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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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Abstract

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 as well as other adult tissue-specific stem cells. Lineage tracing in the 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 the 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 (4-OHT) at gestational day E7.5. Four months after birth, a small YFP+ cell population can be detected in all the major white blood cell lineages, which was stable for 8 months. Transplant of bone marrow cells or Sca1+YFP+ cells from these mice showed continued multi-lineage marking in recipient mice at 4 months. These results demonstrate that Abcg2 expression marks precursors to adult long-term repopulating HSCs at gestational day E7.5 to E8.5 and contributes to a stable subpopulation of HSCs well into adulthood.

Keywords

Abcg2; hematopoietic stem cells; embryo; lineage tracing; precursors of definitive HSCs

Introduction

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 $Abcg2^{CreER}Rosa^{YFP}$ allele in the adult mice confirmed expression of Abcg2

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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 E10.5 in the mid-gestation dorsal aorta with an estimated total of less than 100 of these cells (5). The phenotype and number of precursors of dHSCs (pdHSCs) at earlier developmental stages of 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 $Abcg2^{CreER}$ 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 allows us to do lineage tracing during embryo development under unperturbed conditions.

Materials and Methods

Mice:

Abcg2^{CreERT2}*Rosa*^{EYFP} mice were generated previously in our lab (4). C57BL/6J mice were purchased from Jackson lab. All experiments with mice were done according to protocol approved by the St. Jude Children's Research Hospital Institutional Animal Care and Use Committee.

Tamoxifen treatment of mice:

4-Hydroxytamoxifen (4-OHT, Millipore Sigma, USA) was dissolved in sunflower oil at a concentration of 5 mg/ml. Pregnant *Abcg2*$^{CreERT2/CreERT2+}$ *Rosa*$^{EYFP/EYFP}$ mice were treated with one intraperitoneal injection of 4-OHT at gestational day E7.5 using overnight timed breeding pairs.

Antibody Staining for Flow Cytometry Analysis:

Expression of YFP in different peripheral blood and bone marrow cells was detected by flow cytometry. Peripheral blood samples were stained for B220, CD3, Grl, Macl and Terl 19 using fluorescent conjugated antibodies (B220-PerCP-Cy5.5, CD3-APC, Grl-APC-Cy7, Macl-Alexa700 and Terl 19-PE-Cy7, Becton Dickinson, USA).

Organ collection and processing:

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

Immunofluorescence Microscopy:

14 μm thick tissue sections prepared on a cryostat were immunostained with antibodies specific for 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 Alexa488 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).

Transplant:

Sca1+ cells were first enriched from bone marrow of selected mice that were treated with 4-OHT at E7.5 and are at 9 months of age. 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

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

In our lineage tracing mouse model, the CreERT2 is co-expressed 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 the 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 3 dams. The YFP marking in white blood cells was between 0-6.2% at 1 month, 0.1-4.5% at 4 months and 0-3.5% at 8 months (Fig 1A, 1B). In the majority of mice, the YFP marking was relatively stable between 1 to 8 months (Fig 1A, 1B). The YFP marking occurred in 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 (Fig 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 4 recipient mice at a dose of 7.5×10Λ6 cells each. 4 months after the transplant, all 4 mice had similar or higher YFP+ cells in the peripheral blood compared to the donor bone marrow (Fig 1D). The YFP marking in a second donor (#2887) in the peripheral blood, bone marrow, spleen and thymus was 1.6%, 1.7%, 1.3% and 0.8% respectively. 15,342 sorted Sca1+YFP+ cells were mixed with equal number of sorted Sca1+YFP- cells, along with 2×10Λ5 sorted Sca1- cells, and transplanted into each of 3 recipient mice. 4 months after the transplant, in all 3 recipient mice, >59% of cells in all lineages in peripheral blood were marked by YFP expression (Fig 1E, 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. 2800 sorted Sca1+YFP+ cells were mixed with 2800 sorted Sca1+YFP- cells, along with 2×10Λ5 Sca1- cells and transplanted into two recipient mice. 4 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 shwed that Abcg2 is expressed

primarily in the visceral endoderm but lower expression can also be seen in some mesoderm cells (Supplemental Fig1). In a study using Runx1$^{WT/CreER}$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 contribution of yolk sac cells to adult hematopoiesis (11). However, this interpretation is challenged by the temporal alteration in the emergence of dHSCs in heterozygous Runx1 embryos (9, 10). Our Abcg2-CreER mouse model could complement the Runx1 model in this regard in future studies because Abcg2 expression was not altered.

