Isolation and Characterization of Mesenchymal Stem Cells from Ocular Adipose Tissue and Extra Ocular Muscle

A Thesis

Submitted for the award of the Degree

of

DOCTOR OF PHILOSOPHY

to

Indian Institute of Technology Guwahati

By

Darilang Mawrie

(Roll No: 10610601)

Under the supervision of

Dr. Bithiah Grace Jaganathan

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Indian Institute of Technology Guwahati

Guwahati – 781039, Assam, India

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Guwahati – 781039, Assam, India

TH-2047_10610601

Indian Institute of Technology Guwahati

Department of Biosciences & Bioengineering

CERTIFICATE

This is to certify that the work described in this thesis, entitled "**Isolation and characterization of mesenchymal stem cells from ocular adipose tissue and extra ocular muscle**", done by Darilang Mawrie (Roll No. 10610601) for the award of the Degree of Doctor of Philosophy, is an authentic record of the results obtained from the research carried under my supervision in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India.

The results embodied in this thesis have not been submitted to any other University or institute for the award of any degree.

IIT Guwahati   Dr. Bithiah Grace Jaganathan

December, 2017   (Thesis Supervisor)

Indian Institute of Technology Guwahati

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DECLARATION

I hereby declare that this Ph.D. thesis entitled ***"Isolation and characterization of mesenchymal stem cells from ocular adipose tissue and extra ocular muscle"*** was carried out by me for the degree of Doctor of Philosophy in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India, under the guidance and supervision of **Dr. Bithiah Grace Jaganathan**.

Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature, and acknowledgement of collaborative research and discussions. Dr. Atul Kumar is hereby acknowledged for helping in the phenotyping and gene expression studies and Ms. Renu Sharma for helping in the orbital adipose tissue studies.

IIT, Guwahati — Darilang Mawrie

December, 2017 — (Roll No: 10610601)

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ABREVIATIONS

AD	Alzheimer's disease
ADSC	Adipose derived stromal cell
ADAS	Adipose derived adult stem cell
AF-MSC	Amniotic fluid derived mesenchymal stem cell
AF-NSC	Amniotic fluid derived neural stem cell
ALP	Alkaline phosphatase
arSHED	stem cells from exfoliated deciduous teeth
ATRA	Tretinoin
β-III-tubulin	Neuron-specific β III tubulin
BDNF	Brain-derived neurotrophic factor
b-FGF	Basic fibroblast growth factor
BHA	Butylated hydroxy anisole
BME	β-2-mercaptoethanol
BM-MSC	Bone-marrow derived mesenchymal stem cell
C57BL/6	C 57 Black 6
CD	Cluster of Differentiation
CFU-F	Colony forming unit-fibroblasts
ChAT	Choline esterase
C-MSC	Cartilage mesenchymal stem cell
CNPase	2', 3'-Cyclic-nucleotide 3'-phosphodiesterase
CNTF	Ciliary neurotrophic factor
CPC	Cetyl pyridinium chloride, monohydrate
CSF	Cerebrospinal fluid
DAPI	4', 6 – diamidino-2-phenylindole
DCX	Doublecortin
DBS	Deep brain stimulation
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide

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DFC	Dental follicle cells
DFPC	Dental follicle progenitor cells
DPSC	Dental pulp stem cells
DRD2	Dopamine receptor D2
EB	Embryoid bodies
E-MSC	Ear mesenchymal stem cell
EOM	Extra ocular muscle
EOM-MSC	Extra ocular muscle mesenchymal stem cell
EGF	Epidermal growth factor
ESC	Embryonic stem cell
FBS	Fetal bovine serum
FESEM	Field emission scanning electron microscope
FFA	Free fatty acid
FIT-C	Fluorescein isothiocyanate
GAD56	Glutamate decarboxylase 56
GalC	Galactocerebroside
GD2	Ganglioside 2
GDNF	Glial derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GO	Grave's Ophthalmopathy
GVHD	Graft versus host disease
hAMSC	human amniotic mesenchymal stem cell
hCNS	human central nervous system
HGF	Hepatocyte growth factor
HSF1	Heat shock factor protein 1
IBMX	3-isobutyl-1-methylxanthine
ICH	Intracerebral hemorrhage
IF-M	Intermediate filament M
IGF-1	Insulin-like growth factor-1
iPSC	Induced pluripotent stem cell
ITS	Insulin Transferrin Sodium selenite

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Kv2.1	K^+ channels
KLF4	Kruppel-like factor 4
MAP-2	Microtubule associated protein-2
MAP1b	Major Microtubule-associated protein 1b
MAPC	Multipotent adult progenitor cell
MC-L	Mesenchymal cell-limbal
MESCM	Modified embryonic stem cells medium
MPO	Myeloperoxidase
NES	Nestin
ND1	Neurogenic differentiation 1
NeuN	Neuronal nuclear antigen
NEFL	Neurofilament light polypeptide
NFM/NF	Neurofilament
NF-70	70kDa Neurofilament
NF-200	200kDa Neurofilament
NFH	Neurofilament-H
NGF	Nerve growth factor
NGFR	Neurotrophic growth factor receptor
Nkx6.1	NK 6 homeo box protein 1
NSE	Neuron specific enolase
NSC	Neural Stem cell
NSCM	Neural stem cell differentiation media
NT	Neurotrophin
Nurr1	Nuclear receptor related 1 protein
PD	Parkinson’s disease
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PDLSC	Periodontal ligament stem cells
PDT	Population doubling time
PE	Phycoerythrin
PFP	Preaponeurotic fat pad
PI	Propidium Iodide

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P-MSC	Placental mesenchymal stem cell
PLCSC	Peripheral and limbal corneal stromal cells
PLA	Processed lipo aspirate
OAT-MSC	Ocular adipose tissue derived- -mesenchymal stem cell
Oct-4	Octamer binding transcription factor 4
OFSC	Orbital fat stem cell
Olig2	Oligodendroglial transcription factor 2
RA	Retinoic acid
RE	Retinal extract
RIP	Receptor interacting protein 1
r-MSC	rat-derived mesenchymal stem cell
Sca-1	Stem cell antigen-1
SCAP	Stem cells from apical papilla
Sc	Schwann cells
SC	Spinal cord
SCF	Stem cell factor
SNTX1	Short neurotoxin
Sox-2	Sex determining region Y- box 2
SP	Side population
SSEA-4	Stage specific embryonic antigen 4
TH	Tyrosine hydroxylase
TMRE	Tetramethyl rhodamine ethyl ester perchlorate
TPA	12 – O – tetradecanoylphorbol 13-acetate
TUJ1	Neuron specific Class ш β-tubulin
UC-MSC	Umbilical cord derived mesenchymal stem cell
UTF-1	Undifferentiated embryonic cell-transcription factor 1
VEGF	Vascular endothelial growth factor
WJ-MSC	Wharton's Jelly derived mesenchymal stem cell
µm	micrometer
ng	nano gram

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SYNOPSIS

Stem cells are defined functionally (Melton, 'Stemness' : Definitions, Criteria, an Standards, 2014) as capable of dividing to form a copy of itself. Stem cells can be totipotent, pluripotent, multipotent or unipotent in nature based on their differentiation potential. A totipotent stem cell can form all types of cells while pluripotent cells could generate all cell types excluding extra embryonic trophoblast. Multipotent stem cells differentiate into multiple cell types within one or two germ layer (Sobhani A, *et al,* 2016). Stem cells in an adult are few in number and remain quiescent until required during repair or regeneration. A mature tissue, like the bone marrow harbors a small number of stem cells with a multipotent differentiation potential (J. R. Sanchez-Ramos, 2002). For example the most studied and thoroughly characterized stem cells are the hematopoietic stem cells, and also stem cells of the epidermis and the intestinal epithelium (HALL & WATT, 1989) and the mesenchymal stem cells (MSC). The hematopoietic stem cells are generally localized inside the bone marrow while the mesenchymal stem cells are dispersed in almost all tissue reservoirs that include the bone marrow, skin, lungs, kidneys, intestinal tissue, the dental pulp and various regions of the eye, adipose tissue, umbilical cord blood, umbilical cord, amniotic fluid, Wharton's jelly, and placental tissue. It was Friedenstein *et al* in 1968, who first identified a population of cells capable of forming several colonies isolated from the bone marrow which were fibroblastic, spindle in shape and adherent in nature and they were termed multipotent marrow cells. They expressed CD13, CD29, CD44, CD73, CD90, CD105 and HLA-I on their cell surface and could differentiate into adipocytes, osteocytes and chondrocytes. Besides, the bone marrow MSCs (BM-MSCS) can home to injured sites, secrete several growth factors and cytokines, suppresses inflammatory response and triggers no immunological response. Hence BM-MSC is excessively useful for several therapeutic purposes. However, bone marrow extraction is a painful procedure, when taken from diseased donors, it produces a low yield of stem cells whose proliferative rate also decreases with age of the donor. Therefore several investigations were conducted to identify novel tissue sources where MSC can be easily isolated and expanded. The aim of the current study is to identify stem cells from novel tissues, study their multipotent capacity, differentiate

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them into neuronal lineage and compare them with the bone marrow derived mesenchymal stem cells.

The thesis is organized into different chapters:

1. In the **Review of Literature** chapter, scientific background of the study has been summarized. It highlights the properties of mesenchymal stem cells from the bone marrow and other adult tissues. Bone marrow derived mesenchymal stem cells are multipotent, can home to an injured site, secrete several growth factors and cytokines, can suppress inflammatory action and triggers no immunological response. Several preclinical studies have reported that bone marrow derived MSC differentiate into neural lineages and were transplanted into diseased animal model. Furthermore because neurological disorders often leads to cell death, treatment with pharmacologic drugs can merely attenuate the effects of the disease. Therefore stem cells are used for therapy. However, bone marrow extraction involves a highly invasive procedure. An attempt to identify mesenchymal stem cells from other tissues is required. For instance, orbital adipose tissue was discarded during blepharoplasty. The mesenchymal progenitors isolated from the orbital adipose tissue were both multipotent and have been transplanted into mouse model of ALI and treated to improve corneal transparency. On the other hand, side population (SP) cells isolated from the extra ocular muscle were more proliferative *in vitro* than SP cells from limb muscles. These SP cells were implanted into a dystrophic muscle.

2. **Objectives** – Three objectives were formulated for the study:

a. **Isolation of stem cells from orbital adipose tissue (OAT)**
b. **Isolation of stem cells from extra ocular muscle tissue (EOM)**
c. **Neuronal differentiation of extra ocular muscle tissue derived mesenchymal stem cells**

3. **Materials and Methods** –The methods used for isolation and differentiation of adipose and extra ocular muscle derived MSC are described in this

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section. Different protocols for the differentiation of extra ocular muscle derived mesenchymal into neurons are described in this section.

4. The **Results** chapter describes the findings of the current study. The chapter is subdivided into three sections:

4.1. Isolation of MSC from orbital adipose tissue – Orbital adipose tissue yielded adherent mesenchymal stem cells which can be differentiated into adipocyte/osteocyte lineage cells and confirmed by biochemical assays. Gene expression studies also confirmed the differentiation potential. Central orbital adipose tissue derived mesenchymal stem cell had higher capacity to differentiate into mesenchymal lineage than medial orbital adipose tissue derived MSC. Orbital adipose tissue derived mesenchymal stem cells expressed MSC markers such as CD44, CD49B, CD73, CD90 and CD105 and also express CD34. The gene expression studies reveal they express neurotrophic factors and embryonic transcription factors in their undifferentiated state. Orbital adipose tissue could be differentiated into neuronal lineage which was determined by expression of NGFR, Nestin and β-III-tubulin.

4.2. Isolation of MSC from extra ocular muscle tissue – The extra ocular muscle tissue derived cells resembled the bone marrow derived MSC and were termed extra ocular muscle mesenchymal stem cell (EOM-MSC). The CFU-F forming ability of EOM-MSC was significantly higher than that of BM-MSC, they exhibited a normal karyotype and showed a shorter doubling time of 34 hours compared to BM-MSC whose doubling time was 42 hours. They expressed several mesenchymal markers but not the muscle stem cell marker CD34 and the hematopoietic marker CD45. They readily differentiated into adipocytes, osteocytes and chondrocytes and genes related to mesenchymal differentiation were upregulated. However the capacity to differentiate into mesodermal cells was higher in BM-MSC compared to EOM-MSC. The chapter also highlights the expression of embryonic markers of both cell types. The gene expression analysis studies revealed that OCT4, NANOG and SOX2 expression was 60-100 folds higher compared to BM-MSC. There was no OCT4 expression but SSEA4 was predominantly expressed in spindle-shaped population of EOM-MSC by immunocytochemistry. Further real-time PCR results showed that the mRNA expression of NESTIN was several fold higher than BM-MSC.

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Apoptosis related genes and calcium channel related genes were expressed by both EOM-MSC and BM-MSC. The mitochondrial distribution was both perinuclear and cytoplasmic in EOM-MSC whereas in BM-MSC it was predominantly perinuclear.

4.3. Neuronal differentiation of EOM-MSC - We hypothesized that human extra ocular muscle mesenchymal stem cells have a higher potential to differentiate into neuronal lineage due to their high NESTIN mRNA expression. To check whether EOM-MSC possesses neuroectodermal differentiation potential, EOM-MSC was subjected to differentiation through intermediate stage or direct conversion. When EOM-MSC was supplemented with retinal extract, cell aggregates appeared after 4 days as opposed to 8 days when RE was not supplemented. On the other hand, since EOM-MSC showed a higher CD49B expression, CD49B positive cells have shown to have higher multipotent neurosphere formation capacity. The formation of neurosphere was further confirmed when they stained positive for GFAP. EOM-MSC readily differentiated into neuronal cells as evident from the change in morphology and expression of neuronal specific genes. There was also a significant upregulation of mRNA for neural markers NESTIN (NES) and β-Ш-tubulin in the cells induced with the neuronal media. The clonally derived cells were expanded *in vitro* and were differentiated into neuronal cells. Neuronal differentiation of clonal derived EOM-MSC was confirmed by immunocytochemistry staining when they expressed the neuronal marker NGFR.

5. **Discussion** – The major findings of this study are:

1. Stem cells were isolated from central and medial orbital adipose tissue (OAT). They were adherent and spindle-shaped cells. They were multipotent as they differentiated into adipocytes, osteocytes and chondrocytes. They also differentiated into neuronal lineage which was demonstrated by the expression of NGFR, Nestin and β-III-tubulin. These cells were termed central orbital adipose tissue mesenchymal stem cells (C-OAT-MSC) and medial orbital adipose tissue mesenchymal stem cells (M-OAT-MSC). The C-OAT-MSC had higher a capacity to differentiate into mesenchymal lineage than M-OAT-MSC.

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2. Stem cells were isolated from the extra ocular muscle tissue (EOM). The cells were adherent and spindle in shape. They resembled the BM-MSC and were called the extra ocular muscle mesenchymal stem cell (EOM-MSC). Their population doubling time was lower of 34 hours while the BM-MSC had a population doubling time of 42 hours. They had a higher CFU-F forming capacity than BM-MSC. However their differentiation capacity to mesenchymal lineage was lower compared to the BM-MSC. The transcription factors OCT4, SOX2 and NANOG and the neural progenitor marker NESTIN was more upregulated in EOM-MSC compared to BM-MSC. Finally, the EOM-MSC exhibited both perinuclear and cytoplasmic mitochondrial distribution while the mitochondrial distribution in BM-MSC was predominantly cytoplasmic.
3. EOM-MSC readily differentiated into neurons. Neurosphere were formed in the presence of growth factors and retinal extract. CD49B positive cells had a higher neurosphere forming capacity compared to CD49B negative cells. Neuronal differentiation was further confirmed by the expression of genes involved in differentiation and the expression of specific proteins by immunocytochemical staining.

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REVIEW OF LITERATURE

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1.0. REVIEW OF LITERATURE

1.1. Stem cells

Stem cells are immature cells with an extended self-renewal capacity which are localized in various parts of the body and depending on their origin are either embryonic stem cells or adult stem cells, and are able to differentiate into multiple cell lineage or a single lineage (Lindvall, Kokaia, & Martinez-Serrano, 2004). The basic function of a stem cell is therefore to maintain the number of differentiated cells at a constant level: to replace dead cells or cells that had been lost through injury (Hall & Watt, 1989). Tumors are formed if this balance is lost (Varum *et al.,* 2011). Several tissues of the body be it the bone marrow, the prenatal tissues, adipose tissue, dermis, intestinal tissue and dental pulp also, harbor stem cells. Differentiated cells, on the other hand, are formed via transit amplifying population from a small number of stem cells. This steady state is ensured because the stem cell population is heterogeneous. Therefore for the purpose of repair the multipotent adult stem cells can differentiate into unipotent cells of the residing tissue (Mariano, Teixeira, Marie, & Lepski, 2015). This continual proliferation, however, is limited by telomerase activity in culture. Due to their self-renewal potential and multipotent nature, stem cells are candidates for regenerative medicine (Lv, Tuan, Cheung, & Leung, 2014).

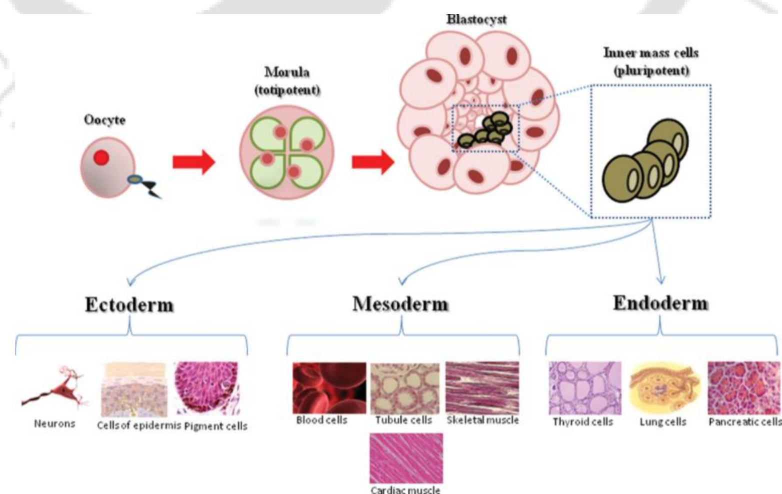

Figure 1.1. *Hierarchy of stem cells (Salem & Thiemermann, 2010).*

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1.2. The bone marrow-derived mesenchymal stem cells and its properties

The bone marrow is a soft spongy tissue that is localized within the hollow interior of long bones (Bala, 2013). Hematopoiesis or production of red blood cells occurs in bone marrow of an adult human. This tissue is composed of heterogeneous population of cells including the hematopoietic stem cells, macrophages, erythrocytes, fibroblasts, adipocytes, and endothelial cells. Stromal cells, which developed from the mesoderm layer of the embryo, and has a multipotent differentiation potential were also present (Salem & Thiemermann, 2010). The mesenchymal stem cells were first isolated from the rat bone marrow by Friedenstein and Petrakova in 1966 (Rodriguez, Elabd, Amri, Ailhaud, & Dani, 2005) and (Kemp, Hows, & Donaldson, 2005) but it was Arnold Caplan (1991) who named these stromal cells as "mesenchymal stem cells or mesenchymal stromal cells"(Murray & Péault, 2015). They were called mesenchymal stem cells because they differentiated into cells of the mesodermal lineage (Falavigna & Costa Da Costa, 2015). They act as supporting cells or stromal cells for the growth and development of hematopoietic stem cells (Mariano *et al.,* 2015). A niche is an environment where a stem cell interact with its environment. Thus a niche is formed within the bone marrow where the HSC and MSC interact with each other via cytokine and paracrine signaling in order for the HSCs to proliferate and differentiate (Branch *et al.,* 2012). MSCs remains in the undifferentiated state as long as the environment signals them not to differentiate. Apart from replenishing themselves and supporting the HSCs, MSCs also differentiates into adipocytes, osteocytes, chondrocytes, cartilage, tendon, muscle and marrow stroma *in vivo* (Pittenger *et al.,* 1999) and also into neuron-like cells when cultured *in vitro* but to a lesser extent than embryonic stem cells. In this way they maintain homeostasis by replacing dead and dysfunctional cells via self-renewal and differentiation (Ma *et al*., 2014). They differentiated into astrocytes and neurons (Hermann *et al.,* 2004) when they were transplanted into an injured mouse brain *in vivo* (Y Zhang & Wang, 2010). They differentiated not only to mesodermal lineage but also to muscle cells (mesoderm), hepatocyte-like cells, kidney and lung and the endothelial cells (endoderm) and the skin (ectoderm) (Herzog, Chai, & Krause, 2003). Therefore the mesenchymal stem cell population is composed both of stem cells and progenitor cells. MSCs were considered equivalent to ESCs (Khang, n.d.) as OCT4 and NANOG

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were localized in the nuclei of the porcine MSC (Y. Liu *et al.,* 2013). Immunomagnetic and physical methods were employed to isolate BM-MSC and they were easily expanded *in vitro* when exposed to growth factors such as cytokines. However Montanaro *et al*, have isolated SP cells from BM by staining the samples with Hoechst dye (Montanaro *et al.,* 2004). The colony forming unit fibroblasts that are formed as a result of isolation appeared to be adherent and proliferated rapidly *in vitro* (Akiyama *et al.,* 2012). They were easily expanded in DMEM media containing 10% FBS when supplemented with L-glutamine at 37°C with 5% CO_2 (Halleux, Sottile, Gasser, & Seuwen, 2001). The BM-MSCs were found to be positive for markers such as STRO-1, CD29, CD73, CD90, CD105, CD146, HLA-2, OCT4, SSEA-4 and CD106 (Bieback & Brinkmann, 2010) but CD34 and CD45 which are typical HSC markers were not expressed by the BM-MSC. Unlike the embryonic stem cells, BM-MSC did not undergo transformation even after culturing them for a longer period *in vitro* nor were there any changes in their differentiation potential into adipocytes, osteocytes and neural cells even after prolonged culture (Okolicsanyi *et al.,* 2015). Therefore these stem are considered safe for cell therapy. When transplanted into another injured tissue they do not trigger any immunological response, they migrate extensively to inflamed sites and exert immunosuppressive and anti-inflammatory response (N. Kim & Cho, 2013) and (Branch *et al.,* 2012). Due to these advantages the MSCs were used for cell replacement therapy via transplantation to treat various complex disorders. However, from each ml of bone marrow extracted only $0.0001\text{-}0.1*10^6$ MSCs were isolated. The production of MSCs was scaled up for clinical purposes but their proliferation rate and cell number decreases with age (Bieback, Schallmoser, Klüter, & Strunk, 2008) and (Kern, Eichler, Stoeve, Klüter, & Bieback, 2006). Also, BM-MSC obtained from the marrow of iliac crest, tibia and femoral (Kemp *et al.,* 2005) involved highly invasive procedure that brought acute pain to the patient which was undesirable by a normal non-diseased donor. These few hurdles limited the role of bone marrow derived MSCs to act as agents for therapeutic purposes. Over the years investigations had been going on to identify novel tissue source that harbor mesenchymal stem like-cells other than the bone marrow. It was assumed that these MSC should have a higher yield/ml and the tissue should be easily taken out with less invasive procedures. Indeed several tissues like the

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umbilical cord, umbilical cord blood, the placental tissue, Wharton's Jelly, adipose tissue, also in cutaneous tissue, dental pulp, hair follicle, synovium and peripheral blood (Crisan *et al.,* 2008) and (Zhang & Wang, 2010) and post-natal tissues such as the brain, liver, spleen, kidney, lungs, thymus and pancreas and the trabecular bone also harbors MSCs (Kuo, Ho, & Lee, 2009). They were even isolated from the menstrual blood, fallopian tube and limbal stroma (G.-G. Li, Zhu, Xie, Chen, & Tseng, 2012) of the eye. Though their basic features were similar, they had differences in the self-renewal capacity, differentiation potential and surface marker expression because each tissue had a different embryonic region. These information will help to determine the source of MSC for designing a particular therapeutic prospect.

1.3. Mesenchymal stem cells from other tissues

1.3.1. MSCs from various fetal tissues

Placenta

Cultures from maternal and fetal placental tissues were established which were mesenchymal in nature and the cells were termed placental-MSC. They exhibited a spindle-shaped morphology in culture and expressed CD73, CD90 and CD105. Their immunosuppressive property must have been influenced by CD200, a marker expressed only in the fetal P-MSC but negligible in maternal P-MSC. HGF, can influence regeneration of adult organs and wound healing, is expressed 100 times more in fetal P-MSC rather than the maternal P-MSC (Zhu *et al.,* 2014).

Amniotic Fluid

Amniotic fluid too acts as a good source of MSC. It is known to harbor only differentiated cells until MSCs were isolated from the trimester AF. AF-MSC were very similar to BM-MSC in that they exhibited spindle-shaped fibroblast-like morphology, expressed surface markers mainly the CD73, CD90, CD105, integrin and MHC-I (Cananzi, Atala, & Coppi, 2014) differentiated into either a mesenchymal cell or endothelial cells. They had a significantly lower population doubling time (25-38 hours) compared to the BM-MSC (30-90 hours) and they maintained a normal karyotype *in vitro* and did not display tumorigenic potential. Though they expressed moderate levels

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of HLAI and HLAII antigens on their surface (Díaz-prado *et al.,* 2011) they exert immunosuppressive, trophic and protective effects like the BM-MSC and therefore holds great promise in healing nerve injuries (Cananzi, Atala, & Coppi, 2014) and (Sessarego *et al.,* 2008). They integrated into the adult brain and migrated towards areas of ischemic damage after being transplanted into the striatum (Cananzi, Atala, & Coppi, 2014).

Umbilical cord blood

The blood from the umbilical vein, the umbilical cord blood (UCB) (Falavigna & Costa Da Costa, 2015) is a medical waste and was known to contain only hematopoietic stem cells. However, umbilical cord mononuclear cells were isolated by negative immunodepletion of CD3+, CD14+, CD38+, CD66b+, and glycophorin A+ cells and serial dilution of the clones (O. K. Lee *et al.,* 2004). The colonies that survived, differentiated into fat, cartilage and bone cells along with neuroglial and hepatocyte-like cells. Interestingly they lacked CD34 and CD45 markers that indicated their non-hematopoietic origin. They expressed GFAP early in differentiation and MAP2 was expressed much later of the differentiation period. Both UCB-MSC and BM-MSC differentiated into hepatocyte cells with no significant difference in potential (K.-D. Lee *et al.,* 2004). An optimum 80 mL blood when stored for not more than 6 hours yielded high mesenchymal stem cell number. As a result, the spindle-shaped UCB-MSC readily differentiated into adipogenic cells with downregulation of the markers CD73, CD90 and CD105 (Sibov *et al.,* 2012). However, several sub-populations of the UCB-MSC exhibited variable degree of neuronal differentiation potential (Divya *et al.,* 2012). The umbilical cord blood however are still not preferred over the peripheral blood (Wexler *et al.,* 2003) since full term deliveries are tedious and time consuming that ultimately leads to a low yield of stem cells (Sibov *et al.,* 2012).

Umbilical cord

Like the UCB, the human umbilical cord (UC) usually does not harbor multipotent MSCs, but clonally derived cells that expressed CD29, CD44, CD73, CD90 and CD105 (Kwon *et al.,* 2016) and that lacked CD34, CD45, CD117 and CD133 (Ding, Chang, Shyu, & Lin, 2015) were later isolated. The spindle-shaped UC-MSC had a population

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doubling time of 26.1±2.2 hours. Their proliferation rate was 11% higher compared to amnion-MSC and chorion-MSC. The morphology of the cells remained consistent until they reached the ninth passage. They had a greater capacity to differentiate into neural cells compared to amniotic and chorion MSCs (Kwon *et al.,* 2016). UC-MSC efficiently differentiated into neurons, astrocytes and oligodendrocytes. They however formed immature adipocytes characterized with small lipid vacuoles and exhibited a delayed and inefficient osteogenic differentiation during mesenchymal differentiation. Banking of UC-MSC had been encouraged but efficient freezing and thawing of the UC-MSC needs to be thoroughly established (Ding *et al.,* 2015). UC-MSC were preferred over BM-MSC to curb the problems of sterility and fertility (Hua *et al.,* 2011).

Wharton's Jelly

MSCs were also isolated from a section of the umbilical cord called the Wharton's Jelly. Hypoxic conditions favored the proliferation rate of WJ-MSCs. However no significant changes were observed in relation to their size, morphology, multipotency and surface protein expression of WJ-MSC in either a hypoxic or normoxic environment (Nekanti, Dastidar, Venugopal, Totey, & Ta, 2010). Messerli *et al,* isolated MSC from umbilical cord WJ of preterm birth which exhibited spindle-shaped morphology. They were highly positive for CD73, CD90 and CD105 but negative for CD14, CD19, CD34, CD45 and HLA-DR markers. They readily differentiated into adipocytes, osteocytes and chondrocytes and also displayed immunosuppressive properties such as the bone marrow and adipose tissue MSC (Yoo *et al.,* 2009). On induction to neural cells the WJ-MSC formed neurosphere and expressed the neural progenitor markers Nestin, Pax6, Musashi and NCAM, and later in the differentiation, expressed mature neuronal markers MAP2, GFAP and MBP (Messerli *et al.,* 2013). WJ is one of the most abundant tissue since it discarded after birth and causes no side effects while collection like the bone marrow and adipose tissue (Kalaszczynska & Ferdyn, 2015). Thereby, several investigations revealed

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that diseases such as cancer, chronic liver disease, cardiovascular disease, nerve, cartilage and tendon injuries can be treated if these cells are translated for clinical applications (D.-W. Kim *et al.,* 2013).

1.3.2. MSCs exist in all the tissues of the human adult

Adipose tissue- another abundant source of mesenchymal stem cells

Adipose tissue had been used for restorative and aesthetic treatments in the 19th century (Wester, 2014). It was previously described as an inactive tissue, like the brain, however only recently was it known to harbor heterogeneous populations of which some are multipotent (Franco Lambert, Fraga Zandonai, Bonatto, Cantarelli Machado, & Pêgas Henriques, 2009). Adipose tissue is of embryonic mesenchyme origin like the bone marrow itself and contains a stroma that is easily isolated (Catherine A. Perrone,* Douglas Tritschler & Raqual Bower,* Bradley K. Yoder, 2003). The cells from the adipose tissue were mainly isolated via liposuction (Peroni *et al*., 2008) which were then digested by collagenase and repeatedly washed via centrifugation. Zuk *et al*, have identified these cells and termed as processed lipoaspirate (PLA) cells (Zuk *et al.,* 2001). The pellet obtained was resuspended in complete culture medium consisting of FBS and antibiotic/antimycotic reagents (Franco Lambert *et al.,* 2009). Human platelet lysate enhanced the isolation of both BM-MSC and AT-MSC in culture compared to culturing them in FBS supplemented growth media as reported by (C. Li *et al.,* 2015). The additive efficiently improved the growth of MSC *in vitro*. Multiple clones were isolated from the PLA which expressed mesenchymal surface antigens and multiple lineage specific genes and proteins, differentiated into mesenchymal cells and neuronal cells, expressed markers which were involved in tissue development, homeostasis and repair of which STRO-1, CD105 and CD166 were consistently expressed (Strem *et al.,* 2005) and (Catherine A. Perrone,* Douglas Tritschler & Raqual Bower,* Bradley K. Yoder, 2003). They were highly positive for the common mesenchymal surface markers CD73, CD90 and CD105. They did not expressed CD34 and CD45 (hematopoietic markers) which indicated they are non-hematopoietic in origin. They also expressed OCT-4, Nodal and UTF-1 which were specifically expressed by the BM-MSC and not generally

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found in other stem cells (Catherine A. Perrone,* Douglas Tritschler & Raqual Bower,* Bradley K. Yoder, 2003). On the other hand, the proliferation rate of AT-MSCs was comparatively higher than those of BM-MSC and C-MSC, and they reached senescence at later passages compared to C-MSC (Peng *et al.,* 2008). AT-MSC displayed the highest CFU-F frequency and showed a 100% isolation success rate compared to the UCB-MSC. AT-MSC and BM-MSC efficiently differentiated into the three mesodermal lineages while UCB-MSC did not form the adipocytes (Kern *et al.,* 2006). Adipose tissue was expanded for long term in culture but longer compared to UCB-MSC (Franco Lambert *et al.,* 2009). Mesenchymal progenitor cells have been also isolated from central and nasal adipose depots (Korn, Kikkawa, & Hicok, 2009). The PLA was also found to secrete paracrine factors and a wide variety of cytokines and growth factors (Gimble *et al.,* 2013) and (Fraser, Wulur, Alfonso, & Hedrick, 2006) which exerted immunomodulatory mechanisms as observed in studies carried out in autoimmune models. Different strategies were proposed to develop therapies and meet with the challenges when using adipose derived stem cells in regenerative medicine (Yarak & Okamoto, 2010). Alternatively, AT-MSC could replace BM-MSC because of the low cost of harvesting and delivery (Franco Lambert *et al.,* 2009). Moreover, unlike the BM, a large volume of tissue (100 ml upto 1liter) was extracted from the adipose tissue through liposuction to obtain more number of multipotent cells with limited morbidity (Rodriguez *et al.,* 2005) and (Yarak & Okamoto, 2010). While another report showed that with about 1 gm of adipose tissue, 5000 adipose MSCs was obtained while only 0.01% of BM-MSCs was isolated from few millimeters of bone marrow aspirates after density gradient centrifugation (Yu Zhang, Khan, Delling, & Tobiasch, 2012). Moreover, 15 million cells per 100 ml lipoaspirate were enough to treat musculoskeletal disorders without culturing them (Murray & Péault, 2015). Perianal fistulas which remained resistant during Crohn's disease were treated with AT-MSC by local injection into the patient (Bernardo *et al.,* 2011). Further, Finnish researchers in the University of Helsinki and Tampere created an ectopic bone within an intramuscular implant that was subsequently transplanted to the injured site while the vascular supply remained intact. Since the AT-MSC could migrate extensively they were applicable in wound injuries where they migrated to injured tissue, proliferated and differentiated to replace lost cells

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and increased tissue angiogenesis. This led to matrix remodeling and activated immune cells which was necessary for esthetic surgery and to treat diseases affecting the ocular and orbital tissues (Wester, 2014). Adipose tissue stem cells also differentiated into neuronal cells, which in turn, could be used for treating neurological disorders. Although AT-MSC differentiated into mature neurons *in vitro* there was no evidence available as to whether the neurons displayed any electrophysiological function (Franco Lambert *et al.,* 2009). Therefore adipose derived stem cells differentiated into mesodermal such as the adipocytes, osteocytes and chondrocytes as well as non-mesodermal and cells such as neurons, endocrine pancreatic cells, hepatocytes, endothelial cells and cardiomyocytes (Schäffler & Büchler, 2007).

The Central Nervous System

The brain was always considered to be incapable of repairing itself especially during injury and cell loss. It was only recently that neural progenitors had been identified within the brain in perivascular niches such as the subventricular zone, the subgranular zone and the dentate gyrus (Ozen, Boix, & Paul, 2012). Mesenchymal stem like cells were also found in these regions that behaved exactly as the BM-MSC.

Intestinal tissue

MSCs were isolated from ductal cell aggregates in expansion medium. Their proliferation rate increased up to 12 fold in earlier passages but decline in subsequent passages. CD13, CD29, CD44, CD49B, CD54, CD90 and CD105 were expressed in these ductal mesenchymal stem cells but not CD45 and CD117. They were considered to be multipotent as they differentiated into mesodermal cells as well as into the hepatocytes and beta cells. The lack of donor supply limits the usage of ductal cells aggregates as a frequent source of MSC. However, new avenues for therapy could be formed using these mesenchymal stem cells if the mesenchymal nature of ductal MSC is established (Seeberger *et al.,* 2006).

Dental Tissue

Stem cells such as the stem cells from exfoliated deciduous tooth (arSHED) and the dental pulp stem cells (DPSC) exhibited multipotent nature, as they differentiated into

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adipocytes, osteocytes and chondrocytes and even Schwann cells. The mesenchymal markers - CD44, CD90 and CD105 and even the caspase3 were expressed by these stem cells. There was no difference in the proliferation rate and population doubling time between the DPSCs and arSHEDs (Kushnerev, Shawcross, Hillarby, & Yates, 2016). Similar results were shown by Huang *et al*, when two sub-populations from the human dental pulp were isolated namely the $Stro1^+/c\text{-}kit^+/CD34^+$ and $Stro1^+/c\text{-}kit^+/CD34^-$ populations. They equally differentiated into the mesodermal lineages and the neurogenic lineage. However, $Stro1^+/c\text{-}kit^+/CD34^-$ did not expressed NESTIN and NGFR while $Stro1^+/c\text{-}kit^+/CD34^+$ expressed both these markers whether in the undifferentiated state or the differentiated state (neurogenic) (Huang, Gronthos, & Shi, 2009). Other multipotent MSCs in the dental region such as the DPSCs, SHEDs, PDLSCs, SCAP also formed the neural cells and odontoblasts. They are therefore essential to regenerate immature teeth as these stem cells are highly reparative and obtained by less invasive methods.

Ear

Ear-mesenchymal stem cells (E-MSC), on the other hand, were reported to have been isolated from the outer ear of wistar rats. The E-MSC assumed a thinner morphology and their proliferation rate was three times more than those of the BM-MSCs. Sca-1 and CD73 were detected on their cell surface and they differentiated into adipocytes, osteocytes and chondrocytes and the genes involved in differentiation such as the C/ebpα, osteocalcin and Col2a respectively were upregulated. Their karyotype remained normal and they did not become tumorigenic when grown in agar though they were maintained only up to the fifth passage in culture. They had a higher capacity to differentiate into chondrocytes compared to adipocytes and osteocytes. (Sart, Schneider, & Agathos, 2009). The E-MSC expressed OCT4, SOX2 and NANOG but their mesenchymal property diminished after subsequent passages (A. Y. Lee *et al.,* 2015).

Similarly MSC had been detected in the bone marrow and adipose tissue of the equine (Iacono *et al.,* 2015).

