Cord blood-derived CD34+ hematopoietic cells with low mitochondrial mass are enriched in hematopoietic repopulating stem cell function

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ABSTRACT

The homeostasis of the hematopoietic stem/progenitor cell pool relies on a fine-tuned balance between self-renewal, differentiation and proliferation. Recent studies have proposed that mitochondria regulate these processes. Although recent work has contributed to understanding the role of mitochondria during stem cell differentiation, it remains unclear whether the mitochondrial content/function affects human hematopoietic stem *versus* progenitor function. We found that mitochondrial mass correlates strongly with mitochondrial membrane potential in CD34+ hematopoietic stem/progenitor cells. We, therefore, sorted cord blood CD34+ cells on the basis of their mitochondrial mass and analyzed the *in vitro* homeostasis and clonogenic potential as well as the *in vivo* repopulating potential of CD34+ cells with high (CD34+ Mito^{High}) *versus* low (CD34+ Mito^{Low}) mitochondrial mass. The CD34+ Mito^{Low} fraction contained 6-fold more CD34+CD38– primitive cells and was enriched in hematopoietic stem cell function, as demonstrated by its significantly greater hematopoietic reconstitution potential in immunodeficient mice. In contrast, the CD34+ Mito^{High} fraction was more enriched in hematopoietic progenitor function with higher *in vitro* clonogenic capacity. *In vitro* differentiation of CD34+ Mito^{Low} cells was significantly delayed as compared to that of CD34+ Mito^{High} cells. The eventual complete differentiation of CD34+ Mito^{Low} cells, which coincided with a robust expansion of the CD34– differentiated progeny, was accompanied by mitochondrial adaptation, as shown by significant increases in ATP production and expression of the mitochondrial genes ND1 and COX2. In conclusion, cord blood CD34+ cells with low levels of mitochondrial mass are enriched in hematopoietic repopulating stem cell function whereas high levels of mitochondrial mass identify hematopoietic progenitors. A mitochondrial response underlies hematopoietic stem/progenitor cell differentiation and proliferation of lineage-committed CD34– cells.

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Introduction

Human hematopoietic stem cells (HSC) and hematopoietic progenitor cells (HPC) are almost exclusively enriched in the CD34+ fraction, which represents a rare cell subset (<1%) in cord blood, bone marrow and mobilized peripheral blood.[1,2] In the setting of clinical transplantation, the dose of total CD34+ cells infused per kilogram of patient's bodyweight is used as a predictor of short-term hematopoietic recovery and establishment of long-term engraftment.[1,3,4] Although distinct surface markers (CD38, CD90, CD45RA, CD133, etc.)[5] have been experimentally used to distinguish between HSC or HPC, *bona fide* segregation of HSC and HPC relies on *in vitro* and *in vivo* functional assays.

It has been extensively demonstrated that the CD34+ fraction is phenotypically and functionally heterogeneous. Experimentally, only 1:10 to 1:4 of CD34+ or CD34+CD38- cells display clonogenic potential. Clinically, different outcomes/hematopoietic recovery are reported in patients with identical underlying disease who undergo hematopoietic stem/progenitor cell (HSPC) transplantation with equal doses of CD34+ cells after receiving identical chemotherapy treatment. It can, therefore, be speculated that genetically identical CD34+ cells within the graft may exhibit cell-to-cell variations not only in the amount of individual gene products but also in metabolic homeostasis/mitochondrial status, resulting in phenotypic and functional diversity.[6] The metabolic status of HSC and HPC becomes crucial during clinical HSPC transplantation since the efficiency of donor-derived HSC/HPC to engraft, survive, home, proliferate and differentiate into multiple lineages in a chemotherapy-induced aplastic patient is markedly influenced by their hypoxic niche, demanding a significant metabolic adaptation to survive and promote rapid and stable hematopoietic reconstitution in chemotherapy-induced aplastic microenvironments.[7,8]

As in other tissues, mitochondria play key roles in HSC/HPC and have recently come under increased scrutiny because compelling evidence has revealed their role in numerous cellular processes, beyond ATP production and apoptosis regulation, and they have recently even been suggested to act as cell-fate or lineage determinants.[9-11] In fact, deregulation of mitochondrial function plays a pathophysio-

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logical role in a range of hematologic diseases, such as inherited dyserythropoiesis, sideroblastic anemias and low-grade myelodysplastic symdromes.[8,12] In addition, transcriptome, epigenetic and proteomic studies in stem cell systems have indicated that specific metabolic/mitochondrial properties are essential for regulating the balance between self-renewal and differentiation.[13,14]

Although recent work has begun to shed light on the mitochondrial response during murine stem cell differentiation,[7,10,15,16] how and to what extent the mitochondrial mass/function contributes to human hematopoietic stem and progenitor function remains poorly understood. Here, we found that mitochondrial mass correlates strongly with mitochondrial membrane potential ($\Delta\Psi$m). This led us to separate cord blood-derived CD34$^+$ cells based on their mitochondrial mass and to analyze the *in vitro* homeostasis and clonogenic potential as well as the *in vivo* repopulating potential of CD34$^+$ cells with high (CD34$^+$ Mito^{High}) *versus* low (CD34$^+$ Mito^{Low}) mitochondrial mass.

