MicroRNAs Contribute to Induced Pluripotent Stem Cell Somatic Donor Memory*

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Induced pluripotent stem cells (iPSCs) maintain during the first few culture passages a set of epigenetic marks and metabolites characteristic of their somatic cell of origin, a concept defined as epigenetic donor memory. These residual somatic features are lost over time after extensive culture passaging. Therefore, epigenetic donor memory may be responsible for the higher differentiation efficiency toward the tissue of origin observed in low passage iPSCs versus high passage iPSC or iPSCs derived from a different tissue source. Remarkably, there are no studies on the relevance of microRNA (miRNA) memory following reprogramming, despite the established role of these molecules in the context of pluripotency and differentiation. Using hematopoietic progenitors cells as a model, we demonstrated that miRNAs play a central role in somatic memory retention in iPSCs. Moreover, the comparison of the miRNA expression profiles among iPSCs from different sources allowed for the detection of a set of candidate miRNAs responsible for the higher differentiation efficiency rates toward blood progenitors observed in low passage iPSCs. Combining bioinformatic predictive algorithms with biological target validation, we identified miR-155 as a key player for the in vitro differentiation of iPSC toward hematopoietic progenitors. In summary, this study reveals that during the initial passages following reprogramming, iPSCs maintained the expression of a miRNA set exclusive to the original somatic population. Hence the use of these miRNAs might hold a direct application toward our understanding of the differentiation process of iPSCs toward hematopoietic progenitor cells.

Background: iPSCs retain marks of their donor population: “somatic donor memory,” which facilitates iPSC differentiation back into their original tissue.

Results: Specific microRNAs maintain their expression in HPC-derived iPSCs promoting their differentiation back to HPCs.

Conclusion: miRNA contributes to iPSC somatic donor memory.

Significance: miRNA donor memory may offer a tool to understand iPSC reprogramming and differentiation toward a particular somatic population.

The discovery of a set of transcription factors with the ability to induce the conversion of any human somatic cell toward an embryonic stem cell (ESC)-like state through a process termed reprogramming has revolutionized the regenerative medicine field (1, 2). Under defined culture conditions, reprogramming yields iPSCs that exhibit morphological and growth properties of ESCs and hence hold the potential to differentiate into any adult somatic lineage (3, 4). Consequently, iPSCs have an epigenomic and metabolomic profile that is much closer to that characterizing ESCs than that observed in their tissue of origin (5, 6) because that somatic cell reprogramming implies the erasure of tissue-specific epigenetic marks to obtain a pluripotent phenotype. Reportedly, however, iPSCs may retain sort of an “epigenetic donor memory,” consisting of specific marks reminiscent of their previous phenotype that escaped the reprogramming process. This may facilitate in vitro differentiation of iPSCs back into their tissue of origin rather than into other cell lineages (7, 8). Interestingly, the donor epigenetic memory observed in iPSCs appears to be gradually lost during in vitro passaging, thus progressively acquiring a more ESC-like phenotype (7–9). Hence we hypothesized that comparing the epigenetic profile of iPSCs cultured for few passages (low passage (LP)-iPSC) with that of their tissue of origin may provide the means to detect key molecules and marks required for maintenance or conversion back toward the donor cell phenotype. Conversely, high passage (HP)-iPSC would reveal the features required to acquire and retain an ESC-like phenotype.

To define donor memory in cultured iPSCs, previous studies have focused on the modification of the DNA methylation profile and chromatin marks as regulators of gene expression during reprogramming (7, 10–12). However, a novel and relevant approach may be that of assessing reprogramming through changes in the expression of noncoding RNAs such as miRNAs. Indeed, although miRNAs represent a minute fraction (~0.01%) of the total RNA mass, they have been postulated to regulate up to 50% of mammalian genes (13, 14). Thus, miRNAs are pro-

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3 The abbreviations used are: ESC, embryonic stem cell; iPSC, induced pluripotent stem cell; HPC, hematopoietic progenitors cell; miRNA or miR, microRNA; LP, low passage; HP, high passage; CB, cord blood; qRT-PCR, quantitative real-time PCR; CB/iPSC, iPSC derived from CB; FIPSC, iPSC derived from fibroblasts; EB, embryoid body; IPA, Ingenuity® pathways analysis.

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cessed from precursor molecules into single-stranded RNAs (~22 nucleotides) with the ability to pair with a long range of mRNAs via targeting their UTR and leading to their transcriptional repression or degradation. In addition, miRNAs can also regulate gene expression through transcriptional silencing, promoter targeting, and translational activation.

Herein we study miRNA donor memory, using as a model the reprogramming of hematopoietic progenitor cells (HPCs). Given that blood progenitors are suitable for reprogramming (15–17) and that successful differentiation from iPSCs to long term repopulating HPCs has not yet been reported (18, 19), we propose that the miRNAs with conserved expression levels between HPCs and their derived LP-iPSCs may play a relevant biological role in determining the ability of these cells to revert toward HPCs in vitro. Applying bioinformatic comparison algorithms on microarray generated data, we detected a set of miRNAs highly expressed in HPCs as well as in the resulting LP-iPSCs, but lost in HP-iPSCs. Moreover, we focused on the role of miR-155 as a candidate playing a key role in the differentiation of iPSCs toward HPCs.

**EXPERIMENTAL PROCEDURES**

**Cord Blood (CB) Collection**—Umbilical CB samples were obtained from the Banc de Sang i Teixits, Hospital Duran i Reynals, Barcelona, Spain.