1.3.3. Stem cells of the eye

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Limbus

The limbus tissue is located at the junction between cornea and conjunctiva. Limbal epithelial tissue were collected from patients that underwent limbal biopsy for ocular surface reconstruction. The limbal tissue was plated on de-epithelized human amniotic membrane cultured in human corneal epithelium medium supplemented with 10% serum. Fibroblastic cells which were spindle and elongated in shape cells emerged from the tissue and were called the limbal mesenchymal cells (MC-L). The population doubling time of MC-L was recorded at 23 hours while the BM-MSC had a population doubling time of 30 hours and had the ability to form CFU-F units. The MC-L expressed mesenchymal markers (CD29, CD71, CD90, CD105 and CD166) and epithelial marker such as Pax6 but were negative for hematopoietic markers (CD11a, CD11c, CD14, CD31, CD34, CD45, and CD138), embryonic markers (SSEA-1, Tra 1-60, and Tra 1-81) and endothelial markers (Flk1, Flt1, and VE-cadherin). These limbal mesenchymal cells differentiated into adipocytes and osteocytes (Polisetty, Fatima, Madhira, Sangwan, & Vemuganti, 2008). In another study mesenchymal stem like cells were also isolated from the corneal limbal stroma (corneal rim) and they were called peripheral and limbal corneal stromal cells (PLCSC). The PLCSCs too, were adherent cells and expressed CD44, CD73, and HLA-ABC, which was not investigated by Polisetty et al. Like the BM-MSC, they also differentiated into all adipocyte, osteocyte and chondrocyte (Branch *et al.,* 2012) thereby fulfilling the criteria to be termed an MSC. Stem cells from the iris pigment epithelia, ciliary body epithelia and choroidal epithelia holds great hope in preclinical studies for cell replacement in retinal and neural injury (Dhamodaran, Subramani, Ponnalagu, Shetty, & Das, 2014).

Orbital Adipose tissue

The BM-MSCs developed 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) and (Branch *et al.,* 2012). Adipose tissue found in all parts of the human adult are derived from the mesoderm but the ocular system contains cells that are derived from both the ectoderm, essentially the neural crest and the mesoderm (Korn *et al.,* 2009). Adipose tissue is ubiquitous found mainly in the bone

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marrow, intra-articular, subcutaneous, visceral depots as well as ectopic sites such as intra-hepatic and intra-muscular (Gimble *et al.,* 2013), were even recorded to be found in the orbital tissue (Korn *et al.,* 2009), (S.-Y. Chen *et al.,* 2014) and (Lin et al., 2013), and were also isolated from the Hoffa's fat pad (Niada *et al.,* 2013).

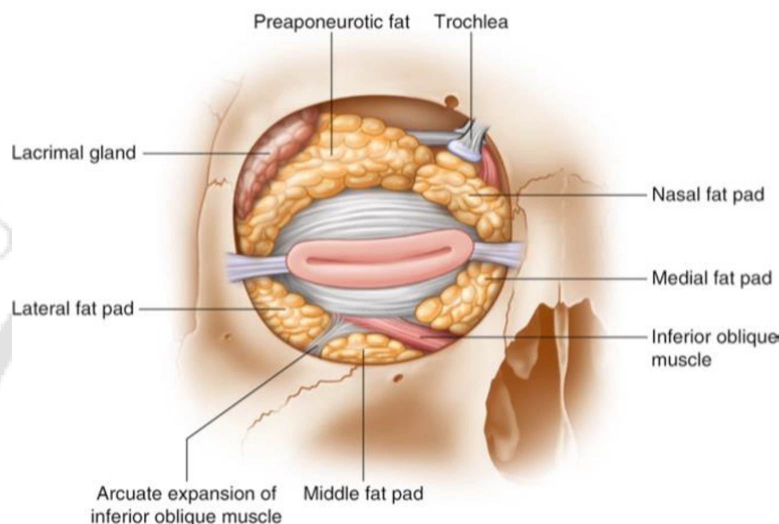

Figure 1.2. Ocular adipose tissue. *The figure represents the localization of the ocular adipose tissue in the human eye which localized in both the lower and upper eyelids (Scheiner et al., 2011).*

Adipose tissue is an energy reservoir that surrounds the eye, extra ocular muscles (EOM), nerves, blood vessels, and the lacrimal gland (Dutton, 2011). During starvation in cats, the FFA content was increased both in the presence of noradrenaline and adrenaline and there was also an increase in the uptake of the palmitic acid in the omental fat, subcutaneous fat and epididymal fat compared to orbital fat and paw fat (Aronovsky, Levarl, Kornblueth, & Wertheimer, 2016). Therefore, the orbital fat has the least metabolic activity since it ensures mechanical protection and maintains the free movement of the ocular globe and intra-orbital structures fat (Ye *et al.,* 2016) compared to omental, epididymal and mesenterial fat and especially the subcutaneous fat whose main function is insulation in order to regulate metabolic activity (Ye *et al.,* 2016). The upper eyelid contains the aponeurotic fat pad (central fat) and the nasal fat pad and the lower eyelid is composed of the medial fat pad, the central fat pad and temporal fat pad.

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Aponeurotic fat (central fat pad) appeared more yellow than the white fat due to the difference in the amount of β-carotenoids present in either tissues (Sires, Saari, Garwin, Hurst, & van Kuijk, 2001). Korn *et al*, were the first to identify adult stem cells from the central and nasal adipose tissue of the upper eyelid. The stem cells derived from both the tissues differentiated into adipocytes and smooth muscle cells with no significant difference in their differentiation capacity. They observed that central ocular adipose tissue derived stem cells expressed NeuN, TH, and β-ш-tubulin (neuronal markers), GFAP (astrocytic marker) and O4 and CNPase (oligodendrocyte markers) when cultured in neurogenic medium. The nasal ocular adipose tissue derived stem cells, on the other hand, expressed β-tubulin ш and O4 markers. Remarkably, nestin was expressed only by the central adipose tissue derived adult stem cells (Korn *et al.,* 2009). CD90 and CD105 were positive in these cells but they were negative for CD11b, CD14, CD31, CD45 and CD106 which coincided with the findings of (Chien *et al.,* 2012) who reported that a wide array of mesenchymal markers such as CD29, CD44, CD49B, CD49D, CD49E, CD58, CD90, CD105 and HLA-ABC were expressed by orbital fat stem cells (OFSC) while CD14, CD31, CD45, CD106, CD117, CD133, CD146, and HLA-DR markers were absent in these cells. Chen *et al*, treated the central and medial orbital tissue with collagenase for 16 hours for better stem cell yield. This enhanced the capacity of stem cells to differentiate into endothelial and mesodermal lineage as compared to cells isolated by the conventional method (S.-Y. Chen *et al.,* 2014) and (Lin *et al*., 2013). Interestingly mesenchymal like progenitor cells were isolated from orbital tissue of a patient that suffered from Grave's orbitopathy. Their behavior was similar to the BM-MSC since they were also spindle in shape and were fibroblastic, formed CFU-F units (Chien *et al.,* 2012) and expressed CD73, CD90, CD105 but CD34 was absent. They also differentiated into lineage specific cells such as the adipocytes, chondrocytes, myocytes and neurons (Kozdon, Fitchett, Rose, Ezra, & Bailly, 2015) and they formed corneal epithelial cells *in vitro* (Chien *et al.,* 2012). These orbital fat stem cells (OFSC) triggered no immune response when injected intravenously (Chien *et al.,* 2012). Due to their immunosuppressive properties, minimal immunogenicity and low tolerance level, healing was initiated when OFSCs were injected into a mice model of ALI using LPS via intra-tracheal injection. On the other

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hand, OFSCs improved the corneal transparency when injected into an injured corneal tissue (Chien *et al.,* 2012). OFSCs were also used to treat inflammatory disorder of the orbit, to heal the wounds of the ocular and periocular tissue, for facial aesthetics, and had similar applications as the ASCs in wound healing of an injured tissue, enhanced antiwrinkle-effect and provide volume-enhancement in periocular tissue (Wester, 2014). There is a need to investigate the mesodermal or neuroectodermal origin of these cells needs so that they can be used as a source of autologous stem cells for ocular surface regeneration. In order to look for new drugs and therapeutics, OFSCs can also be applied to a wide range of eye diseases such as degenerative and heritable disorders. A small volume of fat yield high number of stem cells and involved lower risk and ease of isolation, which perhaps is more advantageous (Chien *et al.,* 2012).

Extra ocular muscle

The human eyeballs are held within the orbital cavity/orbit via six extra ocular muscles namely the medial, lateral, superior and inferior recti and the superior and inferior oblique (Snehasree & Chennai-, 2014). They control various voluntary, saccadic and reflex eye movements. Therefore they possess a unique structure, contractile and regenerative properties different from those of the other skeletal muscles because of which they were classified into a different allotypes of muscle (Pacheco-Pinedo *et al.,* 2009). Hence, on the basis of myosin expression there are three allotypes of muscles – masticatory, limb and EOM. However, the arrangement of skeletal myofiber types in these muscles is conserved because of their shared somatic origin. EOM was developed from pre-chordal mesoderm rather than somites during the embryonic stage and cranial nerves innervate them instead of spinal cord motoneurons (Pacheco-Pinedo *et al.,* 2009). The adult EOM is provided with a rich supply of nerve fibers (Physiology of the Sensorimotor Cooperation of the Eyes), possess heterogeneous myosin myofiber and expressed multiple myosin isoforms (Porter, Khanna, Rao, Li, & Andrade, 2001). The SP cells present in the EOM was successfully implanted into a model having dystrophic muscle (Montanaro *et al.,* 2004) and (Herzog *et al.,* 2003). Apart from the mesenchymal progenitor cells present in the skeletal muscle from which the adipocytes and fibrocytes were formed (Uezumi *et al.,* 2011) there were present side population cells, pericytes,

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CD133+ cells and mesioangioblasts within the muscle stem cell pool (Pacheco-Pinedo *et al.,* 2009). Furthermore, SP cells detected in the EOM were found be higher in number around 15 times more compared to the limb muscles and these SP cells have increased proliferative property *in vitro* thereby generated myotubes with higher efficiency than TA muscles (Pacheco-Pinedo *et al.,* 2009). It was suspected that the role of EOM-SP cells *in vivo* was to maintain a consistent myogenesis, thus the muscle remains spared during Duchenne muscular dystrophy (Pacheco-Pinedo *et al.,* 2009) and (Stuelsatz *et al.,* 2015).

1.4. Mesenchymal stem cells in neural repair

1.4.1. Neurological disorders

A small population of neuroepithelial cells sequentially generates the neuronal and glial cells that comprised the CNS and PNS (Studer, 2014). The CNS basically is incapable in repairing and renewing itself because neurons were mostly considered to be post-mitotic. Therefore, injuries occurring in the CNS are usually permanent which leads to neuronal deterioration and eventually cell death. Different subtypes of neural cells were affected during neurological disorders. The CNS system are composed of neurons, astrocytes and oligodendrocytes. Neurons are again of several subtypes such as the midbrain dopaminergic neurons, serotogenic neurons, GABAergic neurons, glutamate neurons, glycinergic neurons and the neural crest. Therefore one or more of these subtypes were affected during the disease.

Parkinson’s disease (PD)

Parkinson’s disease is characterized by the loss of dopamine neuron in the substantia nigra pars compacta and accumulation of cytoplasmic eosinophilic inclusions called lewy bodies (M.-H. Fu *et al.,* 2015) with no known cause. Extensive death of neurons would have taken place by the time clinical symptoms were detected (Vishwakarma, Bardia, Tiwari, Paspala, & Khan, 2014). PD is characterized by the symptoms of bradykinesia, rigidity and tremor. Usually drugs such as levodopa, DA agonists, monoamine oxidase inhibitors and deep brain simulation were used to attenuate the

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disease (M.-H. Fu *et al.,* 2015) but these treatments would not restore the wide damage that had occurred to the neurons.

Huntington's disease (HD)

During Huntington's disease the main neuron subtype - the GABAergic medium spiny neurons are affected in which the motor neurons are replaced by fibrous astrocytes (Vishwakarma *et al*., 2014). The molecular pathology detected in Huntington's disease is the unstable expansion of cytosine adenine-guanine repeats in the IT15 gene (Studer, 2014). GABAergic neurons have been developed *in vitro*. Huntington's disease could be ameliorated if the injured cells are replaced (Studer, 2014) and (Lindvall & Kokaia, 2006).

Alzheimer's disease (AD)

The impaired formation of new hippocampal neurons from endogenous NSCs in the subgranular zone of the dentate gyrus characterizes the Alzheimer's disease (Vishwakarma *et al*., 2014). The clinical symptoms of AD is characterized by loss of memory and cognitive performance. Acetylcholinesterase inhibitors were given to enhance cholinergic function in order that the disease be alleviated. Transplantation of NSCs alone cannot attenuate the complications caused by AD (Lindvall & Kokaia, 2006).

Amylotrophic sclerosis (AS)

The pathological symptoms of amyotrophic lateral sclerosis is the degeneration and loss of motor neurons in the cerebral cortex, brainstem and spinal cord, leading to fatal paralysis (Y.-P. Wu, Chen, Teng, & Zhang, 2010).

Stroke, demyelination, spinal cord injury, epilepsy and depression are few other neurological disorders.

Earlier no method was available to develop neurons other than from the fetal tissue teratocarcinoma derived cell lines (C N Svendsen *et al.,* 1998). The brain was no longer considered an irreparable tissue since stem cells were also discovered in the CNS in the recent years, though neurons were mostly considered post-mitotic (Johe, 1996). These

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stem cells that only existed in the developing CNS also persisted into adulthood (Graham, Khudyakov, Ellis, & Pevny, 2003) and (Clive N Svendsen & Caldwell, 1999) and (Monnin, Morand-Villeneuve, Michel, Hicks, & Versaux-Botteri, 2007). The neural stem cells (NSC) undergo self-renewal as well as they differentiate into neuron, astrocyte and oligodendrocyte when cultured *in vitro* as well as *in vivo* in the brain (van Praag *et al.,* 2002). Many researchers have reported the existence of NSCs in remote areas of the CNS mainly in the subventricular zone (SVZ), which was first discovered in the mouse brain and the subgranular zone (SG) of the dentate gyrus of the hippocampus in the adult brain (Temple, 2001) and also the spinal cord. For example SVZ or hippocampus was dissected and disintegrated from the host tissue. The dissociated cells were treated with a high concentration of FGF2 and EGF for growth which were later withdrew and treated with other mitogens to induce different neural lineages (Gage, 2000). Though this brought a great relief, however, it was difficult to manipulate and reimplant NSCs taken directly from patient's brain (Scintu *et al.,* 2006) and (Black & Woodbury, 2001). Another reason could be that NSCs were located in very restricted areas of the CNS and in very low numbers. Therefore to treat traumatic, ischemic or degenerative lesions of the CNS would be impossible as neuronal regeneration would not be sufficient. Therefore other alternate neural or non-neural sources had to be considered for cell therapy (Neirinckx, Coste, Rogister, & Wislet-gendebien, 2013), (Suzuki *et al.,* 2004) and (Vishwakarma *et al*., 2014). Several studies therefore had been carried out to direct differentiation of MSCs into the neural lineages *in vitro*. Growth factors, chemical compounds and neurosphere formation or a combination of these, supported differentiation of cells into neural cells and especially neurons in *in vitro*. Firstly, the stem cells were converted into NSCs or neural precursor cells via the generation of neurosphere or grown as monolayer culture (C N Svendsen *et al.,* 1998). The NSC formed were further expanded and later induced for terminal differentiation into either of the neural lineages. On the other hand, the stem cells were directly converted into neurons by the addition of a combination of growth factors, neurotrophic factors and chemical inducers. However, the treatment that came up as a result of clinical research provided no actual recovery except symptomatic relief to the ailing neurological disorders.

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1.4.2. Neurosphere formation

The most common strategy that had been developed by Reynolds and Weiss (Vishwakarma *et al*., 2014) lately to isolate neural progenitors cells *in vitro* was to produce floating spherical cell-clusters called neurosphere, (Beck, Petersen, Felthaus, Schmalz, & Morsczeck, 2011) and the method termed as neurosphere assay. These spherical clusters were simply generated when cells were cultivated in serum free medium and in response to growth factors, EGF and bFGF (Monnin *et al.,* 2007). The neurosphere are heterogeneous that comprises of neural progenitor cells and even differentiated cells. These progenitor cells would subsequently differentiate into any three neural lineages – neuron, astrocyte and oligodendrocyte. Several protocols were developed to generate these neurosphere. Monnin *et al*, devised a media formulation that generated neurosphere from retinal Muller glial cells. Muller cells cultured in DMEM/F12 media supplemented with L-glutamine, ITS, putrescin, progesterone developed neurosphere on the third day of differentiation. The neurosphere were immunoreactive towards nestin and GFAP antibody. However, no spherical clusters appeared even after prolonged culture of the Muller cells in Neurobasal A media containing L-glutamine, B27 and penicillin-streptomycin (Monnin *et al.,* 2007). On the other hand, rat MSC when grown in Neurobasal media supplemented with N2 and bFGF formed spherical clusters which expressed Nestin, neuronal marker such as Tuj1 and MAP2C and GFAP for astrocytes. Further when BDNF was added to the same culture, the cells started to express neuronal marker Tuj1 and MAP2 but the expression of Nestin got diminished (Suzuki *et al.,* 2004). Around 8% of MSCs were converted to neurosphere that contained NSCs. These neurosphere were co-cultured with primary astrocytes derived from human fetal brain tissues using Hermann protocol. They differentiated into neurons that possessed both dendritic and axonal processes, form synapses and were able to fire tetrodotoxin sensitive action potentials (L. Fu *et al.,* 2008). NSC were also isolated by culturing as a monolayer.

1.4.3. Differentiation of BM-MSCs into the neuronal lineage

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Bone marrow stromal cells strictly differentiate into the mesodermal lineages but they also differentiated into myocytes, hepatocytes and glial cells including the neural cells (Gage, 2000). Neuronal differentiation was first detected by the change in the morphology of the BMSC. Initially, the spindle-shaped and flat morphology retracted towards nucleus forming rounded refractive bodies and later developed some neurite-like protrusions. Finally, the cells acquired a neuron-like cell shape (Guan, Xu, Wang, & Lin, 2014) and (Egusa, Schweizer, Wang, Matsuka, & Nishimura, 2005). The shapes of the neurons formed therefore ranges from unipolar and bipolar to branched multipolar cells (Jamous *et al.,* 2010). Neuronal-like cells with varied structures were developed, such as those with short processes while others displayed round bodies with no extensions. Others had nucleus which are contracted and still others exhibited bipolar processes (Guo *et al.,* 2005) and (Deng *et al.,* 2006). Another observation would be that bone marrow derived stem cells also expressed neural markers when differentiated *in vitro*. There was a marked increase of nestin expression in MSC but eventually downregulated in prolonged culture time. However, there was no change in nestin expression in differentiated MSC. Nevertheless undifferentiated BM-MSC moderately expressed several neuronal markers such as DRD2, NSE, MAP1b, NFH and SNTX1 though they did not expressed mature neuronal markers such as synaptophysin, tyrosine hydroxylase and tau (Montzka *et al.,* 2009). A wide range of mRNAs and proteins involved in terminal neuronal differentiation were expressed in undifferentiated MSC (Egusa *et al*., 2005) but Ankeny *et al,* reported that cultured MSCs showed very low levels of nestin and did not expressed Neurofilament protein, NeuN or GFAP (Ankeny, McTigue, & Jakeman, 2004).

BMSC under the influence of EGF or BDNF in experimental conditions formed the neural cells which expressed the neural precursor marker nestin and later expressed the glial cell marker such as the GFAP and Neuron specific nuclear protein (NeuN) (J. Sanchez-Ramos *et al.,* 2000). Rat BM-MSCs were initially preinduced for 24 hours with DMEM supplemented with 20% FBS and β-mercaptoethanol. Later they were induced into neurons grown with DMEM and supplemented with 2% FBS, β-mercaptoethanol, DMSO and BHA (Woodbury D, Schwarz EJ, Prockop DJ, 2000). Mohammad *et al,* adopted the Woodbury protocol that investigated the effect of BHA on differentiation.

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With increased exposure time to BHA, the morphology of MSC was altered, developed processes like those of the neurons and expression of nestin and NF-L was increased, though there was no significant increase of MAP2 expression (Mohammad *et al.,* 2016). On the other hand, when rBM-MSC were initially exposed to IBMX for a period of 2 days they developed into early neuron-like cells and both MAP2 and tyrosine hydroxylase were not initially expressed. Subsequently, cells were treated with a combination of GDNF, IL-lβ, mesencephalic glial cell-conditioned medium and flash-frozen mesencephalic cellular fragments. They detected an increased expression of the markers NSE (early neuronal marker) and MAP2 a and b (mature neuronal marker) and the expression of tyrosine hydroxylase, a dopaminergic neuronal marker, was significantly increased after incubation in induction medium for 15 days (Guo *et al.,* 2005). Hence rBM-MSC can be converted to dopaminergic neurons when exposed to proper inducing agents, which was a significant finding with regards to treating Parkinson's disease. Also, adult human bone marrow MSC efficiently generated NSCs by cultivating neurosphere in which they were later differentiated into glia and neurons in the presence of growth factors such as GDNF or BDNF respectively (Hermann *et al.,* 2004). Scintu *et al*, determined that on addition of chemical compounds TPA, Forkskolin and IBMX plus the growth factor FGF1 the stem cells acquired neural properties within 24 hours of treatment. The BM derived stem cells however took longer to develop into neural cells when only RA and BME was supplemented regardless of cells in both conditions expressed the neural markers NF, TUJ1 and NSE (Scintu *et al.,* 2006). It had been reported that the combination of EGF and bFGF could induced neuronal differentiation *in vitro* mostly in neural stem cells or precursor cells derived from the brain tissue. In serum free media rBM-MSC formed neuronal cells which expressed the NeuN and β-mercaptoethanol also promoted neuronal differentiation. However, when co-cultured with astrocytes GFAP positive cells were detected (Lei *et al.,* 2007). While Nissl bodies also appeared when CSF was added to the culture medium. CSF supported the formation and growth of neural progenitor cells and development of mature neurons and dendrite like structures. Not only that the BM-MSC differentiated into neurons but with them the astroglial and oligodendroglial cells were also formed (Otify, Youssef, Nagy, Marei, & Youssif, 2014). When BM-MSC was co-

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cultured with NSCs they were able to form both NSCs and the neurons but BM-MSC had no effect on the differentiation of the NSCs (Rong *et al.,* 2015). On the other hand, BM-MSC expressed β-ш-tubulin and MAP-2 when they were induced with bFGF followed by addition of steroid hormones (β-estradiol, progesterone and testosterone) (Parivar *et al.,* 2015). MSC from other species exhibited the similar potency to form neural-like cells since 23% of porcine MSC expressed the immature neuronal marker β-tubulin-ш and 29% of the pMSC expressed the mature neuronal marker MAP2 when differentiated into neural-like cells in neurogenic medium that contained bFGF, B27 supplement and EGF for seven days and additionally kept for another seven days with RA (Y. Liu *et al.,* 2013).

MSCs differentiated into neuron-like cells *in vitro* but their electrophysiological properties were not yet determined. However Zeng *et al*, reported that ion channel proteins were expressed and potassium ion current was enhanced when hBMSCs were treated with neurogenic medium containing edaravone, β-mercaptoethanol, EGF and bFGF followed by further treatment with BDNF and NGF (Zeng *et al.,* 2011). A surprising finding manifested the generation of physiologic voltage by naïve BMSC though at lower amplitudes. The peak inward current at depolarized voltage of +40mV was displayed by MSC exposed to neurogenic medium on day 4 of differentiation (Egusa *et al*., 2005). Though many reports have suggested that MSC can differentiate into the neuronal lineages yet there were few who proved otherwise.

1.4.4. Differentiation of MSCs from other tissues into the neuronal lineage

Owing to the painful method involved to obtain BM and the decreased rate of proliferation and multipotency with age of the donor, apparently BM-MSCs cannot be considered the only suitable therapeutic agent for neural repair.

Prenatal stem cells isolated from umbilical cord blood also differentiated into all the three neural lineages such as the neurons, glial cells and oligodendrocytes *in vitro* spontaneously and when exposed to retinoic acid and growth factor, BDNF. The neurons, astrocytes and oligodendrocytes formed, expressed the lineage specific marker β-tubulin-III and GFAP and GalC respectively (Buzańska, Machaj, Zabłocka, Pojda, & Domańska-Janik, 2002). The UC-MSC acquired neuron-like projections and a bigger

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cell body which were highly refractive. They were determined to behave like the neural stem cells when 87.3%±14.7% and 72.6% ±11.8% expressed the NSE and ND1 respectively and 43.1%±10.3% and 69.4%±19.5% cells expressed the neuronal mature markers MAP-2 and NF-M when they differentiated further into neurons respectively (S. Chen *et al.,* 2016). WJ-MSC too spontaneously expressed neurogenic markers in growth culture conditions which was undetectable in BM-MSC except for β-tubulin-III at higher passages. They secreted neurotrophic factors at higher levels, obtained with ease and has a higher proliferation rate compared to BM-MSC that insisted that they might be better candidates for neurodegenerative therapy (Drela *et al.,* 2016). When human amniotic mesenchymal stem cells (hAMSC) were delivered into wistar mouse model affected with intra cerebral hemorrhage the number of ED1+ activated microglia as well as MPO+ and Caspase3 were significantly reduced. Simultaneously increased expression of BDNF and VEGF promoted neurogenesis and angiogenesis that led to an overall neurological recovery in the injured model (Zhou, Zhang, Yan, & Xu, 2016). Therefore they could be considered potent therapeutic agents in treating ICH. Similarly hAMSC expressed vimentin and nestin, both intermediate filaments, spontaneously but BM-MSC expressed vimentin and mildly expressed Nestin. When NSCs were allowed to form in culture greater expression of Nestin and β-tubulin-3 were detected in NSCs generated from hAMSC rather than in NSCs developed from BM-MSCs (Yan *et al.,* 2013). Similarly this is true of AF-MSC isolated from the second trimester AF formed neurosphere more rapidly than BM-MSC. Under non-inducing conditions AF-MSC and BM-MSC expressed vimentin AF-MSC and few BM-MSC expressed nestin and musashi. AF-NSC showed a higher expression of nestin, SOX2 and musashi compared to BM-NSC. The number of AF-NSC positive for GFAP and β-tubulin-III was higher when AF-NSC was differentiated compared to BM-NSC (Yan *et al.,* 2013). Even NEFL and NSE also including β-ш-tubulin significantly increased in canine AF-MSC when differentiated into neurons. In addition, nestin, β-tubulin-III and tyrosine hydroxylase protein levels are also greatly increased (E. Y. Kim, Lee, & Kim, 2014). Also almost 85% of the AF-MSC expressed the neural markers GFAP, Nestin and Neurofilament. They are therefore more advantageous compared to other MSC from bone marrow, umbilical cord blood and human endometrium when cultured in a neurogenic medium

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(Bonaventura *et al.,* 2015). iPS-derived cell lines and ES-derived cell line (HSF1) separately but equally formed electrically active motor neurons. Firstly, embryoid bodies were generated and secondly differentiated into neurons which expressed Nkx6.1 and Olig2 (Karumbayaram *et al.,* 2010). Therefore, human iPS cells could also developed into mature, electrically active neurons when initiated with proper signals. Again, iPSCs formed EBs in absence of FGF. When they were treated with Forkskolin and IBMX followed by exposure to RA and FBS, the EBs significantly expressed higher β-tubulin-III, nestin, NSE, MAP-2, Olig2 and BDNF. The GFAP expression however was found to be lower. Using this protocol 90% iPSCs expressed β-tubulin-III, MAP2 and NSE (Salimi, Nadri, Ghollasi, Khajeh, & Soleimani, 2014). MAPC, a more primitive stem cell population quite similar to the BM-MSC, differentiated into all the three neural lineages while the BM-MSC differentiated into neuroectodermal cells. The neuro-ectodermal markers such as NF-200, MAP-2 and GFAP were highly expressed in MAPC compared to BM-MSC because MAPC is highly pluripotent (Singh, Tripathy, & Nityanand, 2013). Under proper environmental signals, even stem cells isolated from human adult pulp differentiated into neurons which expressed mature neuronal markers both at the mRNA and protein levels. They generated potassium ionic currents indicating them becoming functional. Further when these DPSCs were injected into an avian embryo model where they were observed to display bipolar structures after 48 hours of injection. Later, these stem cells colocalized with both early and late marker, β-Ш tubulin (Arthur, Rychkov, Shi, Koblar, & Gronthos, 2008). Völlner *et al,* for the first time differentiated dental follicle cells into neural progenitors. The neurosphere most efficiently formed when cells were grown in poly-l-lysine substrate. These progenitor cells were further differentiated into specific neural cells in DMEM/F12 media supplemented with ITS, bFGF and RA. The undifferentiated DFCs strongly expressed the early marker nestin and very low levels of specific neuronal marker such as the β-III-tubulin, Neurofilament and NSE. The neurosphere that were formed, on the other hand, were nestin and β-III-tubulin positive. The expression of Neurofilament and NSE was upregulated, nestin was downregulated however β-III-tubulin was constitutively expressed in differentiated follicle cells (Völlner, Ernst, Driemel, & Morsczeck, 2009). These stem cells seemed to be promising for neurological therapy.

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Melanocytes, too, present in scleral and choroid sections formed neuron-like cells obtained from donors. A difficulty was encountered while trying to convert adipose tissue derived stem cells into neural cells. Nevertheless, ADSCs were first reprogrammed into iPSCs with pluripotency genes OCT3/4, KLF4, SOX2 and c-MYC that were further induced into neural cell lineages. The cells that were generated maintained a normal karyotype with no insertional mutagenesis being detected. The reprogrammed ADSCs expressed the neural markers TUJ1, GFAP, RIP and Nurr1 (Qu, Liu, Song, Li, & Ge, 2012). Perrone *et al*, used the Woodbury protocol to differentiate the lipoaspirates into neurons. Firstly, sub-confluent cells were exposed to 20% serum and β-mercaptoethanol for 24 hours after which they were transferred to neurogenic medium as established and maintained for an additional one week. Neuronal differentiation occurred due to change in the morphology of the PLA cells, whereby the cells underwent retraction, formed compact cell bodies with multiple processes. Later during the induction period the cells became more spherical and formed secondary branches. The PLA cells consistently expressed NSE but not NeuN during the induction period. Even the protein levels of NSE were increased. However mature neuronal markers like the MAP2 and NF-70 were not detected since the induction period was kept short. In addition, GFAP and GalC were not expressed. The control PLA cells did not expressed any of the neural markers suggesting that differentiation actually took place if this protocol was utilized (Catherine A. Perrone,* Douglas Tritschler & Raqual Bower,* Bradley K. Yoder, 2003). Nestin, a neural progenitor marker, however, was detected in both the undifferentiated and differentiated PLA cells but the more mature markers such as ChAT or GAD56 were not expressed at all. These PLA cells perhaps have assumed an early neuronal lineage and did not formed the astrocytes and oligodendrocytes. Again, both murine and human adipose derived adult stem cells were capable to form neurons when treated with valproic acid, butylated hydroxyanisole, insulin, Forkskolin, hydrocortisone and KCl. Prior to neuronal induction, human ADAS were first maintained in media that contained DMEM and 20% FBS. The differentiation was improved when both types of ADAS were first treated with EGF and bFGF for a longer period (seven days). The morphology of the ADAS assumed rounded cell bodies which were refractive with multiple extensions when treated with induction media.

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Murine ADASs displayed GFAP, nestin and NeuN while human ADASs expressed IF-M, nestin and NeuN following neuronal induction. A low level of nestin was detected prior to induction (Safford *et al.,* 2002). The disadvantage of this protocol were that the cells became short-lived when cultured in neuronal induction medium for 14 days. Human MSCs isolated from the bone marrow, fat, thymus and spleen expressed neuronal/astroglial and oligodendroglial markers when exposed to chemical inducers or co-cultured with Schwann cells. However these chemical agents initiated the cells to alter their phenotype into neural cells but the differentiation remained stable when co-cultured with Sc cells (Krampera *et al.,* 2007). Therefore, these findings might suggest that mesenchymal stem cells from different tissues too, have wide potential to treat neurological disorders.

1.5. Clinical trials of MSCs from BM and various tissues towards treatment of neurodegenerative disorders

Cell therapy is one of the techniques of regenerative medicine where stem cells are introduced into diseased tissue with or without the application of gene therapy (Wei *et al.,* 2013). Cells lost due to deterioration could be regenerated by transplantation of bone marrow cells that would alleviate neurological defects and also to activate endogenous cells (Gage, 2000) and (C.-J. Chen *et al.,* 2007). Mesenchymal stem cells is useful in cell therapy because it can cross the human leucocyte antigen barrier as it is immune privileged and non-immunogenic and can thereby escape immune recognition. MSC can also secrete neurotrophic factors such as BDNF, VEGF, GM-CSF, FGF2 and TGF-β (Falavigna & Costa Da Costa, 2015). Different types of neurons were affected and damaged depending on the type of neurodegenerative disorder. The current therapies are therefore not yet efficient to rescue the neurons from dying during degeneration (Gincberg, Arien-Zakay, Lazarovici, & Lelkes, 2012). The therapy to be developed should target specific types of neurons affected of the particular neurodegenerative disease.

Neuron replacement therapy is feasible to treat neurodegenerative diseases such as Parkinson's and Huntington diseases as per clinical trials. Therefore the transplantation

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of stem cells particularly MSCs or NSCs themselves, or their differentiated counterpart and mobilization of endogenous stem cells within the adult brain seemed to be an appealing prospect towards finding a cure for neuron disorders (Lindvall *et al.,* 2004).

Initially autologous MSCs were used during a bone marrow transplantation for cancer treatment administered to aid in the engraftment and recovery of hematopoiesis after its ablation (Parekkadan & Milwid, 2010). Consequently, BM-MSCS were tested at different phases of clinical trials to treat diseases of the bone (such as osteogenesis imperfecta), also chronic and acute myocardial infarction, and graft versus host disease (Y Zhang & Wang, 2010) and even cardiovascular and neuronal diseases (N. Kim & Cho, 2013). Even though there had been little success in transplanting bone marrow derived MSCs, some have undergone early termination due to mechanisms that still need to be deciphered. However MSCs still hold a great promise for future application in regenerative medicine and were simultaneously being tested for the treatment of many other new diseases too, such as diabetes, stroke, amyotrophic lateral sclerosis, multiple sclerosis and cancer.

Constitutively GFP-expressed bone marrow from mice were detected in the brain after being transplanted for a long period. The bone marrow cells from the recipient expressed CD45 while 20% GFP+ cells from the CNS did not express the CD45. Then it was assumed that a small population of hardly 0.2-0.3 % GFP+ expressed cells have acquired the neuronal phenotype. They showed neuronal markers NSE and NF within the brain of adult animal (Brazelton, Rossi, Keshet, & Blau, 2000). Clinical trials have been carried out using the bone marrow derived MSCs but there is lack of evidence of differentiation into cells of interest actually taking place *in vivo* within the site of injury. Though this statement stands true, Zhao *et al,* however proved otherwise when they transplanted hBM-MSC into various sites of the ischemic brain of rat model. The hBM-MSC not only grafted into the injured sites but expressed β-III -tubulin, GFAP and GalC respectively. Overall there was no increase in the neurological recovery, although differentiated cells, undifferentiated and immature BM-MSC remained in the grafts (Zhao *et al.,* 2002). Similarly it was demonstrated that when bone marrow derived MSC were transplanted intravenously, they entered the brain and reduced the neurological

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defects caused due to stroke in model rat (J. Chen *et al.,* 2001). Similar effects were observed when BM-MSC were transplanted into human patients. Therefore Mezey *et al,* demonstrated the transplanted bone marrow cells with radiolabeled Y chromosomes were detected in various sections of the brain of four female patients where they were transplanted. These bone marrow entered the brain and differentiated into cells that exhibited neuronal features and expressed neuronal markers NeuN and Kv2.1, though survival rate was very low (Mezey, *et al.,* 2003). On the other hand, the hCNS isolated from fetal brain tissue by flow cytometry were converted into neurosphere in human neurosphere culture media. They were transplanted into adult male Sprague-Dawley rat whose carotid arteries were incised and occluded. The transplanted cells which were present medial to the lesion survived rather those on the vicinity of the lesion site. Though not many transplanted cells at the lesion site survive but the human NSC-derived neurosphere migrated toward the ischemic lesion and the cells were detected to express the immature neuronal marker β-III-tubulin and GFAP (Kelly *et al.,* 2004). Again neurosphere cultures were also transplanted into multiple sclerosis model. The cells entered into demyelinating areas of the CNS and differentiated into mature brain cells. Astrogliosis was significantly reduced and there was a marked decrease in the extent of demyelination and axonal loss in transplanted mice (Pluchino *et al.,* 2003). Even if MSCs were not successfully transplanted into an injured brain, they still secrete neurotrophic factors which can protect the tissue from further damage and loss of neurons (Ankeny *et al.,* 2004). For example when rat BMSC was transplanted to repair the sciatic nerve which was severed in wistar rat model not only was it regenerated, which included axonal outgrowth and remyelination, but also elevated levels of BDNF, GDNF, NGF and CNTF were secreted (C.-J. Chen *et al.,* 2007). When the spinal cord was stimulated with injury BM-MSC was transplanted alone in one group and another case they were transplanted with the administration of bFGF. The neurologic defect was ameliorated after 8 weeks of BM-MSC transplantation especially in the group with additional growth factor, bFGF. Though no neural differentiation took place in injured SC however BM-MSC transplanted with bFGF survived longer than when only BM-MSC was transplanted (K. N. Kim, Oh, Lee, & Yoon, 2006). In another study the BM-MSC transplanted via intrastriatal administration not only survived but expressed

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dominergic protein TH and promoted functional recovery when adult C57BL/6 mice were injected with 1- methyl-4-phenyl-1,2,3,6-tetrahydroyridine that promoted a disorder similar to Parkinson's disease (Y. Li *et al.,* 2001). Neural stem cells have equal response to EGF and bFGF since the neurosphere continued to proliferate in media supplemented with either of the growth factors (Tropepe *et al.,* 1999). However, EGF and FGF have a differential and site specific effects on progenitor cells *in vivo.* FGF2 increased the number of newborn cells, mainly neurons, while EGF reduced the total number of newborn neurons (Kuhn, Winkler, Kempermann, Thal, & Gage, 1997). Similarly BM-MSC and bFGF administered together showed improved recovery compared to when MSC alone was engrafted into rat model induced with spinal cord injury. However the MSC were more inclined to form glial cells instead of neurons (K. N. Kim *et al.,* 2006). BM-MSC also triggered spinal cord cells to form spinal processes when co-cultured with each other and MSC significantly reduced gap lesions after being transplanted to the injured spinal cord tissue of the host. The coordination between the forelimbs and hind limbs movements were improved in the host (S. Wu *et al.,* 2003). This was possible because BM-MSC released some trophic factors that could influence spinal neural differentiation. Following traumatic brain injury MSCs were directly injected into the injured brain during surgery. Neurologic functions improved when traumatic brain patients each received upto 10^{-9} expanded MSCs during the cranial repair operation (Z. X. Zhang, Guan, Zhang, Zhang, & Dai, 2008). Rhesus monkey BM-MSC were initially preinduced with medium containing bFGF for 24 hours and finally induced with medium that contained cryptotanshinone, a neuronal inducer, for 2 hours. When further differentiated into the neuronal lineages the cells displayed the usual morphological changes. Later induced BM-MSC were implanted into the rhesus monkey suffering spinal cord injuries. Normal pain withdrawal reflex on both sides was regained by the treated animals. The monkeys recovered above grade 2 in 2-3 months of post-transplantation but secondary complications were unavoidable. The implanted BM-MSCs which were labelled with Hoechst 33342 were found to express NF, NSE and GFAP (Deng *et al.,* 2006) that indicated their differentiation into neuron and glia. Although transplantation of neural stem cells holds a great promise to treat neurological

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disorders but it still remains a challenge due to immunological response created both in the host and donor cells which is triggered during transplantation.