Design and Methods

Cord blood collection and CD34$^+$ cell isolation and culture

Fresh umbilical cord blood units from healthy neonates were obtained from local hospitals following approval from our local Ethics and Biohazard Board Committee. The cord blood samples were pooled to reduce variability among individual units. Mononuclear cells were isolated using Ficoll-Hypaque and after lysing the red cells (Cytognos, Salamanca, Spain), CD34$^+$ cells were purified by magnetic bead separation using the human CD34 MicroBead kit and the AutoMACS Pro separator (Miltenyi Biotec) as instructed by the manufacturer.[17-19] The purity of the CD34$^+$ fraction was assessed by flow cytometry using an anti-CD34-PE antibody (Miltenyi Biotec), and only CD34$^+$ fractions showing purity higher than 90% were used.[17-19] The CD34$^-$ fraction was irradiated (15 Gy) and used as accessory cells for co-transplantation with CD34$^+$ cells.

MitoTracker staining and cell sorting

CD34$^+$ cells were stained with MitoTracker Red (CMXRos) and MitoTracker Green FM dye (Molecular Probes) for 10 min and 20 min, respectively, according to the manufacturer's guidelines and analyzed by wide confocal cytometry.[6] For functional assays, CD34$^+$ cells were FACS-sorted (FACSAria-II, BD Biosciences) based on MitoTracker Green levels into CD34$^+$ Mito^{High} and CD34$^+$ Mito^{Low} (n=10).

Measurements of ATP and reactive oxygen species

ATP levels were measured using a Cell-Titer-Glo® Luminescent Cell Viability Assay (Promega) according to the manufacturer's guidelines. Briefly, equal numbers of cells (5x10^4/100 μL) were seeded in a 96-well plate and 100 μL of the reaction reagent were added to each well. After 10 min of shaking, the luminescence signal was detected using the GloMax®-Multi Detection System (Promega) and compared against the ATP Standard Curve using ATP disodium salt (Promega).[6]

Reactive oxygen species were measured using the mitochondrial superoxide indicator MitoSOX Red, as previously described.[20] Briefly, CD34$^+$ Mito^{Low} and CD34$^+$ Mito^{High} cells were treated with 3 μM MitoSOX for 20 min and were then washed twice in Hanks balanced salt solution and analyzed by flow cytometry (*Online Supplementary Figure S1A*).

Gene expression by quantitative reverse transcriptase polymerase chain reaction analysis

RNA was extracted using the All Prep DNA/RNA kit (Qiagen) and cDNA was synthesized by using SuperScript™ First-Strand Synthesis System for reverse transcriptase polymerase chain reaction (Invitrogen). The expression of the mitochondrial genes ND1 and COX2 as well as HIF-1α and Meis1 was compared between CD34$^+$ Mito^{high} and CD34$^+$ Mito^{low} cellular fractions by quantitative polymerase chain reaction analysis. Values were normalized to β-actin. The primers used were: ND1-Fw-5'-TGCGAGCAGTAGCCCAAACAATCT-3', ND1-Rw-5'-TTATGGCCAAGGGTCATGATGGCA-3', COX2-Fw-5'-ACAGATGCAATTCCCGGACGTCTA-3', COX2-Rw-5'-GACGATGGGCATGAAACTGTGGTT-3', HIF-1α-Fw-5'-CTGCAACATGGAAGGTATTGCA-3', HIF-1α-Rw-5'-TACCCACACTGAGGTTGGTTACTG-3', Meis1-Fw-5'-AAAAGCGTCACAAAAAGCGT-3', Meis1-Rw-5'-GATGGTGAGTCCCGTGTCTT-3', β-actin-Fw-5-GATGGCCACGGCTGCTT-3' and β-actin-Rw-5'-AGGACTCCATGCCCAGGAA-3'. The polymerase chain reaction conditions were 50°C for 2 min followed by 90°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 60 s.[15] The expression of each gene was analyzed in three independent experiments performed in triplicate.

In vitro liquid culture, cell cycle, and apoptosis analyses of Mito^{High} and Mito^{Low} CD34$^+$ cells

FACS-sorted CD34$^+$ Mito^{High} and CD34$^+$ Mito^{Low} cells were cultured for 40 days on StemSpan media (Stem Cell Technologies) supplemented with early hematopoietic cytokines [stem cell factor (100 ng/mL), FLT3L (100 ng/mL) and interleukin-3 (10ng/mL); PeproTech].[21] To determine the growth kinetics of Mito^{high} and Mito^{low} CD34$^+$ cultures, cells were counted twice a week and replated at a density of 1x10^5 cells/cm^2. Similarly, the maintenance of the CD34$^+$ phenotype was analyzed twice a week as an approach to trace the "default" differentiation of both Mito^{High} and Mito^{Low} CD34$^+$ cells. For cell cycle analysis, Mito^{high} and Mito^{low} CD34$^+$ cells were fixed in 70% ice-cold ethanol and stored at -20°C. Subsequently, cells were washed with ice-cold phosphate-buffered saline and suspended in propidium iodide buffer containing 5 μg of propidium iodide and 100 μg/mL of RNAase. Cell cycle distribution discriminating between quiescent cells (G0/G1) and cycling cells (S/G2/M phase) was analyzed on a FACSCanto-II cytometer using FACSDiva and ModFit software (BD Bioscience).[21] The apoptotic status of Mito^{High} and Mito^{Low} CD34$^+$ cells was assessed using the annexin-V apoptosis detection kit (BD Biosciences) according to the manufacturer's instructions.[21] Briefly, cells were harvested and washed twice with phosphate-buffered saline before staining with annexin V-phycoerythrin and 7-amino actinomycin D. Apoptotic cells were detected by gating the annexin V-positive fraction.[21]

