1 **22-hydroxycholesterol leads to efficient dopaminergic specification of human**

2 **Mesenchymal Stem Cells**

3 **Running title:** 22-HC as neurogenic agent for human MSCs

4 **Abstract:**

5 In the current study, we aim to investigate the neurogenic effect of 22 (R)-

6 hydroxycholesterol (22-HC), on hMSCs obtained from bone marrow, adipose tissue and

7 dental pulp. The effect was evaluated on the basis of detailed morphological and

8 morphometric analysis, expression of genes and proteins associated with maturation of

9 neurons (NF, MAP2, TUJ1, TH), channel ion proteins (Kv4.2 & SCN5A), chemical

10 functionality (DAT), synapse forming tendency of neurons (Synaptophysin) and transcription

11 factors (ngn2 & Pitx3), efficiency of generation of dopaminergic neurons and functional

12 assessment. The percentage of non- DA cells generation (Ach, TPH2, S100 and GFAP

13 positive cells) was also evaluated to confirm selective neurogenic potential of 22-HC. Post

14 analysis, it was observed that 22-HC yields higher percentage of functional DA neurons and

15 has differential effect on various tissue- specific primary human MSCs. This study, which is

16 one of its kinds, may help in improvising the approach of cell based treatment regimes and

17 drug testing in pharmaceutical industry for Parkinson's disease.

18 **Keywords:** Mesenchymal stem cells, Oxysterols, 22 (R)- hydroxycholesterol, dopaminergic

19 neurogenesis, Parkinson's disease

20 **Introduction:**

21 Cholesterol is a type of lipid, which is biosynthesized in all types of animal cells and is an

22 important part of the cell membrane. Human brain consists of as high as ~25% of the lipids[1].

23 Cholesterol, along with other lipids, self- assembles and forms various structures,

24 contributing to the varied functions of the brain[2]. The lipidome profile of the central nervous

25 system (CNS) is associated with neuronal activity, cognitive behaviour and various

26 neurological disorders[3]. While cholesterol is required for dopaminergic (DAergic) neuronal

27 maturity in terms of maintaining synapses and neurotransmission, they are also required for

28 their survival[4-5].

29 Oxysterols are the oxidized derivatives of cholesterols which show their effect in various

30 functioning through lxr-α and lxr-β. Lxr (liver X receptors) are the nuclear receptors which

31 function through their oxysterol ligands and control various activities like cell division, ventral

32 midbrain neurogenesis and DAergic neurogenesis[5]. The study reported by Paolla Sacchetti

33 et al in 2009 states the precedence of various types of oxysterols like 24-HC and 25-HC in

34 DAergic neurogenesis in mice brain. The same study reports the relevance of 22-HC in

35 DAergic neurogenesis in human embryonic stem cell (hESC) lines, H9 and HS181.

36 Oxysterols have also been reported to maintain the balance between neuronal and glial cells

37 generation.

38 However, till date there is no report that states the effect of oxysterols in generation of

39 DAergic neuronal cells from Mesenchymal Stem Cells (MSCs). Lately, MSCs are being

40 considered as very promising candidates for cell replacement therapies in various

41 degenerative diseases, with advantages of the ease of their procurement from tissues like

42 bone marrow (BM), adipose tissue (AD), dental pulp (DP), Wharton's jelly (WJ), etc.,

43 maintenance in the culture, minimal ethical concerns and no immunogenicity. Hence,

44 efficient generation of functional DAergic neurons from MSCs *in vitro* is a pre- requisite to

45 use MSCs for translational purpose.

46 Our aim is to investigate the effect of 22-HC on human Mesenchymal stem cells (hMSCs),

47 which in turn has the most optimum translational implication. Considering the specific action

48 of 22-HC in DAergic neurogenesis and very few evident/ explanatory studies using hESCs or

49 *in vivo* mice models, we ventured to explore its contribution towards generation of DA

50 neurons from hMSCs.

51 Therefore, considering the varied roles and initial studies stating the importance of 22-HC in

52 DAergic neurogenesis and the significance of MSCs in clinical set- up, we designed this

53 study to coax primary MSCs obtained from various tissue sources like BM, AD and DP using

54 22-HC in combination with FGF2 towards DAergic neurons and their characterization at

55 morphological, ultra-structural, transcriptional and translational levels. Induced hMSCs were

56 also assessed for their functionality by calcium ion imaging method. Apart from the analysis

57 of DAergic neuronal cells, presence of any other cell types was also enumerated. Changes

58 occurring in the cells at ultra structural level were also studied. This detailed comparative

59 analysis on a single platform has given the futuristic direction of research in the field of

60 DAergic neurogenesis and regenerative medicine.

61 **MATERIALS & METHODS**:

62 **Ethics Statement:**

63 The study was conducted after receiving ethical clearance from Institutional Ethics

64 Committee (IEC) and Institutional Committee for Stem Cell Research (IC-SCR), AIIMS, New

65 Delhi. All the methods described in this study were performed in accordance with the

66 relevant guidelines and regulations of the Institution.

67 **Cell Culture: Revival and expansion of Bone Marrow- Mesenchymal Stem Cells (BM-**

68 **MSC), Adipose tissue derived Mesenchymal Stem Cells (AD-MSC) and Dental pulp**

69 **derived Mesenchymal Stem Cells (DP-MSC):**

70 Cryopreserved BM- MSC, AD-MSC and DP-MSC were used for the study. Informed consent

71 was obtained from the patients or legal representatives at the time of bone marrow, adipose

72 tissue or extracted tooth/teeth collection for previous research projects. Age of patients

73 ranged from 24 to 40 years for both BM- MSC and AD-MSC and 20-28 years for DP-MSC.

74 Cells of 5 healthy donors of each MSC type, which were cryopreserved at first passage,

75 were revived in DMEM-LG medium (Gibco, USA) with 10% fetal bovine serum (FBS)

76 (Hyclone, USA) and penicillin (100U/ml) streptomycin (100μg/ml) (Gibco, USA) (Expansion

3

77 medium), expanded and characterized. Viability of the revived cells was measured by trypan

78 blue staining (Invitrogen, Life Technologies, USA). Followed by characterization (by surface

79 marker profiling using flow cytometry and trilineage differentiation potential), cells from 3rd to

80 5th passage were used for all the further experiments[6].

81 ***Neuronal Differentiation*:** For neuronal differentiation, induction medium containing

82 Neurobasal media (Gibco, USA), B27 supplement (Gibco, USA), EGF, FGF2 (10ng/ml each)

83 (PeproTech Asia) and 22-HC (2μM) (Sigma, USA), L-Glutamine (Gibco, USA), PenStrep

84 (Gibco, USA) was used. The induction protocol was carried out for 14 days with media

85 change on every 3rd day. After termination of induction period at 14 days, the cells were

86 used for further experiments.