Cell replacement is likely an option depending on the complexity of the neurological disease. For example Parkinson's disease can be easily treated when stem cells were administered to form which is not the case with AD (Vishwakarma, Bardia, Tiwari, Paspala, & Khan, 2014b). Whether or not true differentiation take place after transplantation is a matter of concern since transplantation of a non-neural cell to a damaged neural region could arise due to spontaneous cell fusion (Terada *et al.,* 2002). One advantage that happened was cell fusion did not occur since the cells did not attain a polyploidy state after chromosomal analysis of BM derived lung, kidney and muscle was done (Herzog *et al.,* 2003). BM-MSC were also genetically modified to enhance neuronal differentiation. BDNF transfected BM-MSC proliferated more, exhibited the fibroblastic morphology but secreted higher concentration of BDNF *in vitro*, expressed nestin, NSE and GFAP which later differentiated into nerve cells (Q. Liu *et al.,* 2015). *In vitro* differentiation prior to transplantation is considered safer than direct *in vivo* engraftment although both the naïve MSCs and MSCs differentiated into neural lineages could be implanted into an injured tissue (Yaghoobi & Mahani, 2008) and they displayed a higher survival rate than naïve MSCs within the injured tissue (Deng *et al.,* 2006). This was further supported by the study carried out by (Hayashi *et al.,* 2013) when MSC isolated from parkinsonian macaque were converted to dopaminergic neurons and implanted into brain of the macaque. When bone marrow derived MSC was cultured with retinal explant from rhodopsin knockout mice they were found to be engrafted into the explants and expressed the neural and retinal markers such as Neurofilament 200, GFAP, protein kinase C-α and recoverin but BM-MSC did not expressed the rhodopsin. RPCs are preferred for retinal transplantation (Tomita *et al.,* 2006) though BM-MSC are the best option for treating neurological disorder.

1.6. Neuroprotection

MSCs also secrete neurotrophic factors which prevent further neuronal cell death and even enhance neurogenesis (Joyce *et al.,* 2011). Secretion of neurotrophic factors by

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undifferentiated MSC such as CNTF, BDNF, NGF, NT-3, VEGF, bFGF and HGF reduced the damage to neurons by neuroprotection, neurogenesis and scar inhibition when transplanted to injured host tissue (Montzka *et al.,* 2009). This approach protected the neurons from further dying, instead of simply replacing them with healthy differentiated stem cells that have a short half-life *in vivo*. That is BM-MSC are co-cultured with cortical neurons, secreted BDNF which prevented neuronal cell death as compared to cortical neurons grown in chemically defined medium. When BDNF was blocked the neurons grown with MSC-conditioned medium did not prevent cell death when trophic factor was removed or exposed to NO. The BDNF secreted behaved as a neuroprotective factor (Wilkins *et al.,* 2009).

1.7. Factors involved in neuronal differentiation *in vitro*

Several factors have been known to drive neuronal differentiation *in vitro*. Sanchez-Ramos, mentioned in his review that the agents that initiated neural differentiation *in vitro* are retinoic acid, growth factors, antioxidants, demethylating agent, and noggin (J. R. Sanchez-Ramos, 2002). The effect of different growth factors on neuronal differentiation capacity in BM-MSCs was tested. A slight differentiation occurred when BM-MSC was solely supplemented with EGF but differentiation was upregulated when co-supplemented along with HGF. They have a very important role to direct MSCs to form either the neurons or glial cells. This was supported with the fact that glial and neuronal markers such as MAP2, GFAP, NSE, and NeuN were expressed when the cells were treated with EGF, HGF and VEGF. This stood possible since neural progenitors were available within the BM-MSC pool (Bae *et al.,* 2011). Jin *et al,* also investigated the effects of EGF and HB-EGF which were found to produce the highest expression of nestin, including FGF-2 compared to immature neuronal markers DCX, β-Ш-tubulin and ENCAM. Overall these factors including β-NGF and SCF increased neuronal markers expression but not GFAP. When cells were treated additionally with RA along with EGF and FGF they a assumed a more neuronal-like morphology that could even expressed the NeuN, MAP2 and tau proteins than in culture without RA (Jin, Mao, Batteur, Sun, & Greenberg, 2003). Striatal cells from the adult mice were allowed to

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form floating spherical clusters in presence of EGF but without an adhesive substrate. Within 6-8 DIVs the neurosphere expressed the nestin marker and if further generated can even expressed NSE, Neurofilament and GFAP. When the spheres were grown in substrate in presence of EGF the cultures stopped proliferating. EGF, however could induce the formation of new spheres even from a single cell which later expressed 61±7% NSE expressing cells and GFAP 39±5% respectively. Under serum and feeder cell-free condition in addition to EGF, bFGF and PDGF BM-MSC differentiated into neurons. As a result of the differentiation, neurotransmitters were also expressed of which 30% of the BM-MSC expressed NF-M and GABA, approximately 40-60% expressed NF-M and TH and 60-70% of β-III-tubulin cells expressed the serotonin as a result of differentiation induced for 5 weeks. Therefore, when BM-MSC was treated with cytokines, GABAergic, dopaminergic and serotonergic neurons were generated *in vitro* respectively. However, the neural morphology acquired by MSC gradually disappeared when exposed to medium containing BHA and DMSO (Tao, Rao, & Ma, 2005).

Chemical inducers aids in the development of neural-like features of MSCs *in vitro* and *in vivo* but could prove harmful to the cells. For instance, viability of BM-MSC was reduced when exposed to media that contained DMSO/BHA followed by BME but viability remained unaffected when exposed to ATRA and indomethacin/IBMX. Again, BM-MSC treated with indomethacin/IBMX expressed the higher level of MAP2 and GFAP while NSE expression was not affected by any inducers. Therefore, indomethacin/IBMX were most effective in inducing neuronal differentiation (Mu, Zhao, & Li, 2015). Therefore it was necessary to determine the efficiency of natural growth factors such as IGF, EGF, bFGF and NT-3 to induce neuronal differentiation in BM-MSC.

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2.0. OBJECTIVES OF THE STUDY

1. The clinical use of embryonic stem cells (ES) is hampered by ethical issues (Ma *et al.,* 2014). One key property of ES cells and iPS cells is teratoma formation that may seriously compromise their therapeutic utility (Wei *et al.,* 2013).
2. Mesenchymal stem cells on the other hand are void of any ethical concerns, histocompatibility issues (Ma *et al.,* 2014) and do not form teratoma.
3. However, with bone marrow derived MSCs, the cell number decrease with age, proliferative capacity and differentiation potential decrease as people become older and bone marrow aspiration involves a painful procedure (Falavigna & Costa Da Costa, 2015).
4. Neurodegeneration is one of the major diseases which could benefit from stem cell based therapy.
5. So there is a need to identify novel adult tissue sources which could be utilized for stem cell isolation.
6. EOM is a highly innervated tissue, thus might possess high neuronal differentiation capacity (Porter *et al*., 2001).
7. Several studies show that stem cells isolated from adipose tissue possess multipotent differentiation potential.

Based on these rationale we have formed three objectives:

1. **Isolation of mesenchymal stem cells from orbital adipose tissue**
2. **Isolation of mesenchymal stem cells from extra ocular muscle tissue**
3. **Neuronal differentiation of extra ocular muscle derived mesenchymal stem cells.**

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MATERIALS AND METHODS

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3.0. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Chemicals

Chemical	Supplier
2-Propanol	Merck
3-isobutyl-1-methylxanthine	Sigma Aldrich
Acetone	Merck
Alizarin Red S	Sigma Aldrich
Ascorbic acid-2-phosphate	Sigma Aldrich
B-27	Gibco
Beta-3-glycerophosphate	Sigma Aldrich
Beta-2-mercaptoethanol	Sigma Aldrich
Bovine serum albumin	SRL
Chloroform	Merck
Citrate	Merck
Colchicine	Sigma Aldrich
CPC	Sigma Aldrich
DAPI	Sigma Aldrich
DEPC	Sigma Aldrich
Dexamethasone	Sigma Aldrich
Disodium hydrogen phosphate	Merck
Ethanol	Merck
Forkskolin	Sigma Aldrich
Formaldehyde	Merck
Giemsa Powder	Merck
Glacial Acetic acid	Merck
Glutaraldehyde	Merck
Indomethacin	Sigma Aldrich
Insulin	Sigma Aldrich
Insulin-transferrin-selenium	Gibco

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L-glutamine	Sigma Aldrich
May-Grunwald's powder	Merck
Methanol	Merck
N2 Supplement	Gibco
Nuclease free water	HiMedia
Oil Red O	Sigma Aldrich
Potassium Chloride	Merck
Potassium dihydrogen phosphate	Merck
Propidium Iodide	Sigma Aldrich
Safranin O	Sigma Aldrich, Steinham Germany
Sodium bicarbonate	Sigma Aldrich
Sodium Chloride	Merck
Paraformaldehyde	Merck
Retinoic acid	Gibco
TMRE	Thermoscientific Fischer
Triton X-100	Merck
TRiZol Reagent	Invitrogen

3.1.2. Cell culture media and reagents

DMEM	
-Low glucose (1gm/L)	Sigma Aldrich
-High glucose (4.5gm/L)	Sigma Aldrich
StemPro Chondrogenesis	
Differentiation Kit	Gibco
DMSO	Sigma Aldrich
FBS	Gibco
0.25% Trypsin-EDTA	Gibco
Leukocyte alkaline phosphatase kit	Sigma Aldrich
Neurobasal Media	Gibco
Penicillin Streptomycin	Gibco
PLL	Sigma Aldrich
Trypan Blue Solution (0.4%)	Sigma Aldrich

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3.1.2.1. Cell Culture Media

3.1.2.1. A. Transportation Media for sample collection

Low glucose DMEM (Sigma Aldrich) media, along with 0.1-1% of fetal bovine serum (Gibco) and Penicillin-Streptomycin (Gibco) were added, filtered sterilized with 0.22µm filter and dispensed at a volume of 1 mL in sample vials. The vials were stored at 4°C until extra ocular muscle tissue and ocular adipose tissues were collected. The sample vials, where tissue samples were collected, were always transported in ice-packets.

3.1.2.1. B. Media for maintenance of mesenchymal stem cells

LG-DMEM	
Sodium Bicarbonate	3.7 gm/L
FBS	10%
Penicillin	
-Streptomycin (100X)	1X

3.1.2.1. C. Differentiation Media

Adipogenic Differentiation Media

DMEM-HG supplemented with:

Dexamethasone	1µM
Indomethacin	0.2 mM
IBMX	0.5 mM
Insulin	0.01M

Osteogenic Differentiation Media

DMEM-HG	
FBS	10%
Penicillin	1X
-Streptomycin	
B-Glycerphosphate	10mM
Dexamethasone	0.1 µM
Ascorbic Acid-2-phosphate	0.05 mM

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Chondrogenic Differentiation Media

Basal medium	900 µl
Growth supplement	100 µl

Neurosphere formation media

Neurobasal Media (without L-glutamine and with Phenol Red)

FGF2	10 ng/ml
EGF	10ng/ml
FBS	1%
50U/mL Penicillin -50µg/ml Streptomycin	1X

Retinal Extract from mice

Retinal extract was kindly provided by Veterinary College, Guwahati, India.

Neuronal Differentiation Media

The components of the pre-induction medium

DMEM-HG	
FBS	20%
Penicillin-Streptomycin	1X
L-glutamine	2mM
FGF2	10 ng/mL
B-27	2%
Forkskolin	50 µM
IBMX	250 µM
β-2-Mercaptoethanol	100 µM

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The neuronal induction medium is constituted of:

DMEM high glucose	
FBS	2%
Penicillin-Streptomycin	1X
L-glutamine	2 mM
FGF2	10 ng/ml
Insulin-transferrin-selenium	1%
B-27	2%
Forkskolin	5 µM
IBMX	125 µM
rhBDNF	50 ng/ml
β-2-Mercaptoethanol	100 µM
Retinoic acid	0.5 µM

3.1.2.2. Cell culture reagents

Fibronectin

The final concentration of fibronectin used was 10 ng/cm^2.

Poly-l-lysine

The final concentration of PLL used was 100 ng/cm^2.

Phosphate Buffered Saline (1X)

NaCl	137 mM
KCl	2.7 mM
Na_2HPO_4	10 mM
KH_2PO_4	2 mM

Freezing Mixture

The final composition of freezing mixture contained 10% of DMSO (Sigma) in 90% FBS, filter-sterilized with 0.22 µM filter.

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3.1.3. Antibodies

3.1.3.1. Antibodies used for flow cytometric analysis

All antibodies for flow cytometry were both conjugated with Phycoerythrin (PE) or fluorescein-isothiocyanate (FITC) and purchased from BD Biosciences (BD Pharmingen^{TM}), USA. The details are as follows:

Antibody	Fluorochrome
CD13	PE
CD29	PE
CD34	PE
CD44	FITC
CD45	PE
CD49A	PE
CD49B	FITC
CD49C	PE
CD49D	PE
CD49E	PE
CD49F	PE
CD73	PE
CD90	FITC
CD104	FITC
CD105	PE
CD146	PE
CD200	PE
CD271(NGFR)	PE
HLA-I	FITC
HLA- II	FITC
SSEA4	PE

Table 3.1.3.1. *List of all primary antibodies used for flow cytometry analysis tagged with fluorescent probes.*

3.1.3.2. Antibodies used for immunofluorescence

All primary antibodies immunocytochemistry were from BD biosciences (Heidelberg, Germany) either conjugated with Phycoerythrin (PE) or fluorescein-isothiocyanate (FITC).

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The details are as follows:

Antibodies
CD44
CD73
CD90
CD140A
CD146
TRA 1-60
POU5F1
SSEA-4
MAP2B
NGFR (CD271)
GD2
GFAP
Nestin
β-III-tubulin

Table 3.1.3.2. *The table list all primary antibodies used for immunocytochemical staining.*

The fluorescently conjugated secondary antibody used against the primary antibodies was the Alexa-Fluor 555 (Thermofisher Scientific).

3.1.4. Growth factors

Basic fibroblast growth factor

Epidermal growth factor

Brain-derived neurotrophic factor

3.1.5. Magnetic labelled beads for cell separation

3.1.5.1. EasySep Magnet - Magnet for column-free Immunomagnetic separation. For separating upto $2.5*10^8$ cells (Catalog # 18000, Stem Cell Technologies).

3.1.5.2. EasySep Mouse CD49B positive selection kit – Positive selection. For processing $2*10^9$ cells (Catalog # 18755, Stem Cell Technologies).

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3.1.6. Primers used for quantitative real time PCR

Genes	Forward Primer	Reverse Primer
GAPDH	5'- GGGAAGGTGAAGGTCGGAGT-3'	5'-GGGTCATTGATGGCAACAATA-3'
NESTIN	5'-TGGCTCAGAGGAAGAGTCTGA-3'	5'-TCCCCCATTCACATGCTGTGA-3'
OCT4	5'-CGTGAAGCTGGAGAAGGAGA-3'	5'-CTCAAAGCGGCAGATGGT-3'
NANOG	5'-CAAAGGCAAACAACCCACTT-3'	5'-TCTGGAAC CAGGTCTTCACC-3'
SOX2	5'-GGAGCTTTGCAGGAAGTTTG-3'	5'- CAAGAAGCCTCTCCTTGAA-3'

Table 3.1.6. *The table list the forward and reverse primers used in real time PCR analysis for gene expression studies.*

3.1.7. Preparation of working solutions

Alkaline phosphatase substrate solution

Reagents were part of Leukocyte alkaline phosphatase kit (Sigma-86C-1KT). 250 µl of sodium nitrate solution was mixed with 250 µl FBB alkaline solution and added 10 11.25 ml of de-ionized water. Finally 250 µl of Napthol AS-Bl-Alkaline solution was added and the substrate was ready for use. The reagents were mixed in an amber glass bottle and kept away from light. The solution was prepared fresh before use each time.

Alizarin Red S (2% w/v)

Alizarin Red S	2 gm
Distilled water	100 ml
pH	4.1- 4.3

BSA blocking buffer (5% w/v)

5 gm of BSA was dissolved in PBS. Stored at 4°C. Always prepared fresh.

Carnoy's Fixative

3:1 ratio of Methanol (Merck): acetic acid (Merck)

Citrate acetone formaldehyde fixative

Citrate	25 ml
Acetone	65 ml
37% formaldehyde	8 ml

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The components are mixed properly and stored at 4°C. The fixative was brought to room temperature before use.

Colchicine

1mg/ml stock solution was prepared in methanol and stored at -20°C.

Cold Methanol

Cold methanol was used to fix the colonies for the colony forming unit-assay during May-Grunwald's and Giemsa staining.

DEPC Treatment

Microcentrifuge tubes, microtips and microtip boxes were treated with DEPC against RNAses at a concentration of 0.02% in distilled water overnight.

Ethanol (75% v/v)

75% ethanol was prepared by diluting 100% ethanol in DEPC-treated water.

Giemsa solution (1%)

Weigh 1gm of Giemsa powder and dissolve it 100ml of methanol. Keep the Giemsa solution in a water-bath at 60 degrees Celsius for an hour. Then filter it. We keep it standing overnight.

Glutaraldehyde (0.1%, v/v)

25% of Glutaraldehyde stock is diluted in PBS to a concentration of 0.1 %.

Hypotonic KCl solution

0.075 M of KCl was dissolved in de-ionized water.

May-Grunwald's solution

Weigh out 0.3-0.5 gm of the powdered dye and transfer to a conical flask of 200-250ml capacity. Add 100ml methanol and warm the mixture to 50 degrees Celsius. Allow the flask to cool to 20 degrees Celsius and shake several times during the day. After letting it stand for 24 hours filter the solution. It is then ready for use, no ripening being required.

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Neural Buffered formalin (10%)

Formaldehyde solution is 37%-41% w/v. Formula HCHO, M=30.03g/mol, 1L = 1.08kg (density). Aqueous solution of formaldehyde is formalin (100%). 37% paraformaldehyde is diluted in distilled water to a final concentration of 10%. The pH is set to 7.4.

Oil Red O (1% w/v)

1 gm of Oil Red O powder was dissolved in 100 mL in iso-propanol. The working solution contains 3 parts of 1% Oil Red O solution and 2 parts de-ionized water. The Oil Red O solution was filtered.

Paraformaldehyde (10 %)

90-100% of paraformaldehyde powder was dissolved in PBS. The solution was dissolved by the continually addition of NaOH and stirred over a heating plate or water bath set at 55°C - 65°C till milky appearance of the solution disappears. The pH was set to 7.2 – 7.4. The remaining volume of the solution was made up and the solution was filtered and aliquoted into sample vials (10 ml) and stored at -20°C.

4% working solution of paraformaldehyde solution was prepared by diluting 10% paraformaldehyde in PBS and the pH is maintained at 7.2 - 7.4. The working solution was stored at 4°C in ambered-coloured bottles.

Propidium Iodide (1mg/ml)

The Propidium iodide was dissolved to a concentration of 1 mg/ml with water.

Safranin O Stain (1%)

1 gm of Safranin O powder was dissolved in 100 ml of distilled water and filtered. The stain was kept at room temperature.

TritonX-100 (0.05 %)

Prepared a solution of 0.05 % (v/v) in PBS. Kept at room temperature.

Trypan Blue dye (0.4%)

Dissolve 0.4% (w/v) of trypan blue powder in 1X PBS and filter it.

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60% iso-propanol

60% iso-propanol is prepared by diluting 100% iso-propanol with distilled water. Keep at room temperature.

Cleaning of glass slides for karyotype analysis

Both the sides of the glass coverslides (Riviera) were thoroughly wiped with 70% ethanol. They were also wiped twice with Methanol:acetic acid (3:1) and kept to dry until use.

3.1.8. Materials

Tissue culture plastic plates and flasks were purchased from BD biosciences (Heidelberg, Germany).

3.2. Methods

3.2.1. Isolation of mesenchymal stem cells from ocular tissues

The current study was reviewed and approved by Institute Human Ethics Committee (IHEC) of Indian Institute of Technology Guwahati (IITG), Assam, India.

3.2.1.1. Fibronectin coating

Cell culture vessels were coated with fibronectin at a concentration of 10ng/cm^2 for a time period of one hour at 37°C. After an hour the fibronectin is removed and briefly rinsed with PBS.

3.2.1.2. Isolation of human mesenchymal stem cells from orbital adipose tissue (OAT)

The ocular adipose tissue, was obtained during blepharoplasty when redundant orbital fat tissue was removed from the intra-orbital cavity (Korn *et al.,* 2009), (Chien *et al.,* 2012) and (S.-Y. Chen *et al.,* 2014), in collaboration with the Department of Ophthalmic Plastics and Reconstructive Surgery at Sri Sankaradeva Nethralaya hospital. An approval was given by the local ethical committee to use the patients' ocular tissues for research only after a consent was obtained from the donors or their relatives. The ocular adipose tissue obtained was of the central fat

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and the medial fat of the upper eyelid. The central adipose tissue is bigger and yellow compared to the medial adipose tissue which is paler and smaller in size. These ocular adipose tissue, which were removed immediately after surgery typically were also discarded at that time, were preserved on ice and transported within few hours to be processed (Chen *et al.,* 2014). These tissue were also collected in sample vials containing DMEM low glucose basal media and antibiotics. The tissues were rinsed in sterile PBS containing antibiotics for five minutes and dissociated with forceps. They were then plated in tissue culture plates coated with fibronectin (10ng/cm^2) and cultured in DMEM low glucose basal media containing 10% fetal bovine serum and antibiotics (1X) at 37°C and 5% CO_2. Once cell colonies were formed the tissues were removed. Media was replaced every 3-4 days until the cells reached 70-80% confluency.

3.2.1.3. Isolation of mesenchymal stem cells from human extra ocular muscle tissue (EOM)

Fresh extra ocular muscle tissue was obtained from either female or male patients of the median age of 21 years, who were undergoing strabismus surgery, where the EOM was corrected or resected, under collaboration with the Department of Strabismus of Sri Sankaradeva Nethralaya Hospital. An approval was given by the local ethical committee to use the extra ocular muscle tissue for research only after a consent was obtained from the donors or their relatives. The tissue was collected in transporting medium kept in sample vials and transported via ice packets. The size of the extra ocular muscle was measured to be up to 0.5 – 1 mm in size. The tissue collected was mainly the lateral rectus, medial rectus and the inferior oblique. The samples were processed within twelve to fourteen hours after removal of tissue. The tissue was processed in a sterile petri dish by rinsing it for a period of five minutes in PBS containing twice the concentration of antibiotic. The tissue was dissociated into smaller pieces and plated in six-well plate coated with fibronectin (10ng/cm^2). The samples were grown in low glucose DMEM media supplemented with 10% FBS and penicillin-streptomycin (1X) at 37°C and 5% CO_2. Initially media was replaced after five days of isolation, but later media was replaced after every 3-4 days until colonies were confluent and ready to be harvested. When colonies appeared the tissue was removed from the plate. Once cell colonies were

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formed the tissues were removed. Media was replaced every 3-4 days until the cells reached 70-80% confluency.

3.2.1.4. Clonal cell derivation

EOM-MSC were trypsinized and counted. Depending on the cell count, the cells were diluted hundred times and then ten times before seeding one cell/well in a 96 well plate. The media was replaced every 2-3 days until the cells reach confluency. The presence of one cell/well was confirmed microscopically after few hours and proliferating clones were expanded further.

3.2.2. Maintenance of mesenchymal stem cells in culture

3.2.2.1. Cell harvesting

The stem cells were sub cultured when they reached 70-80% confluency using 0.25% Trypsin and expanded in culture dishes. The cultures are rinsed briefly twice with PBS. Trypsin was added and kept for a period of five to seven minutes. The cells get detached from the culture flask and are obtained for counting in the hemocytometer and seeded accordingly.

3.2.2.2. Cell Count

The cells were diluted with trypan blue using a dilution of (1:1) and counted in the hemocytometer (Neubeaur, Germany). The cells were counted using an inverted microscope (Carl Zeiss) and seeded using the following formula:

Total cell count/ml = N*Dilution factor*V*10^4 /Number of squares

Where,

N = Total number of cells obtained after count

V = Volume of cell suspension

Dilution Factor – the amount of times the cell mix was diluted to obtain a count

Number of squares – the number of squares taken into account for the count

10^4 = the area of the hemocytometer.

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3.2.2.3. Cell cryopreservation

$1*10^5$ – $1*10^6$ cells were used for cryopreservation. Cells were trypsinized and washed at 280*g for seven minutes. The supernatant was removed and to the cell pellet was added 20% DMSO and FBS which together are called the freezing mix. The samples which were frozen in freezing vials and immediately placed in a 4°C cryo-cooler which was later placed in a -80°C and later transferred to liquid Nitrogen equipment.

3.2.2.4. Cell freezing for RNA

$1*10^5$ – $1*10^6$ cells were frozen for RNA. Cells were trypsinized and spun down at 280*g for seven minutes. Then the cell pellet was washed with cold PBS. TRiZol (Invitrogen) was added depending on the cell density obtained and frozen by placing at -80°C until required.

3.2.3. Growth properties of mesenchymal stem cells

3.2.3.1. Population doubling time

EOM-MSC were maintained were passage every 3-4 days. The cell density was maintained at 3000 cells/cm^2. After every passage the cell number was recorded. The EOM-MSC was cultured up to the twelfth passage.

The population doubling time of the EOM-MSC was calculated using the formula:

$$Gr = \ln (Nt) / \log (N0)/t,$$

Where, N (t) = the number of cells at time t

N (0) = the number of cells at time 0

Gr = Growth rate

T = time (usually in hours).

3.2.3.2. Colony forming units – fibroblast

To test the self-renewal capacity of EOM-MSC, cells were seeded in culture dishes at a seeding density of 100 cells per dish. They were cultured for 14 days and media

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change was done after every one week. The media added was low glucose DMEM media supplemented with 20% FBS. After 14 days, staining was done to identify the colonies formed. The staining was done first by fixing the colonies with methanol and subsequently stained with May-Grunwald's and Giemsa stain. Colonies with cells >50 are counted.

3.2.3.3. Study of the morphology of MSCs using Field Emission Scanning Electron Microscope

Cells at a density of 5000 cells/cm^2 were grown on fibronectin coated coverslips. After 24-48 hours the cells were fixed with ice-cold acetone: methanol (1:1) solution overnight at 4°C. Briefly wash away the fixative with PBS. The cells were placed in a moist environment at 37°C for one hour in an incubator. They were then dehydrated with graded series of ethanol (50%, 70%, 90% and 100%). The cells were gold coated with a sputter coater and viewed under Field Emission Scanning Electron Microscope (Zeiss, Sigma).

3.2.3.4. Karyotype Analysis

MSC at 60-70% confluency were treated with colchicine (0.1µg/ml) for 16 hours. The cells were trypsinized and resuspended in 0.56% KCl solution for thirty minutes at 37°C. The reaction was stopped by the addition of 2-3 drops of Carnoy's fixative to the cells and the cells were washed at a speed of 150*g for 5 minutes. The cells were washed twice with glacial acetic acid and methanol mixture (1:3) at a speed of 280*g for five minutes and suspended in 500µl of fixative. The cell mixture was dropped into clean wet slides. The slides were dried; chromosomes were stained with Giemsa and documented.

3.2.4. Differentiation of mesenchymal stem cells into the mesodermal lineage

3.2.4.1. Adipogenic Differentiation

For adipogenic differentiation, 1-2*10^4 cells were seeded at passages 2-4. After 24 hours of seeding, the differentiation media was added to cells. Media was replaced after 3-4 days. Differentiation was kept for 21-28 days. Differentiation was determined by histochemical staining and quantification and by gene expression

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analysis. Expression of ADIPOQ in adipogenic differentiated cells respectively was analyzed by real-time quantitative PCR.

Oil Red O staining

Oil droplets were fixed using 10% formalin for one hour, then washed with 60% isopropanol, then stained with Oil Red O stain for ten minutes and washed thoroughly with distilled water. The Oil Red droplets were enumerated microscopically to determine the number of adipocytes. To further quantify the differentiation, oil red o stain was eluted with 100% isopropanol and the absorbance was read at 500 nm in an ELISA Plate Reader.

3.2.4.2. Osteogenic differentiation

For osteogenic differentiation, $5*10^3$ cells of passages 2-4 were seeded. After 24 hours of seeding, the differentiation media was added to cells. Media was replaced after 3 days. Differentiation was kept for 21-28 days. Osteogenic differentiation was assessed by alkaline phosphatase staining. The cells were enumerated in the microscope to determine the number of differentiated cells. Alizarin Red S staining was done to check the calcium deposition in the osteocytes. Expression of OSTEOCALCIN in osteocyte differentiated cells respectively was analyzed by real-time quantitative PCR.

Alizarin Red S staining

Firstly, cells were fixed with 10% formalin for one hour. Then the cells were stained with Alizarin Red S for ten minutes at room temperature. To further quantify the differentiation, the Alizarin Red S stain was eluted with CPC and diluted accordingly. Absorbance was read at 562 nm.

3.2.4.3. Chondrogenic differentiation

For chondrogenic differentiation, micromass cultures were generated in a 12-well plate according to the manufacturer’s instructions (Thermo fisher Scientific). Media change was done every three days and the differentiated cells were stained with Safranin O after 21 days. Expression of Sox9 was analyzed in chondrogenic differentiated cells by real-time PCR.

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Safranin O staining

For Safranin O staining, cells were fixed with 0.1% glutaraldehyde solution for twenty minutes at room temperature. Then we rinsed the cells with PBS thrice and were quickly rinsed with 1% acetic acid for 10 seconds. Then the cells were stained with Safranin O solution for five minutes. We then rinse again with PBS and kept in distilled water. Photographs of the stained cells were immediately captured.

3.2.5. Neuronal differentiation of mesenchymal stem cells

3.2.5.1. Formation of Neurosphere

EOM-MSC was seeded at density of 1-2*10^5 cells/cm^2 in uncoated culture plates. The cells were supplemented with Neurobasal medium containing bFGF, EGF, at low serum condition (2%) with penicillin-streptomycin and grown at 5% CO_2, 92% N_2 and 3% O_2. Media was replaced after every one week but the growth factors were added at regular intervals that two times per week. In another group retinal extract was additionally added along with the growth factors. The differentiation was carried for 14-21 days.

3.2.5.2. Neuronal differentiation by direct conversion

The cells were plated in poly-l-lysine coated plates at a density of 1.5-2*10^5 cells/cm^2. For neuronal differentiation, culture medium was replaced with pre-induction medium composed of high glucose DMEM, FBS (20%), bFGF2 (10ng/ml), B27 (2%), Forkskolin (50µM), and 3-isobutyl-1-methyl-xanthine (IBMX, 250µM) with 2-β- mercaptoethanol (2-ME, 100 µM) and incubated for 24 hours. To initiate neuronal differentiation, the pre-induction medium was removed, washed with PBS, and replaced with neuronal induction medium containing DMEM, FBS (2%), bFGF2 (10ng/ml), insulin-transferrin-selenium supplements (1%), B27 (2%), Forkskolin (5µM), IBMX (125 µM), BDNF (50ng/ml) with 2-ME (100 µM), and all-trans retinoic acid (RA,0.5 µM). The neuronal induction was added only after 48 hours of addition of pre-induction medium and when the cultures had become confluent. The cells were incubated for an additional 14-21 days.

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3.2.5.3. CD49B separation by magnetic – activated cell sorting

The MACS method allows cells to be separated by incubating with magnetic nanoparticles coated with antibodies against a particular surface antigen. The cells expressing the CD49B antigen attached to the magnetic nanoparticles. Afterwards the cell solution is transferred on a column placed in a strong magnetic field. The cells attached to the nanoparticles (expressing the CD49B) stay on the column, while other cells (not expressing the antigen) flow through.

3.2.5.4. Detection of GFAP using immunofluorescence

The neurosphere was washed briefly with PBS. The cells were first fixed with 4% paraformaldehyde for 20 minutes at room temperature followed by permeabilization with 0.1% Triton X-100 for 15 minutes. The neurosphere were incubated with 2% FBS in PBS for an hour to prevent any nonspecific binding. Finally the neurosphere were incubated with anti-GFAP antibody (1:50 dilution) at 4°C overnight. The following day the antibody was removed and washed briefly in PBS and visualized in a fluorescent microscope.

3.2.6. Expression of surface proteins markers expressed in human mesenchymal stem cells

3.2.6.1. Immunofluorescence staining

Cells were washed with PBS and fixed with neutral-buffered 10% formalin for 20 minutes at room temperature. The cells were permeabilised with 0.05% triton X-100 for 10 minutes, washed and stained with primary antibodies **(Table 3)** which were diluted in 5% BSA solution at 4°C overnight. The following day we briefly washed away the primary antibody with blocking buffer and fluorescently conjugated secondary antibody was added overnight at 4°C. Nuclei were stained with 4’, 6-Diamidino-2-phenylindole, DiHydrochloride (DAPI, 5mg/ml) diluted in 5% BSA at a dilution of 1:2000 in 5% BSA solution for five minutes at room temperature. The cells were finally washed, mounted and documented microscopically (Zeiss, Axio Observer Z1).

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3.2.6.2. Mitochondrial Staining using TMRE

Mitochondria in EOM-MSC were visualized by staining with Tetramethylrhodamine, ethyl ester (TMRE). Briefly, TMRE (100nM) was added to the cells in the culture media and incubated at 37°C for thirty minutes. Stained cells were visualized using Zeiss Axio Observer Z1 inverted microscope (Zeiss, Gottingen, Germany).

3.2.6.3. SSEA4 Staining

The number of cells seeded for SSEA-4 staining were 3000 cells/cm^2. The cells were cultured on fibronectin coated coverslips for 24-48 hours. The cells were washed with PBS and incubated with anti-SSEA4 antibody for one hour. The cells were washed and stained with fluorescent conjugated secondary antibody. The stained cells were microscopically documented.

3.2.7. Phenotypic studies by flow cytometry

Adherent cells were trypsinized and resuspended in freshly prepared ice cold 2% FBS solution in PBS. The cells were washed at a speed of 270*g for 7 minutes. They were stained with fluorescent dye conjugated monoclonal antibodies against human cell surface antigens. The cells were incubated at 4°C for 30 minutes, washed and analyzed with FACS caliber (Becton Dickinson). Propidium Iodide (2 µg/ml) was added before analysis for live/dead separation.

For intracellular markers, the cells were trypsinized and fixed with 4% paraformaldehyde and permeabilised with Triton X-100 (0.1%) and stained with fluorescently conjugated antibodies for one hour at room temperature.

3.2.8. Gene expression studies

3.2.8.1. RNA Isolation

Total cellular RNA was isolated from EOM-MSC and BM-MSC using TRiZol Reagent as per the manufacturer's instruction. Briefly, the cells were lysed with TRiZol. The lysate was mixed thoroughly with the addition of chloroform. The lysate was spun down and after centrifugation, the aqueous phase was collected in a

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fresh DEPC-treated tube to which 100% isopropanol was added. The mixture was pelleted and washed with 75% ethanol after which the pellet was dried. The pellet was finally dissolved in RNase/DNase free water and quantified spectrophotometer.

3.2.8.2. Reverse Transcription (cDNA synthesis)

cDNA was synthesized by reverse transcription using a high capacity cDNA reverse transcription kit (Applied Biosystems, USA). Briefly, 2X reverse transcription master mix was prepared by adding the following kit components for a 15 µl reaction. All the reagents were kept at 4°C and enzymes were stored at -20°C.

Reverse transcription was carried out in a Veriti® Thermal Cycler (Applied Biosystems, USA).

The conditions for reverse transcription were as follows:

Holding	25°C for 10 minutes
Extension	37°C for 120 minutes
Holding	85°C for 5 minutes
Holding	4°C ∞

cDNA was stored in -20°C until use.

3.2.8.3. Real Time Quantitative PCR

The conditions for real-time polymerase chain reaction (PCR) were as follows: initial denaturation at 94°C for 10 minutes followed by 40 cycles of denaturation at 94°C for 15 seconds, primer annealing and extension at 60°C for 45 seconds and a final extension at 72°C for ten minutes using Power SyBr Green real-time PCR master mix using 7500 Real-time PCR System. The gene expression levels in each sample were normalized to their respective GAPDH expression levels. The expression levels were quantified using $\Delta\Delta$ Ct method.

