A SMALL MOLECULE MODULATOR OF PRION PROTEIN INCREASES HUMAN MESENCHYMAL STEM CELL LIFESPAN, EX VIVO EXPANSION AND ENGRAFTMENT TO BONE MARROW IN NOD/SCID MICE

THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY BY

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SUBMITTED June 2014

Acknowledgements

I want to acknowledge my teacher and supervisor **Professor. Ilaria Bellantuono**, for her excellent guidance, co-ordinated supervision and being a constant moral support in guiding me to complete my project while at Sheffield as well as far away from lab, in India. I fondly remember the days in lab when you have acknowledged me by saying 'You are a star!', 'You are my CFU-A queen', 'What will I do without you in the lab', 'Wow, the cells look beautiful-what passage are they?'. I miss working with you. You are and will forever remain a constant source of inspiration in 'my' journey of life.

I would like to dedicate this piece of hard work to my parents **(Dr. Binoy Kumar Mohanty and Mrs. Supriti Mohanty)** and late grandparents (**Kishore Charan Mohanty, Chayamani Devi, Nrusinha Charan Mohanty and Satyabhama Devi**) from whom I have learnt the art of sincerity, perseverance and patience which has helped me throughout the work period. The mere assurance all the way from India, that 'Don't worry, everything will fall in place with time, our blessings are with you', attending my phone calls at awry hours of night to listen to my plight when I was down and most importantly believing in me has been the greatest support from my parents. I am who I am is because of 'You' and thanking you for everything is just not enough. Your love and blessings were and will always remain my constant guide.

My late Badabapa (**Ajaya Kumar Mohanty**) and his entire family will be always remembered fondly for their timely help in need. I would also like to thank each and every **member of my family** for their never ending moral support and blessings from time immemorial. A special mention about my youngest cousin sister **Jigeesha Das**, who has been unknowingly a constant source of positive encouragement. You all inspire me positively in many ways that I cannot describe.

As my husband says 'There are people in your life, you can't thank enough, in fact you don't need to. All you need to do is just love them back. unconditionally'- **Shyamal Misra.** I remember the long nights you were awake just to give me company while I was writing my thesis, to bear my mood-swings when I was fed up thinking 'will I ever

finish my write-up'. Your support has been truly unconditional in every aspect of life. My in-laws (**Dr. Naresh Chandra Misra and Mrs. Ranjita Misra**) without whose support and relentless encouragement, I would not have made this far. My maternal grandparents' in-laws (**Dr. Ram Shankar Rath and Mrs. Suprava Rath**) need a special mention as they have never quit to constantly motivate me to wrap up my PhD work even at their age. I am simply blessed with my new family and counting on all their blessings for years to come.

I would like to express my special thanks to **Manikandan Thangavel**, a silent friend in my life who was by my side from the very first day of my PhD work to cheer and motivate me, especially after having those days of roller-coaster rides at lab.

How can I forget my little sister (**Himanandini Mohanty**) and brother-in-law (**Satwik Patnaik**) to acknowledge? Thank you for being there for me always. My 'Bhaina' (**Kamal Misra**) and 'Didi' (**Jiksha Kamal Misra**) for their timely reminders to support me in submitting my write-up. After parents, you are the only people I count on and I am blessed to have you in my life.

And it would be very unfair not to have mentioned **Juhi Misra - 'my partner in crime and innocence'** whose company as lab mate has evolved into an undefinable and beautiful bond of lifelong friendship. I fondly remember those days of working late in laminar air-flow cabinets and jumping into a piece of dance for a short break, long discussions at Royal Hallamshire cafeteria about work and life in general, the train journeys which comprised only of loud laughter, escape to Meadow hall shopping and movies. I am glad that this has extended to our long lasting skype chats. It was so much fun teaching you and at the same time learning with and from you. Indeed, I have made a beautiful friend for life and cheers to our friendship. Thank you so much for being there for me always.

I would like to acknowledge my mentors '**Subhas Chandra Pattanayak** and **Dr. Niranjan Rout**' for their motivation to pursue a career in research and to go abroad to broaden my horizon in every aspect of life including professional and personal.

I would like to thank my friends **Lucksy Kottam**, **Alessandra Gambardella, Brindha and Sravanth, Mark Baduain, Chandan Kadur, Satyabrata Acharya, Soumya Sudhal and my school friends,** whose timely help and moral support has been enormous. I would like to say a special thank you to my Uncle and Aunty (**Manoranjan Das and Sabitha Sutrave**) and cousins **Sarthak** and **Mahan** from London**,** who have been very warm and welcoming to accommodate me whenever I wanted few days of break from lab. I would like to thank all my colleagues from **DU04**, **DU10** and **DU16** for their tremendous moral support and helping me out with my experiments whenever needed.

Lastly I want to express my sincere gratitude and thanks to all those people who have helped me during my work but whose names I am unable to mention here.

It goes without saying about my gratitude to the **'Almighty-Maa Kundeswari'** whose bountiful blessings are always in disguise……

Declaration

This study was supported by **Biotechnology and Biological Sciences Research Council** (BBSRC). The human bone marrow samples were kindly provided **by Dr. Andrew D Chantry[1], Dr. Sanjeev Madan[2] and Dr. James Fernandes[2]**. Flow cytometry and cell sorting facility of the Medical School is run by **Susan Newton[1]** and **Kay Hopkinson[1]. Steven J Howe[3]** and **Adrian Thrasher[3]** assisted in setting up the lentiviral transduction in the laboratory. The gene expression profiling was funded by Cancer Research UK and was assisted by **W Nicol Keith[4]** and **Claire J Cairney[4]**. Karyotype analyses were performed by **Duncan Baker[5]**. We are grateful to **Orla Gallagher[1], Daren Lough[1]** and **Julia Hough[1]** for their expert technical assistance. We are thankful to **Beining Chen[6]** and **Mark J Thompson[6]** for providing the small molecule 3/689. We thank **Peter Andrews[7]** and **Harry Moore[7]** for helpful discussion.

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List of publications, presentations and posters

Publications

- ***Sindhu T Mohanty*** and Ilaria Bellantuono. 'Intra-femoral injection of human mesenchymal stem cells'. (A chapter to a protocol volume entitled Ageing and Stem cells, Methods and protocols-Methods in Molecular Biology series, 2012)

- ***Sindhu T Mohanty***, Claire Cairney, Andy Chantry, Sanjeev Madan, James Fernandes, Mark J Thompson, Steven J Howe, Adrian Thrasher, Beining Chen, W N Keith and Ilaria Bellantuono. 'A Small Molecule Modulator of Prion Protein Increases Human Mesenchymal Stem Cell Lifespan, Ex Vivo Expansion and Engraftment to Bone Marrow in NOD/SCID Mice'. (Stem cells, 2012)

Oral presentations

- ***Sindhu T Mohanty***, Claire Cairney, Andy Chantry, Sanjeev Madan, James Fernandes, Mark J Thompson, Steven J Howe, Adrian Thrasher, Beining Chen, W N Keith and Ilaria Bellantuono. 'A small molecule modulator of Prion protein increases self-renewal of mesenchymal stem cells and delays their ageing in culture' at the 5th UKMSCN Meeting, Birmingham, UK. (July 2011)

- ***Sindhu T Mohanty***, Claire Cairney, Andy Chantry, Sanjeev Madan, James Fernandes, Mark J Thompson, Steven J Howe, Adrian Thrasher, Beining Chen, W N Keith and Ilaria Bellantuono. 'A small molecule modulator of Prion protein increases self-renewal of mesenchymal stem cells and delays their ageing in culture' at the 2nd Annual Mellanby Centre Research Day, Sheffield, UK. (June 2010)

- ***Sindhu T Mohanty***, Claire Cairney, Andy Chantry, Sanjeev Madan, James Fernandes, Mark J Thompson, Steven J Howe, Adrian Thrasher, Beining Chen, W N Keith and Ilaria Bellantuono. 'A small molecule binding to prion protein increases the number of mesenchymal progenitor cells' at the 1st Annual Mellanby Centre Research Day, Sheffield, UK. (June 2009)

Poster presentations

- ***Sindhu T Mohanty***, Claire Cairney, Andy Chantry, Sanjeev Madan, James Fernandes, Mark J Thompson, Steven J Howe, Adrian Thrasher, Beining Chen, W N Keith and Ilaria Bellantuono. 'A small molecule targeting prion protein enhances mesenchymal stem cells expansion with age and their transplantation ability to bone' at the 1st European Conference on Mesenchymal Stem Cells, Toulouse, France. (November, 2010)

- ***Sindhu T Mohanty***, Claire Cairney, Andy Chantry, Sanjeev Madan, James Fernandes, Mark J Thompson, Steven J Howe, Adrian Thrasher, Beining Chen, W N Keith and Ilaria Bellantuono. 'A small molecule modulator of Prion protein increases self-renewal of mesenchymal stem cells and delays their ageing in culture' at the 4th UKMSCN Meeting, Leeds, UK and was awarded the **best poster prize award.** (March 2010)

- ***Sindhu T Mohanty***, Claire Cairney, Andy Chantry, Sanjeev Madan, James Fernandes, Mark J Thompson, Steven J Howe, Adrian Thrasher, Beining Chen, W N Keith and Ilaria Bellantuono. 'Expression of prion protein decrease in mesenchymal stem cells with age in culture' in 2009 at the 3rd UK Mesenchymal Stem Cell meeting, Octagon Centre, University of Sheffield, UK. (January 2009)

- ***Sindhu T Mohanty***, Claire Cairney, Andy Chantry, Sanjeev Madan, James Fernandes, Mark J Thompson, Steven J Howe, Adrian Thrasher, Beining Chen, W N Keith and Ilaria Bellantuono. 'Mesenchymal progenitor cells undergo increased self-renewal in IL-1ra-/- mouse model of rheumatoid arthritis in the early stages of the disease' at the UKNSCN Inaugural Scientific Conference, Edinburgh Conference Centre at Heriot-Watt University, Edinburgh, UK. (November 2008)

- ***Sindhu T Mohanty***, Claire Cairney, Andy Chantry, Sanjeev Madan, James Fernandes, Mark J Thompson, Steven J Howe, Adrian Thrasher, Beining Chen, W N Keith and Ilaria Bellantuono. 'Expression of prion protein decrease in mesenchymal stem cells with age in culture' at the 2nd SCIBS Initiative Grant Holders' Workshop, Manchester, UK. (November 2008)

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4-OHT	4-hydroxytamoxifen
%	Percentage
^{o}C	Degree Celsius
-/- or KO	Knock-out
+ve/+	Positive
-ve/-	Negative
µCT	Micro-computerised tomography
µg	Microgram
µl	Micro litre
µM	Micro molar
Mm	Millimetre
ml	Millilitre
mM	Millimolar
ng	Nanogram
α	Alpha
B	Beta
γ	Gamma
A	Absorbance
AA	Amino acid
AD	Adipocyte
ALP	Alkaline phosphatase
APS	Ammonium-per-sulphate
ARF	Alternate-reading frame
ATM	Ataxia-telangiectasia mutated
ATR	Ataxia-telangiectasia Rad3 related
B cells	B cells are called as B lymphocytes and are produced in the **B**one marrow
Bak	Bcl-2 antagonist killer
BCA	Bicinchonic acid
BER	Base excision repair

BH2	Bcl-2 homology domain
BM	Bone marrow
BMP-2	Bone morphogenetic protein-2
Bp	Base pair
Brdu	Bromoxyuridine
BSA	Bovine serum albumin
BSE	Bovine spongiform encephalopathy
cAMP/PKA	Cyclic adenosine monophosphate /protein kinase A
CC	Cluster charge
Cbfa1	Core binding factor alpha-1
cm^2	Centimetre square
CaCl2	Calcium chloride
Cd	Core domain
CD	Cluster differentiation
CDKI	Cyclin-dependent kinase inhibitor
cDNA	Complementary Deoxy-ribonucleic acid
CFU	Colony Forming Unit
CFU-A	Colony forming unit adipocyte
CFU-F	Colony forming unit fibroblast
CFU-O	Colony forming unit osteoblast
CFSE	Carboxyfluorescein succinimidyl ester
CGH	Comparative genomic hybridization
CO_2	Carbon dioxide
C_T	Cycle Threshold
Cu-Zn	Copper-Zinc
CXCR4	C-X-C chemokine receptor type-4
CyPrP	Cytosolic PrP
D7-FIB	Fibroblast/epithelial cell marker
Dcx+	Doublecortin
DDR	DNA-damage response
DDT	Dichloro-diphenyl-trichlo-roethane
DIL	Di-alkyl carbocyanine dye

DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulphoxide
DNA	Deoxy-ribonucleic acid
DNA-PKs	DNA-damage sensing protein kinase
ds	Double stranded
E-Cadherin	Epithelial cadherin
ECL	Electro-chemilluminescence
ECM	Extra-cellular matrix
EDTA	Ethylene diamine tetra acetic acid
ERK	Extra-cellular signal related kinase
eGFP	Enhanced green fluroscent protein
ESC	Embryonic stem cell
FACS	Fluorescent activated cell sorting
FBS	Foetal bovine serum
FCS	Fetal Calf serum
FGF	fibroblast growth factor
FISH	Fluorescence in situ hybridisation
FITC	Fluorescein-isothiocyanate conjugated
FSC	Forward scatter
g	Gravitational acceleration
G0	Gap 0 checkpoint of cell cycle where the cell is in resting phase and has stopped cell division
G1	Gap 1 checkpoint of cell cycle to ensure all parameters is ready for DNA synthesis
G2	Gap 2 checkpoint of cell cycle to ensure cell enters the mitosis phase and divides
GAG	Glycosaminoglycans
γH2AX	Gamma-variant of histone 2A protein family
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green Fluorescent protein
GFP+	Green Fluorescent protein positive
GPI	Glycosylphosphatidyl inositol

Grb2	Growth factor receptor bound protein 2
GSK-3	Glycogen synthase kinase
H1-3	α-helix regions
H_2O_2	Hydrogen peroxide
HA/TCP	Hydroxyapatite/tricalcium phosphate
HC	Hydrophobic core region
HCl	Hydrogen chloride
HDM2	E3 ubiquitin protein ligase (MDM2 in mice)
HEK	Human embryonic kidney
hESC	Human ESC
HIV-1	Human immunodeficiency virus
HLA	Human leukocyte antigen
HLA-DR	Human leukocyte antigen-D related that are polymorphic glycoproteins on lymphoid cells
hr	Hours
HRP	Horse radish peroxidase
hMSC	Human mesenchymal stem cells
HS	Heparin sulphate
HSC	Haematopoietic stem cell
Hsp	Heat shock protein
HSPC	Haematopoietic stem and progenitor cell
hTERT	Human telomerase reverse transcriptase
IBMX	3-isobutyl-1-methylxanthine
IDO	Indoleamine 2,3-deoxygenase
IFN-γ	Interferon-γ
IgG1	Immunoglobulin G1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
iPSC	Induced pluripotent stem cells
IR	Ionising radiation
K	Kruppel-like factor (Klf-4)
kDa	Kilo Dalton

Klf-4	Kruppel-like factor
LM	Laminin
KO	Knock out
L	Lin28
LIF	Leukaemia inhibitory factor
Lin	Lineage
LNGFR	Low affinity growth factor receptor
Log	Logarithm
LT	Long term
LT-HSC	Long term-HSC
LV	Lentiviral vector
M	Molar
mAB	Monoclonal antibody
MAPK	Mitogen activated protein kinase
MCAM	Melanoma cell adhesion molecule
M-CSF	Macrophage colony stimulating factor
M_FISH	Multiplex-FISH
MGSC	Mouse mammary gland stem cells
miR	Micro-RNA
MMP	Matrix metalloproteinase
MMR	Mismatch repair
mMSC-CM	Murine-mesenchymal stem cell complete medium
MNC	Mononuclear cells
MOI	Multiplicity of infection
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
mTRF	Mean telomere restriction fragment
N	NANOG
N-cadherin	Neural cadherin
ng	Nanogram
nNOS	Neuronal nitric oxide synthase
nM	Nano molar

Na2PO4	Sodium phosphate
NaCl	Sodium chloride
NER	Nucleotide excision repair
NHEJ	Non- homologous end joining
NMR	Nuclear magnetic resonance
NOD/SCID	Non-obese diabetic/ severe combined immunodeficiency
NSC	Neuronal stem cell
O	Octamer-binding transcription factor-4 (Oct-4)
OB	Osteoblast
OE	Over-expressor
OR	Octa-peptide repeats
Oct-4	Octamer-binding transcription factor
OI	Osteogenesis Imperfecta
Oligo2+	Oligodendrocyte populations in the corpus callosum
ONFH	Osteo necrosis of femoral head
p	Passage number
p3	Early passage
p8	Late passage
p53/p16/p21	Phosphoprotein 53/16 are involved in tumor suppressor pathway
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline-Tween 20
PCR	Polymerase chain reaction
PD	Population doubling
PD/t	Population doubling time
PEG2	Prostaglandin E2
PEI	Polyethylene imine
pRB	Phospho-retinoblastoma protein
Prnp	Human prion gene
PrP	Prion protein
PrP^{C}	Cellular prion protein
PrP^{Sc}	Scrapie prion protein
PTHrP	Parathyroid hormone related protein

RCL	Replication competent lentivirus
rhBMP-2	recombinant bone morphogenetic protein
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-PCR
RT-qPCR	Reverse transcriptase-quantitative PCR
ROS	Reactive Oxygen species
rpm	Rotations per minute
s	SOX-2
ss	single stranded
S	Synthesis phase of cell cycle where DNA replication occurs
SAHF	Senescence associated heterochromatin foci
SASP	Senescence associated secretory phenotype
SDF-1	Stromal-derived factor 1
SDF	Senescence associated DNA-damage foci
SDS	Sodium dodecyel sulphate
SDS-PAGE	Sodium dodecyel sulphate-poly acrylamide gel electrophoresis
SH2	Src homology 2
shRNA	Short hairpin-RNA
shRNA-ns	shRNA-non silencing
SKY	Spectral karyotyping
SMA	Smooth muscle active
SMAD-8	Small mother against Decapentaplegic transcription factor-8
SOD	Superoxide dismutase
Sox2+	Early neural progenitor cells
SPR	Sensor-based PrP binding assay
SSC	Side scatter
SVZ	Sub-ventricular zone
T cells	T cells are called T lymphocytes. The B cells leave BM and mature in thymus
TBBMC	Total body bone mineral content
TDD	Trichothiodystrophy
Terc	Telomerase Ribonucleic acid

Tert	Telomerase reverse transcriptase
Tert-ER	Telomerase reverse transcriptase-oestrogen receptor
TEMED	(N,N,N,N)-tetramethly-ethlyenediamine
TGF-β	Transforming growth factor-β
TR-/-	Telomerase knock-out
TSE	Transmissible spongiform encephalopathy
TNFα	Tumour necrosis factor alpha
UD	Undifferentiated control
UV	Ultra-violet
UT	Untransduced
VAS	Visual analogue scale
VTHS	Virtual high throughput screening
VPA	Valproic acid
WT	Wild type

Summary

Human mesenchymal stem cells (hMSCs) have been shown to have potential in regenerative approaches in bone and blood. Most protocols rely on their *in vitro* expansion prior to clinical use. However, several groups including our own have shown that hMSC lose proliferation and differentiation ability with serial passage in culture, limiting their clinical applications. Cellular prion protein (PrP) has been shown to enhance proliferation and promote self-renewal of hematopoietic, mammary gland and neural stem cells. With this work I tested the hypothesis that PrP decreased with cellular ageing of hMSC and was, at least in part, responsible for the loss of proliferation and differentiation seen with expansion in culture. Here I showed, for the first time, that expression of PrP decreased in hMSC following *ex vivo* expansion. When PrP expression was knocked down, hMSC showed significant reduction in proliferation and differentiation. In contrast hMSC expanded in the presence of small molecule 3/689, which stabilized PrP expression, extended lifespan up to 10 population doublings. These cells showed a 10 fold increase in engraftment levels in bone marrow 5 weeks post-transplant suggesting they were of superior quality. This was due to enhanced protection from DNA damage and enhanced cell cycle progression through upregulation of superoxide dismutase-2 (SOD2). The increase in SOD2 was dependent on PrP expression and suggested increased scavenging of reactive oxygen species as mechanism of action. My data point to PrP as a good target for chemical intervention to delay stem cell ageing.

CHAPTER-1

1.1 Stem cells

A stem cell can be defined as an undifferentiated cell that has the ability to renew its own cell population by undergoing mitotic cell divisions, a process called as self-renewal and also has the ability to undergo differentiation into one or more mature cell types which are responsible for carrying out specialised functions in the body (Lovell-Badge 2001; Gardner 2002; Lakshmipathy and Verfaillie 2005). This dual ability of stem cells enables them to act as a repair system for the body and therefore hold potential in regenerative medicine. In principle, stem cells can be isolated, expanded in culture and administered to the patient for repair of damaged tissues. However, there are many important challenges to be addressed before most stem cells could be used clinically. This includes identification of unique markers for their isolation, the development of *in vitro* culture conditions to obtain sufficient number of stem cells without loss of self-renewal and multi-potent differentiation ability and the advancement in designing protocols for transplantation which would result in their functional integration within the host tissue (Colman and Kind 2000; Stocum 2001). Stem cells from vertebrates can be divided into two major categories: a) Embryonic stem cells (ESC) and b) Adult stem cells.

1.2 Embryonic stem cells

Embryonic stem cells (ESC) are pluripotent stem cells. They can self-renew and differentiate into cells of all three germ layers: ectoderm, mesoderm and endoderm (Thomson, Itskovitz-Eldor et al. 1998; Wobus and Boheler 2005) and are prime

candidates for regenerative medicine. These stem cells have been derived from early embryos of several species including fish (Hong, Winkler et al. 1998), birds (Pain, Clark et al. 1996) and mouse (Brook and Gardner 1997). The human ESC is derived predominantly from the inner cell mass of 4-7 day old embryo called blastocyst (Thomson, Itskovitz-Eldor et al. 1998; Donovan and Gearhart 2001). *In vitro*, these stem cells can be expanded indefinitely and differentiate into several cell types (Fig 1.1) upon exposure to appropriate culture conditions (Semb 2005). However, there are still unsolved problems that prevent the use of ESC for clinical applications. It has been reported that injection of undifferentiated ESC give rise teratomas (Amit, Carpenter et al. 2000; Reubinoff, Pera et al. 2000; Shih, Forman et al. 2007). Attempts to obtain pure population of differentiated cells from ESC have been disappointing due to low efficiency of differentiation which resulted in heterogeneous cell populations still containing undifferentiated ESC (Bieberich, Silva et al. 2004; Mountford 2008). This is most likely due to the fact that all the signals needed for full differentiation are still unknown. Moreover, as ESC come from a different donor, immune-mediated rejection is expected following transplantation (Fairchild, Robertson et al. 2005; Gardner 2007). Last but not the least there are hurdles of ethical issues surrounding the use of embryo (Rippon and Bishop 2004). Establishing an ESC 'line' means destruction of embryo and this is of ethical concern. It is because for some, human life starts once the oocyte fertilises, fundamentally stating that fertilised egg develops into a human being whose rights and interest should be protected. For these reasons, further investigations on other stem cell types such as induced pluripotent stem cells (iPSC) are also warranted in an attempt to overcome some of those hurdles.

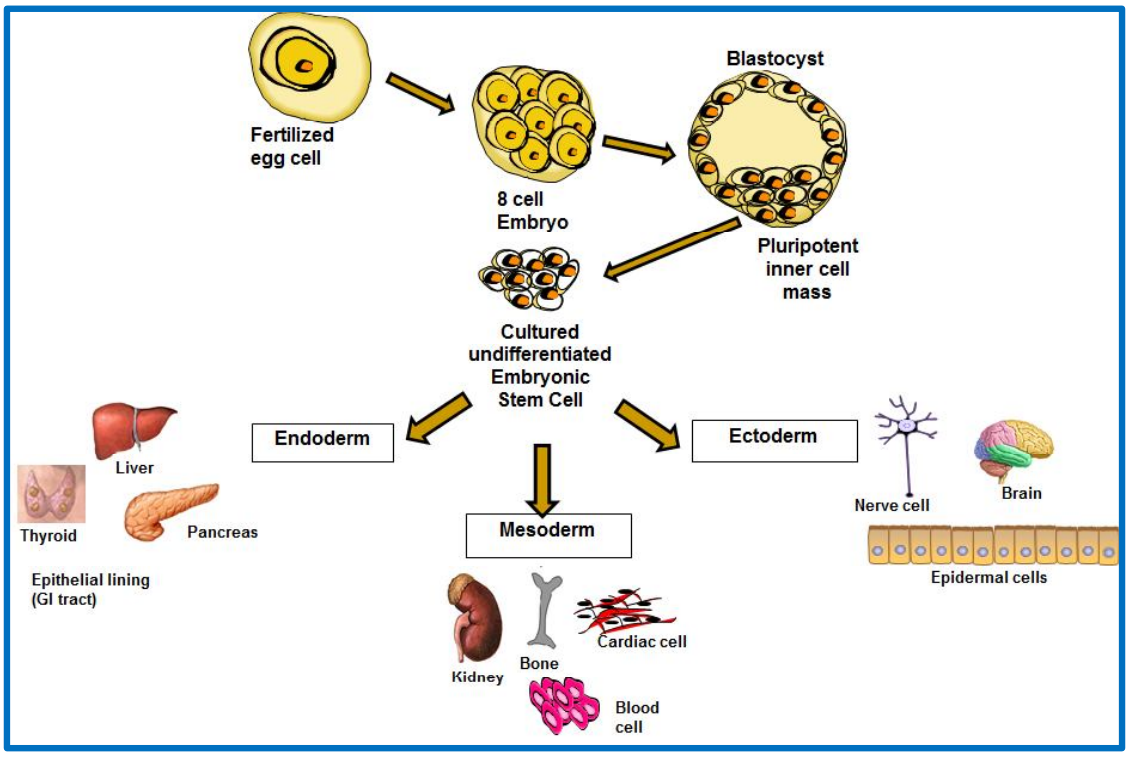


Fig 1.1: Pluripotent nature of embryonic stem cell

(The pictures are adapted and modified from http://www.nlm.nih.gov/medlineplus/)

Induced pluripotent stem cells are a type of pluripotent stem cell that is derived from adult somatic cells by reprogramming their genome into an embryonic stem cell-like state *in vitro*. There are four specific genes: (Oct-4), Sox-2, Kruppel-like factor (Klf-4), c-Myc (Bilic and Izpisua Belmonte 2012) in particular that are sufficient enough to reprogramme adult fibroblasts to iPSCs (Walia, Satija et al. 2012). Table 1.1 shows list of methods employed and reprogramming factors involved in reprogramming of adult somatic cells to iPSCs and their pros and cons (Robinton and Daley 2012). However, the current problem lies in their low efficiency in derivation. Even though rigorous pluripotency tests such as teratoma formation, generation of chimeras and germ line transmission are performed on many iPSC lines, there are discrepancies observed in the outcome of the quality of iPSCs lines obtained (Bilic and Izpisua Belmonte 2012; Robinton and Daley 2012). This is due to the differences in culturing conditions and/or differentiation protocols followed in different laboratories. Additionally there are chances of genomic mutations which may arise during the reprogramming process (Bilic and Izpisua Belmonte 2012). For these reasons adult stem cells are considered alternative to embryonic stem cells.

