Using miRNA-mRNA Interaction Analysis to Link Biologically Relevant miRNAs to Stem Cell Identity Testing for Next-Generation Culturing Development

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ABSTRACT

Therapeutic benefit of stem cells has been demonstrated in multiple disease models and clinical trials. Robust quality assurance is imperative to make advancements in culturing procedures to enable large-scale cell manufacturing without hampering therapeutic potency. MicroRNAs (miRNAs or miRs) are shown to be master regulators of biological processes and are potentially ideal quality markers. We determined miRNA markers differentially expressed under nonclinical multipotent adult progenitor cell (MAPC) and mesenchymal stem cell (MSC) culturing conditions that regulate important stem cell features, such as proliferation and differentiation. These bone marrow-derived stem cell types were selected because they both exert therapeutic functions, but have different proliferative and regenerative capacities. To determine cell-specific marker miRNAs and assess their effects on stem cell qualities, a miRNA and mRNA profiling was performed on MAPCs and MSCs isolated from three shared donors. We applied an Ingenuity Pathway Analysis-based strategy that combined an integrated RNA profile analysis and a biological function analysis to determine the effects of miRNA-mRNA interactions on phenotype. This resulted in the identification of important miRNA markers linked to cell-cycle regulation and development, the most distinctive being MAPC marker miR-204-5p and MSC marker miR-335-5p, for which we provide in vitro validation of its function in differentiation and cell cycle regulation, respectively. Importantly, marker expression is maintained under xeno-free conditions and during bioreactor isolation and expansion of MAPC cultures. In conclusion, the identified biologically relevant miRNA markers can be used to monitor stem cell stability when implementing variations in culturing procedures.

SIGNIFICANCE

Human adult marrow stromal stem cells have shown great potential in addressing unmet health care needs. Quality assurance is imperative to make advancements in large-scale manufacturing procedures. MicroRNAs are master regulators of biological processes and potentially ideal quality markers. MicroRNA and mRNA profiling data of two human adult stem cell types were correlated to biological functions in silico. Doing this provided evidence that differentially expressed microRNAs are involved in regulating specific stem cell features. Furthermore, expression of a selected microRNA panel was maintained in next-generation culturing platforms, demonstrating the robustness of microRNA profiling in stem cell comparability testing.

INTRODUCTION

Regenerative medicine has provided new opportunities to cure injuries and disorders with needs that were previously unmet by conventional medical therapies. Several successful clinical trials have been performed with adult bone marrow-derived stem cell types, including mesenchymal stem cells (MSCs) [1] and MultiStem cells, the clinical-grade allogeneic stem cell product based on multipotent adult progenitor cell (MAPC) technology [2–6]. MAPCs and MSCs can both be isolated from adult bone marrow and expanded as adherent cells. However, after expansion using different procedures and culture media, MAPCs and MSCs are distinct cell types [4, 7–10]. The most pronounced difference in vitro is the elevated proliferative capacity of MAPCs [11, 12] and their ability to undergo up to 70 population doublings (PD), whereas MSCs reach senescence at PD 30–35. Although many MAPC and MSC in
In vitro functions are similar—i.e., immunosuppressive capacity [13–15]—it has been demonstrated that MAPCs and MSCs have different in vivo activities. For instance, MAPCs are more capable than MSCs at reducing inflammatory responses after intracranial injection in a stroke model [16]. Also, MAPCs show a higher capacity to induce blood vessel formation in a Matrigel plug implanted under the skin of nude mice [11]. Taken together, these published data provide evidence that MAPCs and MSCs adopt different phenotypes due to the specific conditions under which they are isolated and cultured and display different functional features in vivo.

Full transcriptional profiling has already proven to be a powerful tool to broaden the knowledge on MAPC and MSC functionality and to better understand the identity and unique characteristics of MAPC cultures. Previous comparative profiling studies [9, 11], resulted in a set of differentially expressed mRNAs. However, the functions attributed to these genes either remained limited to pluripotency determination or required a large set of genes to be enriched. Consequently a relatively large set of mRNA markers is required to obtain a more complete overview of all functionalities of the cell, which is a disadvantage for cell identity testing. In cell comparability testing, a panel of markers based on biological rationale is important to guarantee product stability after, for instance, changes in the manufacturing process [17, 18]. Particularly, for an off-the-shelf allogeneic clinical-grade MAPC product, alterations in expansion platforms will be required to meet cell numbers needed after market approval. This does not only imply a change from two-dimensional (2D) culture platforms to three-dimensional bioreactors, but also moving toward serum-free medium due to batch-to-batch differences, possible adventitious pathogens, ethical considerations and limited availability of serum [19]. It therefore remains a challenge to identify a convenient and relevant panel of robust cell identity markers that underlie functional differences of these cells.

