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Efficiency of Exosome Production Correlates Inversely with the Developmental Maturity of MSC Donor

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Abstract

Mesenchymal stem cells (MSCs) derived from human embryonic stem cells (ESCs) and fetal tissues have been shown to secrete cardioprotective exosome, a protein- and RNA40 containing vesicle. Since the therapeutic efficacy of MSCs is inversely correlated with developmental stage of the donor, we determine if this correlation extended to the cardioprotective MSC exosomes by examining exosomes secreted by MSCs derived from non-embryonic/fetal tissues e.g. umbilical cord.

Unlike ESC- and fetal-MSCs, cord-MSCs have a much smaller proliferative capacity. To circumvent this and produce sufficient MSC exosomes for testing, they were immortalized via *MYC* over-expression. Like ESC-MSCs, *MYC* immortalization of cord MSCs expanded their proliferative capacity to bypass senescence, reduced plastic adherence, enhanced growth rate, and eliminated *in vitro* adipogenic differentiation potential without compromising exosome production. Exosomes produced by immortalized cord-MSCs were cardioprotective, and were equally efficacious in reducing infarct size in a mouse model of myocardial ischemia/reperfusion injury. However, cord MSCs produced the least amount of exosomes followed by fetal- and then ESC-MSC in decreasing order of developmental maturity or youth of the donor tissues, suggesting that the inverse correlation between the therapeutic efficacy of MSC and developmental stage of the donor is underpinned by rate of exosome production.

Keywords: Mesenchymal stem cell; Exosome; Umbilical cord; Myocardial ischemia/Reperfusion injury

Abbreviation: ESC: Embryonic Stem Cell; MSC: Mesenchymal Stem Cell; CM: Conditioned Medium; IS: Infarct Size; AAR: Area at Risk

Introduction

Mesenchymal stem cells (MSCs), often categorized as adult stem cells are multipotent stem cells that could differentiate into at least three cell types namely, adipocytes, chondrocytes and osteocytes [1-6]. They are also reported to be able to differentiate into endothelial, cardiomyocytes and neurons [7-13] with negligible risk of teratoma formation. This wide-ranging differentiation potential were used to rationalize MSC transplantation to treat musculoskeletal injuries, improve cardiac function in cardiovascular disease and ameliorate the severity of graft-versus-host-disease [14]. This together with its readily accessible tissue sources made MSCs one of the most widely tested stem cells in clinics today. In 2010 alone, there were 101 clinical trials using MSCs to treat a variety of disease conditions [15]. Unlike the controversial embryonic stem cells, MSCs could be isolated from many ethically palatable tissues such as bone marrow [16,17], adipose tissue [17,18], liver [19,20], muscle [21,22], amniotic fluid [23,24], placenta [25,26], umbilical cord blood [16,27], dental pulp [28,29]. However, it is generally observed that the biological activity and therapeutic potency of MSCs correlate inversely with developmental stage of the donor [30-48]. Therefore, MSCs from ethically and socially controversial but "young" tissues such as fetal tissues [49] and human embryonic stem cells (ESCs) [50] continue to be investigated for their therapeutic potential. Many studies reported that MSCs derived from these latter tissues are more robust in their proliferative capacity, biological activity and therapeutic potency [20,51-55].

The therapeutic efficacy of MSC transplantations in the treatment of different diseases had been rationalized on the differentiation potential of MSCs to replace lost or injured cells. However, this differentiation-based mechanism has become increasing untenable. In animal models and even patients where MSC transplantation elicited a therapeutic response, <1% of transplanted MSCs reach their target tissue with even less engrafting or differentiating at the injured tissue [56-64]. An alternative and more conciliatory mechanism implicating

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MSC secretion as the therapeutic agent has been proposed [65-73]. Our group demonstrated that MSC secretion alone could improve cardiac function in pig and mouse models of acute and chronic myocardial ischemia [74-76] and identified exosome, a membrane vesicle as the therapeutic factor in the secretion [77,78]. Others subsequently confirmed exosomes as the factor mediating the therapeutic trimester fetal tissues [77,79] for the production of secretion and exosomes. Since developmental stage of the donor is a dominant determinant of the therapeutic potency of MSCs and exosome is the factor mediating this potency, we tested here if exosome production is correlated to the developmental stage of the donor tissue by comparing exosome production from hESC-derived MSC and the developmentally more mature tissues such as the umbilical cord from a full term delivery.

In our hands, MSCs derived from umbilical cord could not be expanded beyond six passages while MSCs derived from human ESCs and fetal tissues could be passaged for more than 20 passages to generate up to 10^19 cells [79,80]. In addition, cord MSCs have a longer doubling time. Therefore, expanding cord MSCs to produce sufficient exosomes for comparative studies with exosomes from either ESC- or fetal MSCs was not feasible. To circumvent this issue, we immortalized the cells by over-expressing *MYC* gene. We had previously use this method to immortalize human ESC-derived MSCs and observed that despite some changes, the immortalized cells retained many fundamental MSC characteristics including the production of cardioprotective exosomes [81].

In this study, we characterized *MYC*-immortalized 109 cord MSCs, their production of exosomes and the efficacy of their exosomes in reducing reperfusion injury in a mouse model of ischemia/reperfusion injury, and compared these against our previous analysis of *MYC*-immortalized ESC-MSCs.