1.3 Adult stem cells

Adult stem cells are an alternative source of stem cells for tissue repair. The primary advantage of adult stem cells over ESC is that they can be derived from the patient's own tissue avoiding the problem of immune rejection (Stocum 2001) and the ethical issues. Comparatively, they are less controversial. However, unlike the ESCs they are

Vector type		Cell types	Factors	Pros	Cons
Integrating	Retroviral	Fibroblasts, neural stem cells, stomach cells, liver cells, keratinocytes, amniotic cells, blood cells and adipose cells	OSKM, OSK, OSK + VPA, or OS + VPA	Reasonably efficient	Genomic integration, incomplete proviral silencing and slow kinetics
	Lentiviral	Fibroblasts and keratinocytes	OSKM or miR302/367 cluster + VPA	Reasonably efficient and transduces dividing and non-dividing cells	Genomic integration and incomplete proviral silencing
	Inducible lentiviral	Fibroblasts, β cells, keratinocytes, blood cells and melanocytes	OSKM or OSKMN	Reasonably efficient and allows controlled expression of factors	Genomic integration and requirement for transactivator expression
Excisable	Transposon	Fibroblasts	OSKM	Reasonably efficient and no genomic integration	Labour-intensive screening of excised lines
	loxP-flanked lentiviral	Fibroblasts	OSK	Reasonably efficient and no genomic integration	Labour-intensive screening of excised lines, and *loxP* sites retained in the genome
Non-integrating	Adenoviral	Fibroblasts and liver cells	OSKM	No genomic integration	Low efficiency
	Plasmid	Fibroblasts	OSNL	Only occasional genomic integration	Low efficiency and occasional vector genomic integration
DNA-free	Sendai virus	Fibroblasts	OSKM	No genomic integration	Sequence-sensitive RNA replicase, and difficulty in purging cells of replicating virus
	Protein	Fibroblasts	OS	No genomic integration, direct delivery of transcription factors and no DNA-related complications	Low efficiency, short half-life, and requirement for large quantities of pure proteins and multiple applications of protein
	Modified mRNA	Fibroblasts	OSKM or OSKML + VPA	No genomic integration, bypasses innate antiviral response, faster reprogramming kinetics, controllable and high efficiency	Requirement for multiple rounds of transfection
	Micro-RNA	Adipose stromal cells and dermal fibroblasts	miR-200c, miR-302s or miR-369s	Efficient, faster reprogramming kinetics than commonly used lentiviral or retroviral vectors, no exogenous transcription factors and no risk of integration	Lower efficiency than other commonly used methods

Table 1.1: Methods and factors considered important for reprogramming of adult somatic cells to induced pluripotent stem cells

(Adapted and modified from (Robinton and Daley 2012)

K - Kruppel-like factor (Klf-4), L - Lin28, M - c-Myc, N - NANOG,O - Octamer-binding transcription factor-4 (Oct-4) ,s - SOX-2, VPA - Valproic acid

multipotent and exhibit a more limited proliferative and differentiation ability. They tend to differentiate mainly in the specialised cells of the tissue where they reside (Jiang, Jahagirdar et al. 2002). They function to maintain tissues under homeostatic conditions and provide a renewal capacity of cells in response to trauma and disease (Fuchs, Tumbar et al. 2004).

Adult stem cells have been reported to exist virtually in all tissues including fat tissue (Hawke and Garry 2001), pancreas (Choi, Ta et al. 2004), kidneys (Rookmaaker, Verhaar et al. 2004), bone marrow (Weissman 2000), skin (Watt 2000), gut (Whitehead, Demmler et al. 1999; Potten, Booth et al. 2003), liver (Alison 2002), mammary gland (Liao, Zhang et al. 2007) and brain (Gage 2000) to name a few.

Two types of adult stem cells that reside in the bone marrow (BM) are the haematopoietic stem cells (HSC), which differentiate into mature haematopoietic/blood cells, and the mesenchymal stem cells (MSC), which differentiate primarily into bone, fat and cartilage. Haematopoietic stem cells are one of the important adult stem cell which has been used routinely in the clinic for over 30 years to regenerate blood tissue (Kapoor 2001) and they are a proof of principle that adult stem cell can be used for tissue repair and thus hold great potential in regenerative medicine (Togel and Westenfelder 2007) . However, although other adult stem cells have shown encouraging results, their clinical use is still far away due to the lack of basic knowledge of their biology. In my thesis, the focus is primarily on mesenchymal stem cells.

1.3.1 Mesenchymal stem cell (MSC)

Mesenchymal stem cells are multipotent adult stem cells that reside within the BM and therefore, are easy to access. They have also been isolated from many tissues such as the adipose tissue (Gronthos, Franklin et al. 2001) and umbilical cord blood (Kim, Kim et al. 2004), amniotic fluid, chorionic villi of placenta (Igura, Zhang et al. 2004), peripheral blood (Zvaifler, Marinova-Mutafchieva et al. 2000), teeth (Miura, Gronthos et al. 2003), fetal liver (Campagnoli, Roberts et al. 2001) and lung (in 't Anker, Noort et al. 2003). About three decades ago, MSCs were identified and characterised in studies by Friedenstein A J et al., (1976) who described them as plastic adherent cells that have ability to form colonies *in vitro* (Friedenstein, Gorskaja et al. 1976). They exhibit self-renewal or proliferation ability and can proliferate up to 40 population doublings (PD) (Friedenstein, Chailakhyan et al. 1987; Bruder, Jaiswal et al. 1997). They can also differentiate to specliased cell types (Fig 1.2) (Friedenstein, Gorskaja et al. 1976).

The three universal criteria proposed to define human MSC (hMSC) *in vitro* by the universal Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (Dominici, Le Blanc et al. 2006) were that hMSCs:

a) Must be phenotypically identified as spindle-shaped cells that adhere to plastic when maintained in standard culture conditions and are capable of forming fibroblast colonies (colony-forming unit-fibroblast, CFU-F),
b) Must express cell surface markers such as cluster of differentiation (CD)105, CD73 and CD90, and must lack expression of CD45 (marker of HSC), CD34 (marker of endothelial cells), CD14 or CD11b, CD79alpha or CD19 and human leukocyte antigen-D related (HLA-DR class II) surface molecules and

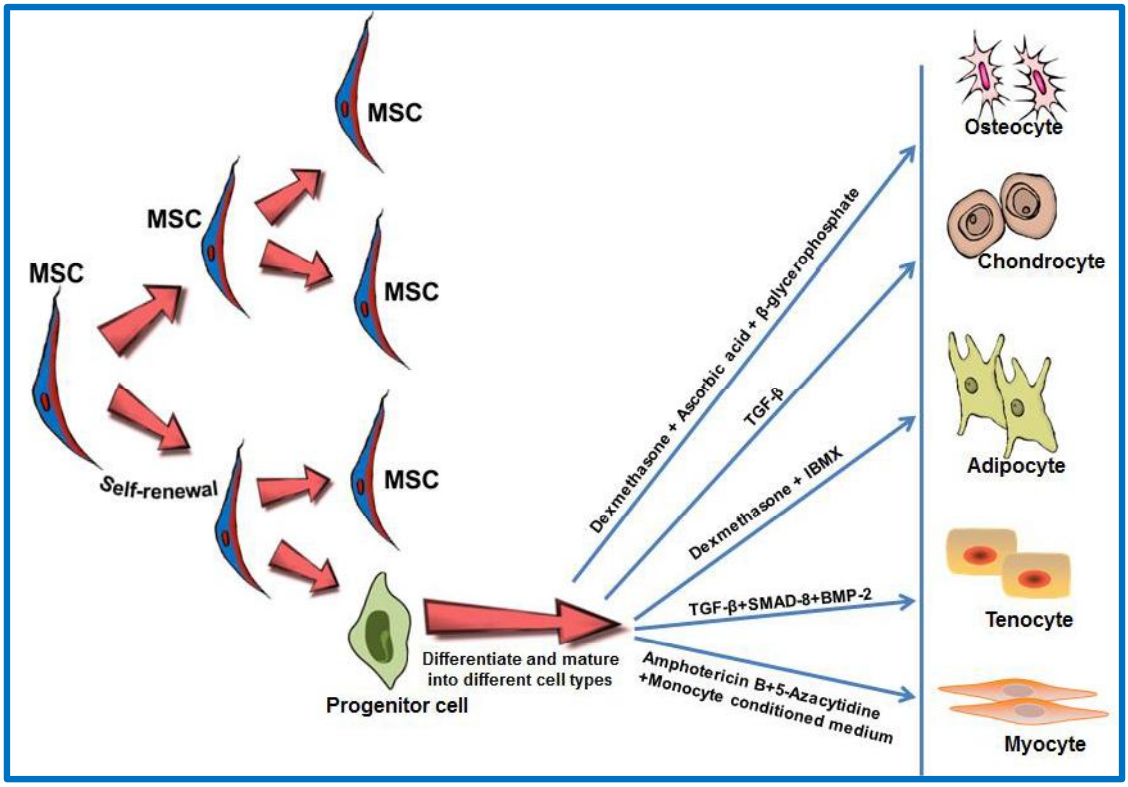


Fig 1.2: Multi-lineage potential of human mesenchymal stem cells (MSC)

IBMX- isobutyl-methyl xanthine; TGF-β- Transforming growth factor-β, SMAD-8-, BMP-2-Bone morphogenetic protein-2

c) Must differentiate to multiple lineages including osteoblasts, chondrocytes and adipocytes that give rise to bone, cartilage and adipose tissue respectively, by addition of supplements such as β-glycerophosphate, ascorbic acid and dexamethasone for inducing osteogenic differentiation (Friedenstein, Gorskaja et al. 1976; Bruder, Jaiswal et al. 1997; Pittenger, Mackay et al. 1999) and isobutyl-methyl xanthine (IBMX) and dexamethasone for inducing adipogenic differentiation (Pittenger, Mackay et al. 1999) *in vitro* (Jaiswal, Haynesworth et al. 1997; Pittenger, Mackay et al. 1999; Barry, Boynton et al. 2001).

However, these set of criteria to define hMSCs have been severely criticized (Bianco, Cao et al. 2013). The reason for the controversy lies in the limited number of retrospective assays used to enumerate and characterize the properties of hMSC. Indeed the limited number of non-specific markers detected *in vitro* and their supposed ability to differentiate to osteoblasts, chondrocytes and adipocytes like cells do not sufficiently differentiate between stem cells and their descendants, the progenitor cells. Progenitor cells also exhibit the potential of undergoing extensive proliferation and differentiation ability *in vitro*, however, unlike stem cell; they contribute very little to long-term tissue regeneration *in vivo*. Stem cells are by definition, cells with the ability to regenerate a tissue *in vivo* and therefore their identification has to reflect this.

1.3.2 Assays for the identification of MSC

The most commonly used assay to assess the number of MSC is based on their clonogenicity. The colony forming unit-fibroblast (CFU-F) assay determines the number

of MSC progenitors based on the ability to generate fibroblast-like cell colonies from a single cell. The CFU-osteoblast (O) and CFU-adipocyte (A) determine the number of MSC progenitors that have osteogenic and adipogenic potential (Fig 1.3). Even when these clonogenic assays are routinely carried out in the laboratory, these assays are not ideal to distinguish between the progenitor cells and the stem cells, which by definition require the ability to regenerate a tissue upon response to injury or following transplantation (Gronthos, Brahim et al. 2002; Bianco, Kuznetsov et al. 2006).

The ectotopic bone formation assay is the best *in vivo* assay available, known to assess the regenerative capacity of MSC. This consists of seeding MSC in an appropriate porous scaffold and implanting this under the skin of a mouse. Five weeks later an ossicle is formed with areas of osteogenesis, chondrogenesis and host haematopoiesis (Daga, Muraglia et al. 2002), allowing assessment of their ability to undergo osteogenic and chondrogenic differentiation and haematopoietic supporting stroma. An alternative *in vivo* assay is transplantation of MSC to their original tissue, the bone marrow. However, high and robust engraftment following transplantation to BM has been difficult to achieve in MSCs. The reasons for this are unclear. Migration of MSC to BM is certainly a reason as engraftment levels were improved when enhanced green fluorescent protein (eGFP)- labelled human MSCs were delivered directly in the murine BM cavity by intra-BM transplantation compared to intravenous transplantation (Muguruma, Yahata et al. 2006). Another possibility is that the right environmental conditions to facilitate engraftment have not been found yet. Alternatively it is possible that few of the transplanted cells are *bonafide* stem cells and therefore they are insufficient to form bone in their own micro-environment where they need to compete

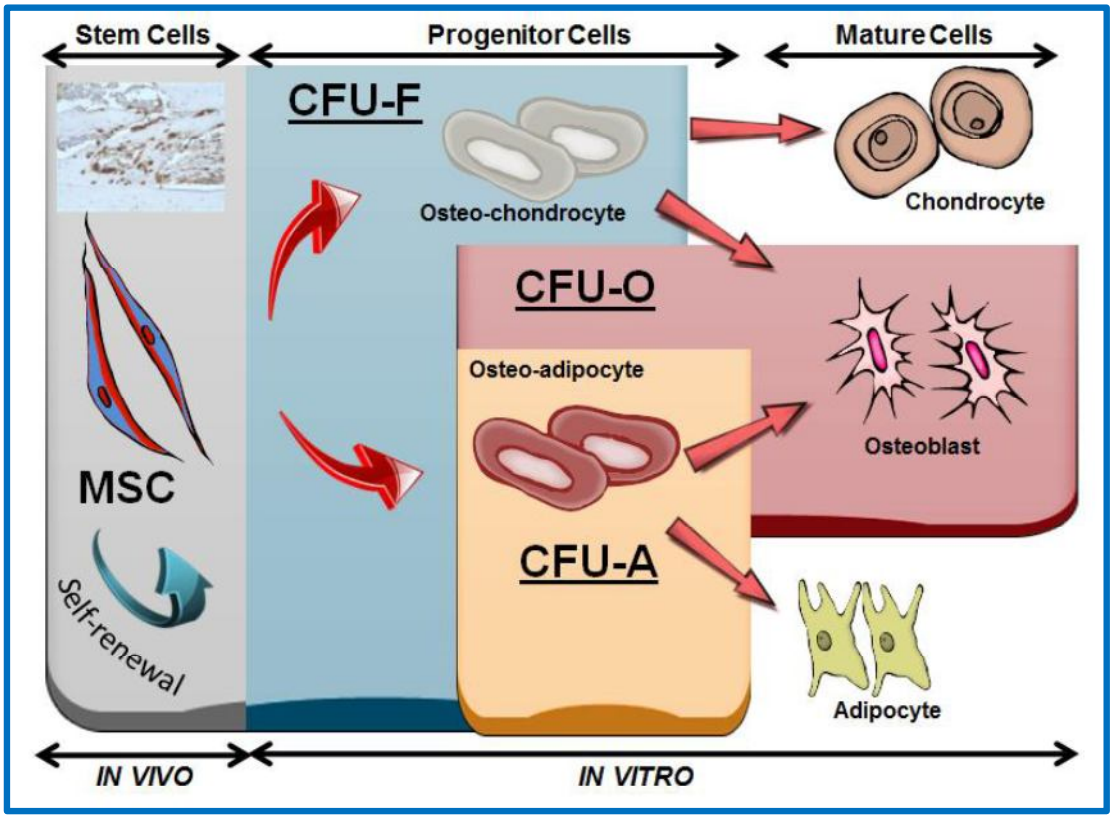


Fig 1.3: Schematic representation of functional assays to determine the self-renewal and differentiation ability of mesenchymal stem cell *in vivo* and *in vitro*

Bone-marrow derived mesenchymal stem cells (MSC) have the potential to differentiate to osteo-adipo and osteo-chondro lineage and give rise to mature cells osteoblasts, adipocytes and chondrocytes. In vitro, the colony forming unit-fibroblast (CFU-F) assay determines the clonogenic potential of bone marrow stromal cells and assays such as the colony forming unit-osteoblast (CFU-O) and colony forming unit-adipocyte (CFU-A) determine the number of MSC progenitors with osteogenic and adipogenic potential respectively. In vivo, the stemness is evaluated by transplantation assay whereby a stem cell when administered into a host is able to regenerate a tissue and reconstitute a stem cell compartment by the donor cells.

with the existing endogenous stem cells. Indeed, it was suggested that following *in vivo* ectotopic transplantation into immunocompromised mouse, only 10% of CFU-F is able to give rise to myelosupportive stroma, bone and adipose tissue (Bianco, Kuznetsov et al. 2006).

To test for the presence of MSC with long term repopulation ability, a secondary transplant assay is important. This consists of re-isolation of cells following primary transplant and administration of these cells to a second host. However, this has been nearly impossible to achieve with MSC and more work is required as these assays are important to define quality of stem cells.

Indeed, functional assays are very important for the validation of surface markers for the prospective isolation of MSC. There are several markers that have been proposed to phenotypically define hMSCs but few of them have been evaluated in a stringent manner considering their ability to regenerate *in vivo*. Although no unique surface markers are available to identify MSC, they are usually identified by a panel of markers which are not specific to MSC but are also expressed by other cell types. Cultured MSCs are usually negative for CD45, indicating their non-haematopoietic nature; CD31, indicating their non-endothelial nature (Pittenger, Mackay et al. 1999; Jones, Kinsey et al. 2002). They also express positive markers for CD105 (endoglin), CD29, CD44, SH3 and SH4 and STRO-1 (Pittenger, Mackay et al. 1999; Deans and Moseley 2000; Jones, Kinsey et al. 2002; Zhou, Huang et al. 2003). However, these markers are not specific to MSCs. For example CD105 is also expressed by endothelial cells. Antigens recognised by SH2

antibody are also expressed by fibroblasts (Deans and Moseley 2000). STRO-1 antibody also binds to glycophorin positive (+ve) and CD19+ve cells.

Even in the ectotopic bone formation assay, when freshly isolated $CD105^{+}$ hMSCs labelled with cell tracker DIL solution along with recombinant bone morphogenetic protein (rhBMP-2) were implanted under the skin of non-obese diabetic/ severe combined immunodeficiency (NOD/SCID) mice using collagen sponge carrier, 2-weeks post-implantation, the harvested implants showed presence of both cortical and trabecular bone formation by micro-computerised tomography (µCT) study (Aslan, Zilberman et al. 2006). Moreover, photomicrographs of stained tissue implants showed that DIL-labelled $CD105^{+}$ MSCs were observed to have morphological characteristics of chondrocytes and lining osteoblasts. However, no secondary transplant was performed suggesting that more robust assays are required to confirm that these cells are MSCs with long term regeneration potential.

More recently, $CD45^{low}CD146^{high}$ in human have shown to enrich the MSC progenitors *in vivo* (Sacchetti, Funari et al. 2007). Human BM has been shown to contain a population of CD45low and CD146high stromal cells. Transplantation of cell populations derived from either a limited number of $CD45^{low}CD146^{high}$ CFU-Fs or single $CD45^{low}CD146^{high}$ CFU-F resulted in the generation of heterotopic ossicle with bone, adipocytes, and stromal cells supporting blood, suggesting their multipotent ability. Moreover the group was able to re-isolate the $CD45^{low}CD146^{high}$ stromal cells as CFU-F from the heterotopic ossicle, which could be secondarily passaged and assayed, suggesting that they were infact self-renewing (Sacchetti, Funari et al. 2007). However,

no secondary transplant was performed in the study. Moreover, Tormin et al.,(2011) had questioned the reliability of CD146 marker as this marker is also expressed by α-smooth muscle active (SMA) positive cells and its expression on MSC is associated with their vascular smooth muscle commitment (Espagnolle, Guilloton et al. 2014).

CD271 is another marker that enriches MSC progenitors (Jones, Kinsey et al. 2002; Quirici, Soligo et al. 2002). The populations of $CD146^{+}$ cells and $CD146^{-/low}$ cells which were also $Lin^{-}/CD271^{+}/CD45^{-}$ gave rise to MSCs cultures that were capable of multi-lineage differentiation and co-expressed markers CD105, CD90, STRO-1, integrin, platelet-derived growth factor-β and nestin. Both sub-populations of cells were transplanted into NOD/SCID mice by heterotopic bone formation assay using hydroxyapatite/tricalcium phosphate (HA/TCP) carrier particles and by intra-femoral injections using GFP-labelled $Lin^{-}/CD271^{+}/CD45^{-}/CD146^{+}$ and $Lin^{-}/CD271^{+}/CD45^{-}/CD146^{-/low}$ cells. The engraftment ability was assessed eight weeks post-transplantation. This functionally similar sub-population of cells were able to generate bone and support HSC *in vivo* which was detected by the presence of bone, fat, fibroblasts, capillaries and invading HSC in heterotopic bone formation assay. Even in the intra-femoral transplantations, both sub-populations of GFP+ cells were detected in perivascular region surrounding the endothelium of vessels, lining the bone, surrounding the adipocytes or as reticular cells in the marrow space. Moreover, bone-lining GFP+ cells also expressed N-cadherin and were localised in close proximity (around 20μm, corresponding to approximately 2 cells) of the bone surface and vasculature. (Tormin, Li et al. 2011). The only difference between these two populations was the location of these populations following transplantation. The $Lin^{-}/CD271^{+}/CD45^{-}/CD146^{-/low}$ cells were

endosteally located and Lin-/$CD271^{+}$/$CD45^{-}$/$CD146^{+}$ cells were perivascularly localized. Although, secondary CFU-Fs were assayed from the primary transplant, no secondary transplant was performed in the study to demonstrate whether they were long-term self-renewing stem cells. A note of caution was mentioned in the study that did not show whether all the Lin^{-}/$CD271^{+}$/$CD45^{-}$/$CD146^{-/low}$ and Lin^{-}/$CD271^{+}$/$CD45^{-}$/$CD146^{+}$ cells identified *in situ* represented CFU-Fs, as some of the cells could belong to the fraction of non-colony forming cells that might still be present even in highly purified cell populations. That is why more work is required to be carried out for precise phenotypic identification of hMSCs.

Considering CD271 marker alone, there is ambiguity about the reliability of this marker. Antibodies for low affinity growth factor receptor (LNGFR), also called CD271, stain BM MSCs. The BM cells adherent after 4hours of culture that showed distinctive 'fibroblast-like' morphology were strongly positive for D7-FIB marker (a fibroblast/epithelial cell marker) along with very low CD45 fluorescence. These D7-FIB+,CD45low cells expressed LNGFR along with CD105, CD10, CD13, CD90, STRO-1 and HLA-DR and also showed multilineage differentiation in culture (Jones, Kinsey et al. 2002). However, when D7-FIB+ve, CD45-ve cells were expanded in culture for up to 4 passages, the expression of LNGFR, HLA-DR and STRO-1 was lost (Jones, Kinsey et al. 2002). Moreover, Quirici N et al., (2002) reported that LNGFR+ve, CD45-ve fraction of MSC were able to successfully show multi-lineage differentiation and CFU-Fs *in vitro*, to the contrary LNGFR-ve, CD45-ve fraction of MSC failed to demonstrate any CFU-F activity, suggesting that its expression is confined to more primitive MSC progenitor population and not a MSC-specific marker.

The best example is shown in a study by Mendez-Ferrer S et al., (2010) who reported the formation of heterotopic ossicles by transplanting single Nestin-green florescent protein positive (GFP+) cells explanted from the murine BM into primary recipient mice. Nestin-GFP+ MSCs obtained from the primary ossicles 2 months post transplantation upon secondary passage in culture generated secondary spheres which when transplanted into secondary mice recipients resulted in the generation of secondary ossicles 8 months post transplants suggesting that Nestin-GFP+ MSCs are indeed *bona fide* stem cells with long-term self-renewal ability (Mendez-Ferrer, Michurina et al. 2010). However, such engraftment ability in an exogenic model is difficult to achieve. Even transplantation of human HSC in NOD/SCID mice give origin to poor engraftment compared to mouse HSC. This is most likely due to differences in homing molecules among the two species. To summarise, studies like this will help investigators to better understand the phenotypic identification of MSC and how this knowledge could be transferred to the human field. A summary of the MSC markers with the type of testing conducted is shown in table 1.2**.**

1.4 Clinical applications of MSC

Due to their differentiation ability to osteoblast and hematopoietic supporting stroma, MSC hold potential in the therapy of bone regeneration especially to correct inherited disorders of bone and in strategy requiring improvement of HSC transplantation.

Marker	Cross-reaction	%cells generating CFU-F	In vivo	Reisolation following transplant	Ref
STRO-1	Erythroblasts, B cells subset	9%	Bone and Hematopoietic micronenvironment	N/D	(Simmons and Torok-Storb 1991; Gronthos, Graves et al. 1994; Gronthos, Zannettino et al. 2003)
STRO-1/VCAM	B cells	50%	Bone formation in 35/64 clones Haematopoietic microenvironment in 11/35 clones	N/D	(Gronthos, Zannettino et al. 2003)
CD105	Endothelial cells, pre B leukemic cells	0.01%	N/D	N/D	(Aslan, Zilberman et al. 2006)
CD271 (LNGFR)	Neural cells	0.16%-1.9%	N/D	N/D	(Quirici, Soligo et al. 2002; Buhring, Battula et al. 2007)
CD45low D7 FIB/CD271	D7FIB-skin fibroblasts	15%	N/D	N/D	(Jones, Kinsey et al. 2002)
SSEA4	Embryonic (carcinoma) stem cells, germ cells	N/D	Bone formation	N/D	(Gang, Bosnakovski et al. 2007)
GD2	Neural cells	N/D	N/D	N/D	(Martinez, Hofmann et al. 2007)
CD45-CD146+	CD146-T cells, melanoma, endothelial cells	2.60%	Bone formation and hematopoietic microenvironment in 2/4 clones	CD146+ cells and CFU-F	(Sacchetti, Funari et al. 2007)

Table 1.2: Markers used for perspective isolation of human mesenchymal stem cell

(Adapted from (Bellantuono, Aldahmash et al. 2009)

CD-Cluster of differentiation; LNGFR- low affinity growth factor receptor; +-positive; N/D-Not done; VCAM-Vascular cell adhesion molecule; SSEA-4-Stage specific embryonic antigen-4

1.4.1 Clinical application of MSC in correcting disorders of bone

One of the first studies, which showed the potential of MSC in the clinical practice, was the study by Quarto et al., (2001). MSC have been used to accelerate repair of bone fracture. The autologous MSC isolated from the BM of three patients were implanted at the site of fracture and to treat large bone fractures (Quarto, Mastrogiacomo et al. 2001). Progressive new bone formation and total integration of the implants with the host bone was reported in the study for 7 years despite the occurrence of cracks in the implants due to bio-ceramic disintegration over the time. However, it was not possible to determine whether the accelerated repair was due to transplanted MSC or the endogenous MSC. Moreover no control group was present in the study.