Previous studies have highlighted the importance of microRNA (miRNA or miR) functionality in stem cells [20–24]. By binding their mRNA targets, miRNAs are able to block translation and lower stability of the transcripts, promoting degradation of the respective mRNAs [25, 26]. Because miRNAs are able to bind multiple targets, they can often function as master regulators of whole molecular networks [27, 28]. Several miRNAs have been described in literature to regulate proliferation and differentiation processes and could be involved in determining differential characteristics between MAPCs and MSCs [8, 29]. Because of their broad functional regulation, miRNAs are potentially ideal quality markers, because a relatively small set could suffice to describe major stem cell functionalities of MAPCs.

In this study we aimed to identify positive and negative miRNA markers for nonclinical MAPC with relevant stem cell related functions that clearly distinguish MAPCs from MSCs. We coupled the analysis of miRNA function and miRNA-mRNA interactions to biological function enrichment analysis using Qiagen Ingenuity Pathway Analysis (IPA) platform (Qiagen, Redwood City, CA, http://www.qiagen.com/ingenuity) [30]. By correlating the mRNA-miRNA integrative transcriptional profile of MAPCs and MSCs to functional gene analysis, a panel of seven negative and six positive MAPC miRNA markers were identified that correlate with phenotypic stem cell differences. Importantly, the miRNA expression profile was maintained during xenobioc-free and bioreactor expansion of MAPC, indicating the utility of these miRNAs as robust and convenient quality markers.

**RESULTS**

**MAPC and MSC mRNA Expression Profiles Correlate With Their Phenotypic Differences**

Both MAPCs and MSCs can be isolated from bone marrow as adherent cells, but have phenotypic differences in vitro and in vivo due to different culture procedures [11, 16]. The most predominant in vitro differences are cell proliferation (Fig. 1A) and morphology (Fig. 1B). MAPCs and MSCs share similar surface markers (Fig. 1C). To identify markers that correlate with the proliferative and morphological differences, comprehensive RNA

**MATERIALS AND METHODS**

**MSC and MAPC Culture**

Human MAPCs were processed as previously described [31]. Briefly, human MAPCs were isolated from a single bone marrow aspirate, purchased from Lonza (Walkersville, MD, http://www.lonza.com) or obtained with consent from a healthy donor, in accordance with the guidelines of the Medical Ethics Committee of the University Hospital of Leuven, and cultured on plastic tissue culture flasks coated with fibronectin (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com). Cell cultures were maintained under low oxygen tension in a humidified atmosphere of 5% CO2. Cells were cultured subconfluent in MAPC culture medium (Dulbecco’s modified Eagle’s medium [DMEM] 1 g/l glucose without L-glutamine [Lonza] supplemented with fetal bovine serum [FBS; Atlas Biologicals, Fort Collins, CO, http://www.atlasbio.com], insulin-transferrin-selenium liquid medium supplement [Lonza], MCDB-201 [Sigma-Aldrich], platelet-derived growth factor and epidermal growth factor [R&D Systems, Minneapolis, MN, https://www.rndsystems.com], dexamethasone [Sigma-Aldrich], penicillin/streptomycin (Lonza), 2-phospho-l-ascorbic acid (Sigma), and linoleic acid–albumin [Sigma-Aldrich]). Cells were passaged every 2–3 days and harvested by using trypsin/EDTA (Lonza). Cells were cryopreserved in 10% FBS-containing DMEM supplemented with 10% dimethyl sulfoxide (Sigma). Human MSCs were isolated on plastic tissue culture flasks from the same bone marrow aspirates as MAPCs. Cell cultures were expanded in a humidified atmosphere of 5% CO2 under normal oxygen tension. MSCs were grown to confluence in Mesenchymal Stem Cell Growth Medium (Lonza) before passaging. For both cell types, cells were counted at every passage, and population doublings were calculated based on the number of cells initially seeded (Ci) and the number of cells harvested (Ch) using the following equation: PDh = PDI + log2(Ch/Ci).

Human MAPCs were expanded on the Quantum cell expansion system (TerumoBCT, Lakewood, CO, https://www.terumobct.com). This closed automated culture system comprises a synthetic hollow-fiber bioreactor connected to sterile closed-loop, computer-controlled media and gas exchangers. The bioreactor contains ∼11,000 fibers, generating an expansion surface area of 2.1 m2. After coating with fibronectin, cells were seeded on the inside of the hollow fibers and expanded in MAPC culture medium. Cells were harvested after 5–6 days by using trypsin/EDTA. For xenobioc-free cultures, cells were expanded in TheraPEAK MSCGM-CD medium (Lonza) or Corning StemGro medium supplemented with MAPC growth factors on Corning CellBIND surfaces and passaged by using TrypLE Select (Thermo Fisher Scientific Life Sciences, Waltham, MA, http://www.thermofisher.com).