Materials and Methods

Derivation of cord MSCs

The collection of umbilical cords of term babies after obstetric delivery was carried out in KK Women's and Children's Hospital under an IRB approved protocol (CIRB 2009/289/D). Umbilical cords were stored in DPBS with 10 μg/ml gentamycin at 4°C during the transfer to the lab. To isolate MSCs, the cord was cut into 3 cm-long pieces, rinsed with DPBS + gentamycin to remove as much blood as possible and then cut lengthwise to remove blood vessels. They were digested with 300 U/ml collagenase, 1 mg/ml hyaluronidase and 3 mM $CaCl_2$ in PBS for 1 h at 37°C with occasional agitation. The cord pieces were then crushed with forceps to release cells from the Wharton's jelly and then digested with 0.05% trypsin-EDTA for 30 min at 37°C before being crushed again with forceps. The cell suspensions were combined, washed and cultured as previously described [75].

Oncogenic transformation of cord MSCs

MYC transformation of cord MSCs was performed using a lentivirus carrying the *CMYC* gene as previously described [81]. Briefly, cord MSCs were plated at 10^6 cells per 10 cm dish and infected with viruses at a MOI of 5 in the presence of 4 μg/ml polybrene overnight. The following day, culture medium was replaced with fresh medium and then 48 h later with medium containing puromycin (2 μg/ml). After 72 h of puromycin treatment, the surviving cells were allowed to expand. Clonal lines from each of three independently infected cell cultures were derived by limiting dilution. When individual clones were expanded to 10^7 cells per clone (or a confluent 15 cm culture dish), the cells were designated p1. Three clonal lines were generated and named CMSC3A1, CMSC3A3 and CMSC3A4 lines, respectively. Integration of the *CMYC* or *GFP* transgene was confirmed by amplifying genomic DNA using specific primers for exon2 and exon3 of *CMYC* respectively: 5'- GCCCCTGGTGCTCCATGAGGAGACACC'-3' and 5'- ACATTCTCCTCGGTGTCCGAGG-3' using the following PCR conditions: one cycle of 94, 2 min; 32 cycles of 94°C, 15 s; 60°C, 30 s; 72°C, 90 s and one cycle of 72°C 5min. The PCR products were resolved on a 1% agarose gel. Differentiation of the MYC-MSCs to adipocytes, chondrocytes and osteocytes was performed using adipogenic, chondrogenic and osteogenic hMSC Differentiation Bullet Kits, respectively (Lonza, Walkersville, MD) according to manufacturer's instructions. Karyotyping by G-banding was performed by the Cytogenetics Laboratory, KKH.

Quantitation of *MYC* RNA transcript by qRT-PCR

20 ηg cellular RNA was converted to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA). The cDNA was then amplified by one cycle of 94°C, 10 min; 40 cycles of 94°C, 15 s; 60°C, 60 s and one cycle of 95°C, 15 s, 60°C 60 s, 95°C, 15 s with primer sets specific for either *MYC* or *ACTB* transcript on the StepOnePlus Real-Time PCR system (Applied Biosystems, Life Technologies).

The *MYC*-specific primer set is 5' ACT TAG TTG CGT TAC ACC C 3' and 5' AAA TAA AGC CAT GCC AAT CTC 3'.

Telomerase activity

Relative telomerase activity was measured by SYBR® Green real time quantitative telomeric repeat amplification protocol assay using a modified method as described by Wege et al. [82]. Briefly, 3×10^6 cells were harvested and cell lysate was prepared using a commercially available mammalian cell extraction kit (Cat K269-500-1, BioVision, Milpitas, CA). The reagents for the PCR amplification was 1 μg of protein cell lysate, 10 μL of 2 X SYBR Green Super Mix (Cat 170-8880, BioRad, Hercules, CA) with 0.1 μg of TS primer (5'-AATCCGTCGAGCAGACTT-3'), 0.1 μg of ACX primer (5'-GCGCGG[CTTACC]3CTAACC-3') and 10 mM EGTA in a total volume of 25 μL. The reaction was first incubated at 25°C for 20 min to allow the telomerase in the cell lysate to elongate the TS primers followed by 2 min incubation at 95°C to inactivate telomerase activity and denature the primers. The telomerase product was amplified by PCR for 40 cycles of 95°C, 30s; 60°C, 90s. The relative telomerase activity was assessed against that of HEK293 cells using the threshold cycle number (or Ct value) for 1 μg protein cell lysate.

Rate of cell cycling

To assess cell cycle rate, 2×10^7 cells were pre-labelled in 2 ml of 10 μM CFDA (Molecular Probe, Eugene, OR) in PBS at 37°C for 15 min, cultured for 24 h and then replated at 5×10^4 cells per well in 6-well coated with gelatin. At 0, 24, 48, and 72 h, cells from duplicate wells were harvested, and fixed in 2% paraformaldehyde, and analyzed on FACSplus (Becton Dickinson; San Jose, CA). The number of cell cycles per 24 h was calculated assuming that each halving of cellular fluorescence represented one cell division. Therefore, the number of cell cycles per 24 h (n) was calculated as n=lg(F-Fn)/lg2 where F is initial average cellular fluorescence and Fn is the average cellular fluorescence after 24 h. The number of cell cycles was then plotted against time to derive the average time per cell cycle.

