Probable impact of age and hypoxia on proliferation and microRNA expression profile of bone marrow-derived human mesenchymal stem cells

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Decline in the therapeutic potential of bone marrow-derived mesenchymal stem cells (MSC) is often seen with older donors as compared to young. Although hypoxia is known as an approach to improve the therapeutic potential of MSC in term of cell proliferation and differentiation capacity, its effects on MSC from aged donors have not been well studied. To evaluate the influence of hypoxia on different age groups, MSC from young (<30 years) and aged (>60 years) donors were expanded under hypoxic (5% O2) and normal (20% O2) culture conditions. MSC from old donors exhibited a reduction in proliferation rate and differentiation potential together with the accumulation of senescence features compared to that of young donors. However, MSC cultured under hypoxic condition showed enhanced self-renewing and proliferation capacity in both age groups as compared to normal condition. Bioinformatic analysis of the gene ontology (GO) and KEGG pathway under hypoxic culture condition identified hypoxia-inducible miRNAs that were found to target transcriptional activity leading to enhanced cell proliferation, migration as well as decrease in growth arrest and apoptosis through the activation of multiple signaling pathways. Overall, differentially expressed miRNA provided additional information to describe the biological changes of young and aged MSCs expansion under hypoxic culture condition at the molecular level. Based on our findings, the therapeutic potential hierarchy of MSC according to donor's age group and culture conditions can be categorized in the following order: young (hypoxia)> young (normoxia) > old aged (hypoxia) > old aged (normoxia).

1   **Probable impact of age and hypoxia on proliferation and microRNA expression profile of**

2   **bone marrow-derived human mesenchymal stem cells**

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23

24   **ABSTRACT**

25 Decline in the therapeutic potential of bone marrow-derived mesenchymal stem cells (MSC) is

26 often seen with older donors as compared to young. Although hypoxia is known as an approach

27 to improve the therapeutic potential of MSC in term of cell proliferation and differentiation

28 capacity, its effects on MSC from aged donors have not been well studied. To evaluate the

29 influence of hypoxia on different age groups, MSC from young (<30 years) and aged (>60 years)

30 donors were expanded under hypoxic (5% O_2) and normal (20% O_2) culture conditions. MSC

31 from old donors exhibited a reduction in proliferation rate and differentiation potential together

32 with the accumulation of senescence features compared to that of young donors. However, MSC

33 cultured under hypoxic condition showed enhanced self-renewing and proliferation capacity in

34 both age groups as compared to normal condition. Bioinformatic analysis of the gene ontology

35 (GO) and KEGG pathway under hypoxic culture condition identified hypoxia-inducible miRNAs

36 that were found to target transcriptional activity leading to enhanced cell proliferation, migration

37 as well as decrease in growth arrest and apoptosis through the activation of multiple signaling

38 pathways. Overall, differentially expressed miRNA provided additional information to describe

39 the biological changes of young and aged MSCs expansion under hypoxic culture condition at

40 the molecular level. Based on our findings, the therapeutic potential hierarchy of MSC according

41 to donor's age group and culture conditions can be categorized in the following order: young

42 (hypoxia)> young (normoxia) > old aged (hypoxia) > old aged (normoxia).

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44

45 **INTRODUCTION**

46   Mesenchymal stem cells derived from bone marrow (BM-MSCs) are adult multipotent

47 stem cells with the self-renewing capacity and the ability to differentiate into cells of various

48 connective tissue lineages. They are regarded as a promising and potential alternative source in

49 the repair of many cells and tissues due to its multilineage differentiation capability into not only

50 mesoderm but also ectodermic and endodermic lineages such as osteoblasts, chondrocytes,

51 adipocytes, neurocytes and myoblasts (Wei et al. 2013; Zhou et al. 2008). MSCs are clinically

52 used in engraftment of post-transplantation or as gene therapy vehicles in osteogenesis

53 imperfecta because of their immunosuppressive capacity and are widely used in the treatment of

54 cardiac disorder, musculoskeletal and cancer (Baxter et al. 2004; Mognetti et al. 2013;

55 Mohyeldin et al. 2010; Oliveira et al. 2012). Outstanding features of MSC are that it can be

56 easily obtained from various sources of adult tissue (adipose and bone-marrow) and postnatal

57 tissues (Wharton-jelly and umbilical cord) and can be expanded *in vitro* (Oliveira et al. 2012;

58 Samsonraj et al. 2013).

59   The challenge for MSC-therapy is that it requires high yield and good quality of stem

60 cells. In order to get sufficient yield, stem cells need to be expanded under prolonged passage,

61 which can lead to deterioration of its self-renewal and differentiation capacity. Prolonged

62 passage has been reported to be directly linked with the shortening of telomere length that leads

63 to decrease in cell proliferation and increase of senescence (Samsonraj et al. 2013). Age is

64 another factor that is associated with progressive loss of cell proliferation resulting in cellular

65 senescence. Biological markers of cellular senescence were highly expressed in MSC from aged

66 donors along with oxidative damage indicators (Reactive oxygen species (ROS) and nitric oxide)

67 (Stenderup et al. 2003; Stolzing et al. 2008). The acceleration of senescence was related with the

68 decrease of cell proliferation and their life span as well as contributing to the accumulation of

69 DNA damage leading to stem cell exhaustion (Rube et al. 2011). These marked the compromised

70 quality of MSCs with age as well as prolonged passage under normal condition. Despite that, in

71   the case of autologous MSC transplantation for severe autoimmune diseases and old age related

72   diseases in aged patients, the use of MSC of aged donor is still highly in demand (Wang et al.

73   2013; Wei et al. 2013).

74   Alternatively, low oxygen content (5% O_2), referred as hypoxia, is regarded as a

75   characteristic feature of their microenvironment *in vivo* that provides microenvironmental

76   stresses to stem cells, allowing them to escape senescence pathways resulting in an increased

77   proliferation rate (Efimenko et al. 2011). Hypoxia is known to stimulate pro-angiogenic effects

78   in stem cells while maintaining the telomere length (Efimenko et al. 2011; Oliveira et al. 2012).

79   Most of our knowledge concerning MSC's differentiation and proliferation potential reduction

80   by age had been obtained from cell cultured under normal oxygen as well as hypoxia conditions.

81   Although the hypoxic condition has been demonstrated as a mode to improve the therapeutic

82   potential of MSC (Cicione et al. 2013; Guan et al. 2012; Peterson et al. 2011; Rosova et al. 2008),

83   little is known about its effect on a specific age group, especially in the involvement and the

84   interplay of miRNAs in the proliferation and renewal properties. As an important class of

85   regulatory noncoding RNAs, miRNAs have been shown to play important roles in the committed

86   differentiation and self-renewal of embryonic stem cells and adult stem cells (Huang et al. 2010).