87 **Neurites' length Analysis:**

88 This was performed as per the already established protocol of the lab. Briefly, induced

89 hMSCs were examined for the morphological changes under inverted microscope. Images

90 were captured at 10X magnification and analysed using SI Viewer software (Tokyo, Japan)

91 for the number and length of neurites, length of axon and area and diameter of the cell body.

92 Respective uninduced hMSCs were used as experimental control[6].

93 **Scanning Electron Microscopy (SEM):**

94 Samples for SEM analysis were processed as per the established protocol of the lab[7].

95 Briefly, hMSCs were cultured and differentiated over cover slips. These samples on

96 coverslips were collected and fixed with Karnovsky fixative (4% paraformaldehyde and 1%

97 glutaraldehyde in 0.1 M Phosphate Buffer (pH 7.4)) for 6-8 hours at 4°C. Dried samples

98 were mounted over aluminium stubs and sputter-coated with gold prior to imaging with

99 EVO18 scanning electron microscope (Zeiss, Oberkochen, Germany) at 5 KVA in secondary

100 electron imaging mode.

101 **Transmission Electron Microscopy (TEM):**

102 Samples for TEM analysis were processed as per the established protocol of the lab[8]. After

103 differentiation of hMSCs into neuronal cells, medium was removed and cells were given a

104 gentle wash using PBS (pH 7.4), followed by fixation of cells by Karnovsky's fixative (4%

105 paraformaldehyde and 1% glutaraldehyde in 0.1 M Phosphate Buffer (pH 7.4)) for 6-8 hours

106 at 4°C. After fixation, cells were washed gently with PBS. Cell number was such that they

107 form a pellet of 100µl upon centrifugation. Water from the cells was removed by treating

108 them with a series of ascending concentrations of the dehydrating agent, ethanol. Ethanol

109 was cleared by treating the cells with xylene. After this, samples were dehydrated in

110 ascending grades of acetone and embedded in araldite CY212. Thin sections (70 nm) were

111 cut with a glass knife and mounted onto nickel grids. They were contrasted with uranyl

112 acetate and lead citrate and viewed under a transmission electron microscope (Tecnai, G 20

113 (FEI)).

Transcriptional Characterization of MSC induced into neuronal cells: Quantitative
reverse transcription- polymerase chain reaction (qRT-PCR):

116 After differentiation, total RNA from all the experimental groups was extracted by phenol-

117 chloroform method as previously described[6]. Single strand cDNA synthesis was performed

118 by using cDNA synthesis kit from Life Technologies (California, USA) according to the

119 manufacturer's protocol. Expression of Nestin, Neurofilament (NF), Microtubule associated

120 protein (MAP2) and Tyrosine hydroxylase (TH) was studied in both induced and uninduced

121 MSC. All these primers were obtained from Sigma (Missouri, USA) (data not shown).

122 qRT-PCR experiments were performed using Realplex real time PCR detection system

123 (Eppendorf, Germany) as previously described (Singh et al., 2017). qRT-PCR was done for

124 Nestin, NF, MAP2, β III tubulin (Tuj1), TH and transcription factors, PitX3 and Ngn2. To

125 study the genes related to dopamine transportation, expression of DAT (dopamine

126 transporter) gene was studied. Apart from these, genes related to various ion channels like

127 Kv4.2 (potassium channel) and SCN5A (sodium channel) were also studied. Expression of

128 nuclear receptors of 22-HC, i.e., LXRα and LXRβ were also studied. Primers of qRT- PCR

129 grade were procured from Sigma (Missouri, USA).

130 The expression of the genes of interest was normalized to that of the housekeeping gene,

131 glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Melting curve was used to confirm

132 the results and data were analyzed using the graph pad prism software. Details of the

133 primers used are given in Supplementary Table 1.

134 **Immunocytochemistry:**

135 The assay was performed as previously described[6] . Briefly, fixed cells were incubated

136 overnight at 4°C with primary monoclonal antibodies against Nestin (1:400), MAP2 (1:250),

137 TH (1:200) and DAT (1:200) (Abcam, USA). After induction period, hMSCs were washed five

138 times with PBS and incubated with fluoro isothiocyanate (FITC) and texas red (TR)

139 conjugated secondary antibodies (1:500 dilution) for 1 hour at room temperature. Finally,

140 after washing five tmes with PBS, cells were counter stained with 4′,6-diamidino-2-

141 phenylindole (DAPI) to visualize the cell nuclei. Cells were washed thrice with PBS to

142 remove excess DAPI stain. Stained cells were examined using a fluorescence microscope

143 equipped with a digital camera (Nikon Eclipse 80i, Japan).

144 **Intracellular staining for Flow Cytometry:**

145 hMSCs after induction with both the induction protocols, were labeled for Nestin (1:100),

146 MAP2 (1:200), TH (1:100), synaptophysin (1:100), DAT (1:100), GFAP (1:100), TPH2

147 (1:150), Ach (1:150) and S100 (1:100) as previously described[6]. Briefly, the cells were

148 incubated with primary antibodies (dilution of 1:100) for 1 hour 20 mins at 4 °C, followed by

149 washing and incubation with secondary antibody labelled with fluorochrome tagged

150 secondary antibody (dilution of 1:400) for 30 mins at room temperature. The cells were then

151 washed and suspended in PBS and acquired on BD LSR II flow cytometer (Becton

152 Dickinson, USA) with minimum of 10,000 events for each sample and analyzed with FACs

153 DIVA software (version 6.1.2). All the antibodies were procured from Abcam, USA.

154 **Immunoblotting:**

6

155 Immunoblotting for the expression of neuronal cell specific proteins was performed with both

156 induced and uninduced control cells, as previously described[6]. Briefly, after preparing whole

157 cell lysates using RIPA buffer (Sigma, USA), the protein quantification was done using

158 Bicinchoninic Acid (BCA) Assay method. Protein extracts (30 µg) were subjected to SDS-

159 PAGE using 12% Tris/HCl SDS (Sodium dodecyl sulphate) gels and transferred onto PVDF

160 membranes (Membrane Technologies, India). After blocking the membranes with 3% BSA,

161 they were incubated with primary antibody against β- actin (Abcam,UK, 1:2500), MAP-2

162 (Abcam, UK, 1:1500) and tyrosine hydroxylase (Santa Cruz, USA, 1:1000) in 1% BSA-

163 phosphate saline buffer (PBS) overnight at 4°C. Post incubation, membranes were washed

164 thrice in PBST and incubated with the appropriate horseradish peroxidase (HRP)-

165 conjugated secondary antibody (1/4,000) (Dako, USA) for 2 hours at room temperature.