When fluorescently labelled MSC were implanted in a rabbit model following fracture at the site of fracture or in the bone marrow cavity of the non-affected tibia, a small proportion of donor cells have been found in the fracture gap suggesting that they at least in part contribute to the tissue repair and can migrate to the site of injury (Shirley, Marsh et al. 2005).

Osteo necrosis of femoral head (ONFH) is a progressive degenerative disease associated with limited blood supply to the bone, leading to collapse of femoral head and the follow-up requires total hip replacement. Randomised clinical study was carried out by two research teams involving 8 and 100 patients respectively, who were diagnosed with ONFH. Patients with ONFH were subjected to core decompression treatment and treatment with autologous MSCs extracted, expanded and implanted into patients. Patients treated with *ex vivo* expansion of autologous MSCs significantly improved in

Harris hip score, visual analogue scale (VAS) score and aided in pain relief and showed decreased necrotic area in femoral head, post implant at different time points compared to core decompression treatment. However, the study did not show complete healing of affected hips (Chang, Tang et al. 2010; Zhao, Cui et al. 2012).

MSC have been also used for the correction of inherited disorders of bone. A study by Horwitz et al., (1999) focuses on osteogenesis imperfecta (OI). OI is a genetic disorder caused by the secretion of a defective form of type I collagen by osteoblasts. It is associated with osteopenia, multiple fractures and severe bone deformities resulting in shortened stature. The trabecular bones of such patients have few numbers of osteoblasts, incomplete calcified area of bone matrix, disorganized formation of new bone and abnormal mineralization. *In vitro* data showed that culture expanded MSC from patients with OI were successfully transduced with wild type complementary deoxy-ribonucleic acid (cDNA) for collagen I protein to express normal form of collagen type I protein (Pochampally, Horwitz et al. 2005). *In vivo*, three children with OI were transplanted with allogeneic BM from human leukocyte antigen (HLA)-identical siblings. On day 216 after transplantation florescence photomicrograph of tetracycline labelled trabecular bone indicated new bone formation and matrix deposition. However, the engraftment levels of donor MSC were very low (1.5%-2%) (Horwitz, Prockop et al. 1999; Horwitz, Prockop et al. 2001; Horwitz, Gordon et al. 2002). A clinical benefit was claimed in the study as in these patients the disease progression slowed down post-transplantation and this was a four-fold increase in bone mineral content and reported less number of fractures compared prior to transplantation. However, it was surprising that 1.5%-2% of osteoblasts could manage to bring about

such important clinical benefit and cast doubts on the real benefit of the treatment. No data regarding disease progression of untreated patients were presented. The reduction in the number of fractures may simply be a physiological process. It is known that the fracture rate in OI is highest in the first six months and decreases thereafter (Bishop 1999). Therefore, although this study was encouraging, it needed to be interpreted with caution and highlights the low level of engraftment of MSC to bone marrow and questions what levels of engraftment are required to obtain clinical benefit. In a second study by Horwitz et al., (2001), the same authors enrolled a pilot clinical trial on five children with OI of which three patients received allogeneic BM therapy and two patients (control) did not receive transplantation. In this study the follow-up post-transplantation was for 36 months. The three patients who received BM infusion showed accelerated growth rates compared to control patients. Even the total body bone mineral content (TBBMC) at the time of treatment was 25%-60% and three months post transplantation, it was increased by 45%-77% compared to control patients. However, with long-term follow-up the growth rate of OI-treated patients slowed down but was still higher than the control groups. It could be suggested that perhaps the integration of competent donor MSCs into the recipient were of osteoblastic lineage that developed to form bone, however, it was still not clear whether they included long-living osteogenic precursors or committed osteoblast with short half-life (Horwitz, Gordon et al. 2001). In another recent study involving a female fetus with multiple intrauterine fractures, diagnosed as OI, underwent transplantation with allogenic HLA-mismatched male fetal liver of a 10 week aborted, first trimester fetus. The MSCs were expanded and injected into the umbellical vein at the 32nd week of gestation. Two year post-transplantation reported only 3 fractures and normal psychomotor development and corrected growth

tendency. However, the study was also conducted with bisphosphonate treatment after 4 months post delivery suggesting that it was really unknown whether the corrected growth tendency was as a result of MSC or bisphosphonates or combination of both (Le Blanc, Gotherstrom et al. 2005). Although these clinical studies are very encouraging, however, they lacked proper controls or showed low levels of engraftment in the long-term follow-up lasting only a few months.

1.4.2 Clinical application of MSC in supporting the maintenance of HSC

Tissue homeostasis requires a defined balance between stem and progenitor cells and the 'niche' plays a major role in ensuring that this balance is maintained. The concept of stem cell 'niche' was proposed initially by Schofield in the year 1978 (Schofield 1978). The stem cell niche comprises of stem cells and heterogeneous population of specialized cell types called as the 'niche cells' within in a special tissue location. The niche cells are commonly the stromal cells, soluble factors, extracellular matrix, neural inputs, vascular network and cell adhesion components. Maintenance and regulation of stem cell number and stem cell fate decisions are immensely dependent on specific cues, molecular cross talk and interactions from hundreds of unique micro-environmental protein combinations of the niche (Engler, Sen et al. 2006; Brack, Conboy et al. 2007; Brack and Rando 2007; LaBarge, Nelson et al. 2009; Kiefer 2011). The cell adhesion and extra-cellular matrix (ECM) molecules of the niche help stem cells to anchor to the niche and facilitate their juxtaposition communication and also play a role in their maintenance (Kiefer 2011). For example, in a study by Shen et al., (2008), it was shown that the endothelial cells in the niche engage Notch signaling pathway to stimulate self-renewal of neural stem cells within the neural sub-ventricular zone. However, when the

adhesion of neural stem cells to endothelial cells was disrupted using integrin receptor blocking antibodies, the proliferation of neural stem cells was altered (Shen, Wang et al. 2008). Suda et al., (2012) suggested that stem cell size is regulated by positive and negative feedback factors from the other cellular components of the niche including the stromal/progenitor cells by paracrine and autocrine mechanisms (Ema and Suda 2012). These interactions can instruct a stem cell to either self-renew in close proximity or commit to differentiation at distant location to regenerate damaged tissues, thereby maintaining tissue homeostasis in the body (Kiefer 2011). The proliferation signal can also come from injured tissue that can activate signaling pathways induced proliferation in their respective stem cells cohorts (Conboy, Conboy et al. 2003). Even the endothelial cells that line the blood vessels in the niche can regulate stem cell proliferation (Ding, Nolan et al. 2010). ECM deposited by endothelial cells promoted endotheliogenesis in MSCs (Datta, Holtorf et al. 2005). All these studies suggest that if the niche components suffer any damage due to disease state or ageing, the stem cell fate and number will be immensely affected, thereby affecting tissue maintenance, repair and regeneration. Therefore, it is important to maintain the quantity and quality of stem cells within a specific niche to support tissue homeostasis.

Among the various stem cell niches, the HSC niche, intestinal stem cell niche and hair follicle stem cell niche are the most well defined niches (Ema and Suda 2012). The BM stem cell niche constitutes of niche cells that includes bone cells at different developmental stages: pre-osteoblasts, osteoblasts, osteocytes; stromal cells; endothelial cells and extra-cellular matrix proteins in a special tissue location, the BM. It also contains stem cells including HSCs and MSCs with a refuge of cell adhesion and

signaling molecules. The developing HSCs are maintained in the BM where they mature and are released into the vasculature. The mesenchymal progenitors (including MSCs and stromal cells) in the BM undergo multipotent differentiation into majority of BM stromal cell lineages including osteoblasts, adipocytes and chondrocytes. Various studies have reported that MSCs localize to perivascular sites (blood vessel wall) (Hirschi and D'Amore 1996; Crisan, Yap et al. 2008) which gives them easy access to all tissues and lends credence to the notion that MSCs are integral to healing of many different tissues. Therefore both HSCs and MSCs share the vascular niche.

In addition the MSCs also serve as 'niche' cells for HSCs and support the maintenance and differentiation of HSC. The early studies by Friedenstein A J et al., (1976), showed that BM-derived MSC were crucial for the support of HSC (Friedenstein, Gorskaja et al. 1976); (Majumdar, Thiede et al. 1998). This was subsequently confirmed by the study of Mendez-Ferrer et al., (2010), who showed that MSCs, defined as nestin expressing cells closely associated with the HSCs in the BM microenvironment and regulated their mobilisation by secreting high levels of transcription factors that are considered important for the maintenance of HSC (Mendez-Ferrer, Michurina et al. 2010). Moreover, *in vivo* depletion of Nestin+ MSCs rapidly reduced the HSC content in the BM while administration of parathyroid hormone doubled the number of Nestin+ MSCs and favoured osteoblast differentiation along with renewal of haematopoietic system, suggesting that MSC and HSC have a common niche and Nestin+ MSCs are required for self-renewal of HSCs (Mendez-Ferrer, Michurina et al. 2010). It has been shown that MSC secrete several cytokines such as interleukin (IL)-6, IL-11, Leukaemia inhibitory factor (LIF) and macrophage colony stimulating factor (M-CSF), all of which are

important for the survival of HSC (Majumdar, Thiede et al. 2000). Moreover, MSC differentiate into osteoblasts, a cell type which has been shown to be involved in supporting the proliferation and survival of long-term repopulating stem cells and in the regulation of the haematopoietic niche (Calvi, Adams et al. 2003; Zhang, Niu et al. 2003). Genetically altered mice producing osteoblast-specific, parathyroid hormone related protein supported the increase in the number of HSC through the jagged-1-Notch signalling pathway (Calvi, Adams et al. 2003). Therefore, it is not unreasonable to suggest that co-transplantation of MSC with HSC could improve efficiency of HSC transplantation when HSC numbers are low or when the microenvironment is damaged. A proof of concept that this may be the case is shown in the study by Muguruma et al., (2006) (Muguruma, Yahata et al. 2006). When eGFP-marked hMSC were transplanted into the tibia of NOD/SCID mice, although at low levels the GFP-MSCs integrated into the functional components of haematopoietic environment and 4-10 weeks later, they differentiated into pericytes, myofibroblasts, stromal cells and endothelial cells (Muguruma Y, Yahata T et al.2006). More importantly this led to a two-fold increase in the number of CD45+ cells when hMSC were co-transplanted with HSC. Moreover, that MSC might enhance engraftment was also supported by another study where only 2 out of 10 mice engrafted when transplanted with low number of HSCs while 8 out of 10 mice showed improved engraftment when co-transplanted with umbilical cord blood cells and MSC (Maitra, Szekely et al. 2004). Co-transplantation of MSC with HSC has been attempted in several clinical trials but with poor results. Autologous MSC were co-transplanted with HSC in patients with breast cancer undergoing intensive chemotherapy. Although patients took less time for haematopoietic uptake, this time was only few days shorter than patients that were transplanted with HSC alone (Koc, Gerson

et al. 2000), questioning the efficiency of MSC therapy in this context. Subsequent studies showed either no engraftment of MSC to BM or very low levels, only detectable by polymerase chain reaction (PCR) (Francois, Bensidhoum et al. 2006). This suggests that lack of efficacy may be due to low levels of MSC engraftment to bone marrow rather than just inefficacy.

1.4.3 Limitations of MSC in clinical application

The reasons behind the low engraftment of MSCs are unknown. One possibility is poor migration. The receptor C-X-C chemokine receptor type-4 (CXCR4) is a specific receptor for the chemokine stromal derived factor-1 (SDF-1) and is used by stem cells for their migration to bone marrow (Brenner, Whiting-Theobald et al. 2004; Kahn, Byk et al. 2004). Indeed low levels of surface receptor CXCR4 (less than 1%) has been seen in MSC (Wynn, Hart et al. 2004) and this seems to be down-regulated especially after prolonged *in vitro* culture (Ploemacher and Brons 1989; Ploemacher 1997; Rombouts and Ploemacher 2003).

A second possibility is that during *in vitro* culture MSC lose part of their stemness with a decrease in their self-renewal and multipotent differentiation capacity. This would lead to decrease frequency of primitive progenitor cells able to engraft in transplantation. Indeed, transplantation of HSC that have been expanded *in vitro* resulted in low level of engraftment as opposed to HSC transplanted without *ex-vivo* expansion. This was shown to be the result of cytokine-activation on HSC when grown in culture leading to loss of stemness (Ahmed, Ings et al. 2004). In our laboratory, it was shown that expansion of hMSC leads to a process of cellular ageing which limits their proliferation and

differentiation ability (Baxter, Wynn et al. 2004). Unfortunately expansion in culture is a major requirement to generate sufficient number of MSCs for clinical applications. Therefore, it is important to understand the mechanisms underlying the loss of proliferation and differentiation and ways to prevent it.

1.5 Ageing

Ageing of cells is primarily characterised by decline in proliferative capacity of the cell resulting in impaired cell function and finally leading to either apoptosis (programmed cell-death) or arrest of the cells to replicate (cellular/replicative senescence) (Wright and Shay 1992; Allsopp, Morin et al. 2003). Although much is known about limited cell proliferation through programmed cell death, not much is known about senescence which contributes to the process of ageing. Replicative senescence was first studied by Hayflick in the year 1961 (Hayflick and Moorhead 1961). The presence of senescent cells have been identified in ageing human skin (Dimri, Lee et al. 1995) and liver (Paradis, Youssef et al. 2001) *in vivo*. Senescent cells are metabolically active cells arrested in the gap1 (G1) phase of the cell cycle. They exhibit enlarged and flatted morphology and express the senescence associated β-galactosidase, higher levels of tumour suppressors and hypo-phosphorylated retinoblastoma protein (Dimri, Lee et al. 1995). Often, they express two major cell cycle cyclin-dependent kinase inhibitors (CDKIs): p21 and p16; both of which are also components of tumor suppressor pathways and are governed by the p53 and retinoblastoma (pRB) proteins respectively (Campisi 2001; Stolzing, Jones et al. 2008). Both these CDKIs are not identical (Figure 1.4). Senescence-inducing signals, including those that trigger a deoxyribonucleic acid

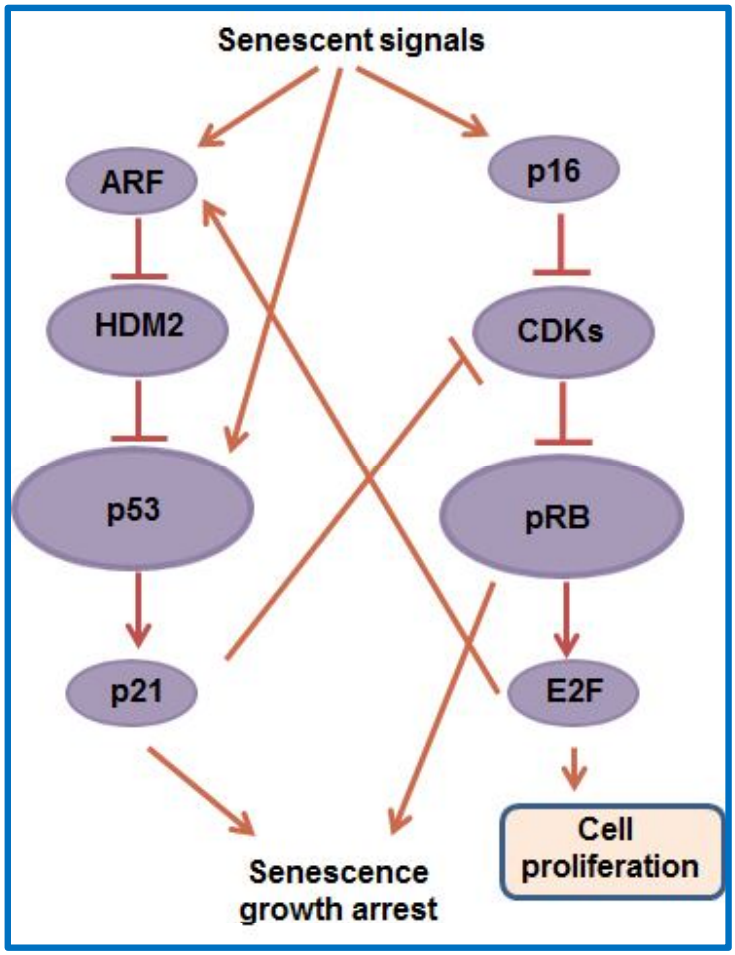


Fig 1.4: Senescence controlled by the p53 and p16–pRB pathways.

(Adapted from (Campisi and d'Adda di Fagagna 2007)

Senescence-inducing signals activate either the p53 or the p16–retinoblastoma protein (pRB) tumour suppressor pathways or both. p53 is negatively regulated by the E3 ubiquitin-protein ligase HDM2, which facilitates its degradation, and HDM2 is negatively regulated by the alternate-reading-frame protein (ARF). Activation of p53 enables immediate growth arrest in part by inducing the expression of p21, a cyclin-dependent kinase inhibitor (CDKI) that suppresses the inactivation of pRB. Senescence signals that engage the p16–pRB pathway activates p16, another CDKI and prevents pRB inactivation. pRB halts cell proliferation by suppressing the activity of E2F, a transcription factor that stimulates the expression of genes required for cell-cycle progression. E2F can also limit proliferation by inducing ARF expression, which engages the p53 pathway.

(DNA)-damage response (DDR), as well as many other stresses, usually engage either the p53 or the p16– retinoblastoma protein (pRB) tumour suppressor pathways or both. The levels of p21 is directly elevated which is induced partly as a result of p53 transactivation (Itahana, Dimri et al. 2001), although p53-independent post transcriptional mechanisms can also contribute to increase p21 levels (Burkhart, Alcorta et al. 1999). It is reported that senescence induced as a result of p53-p21 activation can resume cell division after inactivation of p53 (Beausejour, Krtolica et al. 2003; d'Adda di Fagagna, Reaper et al. 2003), suggesting that expression of p21 is a part of immediate DDR and occurs during transient growth arrest. This gives time for the damaged cells to engage their repairing ability. However, those senescent cells that engage only the p16 pathway cannot resume cell division even after the inactivation of p16 (Beausejour, Krtolica et al. 2003). Moreover it is shown that silencing the activity of p16-pRB pathway in mammary epithelial cells upregulates p53-p21 expression in part as E2F, a transcription factor that stimulates the expression of genes required for cell-cycle progression also stimulates alternate-reading frame (ARF) protein expression (Bates, Phillips et al. 1998; Zhang, Pickering et al. 2006). All these studies suggest that expression of p16 is not part of immediate DDR and does not occur during transient growth arrest. Moreover, not all senescent cells express p16 marker (Beausejour, Krtolica et al. 2003; Itahana, Zou et al. 2003; Krishnamurthy, Torrice et al. 2004) and what factors dictates the induction p16 is not clearly understood (Gil and Peters 2006). The p16-pRB is also important for generating senescence associated heterochromatin foci (SAHF), which silence the genes required for cell division. Cytological markers of SAHFs (Narita, Nunez et al. 2003) and senescence associated DNA-damage foci (SDF) (d'Adda di Fagagna, Reaper et al. 2003; Takai, Smogorzewska et al. 2003) are also used

to identify senescent cells. What factors or mechanism causes a stem cell to choose between apoptosis and senescence is still not known (Gil and Peters 2006). However, such responses are both initiated by DNA damage due to injury or stress or as a result of telomere shortening.

1.5.1 Accumulation of DNA damage independent of telomere shortening

DNA is subjected to various extrinsic and intrinsic insults that lead to accumulation of damage. Exposure to reactive oxygen species (ROS) produced by the metabolic activity of the cell or ultraviolet rays from sun, ionizing radiations (IR) generated by the cosmos, exposure to X-rays and radioactive substances, treatment with specific chemotherapeutic drugs can induce single and double stranded (ds) DNA breaks, interstrand crosslinks, base modifications leading to genomic instability (Blanpain, Mohrin et al. 2011). Although these potential DNA lesions can be repaired by the highly conserved DNA-repair mechanism pathways such as non-homologous end joining (NHEJ), homologous recombination, nucleotide excision repair (NER), base excision repair (BER) and mismatch repair (MMR) pathways; the possibilities of having error-prone repair which may result in small deletions, mutations, chromosomal translocations or nucleotide changes is also unavoidable (Fig 1.5). However, irrespective of the type of DNA lesions and the repair mechanism, when a cell senses DNA damage, it triggers an evolutionary conserved signalling pathway, collectively called as the classical DDR. Activation of DDR, signals the DNA-damage- sensing protein kinases such as ataxia-telangiectasia mutated (ATM), ataxia-telangiectasia Rad3 related (ATR) and DNA-damage sensing protein kinases (DNA-PK) which through a series of phosphorylation steps and stabilization of effector molecule p53 signals a cell to respond to damage by initiating

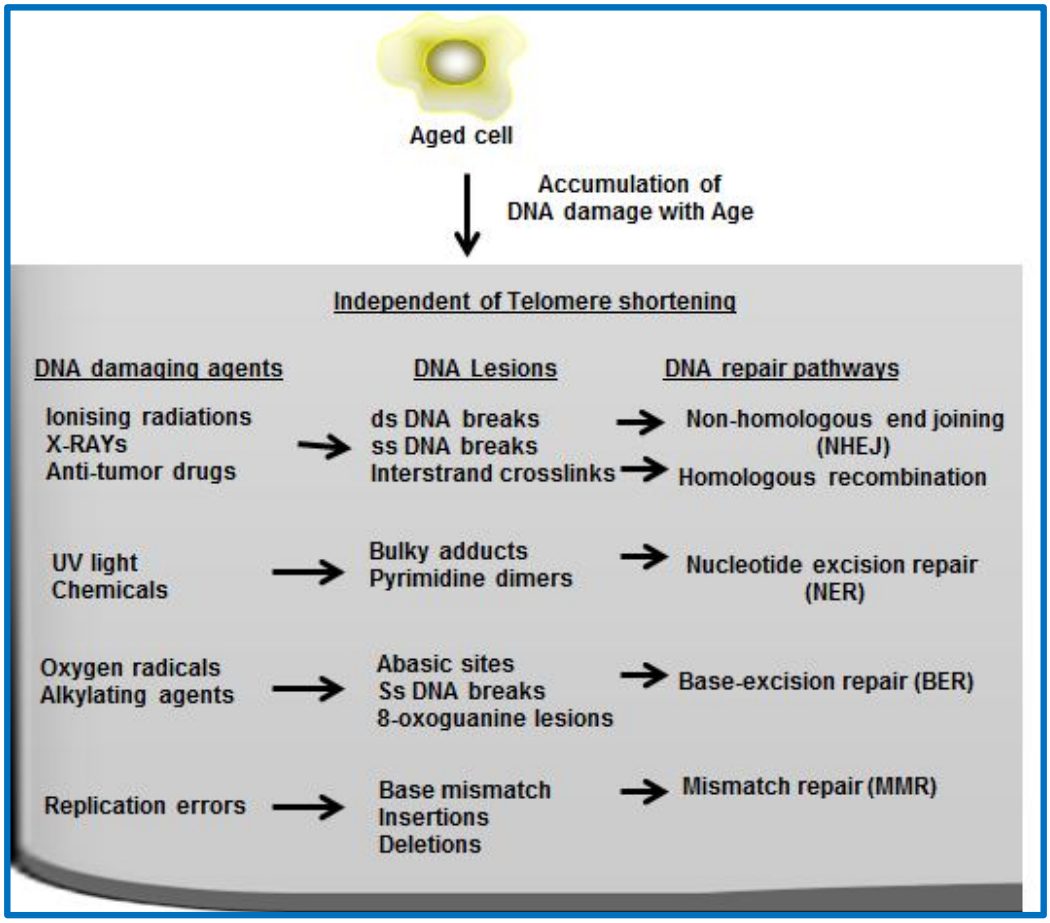


Fig 1.5: The highly conserved DNA-repair pathways triggered upon response to DNA damaging agents

(Adapted and modified from (Blanpain, Mohrin et al. 2011)

DNA- deoxy-ribonucleic acid, ds-double stranded, ss-single stranded, UV-ultra-violet

cell cycle arrest and DNA repair. Depending on the extent of DNA damage, type of cell undergoing DNA damage, the strength and duration of p53 activation and the genes trans-activated by p53, a cell can either undergo apoptosis or senescence (Fig 1.6) (Blanpain, Mohrin et al. 2011).

1.5.2 Telomere-shortening

Another reason behind limited potential of somatic cells to divide after certain period of time was attributed to the length of telomeres. A telomere is a nucleoprotein complex consisting of non-coding TTAGGG double strand repeats, a 3' single strand overhang and associated telomere binding proteins. Their primary function is to protect the end of the chromosome from damage and fusion, degradation and instability (Blackburn 2001; Wright and Shay 2002). Telomere shortening occurs in somatic cells during each cell division due to 'end replication problem' (Harley, Futcher et al. 1990; de Lange 2002), until they become critically very short and become dysfunctional. Dysfunctional telomeres triggers DDR (d'Adda di Fagagna, Reaper et al. 2003; Takai, Smogorzewska et al. 2003) and signal the cells to sense the DNA damage as DNA double stranded breaks resulting in the arrest of cell cycle progression. In some cell types such as germ cells, stem cells and cancer cells (Kim, Piatyszek et al. 1994; Liu, Snow et al. 2000; Wright and Shay 2005) the telomere length is maintained at least part by the enzyme telomerase which consists of two vital components: the telomerase RNA component (Terc) which serves as a template for telomere synthesis and the catabolic subunit of the enzyme which is telomerase reverse transcriptase (Tert). Functionally, the telomerase adds telomeric DNA repeats directly to the ends of the chromosome (Wright and Shay 2005).