Surface antigen analysis

Expression of cell surface antigens on HuES9.E1 and CMSC3A1

MSCs was analyzed using flow cytometry as previously described [81]. The cells were trypsinized for 5 min, centrifuged, resuspended in culture media and incubated in a bacterial culture dish for 1 h in a 37°C, 5% CO2 incubator. The cells were collected, centrifuged, washed in 2% FBS. 2.5×10^5 cells were then incubated with each of the following conjugated monoclonal antibodies: CD29-PE, CD44-FITC, CD49a-PE, CD49e-PE, CD105-FITC, CD166-PE, CD73-FITC, CD34-FITC, CD45-FITC, HLADR-PE, and MHC1-PE (PharMingen, San Diego, CA) for 1 h on ice. After incubation, cells were washed and resuspended in 2% FBS. Nonspecific fluorescence was determined by incubation of similar cell aliquots with isotype-matched mouse monoclonal antibodies (PharMingen, San Diego, CA). Data were analyzed by collecting 20,000 events on a BD FACSCalibur™ Flow Cytometer (BD Biosciences, San Jose, CA) instrument using CELLQuest software.

Illumina gene chip analysis

Total RNA was prepared in technical triplicates from different passages of MSCs using Illumina® TotalPrep RNA Amplification Kit (Ambion, Inc., Austin, TX). The MSCs were HuES9-E1 MSCs at p15 and p16; E1-MYC 21.1 at p3, p4, and p5; E1-MYC 16.3 line at p4, p7, and p8; CMSC3A1 at p4, p5, and p6; CMSC3A3 at p4, p5, and p6; and cord MSCs at p1 and p2. 500 ηg RNA was converted to biotinylated cRNA using the Illumina RNA Amplification Kit (Ambion, Inc., Austin, TX) according to the manufacturer's directions. 750 ηg of the biotinylated cRNA were hybridized to the Sentrix HumanRef-8 Expression BeadChip Version 3 (Illumina, Inc., San Diego, CA). Washing and scanning were performed according to the Illumina BeadStation 500x manual. The data were analyzed using Genespring GX 10. Quantile normalization was performed by a shift to 75th percentile, and the normalized data were baseline transformed to the median of all samples.

Exosome preparation

Exosomes were purified from MSC conditioned medium (CM) by size exclusion using HPLC as previously described [77,81].

Mouse model of myocardial ischemia/reperfusion injury

Cardioprotective effect of the exosomes was tested in a mouse model of ischemia and reperfusion injury. MI was induced by 30 min left coronary artery (LCA) occlusion and subsequent reperfusion. 5 min before reperfusion, mice were intravenously infused with 200 µl saline solution of 0.3 µg exosome protein purified from culture medium conditioned by MYC-MSCs. Control animals were infused with 200 µl saline. After 24 h reperfusion, infarct size (IS) as a percentage of the area at risk (AAR) was assessed using Evans' blue dye injection and TTC staining as described previously [75].

Statistical analysis

Two-way ANOVA with post-hoc Dunnett was used to test the difference in infarct size between groups. Correlation coefficient of each pairs of array was assessed using Pearson correlation test.

Results

Immortalization of cord MSCs

Primary umbilical cord derived MSCs have a limited expansion capacity of about 6 passages in our hands. As such, a single cord preparation would potentially generate only 10^8MSCs against >10^{19} MSCs from a single preparation using human ESCs or fetal tissue. To generate sufficient quantities of exosomes for comparative analyses against those produced by hESC-derived MSCs (HuES9.E1) [80] and the *MYC*-234 immortalized HuES9.E1 or E1MYC [81], primary cord MSCs, p3 were infected with lentivirus carrying the *MYC* oncogene. After puromycin selection, surviving cells were re-plated at low density ranging from 20,000-50,000 cells per 10 cm plate to produce physically well separated colonies and selected colonies were expanded to establish clonal lines. Three colonies from three independent infections were eventually selected to establish CMSC3A1, CMSC3A2 and CMSC3A3 clonal lines. The transformed cells were smaller and rounder with prominent nuclei. They had reduced adherence to plastic culture and reduced contact inhibition at confluence so that the cells formed clusters instead of adhering to the plastic dish as a monolayer (Figure 1A). PCR amplification of genomic DNA revealed that the *MYC* transgene was integrated into the genome (Figure 1B). The level of *MYC* transcripts in CMSC3A1 was higher than that in HuES9.E1 MSCs, but lower than E1MYC16.3 MSCs (Figure 1C). Since *MYC* was reported to promote cell immortalization by activating telomerase to maintain telomeric repeats [83], we determined telomerase activity and observed a concordance between telomerase activity and *MYC* expression level. Telomerase activity in CMSC3A1 cells was higher than that in HuES9.E1 but lower than that in E1MYC16.3 which was established by MYC transformation of HuES9.E1 (Figure 1D). CMSC3A1 also has a normal 46 XY karyotype (Figure 1E). Consistent with the lower telomerase activity, *MYC*-transformed cord MSC lines have a cell cycle of ~13 hours which is longer than the 11 hours for E1MYC16.3 but shorter than the 19 hours for HuES9.E1 (Figure 1F).