Colony-forming unit assay

Primary human clonogenic progenitor assays (n=6) were performed by plating 1000 sorted CD34$^+$ cells into methylcellulose H4434 (Stem Cell Technologies) containing the human growth factors stem cell factor (50 ng/mL), granulocyte-macrophage colony-stimulating factor (10 ng/mL), interleukin-3 (10 ng/mL) and erythropoietin (3 U/mL). Colonies were counted and scored on day 14 of the colony-forming unit (CFU) assay using standard morphological criteria.[21] For secondary replating, all the CFU colonies from the primary methylcellulose culture were harvested, and a single-cell suspension was achieved, washed in Iscove's modified Dulbecco's medium and replated in the secondary CFU assay.

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Mice transplantation and analysis of engraftment

NOD/LtSz-scid IL2Rγ$^{-/-}$ mice (NSG) were housed under sterile conditions. The Animal Care Committee of the University of Granada approved all animal protocols (Ref. UGR-CEEA 2011-361). Mice (n=22) at 8 to 12 weeks of age were sublethally irradiated (2.5 Gy) for 6 to 12 h before transplantation. Mice were anesthetized by isoflurane inhalation, and intrabone marrow transplantation was performed as described in detail elsewhere.[21-23] A total of 2×10^5 Mito^{high} or Mito^{low} CD34$^+$ cells together with 5×10^4 irradiated accessory cells were transplanted in a volume of 30 μL. For pain relief, 0.1 mg/kg buprenorphine and 5 mg/kg carprofen were administered immediately after transplantation and 24 h after.[21-23] Mice were killed 7 weeks after transplantation. Cells from the bone marrow (injected tibia and from the contralateral tibia and femur), spleen, liver and peripheral blood were stained with anti-hHLA-ABC-FITC and anti-hCD45-APC-Cy7 (BD Biosciences) to analyze human chimerism by flow cytometry. All engrafted mice were assessed for multilineage analysis using anti-hCD33-PE for myeloid cells, anti-hCD19-APC for B cells, and anti-hCD34-PE-Cy7 for immature hematopoietic cells (BD Biosciences).[21,22,24]

Statistical analysis

Data are expressed as mean ± SD. Statistical comparisons were performed using a paired Student's t test. Differences were considered statistically significant when the *P* value was <0.05.

Results

FACS sorting of CD34$^+$ cells based on mitochondrial mass

We first analyzed by confocal cytometry the mitochondrial membrane potential (ΔΨm) and the mitochondrial mass using MitoTracker Red (CMXRos) and MitoTracker Green, respectively. A strong correlation was observed between mitochondrial mass and function (ΔΨm) in cord blood-derived CD34$^+$ cells; the greater the mitochondrial content, the higher the ΔΨm (Figure 1A). Accordingly, CD34$^+$ cells were FACS-sorted based on mitochondrial mass (MitoTracker Green) into CD34$^+$ Mito^{High} and CD34$^+$ Mito^{Low} (Figure 1B). Sorted CD34$^+$ Mito^{High} contain 2-fold higher ATP levels (Figure 1C) and also express the mitochondrial-specific genes ND1 and COX2 at a level 2-fold higher than that of CD34$^+$ Mito^{Low} (Figure 1D), confirming that CD34$^+$ Mito^{High} cells have more expression of the mitochondrial-specific genes ND1 and COX2, and produce more ATP.

Hematopoietic stem versus progenitor cell characteristics of metabolically sorted cells

We next tested whether isolation of cord blood-derived CD34$^+$ cells based solely on their mitochondrial mass enriches for stem and/or progenitor cell function. We utilized *in vitro* clonogenic CFU assays as a read-out for HPC function, and bone marrow xenotransplantation assays into NSG mice as an *in vivo* read-out for SCID-repopulating HSC function. Equal numbers of CD34$^+$ Mito^{High} and CD34$^+$ Mito^{Low} were plated in CFU assays and hematopoietic colonies were counted after 14 days. CD34$^+$ Mito^{High} cells displayed higher clonogenic capacity in both primary and secondary CFU compared to CD34$^+$ Mito^{Low} (Figure 2A). Scoring of the CFU revealed no differences in the CFU types (granulocyte-, macrophage-, granulocyte-macrophage-, erythroid- and mix-) obtained from CD34$^+$ Mito^{High} *versus* CD34$^+$ Mito^{Low}. These data suggest that the CD34$^+$ Mito^{High} fraction is enriched in HPC.

In vivo hematopoietic reconstitution studies were undertaken to determine the long-term repopulating capacity of CD34$^+$ Mito^{High} and CD34$^+$ Mito^{Low}. Equal numbers of cells were transplanted into tibiae and mice were sacrificed 7 weeks later for chimerism analysis in multiple hematopoietic organs. Human chimerism was determined by flow cytometry using anti-CD45 and anti-HLA-ABC (Figure 2B). All engrafted mice were assessed for multilineage reconstitution using anti-CD19 (B cells), anti-CD33 (myeloid cells) and anti-CD34 (immature cells) (Figure 2B). The level of engraftment was higher in the mice transplanted with CD34$^+$ Mito^{Low} than in those transplanted with CD34$^+$ Mito^{High} regardless of the tissue analyzed, being more significant in the injected bone marrow (79±18% *versus* 59±17%; $P<0.05$; Figure 2C, left panel). We next characterized the engraftment composition, and found very similar multilineage composition in all tissues reconstituted with CD34$^+$ Mito^{High} or CD34$^+$ Mito^{Low} (Figure 2C, right panel) except for the more immature (CD45$^+$CD34$^+$) hematopoietic fraction which was significantly higher (15±3% *versus* 10±4%; $P<0.05$) in the bone marrow of mice engrafted with CD34$^+$ Mito^{Low} cells.