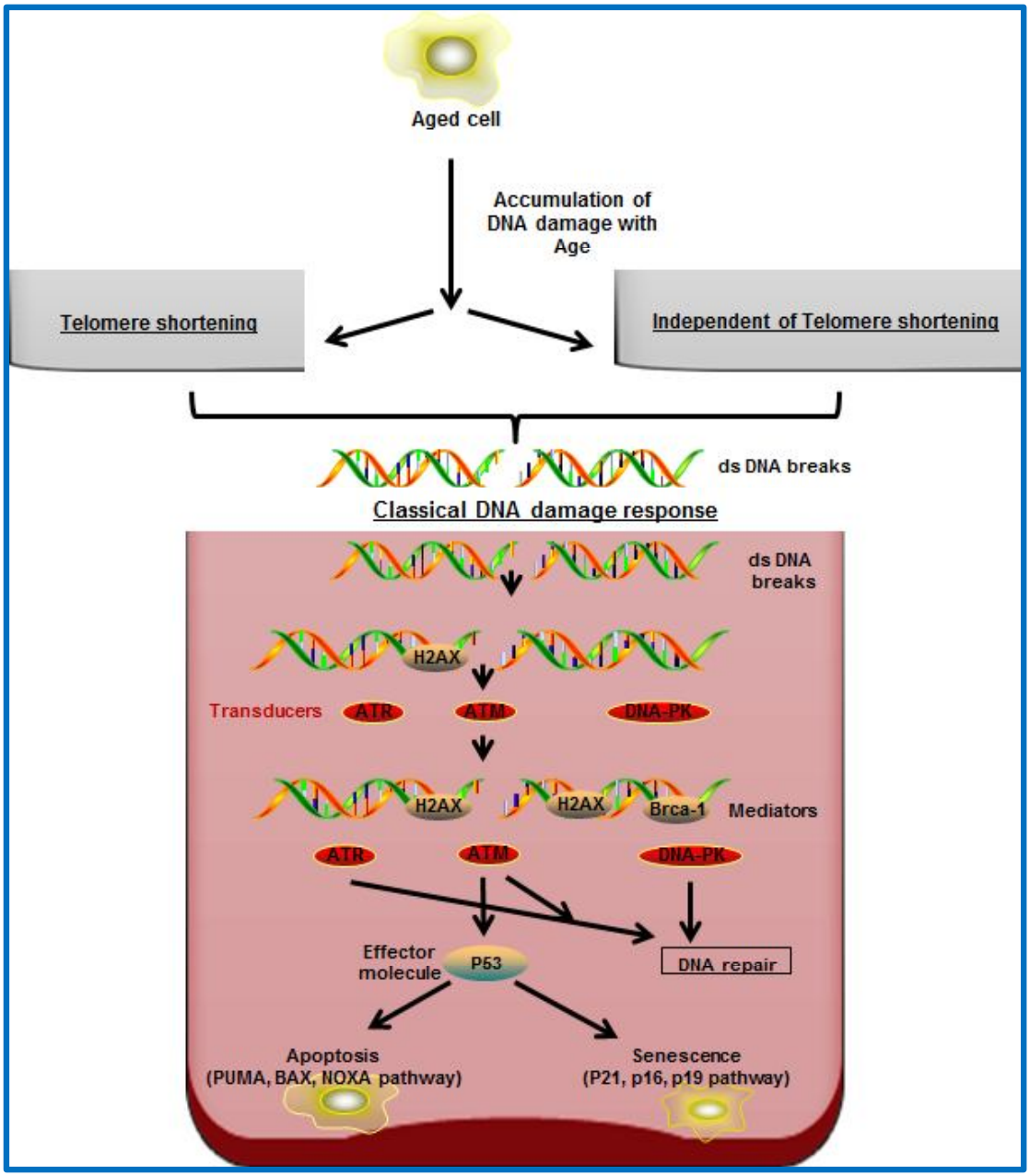


Fig 1.6: The classical DNA-damage response pathways triggered as a result of DNA damage

(Adapted and modified from (Blanpain, Mohrin et al. 2011)

DNA-deoxy-ribonucleic acid, ds-double stranded, ATM-ataxia-telangiectasia mutated, ATR-ataxia-telangiectasia Rad3 related, DNA-PK-DNA-damage sensing protein kinase

1.6 Evidences of stem cell ageing

Evidence of stem cell ageing as a result of intrinsic or extrinsic mechanisms have been reported. Haematopoietic stem cell ageing is the most extensively studied because there are better assays available to assess their self-renewal and differentiation ability. There are evidences in literature which suggest that HSC lose their self-renewal and differentiation capacity with age. Chambers S M et al., (2007) reported that highly purified phenotype-defined HSCs from mice aged 2-21 months old exhibited an increase in stem cell number with age accompanied by a loss in stem cells function (Chambers, Shaw et al. 2007). Rossi D J et al., (2005) showed that self-renewal and differentiation potential of HSC with age resulted in reduced ability to regenerate blood as well as the differentiation was skewed to the myeloid lineage. This was the result of changes intrinsic to the stem cells rather than the BM micro-environment. Moreover the gene expression profiling showed that the genes involved in myeloid commitment were up-regulated and genes involved in lymphoid commitment were down-regulated (Rossi, Bryder et al. 2005). To further support that the skewing to myeloid progenitor was crucial to HSC and had nothing to do with the micro-environment, Sudo K et al., (2000) demonstrated that young mice transplanted with HSC selected as $CD34^{-/low}$ c-$Kit^{+}Sca\text{-}1^{+}$ cells from aged mice donors but not vice-versa, showed blood constitution with strong myeloid skewing, suggesting that functional impairment of HSC occurs with age (Sudo, Ema et al. 2000). However, Ju Z et al., (2007) demonstrated that when BM from 3rd generation aged Terc knockout (Terc-/-) mice, showing signs of impaired B-cell development and accelerated myelopoisis were transplanted into young wild-type mice, their function was rescued suggesting that the micro-environment may also play a role.

Moreover no engraftment ability of HSC was reported when wild type HSC was transplanted into aged Terc-/- mice. Such impairment in engraftment was marginally due to intrinsic defects in HSC. Mostly it was due to the extreme short telomere lengths of mesenchymal progenitor cells in the niche which had minimal ability to support the HSC (Ju, Jiang et al. 2007). These data indicates that the function and engraftment potential of HSC and progenitor cells are severely compromised due to the environmental defects generated by critically short telomeres. However, it is unknown whether such a severe phenotype as the one observed in 3rd generation aged Terc-/- mice models may occur in humans.

A decline in stem cell function with age as a result of local or systemic environmental change was studied by Conboy I M et al., (2005) in muscle stem cells/satellite cell (Conboy, Conboy et al. 2005). The study comprised of sharing a circulatory system (parabiotic pairings) between a young and an old mouse (heterochronic parabionts) as test and between two old mice (isochronic parabionts) as control. Five weeks post parabiosis, muscle injury was introduced by injuring the hind limb muscle of all mice. Post muscle injury, it was observed that the old isochronic parabionts failed to regenerate the muscle, indicated by their poor ability to form myotube and fibrosis at the site of injury, as a result of reduced numbers or inability to differentiate in shared circulation compared to the heterochronic parabionts which showed expression of embryonic myosin heavy chain (a marker of regenerating myotubes) and enhanced regeneration of muscle in old mice. Even in *in vitro* cultures, the regenerative ability of old muscle satellite cell was restored when they were exposed to the serum of young mice and an inhibitory effect was observed when young satellite cell was exposed to the

serum of old mice. These data suggest that factors contained in the systemic circulation affected the ability of stem cells to repair a damaged tissue with age and therefore short intervention by modulating the systemic factors that change with age may provide an insight to maintain tissue homeostasis with increasing age.

Although stem cell express telomerase (Morrison, Prowse et al. 1996), this does not seem to be sufficient to prevent the process of ageing. Shortening of telomeres has been observed in human HSC with donor age and in situation of stress or following BM transplantation (Lee, Kook et al. 1999; Allsopp, Cheshier et al. 2001; Brummendorf, Rufer et al. 2001; Ito, Hirao et al. 2006; Rocci, Ricca et al. 2007). Age-related decline in telomere length was also reported in purified human stem cells of $CD34^{+}CD38^{low}$ phenotype compared to the cells from umbilical cord blood or fetal liver (Vaziri, Dragowska et al. 1994). More direct evidence that stem cell undergo damage leading to organ failure as a result of ageing was demonstrated by using confocal telomere quantitative fluorescence *in situ* hybridization where murine cells with longest telomeres that mapped to the stem cell compartment in several tissues including skin, intestine and brain showed a decline in telomere length with age in those compartments, followed by a decline in stem cell functionality (Flores, Canela et al. 2008). Moreover, i*n vivo* studies have shown that in telomerase knock outs (KO), telomeres undergo accelerated shortening and this is paralleled by a decreased ability to serially transplant the recipient (Allsopp, Morin et al. 2003) suggesting that telomere shortening affects HSC function.

Like telomere shortening occurs with age, similarly in murine HSC, accumulation of DNA damage with age was demonstrated by increased accretion of gamma-H2AX (a

variant of histone 2A protein family) phosphorylation of double stranded breaks in DNA from older mice compared to young mice (Rossi, Bryder et al. 2007). This has been reported in human HSC as well where analysis of CD34+ and CD34- stem/progenitor cells showed an increase of gamma-H2AX foci levels with advancing donor-age associated with age-related decline in telomere length (Chen, Fang et al. 2004). Also HSCs from older donors have shown higher amounts of unrepaired DNA damage and less efficient repair capacities compared to younger donors (Rube, Fricke et al. 2011). It could be proposed that accumulation of DNA damage with age leading to inefficient DNA repair ability results in the decline in stem cell function.

There are several examples showing that accumulation of DNA damage leads to loss of stem cells function. Nijnik A et al., (2007) reported that mice with DNA ligase IV (involved in DNA repair mechanism) mutation, Lig4Y288C showed growth retardation and severely affects the stem cell function *in vitro* and *in vivo* (Nijnik, Woodbine et al. 2007). HSCs from mice lacking the end-processing component of NHEJ DNA-repair pathway, Ku80 and mice expressing mutated form of XPD helicase, an essential component of NER DNA-repair pathway have shown self-renewal defects with poor transplantation ability in those mice with decreased proliferation and impaired lineage differentiation ability (Rossi, Bryder et al. 2007). The study evaluated the effect of deficiencies in NER and NHEJ DNA-repair pathway in young and old mice by knocking out the KU80 and mutate XPD component of the pathways respectively, on stem cell number and function. The frequency of phenotype defined long term (LT)-HSCs ($lineage^{-}$ $cKit^{+}$ $sca\text{-}1^{+}$ $flk2^{-}$ $CD34^{-}$) in the BM of KU80 knockout and XPD mutant mice increased significantly with age which was consistent with LT-HSC reserves from wild

type mice ageing naturally. However, when LT-HSCs from 26week old XPD mutant and XPD mice were competitively transplanted, HSCs from XPD mutant mice showed a significant decline in multi-lineage potential accompanied by progressive stem cell exhaustion by 16 weeks post transplantation. Likewise competitive transplantation of LT-HSCs from KU80 knockout mice were unable to generate mature B and T cells and also showed impaired ability to reconstitute myeloid lineages suggesting that stem cell activity was severely affected. Moreover, those cells were 26-fold less capable to give rise to phenocopies of themselves indicating that even their self-renewal ability was severely attenuated (Rossi, Bryder et al. 2007).

Evidence that stem cell undergoing DNA damage and proliferative stress impacts on tissue homeostasis are well demonstrated by Ruzankina Y et al., (2007). The study showed that conditional depletion of ATR gene (a DNA-damage sensing gene) in mice led to defects in tissue homeostasis resulting in tissue atrophy. The mice appeared normal and only the small proportion of non-recombining cells expressing ATR reconstituted the proliferative tissues one month post-depletion. However, 3 months-post depletion, the mice had developed an ageing phenotype including hair loss and graying indicated by loss of follicle bulge stem cells, kyphosis, osteoporosis, fibrosis of heart and kidney, reduced thymopoiesis and spermatogenesis compared to wild type controls (Ruzankina, Pinzon-Guzman et al. 2007). In patients, mutations in another DNA-damage sensing gene, ATM resulted in plethora of clinical pathologies including neural degeneration, high incidence of cancer, accelerated aging, growth retardation, telangiectasias and pulmonary diseases (Lavin 2008). This was accompanied by thymic and gonadal atrophy, immuno-deficiency, abnormal vasculature and chromosomal

fragility (Raz-Prag, Galron et al. 2011). Moreover, ATM-deficient mice are extremely sensitive to exposure to IR and present neurological abnormalities, decreased T cell numbers, premature hair growing and infertility (Barlow, Hirotsune et al. 1996).

The importance and potential impact of understanding how stem cell age and identifying ways to delay stem cell ageing comes from the study by Jaskelioff M et al., (2011). They engineered a knock-in allele encoding a 4-hydroxytamoxifen (4-OHT)-inducible telomerase reverse transcriptase-oestrogen receptor (Tert-ER) under transcriptional control of endogenous Tert promoter in Tert knock-out mice. 4rth generation homozygous Tert-ER mice exhibited short dysfunctional telomeres, stem cell depletion and signs of tissue atrophy. Telomerase reactivation for only 3 weeks lead to increased proliferation of early neural progenitor cells Sox2+, increased number of Dcx+ (Doublecortin, neuronal marker) new born neurons in sub ventricular zone and Oligo2+ oligodendrocyte populations in the corpus callosum (Jaskelioff, Muller et al. 2011). More importantly study showed rescuing of tissue function. Mice lose sense of smell with age. However mice where telomerase was reactivated returned to have similar sense of smell as that of younger mice. These data establish a strong association between tissue impairment and repair by enhanced stem cells function in aging and suggest that even short chemical intervention allowing amplification or rejuvenation of stem cells may have important impact on tissue function.

1.6.1 Evidence of ageing in mesenchymal stem cells

Whether MSC undergoes a process of ageing, is mainly documented by the evidences which point to important changes occurring in their number of proliferating and differentiating cells assessed mostly by CFU-F and CFU-O assays.

It is very controversial whether the number of CFU-F decreases with age. There are studies which reported no difference in the numbers of CFU-F with age in mice (Brockbank, Ploemacher et al. 1983; Xu, Hendry et al. 1983) and at least as many indicating the number of CFU-F decreases with age in rats (Tsuji, Hughes et al. 1990; Egrise, Martin et al. 1992; Quarto, Thomas et al. 1995). The difference in outcome is most likely due to the way in which CFU-Fs were enumerated or due to the strain of mice used in those studies. Indeed the number of CFU-F decreased with age in Balb/c but not in C57Bl/6 mice. There are even inconsistencies reported in human studies while assessing the number of CFU-F and CFU-O. Such differences in studies are mostly due to the variability in indicating the age gap between the 'young' and 'old' donor groups and the source of bone marrow used. The age of donor cohorts considered as 'young' and 'old' donors differed in different studies. For example no difference in CFU-F number was observed when the 'young' donor age was between 20-40 years (Stenderup, Justesen et al. 2001) as compared to a study where the number of CFU-F decreased when the 'young' donor age was in the range of 7-18 years (Stolzing, Jones et al. 2008). This suggests that majority of changes occur in early days when indices of bone formation are maximum (Szulc, Kaufman et al. 2007) and such changes in the skeletal dynamics may be responsible for the decline in CFU-F observed by Stolzing A et al., (2008). Also the BM were obtained either from the head of the femur of patients

undergoing routine hip replacement or corrective surgery (Oreffo, Bennett et al. 1998; Oreffo, Bord et al. 1998) or from the posterior iliac crest of haematologically healthy donors (Bruder, Jaiswal et al. 1997; Stenderup, Justesen et al. 2001) and more primitive and proliferating MSCs tend to be present in the iliac crest rather than in the periosteum or trabecular bone (Sacchetti, Funari et al. 2007). In some studies patients affected with osteoporosis or osteoarthritis were used. Moreover, the number of sample used in those studies was variable and often small, therefore unlikely to detect a significant difference. Also the definition of CFU-F varied among the investigators. While a cut off of 50 cells with a definite centre of origin was considered as CFU-F by Stolzing A et al., (2008) (Stolzing, Jones et al. 2008), in studies by Stenderup K et al., (2001) a cut off of 16 cells was considered as CFU-F. It could suggested that as stem cells are considered highly proliferative cells, colonies with 50 cells or more is probably a more stringent way of monitoring and representing a stem cell population. This may be the reason why a difference was only found in the study by Stolzing A et al., (2008).

Evidence on decreased differentiation ability of MSC toward osteogenic and adipogenic lineage with age has been contradictory too. While Stolzing A et al., (2008) found that MSCs derived from older cells showed a decline in alkaline phosphatase (ALP) activity upon differentiation to osteogenic lineage compared to younger donors (Stolzing, Jones et al. 2008), Stenderup K et al., (2001) showed that MSCs from young, old and osteoporotic patients maintained a similar osteogenic differentiation ability (Stenderup, Justesen et al. 2001). Bone forming capacity of MSCs has also been assessed by *in vivo* bone formation assays in which subcutaneous implants of MSC were mixed with HA/TCP in syngeneic animals with contrasting results. A decreased bone forming

capacity was observed in MSCs derived from aged rats compared to younger ones in the study by Inoue K et al., (1997) (Inoue, Ohgushi et al. 1997). Mendes S C et al., (2002) demonstrated that when human bone marrow-derived MSC from 53 donors of various age were seeded on calcium phosphate scaffolds and implanted under the skin of nude mice, the ability of cultures to form bone declined with age with 67% of the culture able to form bone at 41-50 years of age, 50% at 51-70 years of age and less that 46% beyond 70 years of age (Mendes, Tibbe et al. 2002). In contrast no difference was observed in the bone forming capacity of MSC obtained from young and older human donors by Stenderup K et al., (2004) (Stenderup, Rosada et al. 2004). The discrepancies in the studies may depend on the passage number when the cells were induced to differentiate with culture passaged for longer periods of time being less good at differentiating.

Similar to HSC, MSCs have also shown to undergo telomere loss and accumulation of DNA damage. The study by Baxter M et al., (2004) showed two interesting findings supporting the notion that MSC undergoes an ageing process. A significant decrease in the mean telomere restriction fragment (mTRF) was observed in MSC from young donors between the primary passage and the end of the culture (Baxter, Wynn et al. 2004).Similar data were reported by other studies too (Banfi, Muraglia et al. 2000; Bianchi, Banfi et al. 2003; Baxter, Wynn et al. 2004; Bonab, Alimoghaddam et al. 2006). Most importantly, when MSC were cultured for an equal number of population doubling, the telomere length in young donors was significantly longer compared to old donors. The average loss *in vivo* was estimated at 17base pair (bp)/year, suggesting that telomere shortening is occurring *in vivo* with the age of the donor (Baxter, Wynn et al. 2004). Indeed the fact that telomere length of MSC obtained from fetal blood, liver and

bone marrow was significantly high ($p<0.01$) compared to adult MSC (Guillot, Gotherstrom et al. 2007), well support this finding in favour of an ageing process of MSC both *in vitro* and *in vivo*.

It is controversial whether MSC similarly to HSC, express telomerase to sufficient levels to delay the ageing process. *In vitro* cultures of human MSC lack the telomerase activity (Zimmermann, Voss et al. 2003) and hTERT expression (Simonsen, Rosada et al. 2002) thus resulting in telomere shortening with serial passaging. Most of the studies showed no expression of telomerase in MSC in cultures (Shi, Gronthos et al. 2002; Simonsen, Rosada et al. 2002; Wright and Shay 2002; Abdallah, Haack-Sorensen et al. 2005). A recent study by Saeed H et al., (2011); demonstrated that Terc-/- mice devoid of telomerase activity and with signs of telomere dysfunction showed a decreased bone mass, MSC number and ability to differentiate, an intrinsic osteoblastic defect and signs of increased senescence following *in vitro* culture (Saeed, Abdallah et al. 2011). This data suggested that MSC may express telomerase *in vivo* but it is not sufficient to maintain telomere with age. It is possible that similar to HSC only very primitive MSC express telomerase *in vivo* that delay telomere shortening with age. Upon isolation in culture, MSC lose part of their stemness and telomerase is switched 'off'. Indeed a study demonstrated that culture expanded human bone marrow stromal cells committed towards osteoblast lineage showed high levels of expression of the osteogenic regulatory gene core binding factor alpha (Cbfa). Telomerase activity was repressed by the expression of Cbfa1 (Isenmann, Cakouros et al. 2007).

Very few studies have reported accumulation of DNA damage in MSCs with age leading to decline in their functional ability. The capacity of NER to repair the damage seemed impaired in late passage MSCs as there was an increase in DNA damage load over time (Alves, Munoz-Najar et al. 2010) which was due to increase in telomere-associated DNA damage and led a significant decrease in their differentiation potential. The telomere-associated DNA damage was demonstrated by co-localization of 53BP1 (a DNA damage marker) staining with a telomeric probe which detected no signal in late passaged MSCs compared to early passage MSCs. Moreover the levels of oxidative stress markers were upregulated in late-passage MSCs. The fact that NER could not repair the DNA damage in long-term culture expanded MSCs was demonstrated by unscheduled DNA synthesis assay. In trichothiodystrophy (TDD) mice, a mutation in the Ercc2 DNA repair gene results in the accumulation of DNA damage associated with features of accelerated aging. It was reported that decrease in bone strength and lack of periosteal apposition in older TTD mice also showed lack of body fat which was due to the impairment in the number and differentiation ability of MSCs in those mice (Nicolaije, Diderich et al. 2012). However careful experiments need to be conducted in MSCs from knock-out models to show how MSC function is diminished if there is DNA damage.

As MSC undergo a process of ageing characterised by decreased proliferation and differentiation ability with the age of the donor along with time in culture, it is very important to understand the molecular mechanisms underlying the process of ageing to find target molecules that would facilitate their long-term self-renewal and

differentiation ability *in vitro* and *in vivo*. One of the possible candidates is the cellular prion protein (PrP).

1.7 Cellular prion protein

Prion protein has been in the limelight over years due to their association with a family of neurodegenerative disease called the transmissible spongiform encephalopathy (TSE) including bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and Creutzfeldt-Jakob disease in human. Stanley. B. Prusiner coined the term 'Prion', an abbreviation for 'proteinaceous infectious particle' and '-on' as an analogy to virion. The specific protein in prion was thus named as Proteinase-resistant protein (PrP). The protein exists in two different isoforms. The normal, non-pathogenic form is called as PrP^{C} (C stands for cellular or common). However, the pathogenic and the infectious form is called as PrP^{Sc} (Sc stands for 'scrapie, a prion disease in animals, primarily in sheep).

The human prion (Prnp) gene is located in the short arm of chromosome 20 and encodes the complete PrP^{C} of 253 amino acids in length (Puckett, Concannon et al. 1991). PrP is synthesized in the rough endoplasmic reticulum and makes its way through the secretory pathways in Golgi complex where it is glycosylated and finally traffics to the cell surface (Cancellotti, Wiseman et al. 2005). Once on the surface, PrP is internalised and degraded very rapidly with a half-life of 3-6 hours (Caughey and Raymond 1991). It is an extra-cellular protein and is anchored to the detergent-resistant areas of plasma-membrane by a glycosylphosphatidyl inositol (GPI) link (Prusiner 1998). This protein is

readily digested by the enzyme proteinase K (Weissmann 2004). The best estimate of the nuclear magnetic resonance (NMR) structures of PrP studied in different species including human, mice, cattle and Syrian hamster shows that structurally, PrP entails one flexible N-terminal octa-peptide region in the extra-cellular space, three α–helices and two-stranded anti-parallel β–sheets flanking the first α–helix (Aguzzi, Sigurdson et al. 2008). The second β–sheet and third α–helixes are connected by a large loop with structural properties. This N- terminus usually appears unstructured consisting of two conserved domains. While the first conserved domain constitutes segment of five repeats of an octameric amino acid sequence, mostly associated with copper binding (Hornshaw, McDermott et al. 1995; Jackson, Murray et al. 2001) and plays a role in prion pathogenesis (Collinge 2001), the second conserved domain is called as the central domain (Cd) and it contains a sequence of positively charged residues (termed as charge cluster) and a hydrophobic and conserved profile (Prusiner 1998) (Fig 1.7A). The Cd domain is the most conserved part of PrP and plays a decisive role in cell homeostasis. Mutations in this part of the Cd domain have shown to alter the endoplasmic regulations of cell and lead to cell degeneration (Hegde, Mastrianni et al. 1998; Stewart, Drisaldi et al. 2001). The last domain is the C-terminal globular domain. The α–helix and β–sheets of this domain are stably linked via a disulphide bond (Riek, Hornemann et al. 1996; Riek, Hornemann et al. 1997; Zahn, Liu et al. 2000). Therefore, PrP presents three isoforms based on their orientations with respect to lumen of the endoplasmic reticulum: an extra-cellular form and two transmembrane isoforms (Fig 1.7B) (Hegde, Mastrianni et al. 1998; (Holscher, Bach et al. 2001).

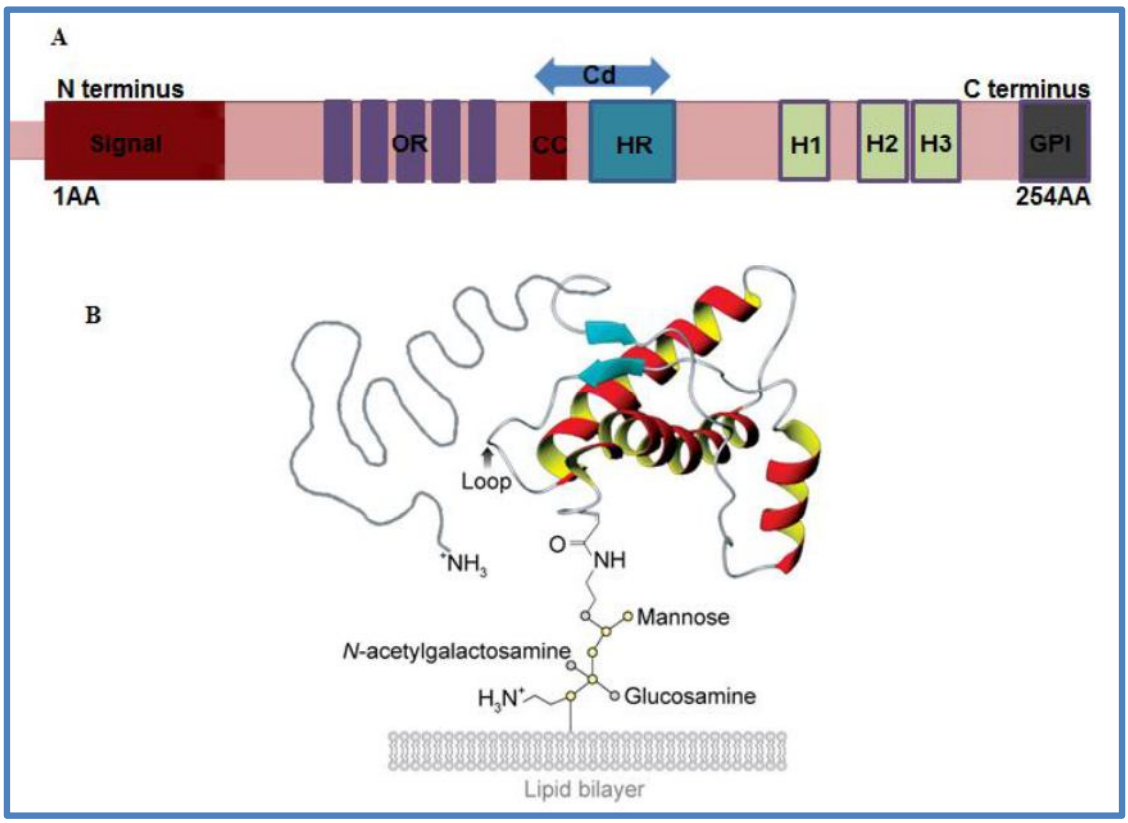


Fig 1.7 Structure of cellular prion protein

Figure A represents primary structure of N terminal tail of cellular prion protein. Figure B represents the tertiary structure of cellular prion protein by NMR spectroscopy. N-terminal tail is indicated by grey, α–helices in red, anti-parallel β–sheets in turquoise. This figure was obtained from (Aguzzi, Sigurdson et al. 2008) and it shows that prion protein is attached to the cell membrane by a glycolipid anchor. It contains a globular domain and an unstructured flexible amino-terminal tail.

