A comparative study of the capability of MSCs isolated from different human tissue sources to differentiate into neuronal stem cells and dopaminergic-like cells

Nidaa Ababneh [Corresp., Equal first author, 1] , Ban Al-Kurdi [Equal first author, 1] , Fatima Jamali [1] , Abdalla Awidi [1, 2, 3]

[1] Cell Therapy Center (CTC), the University of Jordan, Amman, Jordan, Amman, Jordan, Jordan

[2] 2Hemostasis and Thrombosis Laboratory, School of Medicine, the University of Jordan, Amman, Jordan., Amman, Jordan, Jordan

[3] Department of Hematology and Oncology, Jordan University Hospital, Amman, Jordan, Amman, Jordan, Jordan

Corresponding Author: Nidaa Ababneh
Email address: nidaaanwar@gmail.com

Background: Neurodegenerative diseases are characterized by progressive neuronal loss and degeneration. The regeneration of neurons is minimal and neurogenesis is limited only to specific parts of the brain. Several clinical trials have been conducted using Mesenchymal Stem Cells (MSCs) from different sources to establish their safety and efficacy for the treatment of several neurological disorders such as Parkinson's disease, multiple sclerosis and amyotrophic lateral sclerosis. **Aim:** The aim of this study was to provide a comparative view of the capabilities of MSCs, isolated from different human tissue sources to differentiate into neuronal stem cell-like cells (NSCs) and possibly into dopaminergic neural- like cells. **Methods:** Mesenchymal stem cells were isolated from human bone marrow, adipose, and Wharton's Jelly (WJ) tissue samples. Cells were characterized by flow cytometry for their ability to express the most common MSC markers. The differentiation potential was also assessed by differentiating them into osteogenic and adipogenic cell lineages. To evaluate the capacity of these cells to differentiate towards the neural stem cell-like lineage, cells were cultured in media containing small molecules. Cells were utilized for gene expression and immunofluorescence analysis at different time points. **Results:** Our results indicate that we have successfully isolated MSCs from bone marrow, adipose tissue, and Wharton's Jelly. WJ-MSCs showed a slightly higher proliferation rate after 72 hours compared to BM and AT derived MSCs. Gene expression of early neural stem cell markers revealed that WJ-MSCs had higher expression of Nestin and PAX6 compared to BM and AT-MSCs, in addition to LMX expression as an early dopaminergic neural marker. Immunofluorescence analysis also revealed that these cells successfully expressed SOX1, SOX2, Nestin, TUJ1, FOXA2 and TH. **Conclusion:** These results indicate that the protocol utilized has successfully differentiated BM, AT and WJ-MSCs into NSC-like cells. WJ-MSCs possess a higher potential

to transdifferentiate into NSC and dopaminergic-like cells. Thus, it might indicate that this protocol can be used to induce MSC into neuronal lineage, which provides an additional or alternative source of cells to be used in the neurological cell-based therapies.

1 **Title:** A comparative Study of the Capability of MSCs Isolated from Different
2 Human Tissue Sources to Differentiate into Neuronal Stem Cells and
3 Dopaminergic-Like Cells

5 **Running head: Mesenchymal Stem Cells Differentiation Potential into Neuronal Cells**

7 **Authors:**

8 Nidaa A. Ababneh[1,§,*], Ban Al-~~Kk~~urdi[1,§], Fatima Jamali[1], Abdalla Awidi[1,2,3,*]

10 **Affiliations**

11 [1]Cell Therapy Center (CTC), the University of Jordan, Amman, Jordan.

12 [2]Hemostasis and Thrombosis Laboratory, School of Medicine, the University of Jordan, Amman,
13 Jordan.

14 [3]Department of Hematology and Oncology, Jordan University Hospital, Amman, Jordan.

16 [§]These authors contributed equally to this work.

17 [*]Authors to whom correspondence should be addressed:

18 Nidaa A. Ababneh, Cell Therapy Center (CTC), the University of Jordan, Queen Rania Street,
19 Amman 11942, Jordan. *Email:* nidaaanwar@gmail.com; n.ababneh@ju.edu.jo

21 Abdalla Awidi, Cell Therapy Center (CTC), the University of Jordan, Queen Rania Street,
22 Amman 11942, Jordan. *Email*: abdalla.awidi@gmail.com

25   **Abstract:**

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28   specific parts of the brain. Several clinical trials have been conducted using Mesenchymal Stem
29   Cells (MSCs) from different sources to establish their safety and efficacy for the treatment of
30   several neurological disorders such as Parkinson's disease, multiple sclerosis and amyotrophic
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36   Wharton's Jelly (WJ) tissue samples. Cells were characterized by flow cytometry for their ability
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39   these cells to differentiate towards the neural stem cell-like lineage, cells were cultured in media
40   containing small molecules. Cells were utilized for gene expression and immunofluorescence
41   analysis at different time points.

42   **Results:** Our results indicate that we have successfully isolated MSCs from bone marrow,
43   adipose tissue, and Wharton's Jelly. WJ-MSCs showed a slightly higher proliferation rate after
44   72 hours compared to BM and AT derived MSCs. Gene expression of early neural stem cell
45   markers revealed that WJ-MSCs had higher expression of Nestin and PAX6 compared to BM
46   and AT-MSCs, in addition to LMX expression as an early dopaminergic neural marker.
47   Immunofluorescence analysis also revealed that these cells successfully expressed SOX1, SOX2,
48   Nestin, TUJ1, FOXA2 and TH.

49   **Conclusion:** These results indicate that the protocol utilized has successfully differentiated BM,
50   AT and WJ-MSCs into NSC-like cells. WJ-MSCs possess a higher potential to transdifferentiate
51   into NSC and dopaminergic-like cells. Thus, it might indicate that this protocol can be used to
52   induce MSC into neuronal lineage, which provides an additional or alternative source of cells to
53   be used in the neurological cell-based therapies.

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55   **Keywords:** Mesenchymal stem cells, differentiation, neural stem cells, dopaminergic neurons.

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1. Introduction:

Mesenchymal stem cells (MSCs) are a population of cells characterized by their ~~great~~ regenerative capacity and mutlipotent differentiation potential into multiple cell lineages (Ullah et al., 2015). These cells can be easily isolated from different tissue sources with minimal invasive procedures (Ullah et al., 2015). MSCs have been isolated from adipose tissue, bone marrow, Wharton jelly, dental pulp and umbilical cord blood. MSCs have the potential to differentiate into adipogenic, osteogenic and chondrogenic lineages. Some studies have reported the ability of these cells to cross lineage commitment and to differentiate into endodermal and ectodermal cell lineages (Marei et al., 2019; Orbay et al., 2012; Ullah et al., 2015). Additionally, MSCs are hypoimmunogenic and have immunosuppressive properties. All of these characteristics and the fact that MSCs are not burdened by any of the ethical issues or the tumorigenic capacity associated with embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), makes them an attractive choice for tissues engineering and cell replacement therapies (Marei et al., 2019; Musiał-Wysocka et al., 2019; Wang et al., 2018).

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the loss of dopaminergic neurons, resulting in an impairment of the motor function (Alexander G. E, 2004). The loss of these cells makes the PD an attractive model for cell replacement therapies. No specific treatment is currently available to treat PD patients. Surgical therapies as well as different pharmacological treatments have been utilized to relieve some of the PD symptoms (Alexander G. E, 2004; Dauer & Przedborski, 2003; Mu-Hui Fu, Chia-Ling Li, Hsiu-Lien Lin, Pei-Chun Chen, Marcus J. Calkins, Yu-Fan Chang, 2015). However, treatments usually fail after a while, due to the progressive nature of the disease. Searching for a more effective therapeutic strategy is essential to hinder the progression of dopaminergic neurons degeneration.

81 Several clinical trials have been conducted to assess the safety and efficacy of using MSCs for the

82 treatment of graft versus host disease, heart failure, bone and cartilage diseases, neurodegenerative

83 and spinal cord injuries (Musiał-Wysocka et al., 2019; Ul Hassan et al., 2009; Ullah et al., 2015).