The vast majority of CD34$^+$ cells co-express the activation surface marker CD38 which is routinely associated with HPC whereas a very small proportion of CD34$^+$ cells lack CD38 expression and are considered to be enriched in HSC.[5,23,25] We, therefore, analyzed the proportion of CD34$^+$CD38$^-$ cells within the CD34$^+$ Mito^{High} and CD34$^+$ Mito^{Low} fractions (Figure 2D). The CD34$^+$ Mito^{Low} fraction contained 6-fold higher numbers of CD34$^+$CD38$^-$ primitive cells, thus supporting the concept that the CD34$^+$ Mito^{Low} fraction is enriched in repopulating HSC and also explaining, at least in part, the lower progenitor function of CD34$^+$ Mito^{Low} cells (Figure 2A,C). Together, these data indicate that the CD34$^+$ Mito^{Low} fraction is enriched in HSC function.

It has been reported[16] recently that murine long-term HSC are highly enriched in HIF-1α and Meis1. We, therefore, analyzed the expression of these factors on our CD34$^+$ Mito^{High} and Mito^{Low} cells. As shown in *Online Supplementary Figure S1B*, both HIF-1α and Meis1 were up-regulated, by 5- and 4-fold, respectively, in CD34$^+$ Mito^{Low} cells as compared to in CD34$^+$ Mito^{High} cells, confirming that these factors are robustly expressed in the CD34$^+$ cell fraction enriched in repopulating stem cell function.

In vitro homeostasis of CD34$^+$ Mito^{High} and CD34$^+$ Mito^{Low} cells

It remains a challenge to expand CD34$^+$ cells *in vitro* while retaining their stem/progenitor properties.[26] The homeostasis of sorted CD34$^+$ Mito^{High} and CD34$^+$ Mito^{Low} cells was analyzed *in vitro*. We first analyzed the *in vitro* differentiation kinetics of CD34$^+$ Mito^{High} and CD34$^+$ Mito^{Low} cultures by tracing the loss of the CD34 antigen. Interestingly, although the number of CD34$^+$ cells diminished progressively over time, the differentiation of CD34$^+$ Mito^{Low} cultures was significantly delayed as compared to that of CD34$^+$ Mito^{High} (Figure 3A). After 15 days of liquid culture, CD34$^+$ cells were no longer present in either CD34$^+$ Mito^{High} or CD34$^+$ Mito^{Low} cultures (Figure 3A,B). Expansion of CD34$^+$ Mito^{High} and CD34$^+$ Mito^{Low} cultures was moderate during the first 11-15 days of liquid culture (Figure 3B). However, coinciding with the complete differ-

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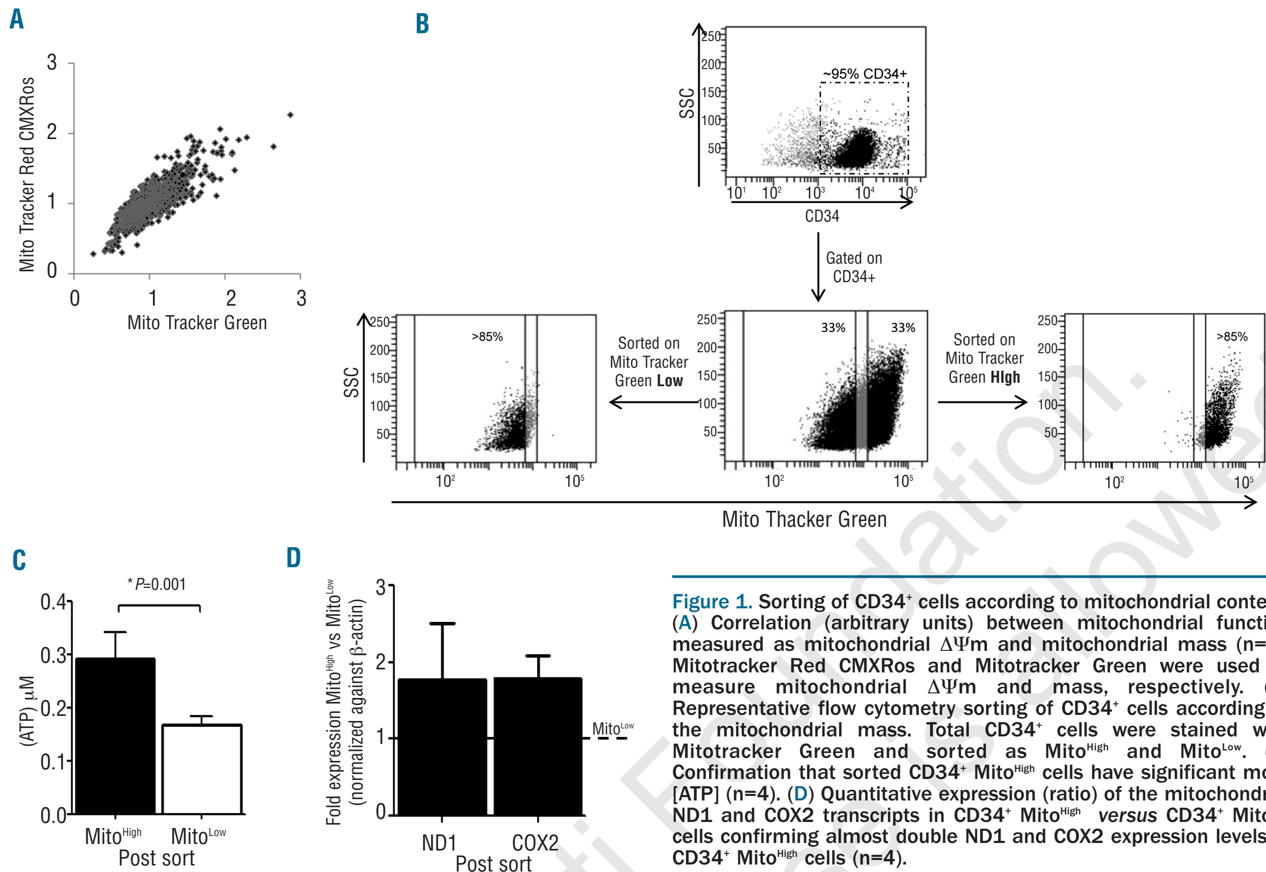