**HPC Purification**—Mononuclear cells were isolated from CB using Lympholyte-H (Cederlane, Burlington, CA) density gradient centrifugation. Then CD133+ or CD34+ cells were positively selected using the Mini-Macs immunomagnetic separation system (Miltenyi Biotec). Purification efficiency was verified by FACS using CD133-PE or CD34-APC (Miltenyi Biotec) antibodies.

**Reprogramming of CD133+ Cells**—CD133+ cells were reprogrammed as previously described (20). Two retroviral vectors (pMSCV-OCT4, pMSCV-SOX2, CBiPSC c) or a polycystronic retroviral vector (pMXs-OSKMG; CBiPSC a and b) were used for this purpose.

**Reprogramming of Human Foreskin Fibroblasts**—Early passage fibroblasts were infected with four retroviruses (pMSCV-OCT4, pMSCV-SOX2, pMSCV-KLF4, and pMSCV-cMYC). Then 10^5 cells were plated in each well of a 6-well dish and on the following day infected with supernatant containing a mix of the four viruses. A second infection was performed 24 h later. At 72 h from the first infection, fibroblasts were plated onto an irradiated human foreskin fibroblast feeder layer and cultured in human ESC medium consisting of Knockout™ DMEM (Invitrogen); 20% Knockout serum replacement (Invitrogen), 1% nonessential amino acids (Lonza), 2 mM L-glutamine (Invitrogen), 0.1 mM 2-β-mercaptoethanol (Invitrogen), and basic FGF (10 ng ml^-1; Chemicon). After 25 days in culture under typical ESC conditions, fibroblast iPSC (FiPSC)-like colonies started to appear. Then colonies identified by their typical morphology features were hand-picked and fully characterized both in vitro and in vivo (data not shown for FiPSC). Both ESCs and iPSCs were maintained in human ESC medium overlaying irradiated human foreskin fibroblast.

**iPSC Characterization**—Pluripotency characteristics and ability to differentiate into the three germ layers were tested by ESC expression markers, in vitro differentiation toward mesoderm, endoderm and ectoderm lineages, and teratoma formation as described elsewhere (15, 21).

**Differentiation of iPSCs into HPCs**—Differentiation of iPSCs and ESCs toward HPCs was performed as previously described (18).

**Flow Cytometry Analysis**—Surface phenotyping was performed by FACS using the following monoclonal antibodies: anti-CD34-PE (Miltenyi Biotec), anti-CD45-APC (Becton, Dickinson), and anti-TRA1–85 FITC (R&D). Gating was performed with matched isotype control monoclonal antibodies. Propidium iodide (2 µg ml^-1) was included in the final wash to exclude dead cells. All analyses were performed on a MoFlo cell sorter (Dako Cytomation) running Summit software.

** Colony Forming Unit Assay**—CD34+ and CD45+ cells obtained following iPSC differentiation, as well CD133+ cells isolated from CB, were diluted in 1 ml of methylcellulose (StemCell Technology) and plated onto a 35-mm dish. Colonies were counted and identified after 14 days and standardized to the initial number of cells seeded.

**Staining of CFU**—CFU granulocyte macrophages were picked and washed in PBS. The cells were then analyzed either by Giemsa May Grünwald staining or by the expression of specific membrane markers. Anti-CD45-APC, anti-CD14-APC, and anti-CD15-FITC (Miltenyi Biotec) antibodies and their corresponding isotype control antibodies were used following the manufacturer’s instructions.

**Generation of miRNA Expression Profiles**—Microarray miRNA expression profiles were obtained using the Affymetrix GeneChip® miRNA 3.0 Array (Affymetrix). For this analysis, 500 ng of total RNA were used. After quality control, data generated were normalized and summarized using the robust multichip analysis (22). Only the 5617 human miRNA probe sets were considered for further analysis, which was performed by subtraction between the different study conditions.

**Heat Map Generation**—A heat map was generated with the 169 miRNA probe sets obtained after comparing the mean values obtained from the analysis of the two CD133+ cell populations with ESC. For this purpose, R functions hclust and heatmap.2 were used to generate the final heat map. Briefly, this software scales normalized data by row and assigns a color scale ranging from green (less abundant) to red (more abundant), as well as taking into account the restrictions imposed by the row and column dendrograms.

**Quantitative Real Time PCR (qRT-PCR)**—Samples were treated in TRizol reagent, and total RNA (including miRNA) was extracted using the miRNA easy kit (Qiagen) following the manufacturer’s instructions. The amount of RNA used to synthesize cDNA varied according to sample from 50 ng to 1 µg. The SuperScript™ III reverse transcriptase (Life Technologies) was used to obtain cDNA for gene expression quantification using previously reported primers (21, 23). A new set of primers was designed for miRNA specific target identification. For miRNA analysis, the NCode Express SYBR GreenER (Life Technologies) kit was used. From 50 ng to 1 µg of total RNA sample was used to quantify miRNA expression using primers designed on Ncode and MirBase miRNA sequences. All qRT-PCRs were performed in an Applied Biosystem 7900 HT Fast
qRT-PCR system thermocycler following the manufacturer's instructions.

