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REGULAR ARTICLE

Human orbital adipose tissue-derived mesenchymal stem cells possess neuroectodermal differentiation and repair ability

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Abstract

Mesenchymal stem cells (MSCs) are used extensively in cell therapy for repair and regeneration of several organs and tissues. Cell therapy is a valuable option to treat neurodegenerative diseases and MSCs have been shown to improve neuronal function through direct differentiation or secretion of neurotrophic factors. In the present study, we isolated and characterized stem cells from medial and central orbital adipose tissue and found that they could be grown in a monolayer culture. The orbital adipose tissue-derived cells were identical to bone marrow-derived MSCs in their cell surface marker expression, gene expression and multilineage differentiation abilities. The orbital adipose-derived MSCs (OAMSCs) express several neurotrophic factors, possess neuroectodermal differentiation ability and secreted factors from OAMSCs abrogated neuronal cell damage induced by oxidative stress. Thus, OAMSCs might be a valuable cell source for treatment of neurological diseases and to reverse oxidative damage in the neuronal cells.

Keywords Stem cells · Neuroprotection · Cell therapy · Neurodegenerative disease · Oxidative damage

Introduction

Mesenchymal stem cells (MSCs) are used for several therapeutic applications to repair and regenerate damaged tissues especially in several cases of neuronal damage. Due to their extensive migration ability to the site of injury (Walker et al. 2009), secretion of neurotrophic factors that promote the survival and regeneration of neuronal cells (Teixeira et al. 2013), MSCs are considered as good therapeutic agents for neuronal repair. Although the resident neural stem cells of the brain promote regeneration, neural stem cells are also subjected to pathological conditions (Li et al. 2008) that affect the neuronal function. In addition, there are conflicting reports on the role of resident brain perivascular-derived MSCs in their neuronal differentiation potential (Lojewski et al. 2015; Paul et al. 2012), which necessitates exogenous administration of stem cells with neuroregenerative potential for efficient neuronal repair.

MSCs generally originate from the mesoderm but the neural crest portions of the head, the facial jaws and associated connective tissues like those of the ocular tissue also give rise to MSCs (Billon et al. 2007; Branch et al. 2012). Adipose tissue found in all parts of the human adult are derived from the mesoderm but the orbital adipose system contains cells that are derived from the ectoderm, essentially the neural crest but not the mesoderm (Johnston et al. 1979) and thus might have high differentiation ability into neuroectodermal lineage cells. Adipose tissue-derived MSCs have high proliferation capacity and studies have reported that the proliferation capacity of these cells did not decline with the age of the patient, which makes it a superior alternative to bone marrow-derived MSCs (Beane et al. 2014; Zuk et al. 2001). Subcutaneous adipose tissue including orbital adipose tissue was found to

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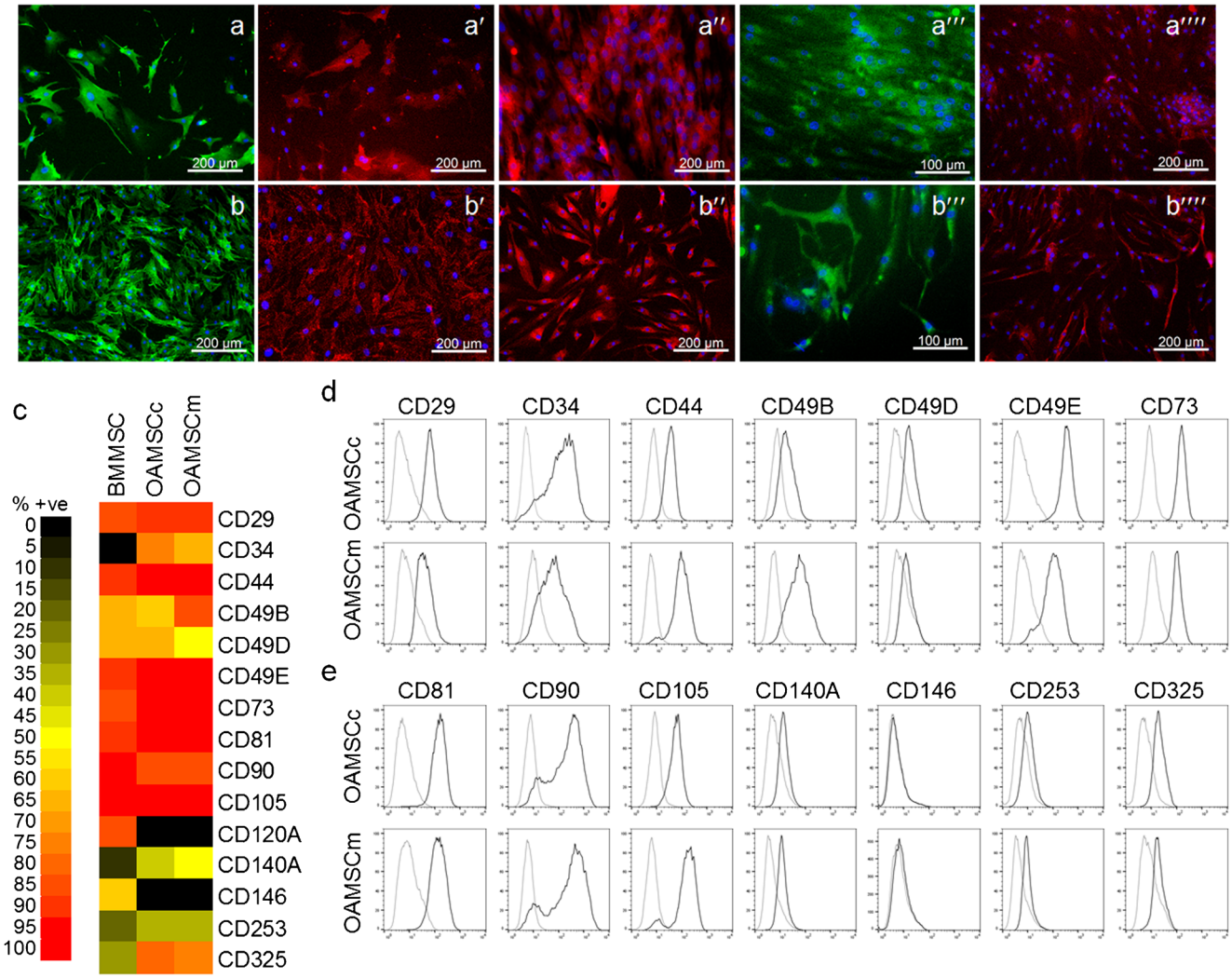
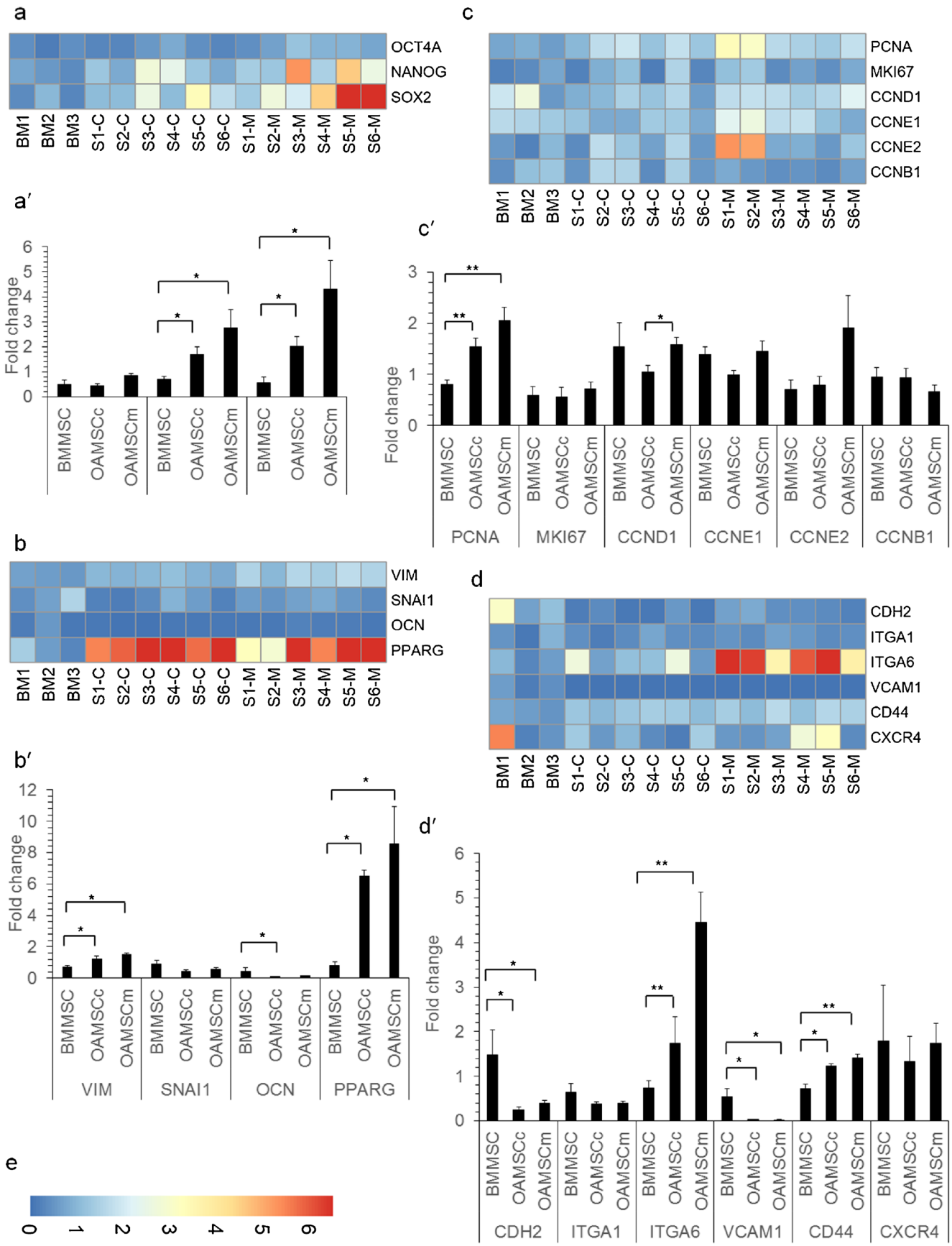