Cd-core domain, OR-octa-peptide repeats, AA-amino acid, CC-cluster charge, HC-hydrophobic core region and H1-3- α-helix regions.

Although the conversion of the PrP^{C} into its infectious form, PrP^{Sc} has been much explored, the exact mechanism is yet to be discovered. Several hypotheses have been proposed concerning the nature of PrP^{Sc} (Aguzzi, Sigurdson et al. 2008). While the 'template-directed refolding model' proposes that the conversion of PrP^{C} to PrP^{Sc} is due to 'conformational modifications' of PrP^{C} gene, the most widely accepted 'protein only' hypothesis is a follow up from the studies by Prusiner S B (Prusiner 1982) and Weissmann C et al., (1993) (Weissmann, Bueler et al. 1993; Weissmann, Bueler et al. 1993) which suggested that the infectious agent that causes TSE is devoid of nucleic acid and is identical to PrP^{Sc} which occurs due to a 'post- translational modifications' of PrP^{C}. A conditional homozygous deletion of Prnp gene that encodes PrP^{C} in mice failed to develop disease upon inoculation with infectious brain homogenate and no signs of infectivity was reported in the brains of those mice (Bueler, Aguzzi et al. 1993; Sailer, Bueler et al. 1994). However, reintroduction of Prnp gene by transgenesis restored the disease progression in Prnp knockout mice (Aguzzi and Polymenidou 2004). Indeed, there are evidences reporting that PrP^{Sc} *in vivo* is not in itself neuro toxic and that there is a lack of co-relation between PrP^{Sc} deposition and disease severity (Lasmezas, Deslys et al. 1997; Hill, Joiner et al. 2000; Mallucci, Dickinson et al. 2003), suggesting that the key substrate in prion pathogenesis is the conversion of PrP^{C} to PrP^{Sc}, rather than the accumulation of PrP^{Sc} only. Hence it is reasonable to suggest that prion disease therapeutics can be targeted either to PrP^{Sc} or to the conversion of PrP^{C} to PrP^{Sc}. Although targeting PrP^{Sc} would seem logical but it is unlikely to have effect on disease progression as it is the end point of a pathogenic conversion process. Alternatively, it is thought that targeting PrP^{C} would be more beneficial as it is the initial substrate that caused the conversion to the infectious form. By biochemical and/or physiochemical

intervention or gene knock out using RNA interference or even using compounds/ small molecules as anti-prion agents would prove potential intervention to study prion disease and might also elicit a clinical application (Bessen, Raymond et al. 1997; Deleault, Lucassen et al. 2003; Safar, Kellings et al. 2005).

1.7.1 Function of prion protein

Although PrP^{C} has been known to play several biological roles, the main function is still unknown. The cellular PrP is expressed in several tissues including lymphoid organs (Ford, Burton et al. 2002), heart, neural tissue with very low levels found in kidney and liver (Miele, Alejo Blanco et al. 2003; Linden, Martins et al. 2008). Across different species, they are also found to be expressed in neural populations of hippocampus, thalamus, neuro-cortex (Harris, Huber et al. 1993; Harris, Lele et al. 1993; Bailly, Haeberle et al. 2004) and in glial cells (Moser, Colello et al. 1995; Laine, Marc et al. 2001; Ford, Burton et al. 2002; Radovanovic, Braun et al. 2005). Immuno-electron microscopy has revealed that this protein in neurons is axonally transported to the nerve terminals, thus making its localization in the synaptic regions (Borchelt, Koliatsos et al. 1994). Light microscopic immuno-histochemistry results have also revealed the presence of PrP in the synaptic region of the olfactory bulb, limbic structures and striato-nigral complex (Sales, Rodolfo et al. 1998). Also when PrP makes it way to the Golgi complex as the mono- and di- glycosylated forms and finally traffics to the cell surface by the process of glycosylation, a small amount of unglycosylated form of the protein remains localized intra-cellularly in the cytoplasm (Cancellotti, Wiseman et al. 2005). The reason for intra-cellular localization may be due to re-translocation of the protein to the proteosome system for degradation (Harris 1999). Indeed PrP has been shown to

undergo sub-cellular trafficking in neuroblastoma cells. Once it reaches the cell surface, it is internalised and subjected to undergo constitutive cycles between the endocytic compartment and the plasma membrane (Shyng, Huber et al. 1993) facilitating late endocytosis of the protein. Prion is known to play a role in numerous functions such as cellular localization and trafficking, copper uptake, oxidative stress, signal transduction, anti and pro-apoptotic role, neuronal morphology and adhesion (Kurschner and Morgan 1995; Pauly and Harris 1998; Kim, Lee et al. 2004).

Prion protein may serve as a receptor for any-extracellular ligand, primarily copper ions as they are known to play an important role in copper metabolism (Pauly and Harris 1998). There have been several evidences in light of this. First, although there are five copper binding sites in the N-terminal region of the protein (Brown, Qin et al. 1997), only two of them serve as highly affinity binders for copper (Jackson, Murray et al. 2001). Also the extra-cellular ligand of PrP binds to copper and stimulates endocytosis via clatherin-mediated pits, which is then imported into the endocytic compartment, and the copper ions dissociate from the PrP before they are again being recycled to the cell surface (Pauly and Harris 1998; Cheng, Lindqvist et al. 2006). In murine N2a neuroblastoma cells, copper ions have been reported to bind to PrP and stimulate endocytosis of PrP in a caveolin-dependent pathway (Alves, Munoz-Najar et al. 2010). Second, since cerebellar cells from $PrP^{-/-}$ mice contain only 20% copper present in cells from WT type animals, it suggests that PrP may be an important copper-binding protein in brain (Brown, Qin et al. 1997). Third, PrP knockout mice showed nearly 50% lower copper concentration in synaptosomal fractions than that in wild type mice (Herms, Tings et al. 1999). While this was associated with regulations of redox levels to

facilitate neural transmission (Herms, Tings et al. 1999), to the contrary Prnp knock-out mice showed defects in synaptic transmission suggesting a functional role of PrP in regulating copper concentration at synaptic regions. Fourth, the N-terminal domain of PrP (Brown, Qin et al. 1997; Pauly and Harris 1998; Cheng, Lindqvist et al. 2006) is known to play a role in maintaining the oxidative stress homeostasis by acting as a ligand for copper intake into the cells thereby up-regulating superoxide dismutase (SOD) activity (Zeng, Watt et al. 2003). The copper-zinc (Cu-Zn) SOD enzyme incorporated less radioactive copper and was enzymatically less active in PrP-/- mice while a transgenic mice overexpressing PrP showed increased uptake of copper and SOD response, suggesting that PrP may have an anti-oxidant role by facilitating the delivery of copper ions to SOD (Brown, Schulz-Schaeffer et al. 1997; Brown and Besinger 1998). Cells that lacked PrP were less viable and more susceptible to oxidative damage. Indeed, introducing PrP to PrP-/- cells rescued cells from undergoing apoptosis via caspase 3/9 pathway by up regulating SOD activity (Sakudo, Lee et al. 2003). Brain lysates from PrP-/- mice showed high levels of oxidative damage to proteins and lipids compared to wild type mice (Klamt, Dal-Pizzol et al. 2001); Wong, Liu et al. 2001). Prnp knock-out mice showed increased sensitivity to oxidative stress (Collinge, Whittington et al. 1994; Herms, Kretzchmar et al. 1995). PrP deficient primary neuronal cells are more susceptible to agents inducing oxidative stress, damaging agents such as hydrogen peroxide (H_2O_2) and this was associated with decrease glutathionine reductase activity (White, Collins et al. 1999). The reason for such phenomenon was explained as a result of decrease Cu/Zn superoxide dismutase-1 (SOD-1) activity (Brown, Schulz-Schaeffer et al. 1997). Moreover, PC12 cells selected for resistance to copper toxicity showed increased PrP expression suggesting that PrP may also be involved in

detoxification of copper (Brown, Schmidt et al. 1998). Even, *in vivo*, the N-terminal region of PrP conferred protection against copper neurotoxicity induced by intrahippocampal copper injection by reducing neuronal cell loss and astrogliosis (Chacon, Barria et al. 2003). All these studies suggest that when PrP is present on the cell surface, it acts as a receptor and functions either as a carrier protein for the delivery of copper ions to intra-cellular targets or it acts as a sink for chelation of extra-cellular copper ions (Pauly and Harris 1998).

There are several studies along with those indicated in the above paragraph that show an important role of PrP in protecting cells against reactive oxygen species (ROS)-mediated DNA damage (Watt, Routledge et al. 2007). For example, lack of PrP expression in cells resulted in increased sensitivity to oxidative stress (Collinge, Whittington et al. 1994; Herms, Kretzchmar et al. 1995; (Brown, Schulz-Schaeffer et al. 1997). Manganese induced mitochondrial dysfunction and ROS generation was significantly attenuated in PrP+/+ cells as compared to PrP-/- cells (Choi, Anantharam et al. 2007). However, it is still unclear how exactly PrP might protect cells from oxidative stress. One possibility is that PrP directly protects cells from ROS as PrP has been shown to portray a copper-dependent SOD activity (Brown, Schmidt et al. 1998; Brown, Clive et al. 2001). Another possibility is that PrP acts indirectly to protect cells from oxidative stress by upregulating Cu-Zn SOD enzyme, that detoxify ROS as mentioned in previous paragraph.

PrP is also associated with other proteins such as neuronal nitric oxide synthase (nNOS) in lipid rafts, which is involved in neuronal development, synaptic plasticity,

regeneration and regulation of transmitter release in wild type mice. nNOS was not associated with lipid rafts in cells lacking PrP (Keshet, Ovadia et al. 1999). However, how PrP influence endocytosis is still unclear.

PrP plays an important role in cell survival and cell death. The detection of Bcl-2 homology domain (BH2) like repeats in N-terminal region of PrP was shown to mediate Bcl-2 interaction with Bax protein and also responsible for protection against Bax-mediated apoptosis (Yin, Oltvai et al. 1994). Additionally, the yeast-two hybrid system demonstrated that the C-terminal portion of Bcl-2 protein, a suppressor of apoptosis interacts with the cellular prion protein (Kurschner and Morgan 1995). Neurons when cultured on Chinese hamster ovary cells transfected to express PrP showed increased neurite growth and neuronal survival as a result of activation of signalling pathways including p59Fyn kinase activity and PI3 kinase/Akt pathway along with up-regulation of Bcl-2 and Bax expression respectively (Chen, Mange et al. 2003). Now Bax-2 is a pro-apoptotic protein and is also a member of the Bcl-2 family. Studies from two groups demonstrated that injection of primary cultures of human neurons with a plasmid encoding Bax gene alone induced Bax-mediated apoptosis within 24 hours of expression whereas neuron co-injected with both Bax and PrP encoding plasmids protected the neurons from undergoing apoptosis (Bounhar, Zhang et al. 2001; Roucou, Guo et al. 2003). Conversely deletion of endogenous PrP with antisense RNA enhanced Bax-mediated apoptosis (Bounhar, Zhang et al. 2001). Moreover, the anti-apoptotic effect of PrP was specific for Bax as PrP did not prevent neuronal apoptosis induced by Bak (a Bcl-2 antagonist killer1)-, t-bid-, staurosporine- or thapsigargin- mediated cell death (Roucou, Giannopoulos et al. 2005), all of which are pro-apoptotic proteins. As PrP

interacts with Bcl-2 gene, it could be suggested that the anti-apoptotic effect of PrP is probably mediated by inhibiting interactions with pro-apoptotic proteins. In this context, Westergard L et al., (2007) has illustrated several possible mechanisms by which this might occur, based on known pathways for Bax-mediated cell death (Fig 1.8) (Danial and Korsmeyer 2004; van Delft and Huang 2006; Westergard, Christensen et al. 2007).

Bax activation involves a series of events including Bax conformational change, mitochondrial translocation, cytochrome c release, ultimately leading to cell death. A study by Roucou X et al., (2005) has confirmed that PrP only inhibits the event of conformational changes in human neurons and in breast carcinoma MCF-7 cells that occurs initially during Bax activation and renders protection against Bax-mediated apoptosis (Roucou and LeBlanc 2005). Moreover, in serum-deprived immortalised hippocampal PrP-null cells, Bax conformational change occurred more rapidly compared to PrP-expressing cells. These evidences are supportive of mechanisms demonstrated in the figures 1.8A and 1.8B. However, there are studies which have shown discrepancies in the mechanisms involving the role of cytosolic PrP in facilitating protection against Bax-mediated apoptosis. There are several studies which show that cytosolic PrP (CyPrP) produces a toxic effect rather than a protective effect to cells (Rambold, Miesbauer et al. 2006). CyPrP produces insoluble aggregates in Chinese hamster ovary cells, green monkey kidney COS-1 cells which are resistant to proteinase K and resemble to that of PrP^{Sc} (Ma and Lindquist 2001; Yedidia, Horonchik et al. 2001). A transgene expressing CyPrP in the cytosol that lacked N-terminal and C-terminal signal peptides, *in vivo*, induced an immense loss of granule cerebellar neurons and gliosis (Ma, Wollmann et al. 2002). To the contrary, in a study by Roucou X et al.,

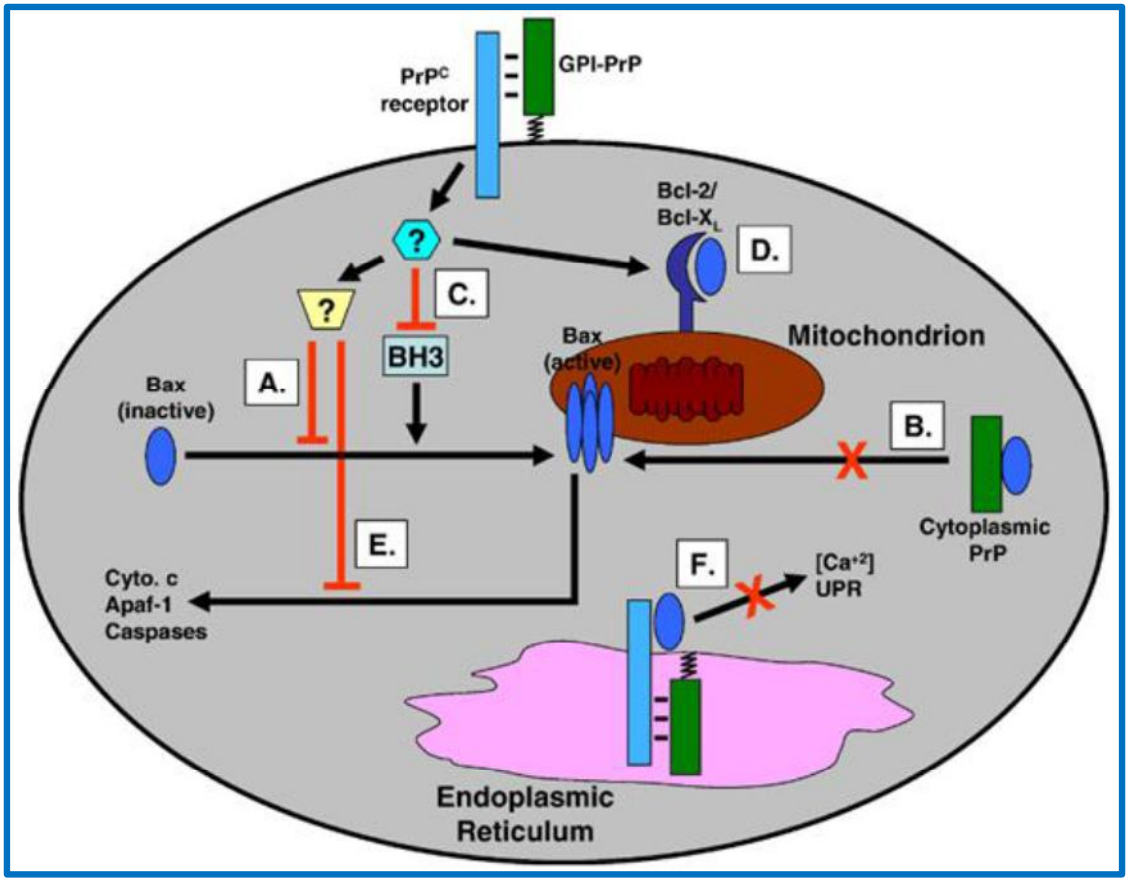


Fig 1.8: Possible mechanisms of PrP in inhibiting Bax-mediated apoptosis

(Adapted from (Westergard, Christensen et al. 2007)

PrP may inhibit Bax-mediated apoptotic pathways at several different points. When PrP is present on the cell surface (GPI-PrP), it may bind to a putative transmembrane receptor and trigger a signal transduction cascade that inhibits Bax mitochondrial translocation, conformational change, or oligomerization, all of which are involved in Bax activation (A). Cytoplasmic PrP may produce similar effects via a direct interaction with Bax (B). PrP may prevent pro-apoptotic, BH3-only proteins (C), or enhance an interaction between Bax and anti-apoptotic, multi-domain proteins such as Bcl-2 and Bcl-XL (D). PrP may suppress downstream events in the Bax activation pathway, such as cytochrome c (cyto. c) release, or activation of Apaf-1 and caspases (E). Finally, PrP in the endoplasmic reticulum may alter Bax function in this organelle, via effects on intracellular calcium and the unfolded protein response (UPR) (F).

(2003), it was shown that an engineered form of cytosolic PrP was not toxic to neuronal cells and protects those cells from undergoing Bax-mediated apoptosis (Roucou, Guo et al. 2003).Even Bounhar Y et al., (2001) reported similar results (Bounhar, Zhang et al. 2001). These studies suggest that PrP may interact directly with Bax, in a manner similar to Bcl-2 as it inhibits Bax-mediated apoptosis. Moreover, it has four identical N-terminal octapeptide repeats which share similarity with Bcl-2 homology domain 2. However, it is still unclear how Bax and PrP interact with each other. Yeast-two hybrid system could not confirm any direct interaction between Bax and PrP (Kurschner and Morgan 1995). Moreover, PrP is mostly present on the cell surface or in luminal vesicular sites and only a small portion is present in the cytosol both *in vitro* (Yedidia, Horonchik et al. 2001) and *in vivo* (Stewart and Harris 2003). Therefore more studies are required to elucidate exact mechanisms underlying the protective role of PrP against Bax-mediated apoptosis.

PrP has been shown to be associated with both intra-cellular and transmembrane signaling by their interactions with signaling molecules such as heat shock protein (Hsp) 60 (Edenhofer, Rieger et al. 1996), stress-inducible protein (Zanata, Lopes et al. 2002), Bcl-2 (Kurschner and Morgan 1995), growth factor receptor bound protein 2 (Grb2) and synapsin1b (Spielhaupter and Schatzl 2001). Antibody-mediated cross-linking of PrP on neuroectodermal cell line (IC11) stimulated P59Fyn signaling by interaction of PrP with lipid raft protein caveolin (Mouillet-Richard, Ermonval et al. 2000). PrP is also known to interact with stress-inducible protein-1 which us mediated by activating the mitogen activated protein kinase (MAPK) signaling pathway which is known to be involved in promoting neural development (Lopes, Hajj et al. 2005). When retinal explants from

neo-natal rats or mice were kept *in vitro* for 24hours and cell death was induced by anisomycin, a PrP-binding peptide activated the cyclic adenosine monophosphate /protein kinase A (cAMP/PKA) and extra-cellular signal related kinase (ERK) pathways and prevented cell death induced by anisomycin in explants from wild type rodents but not from PrP-null mice (Chiarini, Freitas et al. 2002). Therefore, it could be suggested that PrP might act as an unidentified molecule on the cell surface and or presenting it to the signaling receptor(s) might protect cells from apoptosis or sustain their long-term self-renewal.

The function of PrP in promoting cell cycle regulation in proliferating cells has been contradictory. The protein is abundantly expressed on the surface of lymphocytes and its expression *in vitro* promotes lymphocyte activation (Cashman, Loertscher et al. 1990). The PrP-gene is up-regulated in CD8+ cells when they undergo proliferation upon transferring to lymphopaenic mice (Liao, Zhang et al. 2007). PrP over-expressor (OE) mice have also shown to increase cellular proliferation *in vivo* in the adult neurogenic regions of mice compared to the wild type and PrP knock-out mice (Steele, Emsley et al. 2006). Also PrP promotes self-renewal in human HSC (Zhang, Steele et al. 2006). It is directly involved in tumor cell progression in many studies (Pan, Zhao et al. 2006), (McEwan, Windsor et al. 2009). A specific isoform PrP is highly expressed during G1 phase in human glioblastoma cell line (T98G) (Kikuchi, Kakeya et al. 2002). Although there are as many studies indicating association of PrP with cell proliferation, however, there are many studies which do not support this role of PrP. For example, in neo-plastic and viral-transformed cell lines PrP expression promotes growth arrest and terminal differentiation (Kniazeva, Orman et al. 1997; Gougoumas, Vizirianakis et al. 2001),

suggesting that PrP alone is not responsible for promoting cell cycle progression. In this context, neural tissue is of particular interest as the expression of PrP is differentially regulated in proliferating cells. The proliferating cells in the sub-ventricular zone (SVZ) did not express PrP, although in PrP over-expressor mice (Tg20), increasing PrP expression levels positively regulated stem cell proliferation in SVZ zone. To the contrary, in the hippocampus region of PrP knock-out mice, the hippocampal progenitors showed slower proliferation rates but no significant effect of PrP expression was observed in proliferation of hippocampal progenitors (Steele, Emsley et al. 2006). Moreover a study by Kim et al., (2005) showed higher proliferation rates in PrP deficient neural cell lined derived from embryonic hippocampus (Kim, Kim et al. 2005). The discrepancies in these studies suggest that PrP has a cell specific function in proliferating cells of neuronal tissue and it probably acts along with other 'factors' to modulate cell proliferation and differentiation. In absence of these 'factors' PrP inhibits cell proliferation. Indeed PrP has been shown to modulate dual role in cell proliferation and cell polarization in epithelial cells (Morel, Fouquet et al. 2008).

1.7.2 Cellular prion protein and its role in self-renewal of stem cells

Very recently a role in stem cell renewal has emerged in several stem cell types. Prion protein is described as an important marker for HSC (Zhang, Steele et al. 2006; Palmqvist, Pineault et al. 2007). Hox genes are implicated in HSC regulation as well as in leukemia development through translocation with the nucleoporin gene NUP98. In HSCs, a genetically engineered NUP98-HOX fusion gene known to promote HSC expansion ability and block *in vitro* differentiation and leukemia transformation, was able to induce the expression of several genes and especially Prnp gene between 2.1 to

2.3 times and was shown to be involved in cell development, cell proliferation and signal transduction (Palmqvist, Pineault et al. 2007). Also in HSCs, it has been shown that nearly 40% of adult HSC express PrP on the cell surface (Zhang, Steele et al. 2006). Zhang C C et al., (2006) showed that PrP is expressed on the cell surface of bone marrow cell populations that are enriched in long term haematopoietic stem cells. The study demonstrated that HSC from PrP-null bone marrow cells showed significantly less engraftment and impaired self-renewal ability following successive serial transplantations in lethally irradiated mouse recipients. However, retroviral infections of PrP-null bone marrow cells with a vector expressing PrP protein rescued the impaired self-renewal ability of HSC and thus reconstituted the haematopoietic system (Zhang, Steele et al. 2006). This indicated that the self-renewal ability of HSC has been compromised in absence of PrP suggesting that PrP is not only present in HSC but has a functional role in the self-renewal of HSC.

In mammary gland, a population of freshly isolated mammary epithelial cells was fractionated into PrP^{-} (92.5% of total cells), PrP^{med} (5.1%of total cells), PrP^{high} (1.8% of total cells) based of PrP staining. CD49f was used as a marker for *in vivo* repopulating mammary gland epithelial stem cells. Freshly isolated mammary epithelial cells in which sphere initiating cells are enriched in the $CD49f^{+}CD24^{+}PrP^{med}$ fraction were capable of generating nearly 6.1 mammospheres for every 10,000 cells. To the contrary in *in vitro* culture of organoids PrP expression was significantly reduced (Liao, Zhang et al. 2007). This study suggested that PrP may have a role in proliferation of these freshly isolated primitive cells.

In neuronal stem cell (NSC), PrP increases cellular proliferation *in vivo*. After incorporating pulse labels of thymidine analog, BrdUrd, cellular proliferation in adult neurogenic regions (sub-ventricular and hippocampus) of PrP KO, WT and PrP OE mice was assessed. Cellular proliferations significantly increased in PrP-OE mice compared to PrP-KO and WT mice (Steele, Emsley et al. 2006).

Very recently, the role of PrP in human ESC (hESC) in promoting proliferation and deciding the fate of ESC differentiation was studied (Lee and Baskakov 2010; Lee and Baskakov 2013). In hESCs where PrP expression was knocked down and subjected to undergo spontaneous differentiation for 14 days in presence of tetracycline, it was observed that at the end of day 14, markers of ectodermal lineage including growth-associated protein 43, tyrosine hydroxylase and synaptophysin was supressed while markers of endodermal and mesodermal lineage remained unaffected compared to untransfected hESCs and hESCs transfected with a scramble vector. However, in hESCs where PrP was overexpressed the expression of markers of all three germ layers was remarkably down regulated, suggesting that PrP is involved in regulating hESCs differentiation fate and that optimal levels of PrP expression is required for such differentiation.

It is unknown the precise mechanism by which way PrP^{C} promotes self-renewal in stem cells and whether PrP has a role in promoting proliferation and differentiation ability in MSC. MSCs, when cultured *in vitro* have known to decrease their proliferation and differentiation potential along with time. *In vitro* expansion of MSC without loss of self-

renewal is necessary for any clinical application. As prion protein plays an important role in self-renewal of stem cells.

In this project, I will test the hypothesis that:

"PrP is required for the proliferation of hMSC and that a small molecule modulator of PrP can be used to enhance their proliferation and extend their life span in culture, while retaining their ability to differentiate and engraft to bone, thereby delaying the process of replicative ageing following in vitro expansion."

1.8 Aims and Objectives of the project

To test the hypothesis, I will determine whether:

1. PrP expression is decreased in hMSC with time in culture and whether this co-relates with loss of their proliferation and differentiation ability.
2. The expansion ability of hMSC is compromised when the expression of PrP is knocked down.
3. There is a small molecule that can be used to preserve PrP expression with cellular ageing in hMSC and delay hMSC ageing.