**Analysis of miRNA Targets**—The CD34+ cells isolated from CB were plated onto 24-well dishes coated with retronectin (2 × 10^5 each well). Then cells were transfected with a synthetic miR-155 biotinylated at the 5’ end with a spacer 18 (synthesized in Integrated DNA Technologies) (24) using the StemFect™ RNA transfection reagent (StemGent) according to the manufacturer’s directions. At 4 h post-transfection, 500 µl of Iscove’s modified Dulbecco’s medium (10% FBS, 2 mM l-glutamine) supplemented with Flt-3, TPO, and SCF (50 ng ml^-1) was added to each well. Then 24 h post-transfection, cells were detached from the retronectin-coated dish and cultured in suspension for another 24 h. At 48 h post-transfection, cells were again collected, resuspended in 500 µl of cell fraction buffer (5 mM Pipes, pH 8.0, 85 mM KCl, 0.5% Nonidet P-40 (Sigma-Aldrich) with protease and RNase inhibitors), and incubated on ice for 10 min. After centrifugation, the cytoplasmic fraction was transferred into new RNase-free tubes; 50 µl of the sample were aliquoted as input.

**Precipitation and Isolation of miRNA Targets**—Streptavidin MagnesSphere® paramagnetic particles (Promega) were coated with RNase-free BSA (Sigma-Aldrich) and yeast tRNA (Roche Applied Science) for 3 h at 4 °C on rotation. For this purpose, 10 µl of tRNA (10 mg ml^-1) and 10 µl of BSA (10 mg ml^-1) were added to each of 100 µl of beads. The paramagnetic particles were then washed twice with cell fraction buffer and incubated with the cytoplasmic fraction for 2 h at 4 °C on rotation. The cytoplasmic fraction was washed twice with cell fraction buffer, three times with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl (Sigma-Aldrich)), and once with high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris HCl, pH 8.0, 500 mM NaCl). The resulting pellet was resuspended in 200 µl of TRIzol, followed by the addition of 40 µl of chloroform and centrifugation at 12,000 × g for 15 min at 4 °C. The supernatant was precipitated for 1 h at −20 °C using 6 µl of 5 M NaCl and 360 µl of ice-cold 95% ethanol. After centrifugation, the pellet was washed with 70% ethanol and resuspended in 10 µl of RNase-free water. The isolated RNA was then used for downstream analysis by qRT-PCR as described above.

**Lentivirus Production and Infection**—Lentiviral vector over-expressing miR-155 and the corresponding scrambled control were obtained from System Biosciences. Viral particles were produced in HEK 293T cells by co-transfection with the pCMV-VSVG, pRSV-Rev, and pMDLg/pRRE plasmids (Addgene) and concentrated by ultracentrifugation. After infection, GFP-positive cells were selected by FACS sorting.

**Western Blot Analysis**—5 × 10^5 cells were harvested, and equal amounts of cell lysate were separated by 10% SDS-PAGE and transferred onto Immobilon polyvinylidene difluoride membranes (Millipore). For immunochemical detection, blots were incubated with anti-Mecp2 (Cell Signaling 3456; 1:200) and anti-β-actin (Sigma A2172; 1:5000) primary antibodies, followed by the corresponding anti-rabbit and anti-mouse IgG peroxidase-conjugated secondary antibodies (Amersham). Blots were developed using an ECL kit (Amersham).

**RESULTS**

The human hematopoietic system was chosen to study changes in miRNA expression because of its suitability for reprogramming as well as differentiation from iPSCs (16, 18), the well defined characterization of the different cell lineages, and its relevance toward applications in the clinical setting. Hence HPCs isolated from CB were reprogrammed into iPSCs (CBiPSCs), which were then kept in culture for up to 40 passages with *in vitro* differentiation back into HPCs performed at different times according to the number of passages. Thus, two categories of iPSCs were established, LP-CBiPSC (less than 10 passages) and HP-CBiPSC (more than 25 passages), where a passage corresponded to ~7 days in culture. Total miRNA was obtained from each of these populations for expression profile analysis via microarray hybridization (Fig. 1A). Bioinformatic analysis revealed several miRNA candidates to play a key role for the generation of HPCs *in vitro*.

**Generation of iPSCs**—For the generation of iPSCs, CD133+ mononuclear cells from CB units were transduced with retroviral vectors as reported elsewhere (15). After 21 days, cell colonies were identified as displaying a cobblestone appearance with prominent nucleoli and pronounced colony borders, resembling the typical iPSC morphology. Three clones were picked and tested following standard procedures to assess pluripotency and then cultured until they reached either a low (<10) or a high (>25) number of passages (Fig. 1, B–E). As expected, when the expression levels of endogenous and exogenous pluripotency factors were measured, both LP and HP-CBiPSCs had silenced the expression of the transgenes inserted in their genome (Fig. 1F). Altogether, these results indicate that the cells obtained from HPCs can be considered *bona fide* iPSCs after few passages in culture.