Fig. 1 Cell surface marker expression in **a**–**a⁗** OAMSCc and **b**–**b⁗** OAMSCm. Immunocytochemical staining for cell surface receptors **a**, **b** CD44, **a′**, **b′** CD73, **a″**, **b″** CD90, **a‴**, **b‴** CD140a and **a⁗**, **b⁗** SSEA4. Representative microscopic images are shown. **c** Heat map showing percentage positive (%+ve) expression of cell surface markers in OAMSCs compared to BMMSCs as analyzed by flow cytometry. Average expression value is shown. $n = 5$–9 for BMMSCs and $n = 9$–12 for OAMSCs. **d**, **e** Representative flow cytometry histograms showing the expression of cell surface markers **d** CD29, CD34, CD44, CD49B, CD49D, CD49E, CD73; **e** CD81, CD90, CD105, CD140A, CD146, CD253 and CD325. Gray line represents the isotype control and the black line represents the stained sample

yield MSCs with mesodermal and neuroectodermal differentiation ability (Chen et al. 2014; Frese et al. 2016; Korn et al. 2009) and has also been used in clinical medicine. Some of the advantages of utilizing ocular adipose tissue as a source of MSCs are the high success rate of isolation of MSCs compared to umbilical cord blood (Kern et al. 2006) and there are no ethical issues in utilizing the sample for MSCs isolation since the tissue after blepharoplasty is usually discarded. Unlike subcutaneous adipose tissue, MSCs can be isolated from ocular adipose tissue without enzymatic digestion. OAMSCs also express several neurotrophic and neuroregenerative factors that might help in neuronal repair and regeneration. In the current study, we show that MSCs isolated from ocular adipose tissue have gene expression and a cell surface marker profile similar to those of bone marrow-derived MSCs (BMMSCs) and show mesodermal, neuroectodermal differentiation abilities and neuroprotective effect through secretion of neurotrophic factors.

Fig. 2 Expression analysis of self-renewal, mesenchymal, cell cycle and adhesion-related genes in BMMSCs, OAMSCc and OAMSCm. **a**–**d** Heatmaps and **a′**–**d′**) mRNA expression of different gene sets. Total RNA was extracted from OAMSCc and OAMSCm samples and reverse transcribed into cDNA. mRNA expression levels of indicated genes were analyzed by real-time PCR. Values were normalized to GAPDH expression levels in the respective samples. Values represented in the graphs are mean ± SE, $n = 6$–10 independent samples for OAMSCs and $n = 4$ independent samples for BMMSCs, each sample was analyzed in duplicates. $^{*}p < 0.05$, $^{**}p < 0.005$. In the heatmap, BM(1–3) represents BMMSCs samples ($n = 3$ independent samples) and S(1–6)-C represents OAMSCc and S(1–6)-M represents OAMSCm ($n = 6$ independent samples each)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OCT4A
NANOG
SOX2
BM1 BM2 BM3 S1-C S2-C S3-C S4-C S5-C S6-C S1-M S2-M S3-M S4-M S5-M S6-M
a′
Fold change
BMMSC OAMSCc OAMSCm
b
VIM
SNAI1
OCN
PPARG
b′
c
PCNA
MKI67
CCND1
CCNE1
CCNE2
CCNB1
c′
PCNA MKI67 CCND1 CCNE1 CCNE2 CCNB1
d
CDH2
ITGA1
ITGA6
VCAM1
CD44
CXCR4
d′
CDH2 ITGA1 ITGA6 VCAM1 CD44 CXCR4
e
0 1 2 3 4 5 6

Materials and methods

Chemicals and reagents

Dulbecco's modified eagle's medium (DMEM), Hank's balanced salt solution (HBSS), fibronectin, Oil red O, alizarin red S, Safranin O, forskolin and 3-isobutyl-1-methylxanthine, 2-mercaptoethanol, dexamethasone, indomethacin, insulin, β-glycerophosphate and ascorbic acid were purchased from Sigma-Aldrich (Darmstadt, Germany). Tissue culture plates and flasks were from Eppendorf (Hamburg, Germany) and glass bottom dishes for imaging (μ-dish) were from ibidi (Germany). Anti-SSEA4 antibody (clone MC-813-70) was from Chemicon. Fluorescent-conjugated anti-human antibodies for flow cytometry and purified antibodies for immunocytochemistry for CD44 (clone G44-26), CD73 (clone AD2), CD90 (clone 5E10) and CD140a (clone αR1) were from BD biosciences (USA). Anti-Oct4 antibody was from Santa Cruz (Dallas, USA). Anti-human antibodies against CD34 (Clone 581) and tubulin β–III (clone AA10), fetal bovine serum (FBS), recombinant human brain-derived neurotrophic factor (BDNF), recombinant human fibroblast growth factor 2 (FGF2), B27, chondrogenic differentiation media, neurobasal media and neuronal supplements were purchased from Thermofisher Scientific (USA).