3.3. Statistical Analysis

Flow Cytometry data was analyzed using FCS express 5. Karyotype figures were analyzed using Image J software. The cell size was analysed using ImageJ in which each image were calibrated using a calibration micrometer. The length and area of at least 10 cells in the field were taken into consideration and the average size of the cells were calculated. Statistical analysis was performed with SPSS software and the values of $p<0.05$ were considered statistically significant. The difference in gene

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expression between EOM-MSC and BM-MSC was analyzed by Mann Whitney non-parametric variables test. The difference in gene expression between control and osteogenic, adipogenic and chondrogenic and neuronal differentiated EOM-MSC samples were tested using paired samples t-test.

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RESULTS

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4.0. RESULTS

4.1. Isolation and characterization of mesenchymal stem cells from ocular adipose tissue

4.1.1. Isolation of MSC from ocular adipose tissue

The ocular adipose tissue was obtained during blepharoplasty of the upper eyelid when redundant orbital fat tissue was removed from the intra-orbital cavity. The tissues which were discarded after surgery were collected in sample vials and processed by briefly rinsing in PBS containing antibiotics. The tissue was dissociated with the forceps and plated in fibronectin coated plates. For the current study, we have obtained two types of tissues – the ocular central and the medial adipose tissue and the cells isolated from both tissues were compared with each other. The central adipose tissue was larger and yellow in color compared to the medial adipose tissue which is paler and smaller in size **(Figure 4.1.1)**. Adherent and spindle-shaped cells emerged from the central adipose tissue within 4 days of plating, but the cells appeared after two weeks from the medial adipose tissue. Fibroblastic colonies from both the central and medial adipose tissues were subcultured when they became 70-80% confleunt. The cells obtained from central and medial adipose tissue were termed C-OAT-MSC and M-OAT-MSC respectively. Morphologically, both C-OAT-MSC and M-OAT-MSC closely resembled BM-MSC, they were spindle-shaped, adherent cells and proliferated rapidly **(Figure 4.1.2)**. At isolation (P0) the C-OAT-MSC appeared to have a heterogeneous population of spindle-shaped and star-shaped cells **(Figure 4.1.2.C).** There was no significant difference in the morphology and proliferation of OAT-MSC between the left and right central and medial adipose tissues and from passage 1 onwards the cells' morphology appeared to be homogenous and more elongated in shape **(Figure 4.1.2. E & F)**. The medial AT-MSC have a smoother appearance compared to the central AT-MSC.

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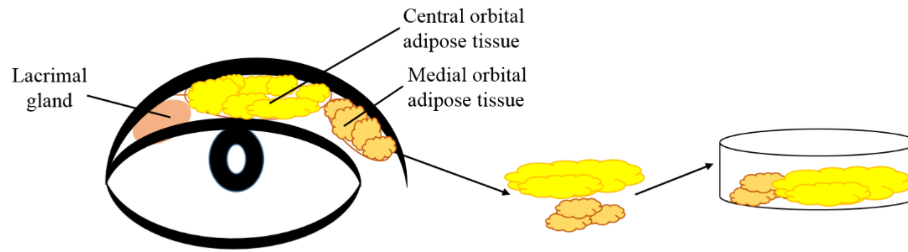

Figure 4.1.1. *A figure representing the ocular adipose tissue from the upper eyelid of the human eye. The central and the medial ocular adipose tissues were collected during blepharoplasty surgery. The tissues were mechanically dissociated and plated in growth media.*

4.1.1.2. Cell Morphology

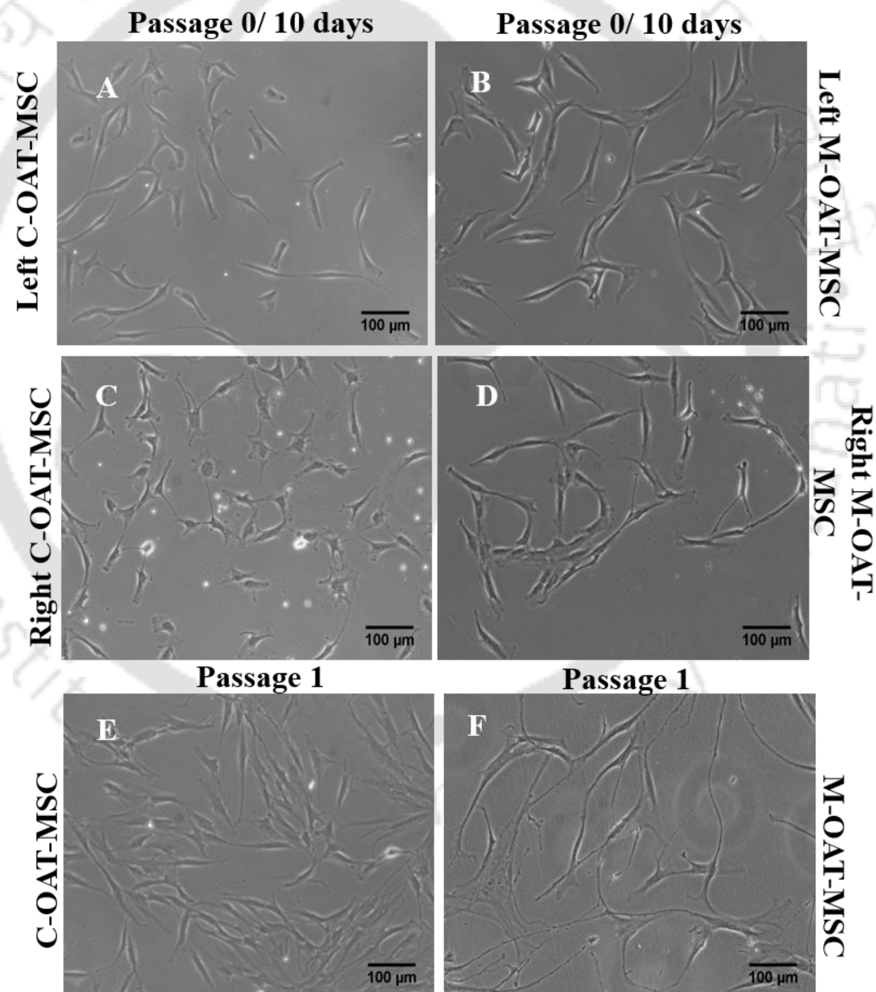

Figure 4.1.2. Morphology of OAT-MSC. OAT-MSC were isolated from adipose tissue of the right and left human eye. *Phase contrast microscopic images of* ***(A)*** *left central OAT-MSC* ***(B)*** *left medial OAT-MSC* ***(C)*** *right central OAT-MSC and* ***(D)*** *right medial OAT-MSC showing spindle shaped adherent cells at isolation (P0). The spindle-shaped cells represents OAT-MSC of* ***(E)*** *the central tissue and* ***(F)*** *of the medial tissue at passage 1. Magnification 10X.*

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Using ImageJ software, the average area of the C-OAT-MSC was recorded to be 102 μm^2. On the other hand, the average area of the M-OAT-MSC was calculated to be 82 μm^2. The C-OAT-MSC were larger in size compared to the M-OAT-MSC.

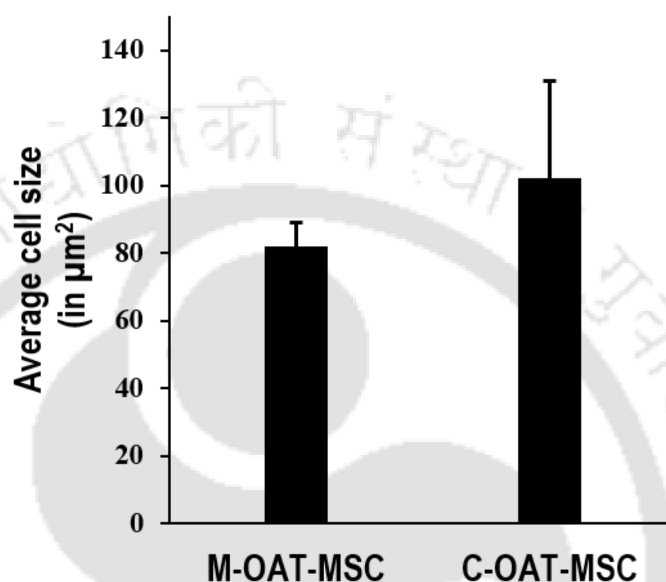

Figure 4.1.3. Cell size in C-OAT-MSC and M-OAT-MSC. *The graph represents the average area of C-OAT-MSC and M-OAT-MSC in* μm^2.

4.1.2. Surface marker expression of OAT-MSC

To characterize the OAT-MSC further, we investigated the expression of MSC markers by flow cytometry. The central and medial OAT-MSC (passage 4) were trypsinized and stained with antibodies against surface markers such as the CD34, CD44, CD49B, CD73, CD90 and CD105. An unstained control was also set to compare with the stained samples. The central and medial OAT-MSC from different donors expressed the markers such as CD44, CD73, CD90 and CD105 and also express CD34 a muscle stem cell marker (**Figure 4.1.4 and 4.1.5**). The C-OAT-MSC expressed CD44 (95.77$\pm$3.10), CD49B (46.83$\pm$25.8), CD73 (98.77$\pm$0.35), CD90 (92.47$\pm$5.67) and CD105 (97.90$\pm$0.53). The M-OAT-MSC expressed CD44 (98.12$\pm$1.20), CD49B (88.1$\pm$4.47), CD73 (98.72$\pm$1.36), CD90 (88.58$\pm$7.46) and CD105 (95.90$\pm$3.16). Unlike BM-MSCs, the C-OAT-MSC and M-OAT-MSC expressed CD34 at 73.57$\pm$19.82 and 67.13$\pm$14.69 respectively.

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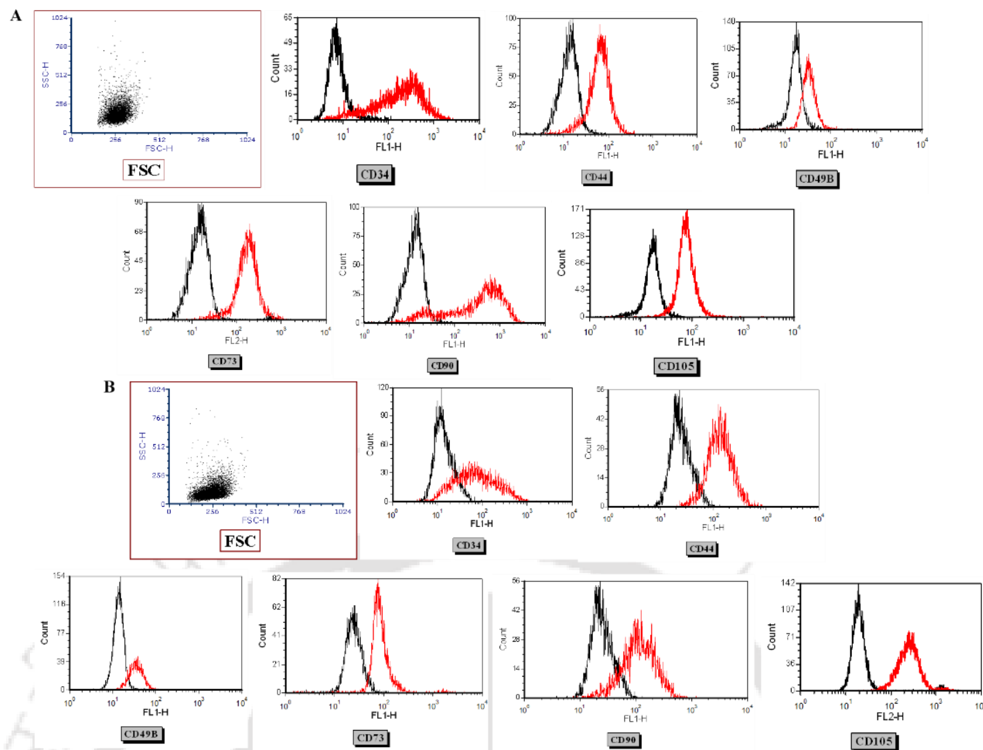

Figure 4.1.4. Surface marker profile of (A) C-OAT-MSC and (B) M-OAT-MSC analyzed by flow cytometry. *The histograms represent the surface marker profiles of OAT-MSC when they were stained with monoclonal antibodies tagged with fluorescent probes. The live vs dead population is identified using the PI dye. In figure the black line in the histogram represents the isotype control while the red line represents the antibody against surface marker. Both C-OAT-MSC and M-OAT-MSC expressed CD44, CD49B, CD73 and CD90 and CD105. There was an expression of CD34 in both the adipose stem cells.*

Marker	% expression level in C-OAT-MSC	% expression level in M-OAT-MSC
CD34	73.57±19.82	67.13±14.69
CD44	95.77±3.10	98.12±1.20
CD49B	46.83±25.8	88.1±4.47
CD73	98.77±0.35	98.72±1.36
CD90	92.47±5.67	88.58±7.46
CD105	97.90±0.53	95.90±3.16
CD120A	0.33±0.01	0.58±0.33

Table 4.1.5. *The percentage expression of surface markers expressed in C-OAT-MSC and M-OAT-MSC. Several markers expressed on the surface of BM-MSC were also expressed by the OAT-MSC.*

By immunocytochemistry staining, the expression of the MSC markers on both the central and medial OAT-MSC was further confirmed. The flow cytometry analysis

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along with the immunocytochemistry results confirmed the expression of CD44, CD73 and CD90 in C-OAT-MSC and M-OAT-MSC. CD140A and CD146 was found to be minimally expressed only in M-OAT-MSC but not in C-OAT-MSC.

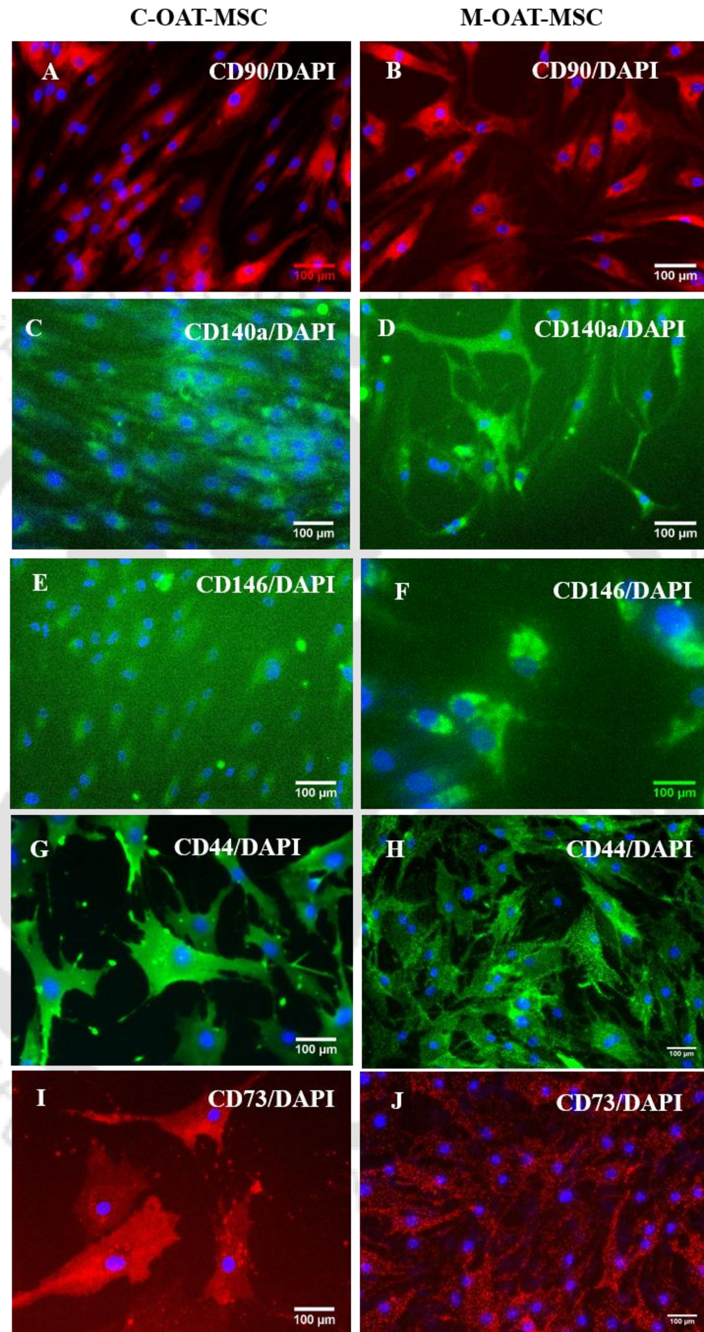

Figure 4.1.6. Surface marker expression of OAT-MSC by immunocytochemistry. *The undifferentiated OAT-MSCs were stained with primary antibody against the antigen and a secondary antibody tagged with a fluorescent probe. The photomicrographs represent CD90 expressed in both (**A**) central (**B**) medial MSC. CD140a was not expressed only in (**C**) central but expressed in (**D**) medial. CD146 was also expressed only in M-OAT-MSC (**E**) central and (**F**) medial. CD44 was expressed in both (**G**) central and medial (**H**) OAT-MSC. CD73 was investigated in central (**I**) and medial (**J**) OAT-MSC. Magnification 20X. Blue stain – DAPI.*

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4.1.3. The expression of embryonic markers in OAT-MSC

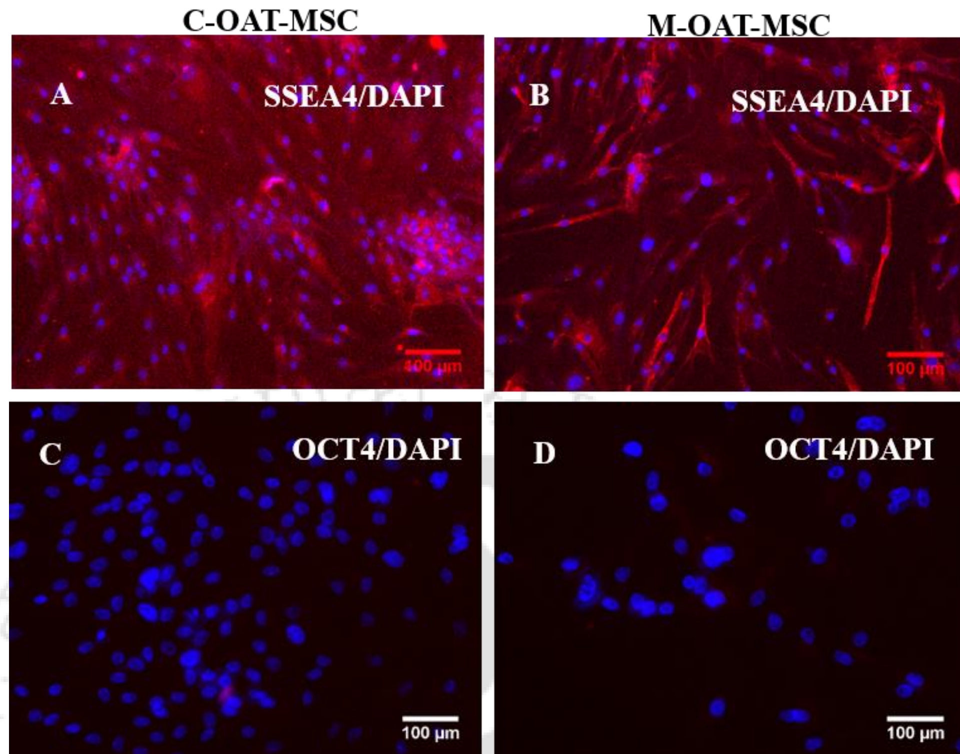

Figure 4.1.7. Expression of embryonic markers in central and medial OAT-MSC by immunocytochemistry. *By immunocytochemistry SSEA-4 an embryonic surface marker was also found to be expressed in spindle shaped cells in* ***(A)*** *central and* ***(B)*** *medial. OCT-4 an embryonic transcription factor however did not appear in both* ***(C)*** *central and* ***(D)*** *medial OAT-MSC in culture. Magnification 10X. Blue stain – DAPI.*

SSEA-4, a marker to define embryonic stem cells was expressed in spindle-shaped cells of the central and medial OAT-MSC. The expression was low in C-OAT-MSC compared to M-OAT-MSC. However OCT-4, an embryonic transcription factor, expression was not seen in either the central or medial OAT-MSCs by immunocytochemistry analysis.

4.1.4. Differentiation of OAT-MSC into Mesodermal Lineage

To check the multilineage differentiation capacity of the central and medial adipose tissue derived MSCs, they were allowed to differentiate into adipocytes, osteocytes and chondrocytes. The cells were incubated in differentiation media for 21 days. During adipogenic differentiation oil droplets accumulated in cells which were stained by Oil Red O. Both medial and central OAT-MSC differentiated into

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adipocytes. Calcium deposition during osteogenic differentiation was detected by Alizarin Red staining and osteogenic differentiation was found in both medial and central OAT-MSC. The micromass which was formed after 21 days of chondrogenic differentiation was detected by safranin O staining (**Figure 4.1.8**).

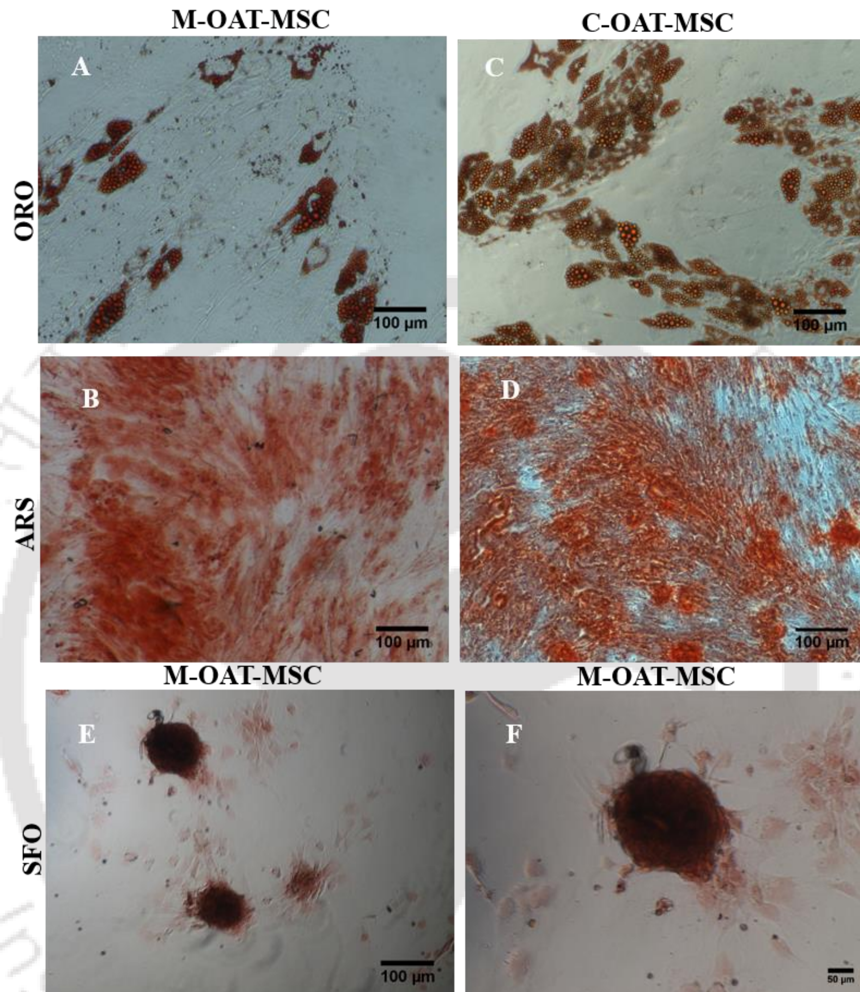

Figure 4.1.8. Mesodermal differentiation of OAT-MSC. *(**A-F**) are C-OAT and M-OAT MSC differentiated into the adipocytes, osteocytes and chondrocytes for 21 days. (**A, D**) adipogenic differentiation was determined by staining with Oil red O, (**B, E**) osteogenic differentiation by staining with alizarin red, and (**E, F**)) chondrogenic differentiation was determined by Safranin O staining done only in M-OAT-MSC. Magnification 10X.*

Both centrally derived and medial derived MSC resembled the BM-MSC in their differentiation capacity into the mesodermal lineage. However, there is a significant difference in the differentiation capacity of the M-OAT-MSC and C-OAT-MSC, where C-OAT-MSC showed higher differentiation capacity compared to M-OAT-MSC (**Figure 4.1.9.**).

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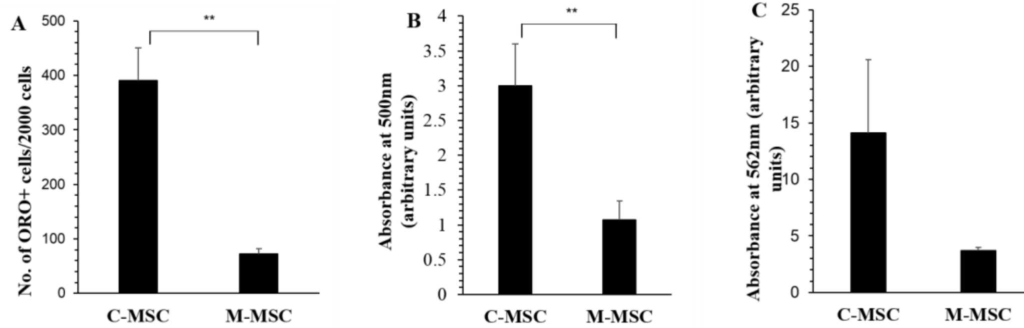

Figure 4.1.9. Quantification of ARS stain for osteocyte differentiation and ORO stain for adipocyte differentiation. *(**A**) No of ORO positive cells were counted after differentiation (**B**) to quantify ORO stain absorbance was measured at 500 nm. (**C**) To quantify ARS stain absorbance was measured at 562 nm. The C-OAT-MSC showed a higher capacity to differentiate into osteocyte and adipocyte compared to the M-OAT-MSC,* ***p<0.001.***

4.1.5. Gene expression analysis

Neurotrophic factors, namely brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), FGF2 and FGF8 produced by MSCs contribute greatly to their beneficial effects against neurodegeneration, whereas neuronal differentiation of MSCs seems not to be the main beneficial property (Gugliandolo *et al*., 2016). Hence we have checked their expression from undifferentiated samples. Both C-OAT and M-OAT MSCs express BDNF, GDNF, FGF8 and bFGF, however they are similar in both cell types. BDNF and FGF2 expression are similar in C-OAT and M-OAT MSC while there is a lower expression of GDNF and FGF8 compared to that of BDNF and FGF2 in both the stem cells. Furthermore to understand the nature of C-OAT-MSC and M-OAT-MSC, the markers that control their self-renewal and pluripotency were analyzed. The expression of embryonic transcription factors such as OCT4, NANOG and SOX-2 and OCT4 were determined by real time PCR. In addition, OCT4 protein was undetectable in both the MSCs unlike their mRNA levels which was upregulated during gene expression analysis (**Figure 4.1.10**).

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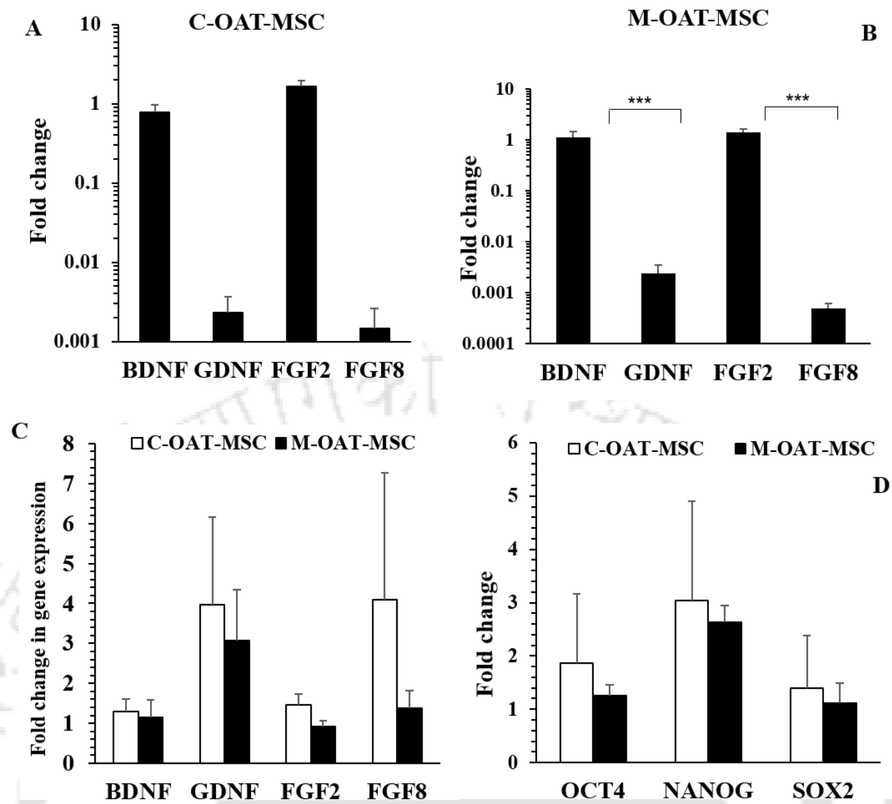

Figure 4.1.10. Gene expression analysis in OAT-MSC. *Expression levels of neurotrophic factors – BDNF, GDNF, FGF8 and FGF2 was analysed by real time PCR and compared with each other. From* ***(A)*** *and* ***(B)*** *there was a similar expression level of BDNF and FGF2 and* ***(C)*** *is a comparison between C-OAT-MSC and M-OAT-MSC* ***(D)*** *Expression levels of transcription factors OCT4, NANOG and SOX2 in C-OAT-MSC and M-OAT-MSC was analysed by real time PCR and compared with each other.*

4.1.6. Neuronal Differentiation of OAT-MSC

The embryonic origin of medial adipose tissue is from the neuroectodermal region (S-Y Chen *et al* and Korn *et al*) whereas the central adipose tissue is derived from the mesodermal layer (Wester 2014). So, we investigated the differentiation potential of C-OAT-MSC and M-OAT-MSC into neuronal lineage. The central and medial OAT-MSC were cultured in neuronal media for 21 days. The differentiation into neuronal lineage was confirmed by the expression of nerve growth factor receptor (NGFR), Nestin and β-III-tubulin **(Figure 4.1.11).**

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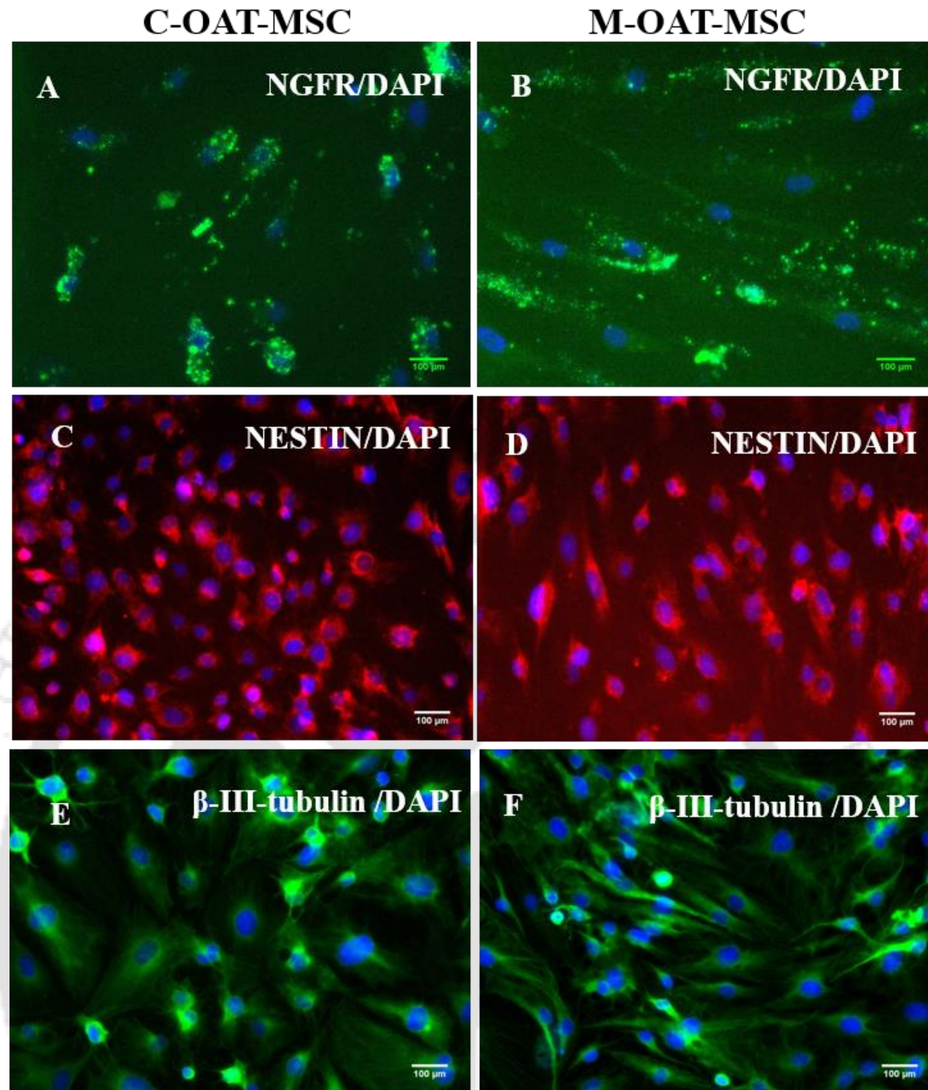

Figure 4.1.11. Neuronal differentiation of OAT-MSC. *Immunofluorescence analysis of neuronal differentiated OAT-MSC showing the expression of NGFR in* ***(A)*** *central and* ***(B)*** *medial OAT derived MSC. The Nestin marker was found to be expressed in* ***(C)*** *central and* ***(D)*** *medial OAT-MSC. β-III-tubulin was expressed in* ***(E)*** *central and* ***(F)*** *medial OAT-MSC after neuronal differentiation in vitro. Representative microscopic images are shown. Blue stain – DAPI.*

C-OAT-MSC and M-OAT-MSC stained positive for NGFR, Nestin and β-III-tubulin suggesting their neuronal differentiation capacity. Thus OAT-derived MSC can be a good source for cell therapy as they have both mesodermal and neuroectodermal differentiation capacity.

Central OAT-MSC might be more suitable for bone tissue engineering due to its high osteogenic differentiation capacity whereas medial OAT-MSC might be used

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for neuronal therapy considering its neuroectodermal origin and neuronal differentiation capacity.

4.2. Isolation and characterization of mesenchymal stem cells from extra ocular muscle tissue

4.2.1. Isolation of mesenchymal stem cells from extra ocular muscle tissue

Extra ocular muscle tissue was obtained from both male and female patients with median age of 21 years, who underwent strabismus correction/surgery. The sample obtained from patients was of the medial rectus, lateral rectus and inferior oblique. The samples were processed within 12-14 hours of collection. Spindle-shaped cells emerged from the tissue within one-two weeks of plating from the tissue. The isolation success percentage was an average of 80%. Media was being replaced after 3-4 days till the culture reached confluency. The cells in colonies appeared spindle in shape and were adherent to the substratum (**Figure 4.2.3.A**). These cells could be cultured up to the twelfth passage without any change in morphology or growth properties (**Figure 4.2.4**). These cells were termed as the extra ocular muscle-mesenchymal stem cells (EOM-MSC). The morphology of these stem cells appeared to be similar to the bone marrow derived mesenchymal stem cells – fibroblastic in shape, adherent and highly proliferative. The EOM-MSC could be cryopreserved, thawed and cultured successfully.