Characterization of *MYC*-immortalized cord MSCs

The surface antigen profile of the *MYC*-transformed cord MSCs, CMSC3A1, was qualitatively similar to that of *MYC*-transformed E1MYC16.3. The cells were $CD29^+$,$CD44^+$, $CD49a^+$ $CD49e^+$, $CD73^+$ $CD105^+$, $CD166^+$, MHC I^-, HLA-DR^-, $CD34^-$ and $CD45^-$ (Figure 2A). The *in vitro* differentiation potential of CMSC3A1 was also similar to that of immortalized human ESC-derived MSC line, E1MYC16.3 [81]. Like E1MYC16.3, CMSC3A1 cells differentiated readily into chondrocytes and osteocytes but not adipocytes (Figure 2B) [84]. During induction of adipogenesis which consisted of 4 cycles of a 6-day treatment of 3 days' exposure to induction medium and 3 days' exposure to maintenance medium, most CMSC3A1 cells like E1MYC16.3 died during exposure to the induction medium. These observations suggested that *MYC*-transformed cord MSCs cannot undergo adipogenic differentiation which is a defining property of MSCs.

Gene expression profile

The genome-wide gene expression profile of HuES9.E1, primary cord-derived MSCs, E1MYC16.3, E1MYC21.1, CMSC3A1 and CMSC3A3 was determined by microarray hybridisation on the Illumina Sentrix HumanRef-8 Expression BeadChip containing more than 24,000 unique features, and assessed for the relatedness between cell types. The expression profile of CMSC3A1 resembled that of *MYC*-transformed human ESC-derived cell lines, E1MYC16.3 (correlation coefficient, r^2= 0.95) more than that of its parental cord-derived MSCs (r^2= 0.92) or HuES9.E 1 (r^2= 0.92) (Fig. 3A) as illustrated by hierarchical clustering (Figure 3B). Upon MYC immortalization, 295 and 377 genes were respectively up- and down-regulated by 2.0 fold in cord-derived CMSC3A lines while only 86 and 120 genes were similarly up- or down-regulated in the human ESC-derived lines. Of these genes, 25 genes were up-regulated and 39 down-regulated genes in both *MYC*-transformed MSCs (Figure 3C).