Samples

Adipose tissue was retrieved from 30 eyelids of 15 patients who underwent routine upper eyelid blepharoplasty either for correction of dermatochalasis or an esthetic indication. Orbital fat that was discarded during the routine blepharoplasty procedure was utilized for this study after informed consent. The surgical technique involved excision of a measured amount of upper lid skin, orbicularis muscle and post septal orbital fat pads after fashioning the skin incision. The post septal preaponeurotic orbital fat was excised by making small incisions over the orbital septum to provide direct access to orbital fat pads. These layers of orbital fat, yellow-colored central fat pad and the white medial fat pad were teased out gently through the small openings of the orbital septum. The orbital fat excision was performed very carefully with sharp scissors to prevent any damage to the adipose cells. This was followed by skin closure with a 6O polypropylene suture. Details of the samples used for the study are given in Electronic Supplementary Material, Table S1. Both medial and central adipose tissues were collected separately in cold HBSS, transported on ice to the lab and processed on the same day. Tissues were mechanically dissociated, treated with or without collagenase and plated in DMEM containing 10% fetal bovine serum (FBS). Colonies of spindle-shaped adherent cells were obtained 1–2 weeks after plating and they were passaged regularly before reaching 90% confluence. Cells at passage 3–5 were used for the experiments.

Flow cytometric analysis

The expression of cell surface markers in both central (OAMSCc) and medial (OAMSCm) orbital adipose tissue-derived MSCs was analyzed by flow cytometry. The cells were trypsinized and stained with indicated fluorescent-conjugated antibodies and analyzed with the flow cytometer (BD FACS calibur). Propidium iodide (Sigma) was added for live/dead separation.

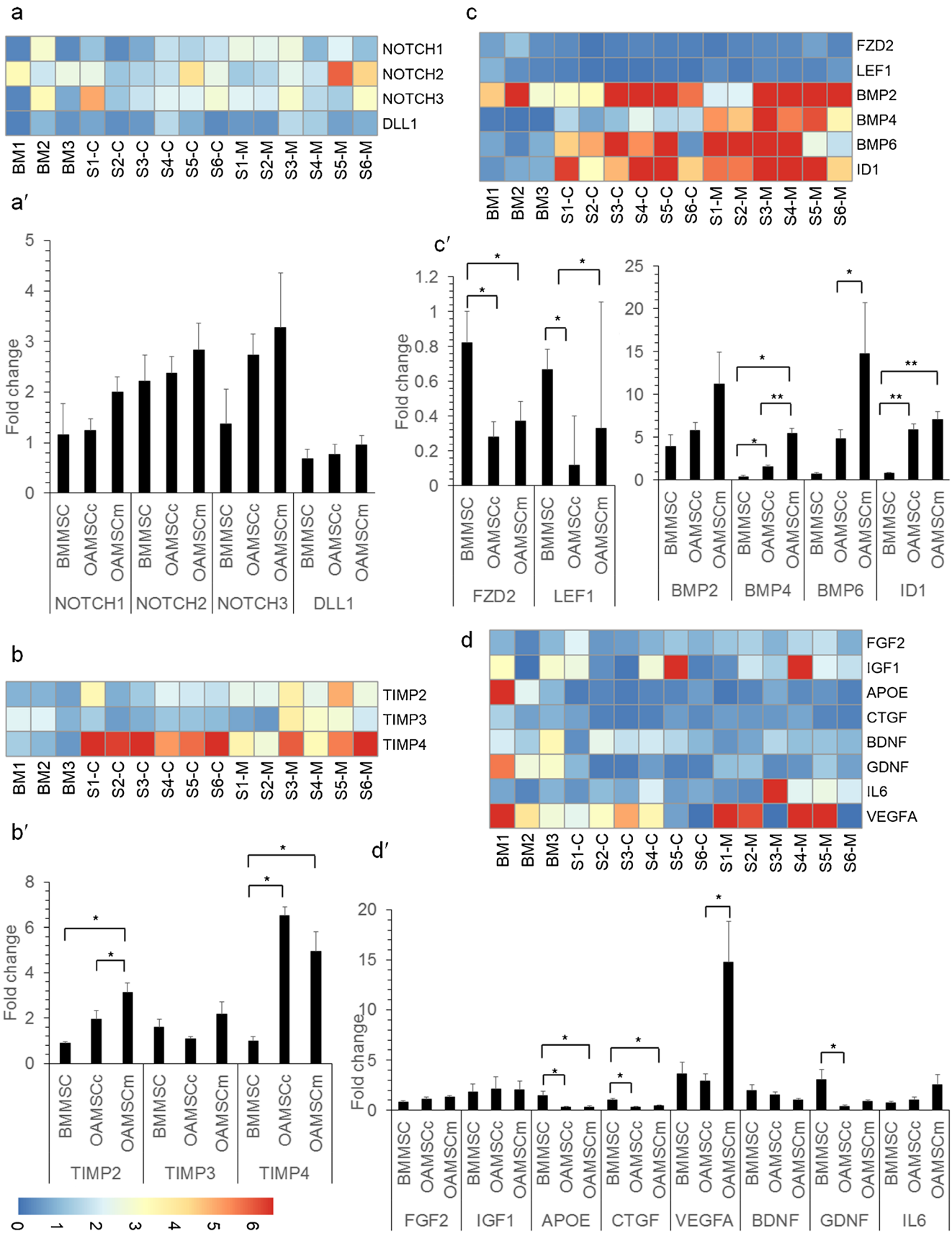
Gene expression analysis

Gene expression analysis was performed by real-time PCR as described previously (Somaiah et al. 2018). Briefly, total RNA was extracted using TriZol reagent (Thermo Fisher Scientific). RNA was reverse transcribed using a high capacity cDNA synthesis kit (Thermo Fisher Scientific) and Oligo dT primers. Expression levels of indicated genes were analyzed by real-time PCR using Power SyBr Green reagents (Thermo Fisher Scientific). Each sample was analyzed in duplicate. The expression levels of each gene were normalized to their respective GAPDH (glyceraldehyde 3-phosphate dehydrogenase) expression levels. The fold change in the expression levels of each gene compared to the respective control was calculated using the ΔΔCt method.

Mesodermal differentiation

OAMSCc and OAMSCm were differentiated into adipogenic, osteogenic and chondrogenic cell types according to the standard protocols. The cells were differentiated into adipocytes and osteoblasts with respective differentiation media as reported earlier (Somaiah et al. 2015). Briefly, the cells were cultured in media supplemented with dexamethasone, isobutyl methyl xanthine, indomethacin and insulin for adipogenic differentiation and dexamethasone, β-glycerophosphate and ascorbic

Fig. 3 Expression analysis of Notch, BMP signaling and secreted factor-related genes in BMMSCs, OAMSCc and OAMSCm. **a**–**d** Heatmaps and **a′**–**d′** mRNA expression profiles of different gene sets analyzed by real-time PCR. Values were normalized to GAPDH expression levels in the respective samples. Values represented in the graphs are mean ± SE, $n = 6$–10 independent samples for OAMSCs and $n = 4$ independent samples for BMMSCs, each sample was analyzed in duplicates. $*p < 0.05$, $**p < 0.005$. In the heatmap, BM(1–3) represents BMMSC samples ($n = 3$ independent samples) and S(1–6)-C represents OAMSCc and S(1–6)-M represents OAMSCm ($n = 6$ independent samples each)


