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Short report

Highly proliferative primitive fetal liver hematopoietic stem cells are fueled by oxidative metabolic pathways

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ABSTRACT

Hematopoietic stem cells (HSCs) in the fetal liver (FL) unlike adult bone marrow (BM) proliferate extensively, posing different metabolic demands. However, metabolic pathways responsible for the production of energy and cellular building blocks in FL HSCs have not been described. Here, we report that FL HSCs use oxygen dependent energy generating pathways significantly more than their BM counterparts. RNA-Seq analysis of E14.5 FL versus BM derived HSCs identified increased expression levels of genes involved in oxidative phosphorylation (OxPhos) and the citric acid cycle (TCA). We demonstrated that FL HSCs contain more mitochondria than BM HSCs, which resulted in increased levels of oxygen consumption and reactive oxygen species (ROS) production. Higher levels of DNA repair and antioxidant pathway gene expression may prevent ROS-mediated (geno)toxicity in FL HSCs. Thus, we here for the first time highlight the underestimated importance of oxygen dependent pathways for generating energy and building blocks in FL HSCs.

1. Introduction

In adult mammals, HSCs give rise to all the blood cells throughout life. During development, HSCs originate in the dorsal aorta, from where they migrate into the FL at E11. The FL is the main site of hematopoiesis until near birth. Around birth, HSCs migrate from the FL to the BM, where they reside during postnatal life. In the FL, HSCs undergo multiple rounds of symmetrical self-renewing cell divisions to give rise to the pool of stem cells required for the lifetime of the organisms. However, in adults, the most primitive HSCs (termed long-term repopulating (LT)-HSCs, that repopulate the hematopoietic system long-term following transplantations, are quiescent, dividing only every 140–180 days in mouse; Wilson et al. 2008; Foudi et al. 2009). BM HSC divisions are asymmetrical generating one HSC and one committed progenitor/short-term repopulating (ST)-HSC (Takano et al. 2004). Nevertheless, quiescent LTR-HSCs can respond very rapidly to stress or damage, and quickly exit from quiescence to regenerate the blood system (Cheng et al. 2000). However, this is associated with aging of LT-HSCs, demonstrated by shortening of telomeres and skewing of LT-HSC differentiation towards the myeloid lineage (Vaziri et al. 1994; Sudo et al. 2000).

Aside from phenotypic differences, functional differences have also been described between adult BM and FL LT-HSCs (Higuchi et al. 2003; Bowie et al. 2007). For instance, FL LT-HSCs display significantly faster expansion kinetics when grafted in adult mice, compared with LT-HSCs from BM of 8-week-old mice (Bowie et al. 2007). Furthermore, FL and early postnatal BM LT-HSCs undergo significantly more symmetrical self-renewing cell divisions compared with 8-week-old adult BM LT-HSCs.

Although it would be logical to hypothesize that extensively proliferating FL LT-HSCs may require a significantly different metabolic activity to create energy and building blocks for cell renewal, few if any studies have addressed this question. Cell metabolism consists of anabolic and catabolic reactions that allow cells to survive, function and synthesize

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new components for cellular division. The generation of cellular energy (ATP), reduction capacity (NADH, NADPH and FADH2) and cellular macromolecules is mainly fueled by the consumption of glucose, glutamine and fatty acids through glycolysis, glutaminolysis and β-oxidation, respectively (Berg et al. 2007; Matés et al. 2009). The function and activity of ATP-generating pathways is known to differ dramatically in stem cells and differentiated progeny. Most stem cells have been shown to use glycolysis for energy production, while more differentiated cells divert glucose to the mitochondria and exhibit a significantly higher rate of OxPhos (Varum et al. 2011; Abu Dawud et al. 2012). This has also been shown for adult BM LT-HSCs. Postnatal BM LT-HSCs depend on glycolysis for energy production, whereas glycolysis decreases upon differentiation and OxPhos increases (Suda et al. 2011; Klimmeck et al. 2012). While TCA cycle-related metabolites such as 2-oxoglutarate, acetyl-CoA, and succinyl-CoA were not detected in LT-HSCs, they were shown to accumulate pyruvate (Takubo et al. 2013). Loss of pyruvate dehydrogenase kinase (*Pdk*) (Takubo et al. 2013) or lactate dehydrogenase (*Ldha*) (Wang et al. 2014), which inhibits glycolysis, results in defects in LT-HSC function. As metabolic pathways used can determine fate changes at the stem cell level (Oburoglu et al. 2014), we believe that understanding the metabolism of proliferative FL LT-HSCs will provide insights into how it might be possible to achieve extensive self-renewal of BM LT-HSCs, and hence, create efficient ex vivo LT-HSC expansion systems.

In this study, we tested the hypothesis that in contrast to adult BM LT-HSCs, which use glycolysis for energy production, FL HSCs may use alternative metabolic pathways to fuel their ability to expand extensively, such as the OxPhos pathway. This hypothesis was based on results of an RNA-sequencing (RNA-seq) study we performed on E14.5 FL and 8-week-old murine LT-HSCs, to gain insight into the molecular differences between fetal and adult HSCs that underlie their functional attributes. In this screen, we surprisingly found that that FL HSCs express significantly higher levels of OxPhos and citric acid cycle (TCA) genes compared with BM HSCs.