CHAPTER-2

2.1 Isolation of MSC from human bone marrow

Human BM was collected either from the iliac crest of patients undergoing osteotomy for reasons other than metabolic disorders, or patients undergoing diagnostic BM, which were subsequently reported normal. The BM was obtained following informed written and/or parental consent in accordance with the protocol approved by the South Sheffield local research ethical committee. The human BM was collected in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (FBS, Hyclone, Thermo Scientific, Northumberland, UK**)** and 0.01% of penicillin/streptomycin (Sigma, Dorset, UK), and 0.1% heparin (Royal Hallamshire Hospital Pharmacy). To obtain mononuclear cells (MNC), BM samples were mixed with equal amount of phosphate buffer saline (PBS, Gibco) and overlaid on Lymphozyten separating medium (density 1.077 g/L; PAA laboratories, Pasching, Austria). The BM was centrifuged at 800 gravitational acceleration (g) for 20 minutes without the application of brakes. The MNC fraction was then washed twice with PBS and the cells were counted in 5% acetic acid using a haemocytometer and were plated at 8000 MNC/cm^2 in MSC medium containing DMEM and 10% FBS (Hyclone). The cells were incubated at 37ºC in 5% carbon dioxide (CO_2) in air. After 48 hours (hr) the non-adherent cells were removed and medium was changed twice weekly till cells were confluent. A list of BM samples used in the project is enlisted in table.2.1.

Donor used	Age	Gender
snbm-17	9yrs 2 months	N/A
snbm-24	14 yrs 7 months	Female
snbm-26	15 yrs 7 months	Male
snbm-28	32 yrs 5 months	Male
snbm-61	16 yrs 1 month	Male
snbm-62	9 yrs 11 months	Male
snbm-70	9 yrs 5 months	Male
snbm-78	54 yrs 10 months	Male
snbm-80	70 yrs 5 months	N/A
snbm-82	80 yrs	Female
snbm-83	74 yrs 2 months	Male
snbm-86	73 yrs 10 months	Female
snbm-99	10 yrs 3 months	Male
snbm-101	65 yrs 9 months	Male
snbm-102	13 yrs 6 months	Male

Table 2.1: Bone marrow samples used in this study

2.2 Culture of hMSC

Human MSCs were cultured in MSC medium and when they reached confluence, the media was removed from the flask and cells were washed once with PBS. The cells were detached by addition of trypsin- ethylenediamine tetra-acetic acid (0.5% trypsin, 1mM EDTA; Gibco, Paisley, UK) and were incubated at 37^{o}C in 5% CO_2 in air for 2 minutes. Cells were then harvested using MSC medium and centrifuged at 800g for 5 minutes at room temperature. Cells were re-suspended in 1ml of MSC medium. Live cells were counted using the trypan blue exclusion method.

For assessment of growth kinetics 1000MSCs/cm^2 were plated and incubated at 37^{o}C in 5% CO_2 in air. This constituted 'one passage' (p1). The number of population doubling was then calculated using the equation:

Number of population doublings = Log N/Log2

where N = the number of cells at confluence / the number of cells seeded at the start of the culture.

The rest of the MSCs were re-plated in MSC medium in a larger flask and incubated at 37^{o}C in 5% CO_2 in air to obtain sufficient number of MSCs to be used for further experiments.

2.3Freezing and defrosting of hMSC

When storage of MSC cells were required, cells were detached by trypsin-EDTA as described in section 2.2 and cells were re-suspended in freezing medium containing 90% fetal calf serum (FCS) and 10% dimethylsulphoxide (DMSO) and quickly were transferred to –80^{o}C for overnight in cryo-freezing container and the day after transferred to liquid nitrogen.

When required, cells were defrosted in a 37^{o}C water bath for few minutes, after which they were transferred into a tube containing cold MSC medium. The mixture was centrifuged at 800g for 5 minutes at room temperature. The cell pellet was re-suspended in MSC medium and counted with 0.4% trypan blue using a haemocytometer and cells were seeded at 8000 cells/cm^{2} incubated in a flask at 37^{o}C in 5% carbon dioxide in air. The cells were subsequently fed twice every week.

2.4 Immuno-staining of hMSC and Fluorescent activated cell sorting (FACS)

Human MSCs (10^{5}) were incubated in 100 µl of PBS plus 5% FCS (Gibco) with the recommended amount of antibody or its respective isotype matched control for 30 minutes on ice (Table 2.2). The cells were then washed twice with 4mls of PBS plus 5% FCS and spun at 800g for 5 minutes at 4^{o}C. The cells were fixed with 500 µl PBS containing 2% paraformaldehyde and 2% FCS and stored at 4^{o}C until FACS analysis was performed. Cells were acquired using FACS calibre (Becton Dickinson, Oxford,

Antigen	Fluorescence	Isotype	Quantity	Company
CD45	Allophycocyanin	Allophycocyanin conjugated Immunoglobulin G1 (IgG1)	1 μg/10^6 cells	Caltag, UK
CD29	R-phycoerythrin	R-phycoerythrin conjugated IgG1	1 μg/10^6 cells	Caltag, UK
CD31	Fluorescein-isothiocyanate (FITC) conjugated	FITC conjugated IgG1	2 μg/10^6 cells	BD Pharmingen
CD34	R-phycoerythrin	R-phycoerythrin conjugated IgG1	1 μg/10^6 cells	Caltag, UK
CD105	Non-conjugated monoclonal antibody (mAb) raised against hMSC (produced in house-100 μl hybridoma supernatant/10^6 cells) plus FITC conjugated goat anti mouse IgG1	Secondary antibody only	1 μg/10^6 cells	Primary Antibody- BD Pharmingen Secondary antibody- Sigma Aldrich, UK

Table 2.2: Antibodies used for the characterisation of MSC

CD-cluster differentiation

UK) and analysed using Cell Quest software.

2.5 Clonogenic Assay - Colony forming unit fibroblast assay (CFU-F)

The CFU-F assay was carried out on hMSCs from established cultures to determine the number of mesenchymal progenitors present in the culture. Cultured hMSCs were plated in 6 well culture plates at the densities of 100 and 200 cells per well (10.5cells/cm^2 and 21.05cells/cm^2) in duplicates in MSC medium. The plates were incubated for 14 days at 37^{o}C in 5% CO_2 in air. At day 14, the cultures were stained by using Wright's Giemsa stain (VWR International, Leicestershire, UK). Briefly, the medium was removed from the culture plates and the plates were washed with non-sterile PBS to remove residual FBS. The plates were then air-dried and the cells were then fixed with methanol (Fisher Scientific, Loughborough, UK) for 5 minutes. The cells were thereafter stained with Wright's Giemsa stain solution for 5 minutes. The plates were then washed under running tap water to wash away any residual stain. The stained colonies appear purple in colour. After the plates have dried completely, the stained purple colored colonies were counted under inverted light microscope. Those colonies comprising of at least 50 cells with a definite centre of origin were considered as CFU-F.

2.6 Clonogenic Assay - Colony forming unit osteoblast assay (CFU-O)

The number of clonogenic progenitors with ostoegenic potential was obtained by plating hMSCs in 6 well culture plates at the densities of 100 and 200 cells per well

(10.5cells/cm^2 and 21.05cells/cm^2) from established hMSC cultures in duplicates in MSC medium supplemented with osteogenic supplements composed of 0.05 mM L-Ascorbic Acid (Sigma Aldrich, St. Louis, USA), 10mM β glycerophosphate (Sigma Aldrich, St. Louis, USA) and 100nM dexamethasone (Sigma Aldrich, St. Louis, USA). Cells were maintained for 14 days at 37^oC in 5% CO_2 in air and fed twice every week. At day 14 colonies were stained for alkaline phosphatase (ALP) enzymatic activity using 86R alkaline phosphatase kit (Sigma Aldrich) according to the manual instructions. Briefly, the medium was removed and the plates were washed twice with PBS. The cells in each plate were then fixed with 500µl of citrate-acetone formaldehyde fixative solution prepared by addition of 2.5ml citrate solution, 6.5ml acetone and 0.8ml of 37% formaldehyde for 30 seconds. To remove any residual fixative the plates were rinsed gently with deionised water. The plates were then incubated with 500µl of alkaline dye mix prepared by addition of dizonium salt (prepared by mixing 100µl of sodium nitrate solution with 100µl of FRV alkaline solution) to 4.5 ml of de-ionized water followed by 100µl of Naphthol AS BI solution; incubated in dark for 15 minutes. The wells were again rinsed with deionised water for 2 minutes, after which 500µl of the haematoxylin (Sigma Aldrich, St. Louis, USA) stain was added for 2 minutes. The plates were finally washed under running tap water to remove any residual stain and were observed under the microscope for the presence of cells expressing alkaline phosphatase, which would appear dark pink in colour. The colonies comprising of at least 40 cells with a definite centre of origin was considered as one CFU-O.

2.7 Clonogenic Assay - Colony forming unit adipocyte assay (CFU-A)

The number of clonogenic progenitors with adipogenic potential was obtained by plating hMSCs in 96 well plates at limiting dilutions (ranging from 10^5 to 6.25 x 10^3 cells, 8 wells/ dilution) in 100ul/well MSC medium and cultured at 37^{o}C in 5% CO_2 in air. After 2 days in culture, the cells were induced with additional 100µl/well MSC medium with adipogenic supplements comprising of 100nM dexamethasone (Sigma Aldrich) and 1 µg/ml 3-isobutyl-1-methylxanthine (IBMX, Sigma Aldrich). The cells were maintained in the adipogenic differentiation medium for two weeks and were fed twice a week. After two weeks exposure to adipogenic induction medium, the cells were stained for Oil red O to detect lipid vacuoles. Briefly, the Oil red O staining involved removal of MSC medium from the wells. The cells were washed with PBS to remove any traces of serum (FBS) and fixed with 10% formaldehyde for an hour. After an hour of incubation, the plates were washed three times with deionised water to remove any excess formaldehyde and incubated with 0.18% Oil Red O solution for 10 minutes, after which the plates were again rinsed with deionised water to remove any excess stain. Cells were finally counterstained with of Haematoxylin solution for 2 minutes and excess stain was washed with deionised water. The plates were viewed under the microscope for the presence of cells containing lipid vacuoles, which appeared as red stained droplets.

The number of positive wells containing lipid vacuole in each dilution was scored. A well was considered to be positive if it contained more than 20 cells containing red lipid vacuoles. The percentage of negative well was then scored at different dilutions of cells.

This data was then used to calculate the number of CFU-A following Poisson distribution (Wu, Liu et al. 2005) and using the formula:

$F_o = e^{-x}$

where F_o = fraction of colony-negative wells at a certain dilution

e = constant whose value is 2.71

x = number of colony forming units per well

2.8 Differentiation Assays

Osteogenic differentiation of hMSC was achieved by plating 1.2 x 10^3 hMSCs/cm^2 in MSC medium supplemented with ostoegenic supplements (0.05 mM L-Ascorbic Acid, 10mM β glycerophosphate and 100nM dexamethasone). Adipogenic differentiation of hMSC was achieved by plating 2.8 x 10^3 hMSCs/cm^2 in MSC medium supplemented with adipogenic supplements (100nM dexamethasone and 1 µg/ml IBMX). Cells were maintained at 37^{o}C in 5% CO_2 in air and fed twice a week. After 2 weeks, total RNA was extracted (see section 2.8.1) and protein lysate (see section 2.9) was collected to perform polymerase chain reaction (see section 2.8.3) and western blotting (see section 2.9) respectively.

2.8.1 Total RNA extraction

Total RNA was extracted using RNAqueous 4PCR kit (Ambion, Warrington, UK) according to manufacturer's instructions. Briefly, 500ul of lysis solution was added to a

cell density of 10^7 hMSCs and vortexed vigorously. Equal volume of 64% ethanol was added to the lysate and vortexed carefully following which the lysate/ethanol mix was poured through a filter cartridge by centrifugation. The filter cartridge was washed once with wash solution #1 and twice with wash solution #2. The RNA was finally eluted from the cartridge by centrifugation in a microfuge at 13,000 rotations per minute (rpm) into a fresh collection tube by adding 60ul pre-heated elution solution. To completely remove traces of DNA, the extracted RNA was treated with DNase 1 and incubated at 37^oC for 30 minutes. The RNA was then treated with DNase inactivation reagent and mixed thoroughly. Then the mixture was centrifuged for a minute to pellet the DNase inactivation reagent and RNA was transferred to a new tube.

The concentration and purity of total RNA obtained was quantified by measuring its absorbance (A) at 260nm and 280nm using a Nano-drop 2000c UV-Vis spectrophotometer (Thermo Scientific, Northumberland, UK). An A_{260} of 1 is equivalent to 40ugRNA/ml. The concentration of RNA was therefore calculated using the equation:

Concentration of RNA = A_{260} x Dilution factor x Extinction co-efficient

where A_{260} = Absorbance at 260nm

Extinction co-efficient = 40 (1 A_{260} = 40ug RNA/ml)

The ratio of A_{260} to A_{280} values was used a measure of RNA purity and the sample was considered of good quality when the ratio was in the range of 1.8 to 2.1.

2.8.2 cDNA preparation

Total RNA was used for reverse transcription using the 1st Strand complementary DNA (cDNA) kit (GE Healthcare, Amersham, UK). Briefly, 2ug of RNA sample was mixed with RNAse-free water to make a final volume of 20ul and heated at 65^{o}C for 10 minutes and then chilled on ice. To the heat-denatured RNA, 11ul of bulk first strand cDNA reaction mix, 1ul dithiothreitol (DTT) solution and 1ul Not I-d (T) 18 primer (1 in 25 dilution in RNAse-free water) was added and mixed thoroughly by pipetting. The mixture was incubated at 37^{o}C for 1 hour. The cDNA was stored at -20^{o}C.

2.8.3 Primer design and real time quantitative PCR

The Taqman primer design system (Primer express, Applied Biosystems) was used to obtain primer sequence in the last 500 base pairs from the polyA tail at the 3` end of the gene. All sequences were checked using blast programmes in NCBI for potential aspecific binding (Genbank-NCBI BLAST, Nucleotide BLAST) and were found to be unique to the gene of interest in humans. The list of primer sequences tested in this project is enlisted in table 2.3. All primers were ordered from Invitrogen (Paisley, UK) and were stored as a concentrated stock of 1mM in DNase RNase free water (Gibco) at -20^{o}C and used at a concentration of 1µM.

For the standard curve known concentrations (50ng, 5ng, 0.5ng and 0.05ng) of human genomic DNA (Promega) were serially diluted and stored in small aliquots allowing only one freeze/thaw cycle per aliquot. Real time- quantitative polymerase chain reaction (RT-qPCR) was performed in triplicates in a 10ul volume containing 2ul

Primers	Sequence
L-32 forward	5'-GGGAGAGACACCGTCTGAACA-3'
L-32 reverse	5'-GAACCACGATGGTCGCTTTC-3'
SOD-2 forward	5’-GGAGTTGCTGGAAGCCATCA-3'
SOD-2 reverse	5’-CAGCCGTCAGCTTCTCCTTAA-3'
LPL forward	5’-TTGTGAAATGCCATGACAAGTCT-3'
LPL reverse	5’-CATGCCGTTCTTTGTTCTGTAGA-3'
RUNX2 forward	5’-CACTATCCAGCCACCTTTACTTACAC-3'
RUNX2 reverse	5’-TAGTGAGTGGTGGCGGACATAC-3'
Osteopontin forward	5’-AATTGCAGTGATTTGCTTTTGC-3'
Osteopontin reverse	5’-GAACTTCCAGAATCAGCCTGTTTAA-3'
Osteocalcin forward	5’-CAATCCGGACTGTGACGAGTT-3'
Osteocalcin reverse	5’-CCTAGACCGGGCCGTAGAAG-3'
PPAR-γ forward	5’-TGGGTGAAACTCTGGGAGATT-3'
PPAR-γ reverse	5’-TTTCTTGTGATATGTTTGCAGACAGT-3'
ALP forward	5’-CCCGTGGCAACTCTATCTTTG-3'
ALP reverse	5’-GCCATACAGGATGGCAGTGA-3'

Table 2.3: Sequence of primers used in real time quantitative PCR

template DNA, 1ul each of forward and reverse primer at 1 micro molar concentration, 5ul SYBR GREEN 2x qPCR Mix (Eurogentec, Belgium) and 1ul distilled water. PCR amplification was performed under the reaction conditions: 1 cycle of 50ºC for 2 min, 95ºC for 10 min; 40 cycles of denaturation at 95ºC for 15 seconds and annealing at 60ºC60ºC for 1 min. This was followed by a dissociation stage where the conditions were 95ºC for 15 seconds, 60ºC for 15 seconds and 95ºC for 15 seconds to generate a melting curve for verification of specificity of amplification product. All this was carried out using 7900HT Real-Time-PCR System (Applied Biosystems) in 384-well PCR-plates (GBO). Every qPCR experiment was conducted 3 times. Analysis was performed using SDS 2.0 software and threshold was set to lie in the middle of logarithm phase and baseline was kept at the default setting of 3-15 cycles. Amounts were calculated using the guidelines under ABI prism 7700 Sequence Detection System protocol using the standard curve method. Logarithm of the average of the cycle threshold (C_T) value was calculated using the slope and intercept of the equation generated by the standard curve. An anti-log was then performed to determine the input amount. The relative expression quantities of all the genes were normalized to housekeeping gene L32 to plot the graphs.

2.9 Protein expression by Western Blotting

2.9.1 Preparation of protein lysates

To obtain protein lysates, MSC were trypsinised (section 2.2) and 3 x 10^6 cells were washed with PBS and spun at 2000 rotation per minute (rpm) for 3 minutes in microfuge. The cell pellet was re-suspended in 500 µl of mammalian cell lysis buffer (Mammalian cell lysis kit, Sigma; containing 250mM Tris- 5mM EDTA, 750mM

sodium chloride, 0.5% sodium dodecyl sulphate, 2.5% deoxycholic acid and 5% Igepal supplemented with 10µl of Proteinase inhibitors cocktail (Sigma-Aldrich, Dorset, UK) containing 4-(2-aminoethyl) benzenesulfonyl fluoride, pepstatin A, bestatin, leupeptin, aprotinin and trans-epoxysuccinyl-L-leucyl-amido(4-guanidino)-butane). The lysate was incubated for 15 minutes on a rotating shaker at 4^{o}C and then spun at 12,000 rpm for 10 minutes. The supernatant was collected and stored at -20^{o}C for further use.

2.9.2 Protein assay by Bradford protein assay

To obtain a standard curve, different concentrations of bovine serum albumin (BSA) (Invitrogen Ltd, Paisley, UK) from 0 to 10µg/ml in mammalian cell lysis buffer (Sigma) were prepared in duplicates in a 96-well plate. Different dilutions of the protein lysate (1:1, 1:2 and 1:5 in mammalian cell lysis buffer in 10µl) were measured in triplicates and then 200µl of a solution containing 1 part copper (II) sulphate in 50 parts of bicinchonic acid (BCA) was added. The plate was incubated at room temperature for 30 minutes and the absorption was read at 562nm by spectrophotometer (Revelation software, Version 4.25).

2.9.3 Protein separation by SDS-PAGE

Protein lysate from each sample (20µg) was diluted in equal volume of 2X laemmli buffer (Gibco). The samples were heated at 95^{o}C for 5 minutes, after which they were quickly transferred on ice and loaded on a 12% Tris-glycine gel. The gel was casted on mini protean 3 cell equipment (Biorad Laboratories, Hempstead, UK) (Table 2.4). Briefly, separating gel was loaded in gel casting stand and allowed to set for 20-30

Separating gel:

REAGENTS	12%
Distilled water	3.35 ml
1.5M Tris-hydrochloric acid (HCL); pH-8.8	2.5 ml
10% sodium dodecyl sulphate (SDS)	100 µl
30% Acrylamide	4 ml
10% Ammonium persulphate (APS)	50 µl
N,N,N'N' Tetra-methylethane-1,2-diamine (TEMED)	5 µl

Stacking gel:

REAGENTS	12%
Distilled water	6.1 ml
0.5M Tris-HCL; pH-6.8	2.5 ml
10% SDS	100 µl
30% Acrylamide	1.3 ml
10% APS	100 µl
TEMED	10 µl

Table 2.4: Reagents for 12% Tris-glycine gel

minutes followed by the addition of stacking gel. A 10 well comb was inserted to prepare wells for loading the samples. The gel was allowed to set at room temperature for 10 minutes and samples were loaded and run at 150V for 60 minutes.

2.9.4 Electro-blotting

Electro-blotting was carried out by wet transfer using nitro-cellulose membrane (Amersham Biosciences, UK). The membrane and gel were inserted between 4 sponges, 3 pieces of 3mm papers (Whatman Schleicher and Schuell, UK) and soaked in transfer buffer (0.25M Tris, 150mM Glycine and 10% methanol; pH 8.3). The membrane was blotted at 70V for 70 minutes.

2.9.5 Antibody detection

After electro-blotting, blocking of the membrane was carried out with 5% BSA in PBS-0.1% Tween 20 (PBS-T; BDH, Poole, UK) for detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 5% Casein (Vector Laboratories, California, USA) in PBS-T for detection of PrP. The membrane was incubated on a shaker at room temperature for 1.5 hour. The anti-prion protein primary monoclonal antibody, SAF32 (Spibio, Massy, France) was diluted in 5% casein/PBS-T at 1:400 and mouse anti-human GAPDH antibody (Abcam, Cambridge) was diluted in 5% BSA/PBS-T at 1:1000. Staining with primary antibody was carried out overnight in blocking solution at 4^{o}C on shaker. The following day, the membrane was washed 5 times for 5 minutes with PBS-T. The membrane was incubated with goat anti-mouse horseradish peroxidase (HRP) conjugated secondary antibody (DAKO, immunoglobulin A/S, Copenhagen,

Denmark) at 1:3000 in the respective blocker solutions for 30 minutes at room temperature on a shaker. The membranes were washed 3 times with PBS-T for 15 minutes each wash. Detection was carried out using enhanced chemiluminescence plus ECL reaction kit (Amersham Bioscience, UK). The membranes were then exposed to ECL hyper film (Amersham Bioscience, UK) for 2 –7 minutes for PrP antibody and 2 minutes for GAPDH antibody. The band size was obtained by linear regression analysis based on the distance migrated by the bands and co-related to the logarithmic molecular weight of the size marker.

2.10 Production of lentiviral particles

2.10.1 Plasmids

Initially lentiviral particles were generated by co-transfection of human embryonic kidney (HEK) 293t cells with in-house available plasmids pCAG_kGP3R (Fig 2.1), pCAG4RTR2 (Fig 2.2), pCAGVSVG (Fig 2.3) and control vector plasmid pCL10.1 MSCV ires GFP (Fig 2.4, kindly donated by Clive Buckle). Lentiviral particles were also generated by co-transfection of human embryonic kidney (HEK) 293t cells with the envelope plasmid pMD.G2 (Fig 2.5) and packaging plasmid pCMVΔR8.91 (Fig 2.6), (produced by L.Naldini and kindly donated by A Thrasher, appendix-I) and plasmid containing the vector of interest as previously described (Thermofisher, Epsom, UK-17). The vector construct p'HRsincpptSEW (Fig 2.7, kindly donated by A Thrasher, appendix-I) was used to express the enhanced green fluorescent protein (eGFP) to label hMSCs for transplantation experiments. A set of 4 pGPIZ shRNAmir (Fig 2.8) each containing a short hairpin sequence to specifically knock down expression of PrP

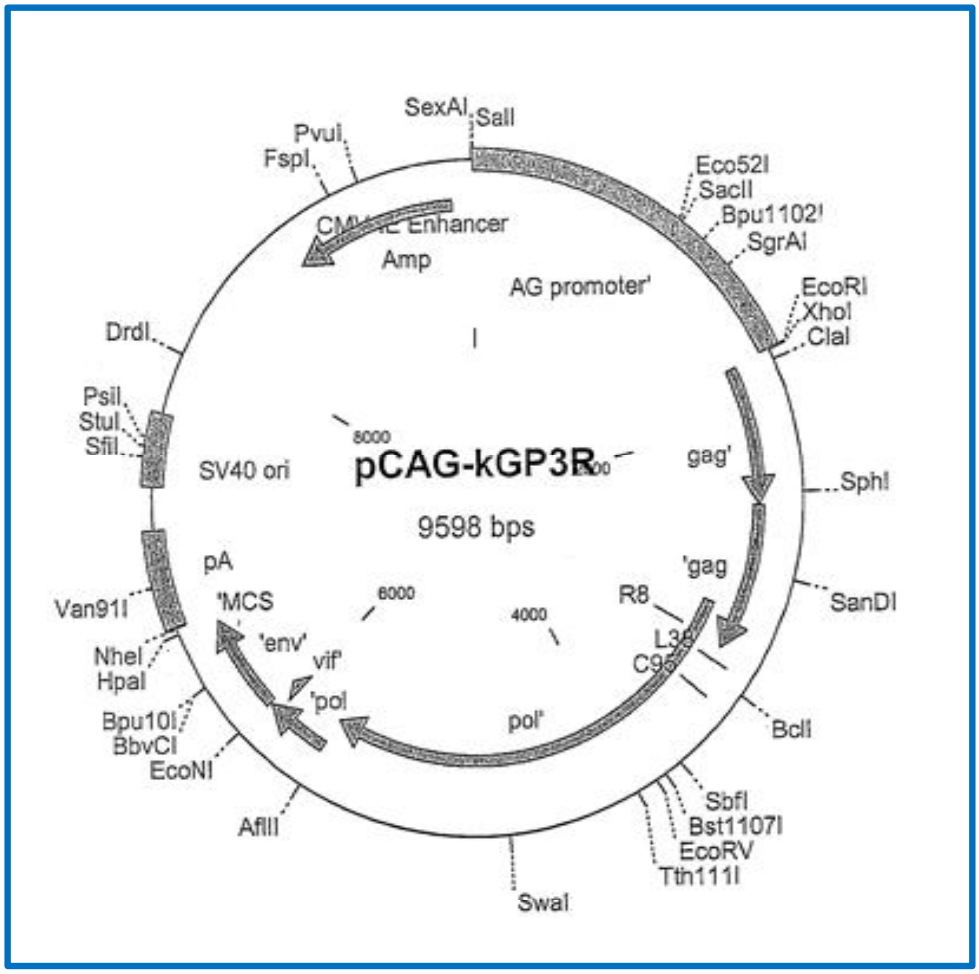


Fig 2.1: Vector construct pCAG-kGP3R

pCAG-kGP3R plasmid contains the structural genes required for replication of the virus.