**Differentiation of CBiPSC Clones**—The CBiPSC clones were differentiated both from LP and HP cells back toward HPCs following a previously published protocol (18). Briefly, iPSCs were first differentiated into embryoid bodies (EBs) and cultured onto OP9 cells under culture conditions supporting hematopoietic differentiation (Fig. 2A). After 14 days, cells were analyzed by FACS for the presence of the CD34 and CD45 hematopoietic markers (Fig. 2B). In addition, the pan-human marker TRA1–85 was used to distinguish human from murine OP9 cells. To validate the efficiency of the differentiation process and compare it to that of iPSCs of different somatic origin, several control cell lines were also differentiated (three of each ESCs, LP-FiPSCs, and HP-FiPSCs). The efficiency of differentiation for the LP-CBiPSC was higher than that of the HP-CBiPSC lines in terms of percentage of differentiated (CD34+ and CD45+) cells. In particular, the percentage of CD45+ cells was higher (p < 0.01) in LP-CBiPSC cells than HP-CBiPSCs for each clone. Interestingly, no significant difference in the efficiency of differentiation was detected between LP-FiPSC and HP-FiPSC, showing that a predisposition to differentiation is not associated *per se* with LP-iPSCs. Moreover the ability of ESC, LP-FiPSC, and HP-FiPSC lines to differentiate toward HPC was comparable to that of HP-CBiPSCs (Fig. 2C). All CD34+ and CD45+ cells were sorted after differentiation and cultured for 16 days in methylcellulose to test their hematopoietic clono-
FIGURE 1. Characterization of iPSCs derived from human CB progenitors, kept in culture for a low (<10, LP) or high (>25, HP) number of passages. A, experimental design. CD133+ cells from CB were reprogrammed to generate CBiPSCs. Then miRNAs were isolated from LP-CBiPSCs, HP-CBiPSCs, and control CD133+ cells, as well as FiPSC and ESC lines. Whole miRNA expression profile was obtained by Affymetrix microarray analysis. B, immunostaining for pluripotency markers on newly generated CBiPSC clones. The surface markers SSEA3, SSEA4, TRA1–81, and TRA1–60 and the transcription factors OCT4, SOX2, and NANOG were analyzed by immunofluorescence. C, high resolution, G-banded karyotype on iPSC diploid metaphases. D, immunofluorescence analysis on in vitro differentiated CBiPSCs. Markers specific for the three germ layers, TUJ1 and GFAP (ectoderm), SMA (mesoderm), and AFP and FOXA2 (endoderm), were used. E, histological sections from CBiPSC teratomas were analyzed by immunohistochemistry. Tissue-specific markers to the three germ layers, TUJ1 and GFAP (ectoderm), SMA and ASA (mesoderm), and AFP and FOXA2 (endoderm), were tested. F, qRT-PCR performed with primers specific for endogenous pluripotency mRNAs and for the corresponding exogenous transgenes (red bar highlighted). The mRNA expression levels were analyzed in all LP-CBiPSCs, HP-CBiPSCs, and ESCs (n = 3).
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The HPCs derived from LP-CBiPSCs were able to generate a higher number of CDU granulocyte macrophages, as confirmed by May-Grünwald/Giemsa and immunofluorescence staining for the CD45, CD14, and CD15 markers (Fig. 2). The HPCs obtained after differentiation were also functionally tested in vivo by transplantation into NSG sublethally irradiated mice. Only mice (2 of 24) injected with LP-CBiPSC-derived HPC cells presented human CD34+ and CD45+ cells at 10 weeks postinjection, as confirmed by human-specific qRT-PCR. However, these cells did not differentiate into human blood lineages but only engrafted in a suitable niche such as the bone marrow (data not shown). Altogether these results support the notion that LP-CBiPSCs have a higher potential to differentiate toward functional blood progenitor cells as compared with their HP counterparts or that of iPSCs derived from different tissues.

miRNA Microarray Analysis—Hence miRNA microarray analysis was performed to test the hypothesis that the differences found between HPCs derived from LP- and HP-CBiPSCs may be partially explained by the maintenance of the expression of a miRNA set specific for CD133+ and then conserved during the initial culture passages in vitro. For this purpose, we compared miRNA expression levels between cell populations along a three-time point analysis: time 0 (T0) comprised the starting population (CD133+ from CB) prior to reprogramming; time 1 (T1) comprised iPSCs derived during the first few culture passages (LP-CBiPSC); and time 2 (T2) iPSCs kept in vitro for an extended period of time (HP-CBiPSC). Expression levels were measured by miRNA microarray analysis with ESCs and HP-FiPSCs used as control lines (we disregarded using LP-FiPSCs because no differences in terms of efficiency of differentiation were detected as compared with HP-FiPSCs). To calculate the change in miRNA expression levels, each miRNA absolute value was Log-scaled, and a fold change of ≥2 between two time points was established as the threshold. Changes in miRNA expression levels could be defined using nine temporal patterns, according to the possible trend combinations between time points. The stretches (Down = D, Equal = E, Up = U) corresponded to a Log-fold change either above or below the established threshold between consecutive time points, that is, consistent with a shift in pattern trend (Fig. 3A). Of the nine temporal patterns possible, we focused on four: DD, ED, UU, and EU. In particular, DD and ED were associated with miRNAs highly expressed in CD133+ cells and which expression levels either decreased when progressing through the three time points or were maintained between times 0 and time 1 and then decreased, respectively. Conversely, EU and EU patterns were related to a sequential enrichment in miRNA expression when progressing through the three time points or to an increase in transcripts between time points 1 and 2, respectively. These four patterns would presumably represent maintenance in miRNA expression levels following reprogramming (i.e., miRNA donor memory). Interestingly, the miRNAs whose expression levels increased along time points were mostly described as miRNAs associated with embryonic pluripotency states (miR-302–367 cluster; miR-183) (25, 26). Conversely, the miRNAs displaying DD and ED expression trends in CBiPSCs were previously associated with differentiation of ESCs or hematopoiesis (Let-7 family, miR-125b, miR-155, and miR-99a) (27–30) or described to be up-regulated in neoplastic diseases (miR-3162 and miR-3921) (31–35) (Fig. 3A). Moreover, the higher expression of these CD133+ cell-specific miRNAs in LP-CBiPSCs versus HP-CBiPSCs (p < 0.01) was confirmed by qRT-PCR (Fig. 3B). This assay was performed in at least three iPSC lines for each cell type, with CBiPSCs used derived from different cord blood units.