Figure 1. **Sorting of CD34$^+$ cells according to mitochondrial content. (A) Correlation (arbitrary units) between mitochondrial function measured as mitochondrial ΔΨm and mitochondrial mass (n=2). Mitotracker Red CMXRos and Mitotracker Green were used to measure mitochondrial ΔΨm and mass, respectively. (B) Representative flow cytometry sorting of CD34$^+$ cells according to the mitochondrial mass. Total CD34$^+$ cells were stained with Mitotracker Green and sorted as Mito^{High} and Mito^{Low}. (C) Confirmation that sorted CD34$^+$ Mito^{High} cells have significant more [ATP] (n=4). (D) Quantitative expression (ratio) of the mitochondrial ND1 and COX2 transcripts in CD34$^+$ Mito^{High} *versus* CD34$^+$ Mito^{Low} cells confirming almost double ND1 and COX2 expression levels in CD34$^+$ Mito^{High} cells (n=4).**

entiation (loss of CD34$^+$ cells) of the CD34$^+$ cultures after 11-15 days, the cell expansion of differentiated (CD34$^-$) Mito^{High} and Mito^{Low} cultures was significantly boosted, especially in the cultures seeded with CD34$^+$ Mito^{Low} cells (Figure 3B). After sorting, >99% of the CD34$^+$ Mito^{Low} cells were quiescent (G0/G1 cell cycle phases) while ~20% of the CD34$^+$ Mito^{High} cells were cycling (Figure 3C), but as CD34$^+$ cells differentiated into CD34$^-$ cells, Mito^{Low} cultures displayed a higher proportion of cycling cells (31±5% *versus* 22±6%, $P<0.05$) and lower apoptotic rate (21±3% *versus* 29±4%, $P<0.05$) than Mito^{High} cultures (Figure 3C,D), explaining the robust increased cell expansion of differentiated CD34$^-$ Mito^{Low} cultures as compared to differentiated CD34$^-$ Mito^{High} cultures (Figure 3B). Finally, the complete differentiation of CD34$^+$ Mito^{Low} cultures by day 15 of liquid culture coupled with their robust expansion from day 15 onwards (Figure 3A,B) was accompanied by a mitochondrial adaptation, as demonstrated by a significant increase in energy (ATP) production (Figure 3E) and expression of the mitochondrial genes ND1 and COX2 (Figure 3F). This suggests that a mitochondrial response underlies the high proliferative activity of the CD34$^+$ Mito^{Low}-differentiated derivatives.

Discussion

Mitochondria are multi-functional organelles that play a vital role in the cell, providing most of the cellular energy and regulating Ca^{2+} homeostasis, cell death, and differentiation; furthermore, they have recently been suggested to act as cell-fate or lineage determinants.[9-11] Hematopoietic tissue homeostasis relies on a finely tuned balance between very dynamic intrinsically and extrinsically regulated processes of self-renewal, differentiation and proliferation. Based on recent transcriptomic, epigenomic and proteomic studies, it has been proposed that the cellular metabolism regulates these processes in different stem cell systems.[14] The metabolic status of human HSC/HPC becomes crucial during clinical HSPC transplantation since the efficiency of donor-derived HSC/HPC to engraft, survive, home, proliferate and differentiate in chemotherapy-induced aplastic patients demands a significant metabolic adaptation to survive and promote rapid and stable hematopoietic reconstitution in chemotherapy-induced aplastic microenvironments.[7,8] Furthermore, deregulation of mitochondrial functions plays a pathophysiological role in several hematologic diseases.[8,12] Although recent work has begun to shed light on the mitochondrial response during stem cell differentiation,[7,10,15,16] how, and to what extent the mitochondrial content affects human hematopoietic stem *versus* progenitor function remains elusive.