84 Different differentiation protocols have been utilized to direct MSCs towards the neuronal lineage.

85 Cell culture media supplemented with FGF2, EGF, BMP-9, retinoic acid, and heparin have been

86 used to induce MSCs derived from adipose tissue to cholinergic and dopaminergic neuronal-like

87 cells (Marei et al., 2018). Additionally, the soluble factors sonic hedgehog (SHH), fibroblast

88 growth factor 8 (FGF8), and basic fibroblast growth factor (bFGF) along with final treatment with

89 BDNF neurotrophic factor have been successfully used to generate functional dopaminergic

90 neurons from WJ, ASC, UC and olfactory Mesenchymal Stem Cells (Boroujeni & Gardaneh,

91 2017; Khademizadeh et al., 2019; Ul Hassan et al., 2009; Yang et al., 2013). Choroid Plexus

92 Epithelial Cell, mesencephalic glial-cell, PA6 stromal cells-derived conditioned media have also

93 been used to induce dopaminergic differentiation on different stem cell types (Boroujeni et al.,

94 2017). Transduction of MSCs with transcription factors required for dopaminergic differentiation

95 such as LMX1, NTN and GDNF using lentiviral or retroviral vectors have been proved to be an

96 efficient way to enhance the differentiation potential of MSCs towards the dopaminergic lineage

97 (Barzilay et al., 2009; Ul Hassan et al., 2009; Yang et al., 2013).

98 Most of the previous studies have shown variations in differentiation potential of different types

99 of MSCs into neuronal lineage. Hence, the aim of this study was to assess and compare the neural

100 dopaminergic differentiation capacity of MSCs isolated from adipose tissue, bone marrow, and

101 Wartons' jelly. Such findings might assist in choosing the appropriate cell source to be utilized in

102 cell replacement and neural regenerative therapies.

103

104 **2. Materials and Methods:**

105 **2.1 Isolation and characterization of MSCs from different tissue sources**

106 This study was conducted after obtaining an International Review Board (IRB/7/2019) approval

107 at the University of Jordan/Cell Therapy Center (CTC). Samples were collected after all donors

108 gave written informed consents.

109 Six samples from each of the following tissue sources were used to isolate MSCs: Adipose tissue,

110 Bone marrow and Warton Jelly tissue. The isolation of stem cells was performed according to the

111 protocols utilized by following protocols, respectively (Bunnell et al., 2008; Gnecchi & Melo,

112 2009; Ranjbaran et al., 2018). The isolated cells obtained from these tissues were cultured in

113 Minimum Essential Medium alpha (α-MEM, Gibco) supplemented with 5% pooled human platelet

114 lysate (hPL), 1% Antibiotic-Antimycotic (Gibco) and 1% Glutamax (Gibco). Cells at 70%-80%

115 confluence were expanded until passages 1-3. Then cells were either used for further experiments

116 or stored in liquid nitrogen.

117 **2.2 Characterization of MSCs**

118 **2.2.1 Flow cytometry**

119 Cells at passage 3 and 70% confluency were utilized for MSC surface markers assessment using

120 Human MSC Analysis Kit (BD, USA). Briefly, cells were detached with 1X TryplE (Gibco) and

121 washed twice with FACS buffer (PBS, 1% FBS). After that, cells were resuspended in FACS

122 buffer and the concentration was adjusted to 1×10^6 cells/ml. Aliquots of 100µl from the cell

123 suspension were placed in test tubes and incubated for 30 minutes in the dark with fluorochrome

124 conjugated antibodies against CD-44, CD-105, CD-73, CD-90 and a negative cocktail mix

125 according to the manufacturer instructions. Cells were then centrifuged at 300xg for 5 minutes and

126 resuspended in 500 µl FACS buffer. Analysis was performed using FACSCanto™ (BD) and the

127 data were analyzed using Diva software.

128 **2.2.2. Multilineage differentiation**

129 Adipogenic differentiation was performed using StemPro Adipogenesis differentiation media

130 (Invitrogen, USA) for 14 days. Cells were washed twice with PBS, fixed and stained with Oil red

131 O stain to confirm the adipogenic differentiation potential. StemPro Osteogenic differentiation kit

132 (Invitrogen, USA) was used to induce ASC differentiation towards the urothelial lineage. After 21

133 days in culture, cells were washed, fixed and stained with Alizarin red stain to verify osteogenic

134 differentiation. Cells under normal culture conditions were used as a negative control.

135 **2.3. Cell proliferation analysis**

136 MSCs were seeded onto 96-well plates at a unified seeding density of 5000 cells/well and cultured

137 under   normal   conditions   for   three   days.   MTT (3(4, 5dimethylthiazolyl2)2, 5-

138 diphenyltetrazolium bromide (ATCC® 301010K) was used to measure the cell proliferation rate

139 after 24, 48 and 72 hours according to the manufacturer's instructions.

140 **2.3. Neural induction**

141 Cells were seeded on Matrigel coated six well plates and coverslips, and in the following day

142 medium was changed into neural induction media consisting of Dulbecco's modified Eagle's

143 medium F12 (DMEM/F12, Gibco) supplemented with 3% Knockout Serum Replacement (KSR,

144 Gibco), 1% Glutamax (Gibco, USA), 1% non-essential amino acid (NEAA, Gibco), 4 ng/mL basic

145   fibroblast growth factor (bFGF, Peprotech), 10 µM SB431542 (Sigma), and 0.5 µM LDN193289

146   (Sigma) for 8 days. Following, cells were passaged at 1:3 split ratio and media was switched every

147   other day as the following: Day 7 and 8 75% neural induction media and 25% of Neuroabasal

148   media consisting of 0.5% B27, 0.5% N2, 100nM LDN, 100ng/mL SHH C24II, 2µM

149   Purmorphamine, 100ng/mL FGF8a, 3µM CHIR-99021. Day 9 and 10 50% neural induction media

150   and 50% Neuroabasal media 0.5% B27, 0.5% N2 100nM LDN 3µM CHIR-99021. Day 11 and 12

151   25% neural induction media and 75% of Neuroabasal media 100nM LDN 3µM CHIR-99021. Day

152   13 onward 3µM CHIR-99021, 10ng/µl BDNF, 10ng/µl GDNF, 1ng/mL TGFb3, 10µM DAPT,

153   200µM Ascorbic Acid and 500µM db-cAMP.

154   **2.4. Gene expression analysis**

155   Total RNA was isolated on day 30 using RNeasy mini kit (Qiagen, USA) according to the

156   manufacturer's instructions. cDNA was synthesized using PrimeScript RT Master Mix (TaKaRa,

157   USA). Quantitative real-time PCR (qPCR) was preformed using iQSYBR mix (BioRad, USA)

158   according to the manufacturer's protocol and using specific forward and reverse primers listed in

159   Table 1. qPCR results were analyzed using the ΔΔCT relative quantification method.

160   **2.5. Immunofluorescence staining of neuronal markers**

161   After 7 and 30 days of induction, cells on coverslips were fixed in 4% formaldehyde for 15 minutes

162   and permeablized with 1X PBS containing 0.1% TritonX-100 for 5 minutes. To prevent

163   nonspecific binding, cells were incubated with blocking solution consisting of 10% normal goat

164   serum and 0.3% TritonX-100 (v/v) in 1X PBS for 60 minutes. Cells were then incubated with the

165   primary antibodies against SOX2, SOX1, TUJ1, Nestin, PAX6, and TH diluted in blocking buffer

166   overnight at 4ºC. Subsequently cells were incubated with appropriate secondary antibodies at 4ºC

167 in the dark and then DAPI staining and mounting onto microscope slides. Cells were imaged using

168 AxioObserever Z1 microscope (Zeiss, Germany).

169 **2.6 Statistical analysis**

170 All the experiments were done at least three times and statistical analysis was performed using

171 GraphPad Prism (Version 6). The data were presented as the mean ± standard error of the mean

172 (SEM). Statistical differences were calculated using One-Way Analysis of Variance (ANOVA 1)

173 and Post-hoc test for comparison between groups. Differences were considered significant at (* P

174 <0.05, ** P <0.01, *** P <0.001).