Further experimental procedures are presented as supplemental online data.
analyses were carried out. Firstly, mRNA screening using Illumina probe microarray analysis was performed on MAPCs and MSCs from three shared donors, followed by IPA biological function enrichment analysis. This web-based bioinformatics platform combines published data on molecular interactions, biological processes, and disease-related functions and thus provides a comprehensive method for RNA marker determination. A total of 2,118 probes showed donor-independent differential expression (fold change $> 2$), corresponding to 1,654 unique genes (set A), of which 914 were lower in MAPCs than MSCs (set B), and 740 were higher in MAPCs than MSCs (set C). To correlate the genes with functional differences between MAPCs and MSCs, these sets were analyzed with IPA core analysis, and enrichment was expressed as $- \log(p)$-values (Fig. 1D). The strongest significant functional enrichment for the total set of genes (set A) was observed for the category "cellular growth and proliferation," and other functions related to cellular proliferation. Besides these, 11 categories related to cellular development (including cell morphology) were found to be enriched. Both MAPC and MSC genes showed enrichment of cellular growth and proliferation (15.24 and 15.12, respectively), and according to IPA, the function had an increased activity in MAPC and decreased activity in MSC upregulated genes (data not shown). When gene sets were taken individually, 10 of the biological functions could be attributed to genes specifically overexpressed in MAPC (set C) or MSC (set B).

Very strong enrichments were found for "cell cycle" and "DNA replication, recombination, and repair" for MAPC genes, whereas MSC genes were not enriched for these functions, which is in line with the observed proliferation rate (Fig. 1A). Functions related to cellular development were enriched only in MSC-upregulated mRNAs, e.g., "tissue development" and "connective tissue development and function." Together, these data show that IPA reveals the cellular phenotypes of MAPCs and MSCs based on their specific mRNA expression patterns.

MAPCs and MSCs Exhibit Unique miRNA Expression Profiles

Given that MAPCs and MSCs have unique mRNA profiles that reflect the phenotypic differences between both cell types, we addressed the question of whether unique miRNA profiles also contribute to these cell phenotypes. Therefore, miRNA profiling was performed with the intent to (a) identify the MAPC and MSC miRNA profiles, (b) correlate miRNA profiles with known phenotypic differences, and (c) use miRNA marker profiling as a quality tool for screening MAPC identity during next-generation expansion techniques such as xeno-free and bioreactor expansion.

The miRNA profile was determined on the same samples that were used for the mRNA profiling. The quantitative polymerase chain reaction (qPCR)-based profiling platform detected 298 miRNAs, of which 124 were differentially expressed (fold change $> 2$), out of which 35 were specifically overexpressed in MAPCs (set C) and 28 in MSCs (set B).
**Figure 2.** miRNA-mRNA interactions correlate to MAPC and MSC differential phenotypes. (A): Schematic representation of bioinformatics analysis approach. mRNA and miRNA profiles were determined by using an Illumina array and TaqMan miRNA assays (Thermo Fisher Scientific Life Sciences), respectively. For mRNA analysis, \(|\text{FC}|\) were calculated, and cut-off was set at 2; for miRNA analysis, FC cut-off was set at 1.5. The differentially regulated mRNAs were taken together or divided in upregulated or downregulated miRNAs for the Qiagen Ingenuity Pathway Analysis (IPA) core analysis. The differentially regulated miRNAs were loaded into IPA, which resulted in 61 unique entries. A target filter analysis was performed, which resulted in 9,997 targeted mRNAs that were experimentally observed or predicted with a high probability. The overlap was determined of the miRNA targets with the differentially regulated mRNAs. Of the mRNAs that overlapped, only those with an inverse correlation were selected. The remaining mRNAs were divided in upregulated or downregulated sets or analyzed together. The analysis was repeated with miRNAs that were not expressed in MAPCs or MSCs and miRNAs that were equally expressed as a control. (B): Significance of enrichment of upregulated and downregulated functional categories in the analyzed mRNA subsets. The table shows \(-\log_{10} p\) values BH corrected (Figure legend continues on next page.)
which 97 had a consistent fold change (FC) > 1.5 in all three donors, with 44 upregulated in MAPC (MAPC miRs) and 53 upregulated in MSC (MSC miRs). The donor independent consistency indicates that these cell types are characterized by unique miRNA patterns.

Analysis of mRNA-miRNA Interactions

We next asked whether within this set of 97 miRNAs, key miRNAs can be identified that specifically contribute to phenotypic differences between MAPCs and MSCs. Because biological function enrichment analysis of mRNA profiles confirmed known phenotypical characteristics, a similar analysis was carried out with mRNA targets of differentially expressed miRNAs. In this way, miRNAs that control the MAPC phenotype were identified through their connection to relevant biological categories (Fig. 2).

Within the set of 97 miRNAs, IPA recognized 61 unique entries, with each entry representing one or more miRNAs with a unique seed sequence. Based on predicted and published information on miRNA-mRNA interactions, IPA identified a total of 9,997 potential miRNA targets of these miRNAs. To select for miRNAs that are both miRNA targets and differentially expressed, the overlap with the mRNA expression data were determined, which included an expression pairing criterion to take into account the predominant inhibitory regulation miRNAs exert on mRNAs. This approach resulted in a set of 707 miRNA targets, with MAPC miRNAs having 395 targets and MSC miRNAs having 312 targets (Fig. 2A).