a
NOTCH1
NOTCH2
NOTCH3
DLL1
BM1 BM2 BM3 S1-C S2-C S3-C S4-C S5-C S6-C S1-M S2-M S3-M S4-M S5-M S6-M
a′
Fold change
BMMSC OAMSCc OAMSCm
NOTCH1 NOTCH2 NOTCH3 DLL1
b
TIMP2
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TIMP2 TIMP3 TIMP4
c
FZD2
LEF1
BMP2
BMP4
BMP6
ID1
c′
FZD2 LEF1 BMP2 BMP4 BMP6 ID1
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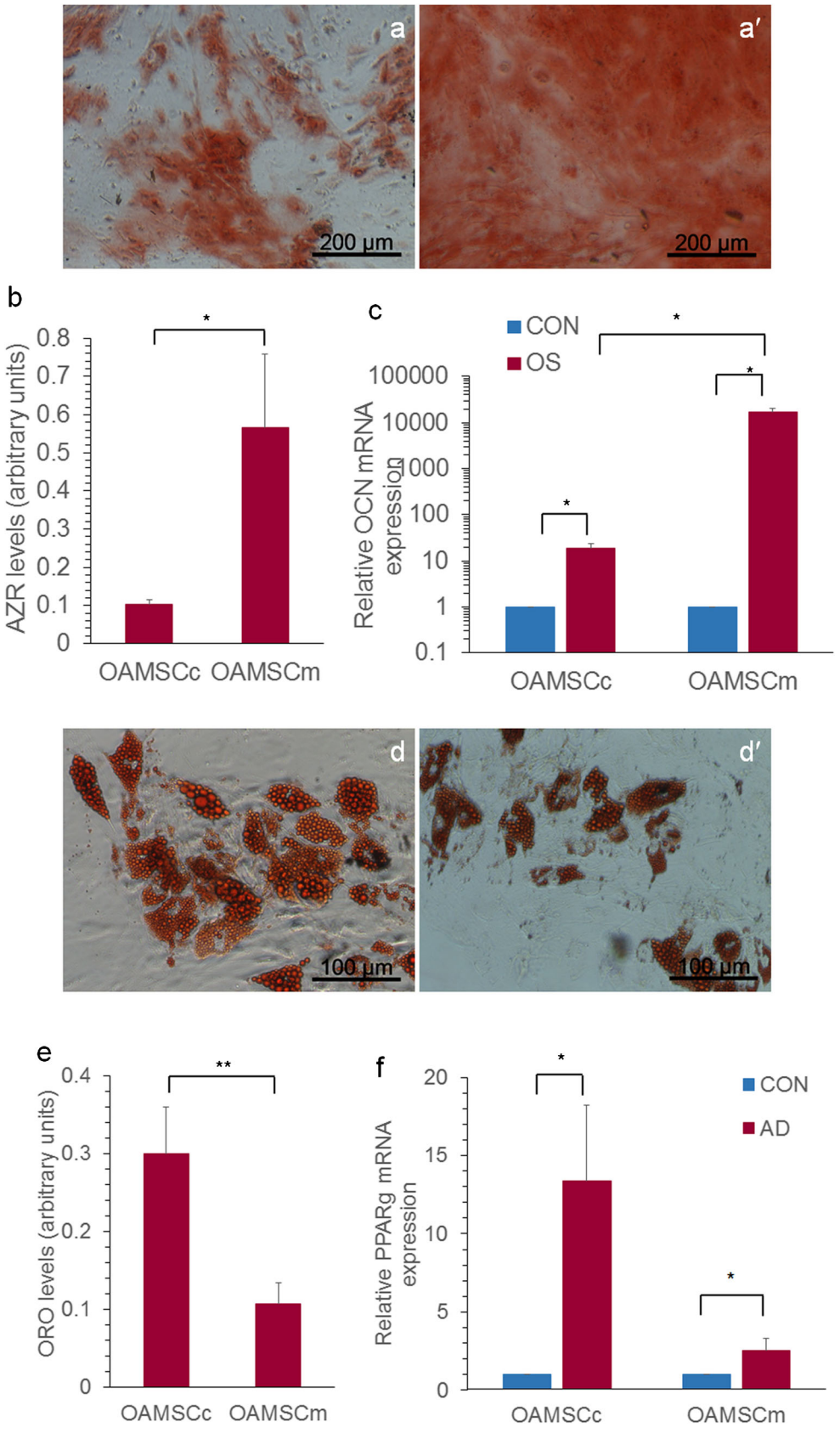
a
200 μm
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AZR levels (arbitrary units)
0.8
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*
OAMSCc OAMSCm
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Relative OCN mRNA expression
100000
10000
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10
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0.4
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Relative PPARg mRNA expression
20
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◀ **Fig. 4** Mesodermal differentiation of OAMSCs. OAMSCc and OAMSCm were induced to differentiate into **a**, **a′**, **b**, **c** osteoblasts and **d**, **d′**, **e**, **f** adipocytes by addition of respective differentiation media. **a**, **a′** Osteogenic differentiation of **a** OAMSCc and **a′** OAMSCm was detected by staining with alizarin red (AZR) and **b** quantified by measuring the absorbance at 562 nm. **c** *OCN* transcript levels in OAMSCc and OAMSCm differentiated into osteoblasts (OS) or respective undifferentiated control cells (CON) were determined by real-time PCR. **d**, **d′** Adipogenic differentiation of **d** OAMSCc and **d′** OAMSCm was detected by staining with oil-red O (ORO) and (e) quantified by measuring the absorbance at 500 nm. **f** *PPARG* transcript levels in OAMSCc and OAMSCm differentiated into adipocytes (AD) or respective undifferentiated control cells (CON) were determined by real-time PCR. Representative microscopic images are shown. Values are mean ± SE, $n = 3$–4 independent samples, each sample had 3–4 technical replicates. $^{*}p < 0.05$, $^{**}p < 0.005$

acid for osteogenic differentiation. Osteogenic differentiation was detected by staining with alizarin red S and adipogenic differentiation was analyzed by oil red O staining. Osteogenic and adipogenic differentiation was also confirmed by analyzing the expression of genes OCN (osteocalcin) and PPARG (peroxisome proliferator-activated receptor gamma), respectively.

Chondrogenic differentiation was performed as reported earlier (Mawrie et al. 2016). Micromass cultures were generated in a multi-well tissue culture plate according to the manufacturer's instructions (Thermo Fisher scientific). Media was changed every 3 days and differentiation into chondrogenic cells was analyzed with Safranin O staining and detecting SOX9 expression after 21 days.

Neuroectodermal differentiation

Neuroectodermal differentiation was performed as per the earlier reported method (Mawrie et al. 2016). The cells were first pre-induced for 24 h in media containing 2% FBS, FGF2, B27, Forskolin, 3-isobutyl-1-methylxanthine (IBMX) and 2-mercaptoethanol (2-ME). The pre-induction media was replaced with neuronal differentiation media containing 2% FBS, FGF2, insulin-transferrin-selenium supplements, B27, Forskolin, IBMX, BDNF, 2-ME and all-trans retinoic acid for 35 days and media change was done every 2 days.

Immunocytochemical staining

For immunocytochemical staining, the cells were fixed with paraformaldehyde (4%), permeabilized with triton X-100 (0.05%) washed and stained with the indicated antibodies. The cells were incubated with primary antibody at 4 °C overnight and stained with fluorescently conjugated secondary antibody for 1–2 h at room temperature. 4′,6-Diamidino-2-phenylindole, dihydrochloride (DAPI) was used to stain the nucleus and the cells were documented microscopically (Axio Observer Z1, Zeiss).

Collection of conditioned media

Cells were grown to confluence and replaced with serum-free media for 6 h. The media was collected, filtered, and used immediately or stored at − 80 °C for later use. Whenever required, the necessary supplements and serum were added to the conditioned media prior to use.

H_2O_2 treatment of neuronal cells

To induce oxidative damage, the neuronal cells were treated with H_2O_2 (100 μM) for 24 h in serum-free media. The treated cells were cultured further in neuronal or conditioned media and collected for gene expression analysis.

Data analysis

Flow cytometric data were analyzed using FlowJo software (FlowJo, LLC). Student's *t* test was used to compare the difference between control and treated samples. Variation in gene expression between the different samples was determined by Mann-Whitney non-parametric variables test using SPSS software.