87   Interestingly, the miRNA expression patterns in MSC when subjected to various factors and

88   culture conditions such as hypoxia and serum deprivation differ considerably (Nie et al. 2011).

89   By preventing the translation of mRNAs, miRNAs are predicted to regulate almost all cell's

90   biological processes, ranging from cell renewal, differentiation, migration, tumorigenesis to cell

91   death, aging and senescence (Di Leva & Croce 2013; Nie et al. 2011; Tome et al. 2011).

92   In this work, we investigated the influence of donor's age on the miRNA expression

93 profiles of BM-MSCs expanded under hypoxic culture and discovered several miRNAs that may

94 potentially act as key regulators.

95

96 **MATERIALS AND METHODS**

97 **Hypoxic culture of human BM-derived MSCs**

98 The primary BM-MSC collection was obtained from Cryo Cord Sdn Bhd and the research

99 approved by the UTAR Scientific and Ethical Review Committee (U/SERC/14/2012), in

100 compliance with international guidelines regarding the use of primary bone marrow stem cell for

101 research and signed consent were obtained prior to study. BM-derived MSC was prepared as

102 described previously (Efimenko et al. 2011). Six subjects (F/M = 4/2/; Age = 19-80 years old)

103 are with no metabolic disease, inherited diseases and other diseases which may affect the current

104 study. MSCs from young ($\leq$30 years old) and aged ($\geq$60 years old) donors were (n=3/group)

105 maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma, USA) supplemented with

106 10% fetal bovine serum (GIBCO, USA) at 37°C, in 5% CO_2 and 20% O_2. Hypoxic (Hx)

107 condition was attained by culturing cells in a gas mixture composed of 94% N_2, 5% CO_2, and 5%

108 O_2 (Ueyama et al. 2012). Cumulative population doubling (CPD) was counted using Typan blue

109 assay. The population doubling was determined using the formula x = log2 (N2)/(N1)/log2,

110 where N2 is the number of harvested cells, and N1 is the number of seeded cells. To calculate the

111 CPD, population doubling in each passage was determined and compared with the population

112 doubling of earlier passages (Stolzing et al. 2008).

113

114 **MSC characterizations**

115 *Immunophenotyping of MSCs*

116 Identification of MSCs was performed using CD90, CD44, CD105 and CD19 MSC surface

117 markers from Flow Cellect kit (Merck, USA). Cells were incubated with 18 μL of antibody

118 working cocktail solution (Anti-human CD105-conjugated with FITC, anti-human CD90-

119 conjugated with PE, anti-human CD44-conjugated with Alexa fluor 647, anti-human CD19-

120 conjugated with PECy5) for 30 minutes in the dark at 4°C and acquired using a BD FACS Canto

121 TM II Flow Cytometer (Becton Dickinson, USA).

122

123 *Multipotent differentiation*

124 The multipotency of MSCs were evaluated using adipogenic and osteogenic assays (Pandey et al.

125 2011). Both MSC groups expanded under normoxic and hypoxic conditions were cultured in

126 low-glucose DMEM containing 10% fetal bovine serum, antibiotics, 100 U/ml penicillin and 100

127 μg/ml streptomycin and 2 mM L-glutamine until 90% confluent. For in vitro differentiation into

128 osteoblasts, cells were induced with osteogenic induction medium (OIM), composed of DMEM

129 supplemented with 10% FBS, 50 μg/mL ascorbate-2 phosphate, 10^8M dexamethasone, and

130 10mM β-glycerophosphate. For in vitro differentiation into adipocytes, cells were induced with

131 adipogenic induction medium (AIM), composed of DMEM supplemented with 10% FBS, 50

132 μg/mL ascorbate-2 phosphate, 10^7 M dexamethasone, 50 μM indomethacin, 0.45 mM 3-isobutyl-

133 1-methyl- xanthine (IBMX) and 10 μg/mL insulin. Change of medium was performed every 3

134 days until 21 days when the matrix mineralization and lipid droplets were fully formed.

135

136 *β-galactosidase staining*

137   Confluent cells were fixed with 2% formaldehyde for 5 minutes and incubated with senescence-

138   associated β-Gal (SA-β-Gal) chromogenic substrate solution (Cell Signaling Technology, USA).

139   The mean percentage of cells expressing β-galactosidase was quantified using ImageJ with an

140   average of 200 cells.

141

142   **Next Generation Sequencing (NGS) of miRNA**

143   Total RNAs including small RNAs were isolated from cells at passage 15 using a miRNeasy kit

144   (Qiagen, USA). The next generation sequencing (NGS) library was prepared using TruSeq small

145   RNA sample prep kit (Illumina, USA). Briefly, 3' and 5' RNAs adapters were ligated to small

146   RNAs molecules, reverse-transcribed and PCR amplified. Prior to amplification, indices for

147   sample multiplexing were incorporated. Samples in the same multiplex group were pooled and

148   run on a 6% SDS-PAGE and validated using an Agilent Technologies Bioanalyzer high

149   sensitivity DNA chip. Data were analyzed using CLC Genomic Workbench 7.0 and sequence

150   with length less than 17 or more than 27 were removed. miRNA sequences were aligned to

151   human genome (*Homo sapiens* GRCh 37.57) and known miRNA database (miRBase-19) of both

152   mature and miRNA precursors (Noren Hooten et al. 2013).

153

154   *Pathway Analysis*

155   Target genes were identified using a target prediction program miRDB. The identified genes

156   were further analyzed using the web-based DAVID Bioinformatic Resource functional

157   annotation tool (http://david.abcc.ncifcrf.gov/) for pathway analysis to identify the gene ontology

158   (GO) and significantly enriched pathways using the Kyoto Encyclopedia of Genes and Genomes

159   (KEGG).

160

161 *MicroRNAs quantification by Real-time PCR analysis*

162 Five miRNAs were selected to validate NGS data. cDNA synthesis and real-time qPCR were

163 performed with miRCURY LNA Universal RT microRNA PCR (Exiqon, Denmark). In brief,

164 RNAs were tailed with a poly (A) sequence at their 3'end and reverse-transcribed into cDNA

165 using a universal poly (T) primer with a 3'end degenerate anchor and a 5'end universal tag. The

166 cDNA products were quantified using SYBR green based real time PCR and locked nucleic acid

167 (LNA) enhanced miRNA specific primers. The qPCR analysis was run on a CFX96^{TM} touch

168 thermocycler (Biorad) (Jensen et al. 2011). Normalization was done using the average value of

169 miR-200a, miR-122-5p and miR-16 evaluated using geNorm algorithms. Primer sequences for

170 qPCR are listed in Table 1.

171

172 *Statistical analysis*

173 Data are expressed as means ± standard deviations. Statistical analyses were performed using

174 one-way analysis of variance (ANOVA), FDR correction and Duncan's post hoc test. The results

175 were taken to be significant at a probability level of p-values <0.05.