IPA Core analysis was carried out on the determined miRNA targets, with distinctions made for total differential expression, mRNAs upregulated in MSCs (i.e., targets of MAPC miRNAs), and miRNAs upregulated in MAPCs (i.e., targets of MSC miRNAs). These core analysis datasets were named datasets D, E, and F, respectively (Fig. 2A), and compared with the core analysis of the mRNA profiles as in Figure 1D, to identify commonly enriched biological functions. Enrichment for targets of miRNAs that were not expressed in both MAPCs and MSCs and miRNAs that were equally expressed in both cell types (datasets G and H) were considered as nonspecific and excluded from the final enrichment overview table (Fig. 2B).

In case miRNAs have a strong influence on the MAPC and MSC phenotypes, core analysis results of the miRNA targets would have to result in similar biological function distinctions as the mRNA sets. In accordance, 19 of 20 biological categories that were enriched in the set of total differentially expressed mRNAs (set A) were also enriched in the set of total differentially expressed miRNA targets (set D), indicating that the miRNA targets represent a relevant subset of the total differential mRNA profile. To distinguish between processes regulated specifically by either MAPC or MSC miRNAs, only categories enriched in either sets B/E or in sets C/F, respectively (Fig. 2B, bold font), were selected.

For the functions cell cycle and DNA replication, recombination, and repair, enrichment was seen for the set of total miRNA targets (set D) with −log(p)-values of 12.62 and 6.33. These enrichments were exclusively seen for targets of MSC miRNAs (set F, −log(p)-values 10.25 and 8.72), which correlates to the results for MAPC upregulated mRNAs (set C) where both classes have a 32.69 and a 31.45 −log(p)-value, respectively. These findings indicate that in MAPCs, the reduced expression of a subset of miRNAs results in the high expression of their mRNA targets, leading to an increased cell division.

Conversely, the biological functions enriched in the set of MSC miRNAs, which are targets of miRNAs upregulated in MAPCs versus MSCs (set E) (Fig. 2B, highlighted in green) relate to development and differentiation of cells and include: “hematological system development and function,” “immune cell trafficking,” “connective tissue development and function,” “cell-to-cell signaling and interaction,” “tissue morphology,” and “skeletal and muscular system development and function,” with −log(p)-values ranging from 4.04 to 9.73. These biological functions were also enriched in the set of mRNAs downregulated in MAPCs (set B), indicating that upregulation of miRNAs in MAPCs suppresses these functions.

In conclusion, by focusing on commonly enriched biological functions of differentially expressed mRNAs and miRNA targets, miRNA-mRNA interactions can be identified that underlie specific properties of different types of stem cells.

Identification of Key Regulatory miRNAs

To identify miRNAs that potentially exert the biggest influence on MAPC and MSC function, a selection was made based on the number of targets of each miRNA and the FC between MAPC and MSC expression measured in the miRNA profile. miRNAs with at least 10 targets and a |FC| difference of 8 were selected for further qPCR analysis in cells from seven donors, including the three donors used for the initial profiling studies. By applying a threshold value of |FC| > 2 in seven donors, qPCR validation resulted in the selection of six key MAPC miRNAs (miR-204-5p, miR-20a-5p, miR-18a-5p, miR-106a-5p, miR-17-5p, and miR-155-5p) and seven key MSC miRNAs (miR-335-5p, miR-145-5p, miR-143-3p, miR-27b-3p, miR-125b-5p, miR-26a-5p, and miR-152-3p) (Fig. 3A, 3B; supplemental online Table 1). IPA core analysis on mRNA targets of key miRNAs, revealed biological function profiles that reflect overall results of the original IPA core analysis (Fig. 3C), albeit that values are less significant than when all differential miRNAs are taken into consideration. Remarkably, ‘Cell Cycle’ is also represented in the E* dataset, which can be explained by the interaction of MAPC miRNAs with genes shown to have an inhibitory function during cell cycle progression (e.g., regulation of CDKN1A by miR-17-5p [32]). The accordance of the IPA analysis of key miRNAs with the original analysis suggests that these key miRNAs are determining factors in controlling the main distinguishable characteristics of the MAPC and MSC phenotypes.

Validation of miRNA Function in Establishment of MAPC Characteristics

Next, we aimed to demonstrate a functional role of the two clearest markers, miR-335 and miR-204, in determining MAPC characteristics (Fig. 4). Previous publications demonstrated a negative correlation between miR-335 overexpression and cell division [33–36]. Because this miRNA correlates with cell cycle regulation and is not expressed in MAPC, we hypothesized that introducing...
Figure 3. Biological function profile of key miRNAs corresponds to the total miRNA profile. (A, B): Relative expression levels of miRNAs to RNU48 were assessed in seven independent donors by using the Exiqon MiRCURY platform. Shown are the miRNAs with a consistent fold change |FC| of 2, including MAPC markers (A) and MSC markers (B). Open bars represent mean value technical replicates (n = 3); colored bars represent the mean value of all donors; error bars represent SD. (C): Qiagen Ingenuity Pathway Analysis (IPA) as performed in Figure 2 was repeated for only the selected miRNA markers. Values represent $2\log_{10}(p)$ values obtained from the IPA core analysis. Columns A, B, and C represent the analysis of the differentially regulated mRNAs; D, E, and F represent the analysis of differentially regulated targets of differentially regulated miRNAs; and $D^*$, $E^*$, and $F^*$ represent the analysis of differentially regulated targets of selected miRNA markers. Abbreviations: A, all differentially regulated in (Figure legend continues on next page.)
miR-335 in MAPC would affect cell-cycle distribution. As predicted, miR-335 mimic transfection in MAPC resulted in more cells in G0–G1 phase than in mimic-control transfected and untreated MAPC, reaching similar levels as in MSC (Fig. 4A).