Results

Expression of mesenchymal stem cell markers in OAMSCs

OAMSCs isolated from central (OAMSCc) and medial (OAMSCm) orbital adipose tissue were analyzed for the expression of mesenchymal stem cell markers. We found that both OAMSCc and OAMSCm were adherent, spindle-shaped cells and have proliferation capacity similar to that of BMMSCs (Electronic Supplementary Material, Fig. S1). Both OAMSCc and OAMSCm expressed cell surface markers CD73, CD81, CD90, CD105 and CD253 similar to that observed in BMMSCs (Fig. 1a–e) as well as several adhesion molecules, integrins CD29, CD44, CD49B, CD49D, CD49E and CD325 (Fig. 1a–e). OAMSCs have cell surface expression of CD34, as also reported by others (Chen et al. 2014; Korn et al. 2009) but unlike BMMSCs, OAMSCs did not express CD120a and CD146 (Fig. 1c, d). Both OAMSCc and OAMSCm showed a low cell surface expression of SSEA4 as identified by immunocytochemical staining (Fig. 1a′′′, b′′′).

We next analyzed the gene expression profile of OAMSCc and OAMSCm and compared with BMMSCs. The transcription factors that confer self-renewal ability such as *OCT4*, *NANOG* and *SOX2* were expressed in both BMMSCs and OAMSCs; however, OAMSCs have significantly higher expression of *NANOG* and *SOX2* compared to BMMSCs (Fig. 2a, a′). Transcripts of mesenchymal gene *VIM*

(Vimentin) were significantly higher in OAMSCs but not *SNAI1* (Snail Family Transcriptional Repressor 1) (Fig. 2b, b′) as compared to BMMSCs. Pertaining to its tissue of origin, BMMSCs showed a significantly high basal level of *OCN* whereas OAMSCs had high *PPARG* transcript levels (Fig. 2b, b′). Proliferation and cell cycle-related genes had similar transcript levels in BMMSCs and OAMSCs except *PCNA* (proliferating cell nuclear antigen), which was significantly higher in both OAMSCc and OAMSCm compared to BMMSCs (Fig. 2c, c′). Also, OAMSCm had significantly high expression of *CCND1* (cyclin D1) compared to OAMSCc (Fig. 2c, c′). Cell adhesion genes were differentially expressed between BMMSCs and OAMSCs except *ITGA1* (integrin subunit alpha 1) and chemokine receptor *CXCR4* (C-X-C motif chemokine receptor 4) that showed similar expression levels. *CDH2* (cadherin 2) and *VCAM1* (vascular cell adhesion molecule 1) expression was significantly higher in BMMSCs whereas transcript levels of *ITGA6* (integrin subunit alpha 6) and *CD44* were significantly higher in OAMSCs compared to BMMSCs (Fig. 2d, d′). However, there was no significant difference in expression level of these genes between OAMSCc and OAMSCm (Fig. 2d, d′).

Among the different genes that represent signaling pathways, genes related to Notch signaling pathway *NOTCH1*, *NOTCH2*, *NOTCH3* and *DLL1* (Delta like canonical Notch ligand 1) had similar expression in BMMSCs and OAMSCs (Fig. 3a, a′). The tissue inhibitor of metalloproteinases *TIMP2* and *TIMP4* had significantly high expression in OAMSCs compared to BMMSCs (Fig. 3b, b′). *FZD2* (frizzled class receptor 2) was significantly high in BMMSCs whereas OAMSCm had significantly high expression of *LEF1* (lymphoid enhancer binding factor 1) (Fig. 3c, c′). OAMSCs had significantly high transcript levels of BMP (bone morphogenetic protein) signaling genes *BMP4*, *ID1* and similar *BMP2* levels compared to BMMSCs (Fig. 3c, c′). *BMP4* and *BMP6* transcript levels were significantly high in OAMSCm compared to OAMSCc (Fig. 3c, c′). We further analyzed the transcript levels of secreted factors that are involved in neuroregeneration and neuroprotection. Expressions of *FGF2*, *IGF1*, *BDNF* and *IL6* (interleukin 6) were comparable between BMMSCs and OAMSCs whereas *APOE* and *CTGF* were expressed significantly high in BMMSCs compared to OAMSCs (Fig. 3d, d′).

Mesodermal and neuroectodermal differentiation of OAMSCs

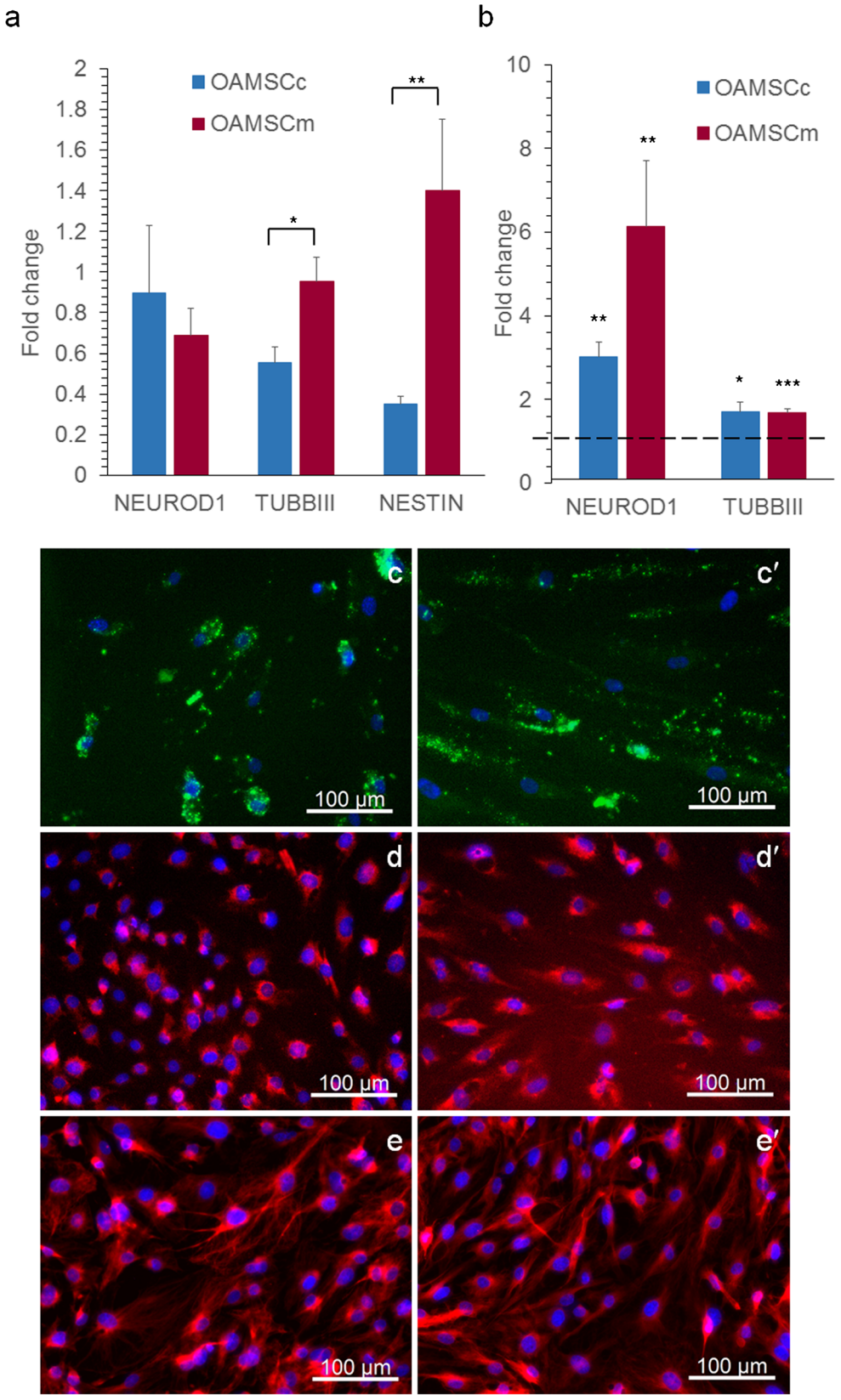
When cultured under mesodermal differentiation conditions, OAMSCs differentiated into adipogenic, osteogenic (Fig. 4) and chondrogenic lineage cells (Electronic Supplementary Material, Fig. S2). Both central and medial tissue-derived OAMSCs stained positive for alizarin red (AZR) after osteogenic differentiation (Fig. 4a, a′, b) and the differentiated cells expressed significantly high levels of osteogenic gene *OCN* (Fig. 4c). The expression of *OCN* was significantly high in OAMSCm compared to OAMSCc, which also correlated with the respective AZR levels (Fig. 4b, c). OAMSCc and OAMSCm differentiated into adipocytes with distinct oil droplet formation (Fig. 4d, d′, e) and expressed significantly high levels of adipogenic specific gene *PPARG* (Fig. 4f). Although, not significant, OAMSCc showed higher levels of *PPARG* expression compared to OAMSCm.