176

177 **RESULTS**

178 *Comparison of BM-MSC of young and aged donors under hypoxic and prolonged passage*

179 *conditions*

180 MSC surface marker expressions showed that CD105, CD90 and CD44 were strongly expressed

181 in young MSC whereas in aged MSC, the markers were present but the expression level was

182 slightly lower. Meanwhile, the expression of CD19 surface marker was not detectable on either

183 young or aged MSC (Fig. 1A) confirming that both groups exhibited phenotype common to

184   MSC. In normal oxygen condition, the proliferation rate of MSC remained relatively high during

185   low passage. As the number of passage increased, the proliferation rate of aged MSC declined

186   significantly. Meanwhile, MSCs subjected to hypoxic treatment exhibited relatively higher

187   proliferation rate in both groups. Overall, aged MSC had significantly lower cumulative

188   population doubling (CPD) compared to young MSC (Fig. 1B), which further showed a

189   comparatively reduced proliferation rate, resulting in growth arrest or early senescence. This was

190   further supported based on morphological assessment where young MSC displayed fibroblast-

191   like morphology with a long-spindle shape whereas substantial alteration of morphology such as

192   loss of their characteristic spindle-shaped morphology and increase in cytoplasmic volume and

193   size (spread-out and polygonal shape) in aged MSC (Fig. 1C). The positive staining of Alizarin

194   Red and Oil Red O confirmed the multilineage differentiation of hypoxic-treated MSCs into

195   osteocytes and adipocytes (Fig. 1D). A decline in the formation of calcium deposition and the

196   number of cells with lipid droplets in hypoxic conditions of MSC from aged donors compared to

197   the young donors was noticed indicating the reduction in their differentiation ability.

198   Differentiation ability was further confirmed with differentiation markers (aP2, adiponectin,

199   RUNX2 and osteopontin) using RT-PCR (Fig.1E). Subsequently, percentage of MSC positive

200   for SA-β-gal increased tremendously with age as a substantial expression of blue color SA-β-gal

201   positive cells were detected in aged MSC compared to young (Fig. 1F). Furthermore, cells

202   expanded under hypoxic condition had a lower senescence expression relative to the cells

203   expanded under normal condition.

204

205   ***miRNAs expression profiles in hypoxia-treated MSC***

206   To screen miRNAs that might be involved in hypoxia implicated cell fate commitment leading to

207   increase of cellular proliferation and differentiation potential, next gene sequencing using

208   Illumina platform was used to compare miRNA expression profiles of MSC between young and

209   aged donors (Fig. 2). On average, 3.2 million effective reads were obtained from young MSC

210   and 4.0 million effective reads from aged MSC with a quality score (>Q30) of 93.3%. The

211   average length of the detected sequences was 21.6 nucleotides after the removal of the 5' and 3'

212   adapter sequences. An average of 46.8% and 38.6% small RNAs were obtained from young and

213   aged MSC respectively using miRBase-19 database. Meanwhile, 53.2% and 61.4% small RNAs

214   were obtained from young and aged MSC respectively using *Homo sapiens* GRCh 37.57 library.

215   Approximately, 27-34% of the small RNAs matched with the known human miRNAs, and the

216   remaining unmatched sequences (65-72%) were classified as novel miRNAs. All the data has

217   been deposited in the geo@ncbi.nlm.nih.gov database (GEO accession number: GSE67630). The

218   distribution of miRNAs that are commonly upregulated and downregulated in young and old

219   MSC donors were illustrated in Venn-diagram (Figure 3 A, B). The overlap miRNAs from each

220   group might contribute to similar phenotype observed in the study. Relative distances between

221   the miRNA profiles of different age groups and hypoxic treatment populations were shown in

222   principal component analyses (PCA) plot (Figure 3C). A total of nine and two miRNAs with fold

223   change >2 were up-regulated in young and aged MSC respectively. Meanwhile, four and 31

224   miRNAs with fold change >2 were down-regulated in young and aged MSC respectively (Table

225   2).

226

227   ***Target genes, gene ontology (GO) and KEGG pathways of differentially expressed miRNAs***

228   To assess the possible biological impact of the differentially expressed miRNAs in MSC, the

229   predicted target genes of miRNAs (fold change>2, p>0.05) were analyzed. A total of 2,118 and

230   3,617 potential targets were identified through this process for differentially expressed miRNAs

231   in young and aged MSCs respectively. In young MSC, the putative target genes of known

232   miRNAs appeared to be involved in a broad range of GO analysis (biological processes) with

233   most of them related to transcriptional categories such as regulation of transcription and

234   inflammatory response to stress (GO:0043619, GO:0043618) (Table 4A), whereas in aged MSC,

235   negative regulation of epithelial to mesenchymal transition (GO:0010719) and differentiation

236   (GO:0010771) were among the top 10 most prominent terms (Table 4B).

237

238   The signaling pathways that were found to be involved in KEGG analysis of MSC from young

239   donors included ErbB signaling, melanogenesis, Wnt, MAPK and GnRH signaling pathways

240   (Table 5). Meanwhile, cell cycle, Wnt, p53 and calcium signaling pathway were the most

241   enriched pathways seen in MSC from aged donors. Cell cycle and p53-signaling pathways are

242   involved in replicative senescence leading to permanent growth arrest. Moreover, pathways

243   involved in various signal transduction and cell-cell interactions such as Wnt and MAPK

244   signaling pathways and others were significantly enriched in both young and aged MSC.

245

246   ***Quantitative RT-PCR validation of selected microRNA***

247   To validate miRNA expression changes in MSC after hypoxic treatment, we performed the RT-

248   qPCR of the five highly dysregulated miRNAs (miR-21, miR-34b, miR-210, miR-19b, miR-33a).

249   The miRNAs were selected for validation based on statistical significance (P<0.05) and their key

250   role in biological or signaling pathways altered in hypoxic treatment with respect to control

251   (normal) culture condition. As illustrated in Fig.4 and Supplementary Table 4, the NGS data

252   correlated well with the RT-qPCR results, indicating the reliability of sequencing based

253   expression analysis.

254

255   **DISCUSSION & CONCLUSIONS**

256   The latest profiling platform, NGS was used to generate miRNA expression profiles and further

257   elaborate the relationship of hypoxia-inducible miRNA in young and aged MSC. The

258   introduction of high throughput sequencing approaches has provided opportunities to generate

259   inclusive sequencing data for the identification and quantification of reproducible known and

260   novel miRNAs compared to previous approaches such as qPCR and microarray. In normal

261   culture condition of prolonged passage, aged MSC displayed a great reduction in viability

262   causing the cells to undergo morphological transformation, senescence and detachment leading

263   to cell death. This is in contrast with young MSC, which retained their MSC characteristics. This

264   finding is showing that MSC isolated from old donors tend to exhibit slower progress on their

265   proliferation, expansion and differential capacity relative to young MSC, similar to a previous

266   study reported by other researchers (Choudhery et al. 2014). Meanwhile, MSC cultured under

267   hypoxic condition showed enhanced self-renewal and proliferation capacity in both age groups

268   compared to normal condition. Nevertheless, young MSC performed better than the aged MSC.