Moreover, as demonstrated by qPCR, targets of miR-335 that were upregulated in the MAPC mRNA profile and are linked to cell cycle regulation—CDC7 [37], TTK [38], CCNF [39], WWC1 [40, 41], and ADGRL2 [42] (supplemental online Fig. 1)—were all

**Figure 4.** miR-335 and miR-204 are functional in cell cycle regulation and adipocyte differentiation in MAPCs. (A): Cell cycle phase determination using propidium iodide, the percentage of cells in G0–G1 phase is significantly increased in MAPCs transfected with miR-335 mimic compared with MAPC and MAPC transfected with mimic ctrl (n = 9). (B): Quantitative polymerase chain reaction (qPCR) showing downregulation of miR-335 targets in cells transfected with miR-335 mimic when normalizing expression to standard MAPCs. miR-335 expression levels validate the transfection of miR-335 mimic in MAPCs (n = 9). (C, D): ORO extraction (n = 4) (C) and bright-field pictures (×10, EVOS-XL, AMG, Thermo Fisher Scientific Life Sciences; scale bar = 200 μm) (D) of adipocyte differentiation of MAPCs transfected with miR-204 inhibitor and inhibitor control. (E): qPCR showing upregulation of miR-204 markers BMP1 and RUNX2 in the absence of miR-204 and a downregulation of adipogenic markers ADIPOQ, PPARG, and FABP4 (n = 4). Statistical analysis of A–C and E: One-way analysis of variance, Tukey’s multiple comparison test, 95% confidence interval; *, p < .05; **, p < .01; ***, p < .001. Abbreviations: ctrl, control; inh, inhibitor; MAPC, multipotent adult progenitor cell; mim, mimic; miR, microRNA; MSC, mesenchymal stem cell; ns, not significant; ORO, Oil Red O; Rel, relative.
downregulated in MAPC transfected with miR-335 mimic (Fig. 4B), of which CDC7, CCNF, and WWC1 significantly. Therefore it can be concluded that upregulation of miR-335 expression in MAPC induces MSC-like stem cell properties.

Conversely, because basal miR-204 levels are higher in MAPC, the effects of miR-204 downregulation by transfecting MAPC with a miR-204 inhibitor were investigated. A number of publications have demonstrated that miR-204 plays a role in cell differentiation [43–47], and although effects may be cell-type-dependent [44], a consistent positive effect of miR-204 on adipocyte differentiation had been described [43, 47]. Consistent with these previous publications, knockdown of miR-204 in MAPC led to reduced adipocyte differentiation of MAPC as demonstrated by Oil-Red-O staining (Fig. 4C, 4D) and qPCR analysis (Fig. 4E). qPCR analysis confirmed significant downregulation of miR-204 expression and a reduced expression of adipocyte markers, which was significant for PPARG and FABP4 when compared with control cells. Furthermore, we measured a significant upregulation of two previously determined targets of miR-204, BMP1 [48] and RUNX2 [43], both of which are involved in differentiation [49, 50] (Fig. 4E). Together, these data show that modulating expression levels of the clearest distinguishing miRNA markers in MAPC affect stem cell identity.

Figure 5. Expansion of established MAPC cultures in xeno-free medium and on the Quantum bioreactor. (A): Proliferation represented as number of population doublings (PD) of established MAPCs, expanded under different conditions to replicative senescence. Standard MAPC were derived from three different donors (A, B, and C) in MAPC medium on fibronectin-coated flasks (MAPC FN), MAPC medium on CellBIND culture flasks (MAPC CB), and further expanded in xeno-free medium on CellBIND culture flasks (MAPC-sw-XF CB). (B): MAPC proliferation of three different donors (A, B, and X) on the Quantum bioreactor. Established MAPCs were seeded onto the bioreactor and harvested after 6 to 7 days. Doubling times were calculated and compared with those of cells expanded on T75 flasks. (C): Surface marker expression assessed by flow cytometry of cells expanded in serum-free medium (MAPC-sw-XF) or on the Quantum (MAPC-sw-Q). Control cells were PD-matched cells obtained from standard expansion on T75 flasks (MAPC). (D, E): MSC (D) and MAPC (E) key miRNA marker expression, assessed by quantitative polymerase chain reaction, is maintained after cell culture variations in separate donors (MAPC, MAPC-sw-XF, and MSC, n = 3; MAPC-sw-Q, n = 2). Each symbol represents the average expression of one miRNA per donor (n = 3). Abbreviations: CB, CellBIND culture flasks; FN, fibronectin-coated flasks; MAPC, multipotent adult progenitor cell; miR, microRNA; MSC, mesenchymal stem cell; PD, population doublings; Q, Quantum; st. dev., standard deviation sw-XF-CB, xeno-free medium on CellBIND culture flasks.