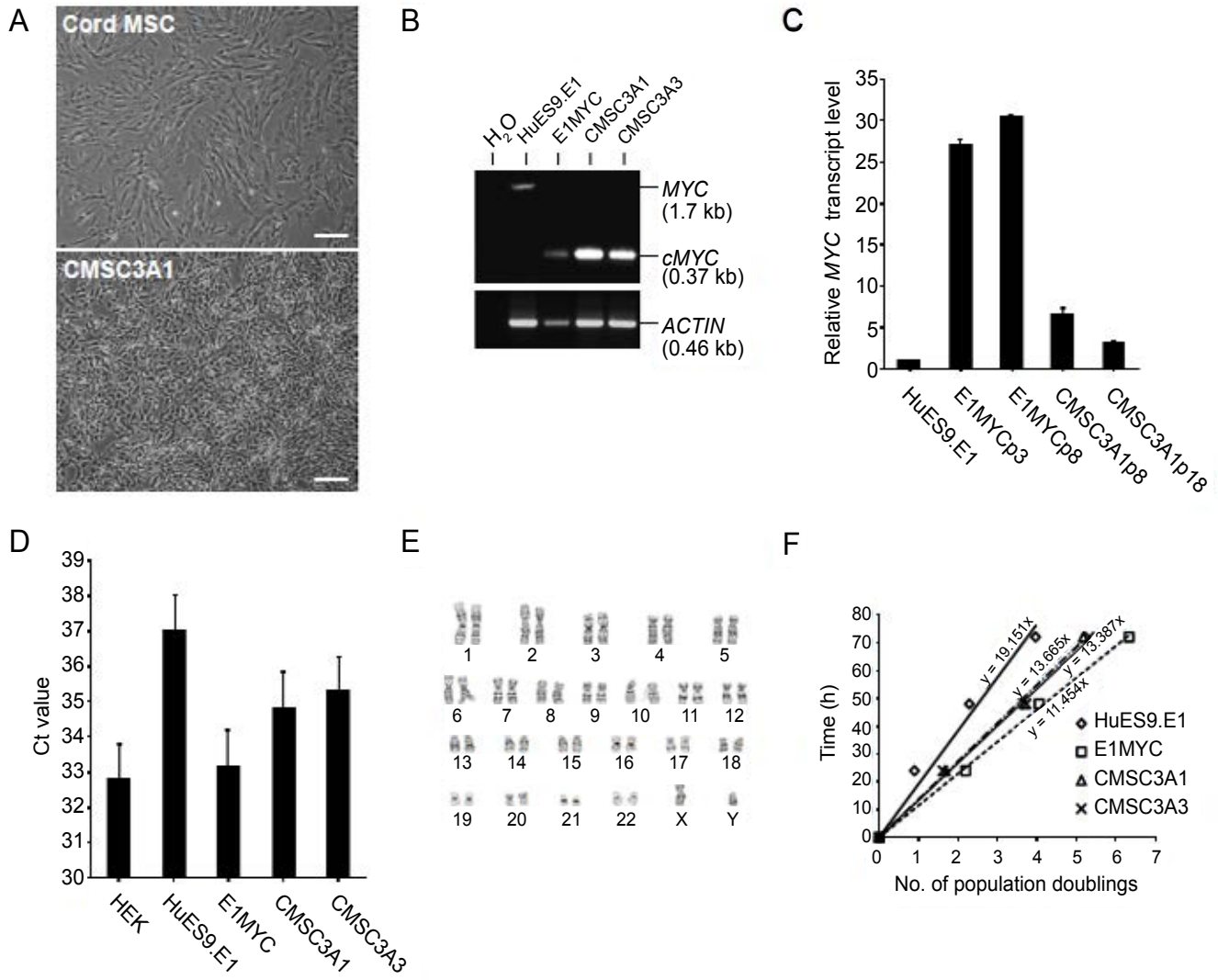

Figure 1: Immortalization of cord MSCs. (A) Cell morphology of parental cord MSC and *MYC*-transfected cord MSCs as observed under phase contrast microscopy, scale bar 100 µm. (B) PCR analysis of cellular DNA from HuES9.E1 MSCs, *MYC*-transfected HuES9.E1 MSCs (E1-MYC), and *MYC*-transfected cord MSCs clones CMSC3A1 and CMSC3A3. DNA was amplified using primers specific for exon 2 and exon 3, respectively. The expected PCR fragment size for the endogenous *MYC* gene was 1.7 kb and for the transfected *MYC* cDNA was 0.37 kb as represented by the amplified fragment from the *MYC* lentivirus. (C) Relative *MYC* transcript level. *MYC* transcript levels in HuES9.E1 (MSCs derived from hESC), E1MYC16.3 (*MYC*-transformed HuES9.E1 MSC line) at p8 and p18, and CMSC3A1 *MYC*-transformed cord MSC line at p8 and p18 were determined by quantitative RT-PCR. The internal reference for each sample was GAPDH transcript. The *MYC* transcript level in each sample was normalized to that in HuES9.E1. (D) Relative telomerase activity. Telomerase activity in each cell type was assayed using 1 µg of cell lysate protein to first extend a TS primer and any extendedproduct was then quantitated by real time PCR. The Ct value reflected the amount of telomerase product and therefore the telomerase activity in the lysate. Note: Ct value is inversely proportional to the template concentration in the PCR reaction. (E) Karyotype analysis of CMSC3A1 by G-banding. (F) Rate of cell cycling. Cells were labelled with CFDA and their fluorescence was monitored over time by flow cytometry. The loss of cellular fluorescence at each time point was used to calculate the number of cell division that the cells have undergone as described in Materials and Methods.

Isolation of exosomes from culture medium conditioned by CMSC3A1

We had previously demonstrated that exosomes secreted by ESC-derived MSCs and their *MYC*-transformed progeny were protective in a mouse model of myocardial ischemia and reperfusion injury [75,77,81]. To test if transformed cord MSCs also produced similar exosomes, CMSC3A1 were grown in a chemically defined medium, the conditioned culture medium (CM) was harvested and exosomes purified as previously described [77,85]. The HPLC protein profile of the CM was similar to that of CM from ESC-derived MSCs and their *MYC*-transformed progeny [77] (Figure 4A) with the fastest eluting fraction having a retention time of about 12 minutes. Dynamic light scattering analysis of this peak revealed the presence of particles with a hydrodynamic radius range of 50-65 ηm. Western blot analysis of this peak also revealed the presence of exosome-associated proteins such as CD9 and CD81 (Figure 4B), suggesting that this peak contains the exosome fraction of the CM. Notably, MYC protein was not detected in any of the exosome fractions.

Cardioprotection by CMSC3A1 exosomes

HPLC-purified exosomes from either E1MYC16.3 or CMSC3A1 was administered intravenously to the mouse model of myocardial ischemia-reperfusion injury at a 0.3 µg per mouse. The area at risk (AAR) as a percentage of left ventricular (LV) area in CMSC3A1