Pre-miRNA Expression Levels—We sought pre-miRNA expression levels to gain insight into the mechanism of memory. Interestingly, the majority of the precursors of miRNA candidates identified as members of temporal patterns EU, UU, ED, or DD showed no variation in their expression levels throughout the three time points. The lack of correlation in expression level changes between miRNA and each corresponding precursor suggests that the miRNA post-transcriptional regulation machinery may play a relevant role during reprogramming. Only few pre-miRNAs adapted their expression trends to the same temporal patterns observed for the corresponding mature miRNAs, that is, miR-1303, miR-1323, and miR-151–5p (EU). The pre-miRNAs belonging to cluster 302 showed a significant increase in expression levels between T0 and T1, followed by a weaker up-regulation toward T2. Regarding temporal patterns ED or DD, only the miR-155 precursor showed a decreased expression throughout the three time points (Fig. 3B). Overall miRNA microarray analysis revealed a set of miRNAs with expression levels maintained between the progenitor CD133+ cells and their derived LP-CBiPSCs, which were then down-regulated after extensive in vitro passaging.

ESC and iPSC Comparison—Further analysis of the data generated by microarrays showed that in regards to changes in miRNA expression, correlation between ESC and LP-CBiPSC (r^2 = 0.67) was lower than that between ESC and HP-CBiPSC (r^2 = 0.79) or ESC and FiPSC (r^2 = 0.81) cell lines, respectively (correlation of Spearman; p < 0.001). We thus inferred that, as reported for miRNAs (6), global miRNA expression is also influenced by residual donor memory. The increase in passage number could reduce the differences between iPSCs and ESCs in miRNA profile. Considering that few progenitors between CD133+ cells and ESCs have been described during the human
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A

miRNA expression level

CD133+ LP-CBIPS HP-CBIPS

B

Mature miRNAs

DD pattern

Let-7a Let-7d Let-7e Let-7i mir-155 mir-99a

mean log2 exp

CD133+ LP-CBIPS HP-CBIPS

Mature miRNAs

ED pattern

Let-7c mir-125b mir-3162-3p mir-320d mir-3921

mean log2 exp

CD133+ LP-CBIPS HP-CBIPS

Precursor of miRNAs

Let-7a Let-7d Let-7e Let-7i mir-155 mir-99a

mean log2 exp

CD133+ LP-CBIPS HP-CBIPS

Precursor of miRNAs

Let-7c mir-125b mir-3162-3p mir-320d mir-3921

mean log2 exp

CD133+ LP-CBIPS HP-CBIPS

C

miR-99a

miR-125b

miR-155

Let-7e

Let-7d

Let-7c

% US

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mesodermal differentiation program (36), we hypothesized that the study of differentially expressed miRNAs between ESC and endogenous HPC would reveal a set of miRNAs playing a putative role during in vitro HPC generation. Using the criteria previously established for the microarray raw data, we detected a set of 169 miRNAs that were differentially expressed when comparing CD133+ and ESC lines. This set was used to generate a cluster analysis including all of the cell lines and aiming toward the detection of similarly expressed miRNAs when comparing LP-CBiPSC and CD133+ populations. The heat map matrix revealed that CD133+ cell lines clustered separately from all the stem cell lines. In addition, HP-CBiPSC clustered with FiPSC, thus strengthening the hypothesis that with an increasing number of passages the miRNA expression profiles become more alike among different iPSC lines. Following this path, we obtained a set of miRNAs displaying similar expression levels in both LP-CBiPSC and CD133+ lines (Fig. 4A). Remarkably, a similar list of candidates including miR-125b, miR-99a, miR-155, and Let-7d was identified when comparing miRNAs differentially expressed between ESC and CD133+ populations (n = 169), as well as those displaying ED and DD patterns for CBiPSCs when using the time point strategy (Fig. 4B).

miRNA Candidate Validation in HPC—To further validate the relevance of these four miRNA roles in the HPC, we followed two strategies. First, we measured their expression levels by qRT-PCR in CD34+/CD45+ cells obtained after differentiation from iPSCs, and second, we followed their expression levels during the lineage maturation of the endogenous HPC population. Interestingly, we observed that HPCs obtained from CBiPSCs presented higher levels of these miRNAs when compared with the other stem cell lines. However, we detected no significant differences in their expression levels between LP and HP CBiPSCs. Regarding the second strategy, we observed that three of these miRNAs were gradually down-regulated along the differentiation of CD133+ cells into CD34+/CD133− and finally into CD34−/CD45+ (Fig. 4D) cells. Contrasting both strategies, only miR-155 followed a similar pattern of expression when comparing in vitro generated HPCs and CD133+ cells (Fig. 4C). In conclusion, using a set of miRNAs differentially expressed between the ESC and CB-derived CD133+ cell lines, we detected four miRNAs that were similarly expressed between LP-CBiPSC and HPCs. From this set, only the miR-155 followed the same expression pattern found in endogenous populations, being down-regulated when progressing along the differentiation path from stem to progenitor to mature blood cells.