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cell properties, thereby proving the importance of miRNAs in establishing the unique MAPC phenotype.

miRNA Markers Are Maintained When Including Cell Culture Variations

In advancing MAPC expansion toward future therapeutic production, novel expansion techniques are being explored, including xenobiotic-free culturing and bioreactor expansion (overview of cell cultures, supplemental online Fig. 2). To investigate MAPC expansion on alternative platforms, established, well-characterized MAPC lines isolated from bone marrow and expanded on T75 flasks in serum containing medium according to standard MAPC expansion procedures [31] were subsequently expanded for multiple passages in xeno-free medium (MAPC-sw-XF) or on the

Figure 6. Isolation and expansion of XF-MAPCs and Q-MAPCs. (A): Mean proliferation of MAPCs, XF-MAPCs, and MSCs. Cells derived from five or six donors were expanded for more than 40 population doublings (MAPCs and XF-MAPCs) or until they reached replicative senescence (MSCs); error bars indicate SD. (B): Proliferation of cells derived from three different donors isolated and expanded on T75 flasks (MAPCs) or on the Quantum bioreactor; error bars indicate SD. (C): Flow cytometric analysis of positive and negative cell surface markers on MAPCs, XF-MAPCs, and Q-MAPCs. MAPC criteria are given in Figure 5C. (D): Proangiogenic activity assay based on human umbilical vein endothelial cell (HUVEC) tube formation. HUVECs were seeded on matrigel in conditioned medium of MAPCs, XF-MAPCs, and MSCs (left) or Q-MAPCs (right). The number of tubes was normalized relative to endothelial basal medium (negative ctrl), endothelial growth medium (positive ctrl), and unconditioned medium were included as controls. (E): Immunosuppression by XF-MAPC and Q-MAPC as illustrated by their capacity to reduce CD3/28 activated T-cell proliferation in a dose-dependent manner with carboxyfluorescein diacetate succinimidyl ester staining as readout. The numbers above the bars indicate the percent suppression relative to positive control (no MAPCs added). Statistical significance is calculated using one-way analysis of variance with Dunnett’s multiple comparison test relative to positive control, 95% confidence interval: **, p < .01; ***, p < .001. Abbreviations: ctrl, control; MAPC, multipotent adult progenitor cell; MSC, mesenchymal stem cell; Neg, negative; PBMC, peripheral blood mononuclear cell; PD, population doublings; Pos, positive; Q-MAPC, Quantum multipotent adult progenitor cell; XF-MAPC, xeno-free medium multipotent adult progenitor cell.
Figure 7. Differences in miRNA profiles between MAPCs and MSCs are maintained in XF-MAPCs and Q-MAPCs. MiRNA profiling of MAPCs, XF-MAPCs, Q-MAPCs, and MSCs samples from different donors is shown. (A): Heat map of two-group comparison of MSCs (group 1) versus MAPCs, XF-MAPCs, and Q-MAPCs (group 2). Blue miRNAs are higher in MAPCs, XF-MAPCs, and Q-MAPCs than in MSC; red miRNAs are higher in MSCs. Markers previously determined in Figure 3 are maintained in this profiling and are highlighted and marked with a hashtag. (B): Dendrogram of unbiased clustering analysis shows three main clusters of cell types, MSCs, XF-MAPCs, and MAPCs and Q-MAPCs combined in one cluster. The MSC cluster is more distinct from the MAPC cluster than XF-MAPC. (C): Heat map of unbiased clustering analysis, performed only with (Figure legend continues on next page.)
Quantum hollow-fiber bioreactor (MAPC-sw-Q) (Fig. 5). Normal cell growth was observed for MAPCs derived from three different donors when transferred to xeno-free conditions for at least 30 days, after which proliferation was slightly reduced compared with serum-containing medium (Fig. 5A). Also, growth kinetics of established MAPCs derived from three different donors that were seeded at standard density on the Quantum bioreactor did not alter significantly between standard plastic expansion and Quantum bioreactor (Fig. 5B).

For xeno-free-expanded and Quantum-expanded MAPCs, typical properties for bone marrow-derived cells [51], such as surface marker expression and differentiation capacity, remained unaltered compared with normal 2D expanded cells (Fig. 5C). Importantly, expression profiles of the identified miRNA markers, as confirmed by qPCR, did not alter profoundly after xeno-free or Quantum expansion (Fig. 5D, 5E), meaning that expression of MAPC miRNAs was maintained and MSC markers were not upregulated. Expression of MSC markers showed some donor-dependent variation, but the strongest markers, including miR-335, miR-145, and miR-143, were consistently the lowest expressed in all three MAPC types (20% or lower of MSC expression levels). For MAPC markers, expression in MAPC-sw-XF and MAPC-sw-Q in general averaged around the MAPC-normalized levels, whereas levels in MSCs were 51% or less of the MAPC normalized expression levels. Although miR-204 expression showed donor-dependent variation, the lowest expression level in MAPC-sw-XF was still 4.5-fold higher than the expression in MSCs, confirming the importance of this miRNA in discriminating MAPCs from MSCs. Together, these data show that when MAPCs isolated according to standard procedure are transferred to xeno-free conditions or to the Quantum bioreactor, their stem cell properties and miRNA profile are maintained.