The low level of marking could reflect inefficient recombination due to either relatively low levels of expression of the recombinant allele in these embryonic HSC precursors or due to inefficient nuclear localization with the single 4-OHT pulse. Alternatively, these marked embryonic HSC precursors may be generating only a minor population of adult HSCs that are competing against a larger fraction of HSCs that arise from precursors that originate later in gestation after the 24 hour 4-OHT washout (11).

Estimation of the number of pdHSCs in early embryos

The total number of dHSCs/RUs within the developing embryo at E12 was estimated to be about 66 using transplant assay (12). It has been difficult to measure the number of pdHSCs at E7.5-E8.5 using transplant based assays because these pdHSCs are not mature enough to reconstitute recipient hematopoiesis. Recent studies have also shown that HSCs could independently arise from the vitelline and umbilical arteries and vasculatures of the placenta and the brain (13-15). Given that cells, including the pdHSCs are digital entities, low marking at E7.5 allows us to estimate the number of pdHSCs at this stage. In several mice, about 0.3-0.8% of cells are marked (Fig 2). If we assume that the 0.3% YFP+ represents marking of a single pdHSC and that the lineage output capacity of all pdHSCs are similar, we could estimate that there are at least 333 pdHSCs at this stage. Even if the 4-OHT were effective as late as E10.5, the number of pdHSCs would be estimated to be at least 333 during E7.5-E10.5. This estimation is consistent with a recent study using multi-colored lineage tracing mouse model showing that approximately 719 pdHSCs were present at E7-E8.5 (16).

Lack of marking in kidney proximal tubule epithelium, hepatocytes and intestinal epithelium

When adult mice were treated with tamoxifen, the kidney proximal tubule cells, the hepatocytes and the intestinal epithelial cells can all be efficiently marked (4). The kidney proximal tubules start to develop from metanephric mesenchyme around E10.5 (17). The hepatocytes develop from the foregut endoderm around E8.0 (18). The small intestinal epithelium development starts at around E9.0 from the gut tube (19). When mice were treated at E7.5 and tissues analyzed at 9 months after birth, no YFP marking in kidney proximal tubules and intestinal epithelium were seen (Fig 3A, B n=4). A small number of cells in the liver are marked by YFP expression (Fig 3C). Costaining with the endothelial marker Pecam showed that majority of these YFP+ cells are located in the blood vessels, showing that they are hematopoietic cells. These results show that the precursor cells to the kidney proximal tubules, hepatocytes and small intestine do not express Abcg2 at E7.5-E8.5 and that the marking in pdHSCs is specific.

In summary, our Abcg2 lineage tracing mouse model showed that pdHSCs are relatively specifically marked at E7.5-8.5 by Abcg2 expression. This mouse model is a useful tool for studying these pdHSCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights:

- Abcg2 expression marks precursors of hematopoietic stem cells at E7.5-8.5
- At least 333 precursors of hematopoietic stem cells exist at E7.5-8.5
- Abcg2 does not mark precursors of Kidney, liver and small intestine at E7.5-8.5