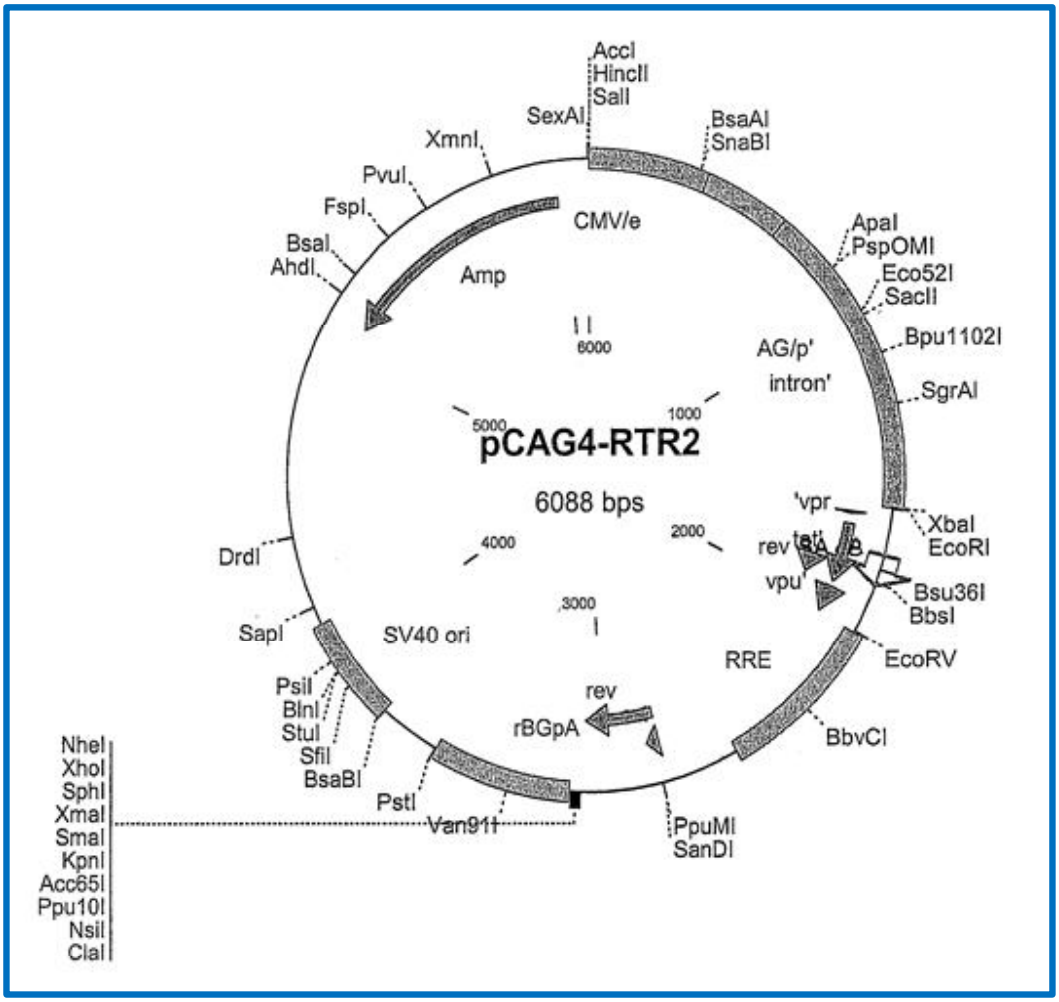


Fig 2.2: Vector construct pCAG-RTR2

pCAG-RTR2 plasmid is the helper plasmid that helps in the transcription and translation of structural genes responsible for the assembly of a new virus within the packaging cell.

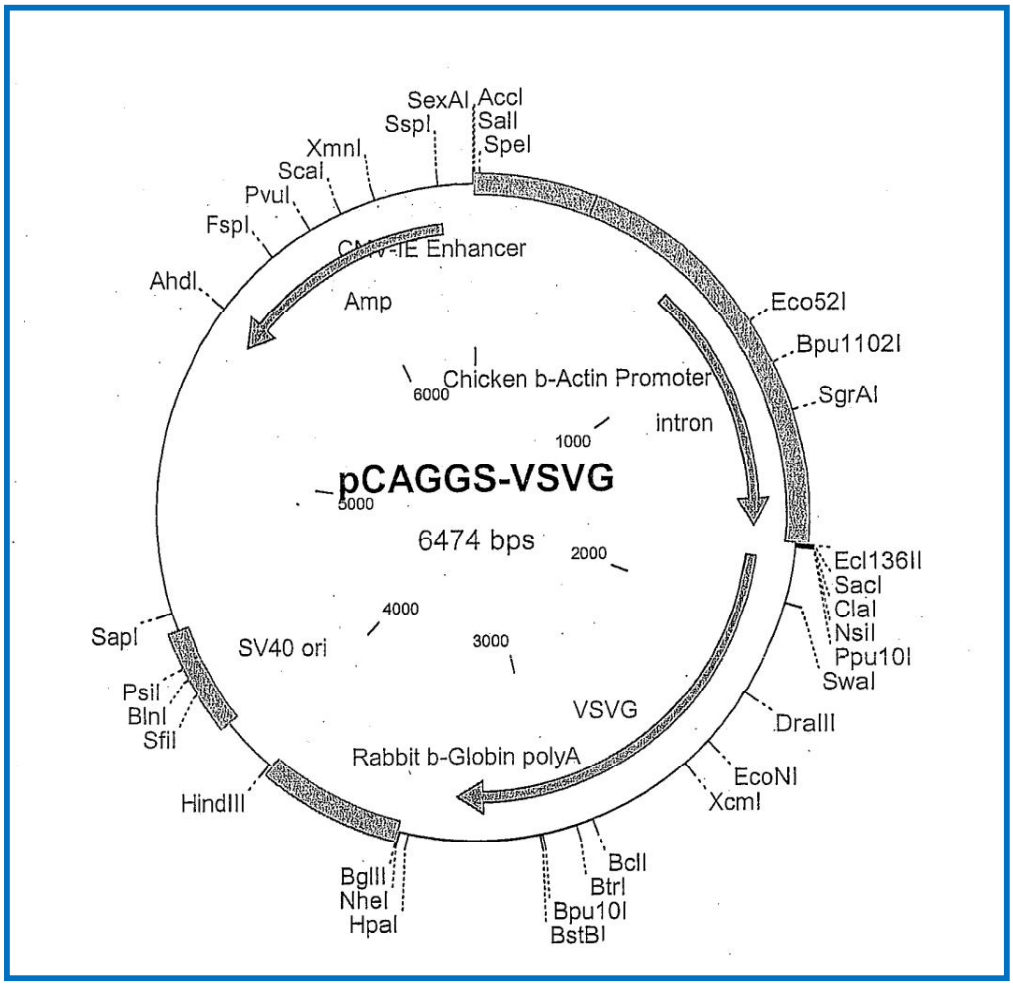


Fig 2.3: Vector construct pCAG.VSVG

pCAG.VSVG plasmid codes for the viral capsid.

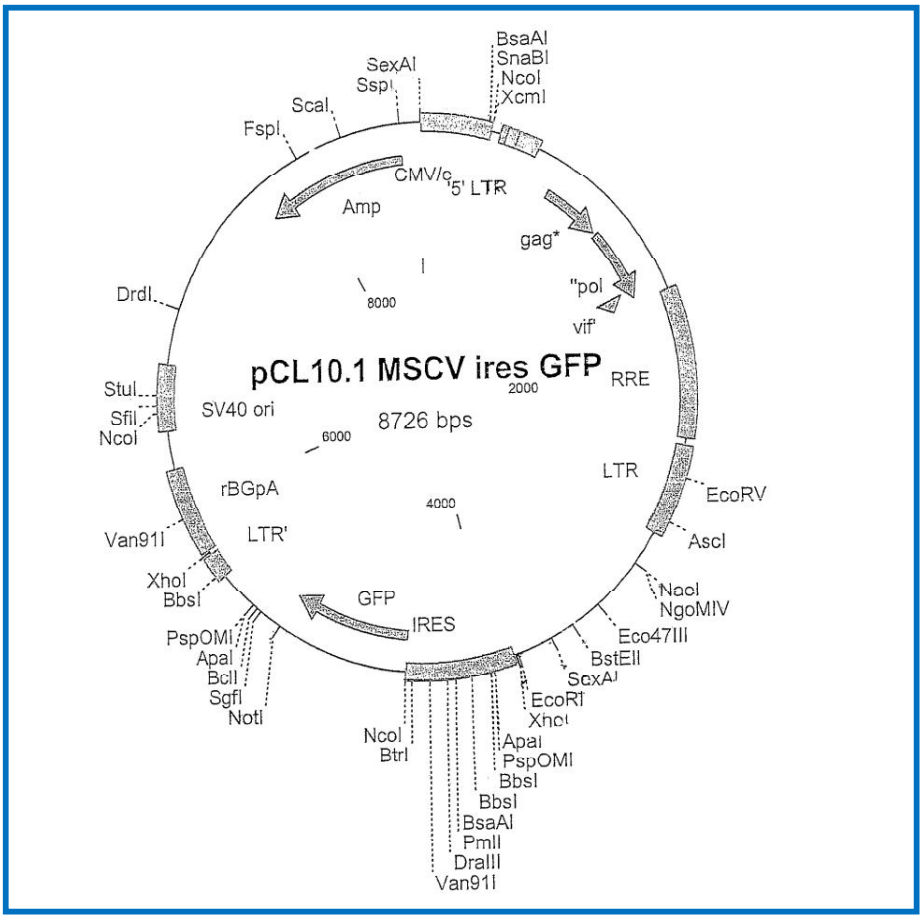


Fig 2.4: Vector construct pCL-10.1MSCV ires GFP

pCL-10.1MSCV ires GFP plasmid codes for the expression of green fluorescent protein.

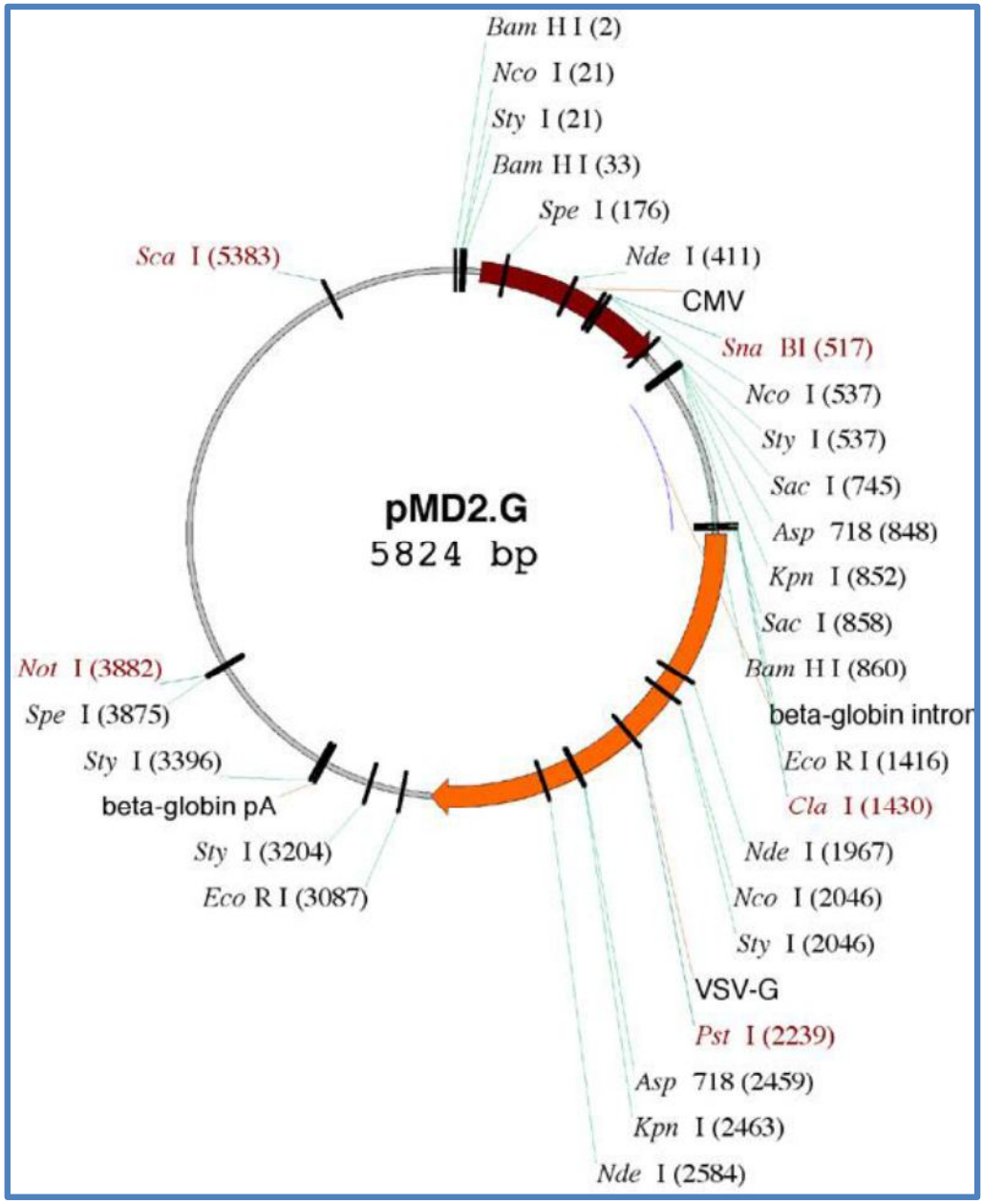


Fig 2.5: Vector construct pMD2.VSVG

pMD2.VSVG plasmid codes for the viral capsid (image courtesy: http://www.addgene.org/12259/).

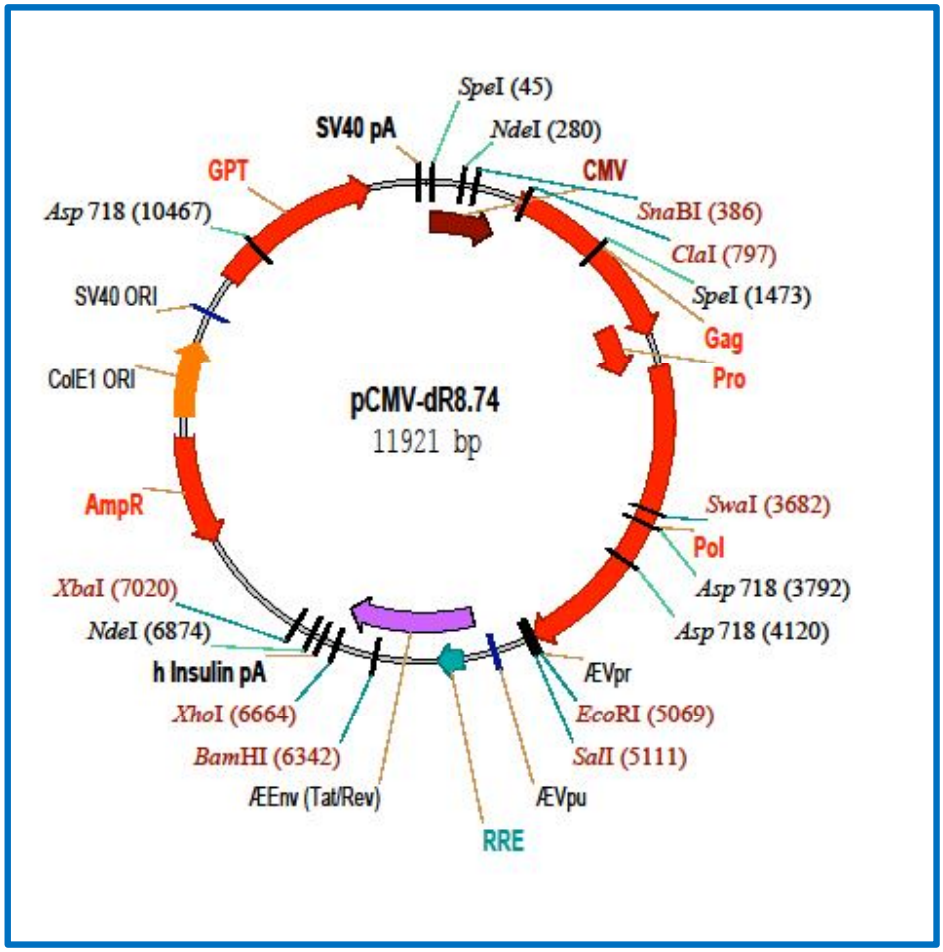


Fig 2.6: Vector construct pCMV-dR8.74

pCMV-dR8.74 contains all the important packaging components Gag, Pol, Rev and Tat in one plasmid that helps in the transcription and packaging of RNA copy of the expression vector into the recombinant pseudo-viral particles.

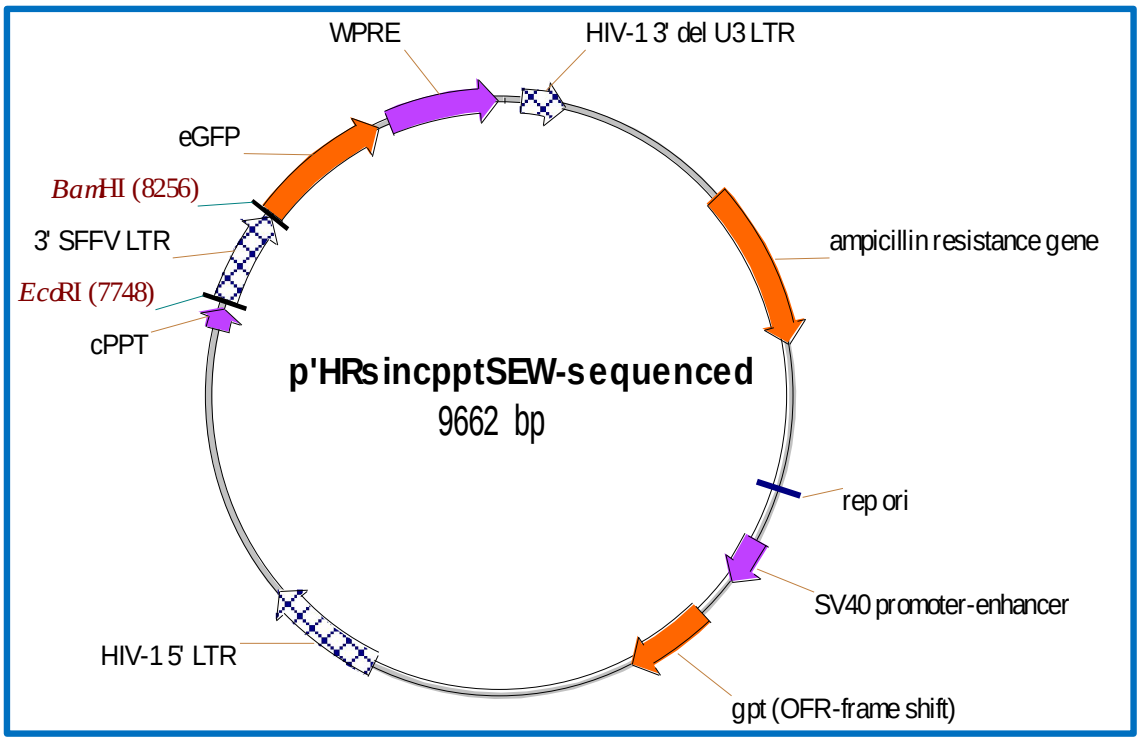


Fig 2.7: Vector construct p'HRsincpptSEW

p'HRsincpptSEW plasmid codes for the expression of enhanced green fluorescent protein.

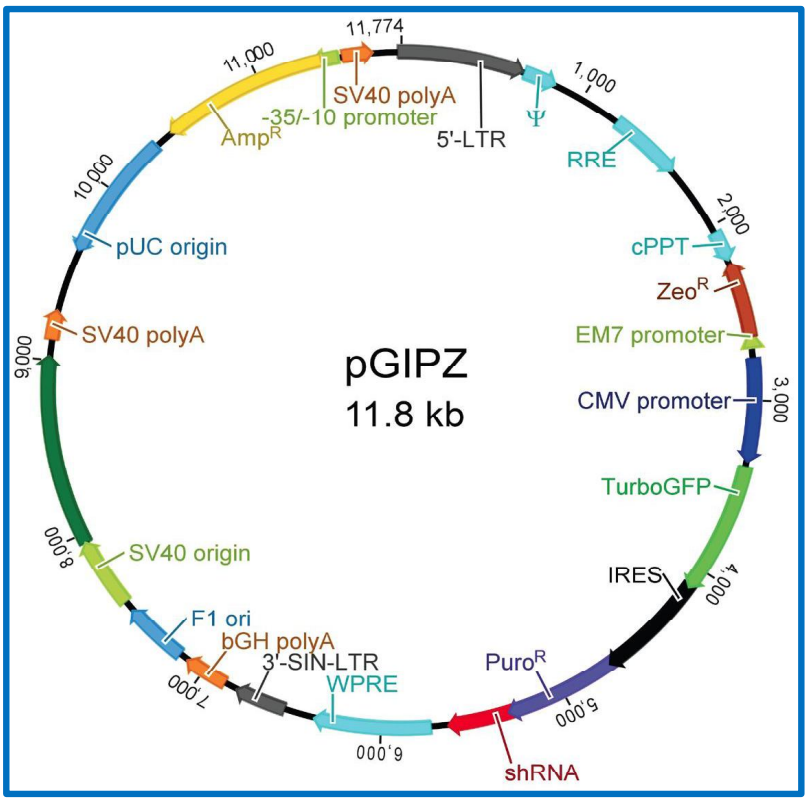


Fig 2.8: Vector construct pGPIZ shRNAmir

pGPIZ shRNAmir contains a short hairpin ribo-nucleic acid (shRNA) sequence to specifically knock down expression of prion protein.

(shRNA-1, shRNA-2, shRNA-3 and shRNA-4), an internal ribosome entry site and TurboGFP (Thermofisher, Epsom, UK) were also used. A similar pGPIZ shRNAmir containing a non-silencing hairpin sequence (shRNA-ns) was used as control. The sequences of the set of 4 shRNAs to knock down PrP expression is enlisted in table 2.5.

2.10.2 Culture of HEK293t cells

Human embryonic kidney (HEK) 293t cells were cultured in DMEM + 10% FCS and incubated at 37°C in 5% CO_2 in air. The media was changed twice every week until the cells were confluent. Once the cells were confluent, the medium was removed and the cells were washed with PBS. The cells were detached using trypsin-EDTA (0.25% trypsin, 1mM EDTA) and collected in DMEM + 10% FCS followed by centrifugation at 2000 rpm for 5 minutes at room temperature. The supernatant was discarded and the cell pellet was re-suspended in DMEM + 10% FCS. The resulting cell suspension was equally distributed in 3 T175 flasks and DMEM + 10% FCS were added to make the total volume up to 20 ml. The flasks were incubated at 37°C in 5% CO_2 in air.

2.10.3 Production of viral particle

2.10.3.1 Production of viral particle using four-plasmid packaging system

Viral particles were produced by co-transfection of HEK 293t cells with in house pCAG_kGP3R, pCAG4RTR2, pCAGVSVG and pCL10.1 MSCV ires GFP plasmids using calcium phosphate precipitation method. Briefly, 2 x 10^6 HEK 293t cells were seeded in $10cm^2$ culture dishes and maintained in D10 medium (DMEM containing 10%

shRNAs	Sequence
shRNA-1	TACATGAAACGATTCAGTG
shRNA-2	ATAAGTATCATGTGGCCTC
shRNA-3	TAGTTTAAAGAAAGGAATG
shRNA-4	TGTTCACTGTGAATATGTC
shRNA-ns	ATCTCGCTTGGGCGAGAGTAAG

Table 2.5: Hairpin sequences of shRNA constructs target the PrP gene sequences located on chromosome 20

(v/v) FBS, penicillin G (50 units/ml), and streptomycin (50 ug/ml)) and incubated at 37^0C in 5% CO_2 for 24 hours. The four plasmids were mixed at 10ug pCAG_kGP3R, 2ug pCAG4RTR2, 2ug pCAGVSVG and 10ug pCL10.1 MSCV ires GFP concentrations in a total volume of 450ul of double-distilled water, following which 50ul of 2.5M calcium chloride ($CaCl_2$) were added. Subsequently, 500 ul of 2X HBSS (280 mM NaCl, 1.5 mM Na2PO4, 50 mM Hepes, pH 7.05) were added drop wise while vortexing the mixture, after which 10 ml D10 medium was added. The culture medium from $10cm^2$ culture dishes were then removed and replaced with D10-DNA-$CaPO_4$ mixture. The cells were incubated for 18hours at 37^oC in 5% CO_2. Following which medium was replaced with fresh D10 medium after washing with 5 ml of PBS. Twenty four hours later the medium containing the freshly produced viral particle (primary viral supernatant) was collected and aliquoted into microcentrifuge tubes and stored at -80°C.

2.10.3.2 Production of viral particle using three-plasmid packaging system

Appropriate amounts of expression plasmids (50µg), envelope plasmid pMD.G2 (17.5ug) and packaging plasmid pCMVΔR8.91 (32.5ug) and 5 ml Opti-MEM (Gibco) were mixed and filtered through a 0.22µm filter. In a separate tube 0.02% v/v of polyethylene imine (PEI, Sigma) was added to 5ml of Opti-MEM and filtered 0.22µm filter. The two solutions were mixed and allowed to stand for 20 minutes. At the end of incubation, 10ml of the PEI/plasmid solution was added to each flask containing the packaging cell line HEK293t and incubated for 4 hours at 37°C in 5% CO_2 in air. The PEI/plasmid solution was then removed and replaced by DMEM + 10% FCS and the cells were incubated for another 24 hours at 37°C in 5% CO_2 in air. Twenty four hours later the medium containing the freshly produced viral particle (primary viral

supernatant) was collected and centrifuged at 3000rpm for 10 minutes. The supernatant was collected and filtered through a 0.22 µm filter and aliquoted into microcentrifuge tubes and stored at -80°C. The primary viral supernatant of vector construct p'HRsincpptSEW (to express eGFP) was only ultra-centrifuged at 19,000 rpm for 2 hours (established in the laboratory by Master student Taneera Ghate). The primary concentrated virus was then aliquoted into microcentrifuge tubes and frozen at -80°C.