**miRNA Profiling for Cell Comparability Testing**

Next, we investigated whether miRNA profiles are also maintained when MAPCs are isolated from bone marrow under xeno-free conditions or on Quantum bioreactor. To this end, miRNA profiling was carried out on MAPCs that were isolated and subsequently expanded in xeno-free medium (XF-MAPC) or after seeding bone marrow on the Quantum followed by subsequent expansion (Q-MAPC) (supplemental online Fig. 2).

Proliferative capacity of XF-MAPC and Q-MAPC was maintained and remained higher than MSC (Fig. 6A, 6B), while other MAPC properties such as cell surface markers (Fig. 6C), trilineage differentiation (supplemental online Fig. 3), proangiogenic (Fig. 6D), and immunosuppressive capacity (Fig. 6E) were also maintained in XF-MAPC and Q-MAPC. These assays all confirm that the tested cell-culture variations did not affect the potency of MAPCs and that, phenotypically, XF-MAPC and Q-MAPC were largely identical.

In order to determine the extent to which marker miRNAs contribute to phenotype consistency of XF-MAPC and Q-MAPC, miRNA profiling was carried out on multiple MAPC, XF-MAPC (both TheraPEAK and StemGro medium), Q-MAPC, and MSC samples. To test whether miRNA markers identified in the initial MAPC versus MSC screen maintain their expression patterns in this independent profiling experiment, a two-group comparison was made, in which MAPC/XF-MAPC/Q-MAPC as one group were compared with MSC samples otherwise (Fig. 7A). The resulting heatmap shows a clear separation between MSC samples and grouped MAPC samples. Of the 81 miRNAs identified in this screen, 42 miRNAs were also differentially expressed in the initial screen (data not shown), including the 13 marker miRNAs previously identified (highlighted and marked with a hashtag in Fig. 7A). These marker miRNAs also show some of the strongest differential expression in this screen, with miR-335 and miR-204 remaining to be clear distinctive markers.

When performing an unbiased clustering analysis of the miRNA profiles, the software independently grouped samples according to cell type, with the largest variance between MAPCs and MSCs and a smaller variance between MAPCs and XF-MAPCs (Fig. 7B). Although XF-MAPCs formed a separate cluster, Q-MAPCs were indistinguishable from MAPCs, indicating that expansion on the Quantum does not lead to significant alterations of MAPC miRNA expression. Importantly, in the two-group comparison shown in Figure 7A, XF-MAPCs clustered together with MAPCs and Q-MAPCs. This indicates that the distinction seen between MAPCs and XF-MAPCs in the unbiased clustering analysis (Fig. 7B) is due to minor changes in the miRNome of XF-MAPC. Despite these changes, XF-MAPCs largely maintain the unique MAPC miRNA expression profile, meaning that XF-MAPCs are highly similar to MAPCs, but are unlike MSCs. When performing an unbiased clustering analysis with only the 13 miRNA markers, the distinction between MSCs and all MAPC types is again clear (Fig. 7C).

When analyzing only the key miRNA markers, XF-MAPC clusters together with MAPCs and Q-MAPCs, meaning that they do not represent the XF-induced minor changes that caused XF-MAPCs to form a separate cluster in the previous unbiased analysis (Fig. 7B). Overall, it can be concluded that miRNAs differentially expressed between MSC and MAPC are highly robust quality markers, irrespective of MAPC culturing procedure, with the additional advantage of being relevant to stem cell biological processes.

**DISCUSSION**

Extensive knowledge of cell identity and proper cell characterization is an absolute necessity during development of stem cell products, with alterations in the established profile potentially influencing potency of the cells. In order to avoid pitfalls during process development, establishment of robust quality markers that remain stable and are clearly linked to stem cell functionalities is pivotal. Because there are only 2,588 mature miRNA species in human, compared with 21,000 protein-coding genes, miRNAs might provide a smaller set of markers sufficient to deliver a full signature of the cells. In this publication, we identified such a limited set of MAPC and MSC miRNAs that can be used as stem cell quality markers and could facilitate development of new stem cells culturing platforms.