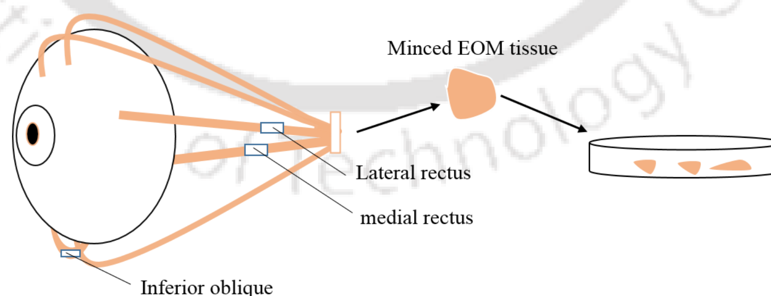

Figure 4.2.1. Extra ocular muscle tissue. *A figure representing a freshly resected extra ocular muscle tissue from human eye. The tissue was collected during strabismus surgery. The tissue was mechanically dissociated and plated in growth media.*

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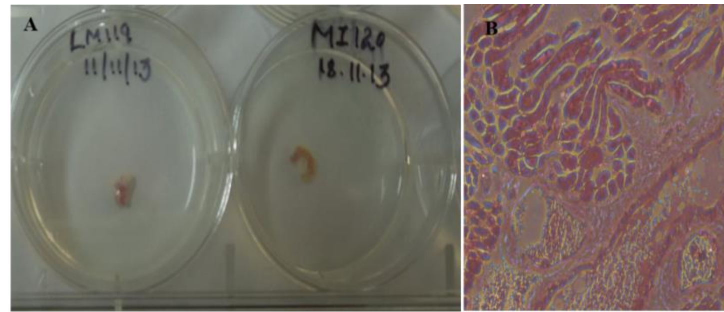

Figure 4.2.2. Extra ocular muscle tissue and tissue section. *(A) EOM-MSC were isolated from freshly resected EOM tissue from the human eye. Figure shows the image of tissue after being processed **(B)** EOM tissue section stained with H&E. Magnification (10X).*

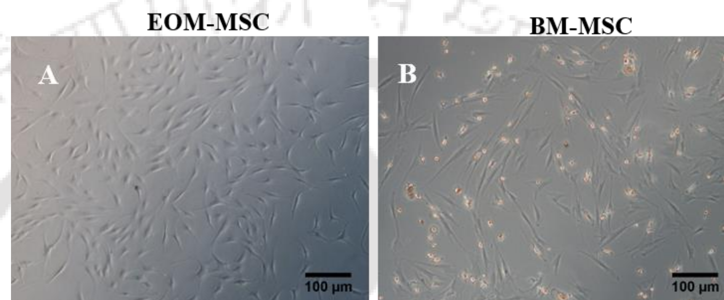

Figure 4.2.3. The morphology of EOM-MSC during isolation. *The figure represents a photomicrographic representation of spindle-shaped adherent **(A)** EOM-MSC and **(B)** BM-MSC taken under an inverted microscope. Magnification 10X. Scale bar 100 µm.*

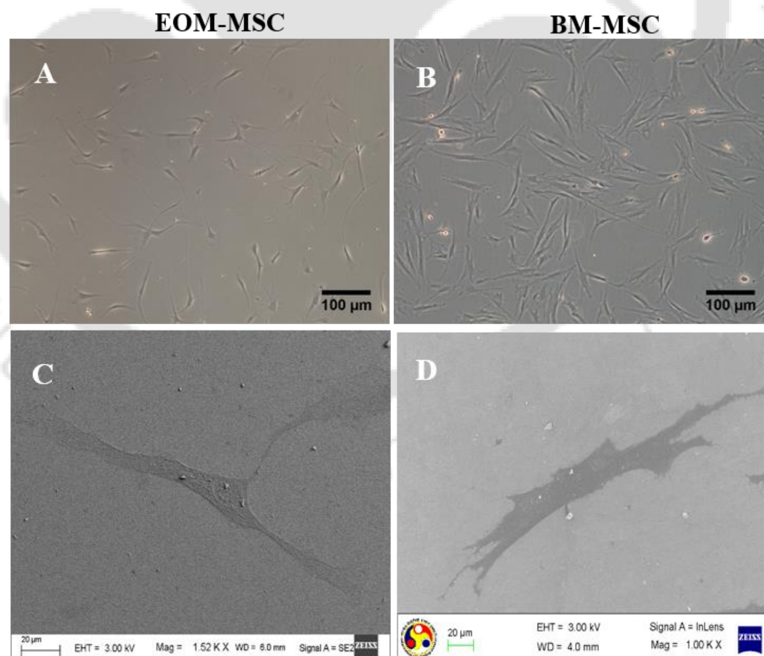

Figure 4.2.4. The morphology of EOM-MSC during expansion. *The images above depict the morphology of the **(A)** EOM-MSC and **(B)** BM-MSC at different passages and **(C) and (D)** are representative FESEM images of EOM-MSC and BM-MSC respectively.*

The size of the EOM-MSC was calculated to have an average area of 85 μm^2 while BM-MSC was calculated to have an average area of 112 μm^2.

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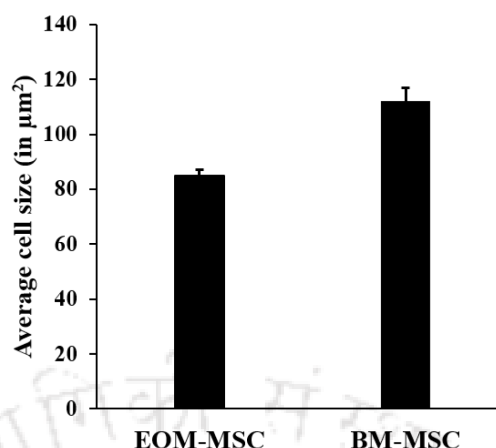

Figure 4.2.5. The average cell size of EOM-MSC and BM-MSC in µm². *The graph represents the average area (µm²) of EOM-MSC vs BM-MSC. The BM-MSC were bigger in size compared to EOM-MSC.*

4.2.2. Growth properties of EOM-MSC

a. Population Doubling Time

The population doubling time was calculated to be 34 hours in EOM-MSC and 42 hours in case of BM-MSC. EOM-MSC proliferated faster than BM-MSC in our culture conditions.

b. Karyotyping

Further to check if there was any alteration in the chromosome number of EOM-MSC due to prolonged culture, karyotyping was performed to assess the chromosome number. Karyotype of EOM-MSC and BM-MSC from different passages was prepared. The karyotype of both EOM-MSC and BM-MSC was found to be normal (2n=46), without any gross abnormality throughout different passages (**Figure 4.2.5**). This was done to determine whether the cells underwent any chromosomal abnormality due to ex vivo expansion that will make these cells unsuitable for therapeutic purposes.

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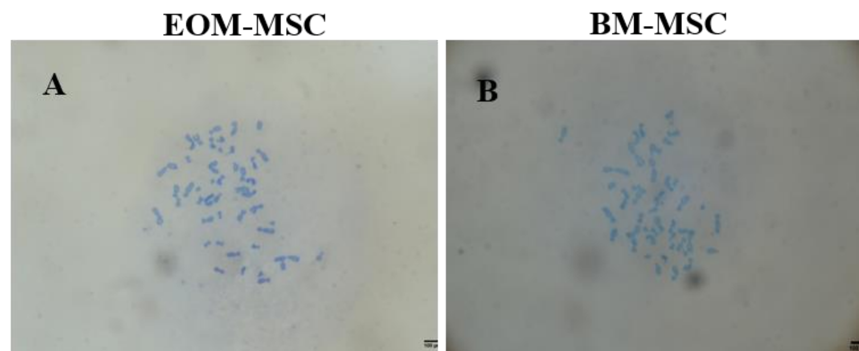

Figure 4.2.6. Karyotype of EOM-MSC. *A metaphase chromosome spread of the* ***(A)*** *EOM-MSC and* ***(B)*** *BM-MSC having normal chromosome number (2n = 46) without any gross abnormality. The microscopic images were taken at 100X with immersion oil from a light microscope. (Scale Bar = 100µm). Magnification 100X.*

With the aid of ImageJ software we identified and arranged the chromosomes according to their shapes and sizes thereby prepared the karyogram of a particular chromosome spread of the EOM-MSC.

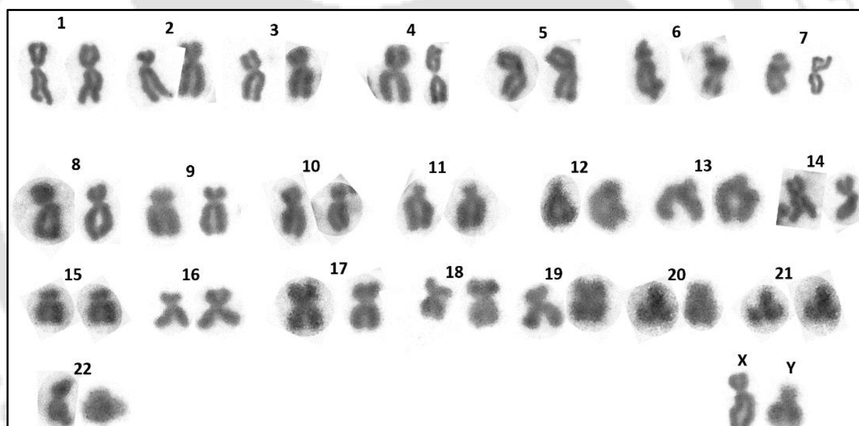

Figure 4.2.7. Karyogram of the EOM-MSC. *Represents the normal karyotype of EOM-MSC (2n=46).*

c. Colony Forming Unit- Fibroblasts

The colony forming unit-fibroblasts is an assay to test the self-renewal potential of a stem cell when plated at low cell-density *in vitro*. When the cultures were supplemented with DMEM medium containing 20% FBS, colonies of cells derived from single cells appeared after one week of seeding. The colonies were cultured for two weeks. The graph plotted below suggested that EOM-MSC had higher CFU-F forming ability compared to BM-MSC.

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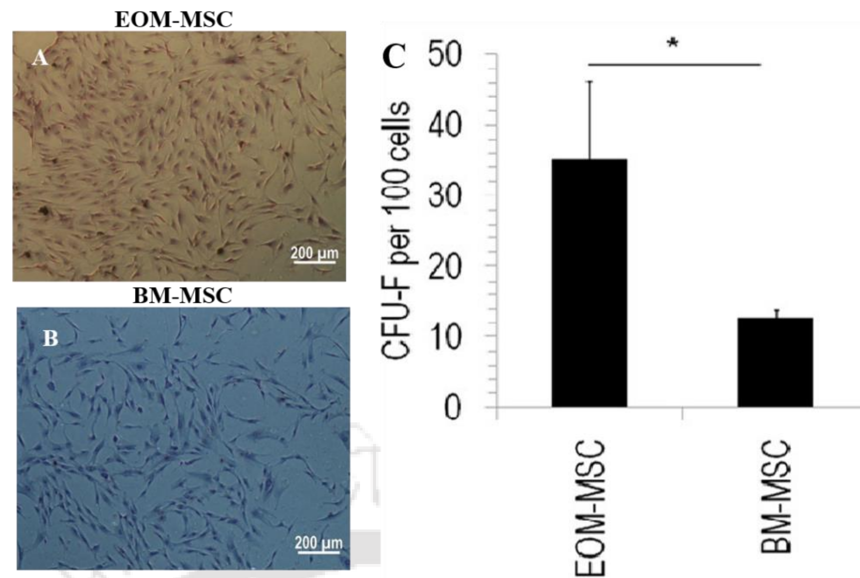

Figure 4.2.8. Colony forming units-fibroblasts of EOM-MSC. *The colonies formed after the EOM-MSC were cultured in DMEM supplemented with 20% FBS. The colonies were stained with May-Grunwald's and Giemsa Staining after 14 days. Photomicrographic images of* ***(A)*** *EOM-MSC and* ***(B)*** *BM-MSC were taken.* ***(C)*** *The graph alongside depicts the CFU-F per 100 cells EOM-MSC vs BM-MSC. The number of colonies (CFU-F) is higher in EOM-MSC compared to BM-MSC. Magnification 5X. (Scale bar = 200µm).*

4.2.3. Differentiation of EOM-MSC into the mesenchymal lineage

To further check their mutipotent differentiation potential, EOM-MSC were differentiated into adipocytes, osteocytes and chondrocytes. EOM-MSC readily differentiated into the adipocytes, osteocytes and chondrocytes after 21-30 days of differentiation. Oil droplets distributed within the adipocytes were stained with Oil Red O at the end point. Osteogenic differentiation was determined by the alkaline phosphatase activity and the calcium deposition was further confirmed after 21-30 days of differentiation by staining with Alizarin Red. The chondrocytes formed a micromass of cells after 21 days and chondrogenic differentiation was detected by Safranin O staining (**Figure 4.2.9, A-H**). Overall the EOM-MSC had a lesser capacity to differentiate into the adipocytes and osteocytes compared to the BM-MSC.

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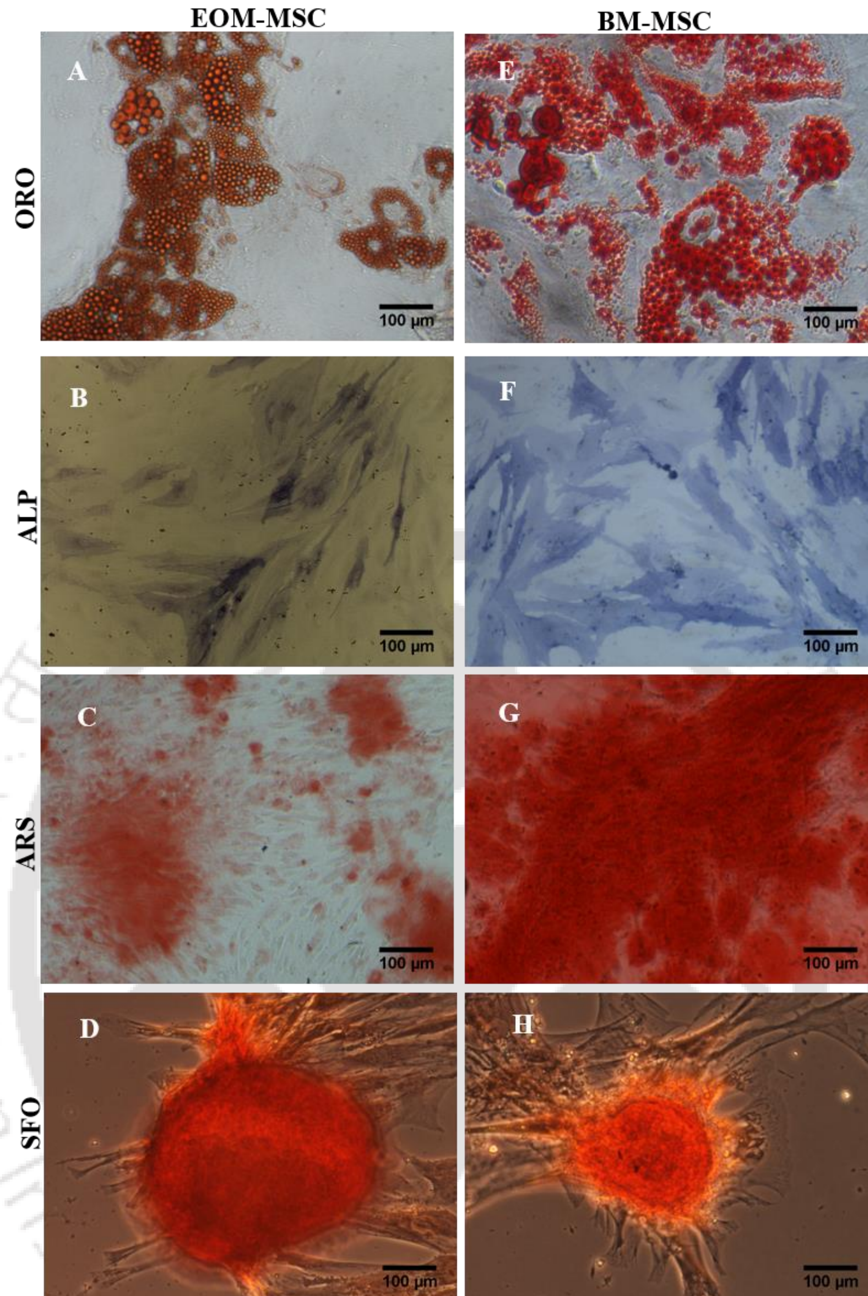

Figure 4.2.9. Mesodermal lineage differentiation of EOM-MSC and BM-MSC. *The image demonstrates the differentiation of EOM-MSC and BM-MSC into adipocytes, osteocytes and chondrocytes for 21-28 days.* ***(A)*** *&* ***(E)*** *represents the fat droplets which are stained with Oil Red O,* ***(B)*** *&* ***(F)*** *represents the osteocytes stained with ALP and* ***(C)*** *&* ***(G)*** *is the calcium deposition during later stages of osteogenic differentiation as detected with Alizarin Red S stain,* ***(D)*** *&* ***(H)*** *represents the chondrocytes stained with safranin O in EOM-MSC & BM-MSC respectively. (Scale Bar = 100 µm and 200 µm). Magnification 10X.*

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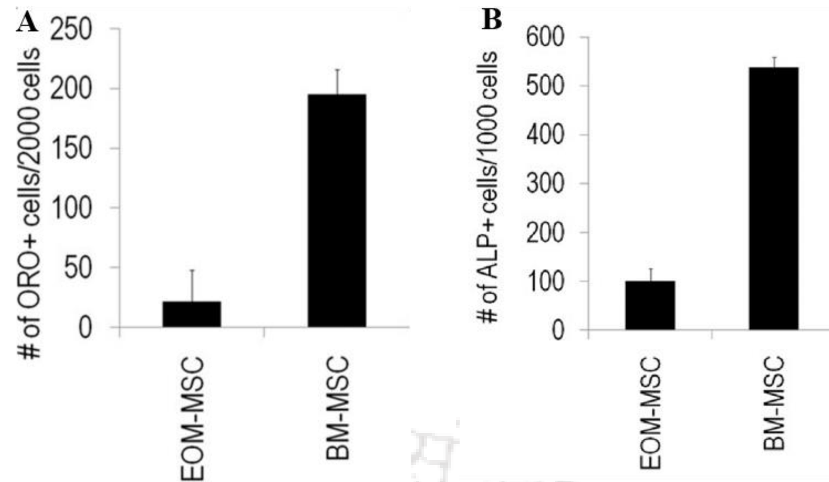

Figure 4.2.10. Mesodermal differentiation of EOM-MSC. *(**A**) The number of ORO positive (ORO) positive cells per 2000 cells and (**B**) alkaline phosphatase positive cells per 1000 cells were calculated for EOM-MSC and BM-MSC after differentiation into respective lineages.* ***Values are Mean±SEM, n=4-7.***

Differentiation into either adipocytes, osteocytes or chondrocytes in both EOM-MSC and BM-MSC was confirmed by gene expression analysis. To identify adipogenic differentiation, transcript levels of ADIPOQ was determined. There was significant upregulation of ADIPOQ in adipo differentiated EOM-MSC compared to control undifferentiated EOM-MSC. However, the relative gene expression levels of ADIPOQ was significantly less in EOM-MSC compared to BM-MSC.

To confirm osteogenic differentiation transcript levels of osteocalcin was determined. There was a significant upregulation of OSTEOCALCIN in osteo differentiated EOM-MSC compared to control undifferentiated EOM-MSC. However, the relative gene expression levels of OSTEOCALCIN was significantly less in EOM-MSC than in BM-MSC.

Similarly, there was a signficant upreguation of SOX9 in chondro differentiated EOM-MSC compared to control undifferentiated EOM-MSC. However, the relative gene expression levels of SOX9 was significantly less in EOM-MSC compared to BM-MSC.

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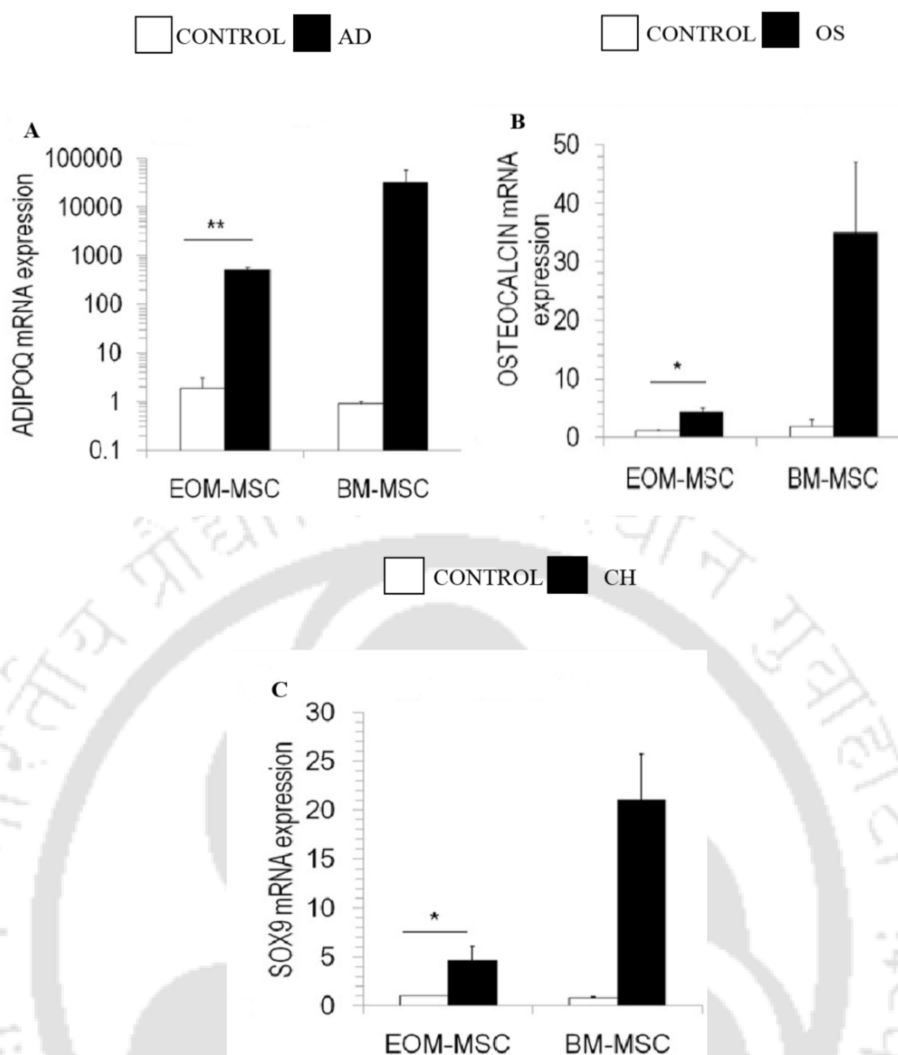

Figure 4.2.11. Genes expressed during mesodermal differentiation analyzed by real time PCR. *Real time PCR was performed to determine the expression levels of **(A)** ADIPOQ, **(B)** OSTEOCALCIN **(C)** SOX-9, in undifferentiated (CONTROL), adipo-differentiated (AD), osteo-dfferentiated (OS) or chondro differentiated (CH) EOM-MSC and BM-MSC respectively. **Values are mean±SEM, n=3**. The expression levels were normalized to GAPDH expression levels in the respective samples. **EOM-MSC and BM-MSC were from passages 3-5 *p<0.05.** ORO- Oil Red O, ALP – Alkaline phosphatse, ARS- Alizarin Red, SFO stain - Safranin O.*

4.2.4. Cell surface marker expression analysis

The EOM-MSC were similar to the BM-MSC in their morphology, growth properties, CFU-F forming potential and differentiation capacity. To further characterize these cells, the cell surface markers expression was analysed. The cells were stained with monoclonal antibody tagged with flourescent molecules against specific antigens and detected in the flow cytometer. The EOM-MSC from different donors were stained for cell surface antigens CD13, CD29, CD34, CD44, CD45,

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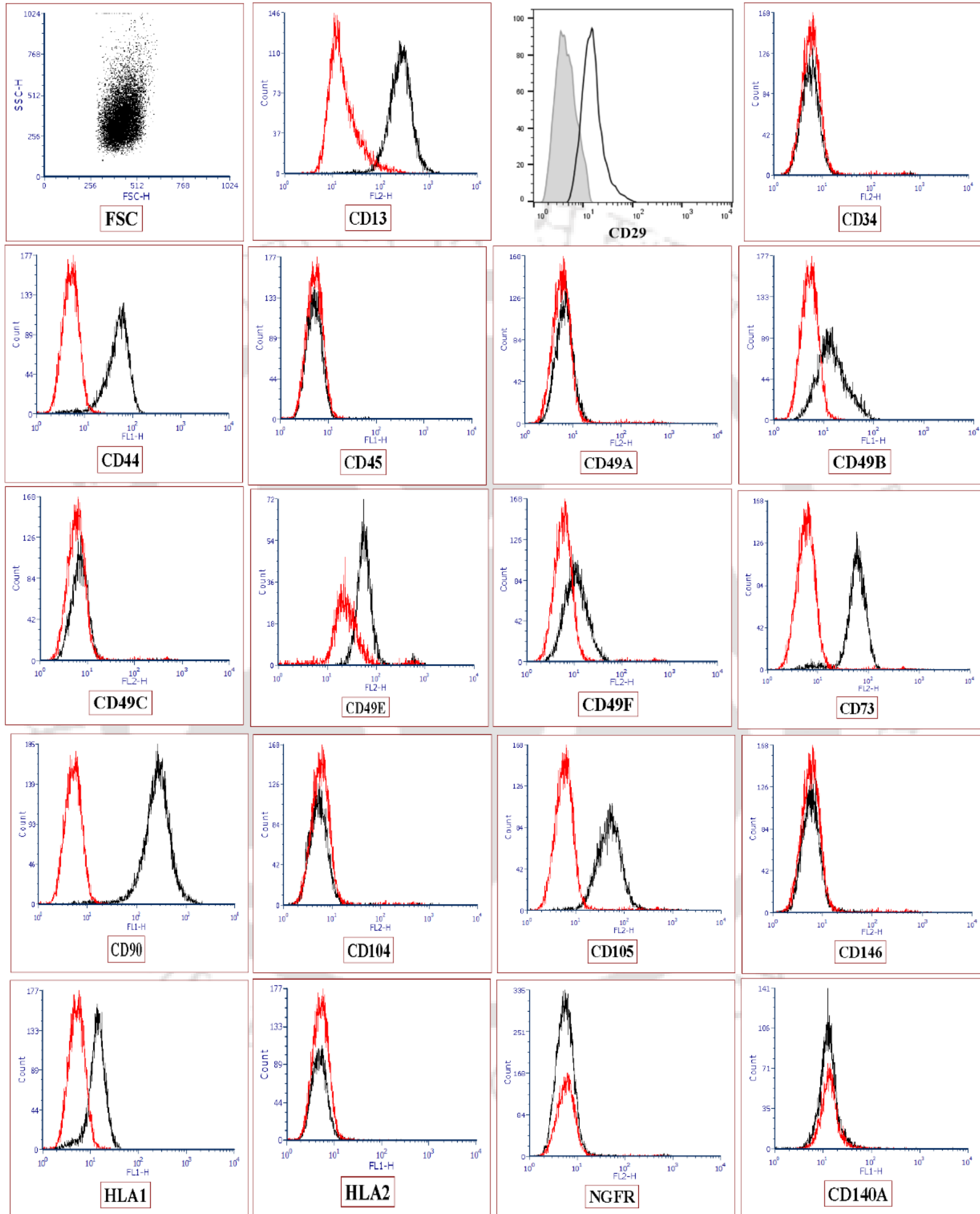
CD49A, CD49B, CD49D, CD49E, CD49F, CD73, CD90, CD104, CD105, CD146, CD200. CD271, HLA Class I, HLA class II, GFAP, MAP2B CD140A.

A



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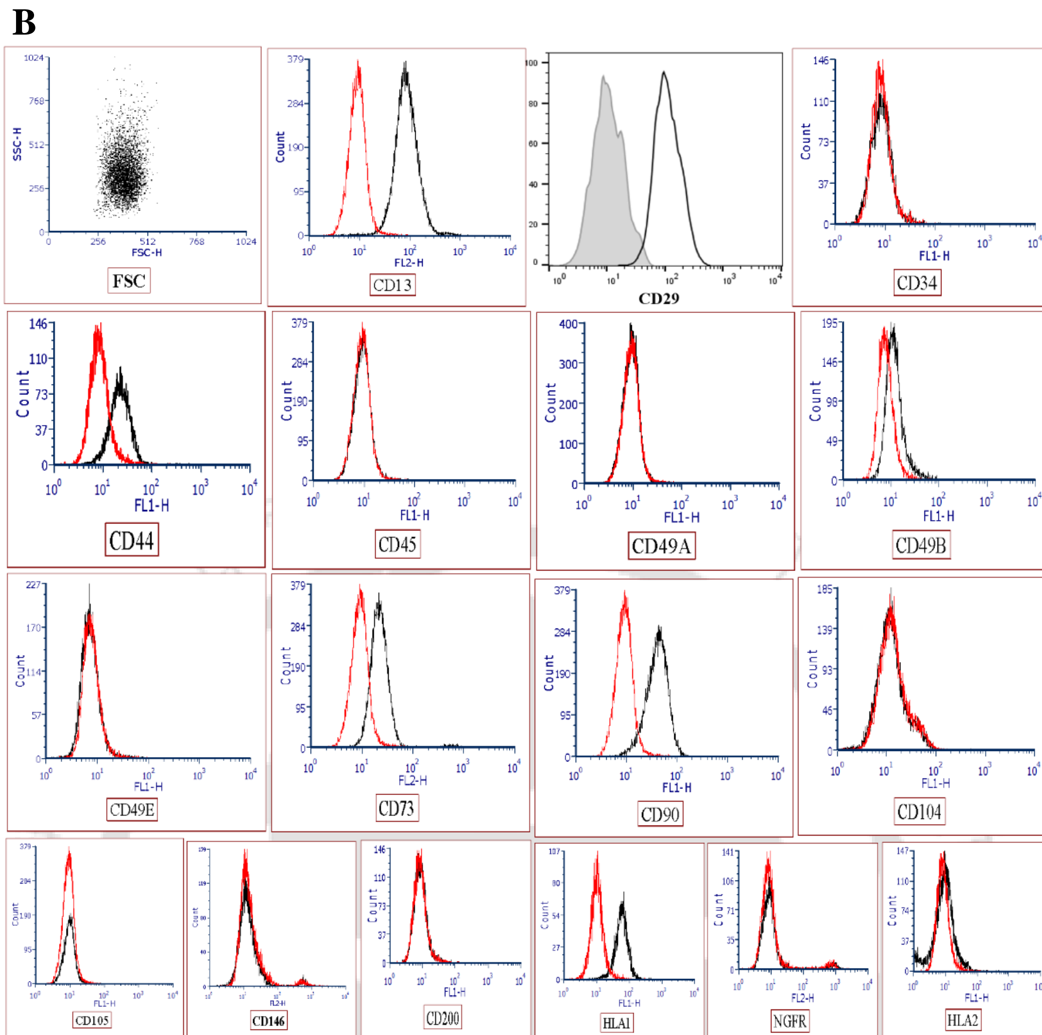

***Figure 4.2.12. Surface marker profile of** (A) **EOM-MSC and** (B) **BM-MSC analyzed by flow cytometry.** The histograms **(A)** and **(B)** represent the surface marker profiles of EOM-MSC and BM-MSC respectively when they were stained with monoclonal antibodies tagged with fluorescent probes. The live vs dead population is identified using the PI dye. In the figures the red line represent the isotype control while the black line represent the antibody against surface marker. Both EOM-MSC and BM-MSC expressed CD13, CD29, CD44, CD49B, CD73, CD90, CD105 and HLA-I but they did not express CD34, CD45, CD49A, CD104, CD146, CD200, NGFR and HLA-II.*

The EOM-MSC expressed CD13 (92.37%±16.34), CD29 (90.1±6.57), CD44 (68.89%±24.79), CD49B (48.93%±24.73), CD49E (73.25%±24.81), CD73 (73.90%±27.73), CD90 (97.4% ±2.96), CD105 (70.17%±26.23), HLA Class I (78%±27.16), but did not express hematopoietic marker CD45, muscle stem cell marker (CD34), neuronal markers CD271 (NGFR) and other markers CD104, CD140A or HLA class II (**Figure 4.2.12**). The expression levels of cell surface markers in EOM-MSC was comparable to that of BM-MSC, except CD49B which was significantly higher in EOM-MSC compared to BM-MSC (48.93%±24.73% in

EOM-MSC versus 1.37%±0.44% in BM-MSC, $p = 0.0285$). The BM-MSCs and EOM-MSC express high levels of CD29 of values 94.14±5.57 and 90.1±6.57 respectively. EOM-MSC also showed (57.3%±2.98) of SSEA4 expression by flow cytometry. The cell surface marker expression profile of EOM-MSC did not change significantly between different donors or over different passages (passage 2-8).

MARKER	% EXPRESSION LEVEL IN EOM-MSC
CD13	92.37%±16.34
CD29	90.1±6.57
CD44	68.89%±24.79
CD49B	48.93%±24.73
CD49E	73.25%±24.81
C73	73.90%±27.73
CD90	97.4%±2.96
CD105	70.17%±26.23
HLAI	78%±27.16

Table 4.2.13. The percentage expression of surface markers expressed in EOM-MSC. *Several markers expressed on the surface of BM-MSC were also expressed by the EOM-MSC. The table shows the percentage expression of mesenchymal markers by EOM-MSC.*

Furthermore, an increased expression of CD90 and CD146 was observed by immunocytochemistry analysis.

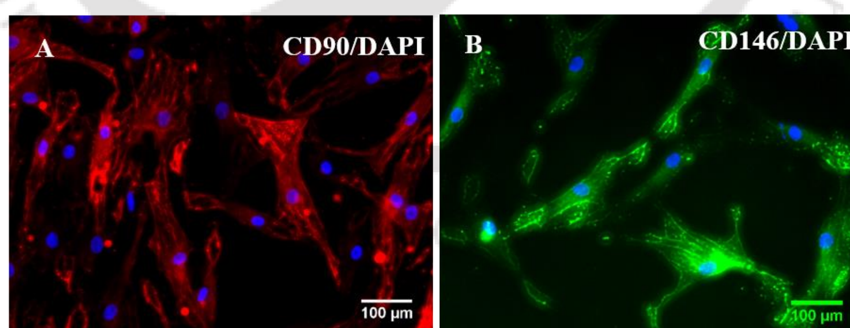

Figure 4.2.14. Surface marker expression of EOM-MSC by immunocytochemistry. *Expression of stem cell markers was tested using immunofluorescence techniques. The undifferentiated EOM-MSCs were stained with primary antibody against the antigen and a secondary antibody tagged with a fluorescent probe. The images were captured by fluorescent microscope (Zeiss) shows **(A)** CD90 positive cells in culture **(B)** CD146 is expressed in undifferentiated EOM-MSC. Magnification 20X. Blue stain – DAPI.*

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4.2.5. Expression of embryonic stem cell marker expression

Furthermore, to better understand the nature of EOM-MSC, the expression markers and genes that define self-renewal and pluripotency were analyzed. Firstly, the expression of embryonic transcription factors OCT4, NANOG and SOX2 were determined by real time PCR. All the samples tested had transcriptional expression of OCT4, NANOG and SOX2 and the level was 60-100 fold higher than that seen in BM-MSC (**Figure 4.2.15**).

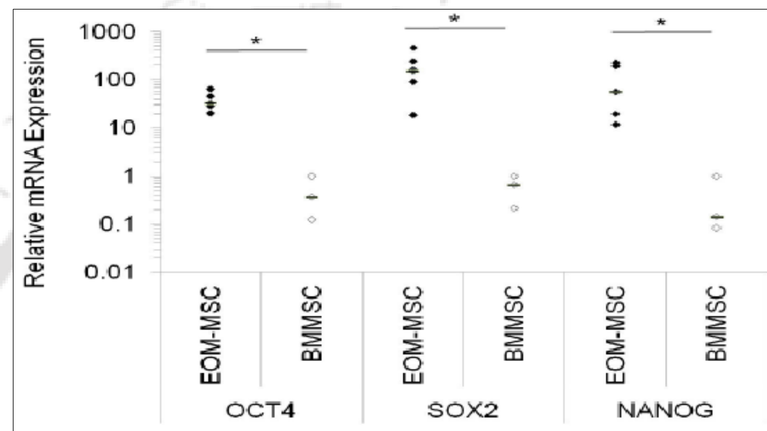

Figure 4.2.15. Relative expression of embryonic markers. *Expression levels of transcription factors OCT4, NANOG and SOX2 and neuronal lineage related genes NESTIN in EOM-MSC was analysed by real time PCR and compared with BM-MSC. Each dot represents an individual donor and the bar represents the median value.*

The cell surface expression of embryonic antigen (stage specific embryonic marker 4) was determined by flow cytometry where 58.1%±2.98 of the marker was found to be expressed in these MSC. Immunocytochemical staining for SSEA4 showed that it was predominantly expressed in high levels by spindle-shaped cells rather than polygonal shaped cells and immunofluorescence images showed that only spindle shaped cells expressed the SSEA-4 (**Figure 4.2.16, A and B**). In addition, OCT4 protein was undetectable in the EOM-MSC in immunocytochemical staining unlike their mRNA levels which was upregulated during gene expression analysis. TRA 1-60 also was not expressed in EOM-MSC by immunocytochemical staining (**Figure 4.2.16, C and D**).

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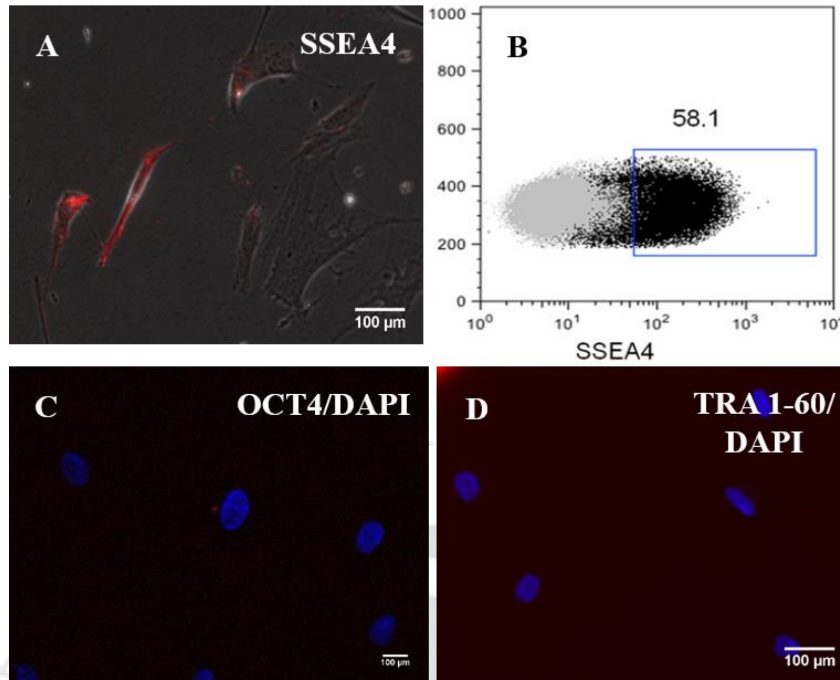

Figure 4.2.16. Embryonic markers expression in EOM-MSC. *A photomicroscopic image of **(A)** spindle shaped EOM-MSC which expressed the SSEA-4 when stained with anti SSEA-4 antibody. The arrow indicates spindle-shaped cells which expressed the SSEA-4 marker. The EOM-MSC was from passage 3. The dot plot **(B)** alongside shows the percentage expression (58.1%) of SSEA-4 in EOM-MSC. **(C)** EOM-MSC did not express OCT-4 in culture **(D)** no expression of TRA 1-60 in the EOM-MSC. Magnification 40X. Blue stain – DAPI.*

4.2.6. Gene expression analysis

To understand EOM-MSC further, gene expression profile of EOM-MSC was analyzed by real time PCR. We have investigated the expression of several mRNA groups in both EOM-MSC and BM-MSC. Among the several genes analyzed, mRNA expression of neuronal marker NESTIN was significantly higher in proliferating EOM-MSC compared to BM-MSC. No significant difference in expression levels of apoptosis related genes BAD or inhibitor of apoptosis cIAP1 and cIAP2 was found between EOM-MSC and BM-MSC. Factors such HIF-α, IL-6 and TNF-α were expressed higher in BM-MSC compared to EOM-MSC. Calcium channel related genes ORAI1, STIM1 and TRPC1expression was similar in both EOM-MSC and BM-MSC. Besides the expression level of neurotrophic factors such as BDNF, GDNF, FGF2 and FGF8 have also been determined. BDNF expression is highest compared to GDNF and FGF8.

