of PBS vehicle control. All mice were then subjected to a temporary ligation of the left anterior descending coronary artery for a duration of 30 min and followed for 1-month post-IR injury. There was no difference in survival between treatment and control groups both before and after ligation (data not shown). In our prior study, using the same tamoxifen and PQ delivery strategy, we observed no cardiac damage in the absence of injury [15].

FIG. 2. The ability of *Abcg2*-derived cells to generate cardiomyocytes diminishes between postnatal day 6 and 14. The total number of ZsGreen-positive cells in adult heart sections (8 weeks old) were scored and normalized to section area: percent ZsGreen-positive cells/area for p1 is 0.20, p6 is 0.26, p14 is 0.21, and p21 is 0.12, $n = 3$, for each time point. $*P < 0.05$ (A). ZsGreen-positive cells were further analyzed for coexpression with dystrophin (cardiomyocytes, C–F) or CD31 (endothelial cells, G–J) following a single dose of tamoxifen at p1 (C, G), p6 (D, H), p14 (E, I), or p21 (F, J). Arrowheads indicate ZsGreen- and dystrophin-double positive cardiomyocytes. The number of dystrophin-positive cardiomyocytes that coexpress ZsGreen when tamoxifen was administered at p1 is 116 ± 33.7 , at p6 is 87 ± 29.2 , at p14 is 6 ± 5.8 , and at p21 is 3 ± 2.5 , $n = 3$ for each time point. $*P < 0.05$ (B). Extensive coexpression of ZsGreen with the endothelial cell marker CD31 (red) is observed in adult heart tissue (8 weeks old) when tamoxifen is given at all time points (p1 to p21, G–J). Nuclei labeled with DAPI (blue) in all images. Scale bars = 50 μm .

FIG. 3. *Abcg2*-labeled cells coexpress the stem cell markers Sca1 and c-kit. ZsGreen-positive cells in adult (8 week old) heart sections were analyzed for coexpression of the stem cell markers Sca1 and c-kit following tamoxifen delivery at p1. ZsGreen-positive (A, B) cells coexpressing Sca1 (red, A') and c-kit (red, B') are indicated with arrowheads. Merged images shown with DAPI (blue, A'' and B''). Scale bars = 10 μm .

We assessed the expression of ZsGreen-positive cells with cardiac and endothelial cell markers 1 month following IR injury in PBS- and PQ-treated animals. We observed no ZsGreen-positive cells coexpressing desmin in any region of the heart in PBS- or PQ-treated groups, indicating an absence of *Abcg2* lineage-positive cardiomyocytes (Fig. 4A, B). We analyzed the *Abcg2* lineage contribution to microvasculature following IR injury by staining with CD31 in the peri-scar region. Overall, capillary density was significantly increased in the border zone in PQ-treated mice compared to PBS-treated mice (Fig. 4C–E; total area CD31 normalized to nuclei per field 127.6 ± 13.8 PQ-treated compared to 80.5 ± 8.3 PBS-treated, $P < 0.05$; $n = 6$). Furthermore, the coexpression of ZsGreen with CD31 in the peri-scar area was increased in PQ-treated mice following IR injury compared to PBS-treated mice (Fig. 4C, D, F; 40.1 ± 5 merged pixels normalized to nuclei per field PQ vs. 27.5 ± 3.8 PBS, $P < 0.05$; $n = 6$), indicating an increase in the number of *Abcg2* lineage-positive endothelial cells. This observation is consistent with our previous finding that exposure to the oxidative stress agent PQ leads to an increase in *Abcg2* lineage-labeled vascular cells in the heart [15].

To determine the physiological effects of the increase in the *Abcg2*-labeled vasculature following pretreatment with PQ and IR injury, we assessed cardiac function 1 month after IR injury. Using echocardiographic analysis of the left ventricle to assess cardiac performance, we observed no significant difference in EF before injury (Supplementary Fig. S4A). One week after IR injury, EF between groups was roughly equivalent and at 1 month EF of the PQ-treated group was greater compared to control but not significantly different (Supplementary Fig. S4A). The EF in the control group continued to decrease significantly between 1 week and 1 month postinjury in contrast to the PQ-treated group, which revealed no significant deterioration at 1 month postinjury. This supports that preconditioning with PQ was protective in preventing continued postinjury functional deterioration and adverse remodeling.