Validating the Role of miR-155—We chose to further validate the role of miR-155 among the four candidates identified in the previous experiments, given that levels of this miRNA displayed a different profile in regards to pre-miRNA expression trends during the three time points analyzed. The decrease in miR-155 precursor expression levels during reprogramming and after the derivation of iPSC from HPC supports the notion that fine regulation of miRNA, and in particular miR-155 expression, is of utmost importance for these processes. Moreover, the miR-155 expression pattern during the differentiation process from iPSC to HPCs resembled the endogenous down-regulation observed from primitive to more mature hematopoietic progenitor populations. Therefore, we searched for miRNA targets reportedly down-regulated in HPCs, and using bioinformatic tools we combined these data with the list of predicted pathways under miR-155 regulation (Fig. 5A). We initially searched for the miR-155 predicted mRNA targets in four different published databases (Pita, PicTar, Target Scan, and EIMMo3). Considering queries present in at least two of the four public algorithms, a list of 356 potential miRNAs targets was generated (Fig. 5A). To minimize the list of possible miR-155 targets to those with a biological role in HPCs, we then ran a pathway analysis of the 356 mRNA targets using the Ingenuity® pathway analysis (IPA) software. This yielded initially a set of 250 different predicted pathways that we then restricted to 20 pathways most related to HPCs and hematopoiesis and displaying the highest p values and ratios as determined by the IPA (Fig. 5B). In parallel, we ran a comparison analysis using the predicted activation state IPA function of GEO published gene expression data for the CB CD133+ (GSM418177 and GSM418178) and ESC (GSM418179, GSM418180, and GSM418181) lines; this analysis retrieved a set of genes and pathways down-regulated in the CD133+ population as compared with ES cells. The association between the pathways inhibited in the CD133+ cells and the list of predicted targets for the miRNA of interest revealed a set of potential downstream effectors for miR-155 in HPCs, including members of the TGFβ, Wnt/β catenin, IGF, DNA methylation, and transcriptional repression signaling pathways.

Validation in Vitro of the Set of miR-155 mRNA Targets—Ultimately, we validated in vitro the set of miR-155 mRNA targets predicted to play a biological role in the context of the HPC transcriptome. Using a biotin-linked probe containing the mature form of the miR-155 construct (24), we aimed to pull down all the targets regulated by this miRNA in HPCs from CB. The mRNAs specifically bound to miR-155 were recovered with streptavidin beads and used to perform qRT-PCR with primers designed to identify the presence of the predicted targets for each of the pathways. We observed a mRNA enrichment for several members of the predicted pathways when compared with total mRNA bound to a nonspecific probe (Fig. 5C). In summary, combining a bioinformatics approach with in vitro experiments, we managed to validate a set of miR-155 targets.

FIGURE 3. Bioinformatic analysis of miRNA expression profiles. A, left panel, theoretical patterns corresponding to miRNA expression levels assessed at three time points along the cell culture timeline, where T0 = CD133+, T1 = LP-CBiPSC, and T2 = HP-CBiPSC. Changes in expression trends are designated as Up-Up (UU), Up-Equal (UE), Up-Down (UD), Equal-Down (ED), Down-Equal (DE), and Down-Down (DD). Highlighted in red are patterns of miRNA highly expressed in CD133+ cells displaying a sequential decrease throughout the three time points analyzed. B, miRNAs fitting their expression level trend to the DD and ED patterns (p < 0.05). Precursor miRNA corresponds to DD and ED miRNA. All the precursors maintained the same level of expression through the three time points, except miR-155. C, a set of miRNA candidates found to fit the temporal DD and ED patterns in the CBiPSC line were further confirmed by qRT-PCR. Expression levels were compared between LP-CBiPSCs (solid red bars) and HP-CBiPSCs (white bars), CD133+ cells (red outline bars) were used as positive controls, whereas HP-FiPSC and ESCs (gray bars) were used as negative controls. The validation of these miRNA candidates was performed in at least three iPSC lines from each cell type (Mann-Whitney statistic test; n > 4; *, p < 0.05; **, p < 0.01; ***, p < 0.001).
FIGURE 4. Identification and validation of differentially expressed miRNAs as assessed by cluster analysis. A, miRNA cluster analysis on CD133+ cells derived from CB, iPSC, and ESC lines. The miRNAs found to be differentially expressed (n = 169) between CD133+ cells and ESC were used to generate the heat map. B, graphical comparison of the two strategies used to identify the miRNAs whose expression was maintained after the CD133+ cell conversion toward ES-like cells. The miRNAs found to have a down-regulation trend (DD and ED) using the three-time point analysis (3 TP) strategy were compared with the 169 miRNAs found to be differentially expressed between CD133+ cells and ESCs (CD133+ versus ESC). The miRNAs found in the intersections of the Venn diagrams are listed. C, qRT-PCR analysis on CD34+/CD45+ cells obtained after LP-CBiPSC (solid red bars) and HP-CBiPSC (white bars) differentiation toward HPCs. CB CD133+ (red outline bars) and CD34+/CD45+ cells isolated after FiPSC or ESC (gray bars) line differentiation were used as controls (n = 3). Graphs display levels of the four miRNAs found to be similarly expressed between CD133+ cells and LP-CBiPSC. D, expression levels of candidate miRNAs (miR-155, miR-125b, miR-99a, and Let-7d) or miRNAs known to be up-regulated during HPC differentiation (miR-183, miR-21, Let-7b) as determined by qRT-PCR in CD133+, CD133+/CD34+, or CD133+/CD34−/CD45+ populations isolated from CB (Mann-Whitney statistic test; biological triplicates are shown; **, p < 0.01).
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A

B

C

miRNA Donor Memory in iPSCs

A

mRNA differentially expressed

356 targets

Predicted pathways downregulated in CD133+

by miR-155 targets

B

Pathways downregulated in CD133+

Chronic Myeloid Leukemia Signaling

T cells receptor signaling

NGF Signaling

DNA methylation and Transcriptional repression

Molecular mechanism of cancer

B Cell Receptor Signaling

ERK5 Signaling

PKC1 Signaling in T Lymphocytes

Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes

Wnt/β catenin pathways

GDNF signaling

DNA Methyl.