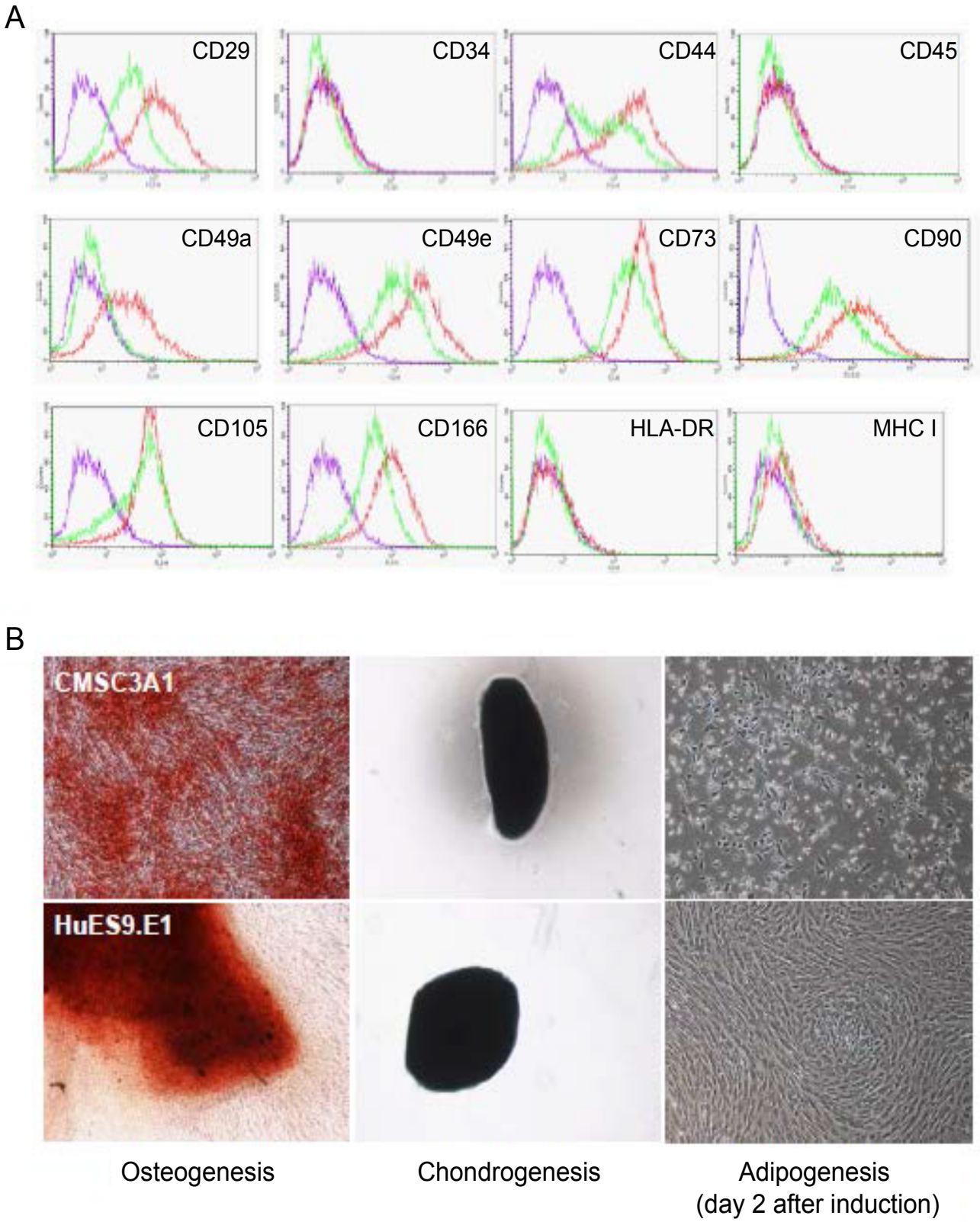

Figure 2: Surface antigen profiling and differentiation potential. (A) CMSC3A1 (green) and HuES9.E1 (red) MSCswere stained with a specific antibody conjugated to a fluorescent dye and analyzed by flow cytometry. Nonspecific fluorescence (purple) was assessed by incubating the cells with isotype-matched mouse monoclonal antibodies. (B) HuES9.E1 and CMSC3A1 MSCs were induced to undergo (left panel) osteogenesis and then stained with von Kossa stain; (middle panel) chondrogenesis and then stained with Alcian blue; (right panel) adipogenesis where CMSC3A1 and HuES9.E1 MSCs were exposed to adipogenesis induction medium for two days.