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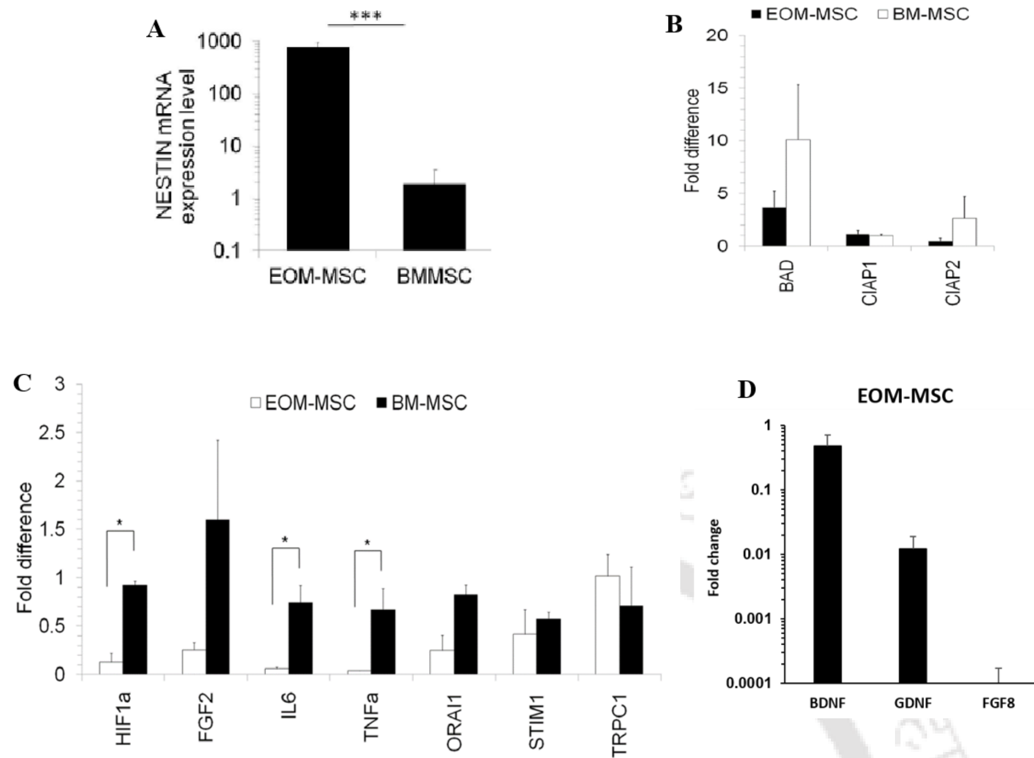

Figure 4.2.17. Gene expression analysis of EOM-MSC. *(**A**) Expression levels of neuronal lineage related genes NESTIN in EOM-MSC was analysed by real time PCR and compared with BM-MSC. Real time PCR analysis results showing significantly higher levels of NESTIN in undifferentiated EOM-MSC than BMMSC.* ***Values are mean+SD, n=4, p<0.0005****. mRNA expression levels of (**B**) apoptosis related genes BAD, cIAP1 and cIAP2 and (**C**) other factors such as HIF- α, FGF2, IL6, TNF- α as well as calcium channel related genes such as ORAI1, STIM1 and TRPC1 were determined in EOM-MSC and BM-MSC by real-time PCR. The expression levels of the genes were normalized to GAPDH expression levels in the respective samples.* ***Values are mean±SD, n = 3-5* p<0.05****. (**D**) Neurotrophic factors such as BDNF, GDNF and FGF8 were analyzed.*

4.2.7. Mitochondrial Distribution in EOM-MSC

Varum *et al,* demonstrated that the mitochondria in undifferentiated/stem cells were distrbuted in the periphery of the nucleus while in more differentiated cells the mitochondria was distributed throughout the cytoplasm. To understand the mitochondrial distribution in EOM-MSC and BM-MSC, the cultured cells were stained with TMRE which stains the active mitochondria in live cells. The mitochondrial distribution was both perinuclear and cytoplasmic in EOM-MSC whereas in BM-MSC it was predominantly perinuclear. This indicates the presence of a heterogenous population in EOM-MSC.

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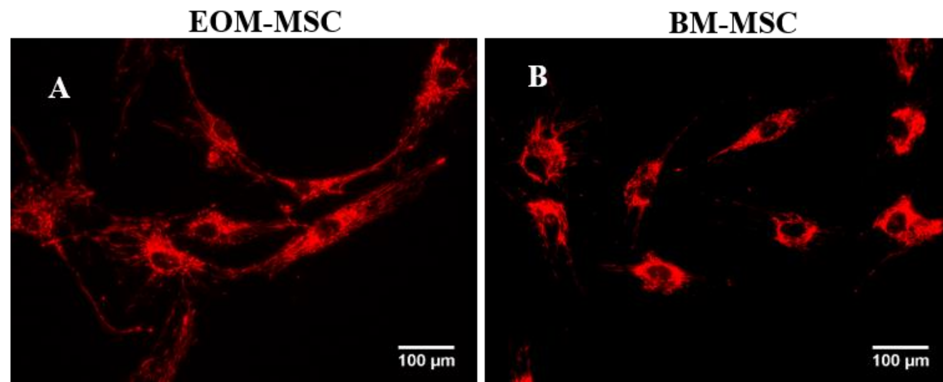

Figure 4.2.18. Mitochondrial localization of EOM-MSC by TMRE staining. *The EOM-MSC and BM-MSC culture were stained with TMRE at 37°C for 30 minutes. The localization of the mitochondria in* ***(A)*** *EOM-MSC and* ***(B)*** *BM-MSC was detected using fluorescence microscope (Zeiss). The mitochondria was distributed both throughout the cytoplasm and the perinuclear region in EOM-MSC while it was localized only at the periphery in BM-MSC. Magnification 10X.*

4.3. Neuronal Differentiation of EOM-MSC

4.3.1. Neurosphere formation by addition of growth factors and RE

The differentiation ability of EOM-MSC into non-mesenchymal neuronal lineage was assessed. Since ocular muscle is a highly innervated tissue, the neuronal differentiation of EOM-MSC was tested. EOM-MSC expressed significantly higher levels of NESTIN compared to BM-MSC which suggested that these cells might possess higher neuronal differentiation capacity compared to BM-MSC (**Figure 4.2.17 A**). EOM-MSC were allowed to differentiate into the neuronal lineage either by indirect conversion through the formation of neurosphere or by direct conversion. The neurosphere assay was carried out to test the presence of neural stem cells which have the potential to differentiate into different neuronal lineages. Firstly, we checked the potential of these cells to form the neurosphere. EOM-MSCs were plated in untreated tissue culture plates and were fed with serum free media in addition to growth factors – bFGF and EGF. Along with these chemical growth factors retinal extract (RE) prepared from mice were also supplemented (**Figure 4.3.1**). The EOM-MSC in neuronal media supplemented with RE formed neurosphere within 4 days of induction. Neurosphere appeared few in number and only after 8 days of induction in cultures which were not supplemented with RE. The neurosphere formation was continued for 9-16 days and the neurosphere were stained for GFAP, an early neuronal marker **(Figure 4.3.4)**.

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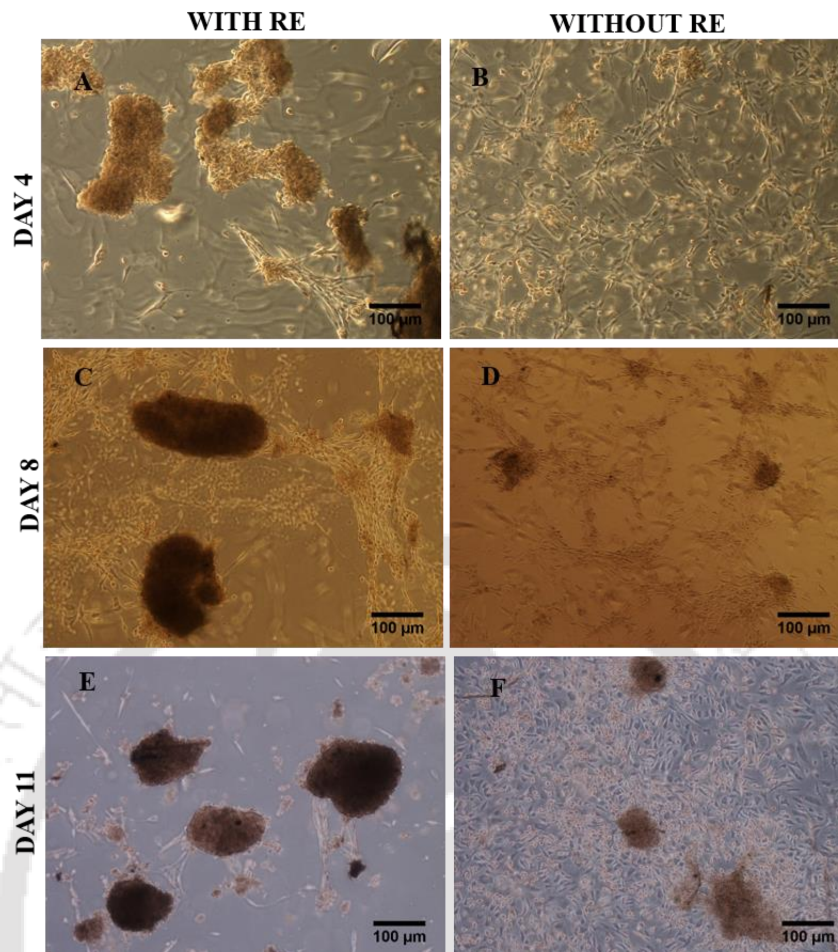

Figure 4.3.1. Neurosphere formation by addition of growth factors supplemented with RE. *Neurosphere formation was induced by addition of growth factors only and in addition with RE. Figures* ***(A)*** *were neurosphere formed within four days of induction supplemented with RE,* ***(B)*** *initiation of neurosphere formation in EOM-MSC without RE within four days of induction* ***(C)*** *neurosphere formed after 8 days of induction supplemented with RE,* ***(D)*** *neurosphere formed after 8 days of induction in neuronal media without RE. The figures* ***(E)*** *represents the neurospheres after 11 days in differentiation media supplemented with retinal extract and* ***(F)*** *neurospheres in differentiation medium without retinal extract supplemnet during 11 days of differentiation. (Scale Bar = 100µm). Magnification 10X.*

Neurosphere formed both in the presence and absence of RE stained positive for GFAP by immunocytochemistry analysis.

4.3.2. CD49B separation and neurosphere formation

CD49B positive and negative populations were separated using magnetic separation. The two populations were seeded in media for neurosphere formation. Neurosphere formation took place in both the CD49B positive and negative (Hmjb *et al.*, 2011) population as was evident from the change in morphology. Cells formed neurosphere like structures within 7 days of induction with differentiation media.

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Since EOM-MSC showed a heterogeneous CD49B expression and CD49B positive cells had been shown to have higher multipotent neurosphere formation capacity (Hmjb *et al.,* 2011).

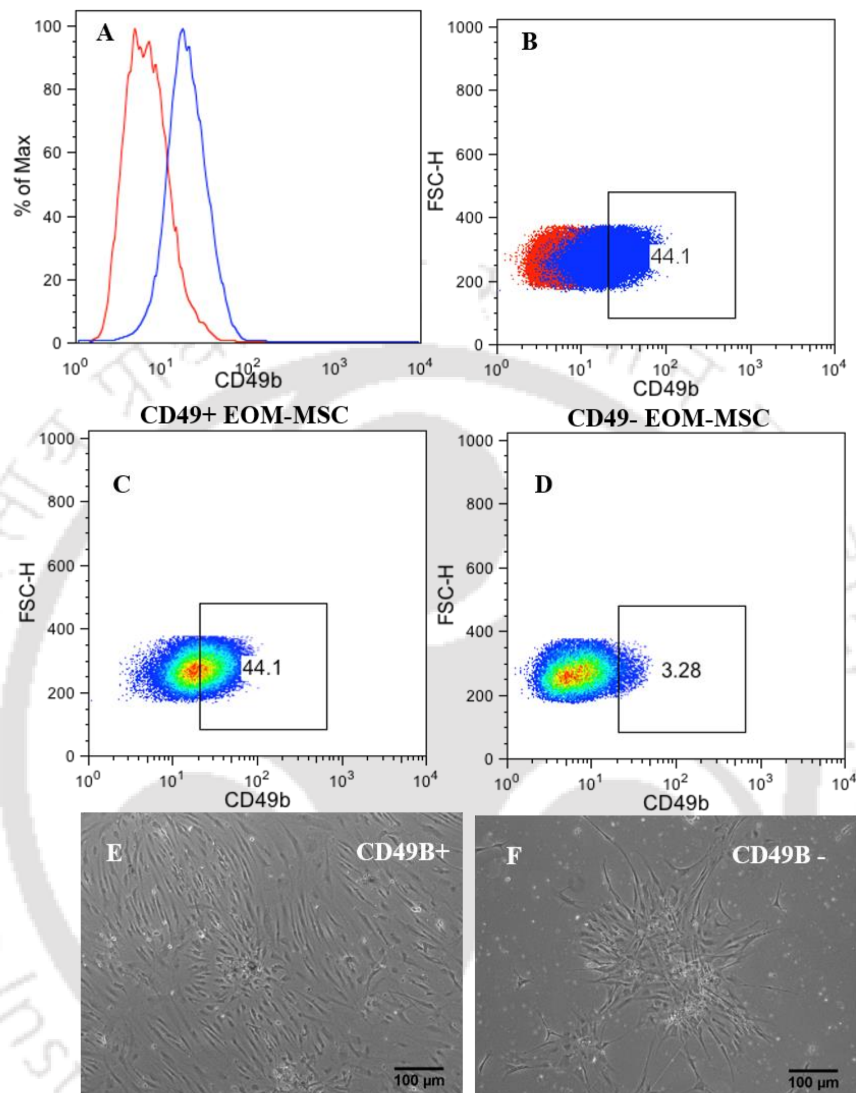

Figure 4.3.2. Separation of CD49B population and neuronal differentiation. *CD49B positive population was separated from the negative population by MACS. After separation both population were subjected to neurosphere formation. The histogram **(A)** flow cytometric plots showing the expression of CD49B in EOM-MSC where the red line indicates the isotype control staining profile vs the blue line that indicates specific antibody staining profile, the plot **(B)** the percentage of cells expressed in EOM-MSC, **(C)** the percentage of population that expressed CD49B after separation, plot **(D)** CD49B negative population after MACS separation **(E)** Neuronal differentiation of CD49B+ **(F)** CD49B- cells after 7 days of induction. Magnification 5X.*

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4.3.3. Morphological changes and surface markers expression in neuronal differentiated EOM-MSC

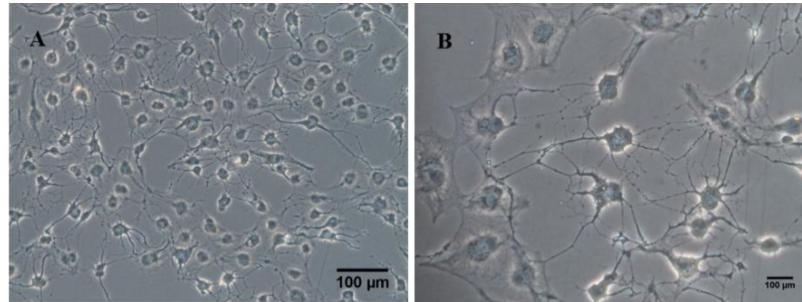

Figure 4.3.3. Change in morphology of the EOM-MSC during neuronal differentiation. *EOM-MSC exhibited bipolar and multipolar extensions after 30 days in neuronal media. Representative images at **(A)** 10X **(B)** 20X.*

EOM-MSC readily differentiated into neuronal cells, which was evident by morphology change and expression of neuronal specific markers by immunocytochemistry. The cells turned into bipolar and multipolar extensions bearing cells from the spindle-shaped.

Neuronal differentiation was accompanied by a decrease in the expression of CD146 **(Fig 4.3.4.B)** compared to proliferating control cells **(4.2.14.B)** but no change in the expression of CD90 was observed **(Fig 4.3.4.A)**. The neuronal differentiation was further confirmed by increase in glial fibrallary acidic protein (GFAP) in the cells at the neurosphere stage and nerve growth factor receptor (NGFR) and microtubule-associated protein 2B (MAP2B) expression in the differentiated cells.

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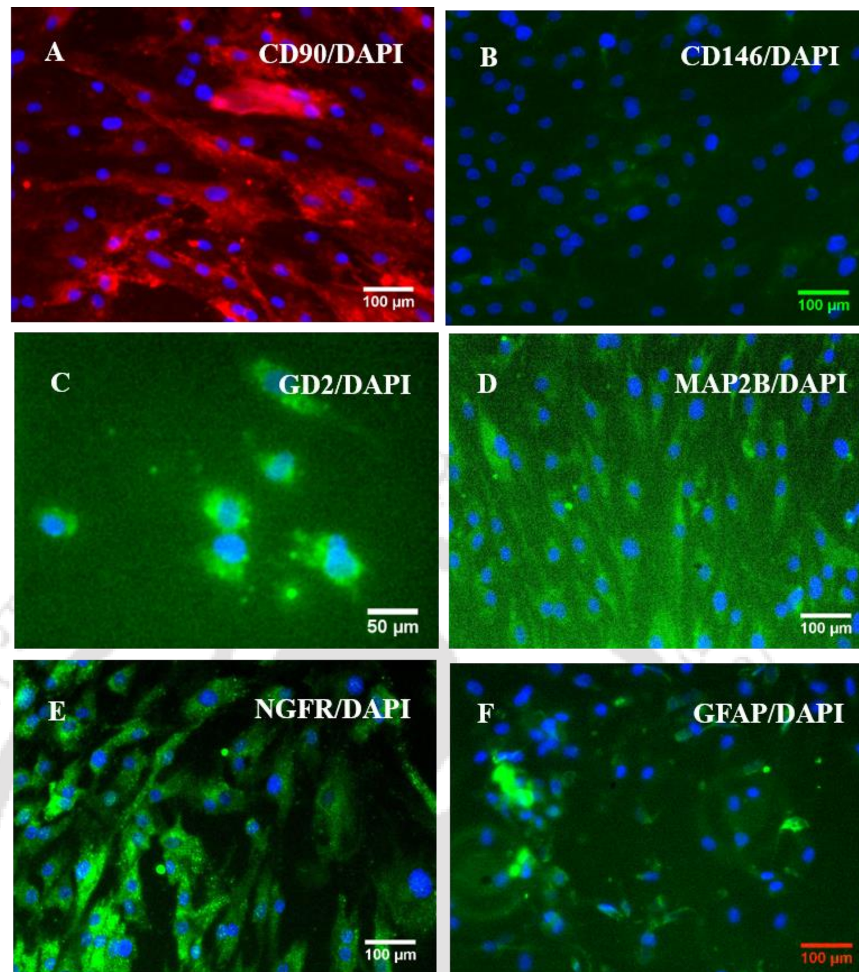

Figure 4.3.4. Neuronal Specific marker expression in neuronal differentiated cells. *Immunofluorescence analysis of neuronal differentiated EOM-MSC showing **(A)** CD90 expression was unaffected in EOM-MSC cultured in neuronal media. **(B)** downregulation of CD146 in cells grown neuronal media. Neuronal differentiated cells exhibited upregulation of neuronal lineage markers **(C)** expression of GD2 was detetected in few differentiated cells **(D)** MAP2B **(E)** NGFR/CD271 and **(F)** GFAP. CD90, CD146 and GFAP staining were performed 9-10 days after addition of neuronal induction media whereas NGFR, MAP2B and GD2 staining were performed after 21 days of neuronal induction. Representative images are shown. Magnification 10X. Blue Stain – DAPI.*

4.3.4. Change in gene expression during neuronal differentiation of EOM-MSC

There was a significant upregulation of NESTIN in cells grown in neuronal media compared to cells grown in growth media. Previously, NESTIN was also expressed in undifferentiated EOM-MSC which was significantly higher compared to BM-MSC. A significant upregulation of mRNA for the neural markers NESTIN (NES) and β-Ш-tubulin (TUBB3) was observed in the cells induced with the neuronal media. Further, the mRNA for neuronal transcription factors Neuronal

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differentiation 1 (NEUROD1) and Paired box 6 (PAX6) were upregulated five-fold and 45-fold respectively in the neuronal differentiating cells. However, the embryonic transcription factors OCT4, SOX2 and NANOG expression were downregulated during neuronal differentiation which was not observed when they were analyzed in the undifferentiated state.

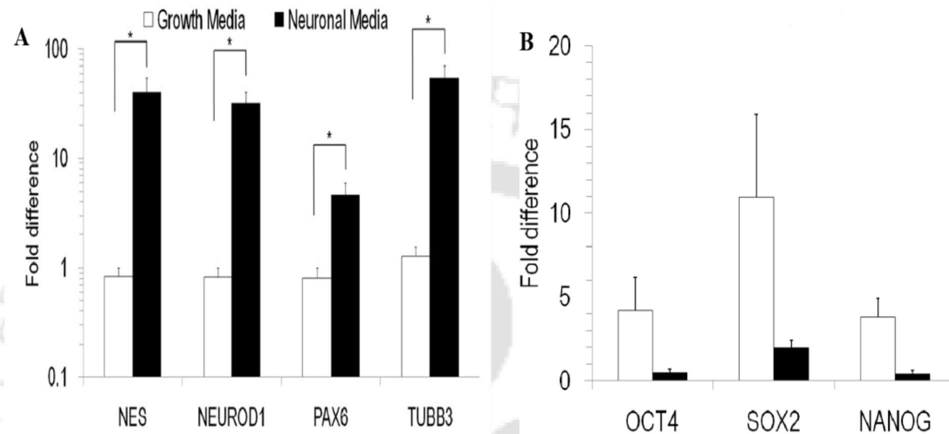

Figure 4.3.5. Gene expression in EOM-MSC when differentiated into neurons detected by real time PCR analysis. *(A) Real-time PCR analysis of neuronal specific genes NES, NEUROD1, PAX6 and TUBB3in EOM-MSC cultured in growth media and neuronal differentiation media for 14 days. The expression levels of the genes were normalized to GAPDH expression levels in the respective samples. Values are Mean±SEM, n = 5, *p<0.05.* ***(B)*** *OCT4, SOX2and NANOG expression during neuronal differentiation. Real time PCR analysis of OCT4, SOX2 and NANOG in EOM-MSC cultured in growth media and neuronal differentiation media for 14 days. The expression levels of the genes were normalized to GAPDH expression levels in the respective samples. Values are Mean±SEM, n = 3-6.*

4.3.5. Neuronal differentiation of clonal-derived EOM-MSC

To understand the neuronal differentiation capacity of EOM-MSC further, single cell derived cell lines were obtained from EOM-MSC. The clonally derived cells were expanded *in vitro* and could be differentiated into neuronal cells in the appropriate differentiation medium for a period of 21 days. There was a change in morphology in clonal derived cells kept for neuronal differentiation. After 21 days neuronal differentiation of clonal derived EOM-MSC was confirmed by expression of neural marker NGFR (CD271) via immunocytochemical staining (**Figure 4.3.6. C, D**).

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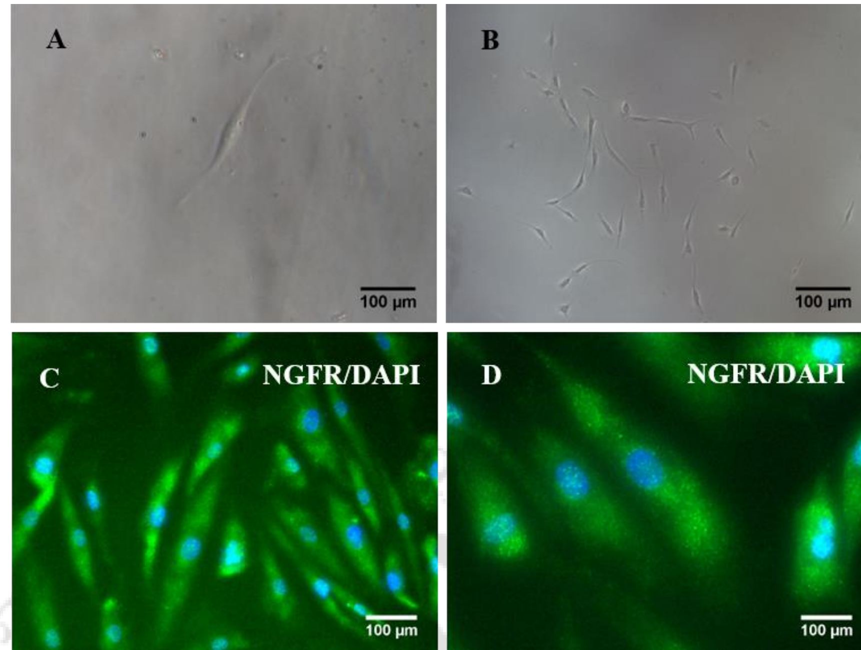

Figure 4.3.6. Neuronal differentiation of clonal derived EOM-MSC. *(**A**) Microscopic image of single cell after 24 hour of plating to isolate single cell derived colonies. (**B**) Single cell derived colony 7 days after plating. (**C, D**) Neuronal differentiation of single cell derived clones of EOM-MSC was determined by staining with NGFR 14 days after neuronal induction. Representative microscopic images are shown. (Scale Bar = 100 µm). Blue stain – DAPI.*

Thus EOM derived MSC possess mesenchymal and neuroectodermal differentiation capacity. Their mesenchymal differentiation ability was significantly less compared to BM-MSC whereas they had higher embryonic transcription factors and nestin gene expression and possess high neuronal differentiation ability which make them suitable candidates for cell therapy to treat neurodegenerative diseases.

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DISCUSSION

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5.0. DISCUSSIONS

5.1. Isolation and characterization of mesenchymal stem cell from ocular adipose tissue

MSC isolated from the BM accounts for just 0.001%-0.01% of nucleated cells (Yu Zhang *et al.,* 2012) whereas it is abundant in adipose tissue (Korn *et al.,* 2009). This was one of the reasons for exploring orbital adipose tissue for stem cells isolation. It was recently reported that abundant stem cells were detected in orbital adipose tissue (Wester, 2014). Korn *et al* were the first to report the existence of multipotent stem cells in the orbital adipose depots. Stem cells were isolated from the orbital fat which was removed in blepharoplasty (Korn *et al.,* 2009). However according to clinical protocol removal of preaponeurotic fat pad is usually avoided while the nasal adipose tissue was preferred (Aronovsky *et al.,* 2016). Unlike Korn *et al* who performed enzymatic digestion of the tissue, we dissociated the tissue with forceps and plated for isolation. This was to avoid damaging stem cells during isolation. A higher number of stem cells was reported to be found in these nasal (medial) adipose tissue which is why it is prominent while the central adipose tissue deteriorates during aging (Aronovsky *et al.,* 2016). We have cultured these mesenchymal stem cells from central and medial adipose tissue only up to four passages. A report suggested that the adult stem cells derived from the orbital adipose tissue could be maintained only to the fourth passage when expanded by the conventional method of isolation (S.-Y. Chen *et al.,* 2014).

The central adipose tissue, also called preaponeurotic fat is surrounded by a membranous sac which are maintained by blood vessels and terminal branches of the supraorbital nerve (Aronovsky *et al.,* 2016). It is bigger and yellower compared to the medial adipose (nasal) tissue which is paler or whiter and smaller (Aronovsky *et al.,* 2016). Different adipose tissues possess different metabolic functions. Aronovsky *et al,* observed the amount of free fatty acid was lowest as well as the mobilization of fat was the least in the orbital adipose tissue during fasting compared to other adipose tissues (Aronovsky *et al.,* 2016).

Mesenchymal progenitor cells from several tissue sites share similarities with those of the bone marrow though BM-MSC had been the most extensively studied stem

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cells. In our study, OAT-MSC displayed a similar cell surface marker profile to that of BM-MSC since they were positive for mesenchymal markers CD44, CD49B, CD73, CD90 and CD105. Chien *et al* also reported that orbital fat stem cell (OFSC) did not express CD31, CD34 and CD45 but were highly positive of CD29, CD90 and CD105. These cells expressed CD34 similar to those that were isolated from different parts of the eye which are express in the initial stages but reduces subsequently in later passages (Korn *et al*). Korn *et al*, also reported that both medial and central OAT-MSC stained positive for CD90 and CD105 (Korn *et al.,* 2009). Though we have not checked the expression of HLA-ABC and HLA-DR in OAT-MSC but ADSC were not known not to express HLA-DR that makes it suitable for allogeneic transplantation (Puissant *et al.,* 2005). The immunophenotype of the OAT-MSC and BM-MSC were similar (Lin et al., 2013) but still its immunomodulatory and anti-inflammatory ability still remains to be deciphered though they have displayed tolerance when injected intravenously during acute lung injury (Chien *et al.,* 2012). There is a wide prospect to explore the mechanisms of immunomodulation and anti-inflammatory effects of these mesenchymal stem like-cells.

Riekstina *et al,* reported that 7.3%±3.0% of adipose tissue derived mesenchymal stem cells (AT-MSC) expressed SSEA4 (Riekstina *et al.,* 2009) still other reports claimed that SSEA4 was not expressed in adipose tissue MSC (Wagner *et al.,* 2005). Our immunostaining results revealed that SSEA4 was expressed in the central as well as the medial OAT-MSCs (**Figure 4.1.7**). There was no expression of OCT4 protein in both central and medial OAT-MSC while expression of CD90 was correlated with the flow cytometry analysis. CD146, an adhesion protein in vascular endothelial cell activity and angiogenesis was also found to be expressed in these stem cells as also reported by (Schäffler & Büchler, 2007).

We have observed a difference in the multipotent capability into adipocyte and osteocyte in the medial and central ocular adipose tissue. The central adipose tissue differentiated more into the mesodermal lineages compared to the medial adipose tissue due to its origin during embryogenesis. The central adipose tissue is largely derived from the mesoderm (Wester, 2014) and the central and temporal adipose tissue of the lower eyelid while S.-Y. Chen *et al* and Korn *et al* stated that greater portions of the medial (nasal) adipose tissue of the both the lower and upper eyelid

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developed from the neural crest and neural crest stem cells. Both the central and medial derived-OAT-MSCs developed a neuronal phenotype and stained positive for CD271 (NGFR), Nestin and β-III-tubulin via immunostaining though Korn *et al* reported that medial (nasal adipose tissue) expressed twofold as much the neural crest markers compared to central adipose tissue (Safford *et al.,* 2002) and (Korn *et al.,* 2009). Topically administered OFSCs into the injured cornea also differentiated into corneal epithelium (Lin *et al*., 2013). BM-MSC are more capable to differentiate into osteocyte and chondrocyte while adipose derived MSC on the other hand possess a higher proliferative capacity and immunomodulatory effects. Thereby it becomes necessary to systemically choose a particular MSC source for specific clinical applications (C. Li *et al.,* 2015).

During corneal injury, corneal transplantation and limbal transplantation presented certain limitation such as GVHD and destruction of healthy limbal stem cells respectively. Stem cell transplantation however is a new therapeutic technique that required a multipotent stem cell and only mesenchymal stem cell are tolerant and non-immunogenic (Lin et al., 2013). Both BM-MSC and ASC can escape immune recognition and it was also reported in the OAT-MSC. OFSCs were topically administered into injured corneal tissue to repair the scarred tissue delivered systemically to treat ALI (Chien *et al.,* 2012). A detailed insight into the characteristics of OAT-MSC is necessary to enhance their capacity to validate them as candidates that can be used for therapeutic purposes for ocular and orbital diseases and especially to cater to our research interest to pave a new way to treat neurodegenerative disorders.

5.2. Isolation and characterization of MSC from extra ocular muscle (EOM) tissue

MSC isolated from the bone marrow are known for their ability to self-renew *in vitro* and differentiate mainly into the adipogenic, osteogenic and chondrogenic lineage. Not only can they self-renew and differentiate, MSCs are also non- immunogenic and secrete inflammatory factors. Therefore, BM-MSC is considered suitable for therapeutic purposes and several studies have enabled MSC to be used effectively for clinical applications. There is an urgent requirement in clinical applications to identify whether MSC reside in other parts of the body other than the bone marrow

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that causes less pain and ease of isolation than BM-MSC for transplantation. Extensive research has been carried out to identify novel tissues to isolate mesenchymal stem cell like cells. MSC have also been largely isolated from adipose tissue (Zuk *et al.,* 2001) and umbilical cord blood (K.-D. Lee *et al.,* 2004). Each of these sources produced MSC which have the potential to differentiate into multiple cell types and MSC from ocular limbal-stroma were also reported to exhibit multipotent differentiation capacity (Branch *et al.,* 2012). These MSC were reported to be isolated from other tissues as well such as the synovial tissue (Ogata *et al.,* 2015), Wharton jelly (Messerli *et al*., 2013), perivascular regions of the brain (Ozen *et al.,* 2012) and deciduous tooth (Kushnerev *et al.,* 2016).

We showed in our current study, extra ocular muscle (EOM) tissue as a novel tissue source of MSC with high neuronal differentiation capacity that could be used both for autologous and allogeneic transplantations. EOMs are important for controlling the movement of the eye but are excised and discarded during corrective strabismus surgery and could be used for MSC isolation (Wright and Hong, 2003). The EOM contain a higher number of stem cells than their counterpart limb muscles (Pacheco-Pinedo *et al.,* 2009) and could differentiate into myogenic cells *in vitro* and the EOM-derived SP cells showed high hematopoietic regenerative capacity after *in vivo* transplantation (Goodell, Brose, Paradis, Conner, & Mulligan, 1996). Our study shows for the first time that multipotent MSCs are present in the human extra-ocular muscle tissues that could be successfully isolated and cultured without compromising their mesenchymal and non-mesenchymal differentiation ability. Spindle shaped cells migrated out of the tissue and formed adherent colonies by merely plating the mechanically dissociated EOM, with an average of 80% of samples yielding expandable colonies of cells. MSCs were isolated from the dental pulp stem cells (DPSC) and stem cells from exfoliated deciduous tooth (SHEDs) using the simple method of mincing the teeth and plate those in DMEM media along with 10% of FBS and antibiotic along with amphotericin (Kushnerev *et al.,* 2016). However, solid tissues, are usually dissociated with enzymes that will most efficiently degrade the tissue and causes minimal damage to their surface receptors such as collagenase 4 and dispase enzymes (Montanaro *et al.,* 2004) which we did not employ in our study. This was done to avoid damage to the stem cells during enzymatic digestion. The tissue was simply rinsed in PBS, was teared into smaller

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pieces with the help of forceps and then were plated in tissue culture dish containing DMEM media supplemented with serum and antibiotic. The size of EOM-MSC appeared to be smaller compared to that of the BM-MSC and the population doubling time of EOM-MSC was 34 hours as opposed to BM-MSC which was 42 hours reported in our study (Cananzi, Atala, & de Coppi, 2014). The MSC can be passaged many times without any change in their properties due to its self-renewal capacity (Ma *et al.,* 2014). The morphology of EOM-MSC did not alter though they were passaged for 12 passages. Our study of the EOM-MSC had been limited to twelve passages while BM-MSC can be cultured extensively in vitro without loss of function or phenotype (Kemp *et al.,* 2005). The karyotype of EOM-MSC (2n=46) did not alter even as they were cultured upto the 12th passage. Similarly isolated marrow mesenchymal cells maintained a normal karyotype and telomerase activity, even at passage 12 (Pittenger *et al.,* 1999).

The MSCs, expresses several surface proteins and there is no single definitive marker that are specific to MSC to aid in their proper enrichment (Maltman, Hardy, & Przyborski, 2011). SSEA4 marker was also expressed at higher levels by amniotic fluid-MSC when isolated by one Soncini's protocol (Díaz-prado *et al.,* 2011). Flow cytometry analysis revealed that SSEA4+ sorted cells maintained a high level of SSEA4 whereas unpurified murine MSCs expressed only 30% (Gang, Bosnakovski, Figueiredo, Visser, & Perlingeiro, 2007). SSEA4 was expressed at very high levels in ESC (Gang *et al.,* 2007) but only spindle-shaped population of the EOM-MSC stained positive for SSEA4 in our study which was also detected by flow cytometry analyses with expression percentage of 58.1%±2.98. The number of SSEA4 positive cells was 6.9%±4.0% in bone marrow and 7.3%±3.0% in adipose tissue as well as in heart and dermis MSC (Riekstina *et al.,* 2009). Some markers are expressed at high levels in culture such as CD90 and expressed even in differentiated cells such as the neurons (Williams & Gagnon, 1982) while the expression of Stro-1 is eventually lost in subsequent passages in placental MSC (Hiwase, Dyson, To, & Lewis, 2009). The EOM-MSC expressed the CD13, CD73, CD90, CD105 and HLA-I markers along with other integrin such as CD29, CD49B and CD49E which was very similar to that of the BM-MSC. Like the BM-MSC they too were negative for muscle stem cell marker, CD34 and hematopoietic marker CD45. EOM-MSC also did not express CD200, PDGFR (CD140a) and neuronal markers such as the

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MAP2B, GFAP and NGFR in the undifferentiated state. The immunophenotype did not display any expression of MAP2B and GFAP throughout passages 3 to 5 but certain reports suggested that BM-MSC even express some neuroectodermal markers in their native state (Tondreau *et al.,* 2005) and CD271 in BM-MSC, AT-MSC and periodontal ligaments MSC and at low levels in placenta-derived MSC (Lv *et al*., 2014) but not MAP2ab (Hermann *et al.,* 2004). This shows that there were no neuronal progenitors in our culture during EOM-MSC isolation. Further the flow cytometric plots determined that HLA-I were highly expressed in both BM-MSC and EOM-MSC but not HLA-II which pointed out to the reason that MSC can escape the immunological effects of the host environment. One of the future prospects is to investigate the immunomodulatory and paracrine functions in EOM-MSC as well which stands to benefit therapy via allogeneic transplantation.