166 Membranes were developed with chemiluminescence detection reagent (Pierce, USA) and

167 acquired by using Gel Imager machine (Fluor Chem E, Cell Biosciences, Australia).

168 **Calcium ion Imaging:**

169 Change in the concentration of calcium ions was studied by calcium ion imaging in hMSCs

170 induced for 12 days in all study groups, as previously described[6]. Briefly, hMSCs upon

171 induction, were stained with 10µM of Fura red AM dye. After washing thrice with HBSS, the

172 cells were activated using 50mM KCl solution. Time lapse recording was made at 488nm

173 and 457nm for 3 minutes. Baseline readings were obtained before adding KCl solution to the

174 cells. The experiment was performed using Leica Confocal Microscope (Model TCS SP8).

175 The ratio of florescence at both the wavelengths was obtained and respective graph was

176 plotted. The experiment was performed on 3 samples each. The data was analysed using

177 Leica LAS AF software.

178 **Data Interpretation and Statistical Analysis**: Means ± SD of independent experiments

179 were analyzed by student's t- test, one way and two way ANOVA test (as per the

180 requirement of data analysis). $P < 0.05$ was considered as statistically significant. Analysis of

181 data was done by using GraphPad Prism 5.00 software (San Diego, California, USA).

182 **Results:**

7

183 **Human Mesenchymal Stem Cells (hMSCs) require higher concentration of 22-HC for**

184 **differentiation into neuronal cells**

185 A higher dose of 22-HC is required to differentiate hMSCs into dopaminergic (DAergic)

186 neuronal cells. As per the already reported dose of 22-HC (0.5µM -1.0µM) with hESC lines

187 by Sacchetti et al., 2009, maximum neurogenic effect was observed at a concentration of

188 0.5µM of 22-HC, determined by the percentage of TUJ1 and TH positive cells. We titrated

189 the dose of 22-HC on BM-MSC from a range of 0.5µM to 3.0µM and evaluated the results by

190 flow cytometric enumeration of MAP2 and TH positive cells. 2 µM and 3 µM concentration of

191 22-HC had similar DAergic neurogenic effect, as was evident from the percentage positivity

192 of MAP2 and TH positive cells (data not shown). Hence, it was decided to continue the

193 induction protocol for 14 days with 2µM of 22-HC, which was higher than the earlier reported

194 dose[5].

195 **22- HC causes neuron-like morphological changes in the human Mesenchymal Stem**

196 **Cells**

197 Upon induction of human Mesenchymal Stem Cells with FGF2 and 22-HC, basic

198 morphological changes were observed. Typical spindle shaped morphology of MSCs started

199 changing to the appearance of distinct cell body and cytoplasmic extensions like neurites

200 and axon. 22-HC induced neurites- lengthening or branching hMSCs of all types. Nucleus of

201 the cell shifted towards periphery and developed cytoplasmic extensions, through axon

202 hillock- like structures on the cells body. The terminals of the induced cells were also

203 observed to have multiple dendritic structures (Figure 1). These preliminary features were

204 confirmed by scanning electron microscopic (SEM) studies. SEM studies showed

205 development of fine neurites at the terminals of the cells post- induction period. Several

206 fields also showed the cell to cell interaction, with extended neurites like extensions. Axon-

207 hillock like structures was also observed clearly in the differentiated neuronal cells (Figure

208 2A i & ii). However, uninduced hMSCs were more flattened with spindle shape and had

209 centralized nucleus.

8

22-HC induces neuronal cells- like features in hMSCs with increase in the average area of cell body, length of neurites and axon

Neuritogenesis and axonogenesis in the induced cells were assessed in detail by measuring the average area of the cell body. Average of the cell body increased from $38.51 \pm 0.8036 \mu m^2$ to 1737 ± 116.3 μm^2 in BM-MSCs, from 43.03 ± 3.064 μm^2 to 1897 ± 130.1 μm^2 in AD-MSCs and from 37.85 ± 1.526 μm^2 to 2430 ± 119.7 μm^2 in DP-MSCs. Average length of the neurites increased from 57.59 ± 11.42 μm to 129.1 ± 6.214 μm in BM-MSCs, from 61.49 ± 6.776 μm to 128.7 ± 5.496 μm in AD-MSCs and from 75.26 ± 10.99 μm to 199.8 ± 13.56 μm in DP-MSCs. When axon length (longest neurite) was calculated, an upsurge from 57.59 ± 11.42 μm to 191.8 ± 16.03 μm in BM-MSCs, from 49.09 ± 6.913 μm to 278.5 ± 10.48 μm in AD-MSCs and from 75.26 ± 10.99 μm to 390.5 ± 25.09 μm in DP-MSCs was observed. There was no significant difference observed in the area of cell body and average length of neurites of induced BM-MSCs and AD-MSCs. On the contrary, length of axon and ratio of axon to cell body was found to be significantly higher in induced AD-MSCs as compared to those in induced BM-MSCs. However, DP-MSCs consistently had higher escalation in all the parameters studied for morphometric analysis of differentiated neuronal cells (Figure 2B i-iv).

22-HC leads to upregulation of DA neuronal cell traits at transcriptional level in hMSCs

Induced hMSCs were characterized for the expression of neuronal and DAergic neuronal specific genes. Post induction a relative upregulation was observed in the expression of NF (3.162 ± 0.2117, 3.274 ± 0.4632 and 29.65 ± 1.353 folds in BM-MSCs, AD-MSCs and DP-MSCs, respectively), TUJ1 (3.144 ± 0.2899, 10.91 ± 0.5304 and 18.06 ± 0.6789 folds in BM-MSCs, AD-MSCs and DP-MSCs, respectively), MAP2 (4.078 ± 0.4473, 9.072 ± 0.6825 and 35.31 ± 0.9012 folds in BM-MSCs, AD-MSCs and DP-MSCs, respectively) and TH (32.66 ± 1.775, 7.882 ± 0.8511 and 41.61 ± 0.6909 folds in BM-MSCs, AD-MSCs and DP-MSCs, respectively). However, there was no significant upregulation observed in transcriptional expression of nestin, except that in DP-MSCs with 1.344 ± 0.1671 folds (Figure 3).

9

238   Mature neuronal markers, TUJ1 and MAP2 followed similar trend, with significantly higher

239   upregulation in AD-MSCs as compared to that in BM-MSCs. This trend was in discordance

240   with the trend observed in rest of the genes. Hence, it may be derived that 22-HC leads to

241   development of mature neuronal cells from AD-MSCs but does not lead to sub- specification

242   of the same into DAergic neuronal cells, as much as it is to other hMSC types under this

243   study.