Common targets

Non common targets

| TGFβ pathway | TGFBR2 | SOS1 | KRAS | FOS | ACVR2A | ACVR2B | SKI | SMAD1 | SMAD3 |
|--------------|--------|------|------|-----|--------|--------|-----|-------|-------|
| Wnt/β catenin | TGFBR2 | ACVR2A | ACVR2B | TCF4 | SOX1 | CSNK1D2 | TLE4 | FZD5 | SOX11 | APC |
| FLT3 Signaling in HPC | SOS1 | KRAS | CREB | RP58K3 | INPP5D | CBL |
| IGF pathway | SOS1 | KRAS | FOS | PRKAR1A | SOS1 | SOS1 | YWHAE |
| GNRH Signaling | SOS1 | KRAS | FOS | CREB | PRKAR1A | MAP3K14 | MAP3K10 | PAK2 |
| DNA Methyl. | | | | | HDAC2 | ME2 | MECP2 |

C

mRNA targets enrichment

Wnt/β catenin

TGFβ pathway

FLT3 signaling

IGF pathway

GNRH pathway

DNA methylation and Transcriptional repression
miRNA Donor Memory in iPSCs

targets with a biological role in the context of the maintenance and regulation of blood cell progenitor differentiation.

Overexpression of miR-155 in HP-FiPSCs—Given these premises, we then sought to ascertain whether overexpression of miR-155 in HP-FiPSCs affected their tendency to differentiate toward HPCs. We first infected HP-FiPSCs with either a lentiviral vector overexpressing pri-miR-155-GFP or a scrambled GFP control. The GFP-positive cells were selected by FACS, and miR-155 overexpression was confirmed by qRT-PCR (Fig. 6A). Notably, miR-155 overexpression did not seem to affect the expression levels of other candidate miRNAs, namely miR-99a, miR-125b, or Let-7d. Next, HP-FiPSC +155 and its control line were differentiated toward HPCs. The efficiency of differentiation was measured by FACS using CD34 and CD45 markers at 7 and 14 days of differentiation (Fig. 6B). Interestingly, HP-FiPSC + miR-155 showed a higher efficiency of differentiation when assessed at day 7 as compared with the control line (p < 0.01). Conversely, overexpression of miR-155 reduced the percentage of CD34+ and CD45+ cells on day 14. Furthermore, miR-155 overexpression impaired the final chromatin reorganization required for cell differentiation process from iPSCs toward HPCs (i.e., iPSCs, EB formation, and Tra1−85+ cells at 7 and 14 days of differentiation). For this purpose, we first measured the levels of expression of candidate miRNAs (miR-155, miR-125b, miR-99a, and Let-7d) and miRNAs known to be up-regulated during HPC differentiation (miR-183, miR-21, and Let-7b) in HP-FiPSC + miR-155 and the corresponding control line. Notably, miR-155 showed a higher level of expression at day 7 (p < 0.01) and was still overexpressed at day 14 in HP-FiPSC + miR-155 as compared with the control (scrambled) cell line (Fig. 6C). Similarly, other miRNA candidates also displayed higher levels of expression at day 7 (p < 0.01) in treated versus control cells. Hence we then analyzed the level of expression of a set of HPC key regulators genes at the established differentiation time points. All of the hematopoietic markers showed higher expression levels in the in HP-FiPSC + miR-155 after 7 days of differentiation (p < 0.05, 0.01, or 0.001) as compared with the scrambled line. Interestingly, by 14 days, the majority of these markers still displayed higher expression in the treated cell line (p < 0.05, 0.01, or 0.001) (Fig. 6D).

miR-155 Overexpression Induces MeCP2 Down-regulation—To explain the discrepancy given by a reduction in the percentage of CD34+ and CD45+ cells after differentiation despite a higher expression level of hematopoietic markers as assessed by qRT-PCR, we decided to investigate the expression of the main miR-155 target based upon our in vitro target validation experiments (Fig. 6E). Namely MeCP2 is a member of the methylcytosine-binding domain family and acts as transcriptional regulator imposing local repressive chromatin structures through recruitment of histone-modifying enzymatic activities. It is highly expressed in HPCs as compared with more differentiated hematopoietic cells, because of its role as a global heterochromatin architecture organizer (37, 38). Herein, overexpression of miR-155 resulted in a clear reduction in MeCP2 protein levels at day 14 of differentiation as assessed by Western blotting in Tra 1−85+ cells (Fig. 6F). Hence our results are consistent with overexpression of miR-155 improving the initial stages of iPSC differentiation toward HPCs in terms of CD34+ and CD45+ cell yield and hematopoietic gene expression levels. This lead to the hypothesis that because our system did not allow for modulation of miR-155 expression levels, the constant overexpression of this miRNA during the differentiation process drove the down-regulation of the chromatin regulator factor MeCP2. This might have partially impaired the final chromatin reorganization required for cell differentiation from a progenitor status to a more committed one.