When grown in normal growth media, the transcript level of *NEUROD1* was similar in OAMSCc and OAMSCm but *NESTIN* and *TUBBIII* were expressed significantly high in OAMSCm compared to OAMSCc (Fig. 5a). Under neuroectodermal differentiation conditions, both OAMSCc and OAMSCm readily differentiated into cells of neuroectodermal-like cells, as identified by significantly increased expression of *NEUROD1* and *TUBBIII* (β-III tubulin) in differentiated cells (Fig. 5b). The differentiation of OAMSCs into neuroectodermal lineage cells was further confirmed by determining the expression of NGFR (nerve growth factor receptor) (Fig. 5c, c′), NESTIN (Fig. 5d, d′) and TUBBIII (Fig. 5e, e′) by immunocytochemical staining in the differentiated cells.

Fig. 5 Neuroectodermal differentiation of OAMSCc and OAMSCm. **a**, **b** ▶ Real-time PCR analysis of neuronal specific genes in OAMSCc and OAMSCm cultured in **a** growth media and **b** neuronal differentiation media. Expression levels of the neuronal genes were normalized to those in cells cultured in growth media. Dotted line represents the expression level in the control cells cultured in growth media. Values are mean ± SE, $n = 3–4$ independent samples, $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$. Immunofluorescence analysis of **c**, **d**, **e** OAMSCc and **c′**, **d′**, **e′** OAMSCm differentiated into neuroectodermal cells showing expression of **c**, **c′** NGFR, **d**, **d′** NESTIN and **e**, **e′** TUBBIII. Representative microscopic images are shown. Each sample had 3–4 technical replicates

Neuroprotective effect of OAMSCs

Previous studies have reported that the neuroregenerative effect of MSCs was mainly due to the paracrine factors secreted by MSCs rather than differentiation into neuroectodermal cells in vivo (Deng et al. 2006; Drago et al. 2013; Maltman et al. 2011; Paul and Anisimov 2013). To investigate the neuroprotective effects of OAMSCs, conditioned media (CM) from OAMSCs (both OAMSCc and OAMSCm) was collected and added to neuroectodermal lineage cells that were treated with H_2O_2. We found TUBBIII expression in both central and medial OAMSCs cultured with neuronal media (Fig. 6a, a′) or CM (Fig. 6b, b′) after treatment with H_2O_2. However, cells treated with CM from both OAMSCc and OAMSCm showed high levels of *NEUROD1* and *NESTIN* expression compared to cells cultured in neuronal media after H_2O_2 treatment (Fig. 6c). H_2O_2-treated neuronal cells had significantly upregulated



expression of antioxidant gene *CAT* (Catalase) but treatment with OAMSC-derived CM significantly downregulated the expression of antioxidant genes *CAT* and *MnSOD* (Manganese superoxide dismutase) (Fig. 6d). Since Bax/Bcl-2

ratio can determine the survival or apoptosis status of cells (Drobny and Kurca 2000), we determined the expression levels of pro-apoptotic gene *BAX* and anti-apoptotic gene *BCL2L1* in CM-treated cells. When treated with CM derived from OAMSCs, the neuronal cells showed significantly decreased expression of *BAX* but significantly increased expression of BCL2L1 compared to cells cultured in neuronal media (Fig. 6e), thus suggesting a neuroprotective effect.

Discussion

Here, we report a detailed characterization of OAMSCs derived from both central and medial fat pad of the orbital tissue. Central and medial adipose tissue-derived MSCs were similar to BMMSCs in their growth properties, morphology and expression of several mesenchymal stem cell markers. Earlier, two groups reported isolation of multipotent stem cells from