244   **22-HC causes increase in the DA neuronal cell specific proteins, corresponding to**

245   **their transcriptional expression**

246   Upon termination of induction period of 14 days, differentiated hMSCs were labelled for

247   neuronal cell specific proteins nestin, MAP-2 and DA sub- specification protein, TH. Induced

248   cells were found to be positive for these markers as compared to uninduced groups. There

249   was a minimum basal expression of MAP2 & TH in uninduced hMSCs, while an upregulated

250   expression was observed post-induction in all the hMSC types (Figure 4). An increased

251   fluorescence intensity in the images support higher expression of MAP2 and TH in

252   differentiated cells. However, among the various hMSCs types, highest fluorescence

253   intensity of these protein markers was observed in case of DP-MSC. Similar trend was also

254   observed when immunoblotting assay was performed in both uninduced and induced

255   groups.

256   **Induction of human Mesenchymal Stem Cells using 22-HC increases the percentage**

257   **of cells positive for dopaminergic neuronal proteins**

258   hMSCs were enumerated for the cells positive for neuronal and DAergic neuronal cells

259   related proteins by flow cytometry. Except in the case of BM-MSCs, there was a significant

260   increase in the percentage of cells positive for nestin (from 9.460 ± 0.964% to 15.16 ±

261   0.479% in AD-MSCs and from 17.84 ± 0.461 to 23.00 ± 1.145% in DP-MSCs). This

262   observation may be attributed to the difference in the mode of action of 22-HC on the three

263   types of hMSCs under this study, especially AD-MSCs. MAP2 positive cells were

264   significantly increased post induction in all hMSC types, under the study. Maximum increase

265   was observed in DP-MSCs (80.20 ± 1.544%), followed by that in BM-MSCs (67.32 ±

266   1.494%) and AD-MSCs (60.14 ± 0.938%). Combining the observations of transcriptional and

267   flow cytometric analysis, it may be stated that 22-HC only led to upregulation of MAP2 gene

268   higher in AD-MSCs as compared to BM-MSCs, but not the number of cells post- induction

269   (Figure 5A (i & ii)) .

270   As far as DAergic specification was concerned, TH positive cells were enumerated. No

271   significant difference was observed in the percentage of cells positive for TH between BM-

272   MSCs and DP-MSCs post neuronal induction (56.94 ± 4.001% and 62.32 ± 4.537%,

273   respectively). Only 33.38 ± 1.148% cells were found to be positive for MAP2 in AD-MSCs.

274   According to the only *in vitro* study by Sachhetti et al., 2009, stating the role of 22-HC in

275   DAergic neuronal specification in hESC lines, there was an increase in TH positive cells from

276   22 to 60% upon treatment with 22-HC; while as high as 62.32 ± 4.537% cells were found to

277   be positive for TH protein in DP-MSCs (considering some specific donor samples, the

278   percentage of TH positive cells was found to be as high as 80%) (Figure 5A (iii)).

279   **Cell Milieu of the induced hMSCs: Presence of Non DA neuronal cells**

280   Cell milieu of the induced hMSCs consisted of cell positive for Non- DAergic proteins like

281   TPH2, S100, GFAP and Ach. Upon enumeration of these markers by flow cytometry, it was

282   observed that the percentage of TPH2 increased significantly in BM-MSC and DP-MSCs

283   only; while it decreased in AD-MSCs (however non- significantly). Similar kind of trend was

284   observed in case of expression of Ach positive cells. However, cells positive for S100

285   increased only in DP-MSCs post-induction. This number was significantly higher than that in

286   AD-MSCs.

287   As per the study reported by Sachhetti et al in 2009, 22-HC leads to a decrease in the GFAP

288   positive cells when hESC lines were treated with it. However, hMSCs do not seem to follow

289   similar trend. Instead, there was a significant increase in the cells positive for GFAP was

290   observed in all hMSC types under study, after induction. This marker also had minimum

291   increase in case of AD-MSCs only. These differences may be attributed to the different

292   pathway(s), being activated by 22-HC in the various types of hMSCs under this study (Figure

293   5B).

11

Increase in the gene expression of transcription factors responsible for maturation and survival of dopaminergic neurons

22-HC also increased the expression of transcription factors (TFs), which are responsible for maturation and survival of the DAergic neurons[11]. Relative fold changes in the expression level of Neurogenin 2 and PitX3 were studied in induced hMSCs, as compared to their uninduced counterparts. It was observed that while BM-MSCs and AD-MSCs showed no significant difference in the expression of PitX3, there was significantly higher upregulation of NGN2 in BM-MSCs (10.68 ± 0.6359 folds) as compared to that in AD-MSCs (8.356 ± 0.2659 folds). Whereas, DP-MSCs maintained the trend with highest upregulation of both the genes (13.75 ± 0.6226 folds of PitX3 and 15.30 ± 0.3902 folds of NGN2 in BM-MSCs, AD-MSCs and DP-MSCs, respectively). These results show that the differentiated hMSCs expressed prototypical midbrain DAergic markers at transcriptional level (Figure 6A (i & ii)).

22-HC improves functional dopaminergic specifications at both gene and protein level

 Functional characterization of differentiated hMSCs was performed at gene level by studying the relative fold change in the mRNA expression level of DAT, Kv4.2 and SCN5A. A constant trend was observed where maximum upregulation of the three genes (10.47 ± 0.3574 folds DAT, 13.97 ± 1.184 folds Kv4.2 and 13.43 ± 0.7351 folds SCN5A) was highest in DP-MSCs, followed by that my BM-MSCs (10.47 ± 0.3574 folds DAT, 13.97 ± 1.184 folds Kv4.2 and 13.43 ± 0.7351 folds SCN5A) and AD-MSCs (7.542 ± 0.4570 folds DAT, 6.944 ± 0.3713 folds Kv4.2 and 7.480 ± 0.4262 folds SCN5A). Extent of upregulation of these functionality related genes was observed to be significantly lower in differentiated AD-MSCs, as compared to that in other hMSC types included in the study (Figure 6B (iii-v)).

Cells positive for DAT and synaptophysin were enumerated by flow cytometry. It was observed that as compared to their control counterparts, the number of cells in differentiated hMSCs positive for both the proteins was significantly higher. While no significant difference was observed between the outcome of BM-MSCs (40.22 ± 2.002%) and AD-MSCs (43.84 ± 0.9368%) for DAT positive cells, DP-MSCs showed significantly higher percentage of DAT positive cells (50.80 ± 0.9121%). Likewise, synaptophysin positive cells were found to be

322 highest in number in differentiated DP-MSCs (54.98 ± 3.751%), followed by that in BM-

323 MSCs (33.02 ± 2.713 %) and AD-MSCs (22.82 ± 1.063%). The difference was significant

324 among all the induced cell types (Figure 6B (i & ii)).

