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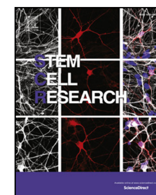

PDK1 regulates definitive HSCs via the FOXO pathway during murine fetal liver hematopoiesis

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ABSTRACT

PDK1 (phosphoinositide dependent kinase-1) plays an important regulatory role in B cells, T cells and platelets. Less is known about how *PDK1* acts in hematopoietic stem cells (HSCs), especially in the fetal liver (FL) during embryonic hematopoiesis, as the FL is the primary fetal hematopoietic organ and the main site of HSC expansion and differentiation. Here, we deleted the *PDK1* gene in hematopoietic cells by crossing *Vav-Cre* transgenic mice with *PDK1*$^{f/f}$ mice. Using a transplantation assay, we found that HSCs from the E15.5 FL of *Vav-Cre;PDK1*$^{f/f}$ embryos are severely impaired compared when compared with HSCs from *PDK1*$^{f/f}$ or *PDK1*$^{f/+}$ FLs. Additionally, we found that there were more FL HSCs in an apoptotic state and active cell cycle in *PDK1*-deficient embryos than in control embryos. By comparing the expression profiles of FL-derived LSKs in *Vav-Cre;PDK1*$^{f/f}$ embryos to the controls, we found that the BH3-only protein PUMA and the cyclin family proteins were expressed higher in the *Vav-Cre;PDK1*$^{f/f}$ group, which may account for the increased apoptosis and activated cell cycle in the deficient HSCs. Furthermore, we demonstrated that the expression of FoxO3a was higher in *PDK1*-deficient LSKs, indicating that the Akt-FoxO3a-PUMA axis may participate in regulating LSKs apoptosis in the E15.5 FL. In contrast, FoxO1 expression was lower in *PDK1*-deficient LSK cells, suggesting that Akt-FoxO1-CCND may regulate the HSC cell cycle. Taken together, our findings support a critical role for *PDK1* in maintaining FL hematopoiesis via regulating apoptosis and cell cycle of definitive hematopoiesis by the Akt-FOXO signaling pathways.

1. Introduction

During HSC development, the expansion and differentiation of HSCs occur in the fetal liver (FL). FL colonization by yolk sac (YS)-derived hematopoietic cells occurs as earlier as E9, followed by the arrival of immature HSCs from p-Sp/AGM at E10. These immature HSCs are capable to arise in situ to HSC and the first HSC appears in the FL at E11.5 either derived from in situ maturation or migrated from AGM. These HSCs will finally move to and reside in the bone marrow (BM) (Cumano and Godin, 2007; Kieusseian et al., 2012). Of note, the number of HSCs increases dramatically from 2 to 3 to 800–1000 in the mouse FL between E11.5 and E15.5, suggesting a unique and powerful mechanism of the FL on the expansion of HSCs. Therefore, identifying novel regulators for HSC development in the FL is vital. The rise of induced pluripotent stem cells (IPS) provides the possibility of obtaining HSCs in vitro, but currently, HSCs with the ability for long-term self-renewal and multi-potential differentiation cannot be obtained by IPS technology. Therefore, a comprehensive and systematic understanding of the essential regulatory mechanism of FL hematopoiesis may provide theoretical basis for the expansion of HSCs in vitro, and the establishment of an accurate signal regulatory network shall be useful for clinical application (Mikkola and Orkin, 2006).

Akt is phosphorylated at two critical residues for its full activation. These residues are a threonine (Thr-308 in Akt1), which is phosphorylated by *PDK1*, and a serine (Ser-473 in Akt1), which is phosphorylated by mTORC2. In addition, *PDK1*, also known as the AGC kinase, is an important downstream molecule of the PI3K signaling pathway (Mora et al., 2004). *PDK1* phosphorylates the conserved T-

Abbreviations: FL, fetal liver; YS, yolk sac; HSCs, hematopoietic stem cells; *PDK1*, phosphoinositide dependent kinase-1; BFU-E, erythroid burst-forming units; GEMM, granulocyte, erythrocyte, monocyte, megakaryocyte- forming unit; CFU-GM, granulocyte-macrophage colony-forming unit

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loop regions of its targets to regulate cell responses to signals such as growth factors, insulin, and other physiological processes involved in cell metabolism, growth, survival and anti-apoptosis (Cantrell, 2001).

The frequent hyper-activation of Akt in cancer cells could be explained in part by the ability of Akt to promote cell survival and inhibit apoptosis. However, it is likely that activation of Akt can also overcome cell-cycle checkpoints and accelerate proliferation. These consequences of Akt activation in combination with its anti-apoptotic activity explain its frequent activation in cancer cells. For instance, Akt activation overcomes a G2/M cell-cycle checkpoint following DNA damage (Kandel et al., 2002). AKT1- null mice are viable but smaller, and accelerated apoptosis occurs in their thymus and testes in response to gamma-irradiation and dexamethasone (Chen et al., 2001).

It has been reported that deletion of *PDK1* affects the hematopoietic system. In the study of hematopoietic lineages, *PDK1* plays an essential role in the B cell antigen receptor-mediated signaling pathway. Deletion of *PDK1* specifically in B cell progenitors leads to abnormal differentiation of pro-B to pre-B and no obvious mature B or T cells in the spleen (Baracho et al., 2014; Park et al., 2013; Hinton et al., 2004). *PDK1* is also involved in the development of T lymphocytes. When *PDK1* is eliminated in the thymus, the differentiation of T lymphocytes is hindered. However, when *PDK1* is partially knocked out, the differentiation of T lymphocytes is normalized, although proliferation is abnormal (Hinton et al., 2004). Additionally, *PDK1* activates PKC to induce NF-kappa B activation and regulates T cell proliferation in the immune response (Lee et al., 2005; Hayashi et al., 2007; Park et al., 2009). *PDK1* is also an important regulator of arterial thrombosis as platelet-specific deletion of *PDK1* leads to thrombocytopenia (Chen et al., 2013). Thus, *PDK1* plays an important role in the biological function of B cells, T cells and platelets. *PDK1* has also been investigated as a therapeutic target in a variety of cancers, including leukemia (Hu et al., 2015).

