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BRIEF COMMUNICATION

Identification of miRNAs from stem cell derived microparticles in umbilical cord blood

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Umbilical cord blood CD34+ (UCB-CD34+) stem cells are clinically used in hematopoietic cell transplantation. However, there are limitations in the use of umbilical cord blood transplants because of the small number of cells and delayed engraftment. To gain a better understanding of functional components of UCB, we have detected and characterized CD34+ microparticles (CD34+MPs) from cord blood units. We collected cord blood units and assessed the numbers of CD34+MPs before and after red blood cell and plasma depletion by SEPAX processing using flow cytometry analysis. In parallel we identified MPs by electron microscopy. CD34+MPs and cells were isolated by MACs sorting. MicroRNAs (miR-106, miR-221, miR-517, miR-519, and miR-221) exhibited a characteristic microRNA profile that was further validated in isolated CD34+MPs. We found that in cord blood, there are CD34+MPs that carry microRNAs.

Cord blood (CB) represents an alternative source of stem cells for hematopoietic stem cell transplantation (HSCT) [1]. Hematopoietic stem cells (HSCs) express the cell surface glycoprotein CD34+, which is associated with stem cell engraftment [1]. Microparticles represent a part of the extracellular vesicles, vary in size (100–1,000 nm) and content [2], and are released from cells during apoptosis or activation [3]. MPs are produced by budding from the plasma membrane after rearrangement of cytoskeleton, which leads to the exposure of phosphatidylserine (PS) [4]. PS on the surface of MPs permits their detection by flow cytometry (FCM) using annexin V. Additional MPs express cell surface markers of their cell origin, which can be detected by FCM. MPs from platelet and endothelial cells have been detected in the peripheral blood of transplanted patients [5,6]. They can function as vectors transferring mRNAs, proteins, and miRNAs [7–9], mediating intercellular communication and interacting with target cells [10]. MicroRNAs (miRNAs) are small RNAs, 19–22 nucleotides in size, that regulate gene expression at the posttranscriptional level. Many studies have reported miRNA expression in hematopoietic cell lines [11–13] and CB cells [14,15]. MPs derived from embryonic or leukemic cells may carry miRNAs [9,10]. MicroRNAs 106, 517c, 519d, and 520h have been detected in umbilical cord blood CD34+ (UCB-CD34+) cells [15,16]. MicroRNAs 106b and 221 are differentially expressed in UCB-CD34+ cells and mobilized peripheral blood (PB) [16–20]. MicroRNA 221 is expressed in erythropoietic progenitor cells in cell cultures of CB CD34+ cells [21]. MicroRNA 221 inhibited engraftment of CD34+ cells in NOD-SCID

AX and MG collected data, analyzed and interpreted the data, and wrote the article. AS and DB analyzed and interpreted the data. AAr analyzed and interpreted the data and wrote the article. AAn reviewed the manuscript, DS directed the research, and reviewed the manuscript.

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mice [22], while miR-519 decreased HL60 cell viability and induced cell apoptosis [12]. In the present study, we detected CD34+ cell-derived MPs in UCB. In addition, we found that these MPs contain miRNAs that may represent a signature for UCB stem cell-derived MPs.

Methods

Human UCB cell collection and processing

Thirty-seven cord blood units were collected in sterile bags containing CPDA-1. Processing of cord blood units was performed in accordance with NETCORD-FACT standards within 48 hours using the automated processing system Sepax (Biosafe SA, Switzerland) [23]. Informed consent was signed by the parents for research use, and the project was approved by the institutional review board of "G. Papanicolaou" Hospital.

Isolation and quantification of MPs

UCB-derived MPs were isolated from plasma by centrifugation at 14,000*g* at 4°C for 30 min [24]. MPs were labeled for flow cytometry with annexin V–fluorescein isothiocyanate (Immunostep S.L.) and anti-CD34-phycoerythrin (Exbio). The instrument was set up with the Megamix beads ranging from 0.1 to 1μm (Biocytex, France). MP immunofluorescence was counted using the Becton Dickinson FACSCantoII and analyzed with the FACSDiva 7.0 software program [25]. The number of MPs was estimated using the algorithm [(events of gated MPs) × (total events of beads in the tube)]/[(events of measured beads by FACs) × (volume of the sample)].