175 **3. Results**

176 **3.1 Isolation and characterization of ADSCs**

177   On the third day of primary culture, cells with fibroblastic morphology were adhered to the

178 tissue culture plate and became confluent within 14 days of initial plating. To validate the stemness

179 of the isolated cells, MSCs from different sources were transdifferentiated into the adipogenic and

180 osteogenic cell lineages. Cells induced with adipogenic media for 14 days exhibited intracellularly

181 localized lipid droplets stained with Oil red O (ORO), which were absent in the negative control

182 (Figure 1A). Following 14 days of osteogenic induction, cells exhibited flattened and more

183 elongated morphology with extracellular deposits. The presence of extracellular calcium

184 phosphate deposits was confirmed with Alizarin Red stain (ARS), which were absent in the

185 uninduced negative controls (Figure 1B). Thus, the cells were successfully differentiated into

186 osteoblasts and pre-adipocytes, confirming the multi-potency of these cells. Flow cytometry

187 analysis showed positive expression of the following MSCs markers: CD-90, CD-105, CD-73 and

188 minimal to no expression of markers in the negative cocktail (Figure 2A).

189   **3.2 Analysis of cellular proliferation**

190   Cellular proliferation was assessed using MTT assay to compare the proliferation rate between

191   MSCs-derived from AT, BM and WJ. After 48 hours, no significant difference in the proliferation

192   rate was observed between the analyzed cell types. However, WJ derived MSCs showed a

193   significantly higher proliferation after 72 hours compared to BM and AT-derived MSCs (P=0.009)

194   (Figure 2B).

195   **3.3. Morphological analysis of MSCs induced towards the neuronal lineage**

196   The morphology of MSCs induced towards the dopaminergic lineage demonstrated several

197   alterations throughout the differentiation process. Initially, the three different MSC types-derived

198   from AT, BM and WJ adhered to the culture flask and exhibited a spindle-shaped morphology.

199   Following splitting and prolonged culture, cells began to change in shape and acquire a more

200   spherical appearance, obtaining a neural-like morphology with the appearance of cellular

201   processes (Figure 3A). This change was more distinguishable in WJ-MSC compared to AT-MSC

202   and BM-MSCs.

203   **3.4. Molecular analysis of neuronal induction**

204   Quantitative real-time PCR was used to evaluate the relative expression of the following neuronal

205   genes: β Tubulin III (TUJ1), Nestin, NKX6.1, SOX2, PAX6 and LMX at the transcriptional levels.

206   Expression levels of the same target genes in undifferentiated original MSCs were considered as

207   the baseline level. The expression of TUJ1 gene, a neuronal marker of immature neurons, was

208   upregulated in all differentiated MSCs without any significant differences between different MSC

209   sources (Figure 3B). Nestin gene expression, a marker of neural progenitors, revealed a significant

210   upregulation in WJ compared to AT (*$P<0.05$) and BM (**$P<0.01$) derived MSCs ((Figure 3B).

211   On the other hand, NKX6.1 is also an early neuronal progenitor marker, was upregulated without

212   any statistical differences between the induced MSC types. SOX2 is a marker for early and

213   intermediate progenitor neuronal cells (Figure 3B). Levels of expression of this gene in WJ derived

214   MSCs was significantly higher compared to BM derived cells (*P<0.05). Additionally, PAX6 an

215   intermediate progenitor marker was significantly upregulated in WJ derived MSCs compared to

216   AT and BM derived MSCs (****P<0.0001). Additionally, we analyzed the expression of LMX1a

217   (LIM homeobox transcription factor 1, alpha) gene associated with the differentiation towards

218   dopaminergic neurons, and we found that WJ derived MSCs expressed significantly higher levels

219   compared to BM and AT derived MSCs (**P<0.01), which suggests that our protocol could direct

220   this types of MSCs towards a dopaminergic-like phenotype (Figure 3B).

221   Gene expression results support the morphological changes seen under the microscope and support

222   the idea that our differentiation protocol successfully generated neural stem/progenitor-like cells

223   from MSCs derived from BM, AT and WJ.

224   **3.5 Assessment of neuronal induction by immunofluorescence**

225   Since the morphological and gene expression results revealed a successful differentiation into the

226   neuronal-like lineage, we confirmed the expression of NESTIN, TUJ1, SOX1, SOX2 and PAX6

227   by immunofluorescent staining. All of these early neuronal progenitor markers were expressed in

228   all type of induced MSCs (Figure 4 & 5). However, a more prominent expression of these markers

229   in WJ-derived MSCs was detected compared to AT and BM derived MSCs. Additionally, cells

230   were stained for TH (Tyrosine Hydroxylase) and FOXA-2 (Forkhead box protein A2) as markers

231   for differentiation towards the dopaminergic lineage. WJ-MSCs showed higher expression levels

232   compared to the other sourdes, which might suggest that WJ represents a more efficient cell source

233   for neuronal cell differentiation (Figure 6).

234

Discussion:

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236   The regeneration of neurons following injury is minimal and neurogenesis is limited to specific

237   parts of the brain (Hess & Borlongan, 2008). Several clinical trials have been conducted using

238   MSCs from different sources to establish their safety and efficacy for the treatment of many

239   neurological disorders such as Parkinson's disease, multiple sclerosis and amyotrophic lateral

240   sclerosis (Boroujeni & Gardaneh, 2017; Karussis et al., 2010; Syková et al., 2017). *In vitro*

241   differentiation studies utilizing MSCs isolated from different tissues have shown variable

242   proliferation and differentiation potential (Alizadeh et al., 2019; Balasubramanian et al., 2013;

243   Datta et al., 2011; Urrutia et al., 2019). These variabilities can significantly impact the clinical

244   outcome. Accordingly, there is a need to provide a clear overview and comparison on the neuronal

245   differentiation potential of MSCs isolated from different sources. Thus, the aim of this study was

246   to provide a comparative view of the capabilities of MSCs isolated from different human tissue

247   sources, to differentiate into neuronal stem cell-like cells and dopaminergic-like cells. The data

248   described here shed the light on the most appropriate MSC source of to be used therapeutically in

249   neural regenerative therapies.

250   Here we confirm that MSCs isolated from adipose tissue, bone marrow, and Wharton-jelly express

251   similar surface markers and they are capable of undergoing multilineage differentiation. The

252   proliferative capacity of MSCs appear to be similar across the three different types of MSCs with

253   minor variations. These variations can result from culture heterogenicity, and different proportions

254   of self-renewing cells in comparison to lineage-specific cells in different tissues. Previous studies

255   have reported different proliferative capabilities of MSCs. Urrutia et al reported higher

256   proliferation rate of AT compared to WJ and BM-derived stem cells (Urrutia et al., 2019). On the

257   other hand Hu et al reported results that contradict the above, in which WJ had higher proliferation

258   rate compared to AT (Hu et al., 2013). Other studies have also reported varying results (Aliborzi

259   et al., 2016; Heidari et al., 2013). Such variabilities might be attributed to differences in isolation

260   and culture methods or differences due to age, sex and health status of the samples donors. The

261   assay of choice used to measure the proliferation capacity plays a critical contribution as well.

262   Such results are of importance for the selection of an appropriate tissue source to derive MSCs for

263   the use in cell-based therapies, which are required in sufficient numbers in a limited time to achieve

264   effective clinical outcomes. Hence, further rigorous analysis must be conducted to clearly identify

265   differences in proliferation capacities, with matched large samples, and utilizing unified consistent

266   methods.

267   Studies in animal models as well as human cell lines have shown that bone morphogenetic protein

268   (BMP) and the Activin/Nodal pathway play a significant role in neural development of embryos

269   as well as neuronal differentiation of different types of stem cells (Park et al., 2017;

270   Wattanapanitch et al., 2014). Several differentiation studies have demonstrated the synergistic

271   inhibition of those two pathways utilizing a small molecules cocktail such as SB 431542, Noggin,

272   LDN 193189 to induce the cells towards the neural progenitor fate that can then be differentiated

273   to a more mature neural cell type (Park et al., 2017; Pauly et al., 2018; Wattanapanitch et al., 2014).