2. Materials and methods

2.1. Animals

Six to 10 weeks old C57BL/6J-CD45.2 mice (Centre d'Elevage R. Janvier, Le Genest-St. Isle, France, http://www.criver.com/) were bred and maintained in the animal facility at KU Leuven, Belgium. During the experiments, mice were maintained in isolator cages, fed with autoclaved acidified water, and irradiated food ad libitum. Animal procedures were performed according to protocols approved by the Institutional Ethics Committee.

2.2. Isolation of LT-HSC from bone marrow and fetal liver

Total adult bone marrow (ABM) cells were flushed from femurs and tibiae of male C57BL/6J mice, pooled, washed twice with phosphate-buffered saline (PBS; Gibco Invitrogen, CA) containing 0.1% bovine serum albumin (BSA; Sigma). Lineage negative cell isolation was performed using lineage cell depletion kit (Miltenyi Biotec, Germany). To isolate LT-HSCs from ABM, resulting Lin^- cells were stained with FITC conjugated anti-Sca-1, PE conjugated anti-c-kit, APC conjugated anti-lineage antibody cocktail (BD Pharmingen, San Diego, CA), PerCP/Cy5.5 conjugated anti-CD150 and APC conjugated anti-CD48 antibodies. Cells were incubated on ice for 30 min.

FL tissues were obtained from C57BL/6J embryos dissected at embryonic day (E) 14.5 (14 days after vaginal plug was observed). Ter-119 positive erythrocytes and erythrocyte progenitors were depleted using MACS columns (Miltenyi Biotec, Germany) and stained with Alexa Fluor 488 conjugated anti-lineage antibody cocktail (containing CD4, CD5, CD8a, CD45R, Ter-119, GR-1 antibodies), PE conjugated anti-CD11b, APC conjugated anti-Sca-1, PE-Cy7 conjugated anti-CD150 and Alexa Fluor 488 conjugated anti-CD48. Cells were incubated on ice for 30 min.

HSCs were sorted by fluorescence-activated cell sorting (FACS), using a FACS ARIAIII (Becton Dickinson).

2.3. RNA sequencing and bioinformatics analysis

To compare the gene expression between ABM and FL HSCs, total RNA was isolated from two biological replicates each of LT-HSCs from E14.5 FL and adult BM using miRNeasy Micro Kit (QIAGEN) and amplified with the Ovation RNA-seq V2 system (NuGen Technologies, CA) as per manufacturer's instructions. Amplified cDNA (1 μg) was sheared using the Covaris system (Covaris, MA) and fragments of 200 bp (180–220 bp) were selected to construct a cDNA library. The libraries were subjected to 2*90 bp paired-end sequencing on an Illumina HiSeq2000 sequencer. Raw reads generated by Illumina HiSeq2000 were subjected to quality control. Sequence reads were pre-processed by the pipeline of BGI (Shenzhen, China) to remove adaptors and filter low quality reads. The high quality reads were aligned to the mouse reference genome mm9 using TopHat2 tool (Kim et al. 2013).

The differential gene expression analysis was performed using the R package DESeq2 (Love et al. 2014) and differentially expressed genes were identified using the following thresholds: false discovery rate (FDR) < 0.05 and |log fold change| > 1.0. Gene-set enrichment analysis (GSEA) was performed to detect significantly enriched gene sets (also called pathways) using the R package GAGE v2.14.4 (Luo et al. 2009). The manually curated gene-set source was downloaded from the Bader lab (http://baderlab.org/GeneSets), which consisted of multiple data repositories, e.g. GO (Ashburner et al. 2000), Reactome (Croft et al. 2011), and Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Ogata et al. 1999). GSEA results were visualized by Enrichment Map v2.1.0 (Merico et al. 2010) and Cytoscape v3.2.1 (Shannon et al. 2003). To further investigate differences on metabolic potential, KEGG metabolic pathway maps were reconstructed to provide an overview of gene expression profiling and molecular interactions within a single pathway by using the R package Pathview (Luo and Brouwer 2013). All sequencing data are deposited at the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) with accession number: E-MTAB-4034.

KEGG pathway analysis was also applied on the published datasets with accession number: GSE37000 (McKinney-Freeman et al. 2012), GSE55525 (Beerman et al. 2014) and E-MTAB-2262 (McKinney-Freeman et al. 2012; Beerman et al. 2014; Cabezas-Wallscheid et al. 2014). In brief, normalized (FRMA method) datasets with accession numbers GSE55525 and GSE37000 were downloaded from In silico DB (https://insilicodb.com) and data sets on HSCs, progenitor and cultured HSCs were analyzed for differentially regulated pathways using KEGG pathway analysis. Normalized read-count for RNA-seq data related to accession number E-MTAB-2262, was downloaded from the published dataset by Cabezas-Wallscheid et al. 2014 and KEGG pathway analysis was performed.

2.4. Quantitative RT-PCR

To confirm levels of expression of some of the genes, independent samples of BM and FL HSCs were sorted to perform qRT-PCR. RNA was isolated from the FACS sorted cells using the miRNeasy micro kit (Qiagen, Hilden, Germany). RNA was amplified and cDNA synthesized using the Ovation RNA-seq V2 system (NuGen Technologies, CA) as per manufacturer's instructions. qRT-PCR was performed using the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). The PCR reactions were carried out on the ViiA7 Real-Time PCR System (Applied Biosystems). A list of primers used for the qRT-PCR can be found in Table 1. Gene expression for different transcripts was calculated relative to β-actin as a housekeeping gene.