Transmission electron microscopy

MPs were fixed and embedded in Epon. Ultrathin sections (600–900 Å) were taken (ultracut Leica EM UC6). Samples were observed under a TEM JEOL 1011microscope at an accelerating voltage of 80 kV.

Scanning electron microscopy

Samples were fixed and mounted on aluminum specimen stubs and sputter-coated with gold–palladium and carbon (Balzers SCD 004 Sputter Coater). All samples were examined by scanning electron microscopy (SEM) in a JEOL JSM- 6390 LV Oxford scanning electron microscope.

Isolation of CD34+ MPs and cells

CD34+MPs and cells were isolated from processed UCB using Magnetic Separation Columns for positive selection (Miltenyi Biotec), following the manufacturer's instructions. UCB-derived MPs isolated from plasma by centrifugation were incubated with CD34 microbeads and FcR blocking reagent at 4°C for 30 min. CD34+MPs were isolated by magnetic separation. The purity of the collected CD34+MPs or cells was estimated by fluorescence-activated cell sorting (FACS) analysis.

miRNA isolation

Total RNAs including miRNAs from CD34+ cells and MPs were isolated using the miRNeasy Micro Kit and serum/plasma kit (Qiagen) according to the manufacturer's instructions. RNA concentration was determined with the Qubit fluorometer (Thermo Fisher Scientific).

Quantitative real-time PCR

cDNA was synthesized using the miScript II RT Kit (Qiagen), and the starting material was 500 pg of total RNA containing miRNA. Quantitative real-time polymerase chain reaction (qPCR) was performed with SYBR FAST qPCR Master Mix kit (Kapa Biosystems). The spike-in control *Caenorhabditis elegans* miR-39 miRNA mimic (Qiagen) was introduced to evaluate the efficiency of the entire procedure. Quantitation was based on the absolute count of template number based on a titration curve starting from 10^{-2} to 10^{-6} molecules of the spike-in control. The values of miRNAs were adjusted using the comparative *Ct* method normalized with miR-39.

The following primers were purchased from Qiagen (miScript primer assays): Hs_miR-106b (MS00003402), Hs_miR-221 (MS00003857), Hs_miR-517c (MS00009954), Hs_miR-519d (MS00004508), and Hs_miR-520h (MS00044926).

Cloning and sequencing

PCR products were extracted from agarose gel using the Qiagen kit. MyTaq DNA polymerase (Bioline) was used to add 3′A overhangs, and subsequently the TOPO cloning reaction was performed (Invitrogen) using the pCR II-TOPO vector. Constructs were transformed into One Shot Mach1-T1 competent *Escherichia coli* cells (Invitrogen). Competent cells were plated on LB-kanamycin agar plates. White colonies were checked for the presence of the insert via colony PCR with vector primers and sequencing of the construct.

Statistics

All statistical analyses were performed with SPSS 15.0 and GraphPad Prism 6.0. Comparisons between groups were made with Pearson's or Spearman's coefficient depending on the normality of the distribution of variables.

Results and discussion

Detection of Ann+/CD34+MPs

UCB processing removes red blood cells and decreases the volume of cord blood units [1]. The number of nucleated cells that a cord blood unit contains is not proportional to the volume. The same applies to CD34 + cells. In many cases there are cord blood units of high volume with a small number of nucleated or/and CD34+ cells and vice versa. Plasma from pre- and post-processed cord blood has been used for MP measurement in flow cytometry [25]. The number of Annexin V+/CD34+MPs per microliter represents their concentration in the plasma. Pre-processed Annexin V +/CD34+MPs are reduced during processing (57.7 ± 8.4 vs. 51.4 ± 6.2) [24], indicating a significant positive correlation ($p = 0.00$, Spearman's $\rho = 0.883$). The number of pre-processed Annexin V+/CD34+MPs is moderately correlated with the starting volume of the cord blood unit before processing ($p = 0.027$, Pearson's