269   Research on the roles of hypoxia-related miRNAs in age-related MSC will have great

270   significance on therapeutic applications of MSC. We therefore hypothesize that these 2 groups of

271   MSCs (young and old age) have distinct etiologies and could present potentially different

272   pathways that might involve and converge in a common biological processes particularly in the

273   proliferation and differentiation processes. Sequencing results revealed differential expression of

274   several hypoxia-inducible miRNAs in young MSC (Table 2). The up-regulation of miR-210,

275   miR-21 and miR-155 as well as down-regulation of let-7i and miR-33a, were among those that

276   were predicted to target mRNAs associated with transcriptional activity leading to enhanced cell

277   proliferation, survival and migration as well as a decrease in growth arrest and apoptosis

278   (Bruning et al. 2011; Clark et al. 2014). Meanwhile, miR-627 (negative regulator of

279   proliferation) and miR-193a (anti-apoptotic regulator) were significantly down-regulated in aged

280   MSC. Moreover, miR-193a was previously found to inhibit ErbB protein translation, which

281   subsequently inhibited cell proliferation and promoted apoptosis in tumor. ErbB signal is

282   important for stem cells development and maintenance (Liang et al. 2015). Our finding

283   demonstrated the involvement of hypoxia in inhibiting miR-627 and miR-193a expression in

284   aged MSC and consequently contributed to increase in proliferation activity. As a whole, this

285   study indicates the possible link of hypoxia-inducible miRNAs in regulating survival and self-

286   renewal, thereby improving the proliferation activity of young and aged MSCs relative to normal

287   oxygen culture condition.

288   Furthermore, it was shown that the age of donor's MSC played a role in the capacity to

289   differentiate into certain lineages. The reduction of adipocyte and osteocyte differentiation was

290   clearly seen in prolonged passage of aged MSC. Previous research reported that shortening of

291   telomere length might be the cause of reduction in differentiation and self-renewal capacity

292   (Zhou et al. 2008). The miRNAs identified in this study that were up-regulated by hypoxia (miR-

293   210, miR-29b) in young MSC and down-regulated (miR-196a, miR-148b) in aged MSC were

294   shown to be involved in promoting osteocytes lineage differentiation. Similarly, miRNAs that

295   were involved in adipocyte differentiation (miR-21) was found to be up-regulated in young and

296   down-regulated in aged MSC. A set of highly down-regulated miRNAs in aged MSC, notably

297   miR-29a and miR-29b (regulated chondrogenic differentiation, cartilage formation and neuronal

298   differentiation) and miR-561 (post-transcriptional regulation of mRNAs) were reported to be

299   associated with human embryonic and other stem cells (Duan et al. 2014; Guerit et al. 2014).

300   Induction and inhibition of these hypoxia-inducible miRNAs may explain their role in the

301   differentiation capacity of MSC. Based on the previous findings and our results, it is highly

302   probable that hypoxic conditioning may promote MSC survival via regulation of various

303   miRNAs.

304   Bioinformatic analysis and target prediction have been used as the main methods to

305   explore the function of miRNAs. The genes possibly regulated by hypoxia-inducible miRNAs

306   are involved in both tumorigenesis and stem cell maintenance such as cell survival,

307   differentiation, angiogenesis, apoptosis, cell cycle regulation, mitochondrial metabolism as well

308   as DNA damage repair (Huang et al. 2010; Kondo et al. 2001; Tsai et al. 2011). MiR-210 and

309   miR-21 were reported to regulate cell proliferation by targeting fibroblast growth factor receptor-

310   like 1 (FGFRL1) (Table 3) (Huang et al. 2010; Tsuchiya et al. 2011). MiR-155 was found to be

311   up-regulated in young MSC and inhibition of miR-155 in senescence cells was reported to cause

312   an elevated level of a protein involved in the TP53 growth arrest pathway (TP53INP1) (Table

313   5B) (Faraonio et al. 2012). Young MSC cultured under hypoxia which showed an up-regulation

314   of miR-155 was able to maintain its proliferative activity and has yet to reach the growth arrest

315   and senescence stage. The results indicated that hypoxia-inducible miRNAs are predicted to

316   target mRNAs and transcriptional activity leading to enhanced cell proliferation and migration as

317   well as a decrease in growth arrest and apoptosis which may serve as a clinical marker for the

318   proliferation and expansion in MSC.

319   A previous finding reported that adipogenic and osteogenic differentiation capacity of

320   MSC diminishes with age (Yu et al. 2011). MiR-210 and miR-29a have been identified as

321   positive regulators of osteocyte differentiation by inhibiting the TGF-beta/activin signaling

322   pathway through the inhibition of the target gene AcvR1b in MSC (He et al. 2013). In our

323   studies, a similar trend could be observed where miR-210 was predicted to target the putative

324   gene TGF-beta that might be involved in promoting osteogenesis and adipogenesis in young

325   MSC. Meanwhile miR-29a was highly down-regulated in aged MSC in which the differentiation

326   into osteocytes was shown to be inhibited.

327   Overall, significantly differentially expressed miRNAs in both young and aged MSCs

328   after hypoxic exposure were mostly shown to target putative genes (Table 3) involving

329   multilineage differentiation, proliferation as well as apoptosis (Table 4). Each differentially

330   expressed miRNAs (up-regulated and down-regulated) with hundreds of predicted target putative

331   genes that were linked with multiple GO and KEGG pathways were combined and hypoxia-

332   related miRNAs were shown and presumed to be the main drivers and regulators of proliferation

333   and differentiation favoring young MSC.

334   Here we reported the different profiles of hypoxia-inducible miRNA signatures between

335   young and aged MSCs, thereby providing additional information on possible link of miRNAs

336   associated with hypoxic condition that increased the proliferation of both young and aged MSCs.

337   These miRNAs can be further used as molecular markers for screening the quality of MSC. In

338   conclusion, our results showed that both young and aged MSCs cultured under hypoxia

339   performed better than normal condition. In this respect, hypoxia enhanced the self-renewal and

340   multi-potent differentiation potential of BM-MSC especially in aged (compromised) MSC. This

341   study not only provided further understanding on the likely effects of age in inducing biological

342　changes in MSC, but more importantly the molecular changes critical to the successful

343　application of MSC in regenerative medicine.