We also quantified the extent of scar tissue in heart sections at the level of the papillary muscle using the Sirius Red–Fast Green staining to identify fibrotic tissue. Mice preconditioned with PQ demonstrated a significant decrease in collagen volume fraction compared to control-treated mice (Supplementary Fig. S4B, C). In summary, the data obtained using the *Abcg2* lineage-tracing model demonstrate that in the adult environment even following injury, the *Abcg2* lineage primarily contributes to the cardiac vascular system.

Discussion

The function of *Abcg2* beyond xenobiotic transport has remained largely unknown because its initial characterization as a multidrug resistant protein in cancer cells [20]. Employment of lineage tracing has allowed for insight into the fate commitment of *Abcg2*-expressing cells in the adult, thus providing a greater understanding of *Abcg2* function as well as its veracity as a marker for adult stem cell populations [13]. In this study, we have undertaken cell fate analysis of *Abcg2*-expressing cells in multiple contexts using a tamoxifen-inducible Cre-lox mouse model (*Abcg2*:CreER^{T2}): first, during embryonic heart development; second, during the early postnatal period, and last, following cardiac injury in

FIG. 4. Oxidative stress pretreatment increases vascular remodeling following ischemia-reperfusion injury. Images show heart sections from PBS-treated (A, C) or PQ-treated (B, D) mice 1 month following IR injury stained for desmin (red, A, B) or CD31 (red, C, D) and imaged with *Abcg2* lineage label (green). Desmin-positive cardiomyocytes are negative for ZsGreen (A, B), whereas CD31-positive endothelial cells are positive for ZsGreen (C, D) in both PBS- and PQ-treated mice. Nuclei are labeled with DAPI (blue) for all images. Capillary density (scored by total CD31-positive area normalized to nuclei per field) in the peri-scar region is increased following IR injury in PQ-pretreated mice (127.6 ± 13.8) compared to control (PBS-treated; 80.5 ± 8.3) mice, $*P < 0.05$ (E). The number of CD31-positive cells coexpressing ZsGreen (*Abcg2*-labeled) was scored by the number of merged pixels normalized to nuclei per field and is increased in the peri-scar region in PQ-treated mice (40.1 ± 5) versus PBS-treated mice (27.5 ± 3.8), $*P < 0.05$ (F). $n = 6$ mice per group for quantitative analysis. Scale bar = 50 μm. IR, ischemia reperfusion; PQ, Paraquat dichloride; PBS, phosphate-buffered saline.

adult mice. Given the low expression of *Abcg2* in a rare cell population and the fact that *Abcg2* is rapidly downregulated with cell differentiation, this inducible lineage-tracing model is advantageous to investigate the function of *Abcg2* in the embryonic and adult heart. We observe a difference in the embryonic versus postnatal differentiation capacity of *Abcg2*-positive progenitor cells. During embryogenesis, the *Abcg2* lineage contributes to cardiomyocytes as well as other cardiovascular cell types, including endothelial cells and VSMCs. In contrast, in adult mice, the *Abcg2* lineage contributes to the cardiac vasculature but does not contribute to cardiomyocytes even following cardiac injury.

Although *Abcg2* is widely expressed during development and cardiac SP cells have been identified in the developing heart [3], the developmental potential of *Abcg2* or cardiac SP cells has not been previously explored. For the first time, we show that endogenous *Abcg2*-expressing cells have the potential to differentiate into cardiomyocytes. In addition to the generation of cardiomyocytes from *Abcg2*-expressing progenitor cells, it is clear that in vivo the *Abcg2* lineage includes other cardiac cell types, including endothelial cells and VSMCs. The embryonic capacity of *Abcg2*-derived cells to differentiate into multiple cardiovascular lineages in vivo is consistent with the differentiation potential of cardiac SP cells in vitro [3–6]. The pattern of *Abcg2*-labeled cells observed during heart development is interesting. Initially, we see the appearance of single *Abcg2*-labeled cells at e13.5 followed by clusters of neighboring cardiomyocytes late in development (e19.5).