PDK1 plays an important role in mouse embryonic development, heart development. However, the role of *PDK1* in the hematopoietic development of embryos is still not clear. To understand the mechanism of HSC expansion in the FL, we generated *PDK1* conditional knockout mice by using *Vav-Cre* transgenic mice (Ogilvy et al., 1999), that have *PDK1* deleted specifically from hematopoietic cells. Our data indicated that *PDK1* plays an important role in definitive hematopoietic stem cells expansion in the fetal liver. *PDK1*-deleted definitive hematopoietic stem cells cannot reconstitute recipient mice. Furthermore, we found that deletion of *PDK1* affects the Akt-FOXO signaling pathway, leads to apoptosis and cell cycle abnormalities in definitive hematopoiesis.

2. Materials and methods

2.1. Animals

All C57BL/6 mice and C57BL/6.SJL (CD45.1) mice were purchased from the State Key Laboratory of Experimental Hematology (SKLEH). All mice were maintained in a specific pathogen-free animal facility at SKLEH, and the Institutional Animal Care and Use Committee (IACUC) of the Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Science, approved all animal procedures for this research project. *Vav-Cre* mice were purchased from Jackson Lab. *PDK1* floxed mice (Lawlor et al., 2002) were kindly provided by Drs. Dario R. Alessi and Wen Ning. Male *Vav-Cre;PDK1*$^{f/+}$ and female *PDK1*$^{f/f}$ mice were used to obtain conditional *Vav-Cre;PDK1*$^{f/f}$ embryos on a CD45.2 background. Noon of the day of vaginal plug discovery following overnight mating was counted as day 0.5.

2.2. Flow cytometry

FL cells were incubated with unconjugated monoclonal antibodies to lineage markers including Ter119, Gr-1, Mac1, B220, CD3, CD4, and CD8, followed by a secondary incubation with streptavidin-conjugated APC-Cy7 and with directly conjugated antibodies to c-Kit, Sca-1, CD48, CD34, FLK2,CD16/32, CD45.1, CD45.2, CD41, CD93, Annexin V, 7AAD, Ki67,CD150 and hochest blue. All monoclonal antibodies were purchased from eBioscience and BD Pharmingen. Data were analyzed with FlowJo software (Tree Star Inc., Ashland, OR).

2.3. Immunofluorescence

For immunofluorescence, LSKs from control and *PDK1*-deleted mice were sorted with FACS according to the manufacturer's instruction. The cells were then fixed with 4% paraformaldehyde solution, permeabilized with 0.25% Triton X-100, washed three times with PBS and blocked with 1% BSA for 30 min at room temperature. The samples were then incubated overnight at 4 °C with primary antibody diluted in 1% BSA. Primary antibodies were detected by incubation with a secondary antibody (donkey anti-rabbit Alexa Fluor 546, Invitrogen) in 1% BSA for 1 h at room temperature. The samples were then washed three times. After 1 μg/ml Hochest was added, the samples were examined under a PerkinElmer UltraVIEW Vox 4D confocal microscope.

2.4. Transplantation assay

Cells from the fetal hematopoietic organs were transplanted together with 1×10^5 nucleated BM cells of CD45.1 mice to promote short-term survival. Female CD45.1 mice were exposed to a split dose of 9 Gy γ-irradiation (^{60}Co). Peripheral blood (PB) of the recipients was collected at the indicated time points. When 0.01 ee control or *PDK1*-deficient CD45.2 E15.5 FL cells were transplanted along with 100,000 CD45.1 wild-type BM cells into irradiated mice. > 10% of CD45.2 cells determined by FACS was considered as successful reconstitution.

2.5. HSC homing assay

2-3ee fetal liver cells from wild type (WT) or *Vav-Cre;PDK1*$^{f/f}$ embryos stained by CFSE were injected into CD45.2 mouse. Recipients were sacrificed to get bone marrow cells and spleen cells 17 h later since fetal liver cells were injected. FACS was performed to examine the CFSE positive bone marrow cells or spleen cells.

2.6. Colony-forming cell (CFC) assay

We labeled the FL cells with CD34, Flk2, Lin, Sca1, c-Kit antibodys and sorted the LT-HSC, ST-HSC and MPP cells by using BD FACSAria III·The sorted LT-HSC, ST-HSCs and MPP from WT and *Vav-Cre;PDK1*$^{f/f}$ mice were cultured in MethoCult GF M3434 medium (Stem Cell Technologies) containing various cytokines to support the hematopoietic progenitors. Colonies were counted after 3–14 days of culture according to the manufacturer's instructions.

2.7. BrdU staining assay

10 mg/ml solution of Brdu in sterile 1× DPBS was injected intraperitoneally into pregnant mice 16 h before embryos were develpmented to E15.5. Each pregnant mice was administrated with 100–200 μg of BrdU solution. After 16 h, fetal livers were dissected and pipetted into single cells with 1 ml syringe, cells were labeled with CD34, Flk2, lineage cocktail, Sca-1 and c-Kit. Then BrdU labeling assays were performed using the APC-BrdU Flow kit (BD Biosciences) according to the manufacturer's instruction.

2.8. In vitro cell growth assay

1600 sorted LSK cells were cultured in 96-well with 3 replications, and the cells were cultured in SFEM supplemented with 50 ng/ml mTPO and 100 ng/ml mSCF. Cell numbers were counted up to the fourth day of culture.

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