274   Here, we utilized a combination of small molecules to direct the differentiation of MSCs towards

275   the neural lineage in a serum-free environment. Small molecules are relatively cheap, stable and

276   have high penetrating capability. Briefly, we employed dual-SMAD inhibition during the initial

277   stage of differentiation. Dual SMAD inhibition was achieved by adding SB-431542 as a TGF-β

278   inhibitor and LDN-193189 as a BMP-inhibitor to induce the neural lineage. Thus, revealed that

279   MSCs from different sources are capable of generating neural stem cell (NSC)-like cells.

280   Following 7 days of the initial induction, MSCs derived from different human tissue-sources

281   changed their morphology into spindled neuronal-like shape. Furthermore, we assessed the

282   expression of a group of NSC markers, including Nestin, Tuj1, Pax6, Sox1, Sox2. Our results point

283   towards the ability of MSCs to differentiate into neural stem cell-like phenotype. These cells are

284   of interest to provide an intermediate neural cell source that can be further differentiated into a

285   more mature state. However, it is important to note that these cells must be further characterized

286   for their purity and the ability to express a comprehensive panel of NSC markers. Furthermore,

287   the ability of these cells to expand with high efficiency in culture must be systematically evaluated.

288   Several different studies have attempted to induce the differentiation of MSCs from different

289   sources towards dopaminergic neurons to assess their ability to be used in cell based therapies for

290   neurodegenerative diseases such as Parkinson's disease (Adib et al., 2015; Tatard et al., 2007;

291   Trzaska et al., 2007). A wide array of small molecules, cytokines and neurotrophic factors, have

292   been used in different differentiation protocols. For instance, brain and glial derived neurotrophic

293   factors, FGF-8, SHH, cAMP, DAPT have been utilized frequently in neuronal induction (Adib et

294   al., 2015; Tatard et al., 2007; Trzaska et al., 2007).

295   Here, we assessed the ability of the generated NSC-like cells to differentiate into dopaminergic

296   neurons. Quantitative Real-time PCR and immunostaining of some dopaminergic-specific markers

297   revealed the higher differentiation potential of WJ derived cells towards the neural lineage

298   compared to AT and BM derived MSCs. It is noteworthy that the utilization of different

299   differentiation protocols, sample pool, as well as different analysis tools might lead to significant

300   variation in the differentiation results. Accordingly, a large-scale study with in depth analysis is

301   required in order to verify the exact potential of each MSCs type.

303 **Conclusion:**

304 In this study, we report that MSCs derived from adipose tissue, bone marrow and Warton's-jelly

305 can be induced to differentiate into neuron-like cells and further matured into dopaminergic-like

306 phenotype. WJ-MSCs showed a higher neuronal differentiation potential compared to AT and BM

307 derived MSCs. The differentiation of MSCs into neural cells might be a realistic goal as evident

308 by the expression of some neuronal markers after cellular induction. However, it is still early to

309 claim that these generated neuronal cells can be used in cell-based therapies, especially that such

310 differentiation protocols dictate that these cells must cross mesodermal lineage towards a

311 neuroectodermal lineage. The efficiency of all trans-differentiation protocols is still debatable and

312 must be comprehensively analyzed in vivo to confirm the terminal differentiation potential.

313

314 **Authorship contribution statement**

315 Ababneh NA: Methodology, Formal analysis and Validation, prepared the figures and wrote the

316 manuscript; Al-Kurdi B: Funding acquisition, Conceptualization, Methodology, Formal analysis

317 and Validation, prepared the figures and wrote the manuscript, Jamali F: Funding acquisition,

318 Conceptualization, Awidi A: Conceptualization, Overall Supervision.

319 **Declaration of Competing Interest**

320 No conflicts of interest associated with this publication.

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466

467 **Figure Legends**

468 *Figure 1*. **Characterization of MSCs by multilineage differentiation analysis.** (A) BM, AT and

469 WJ tissue derived MSCs after 14 days of adipogenic differentiation, showing internal lipid droplets

470 following staining with Oil Red O, which were absent in the undifferentiated controls. (B) MSCs

471 after 14 days of osteogenic differentiation, showing mineral deposition after staining with Alizarin

472 Red stain (ARS), which were absent in the undifferentiated controls.

473 *Figure 2*. **Characterization of MSCs by flow cytometry and comparison of proliferation**

474 **potential.** (A) Flow cytometry analysis of MSCs showed that cells were positive for MSC markers

475 CD-90, CD-105, CD-73 and negative for the negative cocktail mix. (B) Proliferation analysis of

476 MSCs from BM, AT and WJ samples. MTT assay was performed on cells cultured for 24, 48 and

477 72 hours. * $P < 0.05$.

478 *Figure 3*. **Induction of MSCs into neural stem cell-like cells utilizing dual SMAD inhibition.**

479 (A) Phase-contrast images of BM, AT and WJ MSCs, respectively, showing the morphological

480 changes after 30 days in culture. Scale bar: 100 μm. (B) Quantitative real-time PCR was used to

481 assess the expression of TUJ1, Nestin, NKX6.1, SOX2, PAX6 and LMX genes on cultured

482 neurons. Relative gene expression of each gene was normalized to the expression of GAPDH

483 housekeeping gene. Data represents means ± SEM of 3 independent experiments. * $P < 0.05$, **

484 $P < 0.01$, *** $P < 0.001$.

485 *Figure 4*. **Immunofluorescence analysis of neuronal markers at day 7.** Neural stem cells

486 differentiated from MSCs and cultured in neuronal induction media were analyzed by

487 immunofluorescence staining on day 7 for the expression of neural stem/progenitor protein

488 markers (A) SOX2 and Nestin. Scale bar =50 μm. (B) Semi-quantitative analysis of SOX2 and

489   Nestin representing the percent of positive cells. (C) SOX1 and TUJ1, Scale bar =50 μm (D)

490   Semi-quantitative analysis of SOX1 and TUJ1 immunofluorescence representing the percent of

491   positive cells. All experiments were repeated at least three independent times.

492   *Figure 5*. **Immunofluorescence analysis of neuronal markers at day 30.** MSCs derived from

493   bone marrow, adipose and Warton jelly tissue cultured in neuron induction media were analyzed

494   on day 30 for the expression of neural stem/progenitor protein markers (A) SOX2 and Nestin.

495   Scale bar =50 mm. (B) Semi-quantitative analysis of SOX2 and Nestin immunofluorescence

496   representing percentage of positive cells relative to negative control. (C) SOX1 and TUJ1, Scale

497   bar =50 mm (D) Semi-quantitative analysis of SOX2 and Nestin immunofluorescence

498   representing percentage of positive cells. All experiments were repeated at least three times.

499   *Figure 6*. **Immunofluorescence analysis of mature neuronal markers at day 30**. The

500   expression of dopaminergic markers (A) FOXA2 and Tyrosine hydroxylase was analyzed by

501   immunofluorescence staining on cells cultured for 30 days in neuronal induction media. Scale

502   bar =50 mm. (B) Semi-quantitative analysis of FOXA2 and TH immunofluorescence

503   representing the percentage of positive cells. All experiments were repeated at least three times.

504

505

Figure 1

Characterization of MSCs by multilineage differentiation analysis

(A) BM, AT and WJ tissue derived MSCs after 14 days of adipogenic differentiation, showing internal lipid droplets following staining with Oil Red O, which were absent in the undifferentiated controls. (B) MSCs after 14 days of osteogenic differentiation, showing mineral deposition after staining with Alizarin Red stain (ARS), which were absent in the undifferentiated controls.