325 Likewise, immunoflorescence staining revealed the presence of both TH and DAT proteins in

326 all the hMSC types post induction. However, no or very little expression of TH protein could

327 be observed in respective hMSC counterparts (Figure 6C).

328 **Ultra- structural changes, contributing towards functional DA neuronal cell**

329 **specification were observed**

330 Neuronal differentiation of hMSCs is associated with the ultra- structural modifications in the

331 mitochondria[12], dense core vesicles or granules (DCVs)[13] , rough endoplasmic reticulum

332 (RER) (Wu et al., 2017), cytoplasmic filamentous condensation[14-15]and endocytotic

333 vesicles[16].

334 Mitochondria are necessary for neural development, survival, activity, connectivity, neuronal

335 movement, axonal pruning and axonal elongation. Several neuro-developmental and neuro-

336 degenerative diseases are associated with mitochondrial dysfunction. Mitochondrial

337 biogenesis was increased in the *in vitro* differentiated DAergic neuronal cells, as is evident

338 by the increase in the number of mitochondria, with globular cup-like structure and evident

339 cristae.

340 There was an increase in the number of DCVs (specialized secretory organelles which are

341 rich in ADP, ATP, ionized calcium, histamine and neurotransmitters) and RER (extend for

342 several microns as linking network throughout highly branched dendrites and help in

343 axonogenesis and dendritogenesis), attributing towards the increased functionality of the

344 neuronal cells. The increase in the number of DCVs and RER is representative of the

345 increased functionality of the cells. More DCVs may be associated with the chemical

346 functionality of the DAergic neurons in terms of secretion of various neurotransmitters and

347 RER may be associated with the formation of proteins required for enhanced functioning of

348 the cell.

349 Cytoskeletal rearrangement controls cell morphology, regulates growth cone motility

350 (contracted area of the neuronal cell from where neurites originate) and axon guidance and

351 forms scaffold for transport of mitochondria and other organelles. Cytoskeletal condensation

352 is an indicative of neuritogenesis and axonogenesis. Cytoskeletal condensation was

353 observed in all types of hMSCs post differentiation. Microtubules were observed to be

354 arranged in a synchronised pattern after differentiation of hMSCs with 22-HC.

355 Apart from these observations, small endocytotic vesicles were also observed in the ultra-

356 structural study of hMSCs post- induction. These vesicles are an indicative of increased

357 neuronal functionality, as they help in neuronal signalling to regulate cell survival, axon

358 growth and guidance, dendritic branching and cell migration (Figure 7A).

359 **Human Mesenchymal Stem Cells show increase in the calcium ion efflux upon**

360 **treatment with 22-HC**

361 Upon treatment with 50mM of KCl, change in the calcium ion concentration in the cytosol of

362 the *in vitro* generated neuronal cells was observed. There was significant difference in the

363 intracellular calcium ion transients in DAergic neuronal cells generated by treatment with 22-

364 HC in all types of hMSCs, as compared to that in respective uninduced MSCs. In DP-MSCs

365 the change in calcium ion transients was observed to be maximum (58.53 ± 2.622%) as

366 compared to the control (17.93 ± 1.726%). This was followed by that in AD-MSCs (52.07 ±

367 1.831% in differentiated and 18.75 ± 1.711% in control) and least calcium ion transients

368 were observed in BM-MSCs (46.98 ± 2.566% in differentiated and 17.57 ± 1.912% in

369 control). The change in the calcium ion concentration in various study groups have been

370 detailed in the Figure 7B (also see supplementary materials for respective videos).

371 **Liver X receptors (α and β) acknowledge 22-HC differently with different types of**

372 **hMSCs:**

373 LXRα and LXRβ are the nuclear receptors, activated by oxygenated derivatives of

374 cholesterol (22-HC being the natural ligand of LXRs), to accomplish various cellular

375 functions like homeostasis of fatty acid, cholesterols and glucose metabolism. Apart from

376   this, some studies support their role in ventral midbrain dopaminergic neurogenesis. These

377   facts led us to investigate the fold change in the transcriptional expression of both LXRα and

378   LXRβ in hMSCs after treatment with 22-HC for DAergic neuronal differentiation. It was

379   observed that these receptors had different level of expression in different hMSC types after

380   differentiation. AD-MSCs showed highest upregulation of LXRα (5.113 ± 0.1272 folds),

381   followed by that in DP-MSCs (3.710 ± 0.6238 folds), with almost negligible changes in case

382   of BM-MSCs. On the contrary, LXRβ was maximally expressed in DP-MSCs (6.645 ± 0.9002

383   folds), followed by that in AD-MSCs and BM-MSCs, with negligible upregulation (Figure 7C).

384   This difference in the expression of both the receptors in different hMSC types may be

385   attributed to their origin, chief function(s) in the living system and pathway(s) that might have

386   been activated, resulting in DAergic neurogenesis.

387   **Discussion:**

388   Evidences demonstrating the role of oxysterols and their nuclear receptors, LXRα and LXRβ

389   in human midbrain dopaminergic neurogenesis have been reported by Sacchetti et. al.,

390   2009. This study was performed on mouse embryo and human embryonic stem cell lines. It

391   had been observed that after adding 22-HC in the induction cocktail, the percentage of cells

392   positive for TH was increased from 22% to 60% with the subsequent decrease in GFAP

393   positive cells. Thus, the study reports the usage of 22-HC for differentiating hESCs *in vitro*

394   and prospects their use in regenerative medicine and drug testing in future.

395   However, use of hESCs in clinical set up is not recommended at present by several

396   researchers and clinicians due to their immunogenicity and tendency to form teratoma.

397   Hence, MSCs are preferred over hESCs for both clinical and drug testing purposes.

398   Considering the translational aspect of the type of stem cells, in our current study, we have,

399   for the very first time, investigated the effect of 22-HC on DAergic neuronal differentiation of

400   human MSCs, derived from human bone marrow, adipose tissue and dental pulp. The

401   investigation included detailed morphological, morphometric, transcriptional, translational,

402   ultra structural and functional characterization of DAergic neuronal cells derived from these

403   various types of hMSCs under the study.

404   We, hereby, report the use of 22-HC as a novel inducer for efficient *in vitro* generation of

405   DAergic neuronal cells from human MSCs, and their detailed multi- factorial characterization.

406   Our protocol reports as high as 80% MAP2 positive cells after induction of DP-MSCs with

407   22-HC. This is the highest percentage of *in vitro* generated mature neurons reported till now,

408   using any of the established protocol[17-24]. Also our protocol yields the maximum number of

409   TH positive DAergic neuronal cells (72% in case of DP-MSCs), as compared to any of the

410   studies reported till date[5,6,9,14,15]. These statistics are the highest among all the reported

411   studies till date, to the best of our knowledge. DP-MSCs showed maximal efficiency of *in*

412   *vitro* generation of DAergic neuronal cells, followed by BM-MSCs and least being in AD-

413   MSCs. The protocol presented in the current study is not only the most efficient, but also

414   cost- effective, as it includes use of only two inducers for generation of DAergic neuronal

415   cells *in vitro* from hMSCs that can be used for translational purpose.

