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BRIEF COMMUNICATION

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Marking of definitive HSC precursors in E7.5−E8.5 embryos using an Abcg2-CreER lineage-tracing mouse model

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Q3

Abcg2, a member of the ATP-binding cassette transporter family, is expressed in adult hematopoietic stem cells (HSCs) and is required for the side population phenotype of adult bone marrow HSCs and other adult tissue-specific stem cells. Lineage tracing in adult mice using the Abcg2-Cre mouse model showed that Abcg2 marks HSCs, intestinal stem cells, and spermatogonial stem cells. It is unclear whether definitive HSCs or their precursors in early embryonic development can be marked by Abcg2 expression. Here, we treated pregnant Abcg2 Cre/Cre RosaLSL-YFP mice with a single injection of 4-hydroxytamoxifen at embryonic day 7.5. Four months after birth, a small yellow fluorescent protein-positive (YFP$^+$) cell population could be detected in all of the major white blood cell lineages and this was stable for 8 months. Transplant of bone marrow cells or Sca1$^+$YFP$^+$ cells from these mice showed continued multilineage marking in recipient mice at 4 months. These results demonstrate that Abcg2 expression marks precursors to adult long-term repopulating HSCs at E7.5 to E8.5 and contributes to a stable subpopulation of HSCs well into adulthood. © 2018 ISEH – Society for Hematology and Stem Cells. Published by Elsevier Inc. This is an open access article under the CC BY license. (http://creativecommons.org/licenses/by/4.0/)

Abcg2 is a plasma membrane transporter that is expressed in the side population cells of a variety of tissues, including cancer cells, and is required for their SP phenotype [1,2]. In adult mice, virtually all hematopoietic stem cells (HSCs) express Abcg2 [3]. Lineage-tracing studies using an Abcg2$^{\text{CreER}}$Rosa$^{\text{YFP}}$ allele in the adult mice confirmed expression of Abcg2 in adult HSCs and revealed that adult tissue-specific intestinal stem cells and spermatogonial stem cells also express Abcg2 [4]. Definitive HSCs (dHSCs) have been identified at embryonic day 10.5 (E10.5) in the midgestation dorsal aorta with an estimated total of <100 of these cells [5]. The phenotype and number of precursors of dHSCs (pdHSCs) at earlier developmental stages at E7.5−E8.5 is less clear. We have shown that both hematopoiesis and HSC number and functions are normal in Abcg2$^{-/-}$ mice [6]. In our Abcg2$^{\text{CreER}}$ lineage-tracing mouse model, the ires-CreER expression cassette is inserted downstream of the stop codon of Abcg2, so Abcg2 was coexpressed with endogenous Abcg2 [4]. This mouse model allowed us to perform lineage tracing during embryo development under unperturbed conditions.

Methods

Mice

Abcg2$^{\text{CreERT2}}$*Rosa*$^{\text{EYFP}}$ mice were generated previously in our laboratory [4]. C57BL/6J mice were purchased from The Jackson Laboratory. All experiments with mice were performed according to a protocol approved by the St. Jude Children's Research Hospital Institutional Animal Care and Use Committee.

Tamoxifen treatment

4-Hydroxytamoxifen (4-OHT, Millipore Sigma, USA) was dissolved in sunflower oil at a concentration of 5 mg/mL. Pregnant *Abcg2*$^{\text{CreERT2/CreERT2+}}$ *Rosa*$^{\text{EYFP/EYFP}}$ mice were

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treated with one intraperitoneal injection of 4-OHT at E7.5 using overnight timed breeding pairs.

Antibody staining for flow cytometry analysis

Expression of yellow fluorescent protein (YFP) in different peripheral blood and bone marrow cells was detected by flow cytometry. Peripheral blood samples were stained for B220, CD3, Gr1, Mac1, and Ter119 using fluorescent-conjugated antibodies (B220-PerCP-Cy5.5, CD3-APC, Gr1-APC-Cy7, Mac1-Alexa700, and Ter119-PE-Cy7, Becton Dickinson, USA).

Organ collection and processing

Mice were euthanized and intravenously perfused with phosphate-buffered saline followed by 2% paraformaldehyde. The organs were dissected and further fixed overnight at 4°C. Organs were cryopreserved with 30% sucrose and embedded in optimal cutting temperature compound (Tissue-Tek, Sakura, USA).

Immunofluorescence microscopy

Fourteen−micrometer-thick tissue sections prepared on a cryostat were immunostained with antibodies specific for green fluorescent protein (GFP, A11122 rabbit IgG, Invitrogen, USA), Pecam (BD 553070, rat IgG, Becton Dickinson, USA), and Abcg2 (clone BXP53 MC-981, Kamiya Biomedical Company, USA). For Pecam and Abcg2 antibodies, a secondary Alexa Fluor 488 donkey anti-rat antibody was used (A21208, Invitrogen, USA) and, for GFP antibody, a secondary Cy3 donkey anti-rabbit antibody was used (AP18C, Millipore, USA). Images were captured with a confocal laser-scanning microscope (Zeiss).