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B)

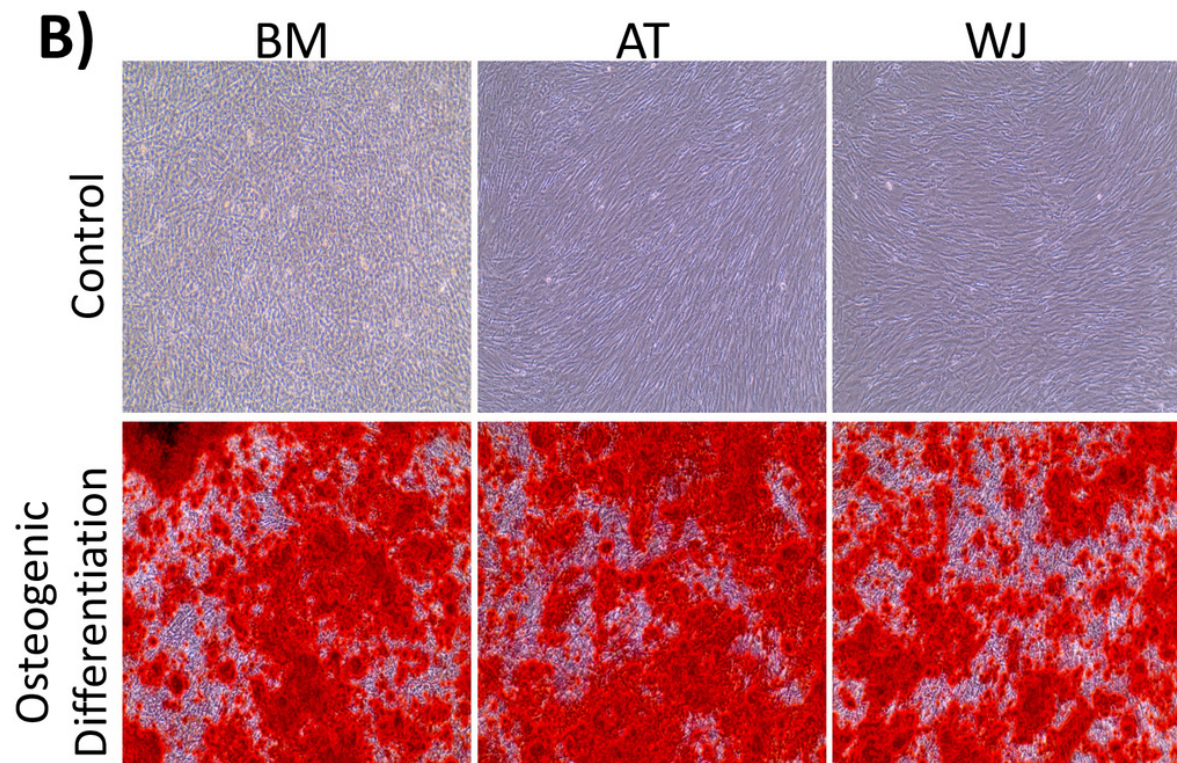
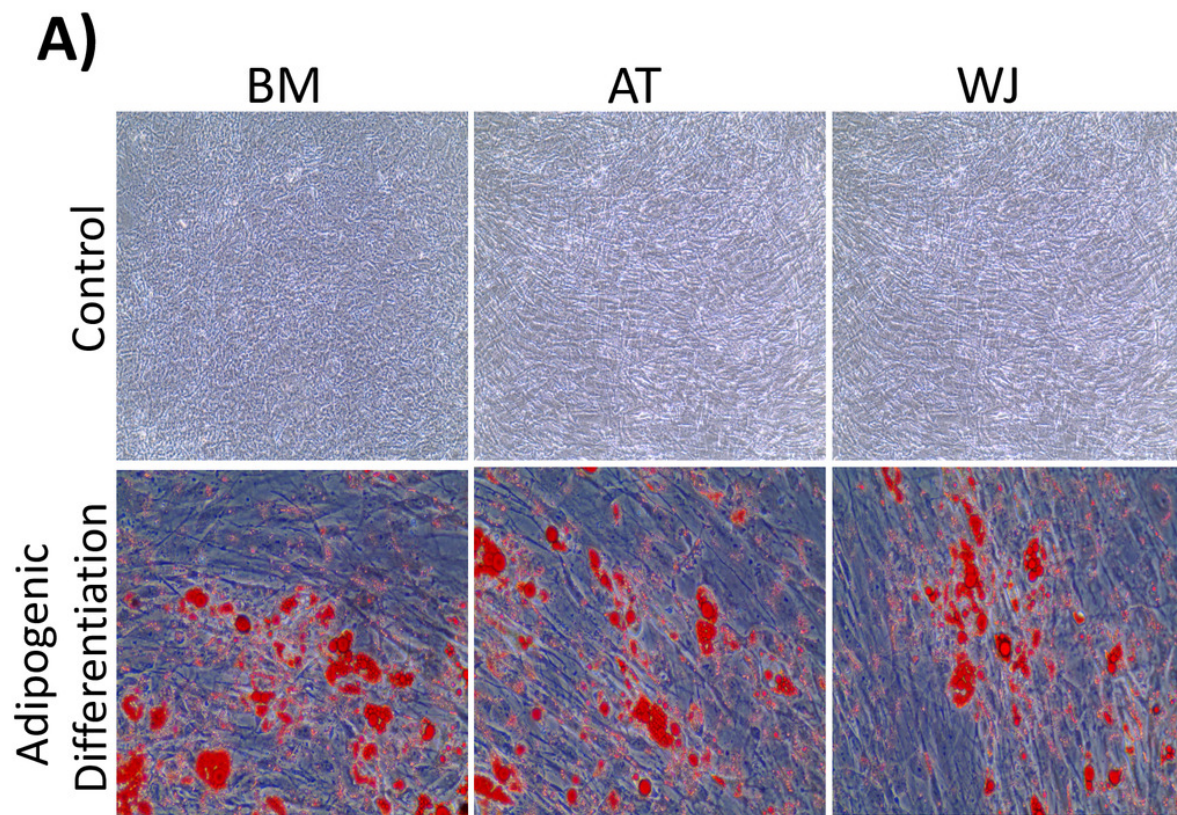
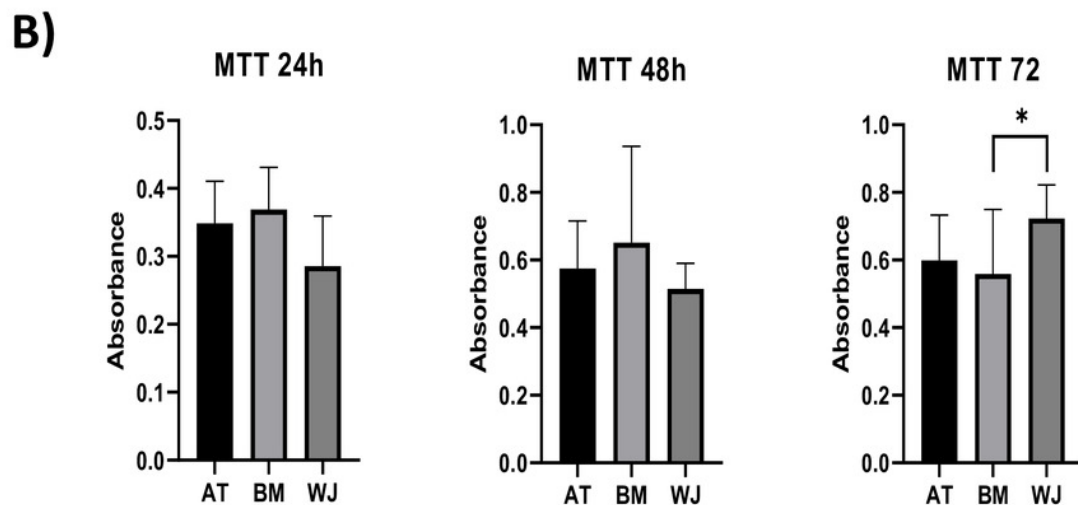
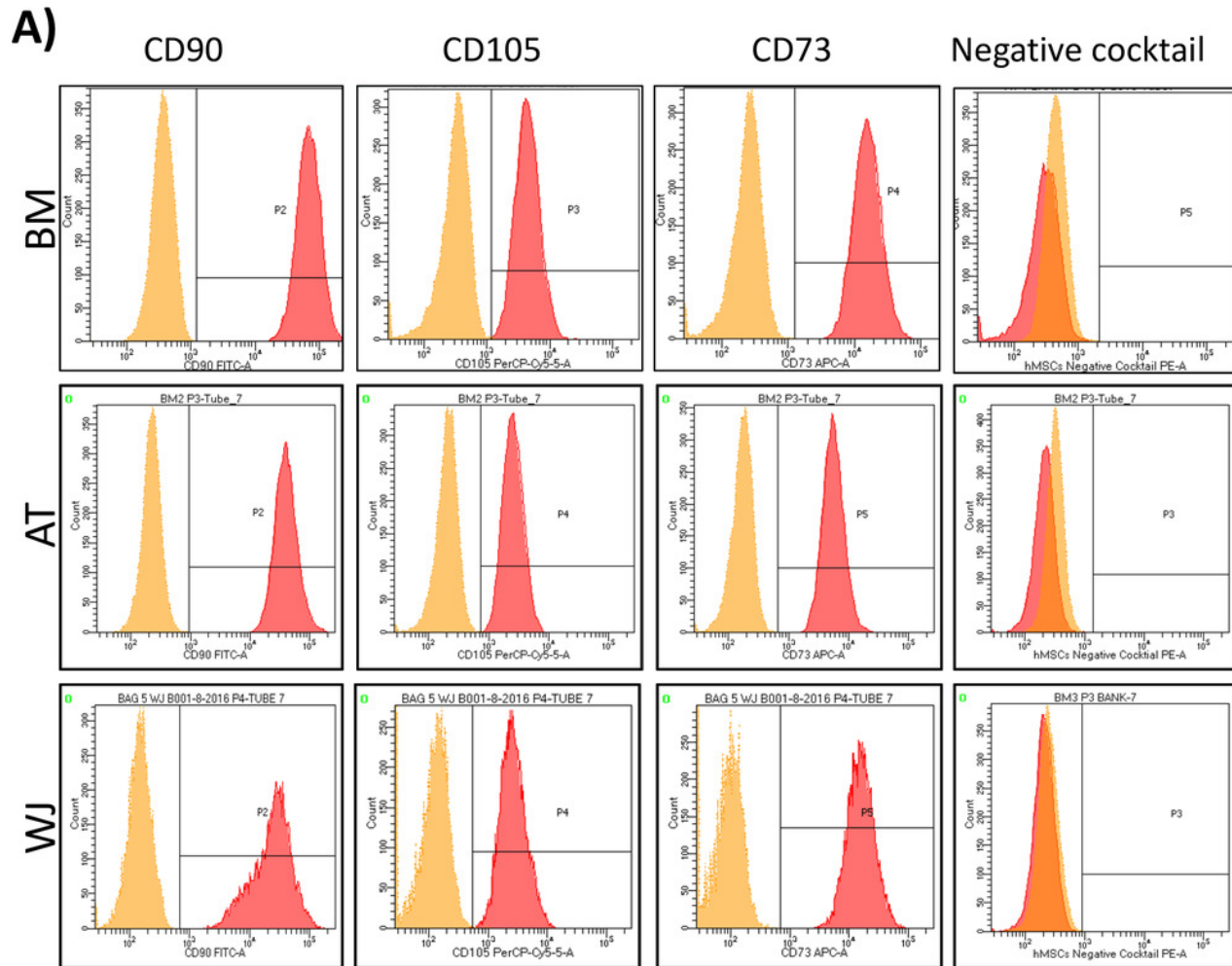