416   We, for the very first time, report the ultra structural changes occurring in hMSCs upon *in*

417   *vitro* differentiation into DAergic neuronal cells. While we have commented on the fine

418   structural changes that appear upon differentiation of hMSCs into neuronal cells, we have

419   also studied in detail the metamorphosis occurring in the cellular components after

420   differentiation.

421   The study also hints that LXRβ plays prominent role in DAergic neurogenesis of hMSCs as

422   compared to LXRα. However, further detailed investigations are required to prove this

423   hypothesis. Also, the noteworthy observations in the difference of transcriptional expression

424   of LXRα in case of AD-MSCs may be reasoned with their constitutive function of fat storage

425   and synthesis of estrogens and androgens[24] in the living system. We hypothesize that LXRα

426   is more upregulated in AD-MSCs, to activate their default pathway(s). As no direct or indirect

427   proof is available to state the exact role of these two receptors in DAergic neurogenesis and

16

428 differential effect on various types of embryonic or adult stem cells, our hypothesis, needs

429 further experimental validation.

430 In conclusion, our study provides comprehensive and robust evidences to state the role 22-

431 HC in generating functional DAergic neuronal cells from hMSCs of varied origins. These in

432 vitro generated cells showed DAergic neuronal specifications at morphological,

433 morphometric, ultrastructural, transcriptional, translational and functional levels, with

434 tremendous clinical and pharmacological application. As reported by Sacchetti et al in 2009,

435 functions of LXRs are conserved in human cells. Our study gives the first evidence that

436 oxysterols (22-HC) causes DAergic neurogenesis and leads to their morphological, genetic

437 and functional changes. Our report has also paved the path to investigate the disparate

438 effect of 22-HC on the three types of hMSCs taken under the study. AD-MSCs have shown

439 off beat expression of LXRα as compared to that of LXRβ. These pathways need to be

440 investigated further to have an insight of the mode and mechanism of action of 22-HC for

441 midbrain DAergic neurogenesis.

442 **References:**

443 1. Courtney and Gary E. Landreth. LXR Regulation of Brain Cholesterol: From
444 Development to Disease Rebecca. Trends in Endocrinology & Metabolism 2016,
445 Volume 27;6:404–414.
446 2. Bozek, K., Wei, Y., Yan, Z., Liu, X., Xiong, J., Sugimoto, M., et al. Organization and
447 evolution of brain lipidome revealed by large-scale analysis of human, chimpanzee,
448 macaque, and mouse tissues. Neuron 2015; 85; 695–702.
449 3. Chan, R.B., Oliveira, T.G., Cortes, E.P., Honig, L.S., Duff, K.E., Small, S.A., et. al.
450 Comparative lipidomic analysis of mouse and human brain with Alzheimer disease. J.
451 Biol. Chem.; 2012; 287, 2678–2688.
452 4. Puchkov, D., and Haucke, V. Greasing the synaptic vesicle cycle by membrane
453 lipids. Trends Cell Biol.; 2013; 23, 493–503.
454 5. Paola Sacchetti, Kyle M Saousa, Anita C Hall, Ernest Arenas et al. Liver X Receptors
455 and oxysterols promote ventral midbrain neurogenesis in vivo and in human
456 embryonic stem cells. Cell Stem Cell 2009; 5; 409- 419.
457 6. Manisha Singh, Anupama Kakkar, Rinkey Sharma, O.P. Kharbanda, Nitika Monga,
458 Manish Kumar, et al. Synergistic Effect of BDNF and FGF2 in Efficient Generation of
459 Functional Dopaminergic Neurons from human Mesenchymal Stem Cells. Sci Rep.
460 2017, Sep 4;7(1):10378. doi: 10.1038/s41598-017-11028-z.
461 7. Krishan G. Jain, Sujata Mohanty, Alok R. Ray, Rajesh Malhotra & Balram Airan,
462 Culture & differentiation of mesenchymal stem cell into osteoblast on degradable
463 biomedical composite scaffold: In vitro study. Indian J Med Res 2015;142;747-758.
464 8. Sudip Sen, Shweta Sharma, Anand Gupta, Noopur Gupta, Himi Singh, Ajoy Roy
465 Choudhury, et al. Molecular characterization of explant cultured human oral mucosal

epithelial cells. Investigative Opthalmology and Investigative Science (IOVS) 2011; 52; 9548-9554.

9. Sushmita Bose Nandy, Sujata Mohanty, Manisha Singh, Madhuri Behari and Balram Airan. Fibroblast Growth Factor-2 alone as an efficient inducer for differentiation of human bone marrow mesenchymal stem cells into dopaminergic neurons. Journal of Biomedical Science 2014, 21:83 doi:10.1186/s12929-014-0083.

10. Trzaska Katarzyna A., King CC, Li KY, Kuzhikandathil EV, Nowycky MC, Ye JH, et al. Brain-derived neurotrophic factor facilitates maturation of mesenchymal stem cell-derived dopamine progenitors to functional neurons. Journal of Neurochemistry 2009;110;3;1058–1069.

11. Sunghoi Hong, Sangmi Chung, Kaka Leung, Insik Hwang, Jisook Moon and Kwang-Soo Kim. Functional Roles of Nurr1, Pitx3, and Lmx1a in Neurogenesis and Phenotype Specification of Dopamine Neurons During In Vitro Differentiation of Embryonic Stem Cells. Stem Cells and Development 2013;00;00,DOI: 10.1089/scd.2013.0406.

12. M Agostini, F Romeo, S Inoue, MV Niklison-Chirou, AJ Elia, D Dinsdale, et al. Metabolic reprogramming during neuronal differentiation Cell Death and Differentiation 2016; 23; 1502–1514.

13. Maria Luisa Malosio, Tiziana Giordano, Andrea Laslop and Jacopo Meldolesi. Dense-core granules: a specific hallmark of the neuronal/neurosecretory cell phenotype. Journal of Cell Science 2004;117; 743-749.

14. Yumei Wu, Christina Whiteus, C. Shan Xu, Kenneth J., Hayworth, Richard J. Weinber, Harald F. Hess et al. Contacts between the endoplasmic reticulum and other membranes in neurons. PNAS 2017, doi: 10.1073/pnas.1701078114.