By comparing MAPCs to MSCs, we identified seven key MAPC-negative markers (miR-335-5p, miR-145-5p, miR-143-3p,
miR-27b-3p, miR-125b-5p, miR-26a-5p, and miR-152-3p) and six key MAPC-positive markers (miR-204-5p, miR-20a-5p, miR-18a-5p, miR-106a-5p, miR-17-5p, and miR-155-5p) (supplemental online Table 1). Previous publications that performed a limited miRNA profiling of MAPC versus MSC, also reported on the differential expression of miR-143 and miR-204 [9] and miR-143, miR-20a, miR-155, miR-106a, and miR-17 [8], confirming the relevance of some of the key miRNAs determined in this study. The differences in miRNA markers between these studies and ours, are probably due to the chosen profiling platform, which is reported to have a substantial impact on the detected miRNAs [52].

Results of the miRNA profiling show an enrichment of cell cycle progression in MAPCs and developmental categories in MSCs. The enrichment for developmental categories is probably best viewed in the perspective of stem cell pluripotency, where MSCs are slightly more committed toward certain lineages than MAPCs, with the latter remaining more immature with a broader differentiation capacity [9, 10]. This enrichment was confirmed when analyzing mRNA-miRNA interactions, indicating that miRNAs influence essential biological categories through suppression of mRNAs.

The balance between proliferation and differentiation is pivotal in stem cell biology. They exert a lot of influence on one another, mostly during the G1 phase of the cell cycle where the decision is made whether to go through another round of division or to slow down proliferation and initiate differentiation [53, 54]. It can be hypothesized that miRNAs control the equilibrium of these two processes and push MAPCs toward proliferation and MSC to differentiation during standard culturing procedures. Many of the miRNAs that are more highly expressed in MAPCs have the ability to downregulate developmental processes, especially miR-204 [43, 45], whereas MSC miRNAs are linked to cell cycle and stemness regulation [33, 34, 36, 55]. We were able to strengthen this hypothesis by providing evidence that miR-335 is functional in cell cycle regulation and miR-204 is able to regulate adipocyte differentiation, showing that at least the two most distinctive markers are able to determine stem cell characteristics. These findings underscore the relevance of monitoring expression of miRNA markers during development of novel expansion procedures.

The majority of the MAPC miRNA markers belong to the miR-17/92 cluster and its paralogs. The miRNA markers belonging to these clusters (miR-20a, miR-18a, miR-106a, and miR-17) are known to perform a pivotal role in both healthy and cancerous tissue in various processes such as cell cycle, proliferation, apoptosis and differentiation [56]. They have also been reported to be downregulated in aging cells, including MSCs [57]. As mentioned previously, miR-204 is known to regulate both osteoblast and adipocyte differentiation in vitro [43–47], but has also been associated with adipose tissue development in vivo [58, 59]. Additionally, the MAPC marker miR-155 has been shown to exert immune-modulatory functions in T cells, B cells, and dendritic cells [60–64].

Many of the MSC miRNA markers are described in the literature to have an important role in regulating cell proliferation, with the best example being miR-335. Several publications elucidate its antiproliferative role in cancerous tissues and cell lines [36, 65] and stem cell populations [33, 34], as well as its involvement in regulating differentiation [66, 67]. In line with these previous publications, we provide evidence for the miR-335-repressive effects on cell cycle progression. Antiproliferative effects have also been described for miR-152 [68], miR-125b [69], miR-26a [70], miR-27b [71], and miR-143 and miR-145 [72]. Notably, many of the MSC miRNAs had already been identified to be MSC markers, confirming the robustness of the profiling analysis and of these miRNAs in defining MSC properties [73].

Coincidentally, a publication reporting on miRNAs influencing proliferation and differentiation in C2C12 myoblasts showed a miRNA profile with considerable overlap with the differential MAPC/MSC profile [29], supporting our claim that the key MAPC and MSC miRNAs are involved in these processes. Furthermore, a study conducted in fibroblast also confirmed the pro-proliferative action of miR-17, miR-106a, miR-20a, and miR-18a and the anti-proliferative action of miR-27b and miR-335 [74].

By comparing miRNA profiles of regular MAPCs with MAPCs cultured on alternative xenobioc-free and bioreactor platforms, we aimed to verify miRNA markers determined in the initial screen. We were able to confirm that xeno-free expanded cells and bioreactor expanded cells maintain all in vitro characteristics of MAPCs, as well as expression of key miRNA markers. We therefore propose that this small panel of miRNAs, based on biological rationale (supplemental online Table 1), can be used as quality markers during future advancements in MAPC process development. Our study also highlights the power of miRNA-mRNA interaction profiling for the identification of robust quality markers in a wide array of cell types.

**Conclusion**

By analyzing mRNA-miRNA interactions in nonclinical MAPCs and MSCs, we identified a panel of miRNA markers with a clear link to stem-cell-related processes. Furthermore, we propose that these newly identified biologically relevant miRNA markers can be used during further process development to monitor stability during variations in culturing procedures.

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**Author Contributions**

M.A.E.C.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; K.G., A.V., and D.C.: collection and/or assembly of data, data analysis and interpretation; S.W.: collection and/or assembly of data; J.P.: final approval of manuscript; R.J.D. and W.A.: conception and design, final approval of manuscript; B.L.T.V.: conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript.

**Disclosure of Potential Conflicts of Interest**

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