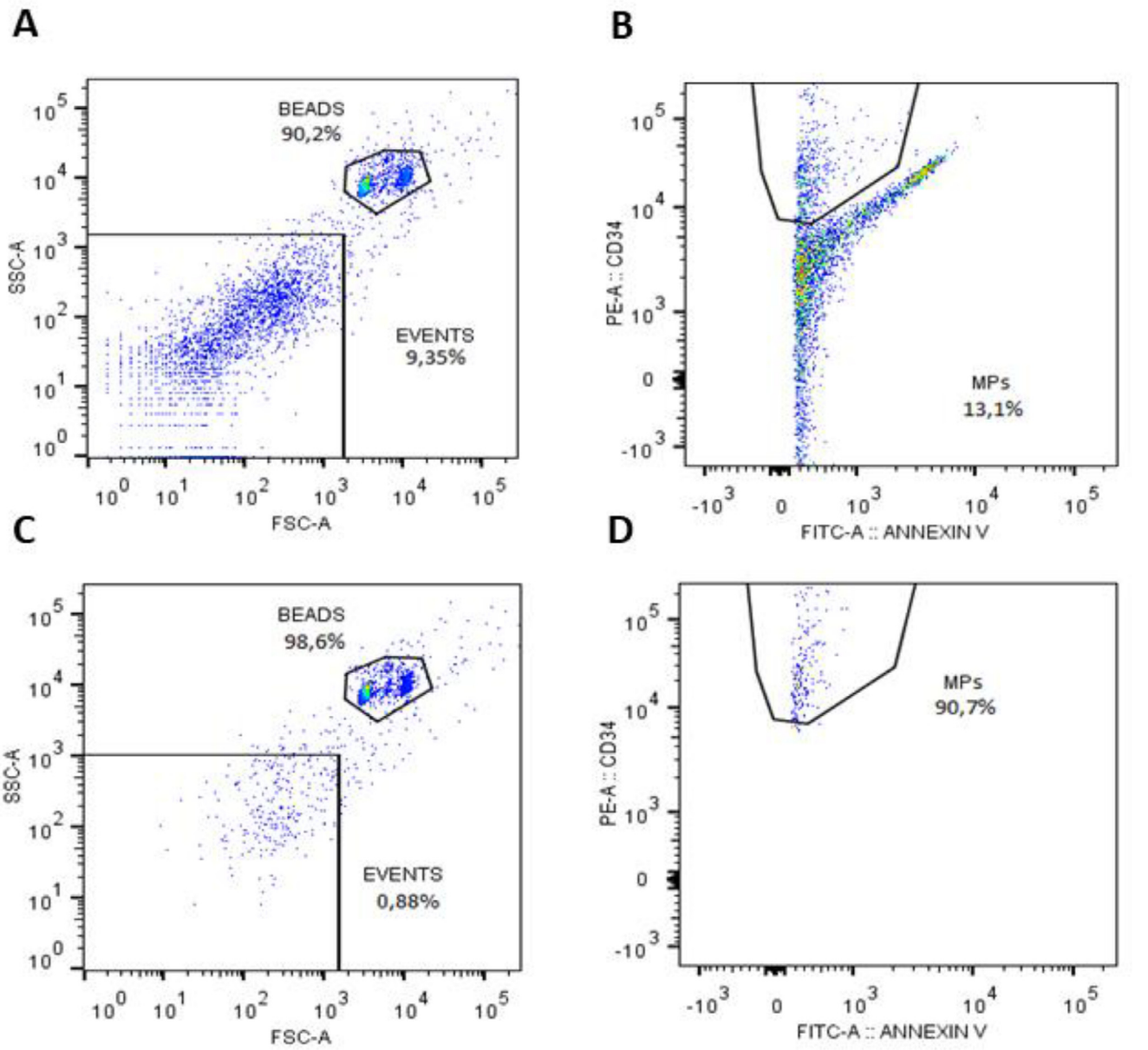

Figure 1. Flow cytometric analysis of microparticles. The forward scatter (FSC) versus side scatter (SSC) histogram was used to define the MPs according to the size of the reference calibrate bead MP gate, including particles from 100 to 1,000 nm in diameter, based on the microsphere sizes used to detect MPs. **(A, C)**. Events defined as MPs were then selected for their annexin V binding and CD34+ expression **(B, D)**. Plots shown are from representative UCB-derived MP analysis before **(A, B)** and after **(C, D)** CD34+ selection by MACS columns.

$\rho = 0.364$) (Figure 1). Annexin V+/CD34+MPs were moderately correlated with the number of pre- and post-processed CD34+ cells respectively ($p = 0.017$, Spearman's $\rho = 0.389$; $p = 0.016$, Spearman's $\rho = 0.393$).