15. Claudia Compagnucci, Fiorella Piemonte, Antonella Sferra, Emanuela Piermarini and Enrico Bertini. The cytoskeletal arrangements necessary to neurogenesis. Oncotarget, 2016; Apr 12;7(15):19414-29.

16. Robert G. Patton, Kai Simons, and Carlos G. Dotti. Axonal and Dendritic Endocytic Pathways in Cultured Neurons. The Journal of Cell Biology 1992;119;1, October 123-137.

17. Trzaska KA, Kuzhikandathil EV, Rameshwar P. Specification of a dopaminergic phenotype from adult human mesenchymal stem cells. Stem Cells 2007, 25: 2797-2808.

18. So Young Chun, Shay Soker, Yu-Jin Jang, Tae Gyun Kwon, and Eun Sang Yoo. Differentiation of Human Dental Pulp Stem Cells into Dopaminergic Neuron-like Cells in Vitro. J Korean Med Sci 2016, 31: 171-177.

19. Keum Seok Bae, Joon Beom Park, Hyun Soo Kim, Dae Sung Kim, Dong Jun Park & Seong Joon Kang. Neuron like differentiation of bone marrow derived mesenchymal stem cells. Yonsei Medical Journal 2010;52(3):401-412.

20. Anna E. Petschnik, Benjamin Fell, Stephan Tiede, Jens K. Habermann, Ralph Pries, Charli Kruse, et al. A novel xenogenic co- culture system to examine neuronal differentiation capability of various adult human stem cells. Plos One 2011, vol.6;9; e24944.

21. Sujeong Jang, Hyong Ho Cho, Yong Bum Cho, Jong Seong Park & Han Seong Jeong. Functional neural differentiation of human adipose tissue derived stem cells using bFGF and Forskolin. BMC Cell Biology 2010,11:25.

22. Myung Soo Cho, Young-Eun Lee, Ji Young Kim, Seungsoo Chung, Yoon Hee Cho, Dae-Sung Kim, et al. Highly efficient and large scale generation of functional dopamine neurons from human embryonic stem cells. PNAS 2008; vol. 105, no. 9; 3392- 3397.

23. Asuka Morizane, Vladimer Darsalia, M. Oktar Guloglu, Tord Hjalt, Manolo Carta, Jia Yi Li et al. A simple method for large scale generation of dopamine neurons from human embryonic stem cells. J. of Neuroscience Research 2010; 88:3467-3478.

24. Linda R. Nelson and Serdar E. Bulun. Estrogen production and action. J Am Acad Dermato. 2001, Volume 45; 3: S116–S124.

Acknowledgement:

Authors' Contributions:

Basic stem cell characterization studies: MS. Conceived, planned and performed the experiments: MS and SM; Electron microscopic studies (conceptualization, performance and analysis): SM, AKD and MS; Data Analysis: MS and SM; Contributed reagents/materials/analysis tool: SM and MS; Arrangement of research funds: BA and SM; Functional studies: MS and SM; Manuscript writing: MS and SM.

Additional Information: Not applicable

Competing Financial Interests: All the authors declare NO competing financial interests.

538   **Figure Legends:**

539   **Figure 1: Morphological changes occurring in hMSCs during various time points (Day**

540   **3, day 5, day 10 and day 12) of neuronal induction** i) BM- MSC; ii) AD- MSC; iii) DP-

541   MSC. The morphology of hMSCs has changed from spindle shaped to perikaryl.

542   Appearance of neuronal morphology starts appearing from 6-7th day of induction.

543   **Figure 2: Morphological and Morphometric Characterization of differentiated hMSCs:**

544   **A. Scanning Electron Microscopic observations depicting minute morphological**

545   **changes occurring in hMSCs after neuronal induction** i) Morphology of hMSCs has

546   changed from spindle shaped to perikaryl. Terminals of the cells show appearance of minute

547   neurites'-like structures, which enhance cell-cell interactions. Here, # denotes neuritis, *

548   denotes cell body and •☐denotes axon hillock ii) Magnified images of differentiated cells,

549   showing the appearance of axon- hillock, neuritogenesis and appearance of terminal

550   neurites, facilitating cellular responses and interactions. **B. Morphometric Analysis of the**

551   **neuronal cells generated in vitro by using 22-HC** i) Area of the cell body under various

552   study groups. The graph depicts significant difference in this parameter between AD-MSC

553   and DP-MSC; ii) Average length of neurites of differentiated cells after induction. The graph

554   shows that the difference in the length of neurites is significantly higher in differentiated DP-

555   MSCs, as compared to that in BM-MSCs and AD-MSCs; iii) Length of axons of cells after

556   neuronal induction. The graph shows that the difference in the axonal length follows similar

557   trend as average length of neurites; iv) Ratio of axon to cell body of cells under various study

558   groups. The graph depicts that this morphological parameter of neuronal cells shows

559   significant difference among all the three types of hMSCs under the study. For all the

560   parameters under the study, five different samples of each type of hMSC were taken (n= 40

561   for each study group). Data was analysed by three independent observers.

562   **Figure 3: qRT- PCR transcriptional analysis of differentiated hMSCs** i) Relative fold

563   change in mRNA expression of nestin in induced hMSCs, as compared to respective

564 uninduced counterparts; ii) Relative fold change in mRNA expression NF in induced hMSCs,

565 as compared to respective uninduced counterparts; iii) Relative fold change in mRNA

566 expression of TUJ1 in induced hMSCs, as compared to respective uninduced counterparts;

567 iv) Relative fold change in mRNA expression of MAP2 in induced hMSCs, as compared to

568 respective uninduced counterparts; v) Relative fold change in mRNA expression of TH in

569 induced hMSCs, as compared to respective uninduced counterparts.

570 **Figure 4: Expression of neuronal cell associated proteins in differentiated hMSCs: A.**

571 **Immunoflorescence Assay** showing expression of nestin, MAP2 and TH protein

572 expression in hMSCs pre- and post- differentiation into DAergic neuronal cells; **B.**

573 **Immunoblotting Assay** for expression of neuronal and DA neuronal cells associated

574 proteins (MAP2 and TH) in uninduced and differentiated hMSCs.

575 **Figure 5: Flow cytometric analysis for cell enumeration: A.** i) Graph depicting the

576 number of nestin positive cells pre- and post- neuronal induction. DP- MSC have maximum

577 number of nestin positive (p*), cells at the beginning of experiments, followed by those in

578 AD-MSC and BM-MSC; ii) Graph depicting the number of MAP2 positive cells pre- and post-

579 neuronal induction. (p*); iii) Graph depicting the number of TH positive cells pre- and post-

580 neuronal induction. **B. Flow cytometric analysis for cell enumeration of non- DAergic**

581 **neuronal cells in the cell- milieu, pre- and post- neuronal differentiation of hMSCs.**

582 **Figure 6: Assessment of genes and proteins associated with neuronal functionality:**

583 **A. qRT- PCR mRNA transcriptional analysis** of differentiated hMSCs for genes associated

584 with functional behaviour of DAergic neuronal cells; **B. Flow cytometric enumeration** of

585 number of cells positive for (i) Dopamine transporter protein, a protein responsible for

586 release of dopamine neurotransmitter through vesicles and (ii) Synaptophysin protein which

587 is responsible for the formation of synapse between two neuronal cells; **C.**

588 **Immunoflorescence assay** to show the expression of TH and DAT proteins in hMSCs pre-

589 and post- differentiation into DAergic neuronal cells.