Here, we report that mitochondrial content correlates strongly with ΔΨm in CD34$^+$ cells. Thus, cord blood-derived CD34$^+$ cells were sorted based solely on their mitochondrial mass, and the *in vitro* homeostasis and clonogenic potential as well as the *in vivo* repopulating potential of CD34$^+$ cells with high (CD34$^+$ Mito^{High}) *versus* low (CD34$^+$ Mito^{Low}) mitochondrial content were analyzed. As expected, the ATP levels and expression of mitochondrial-specific genes were higher in CD34$^+$ Mito^{High} than in CD34$^+$ Mito^{Low} cells. We show for the first time in human CD34$^+$ cells that hematopoietic stem and progeni-

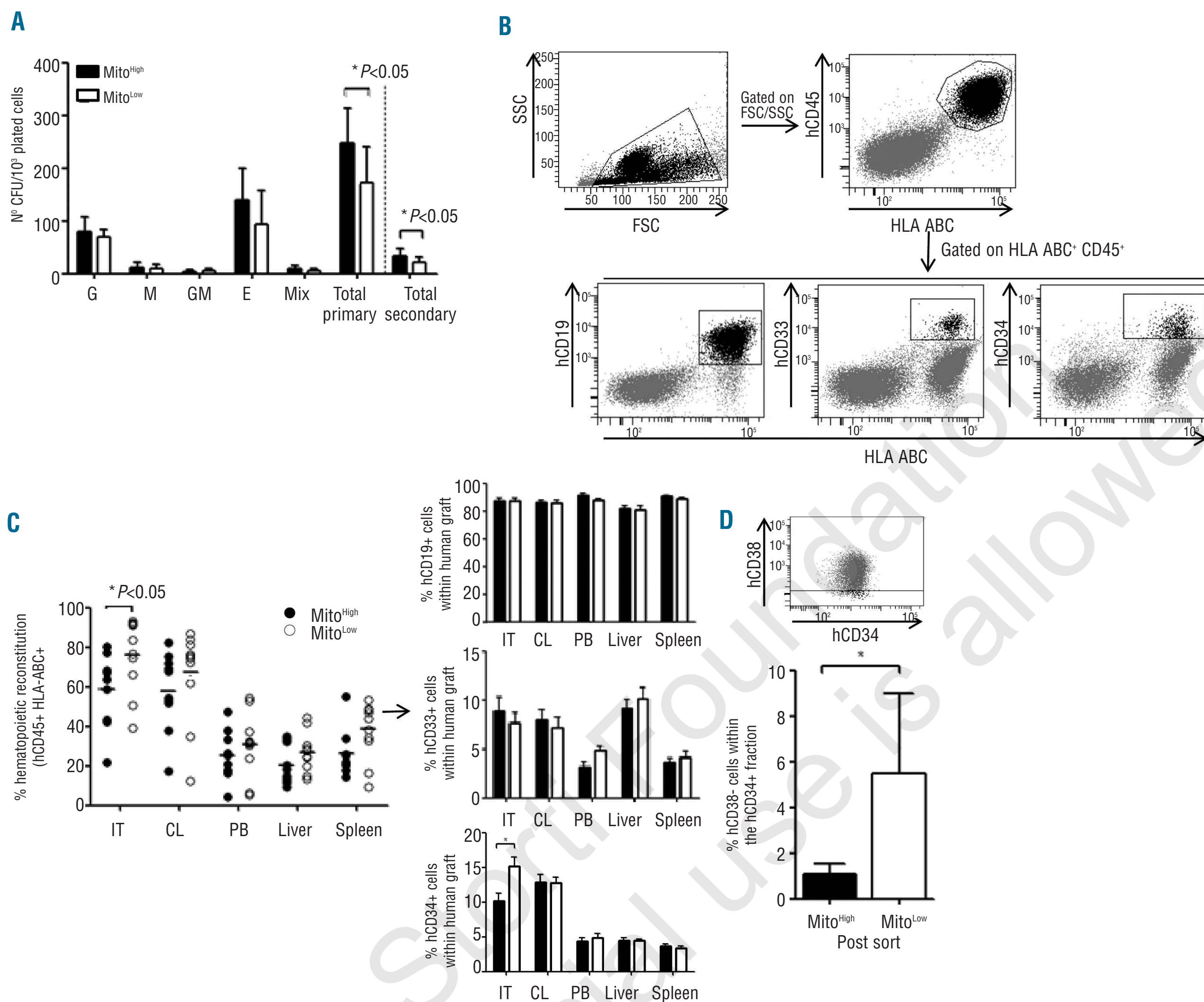

Figure 2. Mito^High and Mito^Low CD34⁺ cells display *in vitro* and *in vivo* functional differences. (A) Clonogenic (CFU) potential of Mito^High *versus* Mito^Low CD34⁺ progenitor cells (n=6). Primary CFU cultures were harvested and re-plated in secondary CFU assays. (B) Representative flow cytometric analysis of human multi-lineage engraftment in NSG mice. Human cells were identified as HLA-ABC⁺ CD45⁺. Within the human graft, myeloid (CD33⁺), B-lymphoid (CD19⁺) and immature (CD34⁺) lineages were analyzed. (C) Levels of human multi-lineage chimerism in injected tibia (IT), contra-lateral tibia (CL), peripheral blood (PB), spleen and liver indicating successful migration of the human CD34⁺ cells from the injected tibia. Each dot/square represents an individual mouse and the horizontal line indicates the mean of each experimental condition. (D) Proportion of CD38⁻ primitive/stem cells within CD34⁺ Mito^High and CD34⁺ Mito^Low fractions (bottom panel). The upper panel depicts a representative flow cytometry analysis of CD38 and CD34 staining.