The EOM-MSC had lesser capacity to differentiate into the mesodermal lineage compared to BM-MSC as seen in the low osteogenic, adipogenic and chondrogenic differentiation ability of these cells which could be related to the nature of tissue from which these cells were derived which might be of advantage during cell therapy for neurological disorders.

OCT4, SOX2 and NANOG are transcription factors which maintain the self-renewal potential of ESC (Niwa, Essentials of STEM CELL BIOLOGY, 2014). Therefore, OCT4 is expressed very strongly in ESCs but expressed at lower levels in BM-MSC. However all the three genes - OCT4, SOX2 and NANOG was 60-100 fold more expressed in EOM-MSC as evident from the real time PCR analysis (**Figure 4.2.15**) in contrast to the lower expression in BM-MSC but there was negligible expression of Oct4 protein in EOM-MSC when tested via immunostaining (**Figure 4.2.16**). The EOM-MSC might contain an immature population which are more ESC-like and express NANOG, OCT4 and SOX2 markers. Riekstina *et al* showed through gene expression analysis that Sox2 was not expressed in BM-MSC while Oct4 and Nanog was demonstrated in the bone marrow, adipose, dermis and heart MSC *(Riekstina et al.,* 2009). However, BM-MSC could still differentiate into neural like cells due to the expression of both Oct4 and Nanog (Y. Liu *et al.,* 2013). Similarly, EOM-MSC was hypothesized that they have a higher capacity to differentiate into neuroectodermal cells. The nestin levels were found to be 800 folds higher in EOM-MSC compared to BM-MSC. Nestin is an intermediate filament expressed in neural

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precursors but as these precursors differentiate to form neurons and glia it was downregulated as they differentiate into cells of specific lineages and other intermediate filaments take charge and most neuro-epithelial cells were nestin positive before neurogenesis can occur (McKay, 1997). Rat BM-MSC lacked nestin expression but were found to progressively increase at higher passages *in vitro* (Montzka *et al.,* 2009) while very few human undifferentiated BM-MSC expressed nestin (Yan *et al.,* 2013) at least only 4% of cells stained positive during immunostaining (Kern *et al.,* 2006) and was also detected by Wagner *et al* where upregulation may be associated with the neuronal differentiation (Wagner *et al.,* 2005).

Mitochondria maintains the self-renewal capacity of stem cells and controls differentiation (Hsu, Wu, Yu, & Wei, 2016). A report suggested that mitochondria in stem cells were immature and characterized by perinuclear localization, whereas the differentiated cells exhibited uniform cytoplasmic distribution (Varum *et al.,* 2011). Further the expression of OCT4 and NANOG was upregulated during the formation of mitochondrial network but their expression was gradually lost when the developed mitochondrial network was formed (Mandal *et al.,* 2015). Mitochondria was distributed in both cytoplasmic and perinuclear position in EOM-MSC which represents a heterogeneous population.

The cell surface expression of CD73, CD90, CD105, integrin and other markers along with the spindle shaped morphology, mesenchymal differentiation capacity suggest that EOM-MSC resemble bone marrow mesenchymal stem cells.

5.3. Differentiation of EOM-MSC into neuroectodermal lineage

BM-MSC are potential candidate for regenerative medicine because they are readily available tissue, can be widely expanded *in vitro* and possess unique immunological properties (Maltman *et al.,* 2011). For the first time we have isolated and characterized MSCs from the extra-ocular muscle that behave similar to the bone marrow MSC. EOM-MSC readily formed neurosphere and differentiated into neurons since they have a greater inclination towards forming neuroectodermal rather than mesodermal cell-lineages. This could partially be due to the origin of the

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tissue. EOM was developed from pre chordal mesoderm while majority of the skeletal muscles developed from the somites (J. C. Chen & Goldhamer, 2003) during the embryonic stage and it is interrupted with a rich supply of cranial nerves instead of spinal cord motoneurons (Pacheco-Pinedo *et al.,* 2009). On this note, these stem cells could be useful for treating neurodegenerative disorders.

Neurosphere was generated as a result of treatment with EGF and bFGF in serum free media (Monnin *et al.,* 2007). bFGF promotes proliferation while inhibiting differentiation (J. C. Chen & Goldhamer, 2003) while EGF along with HGF directs MSCs to form neurons and glia (Bae *et al.,* 2011). Usually the presence of neural stem cells were investigated *in vitro* by dissociating out a part of brain and then the tissue was cultured in presence of mitogens such as the FGF and EGF in either a defined or supplemented medium on a matrix as a substrate binding. Gradually the mitogens were withdrawn from the culture and were replaced with a set of various mitogens so that cells of specific neural lineage could form. The cells formed as a result of these treatment were confirmed using specific antibodies against the neural markers (Gage FH, 2000). Similarly the EOM-MSC were seeded in an untreated substrate and supplemented with the mitogens - bFGF and EGF which initiated the formation of neurosphere. Round-shaped cell aggregates floating in the medium were developed as a result of the treatment. The neurosphere were allowed to grow for 10-20 days and at the end of the time point, they were immunoreactive towards the GFAP antibody. To enhance the formation of neurosphere, we tested the cultures by exposing to retinal extract. EOM-MSC generated neurosphere whether the retinal extract was supplemented to the cultures or not but only in the presence of mitogens. However it took longer time of more than 11 days for the cultures without retinal extract to develop spherical bodies and the cultures exposed to retinal extract formed neurosphere within 4 days of differentiation. In another study, CD49B positive population were separated from the negative population via magnetic activated cell sorting. This was followed by generation of neurosphere by both the population as evident from the change in morphology. On the other hand, they were directly converted into neurons using chemical inducers. hBM-MSC do not form the neuroectodermal cells when converted directly but when the multistep conversion is applied, that is, neurosphere are formed, neural markers were also found to be expressed by the cells (Kern *et al.,* 2006).

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The EOM-MSC differentiated into neuronal lineage when supplemented with growth factors and neurotrophic factors as they were shown to express the neuronal marker CD271, MAP2b, and GD2 but when human BM-MSC were differentiated into neurons, they did not express mature markers such as MAP2ab nor TH and no neural markers such as TUBB4 or GFAP were upregulated as obtained from gene expression studies (Hermann *et al.,* 2004). Our studies had also shown that there was a downregulation in the pluripotent markers such as the OCT4, SOX2 and NANOG. This was supported when SOX2 was constitutively expressed, neuronal differentiation was inhibited and cells remained in their progenitor state but conversely when SOX2 was inhibited neuronal differentiation was initiated in the progenitor cells exhibiting the early neuronal markers (Graham *et al.,* 2003). The neurons expressed markers MAP2b, NGFR and GD2, a neural ganglioside, and the neurosphere expressed the glial marker, GFAP in the neurosphere via immunostaining. CD90 was consistently expressed in both undifferentiated EOM-MSC and the neurons formed as a result of the differentiation but CD146 was no longer expressed in the neurons (**Figure 4.3.4.**). However GD2 was also expressed in undifferentiated BM-MSC and AT-MSC hence not also expressed in neurons. BM-MSC under the influence of EGF or BDNF formed the neural cells which expressed the neural precursor marker, nestin, and later expressed the glial cell marker, GFAP and NeuN (J. Sanchez-Ramos *et al.,* 2000). Neuronal differentiation was further confirmed by the upregulation of NEUROD1, PAX6, TUBB3 and NESTIN in EOM-MSC grown in neuronal media. Though there was an upregulation of genes involved in neuronal differentiation such as Pax6, Tubulin beta4, but the functionality of neurons could be determined only through the expression of connectivity and chemical transmission (Jin *et al.,* 2003). Undifferentiated BM-MSC moderately expressed both early and late neuroectodermal markers (Kern *et al.,* 2006) such as DRD2, NSE, MAP1b, NFH and SNTX1 though there was no expression of mature neuronal markers that include synaptophysin, TH and tau (Montzka *et al.,* 2009). There was an upregulation of nestin levels during differentiation (**Figure 4.3.5**) compared to expression of cells in growth media, however, Montzka *et al* reported that there was no change in nestin expression in differentiated MSC. There was a marked increase of nestin expression in MSC but eventually downregulated in prolonged culture term (Montzka *et al.,*

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2009). Also, cultured BM-MSCs reported very low levels of nestin and did not expressed Neurofilament protein, NeuN or GFAP (Ankeny *et al.,* 2004). This could possibly be one of the reason that EOM-MSC differentiates readily into neurons. There was a significant change in the morphology of the EOM-MSC when differentiated into neurons. The morphology of the BMSC initially turned sharply defined, the spindle-shaped and flat morphology retracted towards nucleus forming rounded refractive bodies and later developed some neurite-like protrusions. Finally, the cells acquire a neuron-like cell shape (Guan *et al.,* 2014) and (Egusa et al., 2005). The shapes of the neurons formed therefore ranges from unipolar and bipolar to branched multipolar cells (Jamous *et al.,* 2010).

Whether BMMSC forms repair neural cell function by transdifferentiation, cell fusion or secretion of trophic factors is still an ongoing debate. They may act in a concerted manner with each other to promote neural cell function in a diseased nervous tissue (Maltman *et al.,* 2011).

When a single cell or clone can differentiate into cells of any lineage such as the mesodermal or neuronal lineage the clone is considered a stem cell (Clive N Svendsen & Caldwell, 1999). Clonal populations were isolated from bone marrow derived mesenchymal stem cells collected from posterior iliac crest. Some clones displayed more than 20 cumulative population doublings and differentiated into adipocytes, osteocytes and chondrocytes but did not investigate differentiation into the neuronal cells (Halleux *et al.,* 2001). Here, clonal population derived from single celled EOM-MSC have been identified (**Figure 4.3.6**). The clones were further expanded in culture and were allowed to differentiate into neuronal cells which eventually expressed the neuronal marker NGFR (CD271).

There are many approaches to treat neurological disorders of which chemical drugs are the main such as levodopa, DA agonists, monoamine oxidase inhibitors and DBS for subsiding the effects of PD. The damaged neurons cannot be repaired by these drugs so restorative approaches are being considered. Henceforth, ESCs were able to differentiate into neurons, astrocytes and oligodendrocytes *in vitro* and as a result has been widely tested for transplantation in various neurodegenerative diseases. However their high capacity of self-renewal and pluripotency can lead to tumor formation therefore a shift towards adult stem cells was considered. iPSCs were also

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used to repair ischemic stroke and PD, but again, has a high risk of tumorigenicity. NSCs on the other hand which have been recently identified are more directed towards neural differentiation and especially into neurons and has a lower risk of tumor formation. These cells cannot be isolated in large numbers and are therefore limited during transplantation. Now what remains are the mesenchymal stem cells which can be isolated from many tissues of the body, lack any ethical concerns and escape the immunological effects of the host. The advantages of BM-MSC is that they can be easily collected from patient's own bone marrow without further CNS damage. Another important feature of BM-MSC is that it can cross the blood brain barrier and migrate throughout the brain. Many studies have reported the neuronal differentiation of BMSC into neurons. However the efficiency of differentiation into neurons is low, and these cells may only be maintained for a few passages. These drawbacks limit the potential application of BMSCs for transplantation (M.-H. Fu *et al.,* 2015).

Though EOM-MSC could differentiate into neuroectodermal lineage, nonetheless, further investigation is necessary to be conducted to determine whether these neuron-like cells possess the functional characteristics of a typical neuron such as excitability, release of neurotransmitters, formation of functional synapses and participation in neuronal network (Arsenijevic *et al.,* 2003). The EOM-MSC can differentiate into both mesodermal and neuroectodermal lineage but they readily differentiate more into the neuroectodermal lineage compared to the mesodermal which shows why they could be useful in neurodegenerative diseases but not disorders related to the bone. However, the electrophysiological property of the neurons formed as a result were not tested in our study, which usually is a functional characteristic of neurons *in vitro* (Hermann *et al.,* 2004). So far these studies reported only morphological data and/or marker expression and no functional properties of the neuroectodermal converted MSCs (Hermann *et al.,* 2004) and (Kern *et al.,* 2006).

MSCs also secrete neurotrophic factors which prevent further neuronal cell death and even enhance neurogenesis (Joyce *et al.,* 2011). The undifferentiated EOM-MSC and OAT-MSC secrete neurotrophic factors namely BDNF, GDNF, FGF8 and FGF2 at different levels which can reduce the damage to neurons by

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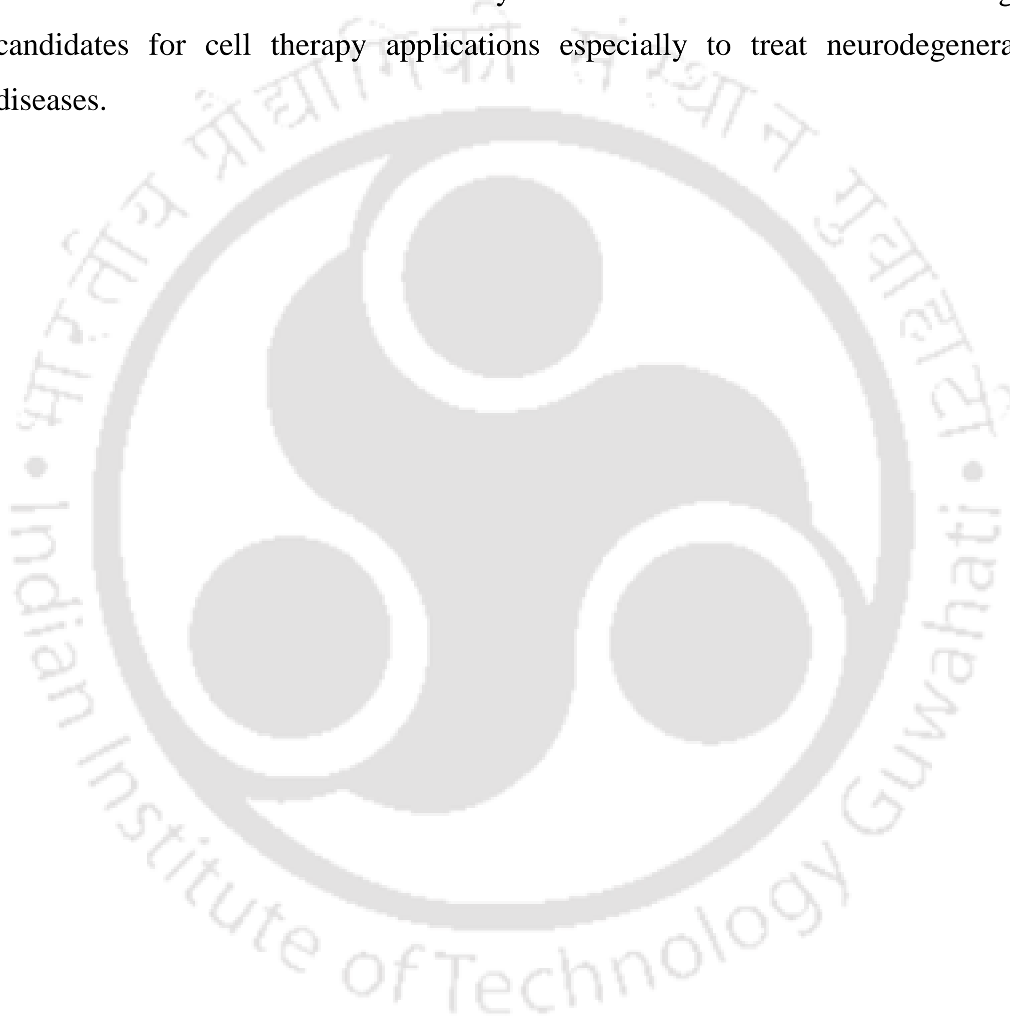
neuroprotection, neurogenesis and scar inhibition when transplanted to injured host tissue (Montzka *et al.,* 2009). This approach protected the neurons from further dying, instead of simply replacing them with healthy differentiated stem cells that have a short half-life *in vivo*.

Thus, we isolated multipotent mesenchymal stem cells with mesodermal and neuroectodermal differentiation abilities from ocular adipose tissue and extra ocular muscle tissue. The ease of availability of tissue and isolation make them good candidates for cell therapy applications especially to treat neurodegenerative diseases.



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REFERENCES

Akiyama, K., You, Y.-O., Yamaza, T., Chen, C., Tang, L., Jin, Y., … Shi, S. (2012). Characterization of bone marrow derived mesenchymal stem cells in suspension. *Stem Cell Research & Therapy*, *3*(5), 40. https://doi.org/10.1186/scrt131.

Ankeny, D. P., McTigue, D. M., & Jakeman, L. B. (2004). Bone marrow transplants provide tissue protection and directional guidance for axons after contusive spinal cord injury in rats. *Experimental Neurology*, *190*(1), 17–31. https://doi.org/10.1016/j.expneurol.2004.05.045.

Aronovsky, E., Levarl, R., Kornblueth, W., & Wertheimer, E. (2016). adipose tissues, 259–264.

Arsenijevic, Y., Taverney, N., Kostic, C., Tekaya, M., Riva, F., Zografos, L., … Munier, F. (2003). Non-neural regions of the adult human eye: A potential source of neurons? *Investigative Ophthalmology and Visual Science*, *44*(2), 799–807. https://doi.org/10.1167/iovs.02-0267.

Arthur, A., Rychkov, G., Shi, S., Koblar, S. A., & Gronthos, S. (2008). Adult human dental pulp stem cells differentiate toward functionally active neurons under appropriate environmental cues. *Stem Cells*, *26*(7), 1787–1795. https://doi.org/10.1634/stemcells.2007-0979.

Bae, K. S., Park, J. B., Kim, H. S., Kim, D. S., Park, D. J., & Kang, S. J. (2011). Neuron-like differentiation of bone marrow-derived mesenchymal stem cells. *Yonsei Medical Journal*, *52*(3), 401–412. https://doi.org/10.3349/ymj.2011.52.3.401.

Bala, A. (2013). Bone Marrow Research in India: A Scientometric Study, 2003-12. *Journal of Bone Marrow Research*, *1*(1). https://doi.org/10.4172/2329-8820.1000108.

Beck, H. C., Petersen, J., Felthaus, O., Schmalz, G., & Morsczeck, C. (2011). Comparison of neurosphere-like cell clusters derived from dental follicle precursor cells and retinal Müller cells. *Neurochemical Research*, *36*(11), 2002–2007. https://doi.org/10.1007/s11064-011-0524-0.

Bernardo, M. E., Cometa, A. M., Pagliara, D., Vinti, L., Rossi, F., Cristantielli, R., … Locatelli, F. (2011). Ex vivo expansion of mesenchymal stromal cells. *Best Practice & Research. Clinical Haematology*, *24*(1), 73–81. https://doi.org/10.1016/j.beha.2010.11.002.

Bieback, K., & Brinkmann, I. (2010). Mesenchymal stromal cells from human perinatal tissues: From biology to cell therapy. *World Journal of Stem Cells*, *2*(4), 81–92. https://doi.org/10.4252/wjsc.v2.i4.81.

Bieback, K., Schallmoser, K., Klüter, H., & Strunk, D. (2008). Clinical protocols for the isolation and expansion of mesenchymal stromal cells. *Transfusion Medicine and Hemotherapy*, *35*(4), 286–294. https://doi.org/10.1159/000141567.

TH-2047_10610601

Billon, N., Iannarelli, P., Monteiro, M. C., Glavieux-Pardanaud, C., Richardson, W. D., Kessaris, N., … Dupin, E. (2007). The generation of adipocytes by the neural crest. *Development (Cambridge, England)*, *134*(12), 2283–92. https://doi.org/10.1242/dev.002642.

Black, I. B., & Woodbury, D. (2001). Adult rat and human bone marrow stromal stem cells differentiate into neurons. *Blood Cells, Molecules & Diseases*, *27*(3), 632–636. https://doi.org/10.1006/bcmd.2001.0423.

Bonaventura, G., Chamayou, S., Liprino, A., Guglielmino, A., Fichera, M., Caruso, M., & Barcellona, M. L. (2015). Different tissue-derived stem cells: A comparison of neural differentiation capability. *PLoS ONE*, *10*(10), 1–26. https://doi.org/10.1371/journal.pone.0140790.

Branch, M. J., Hashmani, K., Dhillon, P., Jones, D. R. E., Dua, H. S., & Hopkinson, A. (2012). Mesenchymal stem cells in the human corneal limbal stroma. *Investigative Ophthalmology & Visual Science*, *53*(9), 5109–16. https://doi.org/10.1167/iovs.11-8673.

Brazelton, T. R., Rossi, F. M., Keshet, G. I., & Blau, H. M. (2000). From marrow to brain: expression of neuronal phenotypes in adult mice. *Science (New York, N.Y.)*, *290*(5497), 1775–9. https://doi.org/10.1126/science.290.5497.1775.

Buzańska, L., Machaj, E. K., Zabłocka, B., Pojda, Z., & Domańska-Janik, K. (2002). Human cord blood-derived cells attain neuronal and glial features in vitro. *Journal of Cell Science*, *115*(Pt 10), 2131–8.

Catherine A. Perrone,* Douglas Tritschler, P. T., & Raqual Bower,* Bradley K. Yoder, and M. E. P. (2003). A Novel Dynein Light Intermediate Chain Colocalizes with the Retrograde Motor for Intraflagellar Transport at Sites of Axoneme Assembly in Chlamydomonas and Mammalian Cells. *Molecular Biology of the Cell*, *14*(May), 2559–2569. https://doi.org/10.1091/mbc.E02.

Chen, C.-J., Ou, Y.-C., Liao, S.-L., Chen, W.-Y., Chen, S.-Y., Wu, C.-W., … Hsu, S.-H. (2007). Transplantation of bone marrow stromal cells for peripheral nerve repair. *Experimental Neurology*, *204*(1), 443–53. https://doi.org/10.1016/j.expneurol.2006.12.004.

Chen, J. C., & Goldhamer, D. J. (2003). Skeletal muscle stem cells. *Reprod Biol Endocrinol*, *1*, 101. https://doi.org/10.1186/1477-7827-1-101.

Chen, J., Li, Y., Wang, L., Zhang, Z., Lu, D., Lu, M., & Chopp, M. (2001). Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats. *Stroke*, *32*(4), 1005–11. https://doi.org/10.1161/01.STR.32.4.1005.

Chen, S.-Y., Mahabole, M., Horesh, E., Wester, S., Goldberg, J. L., & Tseng, S. C. G. (2014). Isolation and characterization of mesenchymal progenitor cells from human orbital adipose tissue. *Investigative Ophthalmology & Visual Science*, *55*(8), 4842–4852. https://doi.org/10.1167/iovs.14-14441.

Chen, S., Zhang, W., Wang, J.-M., Duan, H.-T., Kong, J.-H., Wang, Y.-X., … Song, J. (2016). Differentiation of isolated human umbilical cord mesenchymal stem cells into neural stem cells. *International Journal of Ophthalmology*, *9*(1), 41–7. https://doi.org/10.18240/ijo.2016.01.07.

TH-2047_10610601

Chien, M.-H., Bien, M.-Y., Ku, C.-C., Chang, Y.-C., Pao, H.-Y., Yang, Y.-L., … Ho, J. H. (2012). Systemic human orbital fat-derived stem/stromal cell transplantation ameliorates acute inflammation in lipopolysaccharide-induced acute lung injury. *Critical Care Medicine*, *40*(4), 1245–1253. https://doi.org/10.1097/CCM.0b013e31823bc89a.

Crisan, M., Yap, S., Casteilla, L., Chen, C. W., Corselli, M., Park, T. S., … Péault, B. (2008). A Perivascular Origin for Mesenchymal Stem Cells in Multiple Human Organs. *Cell Stem Cell*, *3*(3), 301–313. https://doi.org/10.1016/j.stem.2008.07.003.

Deng, Y., Liu, X.-G., Liu, Z.-G., Liu, X.-L., Liu, Y., & Zhou, G. (2006). Implantation of BM mesenchymal stem cells into injured spinal cord elicits de novo neurogenesis and functional recovery: evidence from a study in rhesus monkeys. *Cytotherapy*, *8*(3), 210–214. https://doi.org/10.1080/14653240600760808.

Dhamodaran, K., Subramani, M., Ponnalagu, M., Shetty, R., & Das, D. (2014). Ocular stem cells: a status update! *Stem Cell Research & Therapy*, *5*(2), 56. https://doi.org/10.1186/scrt445.

Díaz-prado, S., Muiños-lópez, E., Hermida-gómez, T., Esther, M., Fuentes-boquete, I., Toro, F. J. De, & Francisco, J. (2011). Isolation and characterization of mesenchymal stem cells from human amniotic membrane, *17*(1), 49–59.

Ding, D.-C., Chang, Y.-H., Shyu, W.-C., & Lin, S.-Z. (2015). Human umbilical cord mesenchymal stem cells: a new era for stem cell therapy. *Cell Transplantation*, *24*(3), 339–47. https://doi.org/10.3727/096368915X686841.

Divya, M. S., Roshin, G. E., Divya, T. S., Rasheed, V. A., Santhoshkumar, T. R., Elizabeth, K. E., … Pillai, R. M. (2012). Umbilical cord blood-derived mesenchymal stem cells consist of a unique population of progenitors co-expressing mesenchymal stem cell and neuronal markers capable of instantaneous neuronal differentiation. *Stem Cell Research & Therapy*, *3*(6), 57. https://doi.org/10.1186/scrt148.

Drela, K., Lech, W., Figiel-Dabrowska, A., Zychowicz, M., Mikula, M., Sarnowska, A., & Domanska-Janik, K. (2016). Enhanced neuro-therapeutic potential of Wharton's Jelly-derived mesenchymal stem cells in comparison with bone marrow mesenchymal stem cells culture. *Cytotherapy*, *18*(4), 497–509. https://doi.org/10.1016/j.jcyt.2016.01.006.

Egusa, H., Schweizer, F. E., Wang, C., Matsuka, Y., & Nishimura, I. (2005). Neuronal differentiation of bone marrow-derived stromal stem cells involves suppression of discordant phenotypes through gene silencing. *The Journal of Biological Chemistry*, *280*(25), 23691–23697. https://doi.org/10.1074/jbc.M413796200.

Falavigna, A., & Costa Da Costa, J. (2015). Mesenchymal autologous stem cells. *World Neurosurgery*, *83*(2), 236–250. https://doi.org/10.1016/j.wneu.2013.02.026.

Franco Lambert, A. P., Fraga Zandonai, A., Bonatto, D., Cantarelli Machado, D., & Pêgas Henriques, J. A. (2009). Differentiation of human adipose-derived adult stem cells into neuronal tissue: Does it work? *Differentiation*, *77*(3), 221–228. https://doi.org/10.1016/j.diff.2008.10.016.

TH-2047_10610601

Fraser, J. K., Wulur, I., Alfonso, Z., & Hedrick, M. H. (2006). Fat tissue: an underappreciated source of stem cells for biotechnology. *Trends in Biotechnology*, *24*(4), 150–154. https://doi.org/10.1016/j.tibtech.2006.01.010.

Fu, L., Zhu, L., Huang, Y., Lee, T. D., Forman, S. J., & Shih, C.-C. (2008). Derivation of neural stem cells from mesenchymal stemcells: evidence for a bipotential stem cell population. *Stem Cells and Development*, *17*(6), 1109–1121. https://doi.org/10.1089/scd.2008.0068.

Fu, M.-H., Li, C.-L., Lin, H.-L., Chen, P.-C., Calkins, M. J., Chang, Y.-F., … Yang, S.-H. (2015). Stem cell transplantation therapy in Parkinson's disease. *SpringerPlus*, *4*, 597. https://doi.org/10.1186/s40064-015-1400-1.

Gage, F. H. (2000). Mammalian neural stem cells. *Science*, *287*(5457), 1433–1438. https://doi.org/10.1126/science.287.5457.1433.

Gang, E., Bosnakovski, D., Figueiredo, C., Visser, J., & Perlingeiro, R. (2007). *SSEA-4 identifies mesenchymal stem cells from bone marrow*. *Blood* (Vol. 109). https://doi.org/10.1182/blood-2005-11-010504.The.

Gimble, J. M., Bunnell, B. A., Frazier, T., Rowan, B., Shah, F., Thomas-porch, C., & Wu, X. (2013). Adipose-derived stromal/stem cells, (March), 3–10. https://doi.org/10.4161/org.24279.

Gincberg, G., Arien-Zakay, H., Lazarovici, P., & Lelkes, P. I. (2012). Neural stem cells: Therapeutic potential for neurodegenerative diseases. *British Medical Bulletin*, *104*(1), 7–19. https://doi.org/10.1093/bmb/lds024.

Goodell, M. A., Brose, K., Paradis, G., Conner, A. S., & Mulligan, R. C. (1996). Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *The Journal of Experimental Medicine*, *183*(4), 1797–806. https://doi.org/10.1084/jem.183.4.1797.

Graham, V., Khudyakov, J., Ellis, P., & Pevny, L. (2003). SOX2 functions to maintain neural progenitor identity. *Neuron*, *39*(5), 749–765. https://doi.org/10.1016/S0896-6273(03)00497-5.

Guan, M., Xu, Y., Wang, W., & Lin, S. (2014). Differentiation into neurons of rat bone marrow-derived mesenchymal stem cells. *European Cytokine Network*, *25*(3), 58–63. https://doi.org/10.1684/ecn.2014.0357.

Guo, L., Yin, F., Meng, H.-Q., Ling, L., Hu-He, T.-N., Li, P., … Fan, H.-X. (2005). Differentiation of mesenchymal stem cells into dopaminergic neuron-like cells in vitro. *Biomedical and Environmental Sciences : BES*, *18*(1), 36–42.

HALL, P. A., & WATT, F. M. (1989). Stem cells: the generation and maintenance of cellular diversity. *Development*, *106*(4), 619–633.

Halleux, C., Sottile, V., Gasser, J. a, & Seuwen, K. (2001). Multi-lineage potential of human mesenchymal stem cells following clonal expansion. *Journal of Musculoskeletal & Neuronal Interactions*, *2*(1), 71–76.

Hayashi, T., Wakao, S., Kitada, M., Ose, T., Watabe, H., Kuroda, Y., … Dezawa, M. (2013). Autologous mesenchymal stem cell – derived dopaminergic neurons function in parkinsonian macaques, *123*(1), 272–284. https://doi.org/10.1172/JCI62516.272.

TH-2047_10610601

Hermann, A., Gastl, R., Liebau, S., Popa, M. O., Fiedler, J., Boehm, B. O., … Storch, A. (2004). Efficient generation of neural stem cell-like cells from adult human bone marrow stromal cells. *Journal of Cell Science*, *117*(Pt 19), 4411–4422. https://doi.org/10.1242/jcs.01307.

Herzog, E. L., Chai, L., & Krause, D. S. (2003). Review in translational hematology Plasticity of marrow-derived stem cells. *Stem Cells*, *102*(10), 3483–3493. https://doi.org/10.1182/blood-2003-05-1664.Supported.

Hiwase, S. D., Dyson, P. G., To, L. B., & Lewis, I. D. (2009). Cotransplantation of placental mesenchymal stromal cells enhances single and double cord blood engraftment in nonobese diabetic/severe combined immune deficient mice. *Stem Cells*, *27*(9), 2293–2300. https://doi.org/10.1002/stem.157.

Hmjb, O., Nvmujqpufou, B. S. F., Dvmuvsf, J. O., Joseph, N. M., He, S., Quintana, E., … Morrison, S. J. (2011). &Oufsjd Hmjb Bsf Nvmujqpufou Jo Dvmuvsf Cvu Qsjnbsjmz Gpsn Hmjb Jo Uif Bevmu Spefou Hvu, *121*(9). https://doi.org/10.1172/JCI58186DS1.

Hsu, Y.-C., Wu, Y.-T., Yu, T.-H., & Wei, Y.-H. (2016). Mitochondria in mesenchymal stem cell biology and cell therapy: From cellular differentiation to mitochondrial transfer. *Seminars in Cell & Developmental Biology*, *52*(February), 119–131. https://doi.org/10.1016/j.semcdb.2016.02.011.

Hua, J., Qiu, P., Zhu, H., Cao, H., Wang, F., & Li, W. (2011). Multipotent mesenchymal stem cells (MSCs) from human umbilical cord: Potential differentiation of germ cells. *African Journal of Biochemistry Research*, *5*(April), 113–123.

Huang, G. T.-J., Gronthos, S., & Shi, S. (2009). Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. *Journal of Dental Research*, *88*(9), 792–806. https://doi.org/10.1177/0022034509340867.

Iacono, E., Merlo, B., Romagnoli, N., Rossi, B., Ricci, F., & Spadari, A. (2015). Equine Bone Marrow and Adipose Tissue Mesenchymal Stem Cells: Cytofluorimetric Characterization, In Vitro Differentiation, and Clinical Application. *Journal of Equine Veterinary Science*, *35*(2), 130–140. https://doi.org/10.1016/j.jevs.2014.12.010.

Jamous, M., Al-Zoubi, A., Khabaz, M. N., Khaledi, R., Khateeb, M. Al, & Al-Zoubi, Z. (2010). Purification of mouse bone marrow-derived stem cells promotes ex vivo neuronal differentiation. *Cell Transplantation*, *19*(2), 193–202. https://doi.org/10.3727/096368910X492599.

Jin, K., Mao, X. O., Batteur, S., Sun, Y., & Greenberg, D. A. (2003). Induction of neuronal markers in bone marrow cells: Differential effects of growth factors and patterns of intracellular expression. *Experimental Neurology*, *184*(1), 78–89. https://doi.org/10.1016/S0014-4886(03)00133-X.

Johe, K. K. (1996). Single factors direct the differentiation of stem cells from the fetal and adult central nervous system, 3129–3140.

TH-2047_10610601

Joyce, N., Annett, G., Wirthlin, L., Olson, S., Bauer, G., & Nolta, A. (2011). Mesenchymal stem cells for the treatment of neurodegenerative disease. *Regenerative Medicine*, *5*(6), 933–946. https://doi.org/10.2217/rme.10.72.Mesenchymal.

Kalaszczynska, I., & Ferdyn, K. (2015). Wharton ' s Jelly Derived Mesenchymal Stem Cells : Future of Regenerative Medicine ? Recent Findings and Clinical Significance. *BioMed Research International*, *2015*(430847), 1–11. https://doi.org/10.1155/2015/430847.

Karumbayaram, S., Novitch, B. G., Patterson, M., Umbach, J. A., Richter, L., Conway, A., … Lowry, W. E. (2010). Directed Differentiation of Human Induced Pluripotent Stem Cells Generates Active Motor Neurons. *Stem Cells*, *27*(4), 806–811. https://doi.org/10.1002/stem.31.Directed.

Kelly, S., Bliss, T. M., Shah, A. K., Sun, G. H., Ma, M., Foo, W. C., … Steinberg, G. K. (2004). Transplanted human fetal neural stem cells survive, migrate, and differentiate in ischemic rat cerebral cortex. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(32), 11839–44. https://doi.org/10.1073/pnas.0404474101.

Kemp, K. C., Hows, J., & Donaldson, C. (2005). Bone marrow-derived mesenchymal stem cells. *Leukemia & Lymphoma*, *46*(11), 1531–1544. https://doi.org/10.1080/10428190500215076.

Kern, S., Eichler, H., Stoeve, J., Klüter, H., & Bieback, K. (2006). Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells*, *24*(5), 1294–1301. https://doi.org/10.1634/stemcells.2005-0342.

Khang, G. (n.d.). Introduction.

Kim, D.-W., Staples, M., Shinozuka, K., Pantcheva, P., Kang, S.-D., & Borlongan, C. (2013). Wharton's Jelly-Derived Mesenchymal Stem Cells: Phenotypic Characterization and Optimizing Their Therapeutic Potential for Clinical Applications. *International Journal of Molecular Sciences*, *14*(6), 11692–11712. https://doi.org/10.3390/ijms140611692.

Kim, E. Y., Lee, K. B., & Kim, M. K. (2014). The potential of mesenchymal stem cells derived from amniotic membrane and amniotic fluid for neuronal regenerative therapy. *BMB Reports*, *47*(3), 135–140. https://doi.org/10.5483/BMBRep.2014.47.3.289.

Kim, K. N., Oh, S. H., Lee, K. H., & Yoon, D. H. (2006). Effect of human mesenchymal stem cell transplantation combined with growth factor infusion in the repair of injured spinal cord. *Acta Neurochir Suppl*, *99*, 133–136.

Kim, N., & Cho, S.-G. (2013). Clinical applications of mesenchymal stem cells. *The Korean Journal of Internal Medicine*, *28*(4), 387–402. https://doi.org/10.3904/kjim.2013.28.4.387.

Korn, B. S., Kikkawa, D. O., & Hicok, K. C. (2009). Identification and characterization of adult stem cells from human orbital adipose tissue. *Ophthalmic Plastic and Reconstructive Surgery*, *25*(1), 27–32. https://doi.org/10.1097/IOP.0b013e3181912292.

TH-2047_10610601

Kozdon, K., Fitchett, C., Rose, G. E., Ezra, D. G., & Bailly, M. (2015). Mesenchymal stem cell-like properties of orbital fibroblasts in graves??? orbitopathy. *Investigative Ophthalmology and Visual Science*, *56*(10), 5743–5750. https://doi.org/10.1167/iovs.15-16580.

Krampera, M., Marconi, S., Pasini, A., Galiè, M., Rigotti, G., Mosna, F., … Bonetti, B. (2007). Induction of neural-like differentiation in human mesenchymal stem cells derived from bone marrow, fat, spleen and thymus. *Bone*, *40*(2), 382–390. https://doi.org/10.1016/j.bone.2006.09.006.