344

345　**ACKNOWLEDGEMENTS**

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MSC characterization

A) Immunophenotyping of BM-MSC from young and aged donors (n=3). Representative graphs were all positive for CD105, CD90, CD44 and negative for CD19; B) Cumulative population doubling (CPD) of MSC expanded under normal and hypoxic conditions at p15; C) BM-MSC morphology during culture expansion under normal and hypoxic at low passage (P5) and high passage (P15). Arrow indicates differentiated cells that have changed in size and morphology with evidence of cytoplasm spreading (n=3). (Magnification of x10 scale bar =100 μm); D) Mesodermal differentiation of young and aged MSCs post exposure to hypoxia. Cells from young and aged MSCs (p15) were cultured in standard medium (control), osteogenic induction medium and adipogenic induction medium respectively. (Magnification of x20 scale bar =100 μm); E) Representative reverse-transcription polymerase chain reaction run on 2% of agarose gel showing expression of aP2 and adiponectin by adipocytes and RUNX2 and osteopontin by osteocytes cells, induced from human bone-marrow mesenchymal stem cells. Control cells without induction medium treatment either did not show any expression or showed lower level of expression of the above markers than the differentiated cells; F) Senescence-associated β-Gal activity in MSC of normal vs Hypoxic at p15. Results are considered as significantly changed when *$p< 0.05$ using one-way ANOVA.
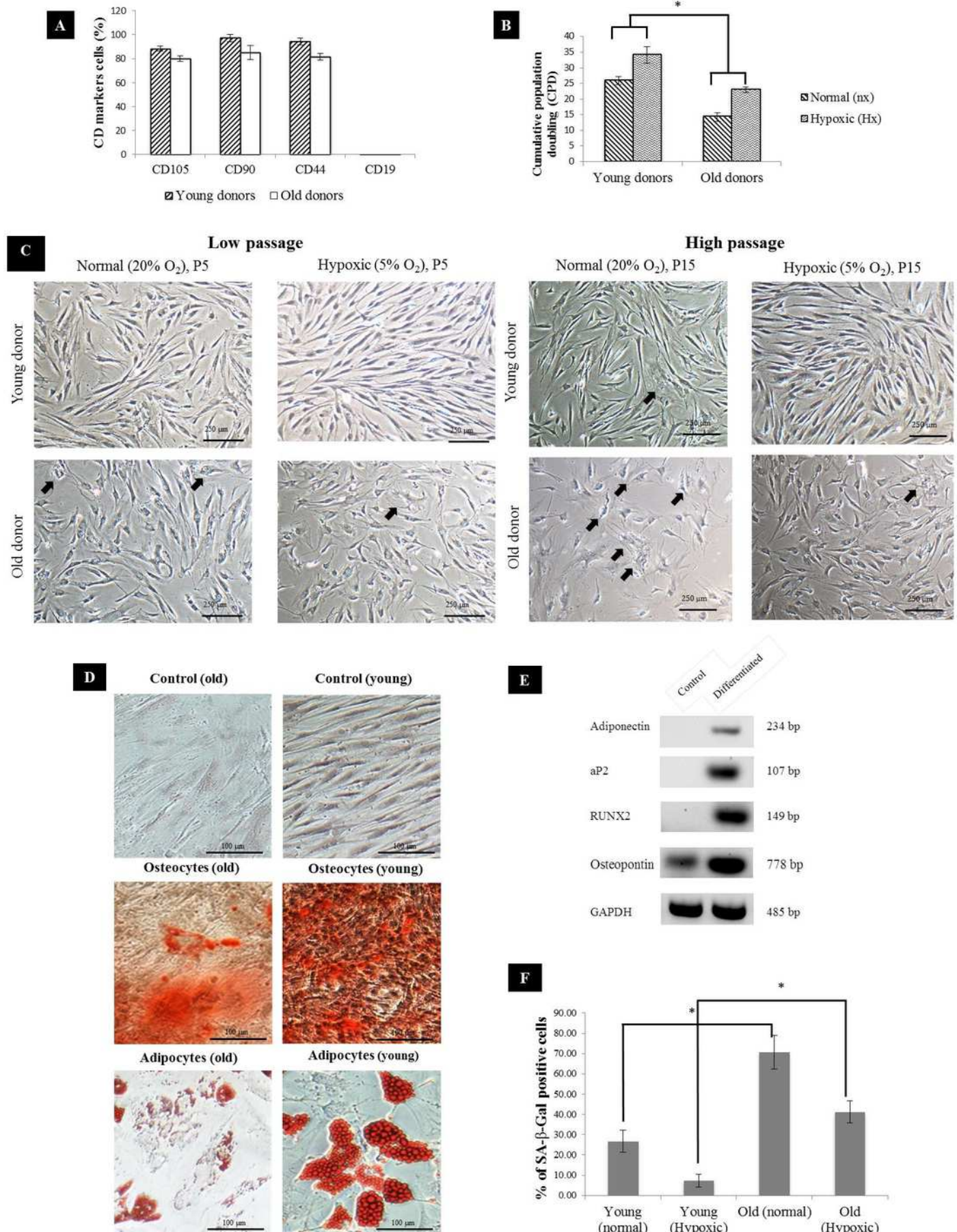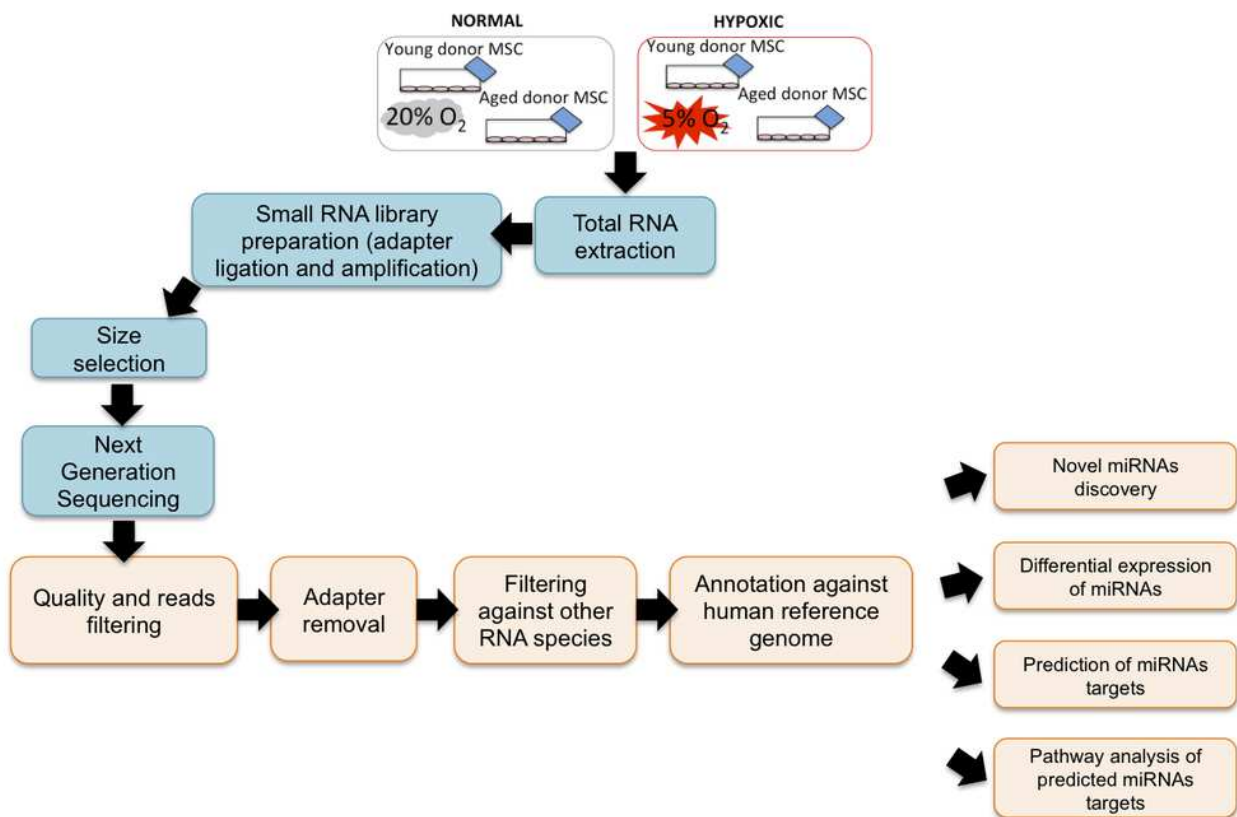
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NGS Workflow