tor cell functions segregate partially between metabolically sorted cells. The CD34⁺ Mito^Low fraction displayed significantly higher *in vivo* reconstitution potential upon inoculation in NSG mice whereas the CD34⁺ Mito^High fraction was more enriched in progenitor function with higher *in vitro* clonogenic capacity. This is in line with the "dogma" that stem cells are usually quiescent and contain low numbers of mitochondria whereas progenitors are more proliferative and contain higher numbers of mitochondria.[9,10] The vast majority of CD34⁺ cells co-express the activation surface marker CD38 which is associated with HPC whereas a very small proportion of CD34⁺ cells lack CD38 expression and are considered to be enriched in HSC.[5,23,25] Accordingly, the proportion of CD34⁺CD38⁻ primitive cells was 6-fold higher in the CD34⁺ Mito^Low than in the CD34⁺ Mito^High fraction, supporting the concept that the CD34⁺ Mito^Low fraction is enriched in repopulating HSC function, and also explaining, at least in part, the lower progenitor function of CD34⁺ Mito^Low cells. Our data confirm previous findings in mice showing that the Mito^Low cellular fraction of murine bone marrow is highly enriched in repopulating HSC, partially due to the enrichment of long-term HSC in this Mito^Low fraction.[16] Furthermore, Simsek *et al.*[16] recently reported that murine long-term HSC are highly enriched in Meis1, a HSC-associated transcriptional factor required for definitive hematopoiesis, and in HIF-1α, a master regulator of glycolysis and mitochondrial respiration, including a metabolic shift toward anaerobic glycolysis. In line with this study, the expression of both HIF-1α and Meis1 was found to be robustly up-regulated in CD34⁺ Mito^Low cells as compared to in CD34⁺ Mito^High cells, confirming that these factors are robustly expressed in the CD34 cell fraction enriched in repopulating stem cell function. Interestingly, mitochondrial content enriches for either HSC or HPC but it does not seem to act as a determinant in progenitor/lineage com-

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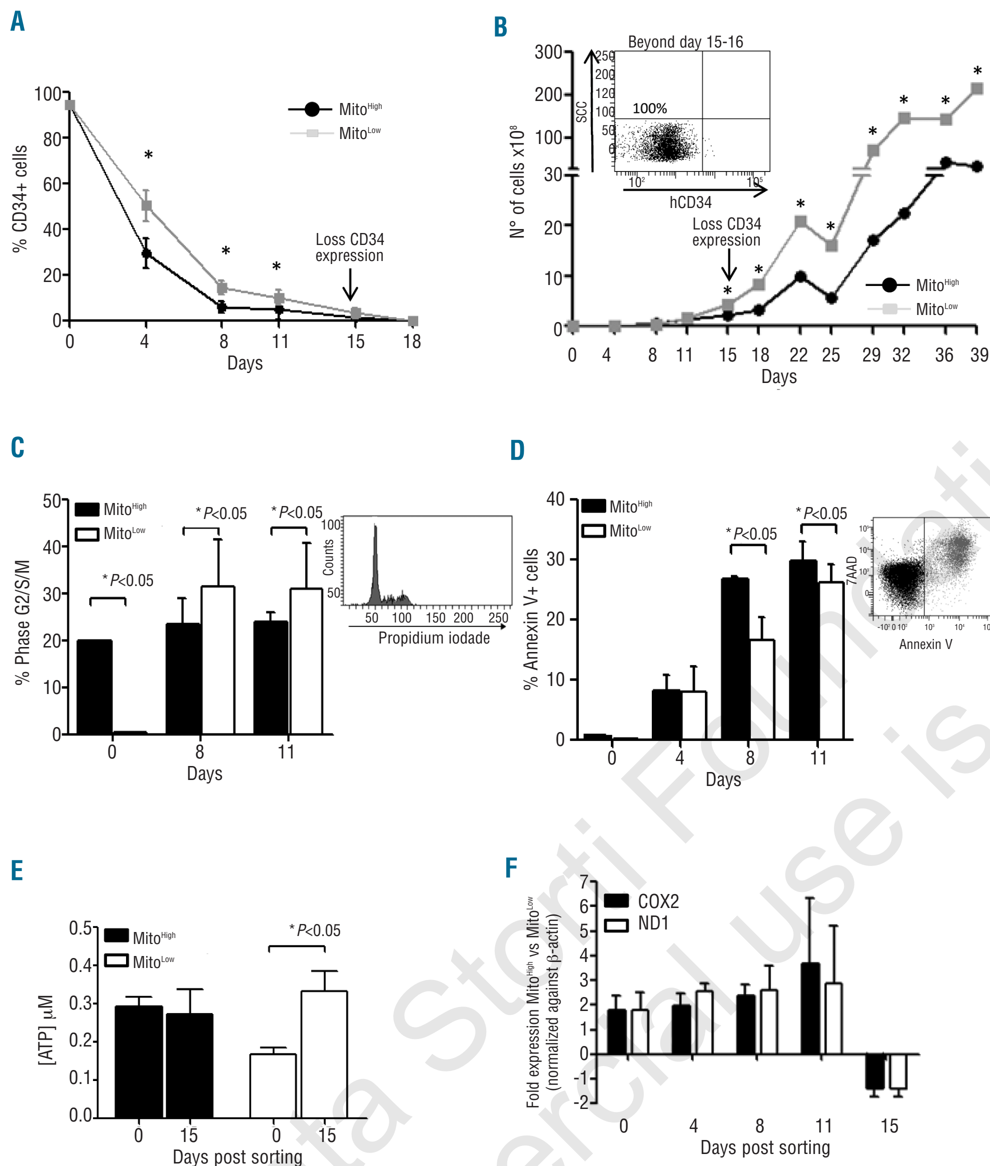