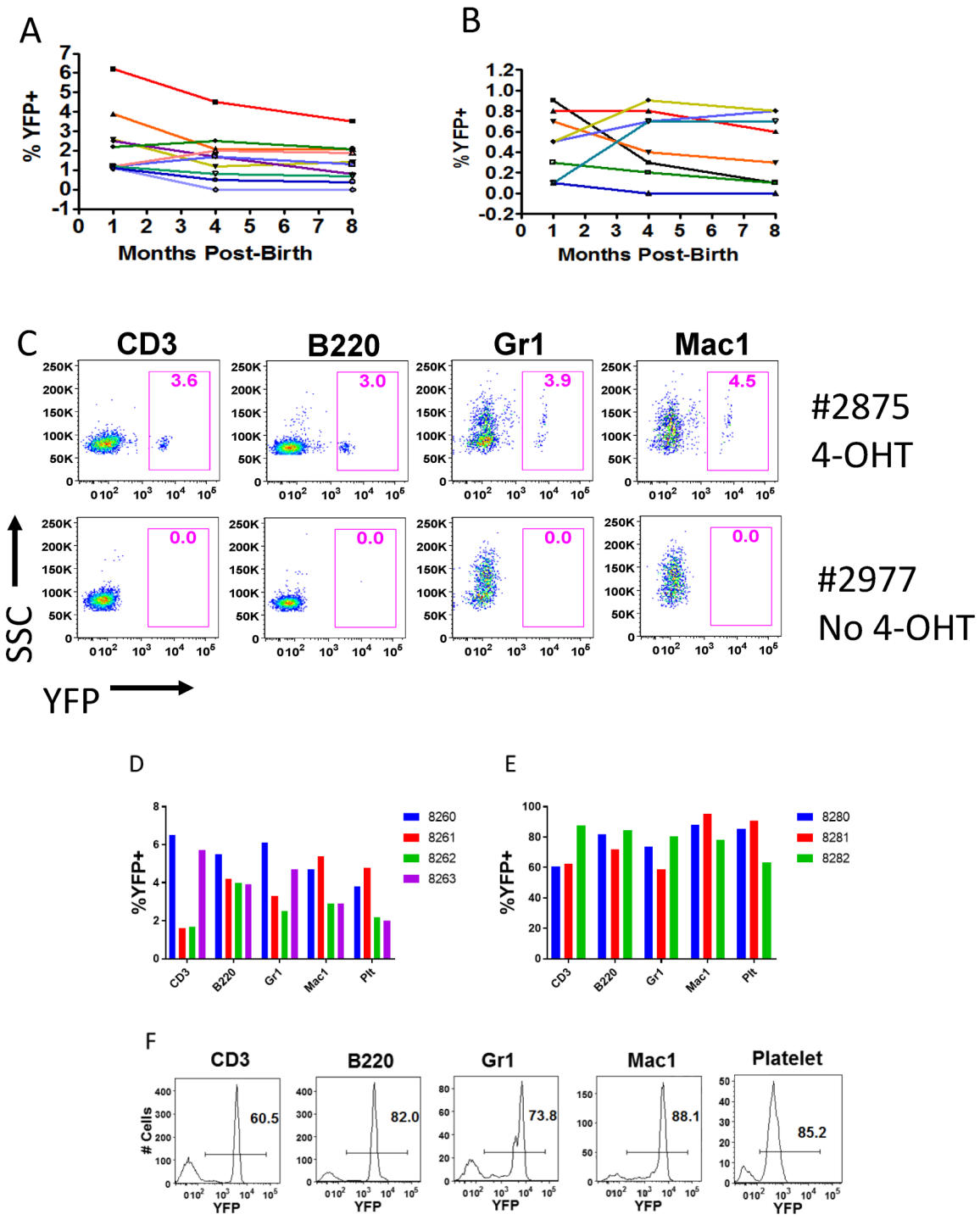

Fig 1. Abcg2 expression marks E7.5-E8.5 pdHSCs.

Pregnant *Abcg2*^{CreERT2}*Rosa*^{EYFP} mice were treated with a single injection of 4-OHT at E7.5. Fetuses were allowed to be born and grew to adult. Peripheral blood was taken at various time points and analyzed for YFP expression in the white blood cell lineages. Mice that had >1% YFP+ white blood cells at one month are graphed in A, and mice that had <1% YFP+ white blood cells at 1 month are graphed in B. YFP expression in white blood

cell lineages from a representative mouse is shown in C. One *Abcg2*^{CreERT2}*Rosa*^{EYFP} mouse that did not receive 4-OHT showed absolutely no YFP expression (C, lower panels). Total bone marrow cells (D) or sorted Sca1+YFP+ cells (E) from mice treated with 4-OHT at E7.5 when 9 months old were transplanted into lethally irradiated recipient mice and YFP expression was analyzed in peripheral blood cell lineages 4 months after transplantation. YFP expression in lineages from one representative recipient mouse is shown in F.