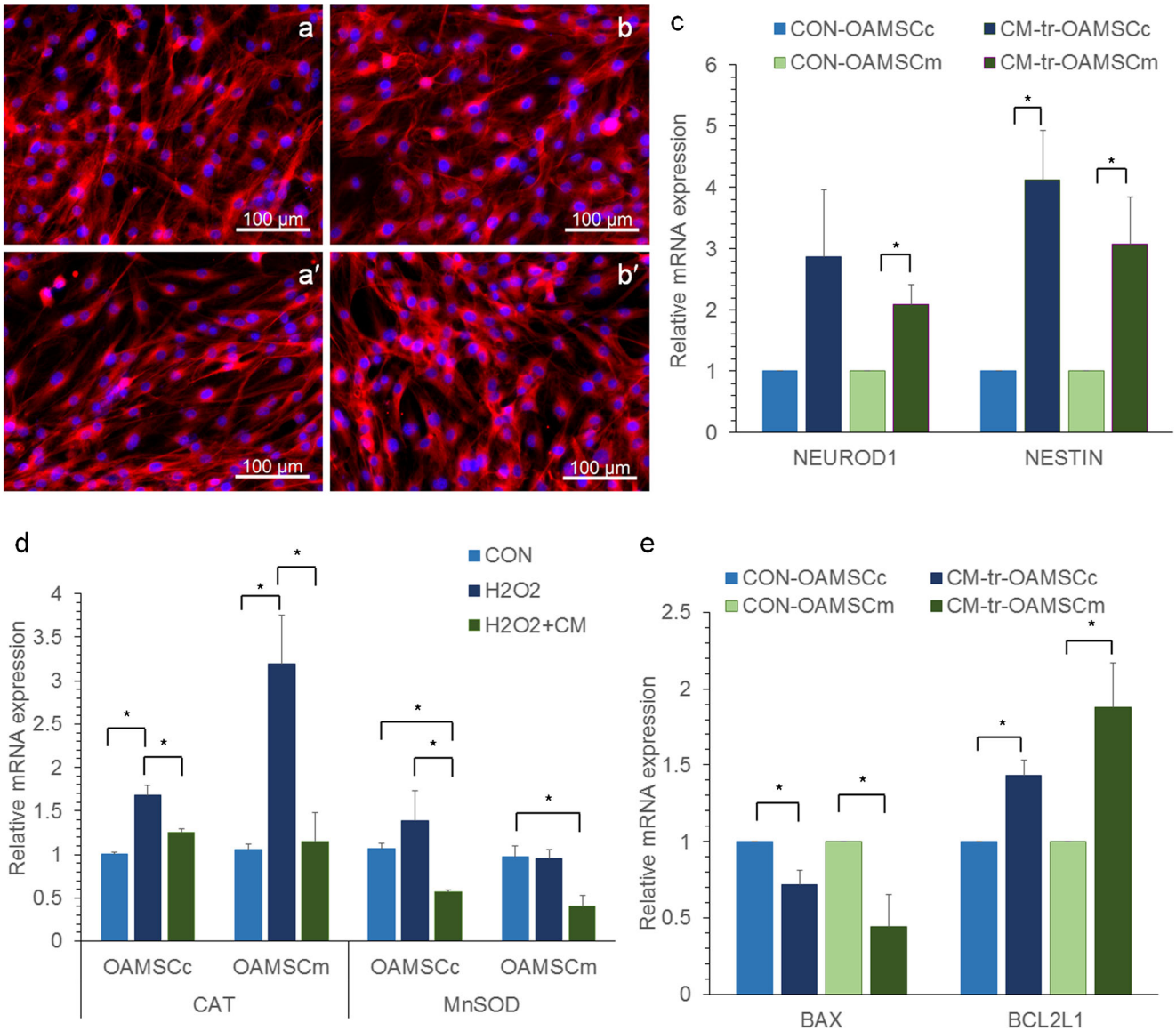


Fig. 6 Neuroprotective effect of OAMSCc and OAMSCm derived conditioned media. Neuroectodermal differentiated OAMSCc and OAMSCm were treated with H_2O_2 (100 μM) for 24 h and cultured in conditioned media (CM) collected from OAMSCs or neuronal differentiation media for a further 3 days. **a**–**b′** Immunofluorescence analysis of TUBBIII expression in **a** control OAMSCc, **a′** control OAMSCm and **b** CM-treated OAMSCc, **b′** CM-treated OAMSCm neuroectodermal cells after H_2O_2 treatment. Representative microscopic images are shown. **c** Real-time PCR analysis of neuroectodermal lineage cells cultured in neuronal media (CON) or conditioned media (CM-tr) collected from OAMSCc or OAMSCm for the expression of neuronal specific genes *NEUROD1* and *NESTIN*. **d** Expression of *CAT* and *MnSOD* genes in untreated neuroectodermal cells (CON), cells treated with H_2O_2, cultured in neuronal media (H2O2) or conditioned media from OAMSC after treatment with H_2O_2 (H2O2 + CM). **e** Expression of apoptosis-related genes *BAX*, *BCL2L1* in control (CON) and conditioned media (CM-tr) treated neuroectodermal cells. Values are mean ± SE, $n = 3$–4 samples, each analyzed in duplicates. *$p < 0.05$

orbital adipose tissue (Chen et al. 2014; Korn et al. 2009). Korn et al. reported that orbital adipose-derived stem cells expressed CD90 and CD105 (Korn et al. 2009), which are characteristic mesenchymal stem cell markers as seen also in our study. CD34, which was generally not expressed in BMMSCs, was expressed in OAMSCs and similar results have been reported by others in MSCs derived from other tissues (Togarrati et al. 2017) and orbital adipose tissue (Chen et al. 2014; Korn et al. 2009). We also found that CD146, which is generally expressed in BMMSCs, was not expressed by OAMSCs. Gene expression analysis showed an extensive similarity between the BMMSCs and OAMSCs. The important differences were in the expression levels of *NANOG* and *SOX2*, which were significantly high in OAMSCs. As BMMSCs are bone marrow derived that differentiate mainly into bone cells in vivo, they showed high basal levels of *OCN* compared to OAMSCs, whereas the adipose-derived OAMSCs had high levels of adipogenic specific gene *PPARG*. OAMSCs have trilineage mesodermal differentiation ability (Chen et al. 2014), where both central and medial OAMSCs differentiated efficiently into adipogenic, osteogenic and chondrogenic cells. Interestingly, the basal expression of *OCN* and *PPARG,* the osteogenic and adipogenic genes respectively, were similar in central and medial OAMSCs; however, osteogenic differentiation ability was significantly higher in OAMSCm compared to OAMSCc. Although we found *OCT4*, *NANOG*, *SOX2* transcripts in OAMSCs, we did not find expression of OCT4 protein (Electronic Supplementary Material, Fig. S3) as reported by Chen et al. (2014). A point to note here is that Chen et al. reported cytoplasmic as well as low nuclear OCT4 expression in OAMSCs; however, OCT4 being a transcription factor has nuclear but not cytoplasmic localization in pluripotent stem cells (Hu et al. 2010).

Considering that cell therapy is one of the most potential options for neuronal repair after neuronal injury in brain or spinal cord (Irion et al. 2017), we tested our cells for their neuronal differentiation ability. Both OAMSCc and OAMSCm could differentiate into neuroectodermal cells. Although OAMSCm had significantly higher expression of *NESTIN* and *TUBBII* compared to OAMSCc before the induction of neuroectodermal differentiation, the differentiation of either cell types into neuroectodermal lineage was similar. Nevertheless, studies have shown that only a few MSCs transdifferentiated into neuronal cells in vivo after transplantation and neuronal regeneration after MSC injection might mainly be due to secreted factors (Hermann et al. 2010; Maisel et al. 2010). Montzka et al. reported that secretion of neurotrophic factors BDNF, NGF, VEGF, FGF2 by undifferentiated MSC reduced the damage to neurons by neuroprotection, neurogenesis and scar inhibition when transplanted into injured host tissue (Montzka et al. 2009). We found expression of neurotrophic factors *BDNF*, *GDNF* and *FGF2* (Cova et al. 2010) and neuroprotective factors *APOE* (Polazzi et al. 2015), *IL6* (Jung et al. 2011) and *VEGFA* (Sun et al. 2003) in both OAMSCc and OAMSCm. These secreted neurotrophic factors were reported to produce a neurogenic effect on resident neural stem cells during damage (Maltman et al. 2011; Teixeira et al. 2013; Uccelli et al. 2011). Neuronal damage is also caused by oxidative stress during inflammatory brain diseases (Gilgun-Sherki et al. 2004; Smith et al. 1999) and we found that the secreted factors from OAMSCs such as BDNF, GDNF, FGF2, IL6, etc. could help in the recovery of neuronal cells after exposure to oxidative stress (Lanza et al. 2009), by reversing the oxidative damage. The neuroprotective effect of OAMSC-derived CM might also be due to modulation of pro-apoptotic *BAX* and anti-apoptotic *BCL2L1* expression after oxidative damage (Wang et al. 2016).

Thus, our results show that orbital adipose-derived mesenchymal stem cells could be utilized for treatment of neuronal damage as they possess neuroectodermal differentiation ability, neuroprotective effect, abrogates neuronal damage caused by oxidative stress and might activate the endogenous repair mechanism.

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Authors' contributions KB and BGJ conceived the study. DM, AK and BGJ designed the study. KB performed blepharoplasty surgery. DM, AS, RS and AK performed experiments. DM, KB, JB, HB, ND and BGJ analyzed the data. DM, KB and BGJ wrote the manuscript. All the authors approved the final version of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical statement All procedures involving human samples were in accordance with the Helsinki convention and approved by the ethics committee of the Indian Institute of Technology Guwahati and Sri Sankaradeva Nethralaya, Guwahati. The study was carried out in accordance with the human ethics committee guidelines and written informed consent was obtained from all the patients involved in the study.