Figure 3. ***In vitro*** **homeostasis of CD34+ Mito^High and CD34+ Mito^Low cells. (A) Loss of CD34 antigen over time in liquid cultures seeded with Mito^High *versus* Mito^Low CD34+ cells (n=6). (B) *In vitro* expansion of liquid cultures initially seeded with Mito^High *versus* Mito^Low CD34+ (n=6). Note that after day 15 there are no residual CD34+ cells in the culture. From day 15 onwards the entire culture is comprised of differentiated CD34- cells (flow cytometry panel as inset). (n=6). (C) Proportion of cycling cells (S/G2/M cell cycle phase) measured at the indicated time points in Mito^High *versus* Mito^Low CD34+ cultures (n=2). (D) Proportion of apoptotic cells (annexin V+) measured at the indicated time points in Mito^High *versus* Mito^Low CD34+ cultures (n=2). (E-F) *In vitro* differentiation of CD34+ Mito^Low cultures (loss of CD34 antigen) is associated with increased ATP production (E) and higher levels of ND1 and COX2 expression (F).**

mitment since the multilineage composition in both CFU and repopulating assays was very similar between CD34+ Mito^High and CD34+ Mito^Low cells. These data suggest that mitochondrial mass/activity determines global *in vitro* clonogenic potential and *in vivo* repopulating function but does not impair/skew normal developmental stem cell fate/hematopoietic lineage commitment.

Homeostasis of CD34+ Mito^High and CD34+ Mito^Low cultures was further analyzed *in vitro*. The differentiation of CD34+ Mito^Low cultures was significantly delayed as compared to that of CD34+ Mito^High, but CD34+ cells were no longer present in either CD34+ Mito^High or CD34+ Mito^Low cultures after 15 days. Expansion of CD34+ Mito^High and CD34+ Mito^Low cultures was limited during the first 15 days; however, coinciding with the complete differentiation of the CD34+ cultures after 15 days, the expansion of differentiated (CD34-) Mito^High and Mito^Low cells was significantly boosted, especially in the cultures seeded with CD34+ Mito^Low cells. The fact that the CD34+ Mito^Low cells display a delayed differentiation coupled to a much higher expansion rate of their CD34- differentiated cells supports the better hematopoietic engraftment of NSG mice transplanted with the CD34+ Mito^Low fraction because efficient *in vivo* hematopoietic reconstitution relies on a finely tuned balance between low proliferative/self-renewing CD34+ HSC and rapidly amplifying progenitors, many of which are far lineage-committed and lack CD34 expression.

Mitochondrial biogenesis during the differentiation of embryonic stem cells has recently been studied.[11,27] Undifferentiated embryonic stem cells have a low mitochondrial content and low levels of ATP. Upon differentiation the mitochondrial content increases and mitochondrial biogenesis is activated to promote the synthesis of increased levels of ATP which seem to be required for the homeostasis of the differentiated cells.[28-30] Similarly, we found that the complete differentiation of CD34+ Mito^Low cells, which coincided with a robust expansion of the differentiated progeny, was accompanied by mitochondrial adaptation as demonstrated by a significant increase in ATP production and expression of the mitochondrial

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genes ND1 and COX2. This suggests that HSPC differentiation and proliferation of rapidly amplifying lineage-committed CD34⁻ cells are processes demanding up-regulation of mitochondrial content and biogenesis, and these properties seem to be conserved among embryonic stem cells and HSC/HPC.

Increased bioenergetics and mitochondrial activity come at an increased risk of oxidative damage, primarily in the form of reactive oxygen species (ROS) which are mainly generated by the mitochondria.[10,31] Various recent studies in mice have provided evidence for a link between intracellular ROS levels and preservation of stem cell function, with increased levels of ROS being associated with reduced repopulating stem cell activity.[32,33] Stem cells have strategies to lessen the negative impact of ROS such as lowering the numbers of mitochondria and ATP generation by promoting glycolysis over oxidative phosphorylation.[29,34] In this study, human $CD34^+$ $Mito^{Low}$ cells which were shown to be more enriched in HSC function displayed significantly lower levels of ROS than $CD34^+$ $Mito^{High}$ cells (*Online Supplementary Figure S1A*) which are more enriched in HPC function, suggesting that HSC function may be enriched in the $CD34^+$ $Mito^{Low}$ fraction as a strategy to lessen the negative impact of ROS, which can induce cell damage/death. Finally, during mitosis mitochondria may be segregated to daughter cells either symmetrically or asymmetrically. Asymmetric cellular distribution of mitochondria is a dynamic process which has been observed in some species and may play a role in cell-fate determination, differentiation and self-renewal.[11,27,35-37] Whether symmetric or asymmetric mitochondrial segregation occurs in human $CD34^+$ cells is unknown. It would be worth studying in future work whether asymmetric mitochondrial segregation could be an underlying mechanism explaining why phenotypically identical $CD34^+$ cells may exhibit cell-to-cell variations in mitochondrial content resulting in functional diversity.

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