Figure 2: Workflow of a miRNA next generation sequencing procedure and data mining.

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Venn-diagram and PCA plot

Figure 3: A) Venn-diagram illustrating groups of miRs that are commonly upregulated and downregulated in young donors, B) Venn-diagram illustrating groups of miRs that are commonly upregulated and downregulated in old donors, C) Principal component analyses indicating the relative distances between the miRNA profiles of different age groups and hypoxic treatment populations.

Young vs old donors MSC cultured under normoxia

Young vs old donors MSC cultured under hypoxia
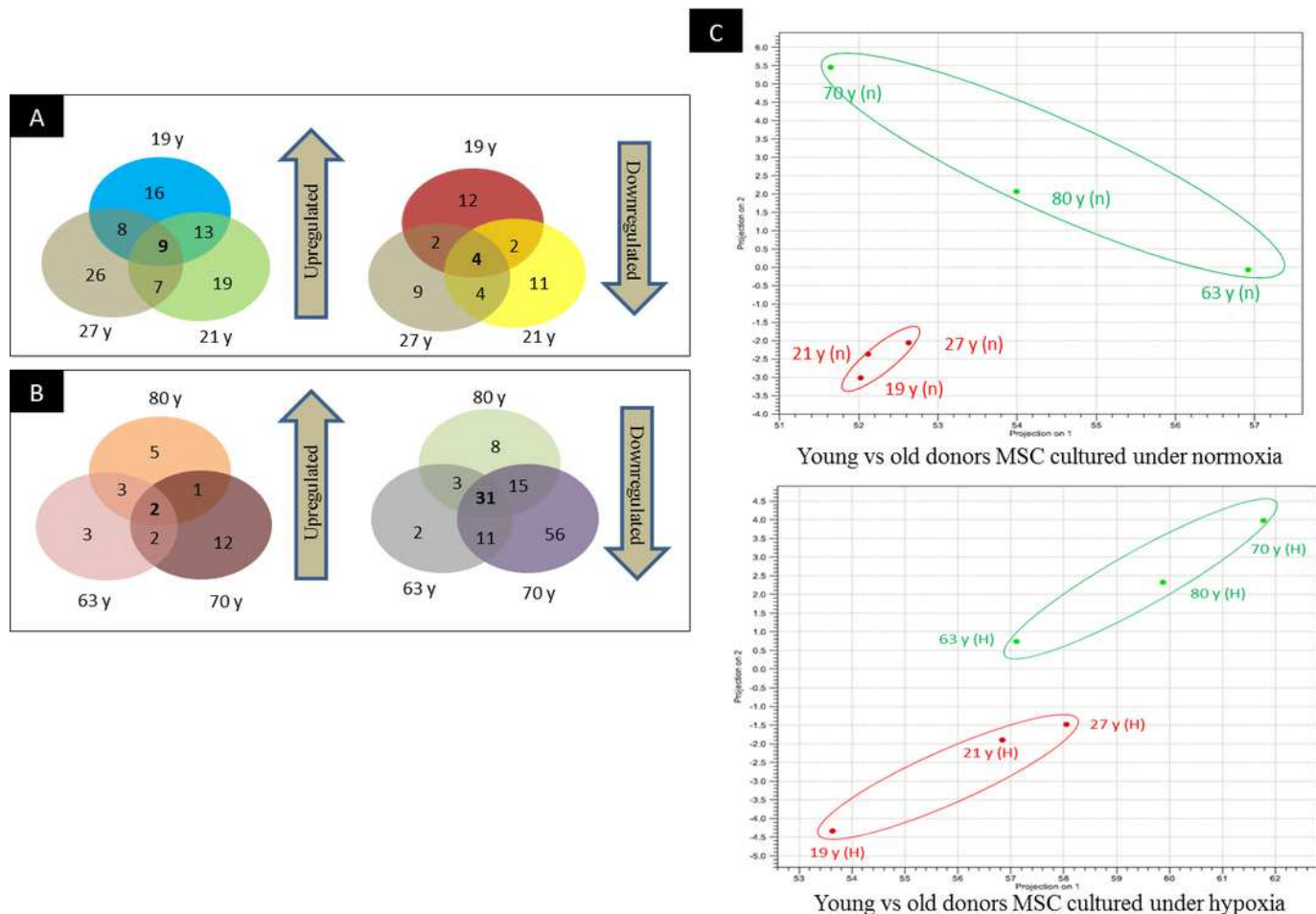
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qPCR validation and correlation plot