590   **Figure 7: A. Transmission Electron Microscopic observations depicting ultra-**

591   **structural changes occurring in hMSCs after neuronal induction** i) Ultra- structural

592   composition of hMSCs has changed on various parameters. There was observed increased

593   mitogenesis, increase in dense core vesicles, rough endoplasmic reticulum, cytoskeletal

594   condensation and endocytotic vesicles. The genesis of these cellular organelles may be

595   associated with the increased functionality of the terminally differentiated hMSCs. ii) Part of

596   the cell membrane showing the release of vesicles (probably synaptic vesicles) by

597   exocytosis, contributing to the chemical functionality of the DAergic neuronal cells. In this

598   figure, # represent rough endoplasmic reticulum, * represent mitochondria, ☐ represent

599   endocytotic vacuoles, • represent cytoskeletal condensation and ☐ represent dense core

600   vesicles. å represent the exocytotic vesicles released from differentiated DP-MSCs. **B.**

601   **Calcium ion imaging analysis by Fura red- AM ratiometric dye:** i) Line graphs showing

602   the changes in the Ca2+ transients upon depolarization with KCl in cells of all study groups.

603   The uninduced hMSC did not show any change in the intracellular Ca2+ concentration after

604   depolarization. The red arrows indicate the time point of addition of KCl for depolarization in

605   the cell culture; ii) Graph showing the change in the percentage increase in the calcium ion

606   concentration in hMSCs obtained from various tissue sources after DAergic neuronal

607   induction by 22-HC. **C. qRT-PCR transcriptional Analysis of liver X receptors α and β**,

608   which are the main nuclear receptors, reported till date, responsible for DAergic

609   neurogenesis by oxysterols (22-HC). Our results suggest a prominent role of LXRβ in

610   DAergic neurogenesis with 22-HC as inducer DP-MSC only. LXRα does not seem to play

611   much distinct role in this process. However, least upregulation of both of these genes was

612   observed in BM-MSCs.

.No.	Gene	Primer sequence	Annealing Temperature	Amplicon size
1.	GAPDH	5'-GACAAGCTTCCCGTTCTCAG-3' 5'-GAGTCAACGGATTTGGTCGTCGT-3'	57.0°C	198bps
2.	Nestin	5'-GCCCTGACCACTCCAGTTTA-3' 5'-GGAGTCCTGGATTTCCTTCC-3'	57.5°C	198bps
3.	NF	5'-AGGGATCCAGGAAGGAAGA -3' 5'-TGACAACGCCTTTCTCCTCT-3'	55.0°C	117bps
4.	MAP2	5'-ACTCCCTCTACAGCTGAGCC-3' 5'-TGCCCAAACAAAGCTAGGGG-3'	55.2°C	182bps
5.	Tuj1	5'-GCAAGGTGCGTGAGGAGTAT-3' 5'-TCGTTGTCGATGCAGTAGGT-3'	60.9°C	157bps
6.	TH	5'-GGTCGCGCTGCCTGTACT-3' 5'-TCATCACCTGGTCACCAAGTT-3'	53.9°C	125bps
7.	Ngn2	5'-ACCACCACAACACACGAGAC-3' 5'-TTGCTGTGACTTTGGCCTGT-3'	62.0°C	196bps
8.	Pitx3	5'-GGAGTCTGCCTGTTGCAGGA-3' 5'-CGAGGCCTTTTCTGAGTCGC-3'	59.9°C	173bps
9.	DAT	5'-TCCAGTGGAGACAGCTCGG-3' 5'-GCTGAAGTAGAGCAGCACGA-3'	59.1°C	174bps
10.	Kv4.2	5'-AGTATTTGCTTCGCCTGGCT-3' 5'-TCGGAGTGTGACAAAGGCTC-3'	60.4°C	152bps
11.	SCN5A	5'-CAGGCCTTTGACGTCACCAT-3' 5'-GAGCACAGTGCCTGTGAAGAT-3'	56.7°C	147bps
12.	LXRα	5'- CAGCCCGGAACAACTGGGCA-3' 5'- AGCATCACCGTGACTCGAAGCC-3'	63.2°C	99bps
13.	LXRβ	5'- AGCACAGACTGGGGCGTCCT-3' 5'- GCCCCACAGGTGACTGCGAC-3'	63.5°C	103bps

1

	Uninduced	22-HC	Uninduced	22-HC	Uninduced	22-HC
D3						
D5						
D10						
D12						
	BM-MSC		AD-MSC		DP-MSC	

A.

	BM- MSC	AD- MSC	DP- MSC

(i)

Uninduced

22-HC

(ii)

B.

(i)

(ii)

(iii)

(iv)

A.

	Uninduced	22-HC	Uninduced	22-HC	Uninduced	22-HC
Nestin						
MAP2						
TH						
	BM-MSC		AD-MSC		DP-MSC	

B.

	Uninduced			22-HC		
	BM-MSC	AD-MSC	DP-MSC	BM-MSC	AD-MSC	DP-MSC
TH, 57kDa						
MAP2, 70kDa						
β Actin, 42kDa						

A.

(i) Nestin

(ii) MAP2

(iii) TH

B.

TPH2

S100

GFAP

Ach

A.

(i) NGN2 p<0.0001

(ii) PitX3

(iii) SCN5A

(iv) Kv4.2

(v) DAT

B.

i) DAT

ii) Synaptophysin

Legend: UI, 22-HC

C.

BM-MSC   AD-MSC   DP-MSC

Uninduced

22-HC

A.

(i)

BM- MSC   AD- MSC   DP- MSC

Uninduced

22-HC

(ii)

B.

(i)

BM-MSC   AD-MSC   DP-MSC

Uninduced

22-HC

(ii)

% Increase in Ca ion concentration

UI BM-MSC   BM-MSC (22-HC)   UI AD-MSC   AD-MSC (22-HC)   UI DP-MSC   DP-MSC (22-HC)

Study Groups

ns   *   **   ***

C.

LXRα   LXRβ

Fold Change in gene expression

BM-MSC   AD-MSC   DP-MSC

Cell Type