Furthermore, MPs from plasma of pos-tprocessed CB have been observed using electron microscopy. Transmission electron microscopy (TEM) revealed that MPs were mostly spherical structures varying in size (range: 100–300 nm). In our system we did not detect subcellular organelles or any cytoskeletal structure such as actin filaments or microtubules (Figure 2A). Considering that plasma is rich in MPs derived from different cell types, CD34+MPs were isolated using the magnetic activated sorting system (MACS). The purity of CD34+MPs, confirmed by FACS analysis, was 99%. For further confirmation, purified CD34+MPs were visualized by TEM and SEM before proceeding with miRNA isolation (Figure 2B,C).

miRNA levels in CD34+ cells and CD34+ cell-derived MPs

MPs are highly enriched in mRNA and participate in a number of physiological processes. MPs isolated from embryonic, mesenchymal, and peripheral blood cells,

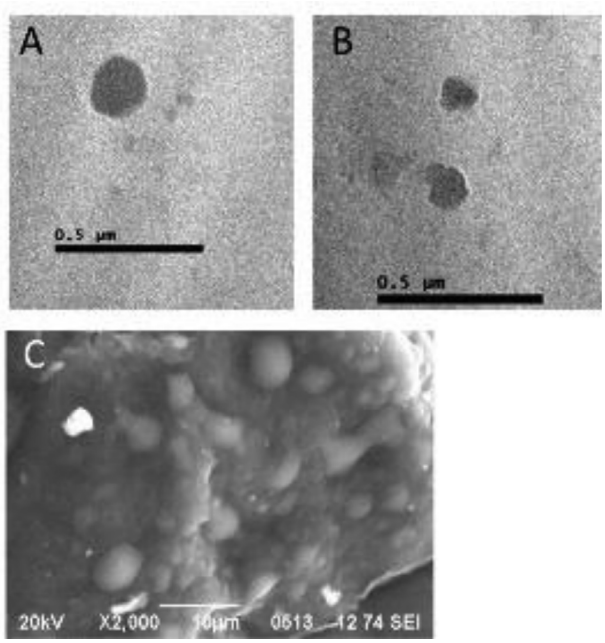

Figure 2. Electron microscopy analysis. MPs were isolated with the MACs systems as indicated under Methods. MPs were visualized by TEM **(A, B)** and SEM **(C)**. The electron micrograph in **(A)** represents MPs before, and that in **(B)**, after CD34+ MACs selection. Scale bar = 500 nm **(A, B)** and 10 μm **(C)**, respectively.

have been demonstrated that carry miRNAs, indicating a possible role in hematopoiesis [20,25]. Based on this we examined the existence of hematopoiesis-specific miRNAs in Annexin V+CD34+MPs using CD34+cells as a control.

Initially, we found that all the microRNAs studied are expressed in both CD34+cells and CD34+MPs. To confirm the presence of the studied miRNAs, the qPCR products were cloned and sequenced. Sanger sequencing verified that miR-106b, miR-221, miR-517c, miR-519d and miR-520h indeed exist in CD34+ cells and MPs (Figure 3).

MicroRNAs 106b and 221 are the most prevalent in CD34+ cells and MPs. Our results confirm previous observations regarding miR-106b expression in CD34+ cells [17]. Given that the miR-106b family is implicated in regulating cell cycle progression, its expression in CD34+ cells and MPs could be related to the self-renewal process. In our study, miR-221 also exhibited significant expression in CD34+ cells and MPs. However, this finding is not consistent with other results indicating that CD34+ cells from cord blood and bone marrow express miR-221 at very low levels compared with peripheral blood CD34+cells [17]. MicroRNA 221 is a key molecule in erythropoiesis [18]. Two independent studies have associated its