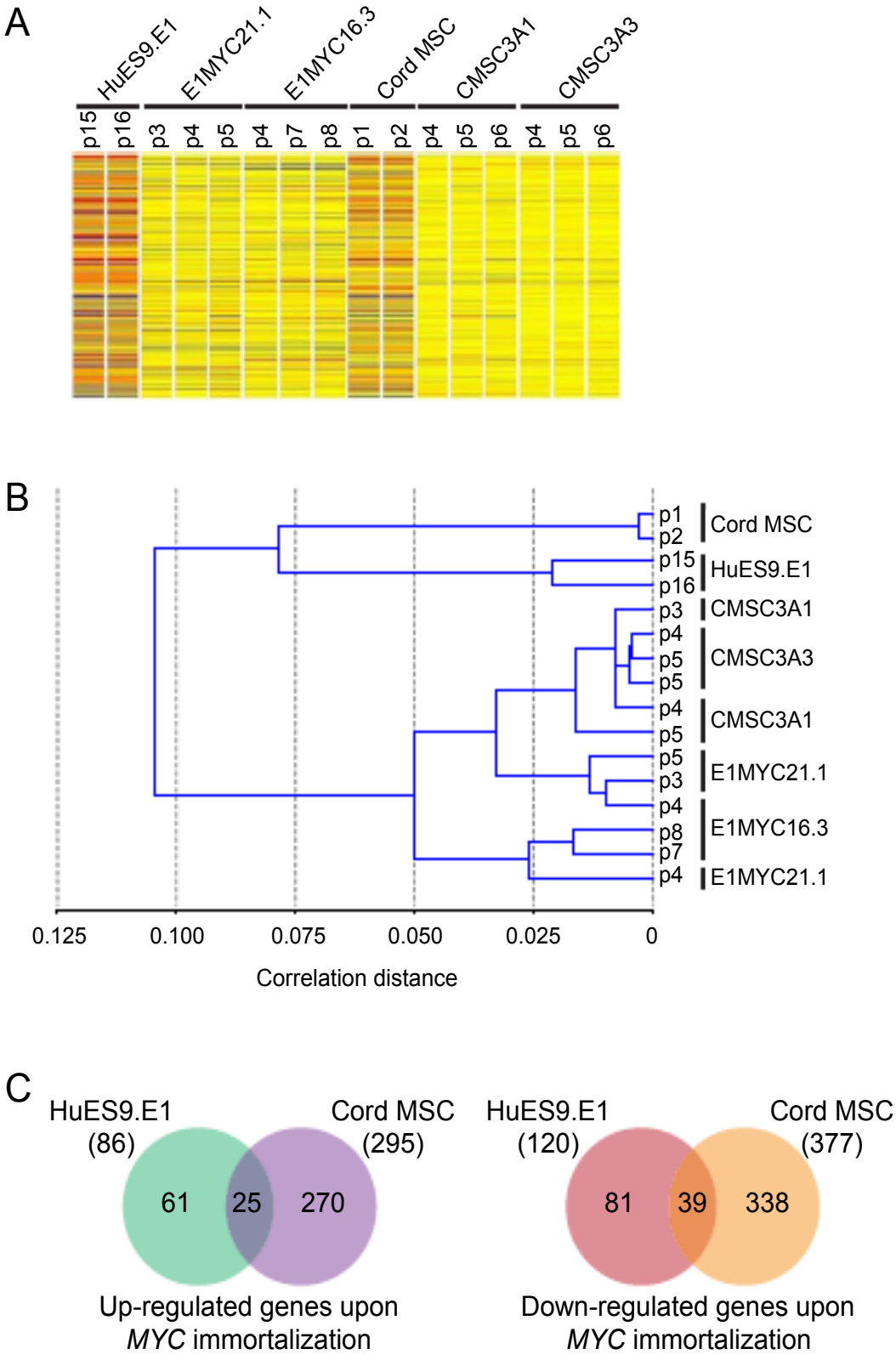

Figure 3: Gene expression profile of MSCs and their *MYC*-transformed progenies. (A) A heat map of the gene expression profiles of hESC- and cord-derived MSCs and their *MYC*-transformed progenies at different passage numbers. (B) Hierarchical clustering of expressed genes by the various MSC lines. (C) Distribution of up674 regulated (left panel) and down-regulated (right panel) genes upon *MYC* immortalization of HuES9.E1 and cord MSCs, represented by Venn diagrams. Fold change > 2.0.

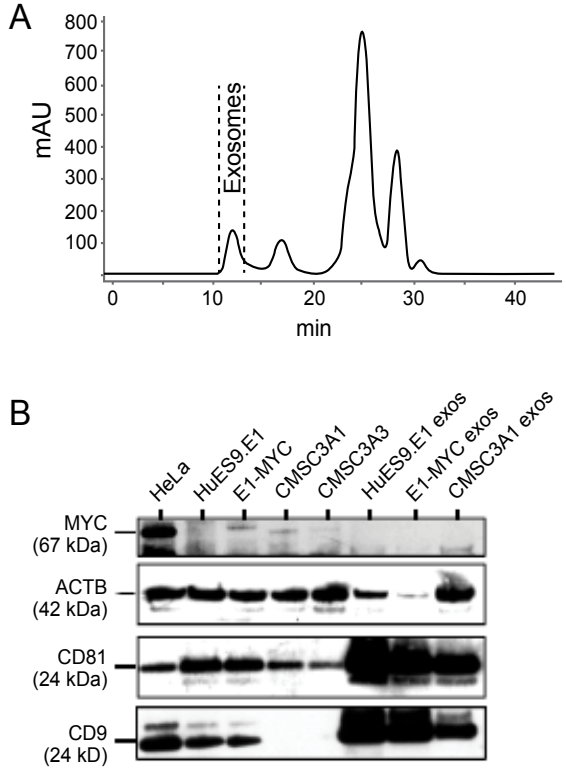

Figure 4: Analysis of secretion. (A) HPLC fractionation of CMSC3A1 conditioned medium. CMSC3A1 conditioned medium was fractionated on a HPLC using BioSep S4000, 7.8 mm x 30 cm column. The components in CM were eluted with 20 mM phosphate buffer with 150 mM of NaCl at pH 7.2. The elution mode was isocratic and the run time was 40 minutes. The eluent was monitored for UV absorbance at 220 ηm. Each eluting peak was then analyzed by light scattering. The fastest eluting peak was labelled as the exosome fraction and collected for subsequent analysis. (B) Western blot analysis. Proteins from the lysates of the various MSC lines as well as their HPLC-686 purified exosomes were separated on SDS-PAGE and probed with anti-MYC, anti-ACTB, anti-CD81 and anti- CD9 antibodies.

exosome, E1MYC16.3 exosome or the saline-treated control group sectioned, stained and measured as previously reported for E1MYC 16.3 was similar (Figure 5A). The relative infarct size (IS/AAR) in mice treated with E1MYC16.3 exosomes or CMSC3A1 exosomes was 22.6 ± 4.5%, and 19.8 ± 2.9%, respectively and their relative infarct sizes were significantly lower than the relative infarct size of 38.5 ± 5.6% in saline-treated mice (P<0.002 and P<0.001, respectively) (Figure 5B).