Transplantation

$Sca1^+$ cells were first enriched from bone marrow of selected mice that were treated with 4-OHT at E7.5 and were 9 months of age at that time. YFP^+ cells were then sorted and transplanted along with $Sca1^-$ cells into lethally irradiated (1100 rad) C57BL/6J recipient mice. Bone marrow nucleated cells were also transplanted from one donor mouse.

Results and discussion

Single pulse treatment of mice with 4-OHT at E7.5 marks pdHSCs

In our lineage-tracing mouse model, the CreERT2 was coexpressed with endogenous Abcg2 because the Ires-CreERT2 expression cassette is inserted downstream of the Abcg2 coding sequence [4]. Upon exposure to 4-OHT, the Cre translocates to the nucleus and deletes the stop element upstream of the EYFP transgene, which leads to ubiquitous, permanent expression of YFP in all progenies. We have also shown that HSC development was normal in the $Abcg2^{-/-}$ mice [6], so hematopoietic development is most likely unperturbed in the $Abcg2^{CreERT2}Rosa^{EYFP}$ mouse model. To limit the length of exposure of 4-OHT to a stringent short time window, we treated pregnant homozygous $Abcg2^{CreERT2}Rosa^{EYFP}$ females with a single 1 mg injection of 4-OHT at E7.5. It has been shown that this treatment does not mark cells beyond 24 hours due to the short half-life of 4-OHT [7,8]. A total of 18 live pups were born from three dams. The YFP marking in white blood cells was 0−6.2% at 1 month, 0.1−4.5% at 4 months, and 0−3.5% at 8 months (Figures 1A and 1B). In the majority of mice, the YFP marking was relatively stable between 1 and 8 months (Figures 1A and 1B). The YFP marking occurred in the $CD3^+$, $B220^+$, $Gr1^+$, and $Mac1^+$ lineages, suggesting pdHSC marking. None of the mice not exposed to 4-OHT had any YFP expression in the peripheral blood cells (Figure 1C, lower panels).

Bone marrow cells from mouse #2888, which had YFP marking in the peripheral blood, bone marrow, spleen, and thymus of 1.7%, 1.3%, 1.5%, and 2%, respectively, at 9 months were transplanted into four recipient mice at a dose of 7.5×10^6 cells each. Four months after the transplantation, all four mice had similar or higher YFP^+ cells in the peripheral blood compared with the donor bone marrow (Figure 1D). The YFP marking in a second donor (mouse #2887) in the peripheral blood, bone marrow, spleen, and thymus was 1.6%, 1.7%, 1.3%, and 0.8%, respectively. A total of 15,342 sorted $Sca1^+YFP^+$ cells were mixed with an equal number of sorted $Sca1^+YFP^-$ cells, along with 2×10^5 sorted $Sca1^-$ cells and transplanted into each of three recipient mice. Four months after the transplantation, >59% of cells in all lineages in peripheral blood were marked by YFP expression in all three recipient mice (Figures 1E and 1F). The third mouse (#2873) had YFP marking in the peripheral blood, bone marrow, spleen, and thymus of 0.9%, 0.4%, 0.8%, and 0.8%, respectively. A total of 2800 sorted $Sca1^+YFP^+$ cells were mixed with 2800 sorted $Sca1^+YFP^-$ cells, along with 2×10^5 $Sca1^-$ cells, and transplanted into two recipient mice. Four months later, the YFP marking in the peripheral blood mononuclear cells was 13.4% and 88.4%. These results suggest that Abcg2 is expressed in pdHSCs at E7.5−E8.5. Immunofluorescence staining of E7.5 embryo sections showed that Abcg2 is expressed primarily in the visceral endoderm, but lower expression can also be seen in some mesoderm cells (Supplementary Figure E1, online only, available at www.exphem.org). In a study using the $Runx1^{WT/CreER}$ mouse line, 1−10% of marking in all adult lineages were seen when mice were treated with 4-OHT at E7.5, which was interpreted as a contribution of yolk sac cells to adult hematopoiesis [9]. However, this interpretation is challenged by the temporal alteration in the emergence of dHSCs in heterozygous Runx1 embryos [10,11]. Q4 Our Abcg2-CreER mouse model could complement the Runx1 model in future studies because Abcg2 expression was not altered.

The low level of marking could reflect inefficient recombination because of either relatively low levels of expression of the recombinant allele in these