DISCUSSION

Using human blood progenitors as a model for obtaining and subsequently differentiating iPSCs, we addressed the question of how the miRNA profile of the somatic cells was gradually reset toward an ESC-like profile while progressing through subsequent culture passages. We hypothesized that during the first few cell passages, a set of miRNAs expressed in reprogrammed iPSCs would be similar to those observed in the somatic cell of origin as a form of tissue-specific miRNA donor memory. Moreover, this cell origin-specific miRNA expression profile would then be progressively lost in negative correlation with time spent in culture. This so-called phenomenon of postprogramming molecular memory has already been described for the metabolome and the epigenome of iPSCs (7, 8, 39). In this regard, the metabolite profiles and the epigenetic marks of the derived iPSCs adjust further toward ESC-like patterns as compared with the somatic cell of origin, the longer they remain in culture. This observation led to the discovery that reprogrammed cells are prone to differentiating back into the lineage from which they were originally derived. Thus, the donor memory emerges as an interesting source of information to detect candidate molecules suitable for understanding the differentiation mechanisms for the generation of somatic progenitor cells in vitro.

It has been recently proposed that the developmental proximity between ESCs and the somatic tissue to be reprogrammed influences the reprogramming efficiency as well as the differen-

FIGURE 5. Prediction and validation of the miR-155 targets. A, bioinformatic identification of miR-155 targets. The lists of targets predicted by four different databases were compared, and 356 final miRNAs were selected as query of at least two of the four bioinformatic algorithms. The miR-155 predicted targets were compared by IPA software against published gene expression data of differentially expressed mRNAs between cord blood CD133+ cells and ESCs. B, network of predicted pathways related with hematopoiesis and maintenance of HPCs and modulated by miR-155, based upon IPA predicted target core analysis. Four of the pathways (green) were detected as down-regulated in CD133+ cells when compared with ESCs. C, target enrichment analysis after mRNA pulldown using a biotinylated miR-155 probe in HPCs. The expression levels of each target are depicted as the ratio between the total RNA (input) and the enriched fraction using the biotinylated miR-155 and a control probe. Each gene is associated with the corresponding signaling pathways.
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The relevance of miRNAs in the context of the hematopoietic system has been extensively described, both during hematopoiesis as well as related to the occurrence of blood malignancies (41, 45). In this regard, miRNAs have a crucial role during the differentiation of iPSCs into HPCs. Similar to that of translation regulators, such as miRNAs, could play a relevant role during the differentiation of iPSCs into HPCs. Interestingly, the promoter demethylation of blood-specific genes after cell reprogramming was not necessarily involved in gene activation or up-regulation in 19.9% of gene promoters studied (42). This implies that translation regulators, such as miRNAs, could play a relevant role during the differentiation of iPSCs into HPCs.

The relevance of miRNAs in the context of the hematologic system has been extensively described, both during hematopoiesis as well as related to the occurrence of blood malignancies (41, 45). In this regard, miRNAs have a crucial role during the differentiation of self-renewal (41). Several studies have reported a group of miRNAs up-regulated in HPCs isolated from different sources, including those observed in our study (27, 31, 46, 47). However, although miR-155 was also identified in previous studies, herein we validated for the first time its miRNA targets in the context of maintenance of the HPC phenotype. Thus, our study supports the notion that miRNAs playing a key role in blood progenitors, as predicted by bioinformatics, are targeted by miR-155 in HPCs. Concordantly, the constitutive overexpression of miR-155 in the early stem progenitor stage has been reported to block their differentiation toward more mature cells, hence highlighting a crucial role for this molecule in both myeloid and erythroid differentiation of human HPCs. High levels of expression seem to be related to maintenance of the pluripotent state, whereas a decrease may allow differentiation of HPCs to a more mature progenitor status (31). In agreement, studies on ESCs and HPCs have provided evidence for fine epigenetic regulation during stem cell fate decision and HPC maturation or maintenance (48, 49). However, the mechanism by which a particular epigenetic status could determine HPC fate decision or its hematopoietic function has still to be elucidated. In this regard, HPCs display active modeling of chromatin structures in the undifferentiated state of the cells where the transcriptional repressor MeCP2 or the enzymes for de novo DNA methylation, DNMT3α or DNMT3β, are expressed at a higher level when compared with committed blood cells (50, 51). Altogether, these studies suggest that a fine regulation of miR-155 in HPCs is required for cell fate determination, considering its role as a MeCP2 modulator as well as in DNA methylation and transcription repression pathways. Hence an inducible expression system would yield a more reliable system to establish the correct timing for the expression of miR-155 to drive differentiation from iPSCs to HPCs.

In summary, the use of a well established differentiation model such as the hematopoietic system allowed us to identify individual miRNAs with a relevant biological role in HPC differentiation or maintenance. For this purpose we tracked the miRNA expression profile of HPCs after their reprogramming into iPSCs. During the first few passages in vitro, iPSCs maintained the expression of a set of miRNAs that had been demonstrated to play a key role in the maintenance of the HPC phenotype. In particular, we found miR-155 as a key regulator in HPCs, presumably by acting as modulator in a broad range of pathways specific for hematopoietic endogenous progenitor cells. Additional studies are required to further elucidate the molecular mechanisms driving acquisition and loss of what we have termed miRNA donor memory. The present study may be
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