Figure 2

Characterization of MSCs by flow cytometry and comparison of proliferation potential.

(A) Flow cytometry analysis of MSCs showed that cells were positive for MSC markers CD-90, CD-105, CD-73 and negative for the negative cocktail mix. (B) Proliferation analysis of MSCs from BM, AT and WJ samples. MTT assay was performed on cells cultured for 24, 48 and 72 hours. * P <0.05.

A)

B)

Figure 3

Induction of MSCs into neural stem cell-like cells utilizing dual SMAD inhibition

(A) Phase-contrast images of BM, AT and WJ MSCs, respectively, showing the morphological changes after 30 days in culture. Scale bar: 100 μm. (B) Quantitative real-time PCR was used to assess the expression of TUJ1, Nestin, NKX6.1, SOX2, PAX6 and LMX genes on cultured neurons. Relative gene expression of each gene was normalized to the expression of GAPDH housekeeping gene. Data represents means ± SEM of 3 independent experiments. * P <0.05, ** P <0.01, *** P <0.001.

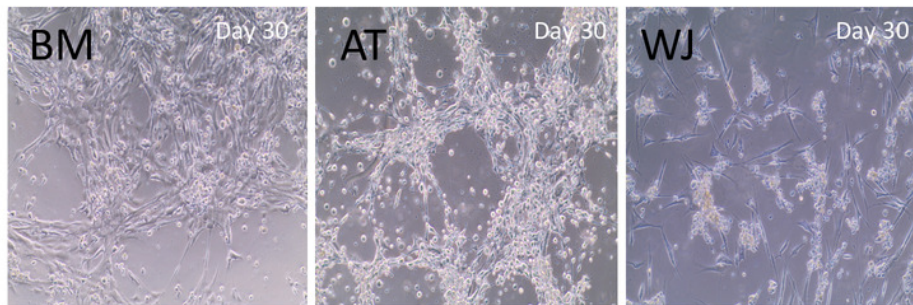
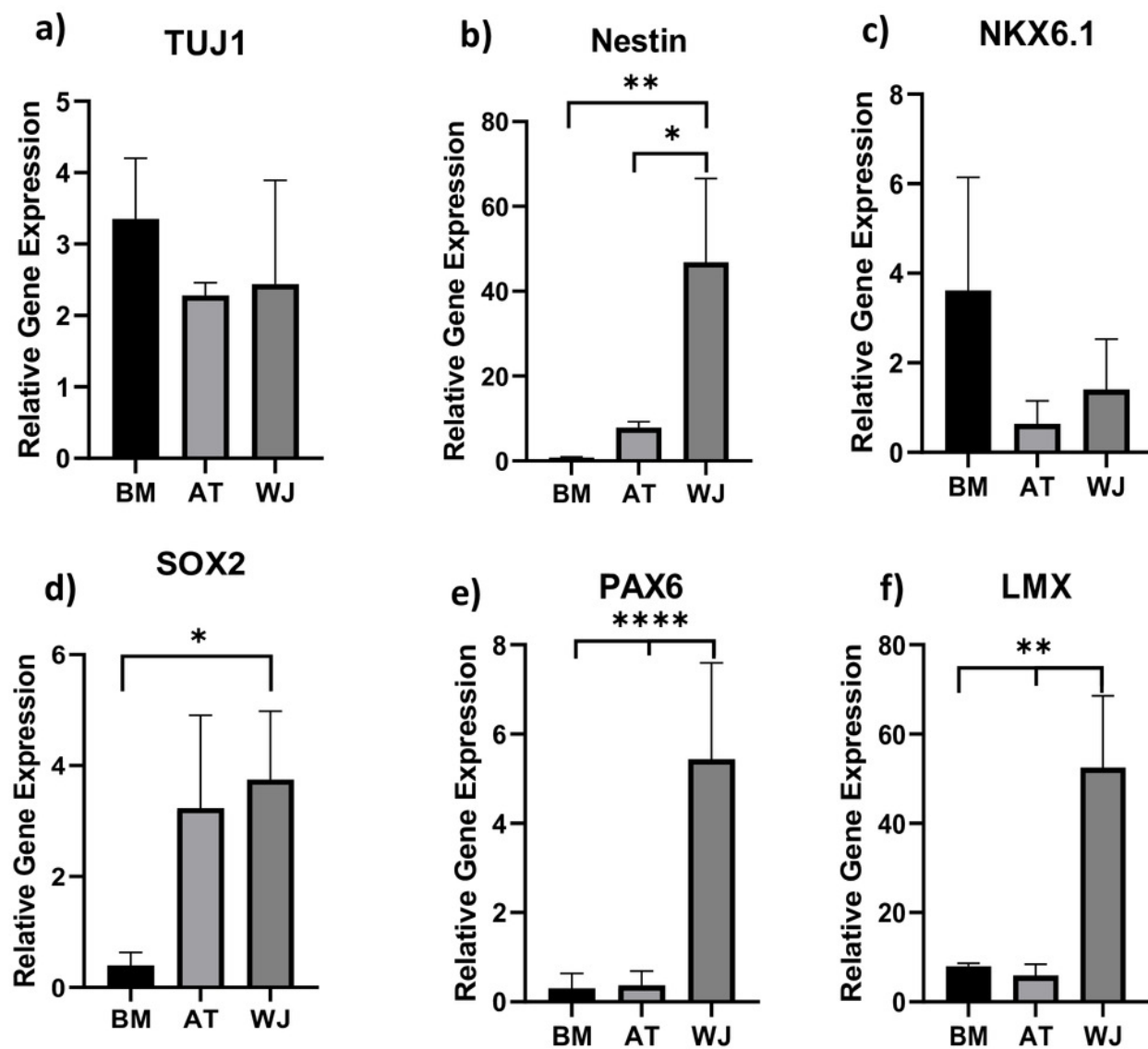



Figure 4

Immunofluorescence analysis of neuronal markers at day 7

Neural stem cells differentiated from MSCs and cultured in neuronal induction media were analyzed by immunofluorescence staining on day 7 for the expression of neural stem/progenitor protein markers (A) SOX2 and Nestin. Scale bar =50 μm. (B) Semi-quantitative analysis of SOX2 and Nestin representing the percent of positive cells. (C) SOX1 and TUJ1, Scale bar =50 μm (D) Semi-quantitative analysis of SOX1 and TUJ1 immunofluorescence representing the percent of positive cells. All experiments were repeated at least three independent times.