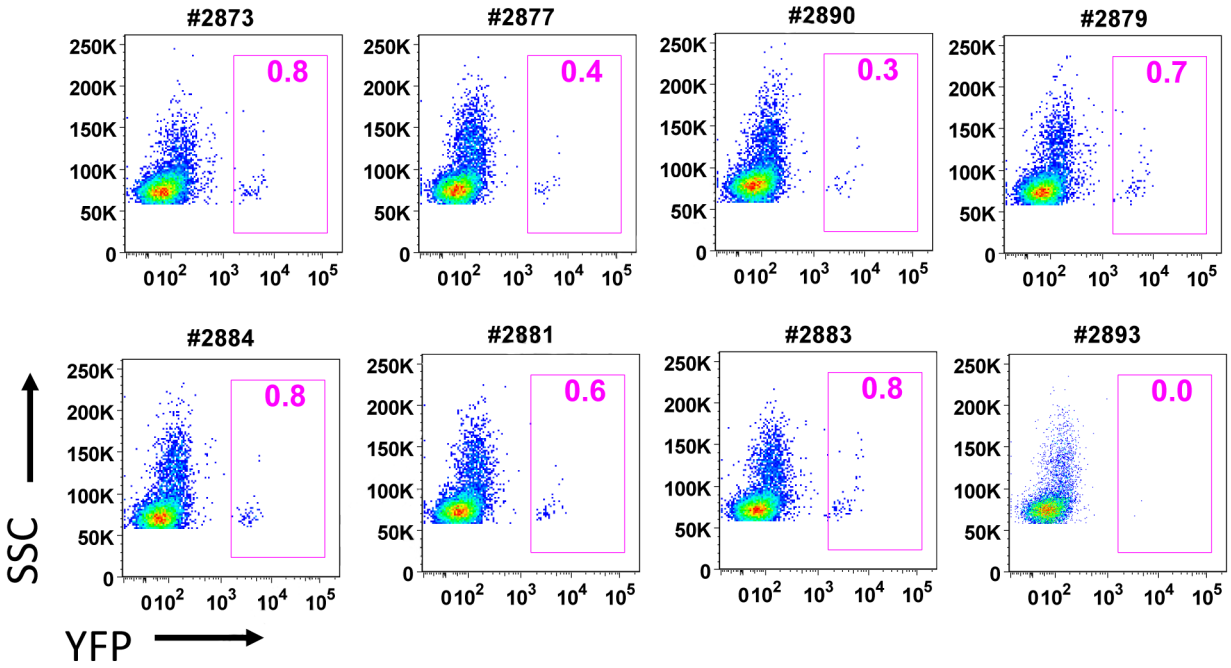

Fig 2. Low YFP marking in the blood allows estimation of the number of pdHSCs at E7.5-E8.5.

Abcg2^{CreERT2}*Rosa*^{EYFP} mice were treated with a single injection of 4-OHT at E7.5. Fetuses were allowed to be born and grew to adult. YFP expression in white blood cells at 8 months are shown for 8 mice.

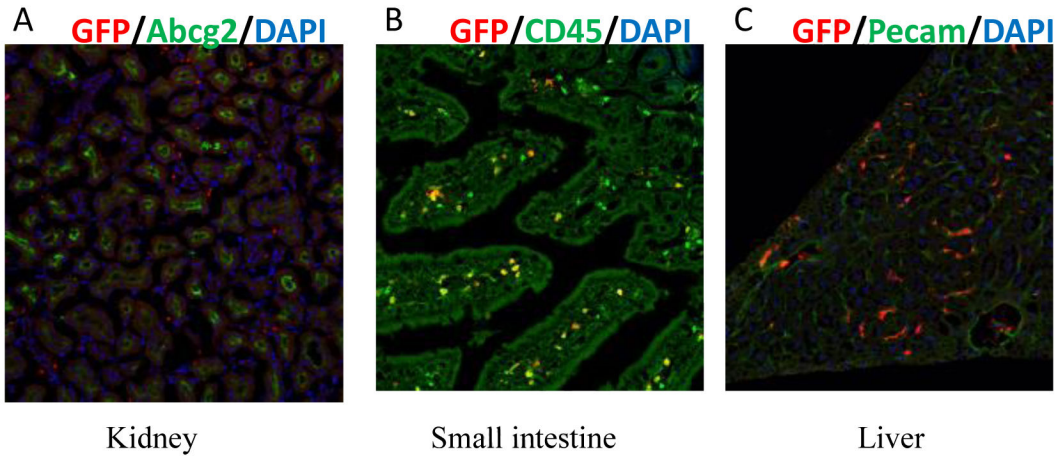

Fig 3. Lack of marking in kidney proximal tubules, hepatocytes and small intestine epithelial cells.

Abcg2^{CreERT2}*Rosa*^{EYFP} mice were treated with a single injection of 4-OHT at E7.5. Fetuses were allowed to be born and grew to adult and tissue sections were stained with anti-GFP antibody, anti Abcg2 antibody Bxp-53 and DAPI. Kidney (A), small intestine (B), liver (C) all at 20X magnification.