Kuhn, H. G., Winkler, J., Kempermann, G., Thal, L. J., & Gage, F. H. (1997). Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *17*(15), 5820–5829.

Kuo, T. K., Ho, J. H., & Lee, O. K. (2009). Mesenchymal stem cell therapy for nonmusculoskeletal diseases: Emerging applications. *Cell Transplantation*, *18*(9), 1013–1028. https://doi.org/10.3727/096368909X471206.

Kushnerev, E., Shawcross, S. G., Hillarby, M. C., & Yates, J. M. (2016). High-plasticity mesenchymal stem cells isolated from adult-retained primary teeth and autogenous adult tooth pulp - A potential source for regenerative therapies? *Archives of Oral Biology*, *62*, 43–48. https://doi.org/10.1016/j.archoralbio.2015.11.009.

Kwon, A., Kim, Y., Kim, M., Kim, J. M. J., Choi, H., Jekarl, D. W., … Park, I. Y. (2016). Tissue-specific Differentiation Potency of Mesenchymal Stromal Cells from Perinatal Tissues. *Scientific Reports*, *6*, 23544. https://doi.org/10.1038/srep23544.

Lee, A. Y., Lee, J., Kim, C. L., Lee, K. S., Lee, S. H., Gu, N. Y., … Cha, S. H. (2015). Comparative studies on proliferation, molecular markers and differentiation potential of mesenchymal stem cells from various tissues (adipose, bone marrow, ear skin, abdominal skin, and lung) and maintenance of multipotency during serial passages in minia. *Research in Veterinary Science*, *100*, 115–124. https://doi.org/10.1016/j.rvsc.2015.03.010.

Lee, K.-D., Kuo, T. K.-C., Whang-Peng, J., Chung, Y.-F., Lin, C.-T., Chou, S.-H., … Lee, O. K.-S. (2004). In vitro hepatic differentiation of human mesenchymal stem cells. *Hepatology (Baltimore, Md.)*, *40*(6), 1275–1284. https://doi.org/10.1002/hep.20469.

Lee, O. K., Kuo, T. K., Chen, W., Lee, K., Hsieh, S., & Chen, T. (2004). Isolation of multipotent mesenchymal stem cells from umbilical cord blood. *Stem Cells*, *103*(5), 1669–1675. https://doi.org/10.1182/blood-2003-05-1670.Supported.

Lei, Z., Yongda, L., Jun, M., Yingyu, S., Shaoju, Z., Xinwen, Z., & Mingxue, Z. (2007). Culture and neural differentiation of rat bone marrow mesenchymal stem cells in vitro. *Cell Biology International*, *31*(9), 916–923. https://doi.org/10.1016/j.cellbi.2007.02.006.

TH-2047_10610601

Li, C., Wu, X., Tong, J., Yang, X., Zhao, J., Zheng, Q., … Ma, Z. (2015). Comparative analysis of human mesenchymal stem cells from bone marrow and adipose tissue under xeno-free conditions for cell therapy. *Stem Cell Research & Therapy*, *6*(1), 55. https://doi.org/10.1186/s13287-015-0066-5.

Li, G.-G., Zhu, Y.-T., Xie, H.-T., Chen, S.-Y., & Tseng, S. C. G. (2012). Mesenchymal stem cells derived from human limbal niche cells. *Investigative Ophthalmology & Visual Science*, *53*(9), 5686–97. https://doi.org/10.1167/iovs.12-10300.

Li, Y., Chen, J., Wang, L., Zhang, L., Lu, M., & Chopp, M. (2001). Intracerebral transplantation of bone marrow stromal cells in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. *Neuroscience Letters*, *316*(2), 67–70.

Lin, K.-J., Loi, M.-X., Lien, G.-S., Cheng, C.-F., Pao, H.-Y., Chang, Y.-C., … Ho, J. H.-C. (2013). Topical administration of orbital fat-derived stem cells promotes corneal tissue regeneration. *Stem Cell Research & Therapy*, *4*(3), 72. https://doi.org/10.1186/scrt223.

Lindvall, O., & Kokaia, Z. (2006). Stem cells for the treatment of neurological disorders. *Nature*, *441*(7097), 1094–1096. https://doi.org/10.1038/nature04960.

Lindvall, O., Kokaia, Z., & Martinez-Serrano, A. (2004). Stem cell therapy for human neurodegenerative disorders–how to make it work. *Nature Medicine*, *10*(July), S42–S50. https://doi.org/10.1038/nm1064.

Liu, Q., Cheng, G., Wang, Z., Zhan, S., Xiong, B., & Zhao, X. (2015). Bone marrow-derived mesenchymal stem cells differentiate into nerve-like cells in vitro after transfection with brain-derived neurotrophic factor gene. *In Vitro Cellular & Developmental Biology. Animal*, *51*(3), 319–27. https://doi.org/10.1007/s11626-015-9875-1.

Liu, Y., Liu, L., Ma, X., Yin, Y., Tang, B., & Li, Z. (2013). Characteristics and neural-like differentiation of mesenchymal stem cells derived from foetal porcine bone marrow. *Bioscience Reports*, 351–360. https://doi.org/10.1042/BSR20120023.

Lv, F.-J., Tuan, R. S., Cheung, K. M. C., & Leung, V. Y. L. (2014). Concise Review: The Surface Markers and Identity of Human Mesenchymal Stem Cells. *Stem Cells*, *32*(6), 1408–1419. https://doi.org/10.1002/stem.1681.

Ma, S., Xie, N., Li, W., Yuan, B., Shi, Y., & Wang, Y. (2014). Immunobiology of mesenchymal stem cells. *Cell Death and Differentiation*, *21*(2), 216–25. https://doi.org/10.1038/cdd.2013.158.

Maltman, D. J., Hardy, S. A., & Przyborski, S. A. (2011). Role of mesenchymal stem cells in neurogenesis and nervous system repair. *Neurochemistry International*, *59*(3), 347–356. https://doi.org/10.1016/j.neuint.2011.06.008.

Mandal, S., Lindgren, A. G., Srivastava, A. S., Clark, A. T., Banerjee, U., & Angeles, C. L. (2015). HHS Public Access, *29*(3), 486–495. https://doi.org/10.1002/stem.590.Mitochondrial.

Mariano, E. D., Teixeira, M. J., Marie, S. K. N., & Lepski, G. (2015). Adult stem cells in neural repair: Current options, limitations and perspectives. *World Journal of Stem Cells*, *7*(2), 477–82. https://doi.org/10.4252/wjsc.v7.i2.477.

TH-2047_10610601

McKay, R. (1997). Stem cells in the central nervous system. *Science (New York, N.Y.)*, *276*(5309), 66–71. https://doi.org/10.1126/science.276.5309.66.

Messerli, M., Wagner, A., Sager, R., Mueller, M., Baumann, M., Surbek, D. V., & Schoeberlein, A. (2013). Stem Cells From Umbilical Cord Wharton's Jelly From Preterm Birth Have Neuroglial Differentiation Potential. *Reproductive Sciences*, *20*(12), 1455–1464. https://doi.org/10.1177/1933719113488443.

Mohammad, M., Yaseen, N., Al-Joubory, A., Abdullah, R., Mahmood, N., Ahmed, A. A., & Al-Shammari, A. (2016). Production of Neural Progenitors from Bone Marrow Mesenchymal Stem Cells. *Stem Cell Discovery*, *6*(1), 1–12. https://doi.org/10.4236/scd.2016.61001.

Monnin, J., Morand-Villeneuve, N., Michel, G., Hicks, D., & Versaux-Botteri, C. (2007). Production of neurospheres from mammalian Müller cells in culture. *Neuroscience Letters*, *421*(1), 22–26. https://doi.org/10.1016/j.neulet.2007.04.073.

Montanaro, F., Liadaki, K., Schienda, J., Flint, A., Gussoni, E., & Kunkel, L. M. (2004). Demystifying SP cell purification: Viability, yield, and phenotype are defined by isolation parameters. *Experimental Cell Research*, *298*(1), 144–154. https://doi.org/10.1016/j.yexcr.2004.04.010.

Montzka, K., Lassonczyk, N., Tschöke, B., Neuss, S., Führmann, T., Franzen, R., … Wöltje, M. (2009). Neural differentiation potential of human bone marrow-derived mesenchymal stromal cells: misleading marker gene expression. *BMC Neuroscience*, *10*(1), 16. https://doi.org/10.1186/1471-2202-10-16.

Mu, M. W., Zhao, Z. Y., & Li, C. G. (2015). Comparative study of neural differentiation of bone marrow mesenchymal stem cells by different induction methods. *Genetics and Molecular Research : GMR*, *14*(4), 14169–14176. https://doi.org/10.4238/2015.October.29.39.

Murray, I. R., & Péault, B. (2015). Q&A: Mesenchymal stem cells — where do they come from and is it important? *BMC Biology*, *13*(1), 99. https://doi.org/10.1186/s12915-015-0212-7.

Neirinckx, V., Coste, C., Rogister, B., & Wislet-gendebien, S. (2013). Neural Fate of Mesenchymal Stem Cells and Neural Crest Stem Cells : Which Ways to Get Neurons for Cell Therapy Purpose ? In *Trends in Cell Signaling Pathways in Neuronal Fate Decision* (pp. 327–357). https://doi.org/http://dx.doi.org/10.5772/53260.

Nekanti, U., Dastidar, S., Venugopal, P., Totey, S., & Ta, M. (2010). Increased proliferation and analysis of differential gene expression in human Wharton's jelly-derived mesenchymal stromal cells under hypoxia. *International Journal of Biological Sciences*, *6*(5), 499–512. https://doi.org/10.7150/ijbs.6.499.

Niada, S., Ferreira, L. M., Arrigoni, E., Addis, A., Campagnol, M., Broccaioli, E., & Brini, A. T. (2013). Porcine adipose-derived stem cells from buccal fat pad and subcutaneous adipose tissue for future preclinical studies in oral surgery. *Stem Cell Research & Therapy*, *4*(6), 148. https://doi.org/10.1186/scrt359.

TH-2047_10610601

Ogata, Y., Mabuchi, Y., Yoshida, M., Suto, E. G., Suzuki, N., Muneta, T., … Akazawa, C. (2015). Purified Human Synovium Mesenchymal Stem Cells as a Good Resource for Cartilage Regeneration. *PloS One*, *10*(6), e0129096. https://doi.org/10.1371/journal.pone.0129096.

Okolicsanyi, R. K., Camilleri, E. T., Oikari, L. E., Yu, C., Cool, S. M., Van Wijnen, A. J., … Haupt, L. M. (2015). Human mesenchymal stem cells retain multilineage differentiation capacity including neural marker expression after extended in vitro expansion. *PLoS ONE*, *10*(9), 1–29. https://doi.org/10.1371/journal.pone.0137255.

Otify, D. Y., Youssef, E., Nagy, N. B., Marei, M. K., & Youssif, M. I. (2014). Transdifferentiation of Bone Marrow Mesenchymal Stem Cells into Neural Cells via Cerebrospinal Fluid. *Biomedicine and Biotechnology*, *2*(4), 66–79. https://doi.org/10.12691/BB-2-4-2.

Ozen, I., Boix, J., & Paul, G. (2012). Perivascular mesenchymal stem cells in the adult human brain: a future target for neuroregeneration? *Clinical and Translational Medicine*, *1*(1), 30. https://doi.org/10.1186/2001-1326-1-30.

Pacheco-Pinedo, E. C., Budak, M. T., Zeiger, U., Jørgensen, L. H., Bogdanovich, S., Schrøder, H. D., … Khurana, T. S. (2009). Transcriptional and functional differences in stem cell populations isolated from extraocular and limb muscles. *Physiological Genomics*, *37*(1), 35–42. https://doi.org/10.1152/physiolgenomics.00051.2008.

Parekkadan, B., & Milwid, J. J. (2010). Mesenchymal Stem Cells as Therapeutics. *Annu Rev Biomed Eng*, *12*, 87–117. https://doi.org/10.1146/annurev-bioeng-070909-105309.Mesenchymal.

Parivar, K., Ph, D., Baharara, J., Ph, D., Sheikholeslami, A., & Sc, M. (2015). Neural Differentiation of Mouse Bone Marrow-Derived Mesenchymal Stem Cells Treated with Sex Steroid Hormones and Basic Fibroblast Growth Factor, *17*(1), 27–36.

Peng, L., Jia, Z., Yin, X., Zhang, X., Liu, Y., Chen, P., … Zhou, C. (2008). Comparative analysis of mesenchymal stem cells from bone marrow, cartilage, and adipose tissue. *Stem Cells and Development*, *17*(4), 761–773. https://doi.org/10.1089/scd.2007.0217.

Peroni, D., Scambi, I., Pasini, A., Lisi, V., Bifari, F., Krampera, M., … Galiè, M. (2008). Stem molecular signature of adipose-derived stromal cells. *Experimental Cell Research*, *314*(3), 603–615. https://doi.org/10.1016/j.yexcr.2007.10.007.

Pittenger, M. F., Mackay, A. M., Beck, S., Jaiswal, R. K., Douglas, R., Mosca, J. D., … Marshak, D. (1999). Multilineage Potential of Adult Human Mesenchymal Stem Cells. *Science*, *284*(April), 143–147. https://doi.org/10.1126/science.284.5411.143.

Pluchino, S., Quattrini, A., Brambilla, E., Gritti, A., Salani, G., Dina, G., … Martino, G. (2003). Injection of adult neurospheres induces recovery in a chronic model of multiple sclerosis. *Nature*, *422*(6933), 688–694. https://doi.org/10.1227/01.neu.0000309408.99477.a5.

TH-2047_10610601

Polisetty, N., Fatima, A., Madhira, S. L., Sangwan, V. S., & Vemuganti, G. K. (2008). Mesenchymal cells from limbal stroma of human eye. *Molecular Vision*, *14*(February), 431–442. https://doi.org/v14/a53 [pii].

Porter, J. D., Khanna, S., Rao, J. S., Li, J., & Andrade, F. H. (2001). Extraocular muscle is defined by a fundamentally distinct gene expression profile. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(21), 12062–7. https://doi.org/10.1073/pnas.211257298.

Puissant, B., Barreau, C., Bourin, P., Clavel, C., Corre, J., Bousquet, C., … Blancher, A. (2005). Immunomodulatory effect of human adipose tissue-derived adult stem cells: Comparison with bone marrow mesenchymal stem cells. *British Journal of Haematology*, *129*(1), 118–129. https://doi.org/10.1111/j.1365-2141.2005.05409.x.

Qu, X., Liu, T., Song, K., Li, X., & Ge, D. (2012). Induced Pluripotent Stem Cells Generated from Human Adipose-Derived Stem Cells Using a Non-Viral Polycistronic Plasmid in Feeder-Free Conditions. *PLoS ONE*, *7*(10). https://doi.org/10.1371/journal.pone.0048161.

Riekstina, U., Cakstina, I., Parfejevs, V., Hoogduijn, M., Jankovskis, G., Muiznieks, I., … Ancans, J. (2009). *Embryonic stem cell marker expression pattern in human mesenchymal stem cells derived from bone marrow, adipose tissue, heart and dermis*. *Stem Cell Reviews and Reports* (Vol. 5). https://doi.org/10.1007/s12015-009-9094-9.

Rodriguez, A. M., Elabd, C., Amri, E. Z., Ailhaud, G., & Dani, C. (2005). The human adipose tissue is a source of multipotent stem cells. *Biochimie*, *87*(1 SPEC. ISS.), 125–128. https://doi.org/10.1016/j.biochi.2004.11.007.

Rong, J. U., Wen, Z., Rong, W. U., Zhichun, F., Ju, R., Zeng, W., … Feng, Z. (2015). Interaction between neural stem cells and bone marrow derived-mesenchymal stem cells during differentiation. *Biomed Rep*, *3*(2), 242–246. https://doi.org/10.3892/br.2014.405.

Safford, K. M., Hicok, K. C., Safford, S. D., Halvorsen, Y. C., Wilkison, W. O., Gimble, J. M., & Rice, H. E. (2002). Neurogenic differentiation of murine and human adipose-derived stromal cells. *Biochemical and Biophysical Research Communications*, *294*(2), 371–379. https://doi.org/10.1016/S0006-291X(02)00469-2.

Salem, H. K., & Thiemermann, C. (2010). Mesenchymal stromal cells: Current understanding and clinical status. *Stem Cells*, *28*(3), 585–596. https://doi.org/10.1002/stem.269.

Salimi, A., Nadri, S., Ghollasi, M., Khajeh, K., & Soleimani, M. (2014). Comparison of different protocols for neural differentiation of human induced pluripotent stem cells. *Molecular Biology Reports*, *41*(3), 1713–1721. https://doi.org/10.1007/s11033-014-3020-1.

Sanchez-Ramos, J. R. (2002). Neural cells derived from adult bone marrow and umbilical cord blood. *Journal of Neuroscience Research*, *69*(6), 880–893. https://doi.org/10.1002/jnr.10337.

TH-2047_10610601

Sanchez-Ramos, J., Song, S., Cardozo-Pelaez, F., Hazzi, C., Stedeford, T., Willing, A., … Sanberg, P. R. (2000). Adult bone marrow stromal cells differentiate into neural cells in vitro. *Experimental Neurology*, *164*(2), 247–256. https://doi.org/10.1006/exnr.2000.7389.

Sart, S., Schneider, Y. J., & Agathos, S. N. (2009). Ear mesenchymal stem cells: An efficient adult multipotent cell population fit for rapid and scalable expansion. *Journal of Biotechnology*, *139*(4), 291–299. https://doi.org/10.1016/j.jbiotec.2008.12.011.

Schäffler, A., & Büchler, C. (2007). Concise review: adipose tissue-derived stromal cells--basic and clinical implications for novel cell-based therapies. *Stem Cells*, *25*(4), 818–827. https://doi.org/10.1634/stemcells.2006-0589.

Scheiner, A. J., Baker, S. S., Georgescu, D., Belsare, G., Mccann, J. D., Anderson, R. L., … Kulbersh, J. S. (2011). Master Techniques in Blepharoplasty and Periorbital Rejuvenation, *l*, 109–123. https://doi.org/10.1007/978-1-4614-0067-7.

Scintu, F., Reali, C., Pillai, R., Badiali, M., Sanna, M. A., Argiolu, F., … Sogos, V. (2006). Differentiation of human bone marrow stem cells into cells with a neural phenotype: diverse effects of two specific treatments. *BMC Neuroscience*, *7*(14), 1–12. https://doi.org/10.1186/1471-2202-7-14.

Seeberger, K. L., Dufour, J. M., Shapiro, A. M. J., Lakey, J. R. T., Rajotte, R. V, & Korbutt, G. S. (2006). Expansion of mesenchymal stem cells from human pancreatic ductal epithelium. *Laboratory Investigation; a Journal of Technical Methods and Pathology*, *86*(2), 141–53. https://doi.org/10.1038/labinvest.3700377.

Sessarego, N., Parodi, A., Podestà, M., Benvenuto, F., Mogni, M., Raviolo, V., … Frassoni, F. (2008). Multipotent mesenchymal stromal cells from amniotic fluid: Solid perspectives for clinical application. *Haematologica*, *93*(3), 339–346. https://doi.org/10.3324/haematol.11869.

Sibov, T. T., Severino, P., Marti, L. C., Pavon, L. F., Oliveira, D. M., Tobo, P. R., … Moreira-Filho, C. A. (2012). Mesenchymal stem cells from umbilical cord blood: Parameters for isolation, characterization and adipogenic differentiation. *Cytotechnology*, *64*(5), 511–521. https://doi.org/10.1007/s10616-012-9428-3.

Singh, S. P., Tripathy, N. K., & Nityanand, S. (2013). Comparison of phenotypic markers and neural differentiation potential of multipotent adult progenitor cells and mesenchymal stem cells. *World Journal of Stem Cells*, *5*(2), 53–60. https://doi.org/10.4252/wjsc.v5.i2.53.

Sires, B. S., Saari, J. C., Garwin, G. G., Hurst, J. S., & van Kuijk, F. J. (2001). The color difference in orbital fat. *Arch Ophthalmol*, *119*(6), 868–871. https://doi.org/10.1001/archopht.119.6.868.

Snehasree, S., & Chennai-, S. D. C. (2014). Extraocular Muscles – A Review. *Journal of Pharmaceutical Sciences and Research*, *6*(4), 217–219.

Strem, B. M., Hicok, K. C., Zhu, M., Wulur, I., Alfonso, Z., Schreiber, R. E., … Hedrick, M. H. (2005). Multipotential differentiation of adipose tissue-derived stem cells. *Keio J.Med.*, *54*(0022–9717 (Print)), 132–141. https://doi.org/10.2302/kjm.54.132.

TH-2047_10610601

Stuelsatz, P., Shearer, A., Li, Y., Muir, L. A., Ieronimakis, N., Shen, Q. W., … Yablonka-Reuveni, Z. (2015). Extraocular muscle satellite cells are high performance myo-engines retaining efficient regenerative capacity in dystrophin deficiency. *Developmental Biology*, *397*(1), 31–44. https://doi.org/10.1016/j.ydbio.2014.08.035.

Suzuki, H., Taguchi, T., Tanaka, H., Kataoka, H., Li, Z., Muramatsu, K., … Kawai, S. (2004). Neurospheres induced from bone marrow stromal cells are multipotent for differentiation into neuron, astrocyte, and oligodendrocyte phenotypes. *Biochemical and Biophysical Research Communications*, *322*(3), 918–922. https://doi.org/10.1016/j.bbrc.2004.07.201.

Svendsen, C. N., & Caldwell, M. A. (1999). SYMPOSIUM : Neural Stem Cells Human Neural Stem Cells : Isolation , Expansion and Transplantation. *Brain Pathology (Zurich, Switzerland)*, *513*, 499–513.

Svendsen, C. N., ter Borg, M. G., Armstrong, R. J., Rosser, a E., Chandran, S., Ostenfeld, T., & Caldwell, M. a. (1998). A new method for the rapid and long term growth of human neural precursor cells. *Journal of Neuroscience Methods*, *85*(2), 141–152. https://doi.org/10.1016/S0165-0270(98)00126-5.

Tao, H., Rao, R., & Ma, D. D. F. (2005). Cytokine-induced stable neuronal differentiation of human bone marrow mesenchymal stem cells in a serum / feeder cell- free condition, 423–433.

Temple, S. (2001). The development of neural stem cells. *Nature*, *414*(6859), 112–117. https://doi.org/10.1038/35102174.

Terada, N., Hamazaki, T., Oka, M., Hoki, M., Mastalerz, D. M., Nakano, Y., … Scott, E. W. (2002). Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. *Nature*, *416*(6880), 542–545. https://doi.org/10.1038/nature730.

Tomita, M., MORI, T., Maruyama, K., Zahir, T., WARD, M., Umezawa, A., … Tomita M, Mori T, Maruyama K, Zahir T, Ward M, Umezawa A, Y. M. (2006). A Comparison of Neural Differentiation and Retinal Transplantation with Bone Marrow-Derived Cells and Retinal Progenitor Cells. *… Cells*, *24*(10), 2270–2278. https://doi.org/10.1634/stemcells.2005-0507.S.

Tondreau, T., Meuleman, N., Delforge, A., Dejeneffe, M., Leroy, R., Massy, M., … Lagneaux, L. (2005). Mesenchymal stem cells derived from CD133-positive cells in mobilized peripheral blood and cord blood: proliferation, Oct4 expression, and plasticity. *Stem Cells*, *23*, 1105–1112. https://doi.org/10.1634/stemcells.2004-0330.

Tropepe, V., Sibilia, M., Ciruna, B. G., Rossant, J., Wagner, E. F., & van der Kooy, D. (1999). Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon. *Developmental Biology*, *208*(1), 166–88. https://doi.org/10.1006/dbio.1998.9192.

Uezumi, A., Ito, T., Morikawa, D., Shimizu, N., Yoneda, T., Segawa, M., … Fukada, S. (2011). Fibrosis and adipogenesis originate from a common mesenchymal progenitor in skeletal muscle. *Journal of Cell Science*, *124*(Pt 21), 3654–64. https://doi.org/10.1242/jcs.086629.

TH-2047_10610601

van Praag, H., Schinder, A. F., Christie, B. R., Toni, N., Palmer, T. D., & Gage, F. H. (2002). Functional neurogenesis in the adult hippocampus. *Nature*, *415*(6875), 1030–4. https://doi.org/10.1038/4151030a.

Varum, S., Rodrigues, A. S., Moura, M. B., Momcilovic, O., Easley IV, C. A., Ramalho-Santos, J., … Schatten, G. (2011). *Energy metabolism in human pluripotent stem cells and their differentiated counterparts*. *PLoS ONE* (Vol. 6). https://doi.org/10.1371/journal.pone.0020914.

Vishwakarma, S. K., Bardia, A., Tiwari, S. K., Paspala, S. A. B., & Khan, A. A. (2014). Current concept in neural regeneration research: NSCs isolation, characterization and transplantation in various neurodegenerative diseases and stroke: A review. *Journal of Advanced Research*. https://doi.org/10.1016/j.jare.2013.04.005.

Völlner, F., Ernst, W., Driemel, O., & Morsczeck, C. (2009). A two-step strategy for neuronal differentiation in vitro of human dental follicle cells. *Differentiation*, *77*(5), 433–441. https://doi.org/10.1016/j.diff.2009.03.002.

Wagner, W., Wein, F., Seckinger, A., Frankhauser, M., Wirkner, U., Krause, U., … Ho, A. D. (2005). Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. *Experimental Hematology*, *33*(11), 1402–1416. https://doi.org/10.1016/j.exphem.2005.07.003.

Wei, X., Yang, X., Han, Z., Qu, F., Shao, L., & Shi, Y. (2013). Mesenchymal stem cells: a new trend for cell therapy. *Acta Pharmacologica Sinica*, *34*(6), 747–54. https://doi.org/10.1038/aps.2013.50.

Wester, S. (2014). Orbital Stem Cells. *Current Ophthalmology Reports*, *2*(3), 107–115. https://doi.org/10.1007/s40135-014-0044-6.

Wexler, S. A., Donaldson, C., Denning-Kendall, P., Rice, C., Bradley, B., & Hows, J. M. (2003). Adult bone marrow is a rich source of human mesenchymal “stem” cells but umbilical cord and mobilized adult blood are not. *Br.J.Haematol.*, *121*(2), 368–374.

Wilkins, A., Kemp, K., Ginty, M., Hares, K., Mallam, E., & Scolding, N. (2009). Human bone marrow-derived mesenchymal stem cells secrete brain-derived neurotrophic factor which promotes neuronal survival in vitro. *Stem Cell Research*, *3*(1), 63–70. https://doi.org/10.1016/j.scr.2009.02.006.

Williams, a F., & Gagnon, J. (1982). Neuronal cell Thy-1 glycoprotein: homology with immunoglobulin. *Science (New York, N.Y.)*, *216*(4547), 696–703. https://doi.org/10.1126/science.6177036.

Woodbury D, Schwarz EJ, Prockop DJ, B. I. (2000). Adult rat and human bone marrow stromal cells differentiate into neurons. *Journal of Neuroscience Research*, *370*(61), 364–370.

Wu, S., Suzuki, Y., Ejiri, Y., Noda, T., Bai, H., Kitada, M., … Ide, C. (2003). Bone marrow stromal cells enhance differentiation of cocultured neurosphere cells and promote regeneration of injured spinal cord. *Journal of Neuroscience Research*, *72*(3), 343–351. https://doi.org/10.1002/jnr.10587.

TH-2047_10610601

Wu, Y.-P., Chen, W.-S., Teng, C., & Zhang, N. (2010). Stem Cells for the Treatment of Neurodegenerative Diseases. *Molecules*, *15*(10), 6743–6758. https://doi.org/10.3390/molecules15106743.

Yaghoobi, M. M., & Mahani, M. T. (2008). NGF and BDNF expression drop off in neurally differentiated bone marrow stromal stem cells. *Brain Research*, *1203*, 26–31. https://doi.org/10.1016/j.brainres.2008.01.086.

Yan, Z.-J., Hu, Y.-Q., Zhang, H.-T., Zhang, P., Xiao, Z.-Y., Sun, X.-L., … Xu, R.-X. (2013). Comparison of the neural differentiation potential of human mesenchymal stem cells from amniotic fluid and adult bone marrow. *Cellular and Molecular Neurobiology*, *33*(4), 465–75. https://doi.org/10.1007/s10571-013-9922-y.

Yarak, S., & Okamoto, O. K. (2010). Human adipose-derived stem cells: current challenges and clinical perspectives. *Anais Brasileiros de Dermatologia*, *85*(5), 647–656. https://doi.org/10.1590/S0365-05962010000500008.

Ye, X., Liao, C., Liu, G., Xu, Y., Tan, J., & Song, Z. (2016). Age-Related Changes in the Regenerative Potential of Adipose-Derived Stem Cells Isolated from the Prominent Fat Pads in Human Lower Eyelids, 1–16. https://doi.org/10.1371/journal.pone.0166590.

Yoo, K. H., Jang, I. K., Lee, M. W., Kim, H. E., Yang, M. S., Eom, Y., … Koo, H. H. (2009). Comparison of immunomodulatory properties of mesenchymal stem cells derived from adult human tissues. *Cellular Immunology*, *259*(2), 150–156. https://doi.org/10.1016/j.cellimm.2009.06.010.

Zeng, R., Wang, L.-W., Hu, Z.-B., Guo, W.-T., Wei, J.-S., Lin, H., … Yang, L.-J. (2011). Differentiation of human bone marrow mesenchymal stem cells into neuron-like cells in vitro. *Spine*, *36*(13), 997–1005. https://doi.org/10.1097/BRS.0b013e3181eab764.

Zhang, Y., Khan, D., Delling, J., & Tobiasch, E. (2012). Mechanisms underlying the osteo- and adipo-differentiation of human mesenchymal stem cells. *TheScientificWorldJournal*, *2012*, 793823. https://doi.org/10.1100/2012/793823.

Zhang, Y., & Wang, W. (2010). Effects of bone marrow mesenchymal stem cell transplantation on light-damaged retina. *Investigative Ophthalmology & Visual Science*, *51*(7), 3742–3748. https://doi.org/10.1167/iovs.08-3314.

Zhang, Z. X., Guan, L. X., Zhang, K., Zhang, Q., & Dai, L. J. (2008). A combined procedure to deliver autologous mesenchymal stromal cells to patients with traumatic brain injury. *Cytotherapy*, *10*(2), 134–139. https://doi.org/10.1080/14653240701883061\r790469523 [pii].

Zhao, L.-R., Duan, W.-M., Reyes, M., Keene, C. D., Verfaillie, C. M., & Low, W. C. (2002). Human bone marrow stem cells exhibit neural phenotypes and ameliorate neurological deficits after grafting into the ischemic brain of rats. *Experimental Neurology*, *174*(1), 11–20. https://doi.org/10.1006/exnr.2001.7853.

Zhou, H., Zhang, H., Yan, Z., & Xu, R. (2016). Transplantation of human amniotic mesenchymal stem cells promotes neurological recovery in an intracerebral hemorrhage rat model. *Biochemical and Biophysical Research Communications*, *475*(2), 202–208. https://doi.org/10.1016/j.bbrc.2016.05.075.

TH-2047_10610601

Zhu, Y., Yang, Y., Zhang, Y., Hao, G., Liu, T., Wang, L., … Li, Y. (2014). Placental mesenchymal stem cells of fetal and maternal origins demonstrate different therapeutic potentials. *Stem Cell Research & Therapy*, *5*(2), 48. https://doi.org/10.1186/scrt436.

Zuk, P. a, Ph, D., Zhu, M. I. N., Mizuno, H., Benhaim, P., & Lorenz, H. P. (2001). Multilineage Cells from Human Adipose Tissue : Implications for Cell-Based Therapies. *Tissue Engineering*, *7*(2), 211–228. https://doi.org/10.1089/107632701300062859.

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PUBLICATIONS AND CONFERENCES

PUBLICATIONS

1. **Darilang Mawrie***, Atul Kumar*, Damaris Magdalene, Jina Bhattacharyya, Bithiah Grace Jaganathan. (2016) Mesenchymal stem cells from human extra ocular muscle harbor neuroectodermal differentiation potential. *PLoS ONE*, **11**(6). 1 – 15.

   * Equal contribution

2. Chinnapaka Somaiah, Atul Kumar, **Darilang Mawrie**, Amit Sharma, Suraj Dasharath Patil, Jina Bhattacharyya, Rajaram Swaminathan, Bithiah Grace Jaganathan. (2015). Collagen promotes higher adhesion, survival and proliferation of mesenchymal stem cells. *PLoS ONE*, **10**(12), 1–15.

CONFERENCE PARTICIPATION

1. Presented a poster on the topic "Isolation and Characterization of stem cells from Extra Ocular Muscle Tissue" in the 3rd International Conference on Stem Cells and Cancer – Proliferation, Differentiation and Apoptosis (ICSCC – 2012), held from 27th – 30th October 2012, at PGIMER Auditorium, Dr. Ram Manohar Lohia Hospital, New Delhi, India.

2. Presented a poster on the topic "Isolation and Characterization of stem cells from the Extra Ocular Muscle Tissue" at the XXXVII All India Cell Biology Conference on Cell Dynamics and Cell Fate organized by Institute for Stem Cell Biology & Regenerative Medicine (inStem) at the J.N. Tata Auditorium, Indian Institute of Science, Bangalore (India) from December 22-24, 2013.

3. Presented a poster on the topic "Characterization of Stem Cells Isolated from the Extra Ocular Muscle Tissue" at the 5th International Conference on Stem Cells and Cancer – Proliferation, Differentiation and Apoptosis, 2014 held at JNU, New Delhi, India from the 8th - 10th November, 2014.

4. Presented an oral talk in the International Conference on Molecular Signaling: Recent Trends in Biosciences organized by the Department of Zoology, North- Eastern Hill University, Shillong – 793022 from November 20 – 22, 2015.

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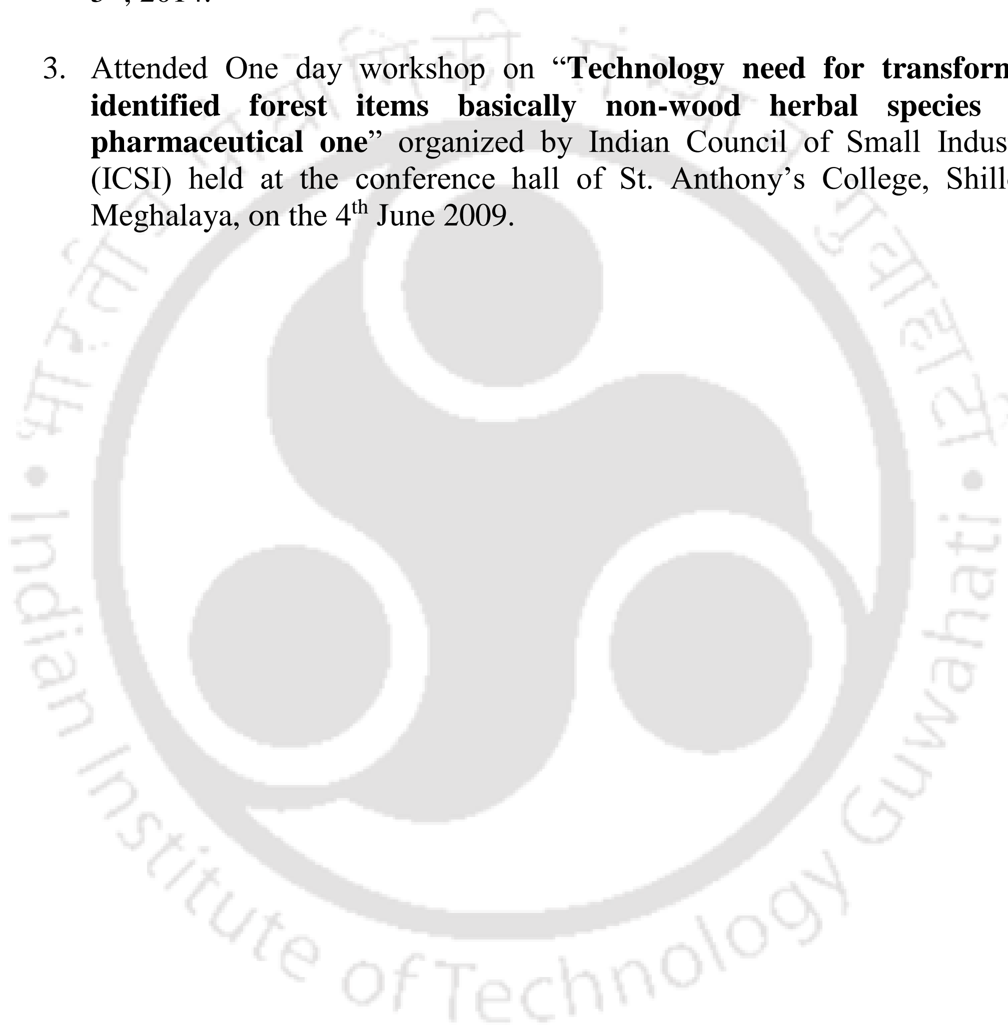
SEMINARS AND WORKSHOPS

1. Participated in "Biotech Hub Symposium – 2014" organized by Biotech Hub, Center for Environment, Indian Institute of Technology Guwahati, which was held on the 2nd December, 2014.

2. Participated in the National Conference on "**Recent Advances in Cancer Biology and Therapeutics – 2014 (RACBT)"** organized by Department of Biotechnology, Indian Institute of Technology Guwahati held on December 5th, 2014.

3. Attended One day workshop on "**Technology need for transforming identified forest items basically non-wood herbal species into pharmaceutical one**" organized by Indian Council of Small Industries (ICSI) held at the conference hall of St. Anthony's College, Shillong, Meghalaya, on the 4th June 2009.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Dedicated to

My loving parents,
Mr. Phain Sing Kharnaior
&
Mrs. Aitimai Mawrie

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