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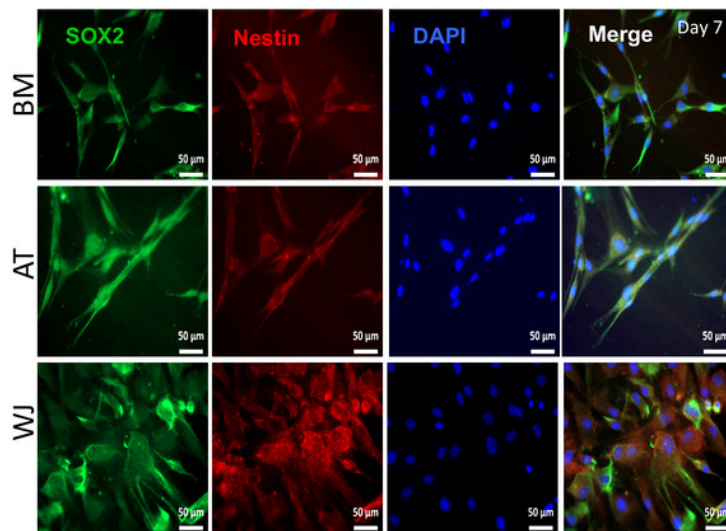
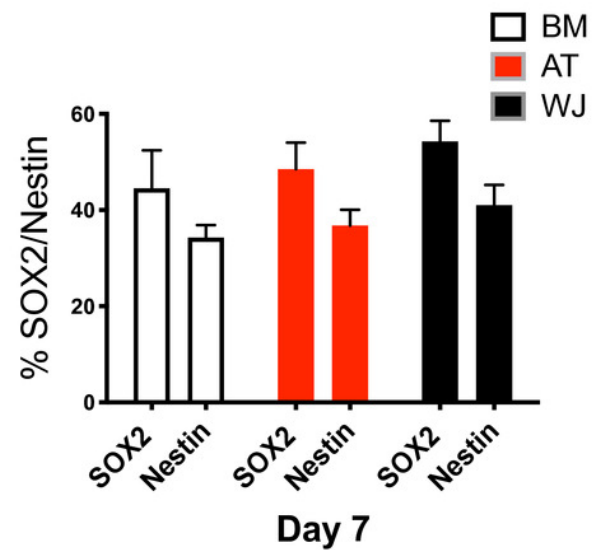
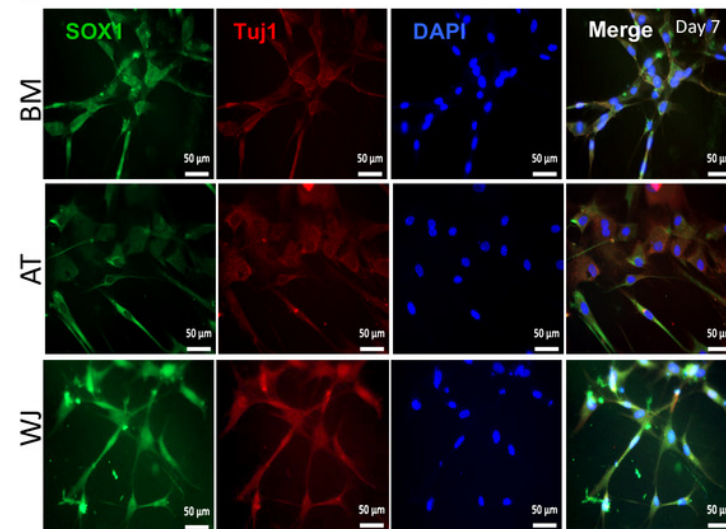

B)

C)

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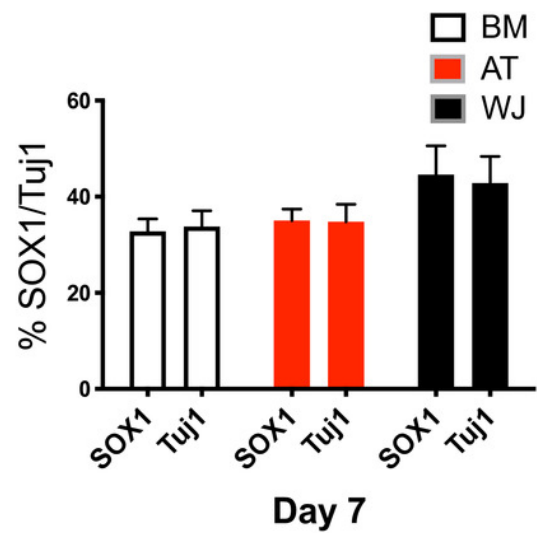
Figure 5

Immunofluorescence analysis of neuronal markers at day 30

MSCs derived from bone marrow, adipose and Warton jelly tissue cultured in neuron induction media were analyzed on day 30 for the expression of neural stem/progenitor protein markers (A) SOX2 and Nestin. Scale bar =50 mm. (B) Semi-quantitative analysis of SOX2 and Nestin immunofluorescence representing percentage of positive cells relative to negative control. (C) SOX1 and TUJ1, Scale bar =50 mm (D) Semi-quantitative analysis of SOX2 and Nestin immunofluorescence representing percentage of positive cells. All experiments were repeated at least three times.
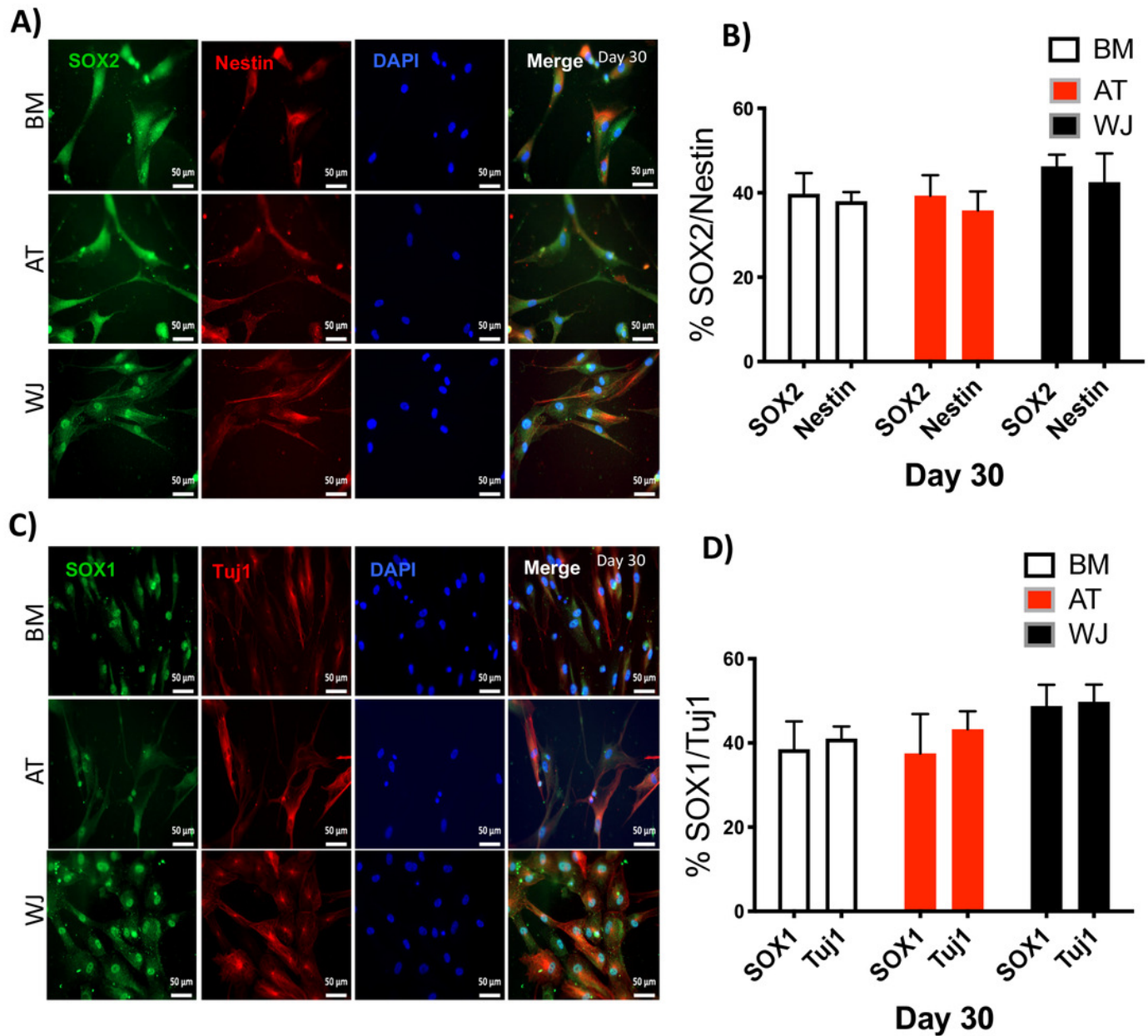
Figure 6