Figure 4: Reproducibility of NGS and comparison of miRNA expression between NGS and qPCR analysis. A) qPCR analysis in MSC of young and old age donors, B) Correlation plot comparing the Fold change values of qPCR with the log2 of the NGS in young donors, C) Correlation plot comparing the Fold change values of qPCR with the log2 of the NGS in old donors. Corresponding R2 values were determined by linear regression analysis.
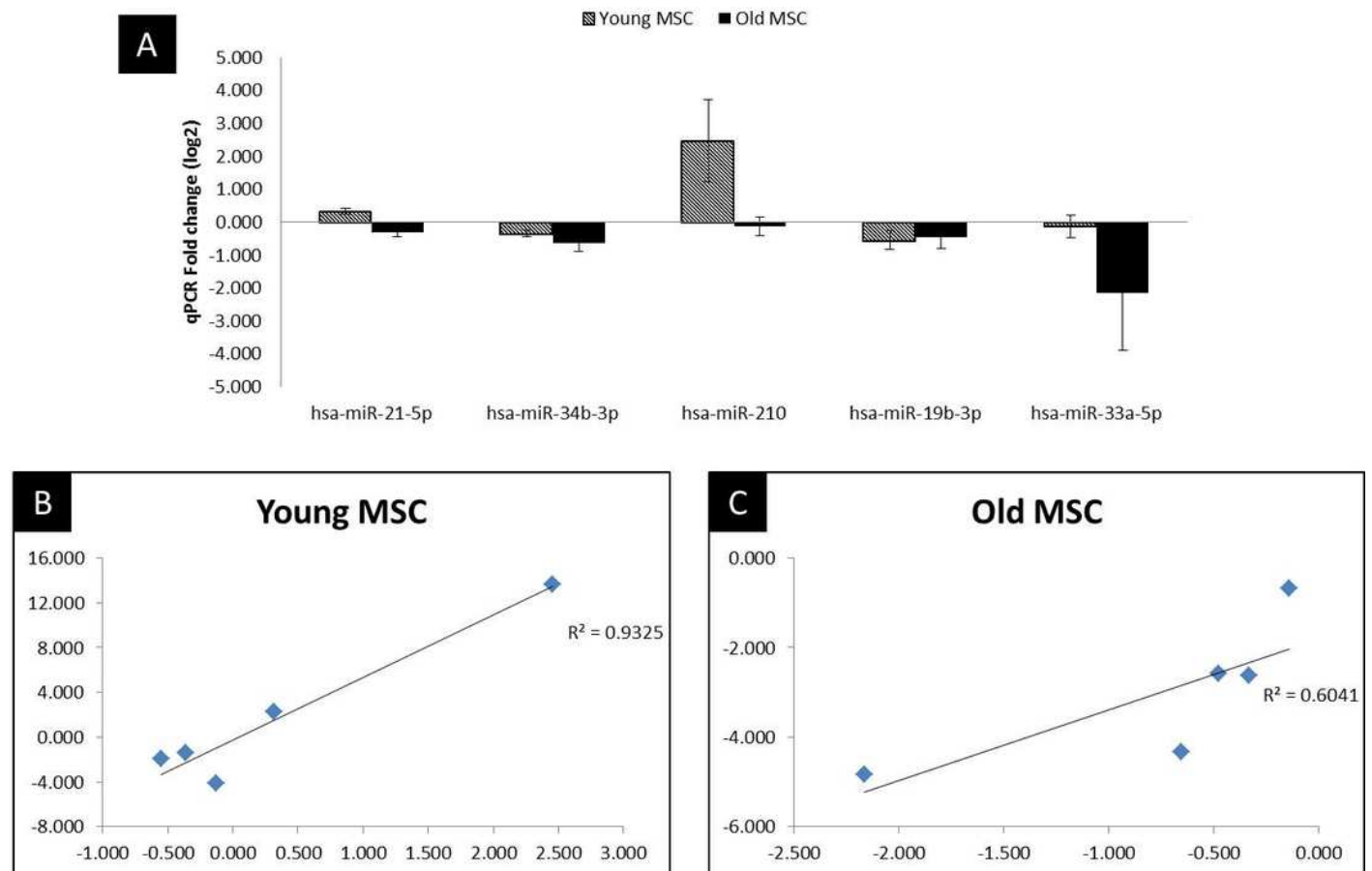
Table 1(on next page)

Table 1

The accession number and target sequence of the primers used in the quantitative real-time PCR assay.

1   **Table 1.** The accession number and target sequence of the primers used in the quantitative real-

2   time PCR assay.

Name	Accession number	Target Sequence
hsa-miR-200a-3p (Reference)	MIMAT0000682	UAACACUGUCUGGUAACGAUGU
hsa-miR-122-5p (Reference)	MIMAT0000421	UGGAGUGUGACAAUGGUGUUUG
hsa-miR-34b-3p	MIMAT0004676	CAAUCACUAACUCCACUGCCAU
hsa-miR-210	MIMAT0000267	CUGUGCGUGUGACAGCGGCUGA
hsa-miR-19b-3p	MIMAT0000074	UGUGCAAAUCCAUGCAAAACUGA
hsa-miR-33a-5p	MIMAT0000091	GUGCAUUGUAGUUGCAUUGCA
hsa-miR-21-5p	MIMAT0000076	UAGCUUAUCAGACUGAUGUUGA

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Table 2(on next page)

Table 2

List of differentially expressed miRNAs of MSC from young and aged donors cultured under hypoxic (Hx) culture condition relative to normal (nx) culture condition.

1 Table 2. List of differentially expressed miRNAs of MSC from young and aged donors cultured

2 under hypoxic (Hx) culture condition relative to normal (nx) culture condition.

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miRNA of young MSC	Fold change > 2 (Hx/nx)	p-value
mir-210	13.64	0.0119
mir-423	2.58	0.0105
mir-1468	2.30	0.0243
mir-21	2.26	0.0197
mir-3605	2.21	0.0061
mir-625	2.20	0.0709
mir-155	2.08	0.0071
mir-3065	2.07	0.0082
mir-138-1	2.06	0.0034
mir-424	-2.85	0.0073
let-7i	-3.22	0.0038
mir-655	-3.67	0.0323
mir-33a	-4.15	0.0182

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miRNA of aged MSC	Fold change > 2 (Hx/nx)	p-value
mir-7977	2.55	0.0350
mir-195	2.15	0.0061
mir-19b	-2.58	0.0280

mir-21	-2.61	0.0089
mir-99a	-2.74	0.0168
mir-708	-3.17	0.0030
mir-1185-1	-3.23	0.0048
mir-590	-3.24	0.0102
mir-455	-3.46	0.0039
mir-374a	-3.53	0.0315
mir-381	-3.53	0.0340
mir-126	-3.87	0.0019
mir-196a-2	-3.99	0.0340
mir-136	-4.03	0.0081
mir-100	-4.29	0.0062
mir-34b	-4.33	0.0159
mir-542	-4.35	0.0277
mir-376a-1	-4.35	0.0307
mir-1197	-4.53	0.0053
let-7f-2	-4.69	0.0045
mir-1228	-4.70	0.0091
mir-2355	-4.73	0.0143
mir-148b	-4.79	0.0729
mir-33a	-4.84	0.0295
mir-551b	-4.88	0.0013
mir-483	-6.29	0.0061

mir-29a	-6.40	0.0003
mir-378a	-6.56	0.0114
mir-376b	-8.12	0.0039
mir-561	-8.28	0.0006
mir-29b-1//mir-29b-2	-9.30	0.0340
mir-193a	-11.88	0.0315
mir-627	-15.00	0.0483

6   ($P<0.05$) with at least two-fold change

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Table 3(on next page)