References

Beane OS, Fonseca VC, Cooper LL, Koren G, Darling EM (2014) Impact of aging on the regenerative properties of bone marrow-, muscle-, and adipose-derived mesenchymal stem/stromal cells. PLoS One 9: e115963. https://doi.org/10.1371/journal.pone.0115963

Billon N et al (2007) The generation of adipocytes by the neural crest. Development 134:2283–2292. https://doi.org/10.1242/dev.002642

Branch MJ, Hashmani K, Dhillon P, DRE J, Dua HS, Hopkinson A (2012) Mesenchymal Stem Cells in the Human Corneal Limbal

Stroma. Invest Ophthalmol Vis Sci 53:5109–5116. https://doi.org/10.1167/iovs.11-8673

Chen SY, Mahabole M, Horesh E, Wester S, Goldberg JL, Tseng SCG (2014) Isolation and Characterization of Mesenchymal Progenitor Cells From Human Orbital Adipose Tissue. Invest Ophthalmol Vis Sci 55:4842–4852. https://doi.org/10.1167/iovs.14-14441

Cova L et al (2010) Multiple neurogenic and neurorescue effects of human mesenchymal stem cell after transplantation in an experimental model of Parkinson's disease. Brain Res 1311:12–27. https://doi.org/10.1016/j.brainres.2009.11.041

Deng J, Petersen BE, Steindler DA, Jorgensen ML, Laywell ED (2006) Mesenchymal stem cells spontaneously express neural proteins in culture and are neurogenic after transplantation. Stem Cells 24:1054–1064. https://doi.org/10.1634/stemcells.2005-0370

Drago D, Cossetti C, IraciN GE, Musco G, Bachi A, Pluchino S (2013) The stem cell secretome and its role in brain repair. Biochimie 95:2271–2285. https://doi.org/10.1016/j.biochi.2013.06.020

Drobny M, Kurca E (2000) Possible extrapyramidal system degradation in Parkinson's disease. Brain Res Bull 53:425–430. https://doi.org/10.1016/S0361-9230(00)00367-1

Frese L, Dijkman PE, Hoerstrup SP (2016) Adipose Tissue-Derived Stem Cells in Regenerative Medicine. Transfus Med Hemother 43:268–274. https://doi.org/10.1159/000448180

Gilgun-Sherki Y, Melamed E, Offen D (2004) The role of oxidative stress in the pathogenesis of multiple sclerosis: The need for effective antioxidant therapy. J Neurol 251:261–268. https://doi.org/10.1007/s00415-004-0348-9

Hermann A et al (2010) Age-dependent neuroectodermal differentiation capacity of human mesenchymal stromal cells: limitations for autologous cell replacement strategies. Cytotherapy 12:17–30. https://doi.org/10.3109/14653240903313941

Hu BY, Weick JP, Yu JY, Ma LX, Zhang XQ, Thomson JA, Zhang SC (2010) Neural differentiation of human induced pluripotent stem cells follows developmental principles but with variable potency. Proc Natl Acad Sci U S A 107:4335–4340. https://doi.org/10.1073/pnas.0910012107

Irion S, Zabierowski SE, Tomishima MJ (2017) Bringing Neural Cell Therapies to the Clinic: Past and Future Strategies. Mol Ther Methods Clin Dev 4:72–82. https://doi.org/10.1016/j.omtm.2016.11.005

Johnston MC, Noden DM, Hazelton RD, Coulombre JL, Coulombre AJ (1979) Origins of avian ocular and periocular tissues. Exp Eye Res 29:27–43. https://doi.org/10.1016/0014-4835(79)90164-7

Jung JE, Kim GS, Chan PH (2011) Neuroprotection by Interleukin-6 is mediated by signal transducer and activator of transcription 3 and Antioxidative signaling in ischemic stroke. Stroke 42:3574–U3371. https://doi.org/10.1161/Strokeaha.111.626648

Kern S, Eichler H, Stoeve J, Kluter H, Bieback K (2006) Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells 24:1294–1301. https://doi.org/10.1634/stemcells.2005-0342

Korn BS, Kikkawa DO, Hicok KC (2009) Identification and Characterization of Adult Stem Cells From Human Orbital Adipose Tissue. Ophthal Plast Reconstr Surg 25:27–32. https://doi.org/10.1097/IOP.0b013e3181912292

Lanza C et al (2009) Neuroprotective mesenchymal stem cells are endowed with a potent antioxidant effect in vivo. J Neurochem 110:1674–1684. https://doi.org/10.1111/j.1471-4159.2009.06268.x

LiJYet a (2008) Lewy bodies in grafted neurons in subjects with Parkinson's disease suggest host-to-graft disease propagation. Nat Med 14:501–503. https://doi.org/10.1038/nm1746

Lojewski X et al (2015) Perivascular Mesenchymal Stem Cells From the Adult Human Brain Harbor No Instrinsic Neuroectodermal but High Mesodermal Differentiation Potential. Stem Cells Transl Med 4:1223–1233. https://doi.org/10.5966/sctm.2015-0057

Maisel M et al (2010) Genome-wide expression profiling and functional network analysis upon neuroectodermal conversion of human mesenchymal stem cells suggest HIF-1 and miR-124a as important regulators. Exp Cell Res 316:2760–2778. https://doi.org/10.1016/j.yexcr.2010.06.012

Maltman DJ, Hardy SA, Przyborski SA (2011) Role of mesenchymal stem cells in neurogenesis and nervous system repair. Neurochem Int 59:347–356. https://doi.org/10.1016/j.neuint.2011.06.008

Mawrie D, Kumar A, Magdalene D, Bhattacharyya J, Jaganathan BG (2016) Mesenchymal stem cells from human extra ocular Muscle Harbor Neuroectodermal differentiation potential. PLoS One 11:e0156697. https://doi.org/10.1371/journal.pone.0156697

Montzka K et al (2009) Neural differentiation potential of human bone marrow-derived mesenchymal stromal cells: misleading marker gene expression. BMC Neurosci 10:16. https://doi.org/10.1186/1471-2202-10-16

Paul G, Anisimov SV (2013) The secretome of mesenchymal stem cells: Potential implications for neuroregeneration. Biochimie 95:2246–2256. https://doi.org/10.1016/j.biochi.2013.07.013

Paul G et al (2012) The adult human brain harbors multipotent perivascular mesenchymal stem cells. PLoS One 7:e35577. https://doi.org/10.1371/journal.pone.0035577

PolazziE, MengoniI, Pena-AltamiraE, MassenzioF, VirgiliM, PetrallaS, MontiB (2015) Neuronal regulation of neuroprotective microglial apolipoprotein E secretion in rat in vitro models of brain pathophysiology. J Neuropathol Exp Neurol 74:818–834. https://doi.org/10.1097/Nen.0000000000000222

Smith KJ, Kapoor R, Felts PA (1999) Demyelination: The role of reactive oxygen and nitrogen species. Brain Pathol 9:69–92

Somaiah C et al (2015) Collagen promotes higher adhesion, survival and proliferation of mesenchymal stem cells. PLoS One 10:e0145068. https://doi.org/10.1371/journal.pone.0145068

Somaiah C et al (2018) Mesenchymal stem cells show functional defect and decreased anti-cancer effect after exposure to chemotherapeutic drugs. J Biomed Sci 25:5. https://doi.org/10.1186/S12929-018-0407-7

Sun YJ, Jin KL, Xie L, Childs J, Mao XO, Logvinova A, Greenberg DA (2003) VEGF-induced neuroprotection, neurogenesis, and angiogenesis after focal cerebral ischemia. J Clin Investig 111:1843–1851. https://doi.org/10.1172/Jci200317977

Teixeira FG, Carvalho MM, Sousa N, Salgado AJ (2013) Mesenchymal stem cells secretome: a new paradigm for central nervous system regeneration? Cell Mol Life Sci 70:3871–3882. https://doi.org/10.1007/s00018-013-1290-8

Togarrati PP et al (2017) Identification and characterization of a rich population of CD34(+) mesenchymal stem/stromal cells in human parotid, sublingual and submandibular glands. Sci Rep 7:3484z. https://doi.org/10.1038/S41598-017-03681-1

Uccelli A, Benvenuto F, Laroni A, Giunti D (2011) Neuroprotective features of mesenchymal stem cells. Best Pract Res Clin Haematol 24:59–64. https://doi.org/10.1016/j.beha.2011.01.004

Walker PA, Shah SK, Harting MT, Cox CS (2009) Progenitor cell therapies for traumatic brain injury: barriers and opportunities in translation. Dis Model Mech 2:23–38. https://doi.org/10.1242/dmm.001198

Wang XQ et al (2016) Neuroprotection effect of Y-27632 against H2O2-induced cell apoptosis of primary cultured cortical neuron. RSC Adv 6:49187–49197. https://doi.org/10.1039/c6ra03284b

Zuk PA et al (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7:211–228. https://doi.org/10.1089/107632701300062859