The potential for *Abcg2*-positive stem cells to differentiate into cardiomyocytes seems to be largely restricted to embryogenesis. Approximately, 1% of adult cardiomyocytes are labeled when Cre recombination is induced at p1. This is considerably less than when *Abcg2*-expressing cells are labeled during embryogenesis. The capacity of *Abcg2*-labeled cells to differentiate to cardiomyocytes decreases rapidly in neonatal mice. By p6, it is already ~25% compared to p1 and is essentially completely extinguished by p21. Interestingly, this seems to correspond to the window of cardiomyocyte proliferation and regenerative capacity [21]. Prior studies have demonstrated that cardiac *Abcg2* expression as well as the cardiac SP cell population decreases with age [3,6]. The postnatal decrease in the capacity of *Abcg2*-expressing cells to contribute to cardiomyocytes may be explained by the decrease in new cardiomyocyte generation during the neonatal period or by the decrease in cardiac SP cells.

The present study is the first time the potential of the endogenous *Abcg2* lineage has been evaluated following cardiac injury. Surprisingly in response to IR, the *Abcg2* lineage does not contribute to cardiomyocyte renewal. Previously, Oyama et al. demonstrated that isolated neonatal cardiac SP cells could migrate to the site of injury and were able to differentiate into cardiomyocytes, endothelial cells, smooth muscle cells, and fibroblasts in vivo when transplanted following cardiac injury [6]. Similarly, Liang et al. further subfractionated the cardiac SP population based on the expression of Sca1 and CD31 (Sca1+, CD31–) and demonstrated the ability of these cells to migrate to the site of injury and differentiate into cardiomyocytes and endothelial cells following cardiac injury [7]. There are several possibilities that could explain the differences between these results and our results. Both of these results depend on the isolation of cardiac SP cells and transplantation into recipient animals; the

manipulation of isolated SP cells may alter their properties to favor cardiomyogenic differentiation. Second, Oyama et al. used cardiac SP cells isolated from neonatal rats. Consistently, our data suggest that the cardiomyocyte differentiation potential of the *Abcg2* lineage decreases with age; thus, again highlighting the differing capacity of young and adult cardiac SP cells. The regenerative capacity of *Abcg2*-derived cells may parallel or even overlap with the ability of other endogenous stem cell populations (c-kit or Sca1 positive) to contribute to cardiomyocytes following injury. However, the regenerative capacity of these cells has been controversial and recent data suggest that it may also be limited [22]. Last, it is possible that the level of CreER^{T2} expression from the *Abcg2* locus is below a sufficient threshold to induce recombination in all adult cardiac SP cells, which may leave a SP cell fraction unstudied in our current model.

Although the *Abcg2* lineage-tracing studies did not show evidence of *Abcg2*-derived cardiomyocyte regeneration or proliferation postinjury, this does not eliminate an important physiological role for *Abcg2*-derived cells in cardiac remodeling. Our studies have previously established a cytoprotective role for *Abcg2* in response to oxidative stress [11,15]. Other studies utilizing the *Abcg2*-null mice have shown decreased cardiac function and increased mortality in two different myocardial infarction models, which has been attributed in part to impaired survival of microvascular endothelial cells under oxidative stress [9,10]. Our current study confirmed that *Abcg2* contributes to vascular remodeling following injury and plays an important cytoprotective role in response to oxidative stress.

In summary, utilizing an inducible lineage-tracing technique, we have described the cell fate of *Abcg2*-expressing cells in cardiac development and adulthood. We have shown that during embryogenesis, the *Abcg2* lineage gives rise to multiple cardiovascular cell populations including cardiomyocytes, endothelial cells, and VSMCs. During the postnatal period, the cardiogenic capacity of the *Abcg2*-expressing cells diminishes, even in response to myocardial injury, however, the endothelial capacity remains. Preconditioning with an oxidative stress agent was confirmed to promote favorable remodeling postmyocardial injury. These data support the idea that the *Abcg2*-derived lineages play an important role in the cardiovascular development. In adulthood, the vascular contribution remains important in providing protective effects of ventricular remodeling postinjury.

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Author Disclosure Statement

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Address correspondence to:

Dr. Cindy M. Martin

Department of Medicine

Lillehei Heart Institute

University of Minnesota

Cancer and Cardiovascular Research Building

2231 6th Street SE

Minneapolis, MN 55455

E-mail: cmmartin@umn.edu

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