Table 3

The top 5 of highly predicted target genes of differentially expressed miRNAs in young and

aged MSCs identified using target prediction program miRDB

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2   Table 3. The top 5 of highly predicted target genes of differentially expressed miRNAs in young

3   and aged MSCs identified using target prediction program miRDB

Gene symbol (Young MSC)	Gene description
IGF2	Insulin-like growth factor 2 (somatomedin A)
FGFRL1	Fibroblast growth factor receptor-like 1
PTPN21	Protein tyrosine phosphatase, non-receptor type 21
ISCU	Iron-sulfur cluster scaffold homolog (E. coli)
SLC25A26	Solute carrier family 25, member 26
Gene symbol (Aged MSC)	Gene description
FGF2	Fibroblast growth factor 2 (basic)
MTMR3	Myotubularin related protein 3
CASK	Calcium/calmodulin-dependent serine protein kinase (MAGUK family)
WEE1	WEE1 homolog (S. pombe)
CCNE1	Cyclin E1

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Table 4(on next page)

Table 4

The top 10 of highly enriched gene ontology (GO) terms of biological processes in predicted targets miRNAs (fold change > 2) of MSC from young and aged donors cultured under hypoxic (Hx) culture condition relative to normal (nx) culture condition. A) Top 10 of highly enriched GO (biological processes) in MSC from young donors, B) Top 10 of highly enriched GO (biological processes) in MSC from aged donors

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2 Table 4. The top 10 of highly enriched gene ontology (GO) terms of biological processes in

3 predicted targets miRNAs (fold change > 2) of MSC from young and aged donors cultured under

4 hypoxic (Hx) culture condition relative to normal (nx) culture condition.

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6 A) Top 10 of highly enriched GO (biological processes) in MSC from young donor

Function	p-value	Count	%	Fold enrichment
Regulation of transcription from RNA polymerase II promoter in response to oxidative stress GO:0043619	3.70E-02	3	0.2	9
Polyol transport GO:0015791	9.50E-03	4	0.3	8
Synaptic vesicle maturation GO:0016188	5.80E-02	3	0.2	7.2
Protein palmitoylation GO:0018345	5.80E-02	3	0.2	7.2
Healing during inflammatory response GO:0002246	5.80E-02	3	0.2	7.2
Regulation of transcription from RNA polymerase II promoter in response to stress GO:0043618	5.80E-02	3	0.2	7.2
Nuclear pore organization GO:0006999	5.80E-	3	0.2	7.2

Function	p-value	Count	%	Fold enrichment
	02			
Regulation of transcription in response to stress GO:0043620	5.80E-02	3	0.2	7.2
Definitive hemopoiesis GO:0060216	1.60E-02	4	0.3	6.9
Embryonic camera-type eye morphogenesis GO:0048596	2.30E-02	4	0.3	6

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8 B) Top 10 of highly enriched GO (biological processes) in MSC from aged donor

Function	p-value	Count	%	Fold enrichment
Negative regulation of epithelial to mesenchymal transition GO:0010719	4.00E-02	2	0.6	49.2
Voluntary musculoskeletal movement GO:0050882	4.00E-02	2	0.6	49.2
Negative regulation of cell morphogenesis involved in differentiation GO:0010771	6.00E-02	2	0.6	32.8
Epithelial cell maturation GO:0002070	9.70E-02	2	0.6	19.7
Regulation of calcium ion-dependent exocytosis GO:0017158	1.30E-02	3	0.8	16.4
Vesicle transport along microtubule GO:0047496	1.30E-02	3	0.8	16.4

Regulation of epithelial to mesenchymal transition GO:0010717	1.30E-02	3	0.8	16.4
Protein ubiquitination during ubiquitin-dependent protein catabolic process GO:0042787	4.50E-03	4	1.1	11.6
Cerebral cortex cell migration GO:0021795	3.20E-02	3	0.8	10.5
Bone resorption GO:0045453	3.60E-02	3	0.8	9.8

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Table 5(on next page)

Table 5

KEGG pathways enrichment of miRNA target predicted genes (fold change > 2) of MSC from young and aged donors cultured under hypoxic (Hx) culture condition relative to normal (nx) culture condition. A) KEGG pathways enrichment of miRNA target predicted genes of young MSC, B) KEGG pathways enrichment of miRNA target predicted genes of aged MSC.

1 Table 5. KEGG pathways enrichment of miRNA target predicted genes (fold change > 2) of

2 MSC from young and aged donors cultured under hypoxic (Hx) culture condition relative to

3 normal (nx) culture condition.

4 A) KEGG pathways enrichment of miRNA target predicted genes of young MSC

Pathways	Count	%	P-Value	Fold Enrichment	Benjamini	FDR
GnRH signaling pathway	17	1.1	1.30E-03	2.4	2.00E-01	1.50E+00
Melanogenesis	16	1	3.80E-03	2.3	2.00E-01	4.50E+00
Vascular smooth muscle contraction	16	1	1.20E-02	2	4.00E-01	1.40E+01
Type II diabetes mellitus	9	0.6	1.60E-02	2.7	4.30E-01	1.80E+01
Gap junction	13	0.9	2.20E-02	2.1	4.20E-01	2.40E+01
MAPK signaling pathway	29	1.9	2.20E-02	1.5	4.70E-01	2.30E+01
Wnt signaling pathway	18	1.2	3.70E-02	1.7	5.20E-01	3.70E+01
ErbB signaling pathway	12	0.8	4.20E-02	1.9	5.20E-01	4.10E+01
Adherens junction	11	0.7	4.40E-	2	5.00E-01	4.20E+01

			02			
Long-term potentiation	10	0.7	4.90E-02	2.1	5.10E-01	4.60E+01

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6   B) KEGG pathways enrichment of miRNA target predicted genes of aged MSC

Pathways	Count	%	P-Value	Fold Enrichment	Benjamini	FDR
Insulin signaling pathway	10	2.8	1.70E-03	3.6	1.90E-01	2.00E+00
Wnt signaling pathway	9	2.5	1.20E-02	2.9	3.10E-01	1.30E+01
p53 signaling pathway	6	1.7	1.30E-02	4.2	2.60E-01	1.40E+01
B cell receptor signaling pathway	6	1.7	1.90E-02	3.8	3.10E-01	2.00E+01
Cell cycle	7	2	4.40E-02	2.7	3.20E-01	4.00E+01
MAPK signaling pathway	11	3.1	4.80E-02	2	3.20E-01	4.30E+01
Calcium signaling pathway	8	2.2	7.10E-02	2.2	3.80E-01	5.70E+01
Type II diabetes mellitus	4	1.1	7.20E-	4.1	3.60E-	5.80E+0

			02		01	1
mTOR signaling pathway	4	1.1	9.10E-02	3.7	4.20E-01	6.70E+01

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