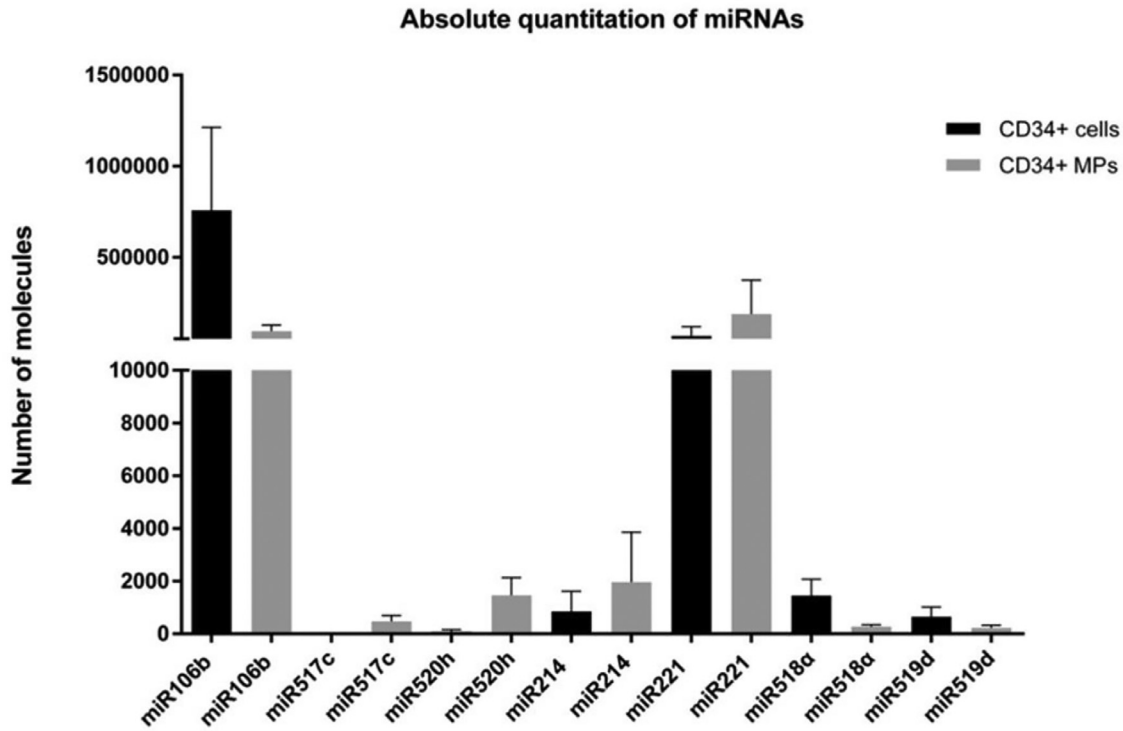

Figure 3. Quantitation of selected miRNAs in CD34+ MPs and cells. The *y* axis indicates the absolute number of miRNAs. The values of miRNAs were adjusted using the comparative *Ct* method normalized with the *Caenorhabditis elegans* miR-39 miRNA mimic. Values presented are the averages of five independent experiments. Data are presented as the mean with SEM.

expression with erythropoiesis inhibition and revealed that the downregulation of miR-221 promotes positive regulation of erythropoiesis via translational induction of c-KIT [17,22]. MicroRNAs 106 and 221 commonly target the cadherin gene [26], which plays a role in cell–cell adhesion and signaling. MicroRNAs 517c, 519d, and 520h, which form a cluster on chromosome 19q13, were found to be highly expressed in UCB CD34+cells [17] compared with bone marrow CD34 +cells [15]. In accordance, we found that miR-517c, miR-519d, and miR-520h are expressed at low levels in UCB CD34+ cells and MPs. Their expression level was higher in CD34+ MPs compared with the cell of origin. It has been proposed that miR-520h induces differentiation of HSCs into progenitor cells (HPCs) by targeting the ABCG2 gene, which is involved in stemness homeostasis. The high expression of miR-520h in CD34+CD38-HSCs from UCB compared with the underexpression in CD34+-committed HPCs confirms this claim [16].

Molecular analysis confirmed the identification of line-specific miRNAs in CD34+ cells and MPs from post-processed CB samples. Moreover, miRNA expression in CD34+ MPs follows the same pattern as in CD34+ cells, indicating that miRNAs from CD34+ MPs mirror those of CD34+ mother cells, at least those analyzed. Thus, miRNAs in stem cell-derived MPs could serve as possible candidate biomarkers in CB.

To our knowledge, this is the first study identifying CD34+MPs in CB. We have also proved the presence of miRNAs in CD34+MPs. However, functional studies need to be carried out to elucidate the role of these MPs and their potential role in hematopoietic stem cell transplantation.

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Conflict of interest disclosure

The authors declare no competing financial interests.

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