Discussion

Human cord-MSCs, like human ESC derived-MSCs could be immortalized by over expression of *MYC* gene to increase telomerase activity, enhance rate of proliferation and bypass senescence. The immortalized MSCs retained many of the MSC characteristics. Their genome-wide gene expression profile was highly similar to that of their parental cells with a correlation coefficient of 0.92. However, we observed this gene expression profile was more similar to that of *MYC*-immortalized ESC-MSCs with correlation coefficient of 0.95. The immortalized cells also have the characteristic MSC surface antigen profile: $CD29^+$, $CD44^+$, $CD49a^+$ $CD49e^+$, $CD105^+$, CD166+, MHC I^-, HLA-DR^-, $CD34^-$ and $CD45^-$. However, in contrast to a previous report that observed no fundamental changes in MSC properties after *MYC* immortalization [86], several MSC features were altered. These alteration included a reduced adherence to plastic and a failure to undergo adipogenesis, and were similarly observed in ESC-MSCs after *MYC* immortalization [81]. Notwithstanding these changes, the *MYC*-immortalized cord MSCs like the *MYC*-immortalized ESC-MSCs retained a normal karyotype and the potential albeit limited to differentiate, suggesting a non-tumorigenic phenotype. Indeed, *MYC*-immortalized ESC-MSCs failed to engraft when transplanted in immune compromised mice (unpublished data). In addition, *MYC*-immortalized cord MSCs also secreted exosomes that were equally efficacious as those from *MYC*-immortalized ESC MSCs and could reduce infarct size in a mouse model of ischemia/reperfusion injury [81]. As ESC-MSC exosome-mediated reduction in infarct size was previously shown to correlate with an improvement in cardiac function, ATP/ADP and NADH/NAD ratios, immune cell infiltration and survival signalling [78], the similar reduction mediated by exosomes from *MYC*-immortalized cord MSCs implied that they have similar efficacy in restoring cardiac function.

The most notable difference between the *MYC*-immortalized cord- and ESC-MSCs was the exosome yield. A liter of culture medium conditioned by *MYC*-immortalized cord MSCs yielded 177 µg exosomes while that conditioned by *MYC*-immortalized ESC MSCs yielded 1282 µg exosomes. This exosome yield for *MYC*-immortalized cord- MSCs (177 µg per liter) was even lower than that of non-immortalized ESC-MSCs and fetal-MSCs which produced ~500 and ~800 µg per liter of conditioned medium respectively (unpublished data) but was comparable to *MYC*-immortalized adult bone marrow-MSCs (unpublished data). We generally observed that after *MYC* immortalization, MSCs produced more exosomes and we attributed this to the smaller cell size of the immortalized MSCs which resulted in

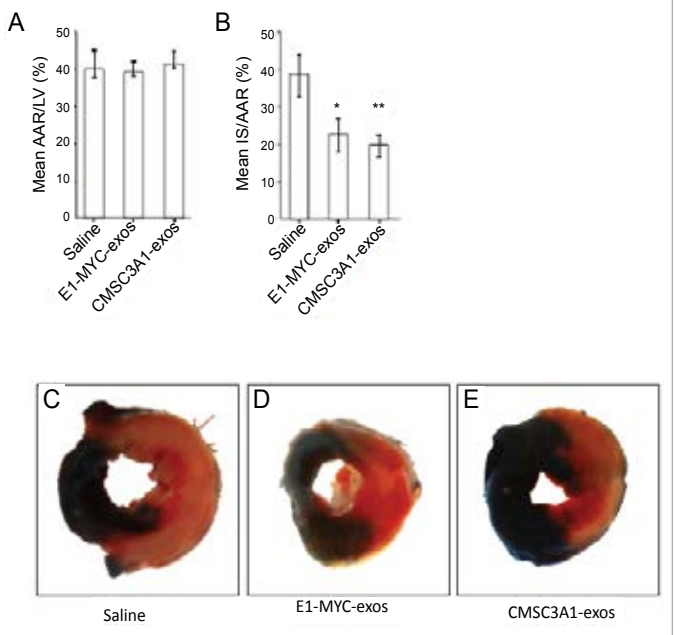

Figure 5: Cardioprotection by CMSC3A1 exosomes. 0.3 µg HPLC-purified exosomes from either E1MYC16.3 or CMSC3A1 was administered intravenously to a mouse model of acute myocardial/ischemia reperfusion injury five minutes before reperfusion. Infarct size (IS) as a percentage of the area at risk (AAR) upon treatment with saline (n = 10), E1MYC16.3 exosomes (n = 4) and CMSC3A1 (n = 4) were measured (A,B). (*, P < 0.002 and **, P < 0.001). (C–E) Representative pictures of Evan blue (blue) and TTC (pink) staining on hearts of mice treated with (C) saline, (D) E1-MYC-exos, or (E) CMSC3A1-exos.

a higher cell density for each confluent culture. The inverse correlation between the exosome production with developmental maturity of the donor tissues supported the correlation between therapeutic efficacy of MSC secretion and therefore MSCs with the "youthness" of the donor. It is possible that the composition of the exosomes from MSCs of donors at different developmental stages is different. However, this difference in composition is not likely to contribute to the efficacy of the exosomes from MSCs of different donors as we observed that similar efficacy was achieved with the same exosome dosage. We therefore propose that the correlation between MSC therapeutic efficacy and developmental stage of the donor is underpinned by exosome production.

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