Immunofluorescence analysis of mature neuronal markers at day 30

The expression of dopaminergic markers (A) FOXA2 and Tyrosine hydroxylase was analyzed by immunofluorescence staining on cells cultured for 30 days in neuronal induction media. Scale bar =50 mm. (B) Semi-quantitative analysis of FOXA2 and TH immunofluorescence representing the percentage of positive cells. All experiments were repeated at least three times
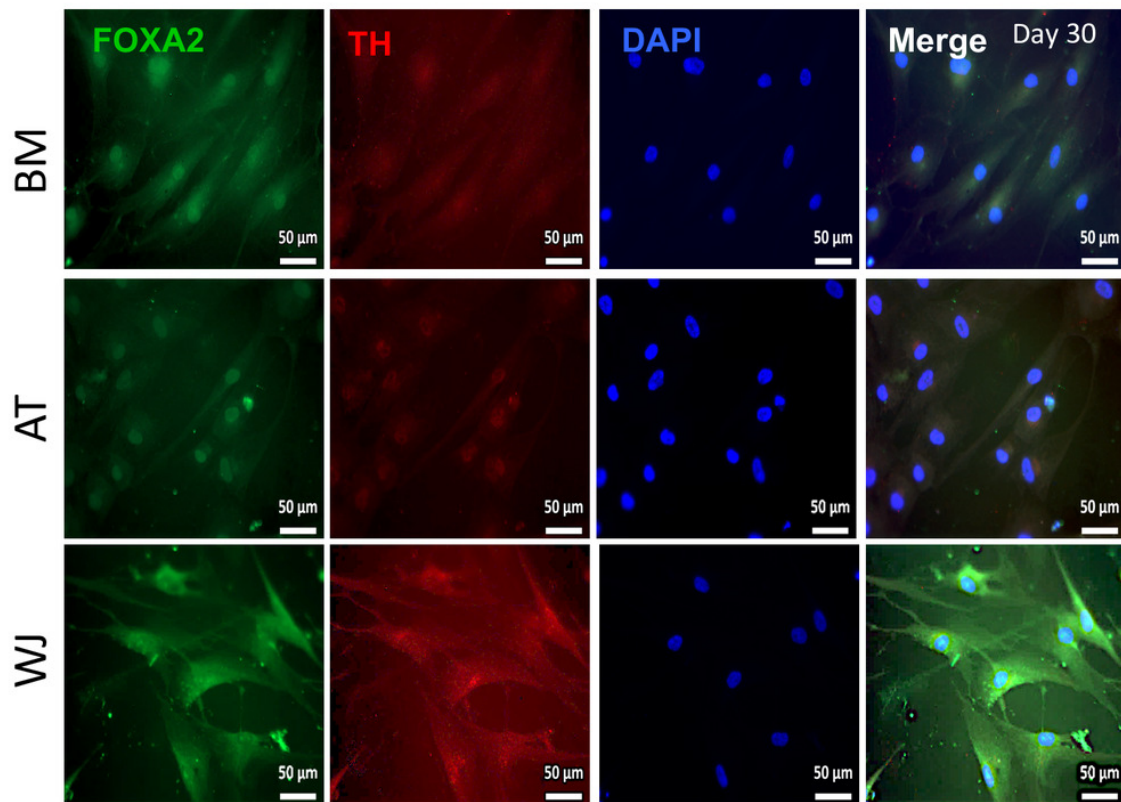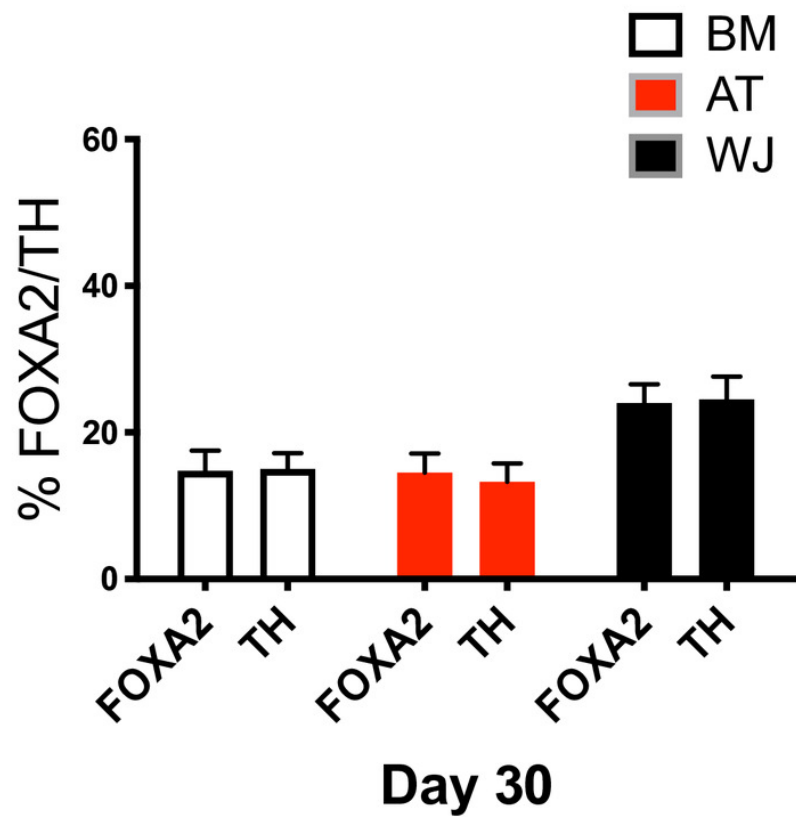
Table 1: qPCR Primer sequence.

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Gene Name	Forward (5→3)	Reverse (5→3)
GAPDH	CCTGTTCGACAGTCAGCCG	CGACCAAATCCGTTGACTCC
NKX6.1	ATTCGTTGGGGATGACAGAG	CCGAGTCCTGCTTCTTCTTG
Nestin	AGAAACAGGGCCTACAGAGC	GAGGGAAGTCTTGGAGCCAC
Sox-2	TAGAGCTAGACTCCGGGCGAT	TTGCCTTAAACAAGACCACGAAA
Pax6-	CGGAGTGAATCA GCTCGGTG	CCGCTTATACTGGGCTATTTTGC
TUJ	GCGAGATGTACGAAGACGAC	TTTAGACACTGCTGGCTTCG
LMX1A	AGGAAGGCAA GGACCATAAGC	ATGCTCGCCTCTGTTGAGTTG