2.10.4 Determination of viral transducing units/ml

The number of viral particles in the primary viral supernatant was determined by titration experiment. The HEK293t cells were seeded at a density of $5x10^4$ cells per well in a 12 well plate. Viral dilutions of 1:50 and 1:500 of pCL10.1 MSCV ires GFP, shRNA-1, shRNA-2, shRNA-3, shRNA-4 and shRNA-1-2-3-4 were inoculated onto the 293t cells and incubated at 37°C in a 5% CO_2 in air for 48 hours. A well was maintained as an untransduced negative control. The cells were then analysed by FACS analysis for eGFP expression. For the vector construct p'HRsincpptSEW, data was followed as established in our laboratory (kindly given by Taneera Ghate). *Briefly,* Forty eight hours after addition of the virus, the cells were detached as described in Section 2.2. The cell pellet was re-suspended in 500µl of 2% paraformaldehyde (VWR) in PBS + 2% FBS. The cells were acquired using FACS calibre (Becton, Dikinson) and analysed using the Cell Quest software. The number of viral particles/ml contained in the viral supernatant was calculated by the following formula:

No. of viral particles = (% positive cells) x (dilution) x (no. Of cells seeded)/100

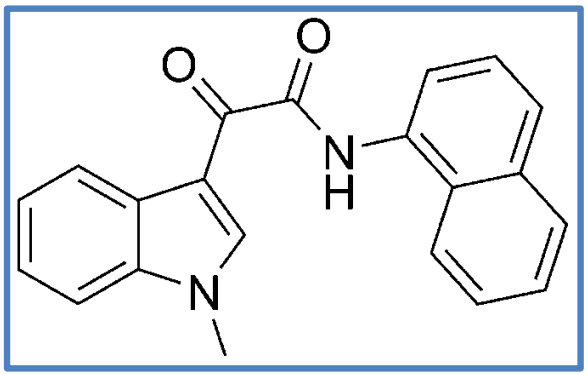
2.10.5 Transduction of hMSC

Human MSCs were seeded at a density of $5x10^4$ cells per well in a twelve well plate in MSC medium. Cultures were initially inoculated with dilutions of viral supernatant and incubated at 37°C in 5% CO_2 in air for 8 hours after which the media was removed and fresh MSC medium was added. The cells were further incubated for 48 hours and then analysed for the expression of eGFP by fluorescence activated cell sorter analysis (FACS). The multiplicity of infection (MOI) was calculated by considering the viral dilutions at which 5-30% of cells were positive for eGFP:

MOI = No. of viral particles seeded/ml / No. of cells seeded

2.11 Small molecule 3000689 (3/689)

Small molecule 3000689 (3/689) and 3000165 (3/165) (kindly donated by Beining Chen, appendix-I, Fig.2.9) are members of the indole-3-glyoxlnapthylamide family of anti-prion compounds. Their synthesis and characterisation was described in the study by Thompson et al., (2009) and Chen (2010) (Thompson, Borsenberger et al. 2009). Small molecule 3/689 and 3/165 were dissolved in dimethylsulfoxide (DMSO) at 3.89mg/ml and 2.61mg/ml respectively and then diluted further in MSC medium for *in vitro* and *in vivo* studies. Cultures were fed twice weekly.



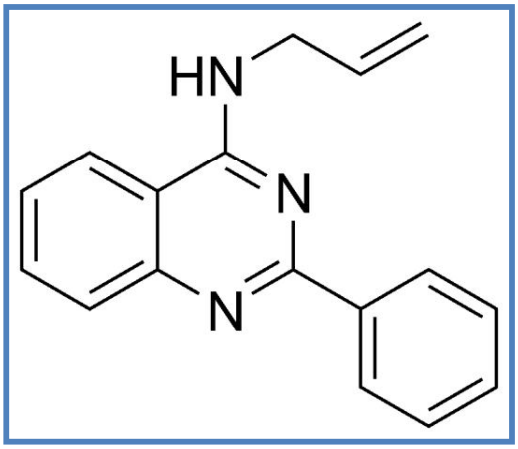


Fig 2.9 Chemical structure of small molecule 3/689 and 3/165

2.12 Transplantation of hMSC by intra-femoral injection

Human MSCs were transduced at a multiplicity of infection of 40, followed by FACS (previously described in section 2.10) to express eGFP. NOD/LtSz-Prkdcscid (NOD/SCID-Non-obese diabetic/severe compromise immuno-deficient) mice aged 5-6 weeks were injected with $5x10^5$ hMSC expressing eGFP by intra-femoral injection according to the approved home office license. Briefly, the mice were anesthetised by injecting 100ul ketamine-rompun mix (0.5ml of ketasal, 0.25ml of rompun and 4.3ml of sterile distilled water) /10 gram mouse (weight), intra-peritoneally. A small deep incision was made using a sterile scalpel blade above the knee joint and the kneecap was exposed slowly using sterile forceps by separating the tissue around without damaging any blood vessels. Through the groove of the kneecap in the femur about half a centimetre deep hole was drilled. Using a Hamilton syringe 5ul hMSCs were gently injected (avoiding bubble formation) into the hole. After injecting, the hole was immediately closed with bone wax using a sterile scalpel blade and finally sutured (appendix-II, Fig.2.10). Mice were sacrificed 3 days and 5 weeks later and analysed for the content of eGFP+CFU-F and eGFP+CFU-O and number of eGFP+ cells respectively.

2.12.1 Detection of eGFP+CFU-F and eGFP+CFU-O colonies

Mice were sacrificed 3 days post-transplant. Using sterile forceps and scissors, the femur was dissected and transferred in growth medium consisting of Modified Eagle's Medium (DMEM) (Gibco, Paisley, UK) supplemented with 10% Fetal bovine serum (FBS)

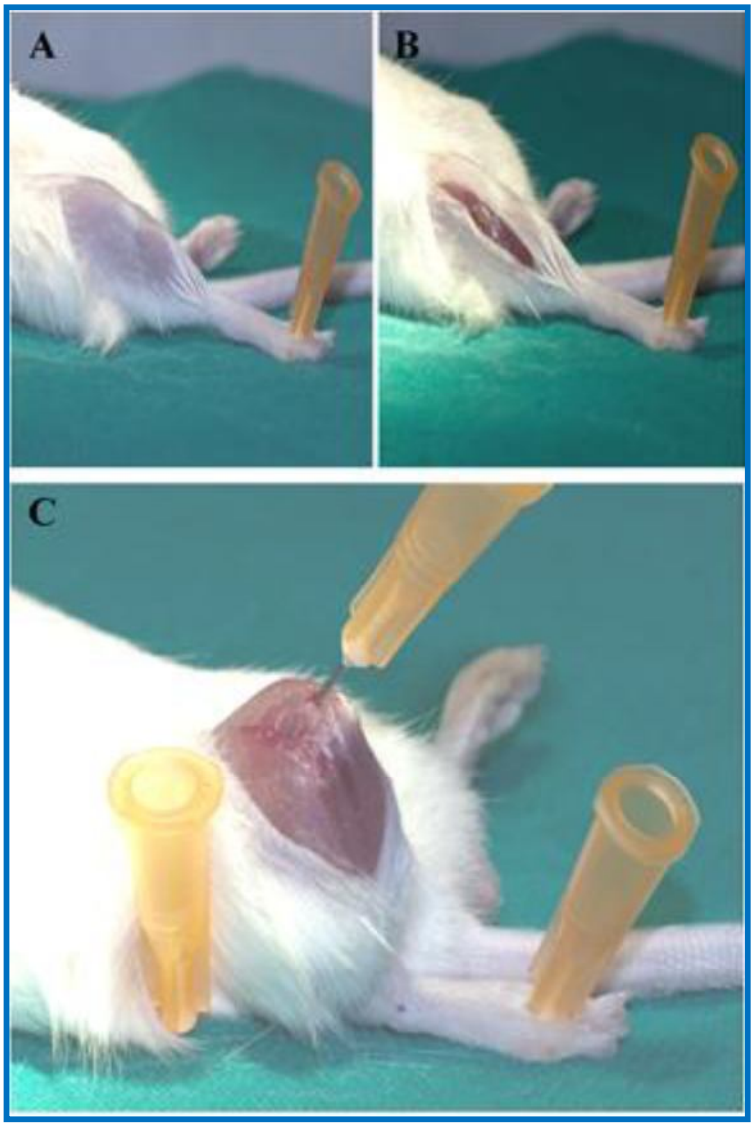


Fig 2.10: Surgical procedure involving intra-femoral injections of eGFP labelled hMSC cells.

Figure A shows a representative image of area where the fur of the mouse has been shaved at the knee joint using an electrical shaver. Figure B shows a 1cm deep incision using a sterile scalpel blade aside of the knee joint and Figure C shows the position where the hole is drilled gently through the groove of the knee-cap to allow injection of hMSC cells.

(Gibco), 10% sodium citrate (Royal Hallamshire Hospital pharmacy, Sheffield, UK) and 0.25% penicillin-streptomycin (Gibco). The bones were then cleaned using sterile forceps, scissors and sterile gauze cloth in a laminar air flow cabinet. Once the bones were cleaned, the ends were trimmed to facilitate the flushing of BM cells from the bone. Using a sterile syringe and 21G needle, the bone marrow was flushed into mMSC-CM (Murine-mesenchymal stem cell complete medium, Stem Cell Technologies, Vancouver, Canada). Bone marrow cells were counted using 3% acetic acid solution to lyse red cells. For CFU-F and CFU-O assays, BM cells were plated at 2.5 x 10^5 cells per well in human MSC medium for the former assay and MSC medium supplemented with osteogenic supplements for the latter assay and incubated for 14 days at 37°C. At day 14, CFU-F and CFU-O were scored using Leica DMI4000B inverted fluorescence microscope where eGFP colonies (green colonies) comprising of at least 50 cells with a definite centre of origin were considered as CFU-F and eGFP colonies (green colonies) comprising of at least 40 cells with a definite centre of origin was considered as one CFU-O respectively. Later, to confirm that eGFP+ CFU-F and CFU-O were colonies, they were stained with Wright's Geimsa stain (section 2.5) and alkaline phosphatase (section 2.6) respectively and rescored by overlaying with the imaging visualisation seen in the eGFP staining.

2.12.2 Immuno staining to access hMSCs expressing eGFP

For assessing long term engraftment ability of hMSC post-transplantation, mice were sacrificed at 5 weeks later and femurs were fixed with 10% formalin. Following rapid decalcification in Surgipath decalcifier II (Leica Microsystems, Milton Keynes, UK) for

2 hours, tissues were embedded in wax and 3 µm sections were cut using a Leica Microsystems microtome (Leica Microsystems) and stained for the detection of eGFP. Briefly, sections were fixed with 99% ethanol for 5 minutes and blocked with 3% H_2O_2 for 10 minutes. They were then blocked with 10% goat serum (Dako, Ely, UK). The primary rabbit anti-GFP antibody (Invitrogen, Paisley, UK) was used at 1:600 overnight at 4^0C and the goat anti-rabbit HRP secondary antibody at 1:400 (Insight Biotech, Wembley, UK) for 45 minutes at room temperature. Detection was carried out using Vector NovaRED substrate kit (Vector Laboratories Ltd, Peterborough, UK) and counterstained by Gill's haematoxylin stain (VWR International, Lutterworth, UK). Eight sections per animal were scored for the presence of eGFP+ cells. A detailed description of immuno-staining procedure is mention in appendix-II.

2.13 Cell cycle analysis

For cell cycle analysis 10^5 hMSCs were maintained for three days in culture in presence of 3/689 or di-methyl sulfoxide (DMSO). After 3 days, hMSCs maintained in presence or absence of 3/689 was trypsinised and re-seeded at 4000/cm^2. The following day cells were washed in PBS and incubated with Carboxyfluorescein succinimidyl ester (CFSE) at 1µM using the Cell Trace CFSE Cell proliferation kit (Invitrogen, Paisley, UK) in hMSC medium for 15 min. Medium was then discarded and replaced with fresh MSC medium. Cells were incubated and analysed for 1 and 5 days at the end of which hMSC were harvested and analysed by FACS using Cell Quest software.

2.14 DNA damage detection

For the induction and detection of DNA damage hMSCs (2000 cells/cm^2) were maintained for three days in culture in presence of 3/689 or DMSO prior to being exposed to hydrogen peroxide (H_2O_2) at 75uM for 1hour. Detection of phosphorylation of histone H2AX on Ser 139 (γH2AX) was carried out using the γH2AX phosphorylation assay kit (Millipore, Watford, UK) according to manufacturer's instruction. Briefly, hMSCs were trypsinised (section 2.2) and 10^5 hMSCs were re-suspended in 50ul of 1X Fixation solution and incubated on ice for 20 minutes. The cells were then washed twice with PBS to remove traces of fixative. Fifty microliter of 1X Permeabilization solution was added to 10^5 hMSCs, followed by addition of 3.5ul of either anti-phosho-Histone H2A.X (Ser 139), FITC conjugate or the isotype control mouse IgG-FITC conjugate and incubated on ice for 20 minutes. After incubation on ice, 100ul of IX wash solution per well was added to wash away excess FITC labelled antibody. The cells were then pelleted and supernatant was discarded. The cell pellet was re-suspended in 150µl of 2% paraformaldehyde in PBS+5%FCS and were analysed by FACS using Cell Quest software.

2.15 Microarray

This was kindly performed by Claire J Cairney and W Nicol Keith, University of Glasgow. Total RNA was extracted from hMSC cultures (n=3/group) which were expanded in the presence or absence of 3/689 at 10uM and harvested after 2 passages or 8 passages using the Nucleospin II RNA extraction kit (Macherey-Nagel, Duren,

Germany) following manufacturers' instructions. RNA was amplified and labelled using the Agilent Low RNA Input Linear Amplification Kit PLUS, One-Colour and hybridised to Agilent whole human genome 4 × 44K gene expression arrays as per manufacturers' instructions (kindly helped by Claire J Cairney and W Nicol Keith, University of Glasgow). Raw data was extracted from scanned images using Agilent feature extraction software (Agilent Technologies, Santa Clara, CA). All array data were then imported into GeneSpring GX (version 11, Agilent Technologies, Santa Clara, CA) and normalised to the 75th percentile. Differentially expressed genes were obtained using GeneSpring GX by paired t test with $p<0.05$. The data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE31205 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31205, appendix-I). The network analysis was performed using Metacore software (GeneGo Inc.). Figure 2.11 shows a schematic representation of the microarray experimental plan.

2.16 Statistical analysis

All experiments were analysed using student's t-tests or one way ANOVA – and Bonferroni's for multiple comparisons post-test. All results are expressed as the mean ± SEM. Significant p values were less than 0.5 with *P<0.05, **P<0.01, ***P<0.001.

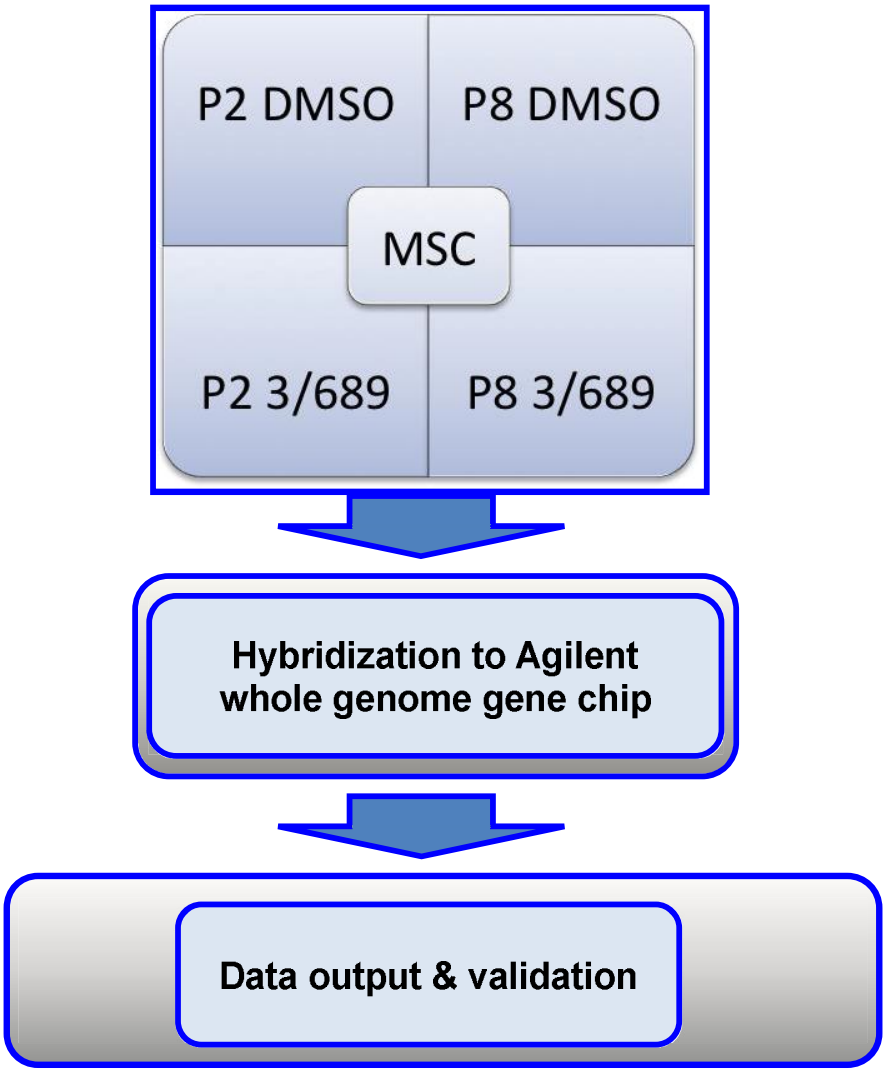


Fig 2.11: A schematic representation of Microarray experimental procedure

CHAPTER-3

3.1 Introduction

In vitro, during long-term expansion, MSCs have shown to undergo changes which are typically associated with cellular ageing. They acquire enlarged morphological appearance, show signs of reduced proliferation and differentiation ability and ultimately undergo replicative senescence (Baxter, Wynn et al. 2004; Rossi, Bryder et al. 2005; Molofsky, Slutsky et al. 2006; Wagner, Bork et al. 2009; Yew, Chiu et al. 2011). More importantly, these changes have been associated with reduced ability to engraft following transplantation (Rombouts and Ploemacher 2003). Therefore it is important to understand the mechanisms leading to cellular ageing in MSC as this may have important clinical implications and lead to new interventions to delay cellular ageing.

Prion protein (PrP) has been shown to promote self-renewal in variety of stem cells (Zhang, Steele et al. 2006). However, the functional roles of PrP in hMSCs have never been investigated. In this chapter, I have used hMSC aged in culture as a model system of cellular ageing to test the hypothesis that hMSC undergoing cellular ageing show decreased PrP expression. I have assessed this by determining whether loss of hMSC proliferation and differentiation ability is associated with decreased PrP expression and whether PrP expression is a unique property of undifferentiated hMSC.

3.2 Isolation and characterisation of hMSC cultures

Human MSCs were derived from human BM from three healthy donors (snbm-24, snbm-26 and snbm-28) and the ages of the donors are mentioned in table 2.1. Once the hMSC cultures were established (described in materials and methods section 2.2), at passage p2 (equivalent to 12-14 PDs) cells were stained for CD45, CD31, CD29, CD34 and SH2 antibody (CD105) and were analysed by flow cytometry (section 2.4) to determine whether cultures had antigenic profile typical of MSC. As expected all the hMSC cultures were negative for the expression of CD45 (haematopoietic), CD31 (endothelial), CD34 expression and positive for CD29 and CD105 expression (Fig 3.1).

3.3 Differentiation of hMSC cultures to osteogenic and adipogenic lineage

To determine whether hMSC cultures (n=3) exposed to osteogenic and adipogenic supplements for 2 weeks were undergoing differentiation, the cultures were assessed for the level of expression of osteogenic markers: osteocalcin and alkaline phosphatase staining and adipogenic markers: PPAR-γ and Oil red O staining in hMSC cultures. Analysis of the expression of genes by RT-PCR were normalised to L-32 and the calculations were carried out as described in section 2.8.

Cultures not exposed to differentiation supplements served as undifferentiated control (UD, Fig 3.2A and Fig 3.3A). Cultures exposed to osteogenic differentiation medium (OB) showed up-regulation of alkaline phosphatase staining (Fig 3.2B) and osteocalcin (Fig 3.2C) compared to undifferentiated control, confirming that they had undergone

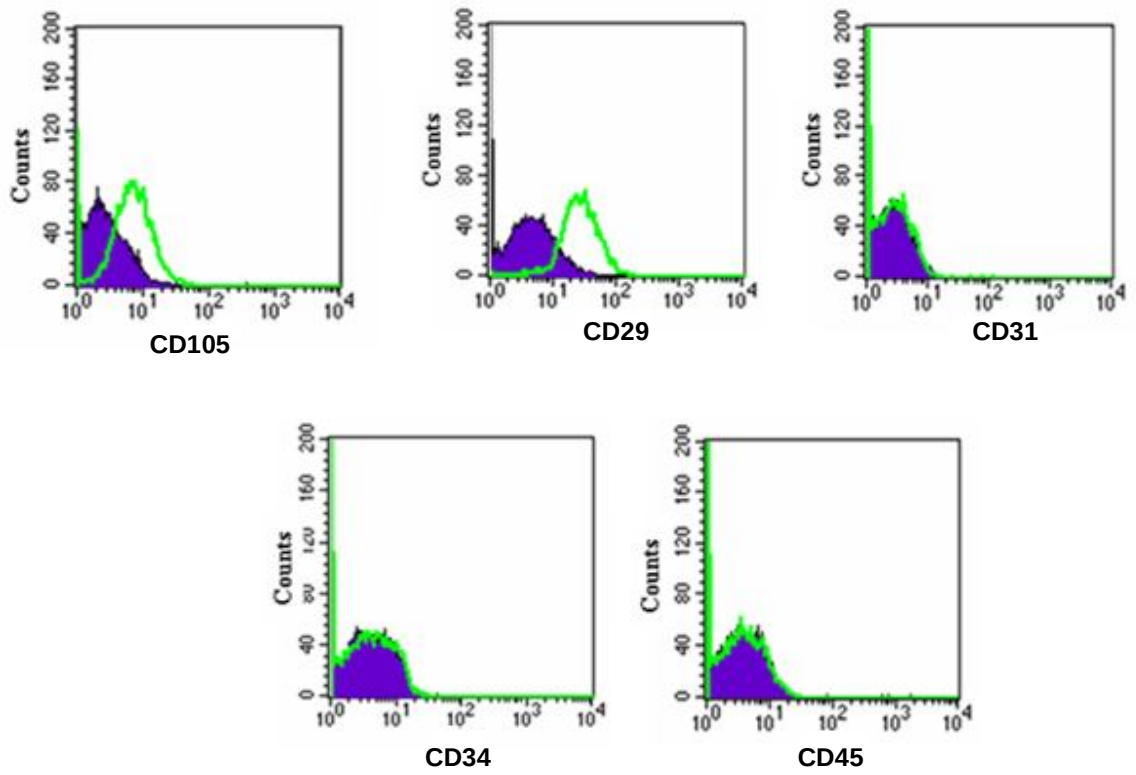


Fig 3.1: A representative example of the antigenic profile of human MSC cultures isolated from bone marrow

Purple histogram represents cells stained with isotype controls and green histogram represents cells stained with antibodies specific for CD29, CD34, CD105, CD31 and CD45.

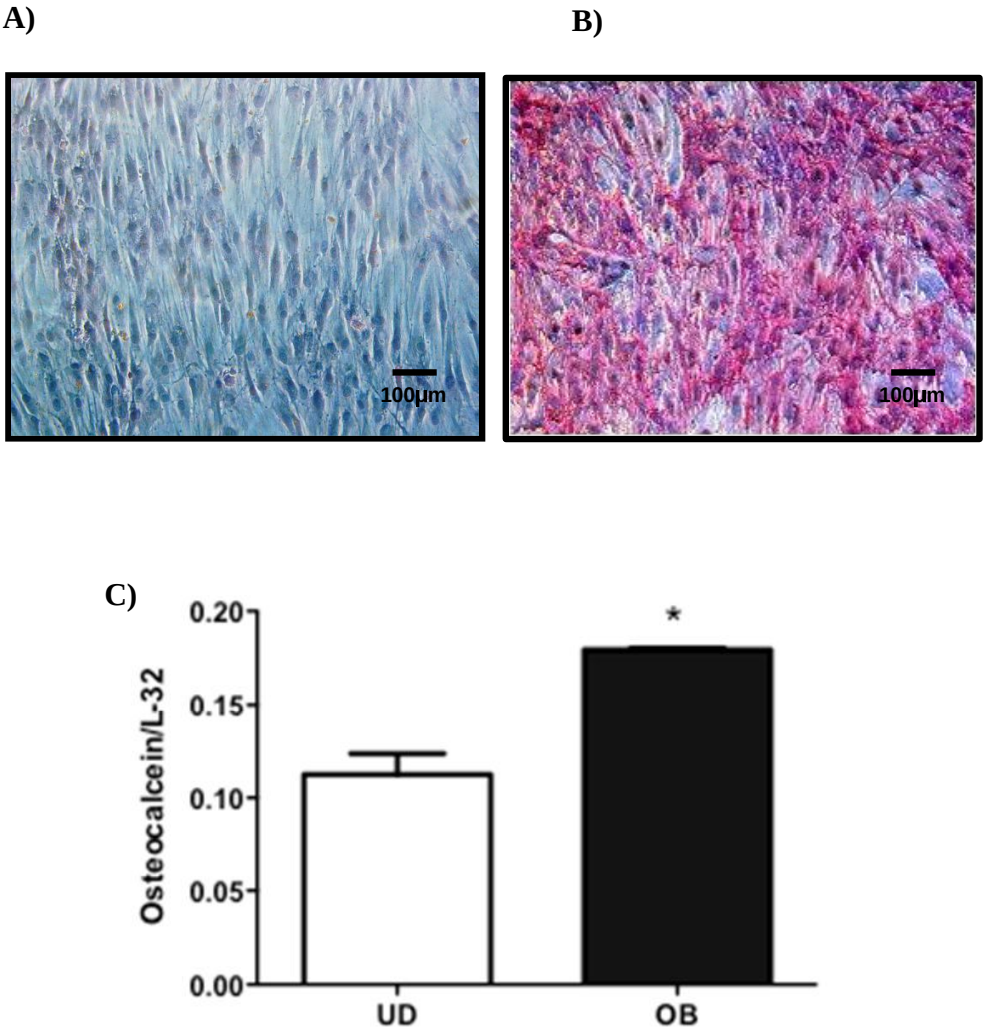


Fig 3.2: hMSC cultures show signs of osteogenic differentiation

*Figure A and B are representative examples of undifferentiated hMSC and hMSC exposed to osteogenic differentiation supplements and stained for alkaline phosphatase activity. Figure C shows relative gene expression of osteogenic marker Osteocalcin normalised to L-32 at day 14 hMSC cultures differentiated to osteogenic lineage (OB) compared to undifferentiated hMSC culture (UD) assessed by real-time polymerase chain reaction (RT-PCR) (n=3). Data presented as mean ± SEM and analysed by student's t test, *p-0.0296. Images were taken using a light microscope Leica Leits DMRB at 10x magnification.*

osteogenic differentiation. Similarly, hMSC cultures exposed to adipogenic supplements (AD), showed an increased expression of adipogenic marker PPAR-γ (Fig3.3C) by RT-PCR and formation of Oil Red O lipid vacuoles (Fig 3.3B) compared to undifferentiated control, confirming that they had undergone adipogenic differentiation.

3.4 Detection of PrP expression

To determine whether hMSC expressed cellular PrP and whether differentiation affected its expression, the same hMSC cultures (n=3) established from the BM were assayed for the expression of PrP after 19-23 PD and following exposure to adipogenic and osteogenic supplements, by RT-PCR and western blotting.

3.4.1 Detection of PrP expression by RT-PCR

Undifferentiated hMSC cultures expressed PrP. Moreover, after confirming that hMSC cultures were differentiated to osteogenic and adipogenic lineage, the level of PrP expression was then determined in differentiated hMSC cultures at similar PDs by RT-PCR. All the differentiated cultures expressed PrP and if anything, an increase in the level of PrP expression was seen as compared to undifferentiated hMSC cultures, although it was not statistically significant (Fig 3.4).

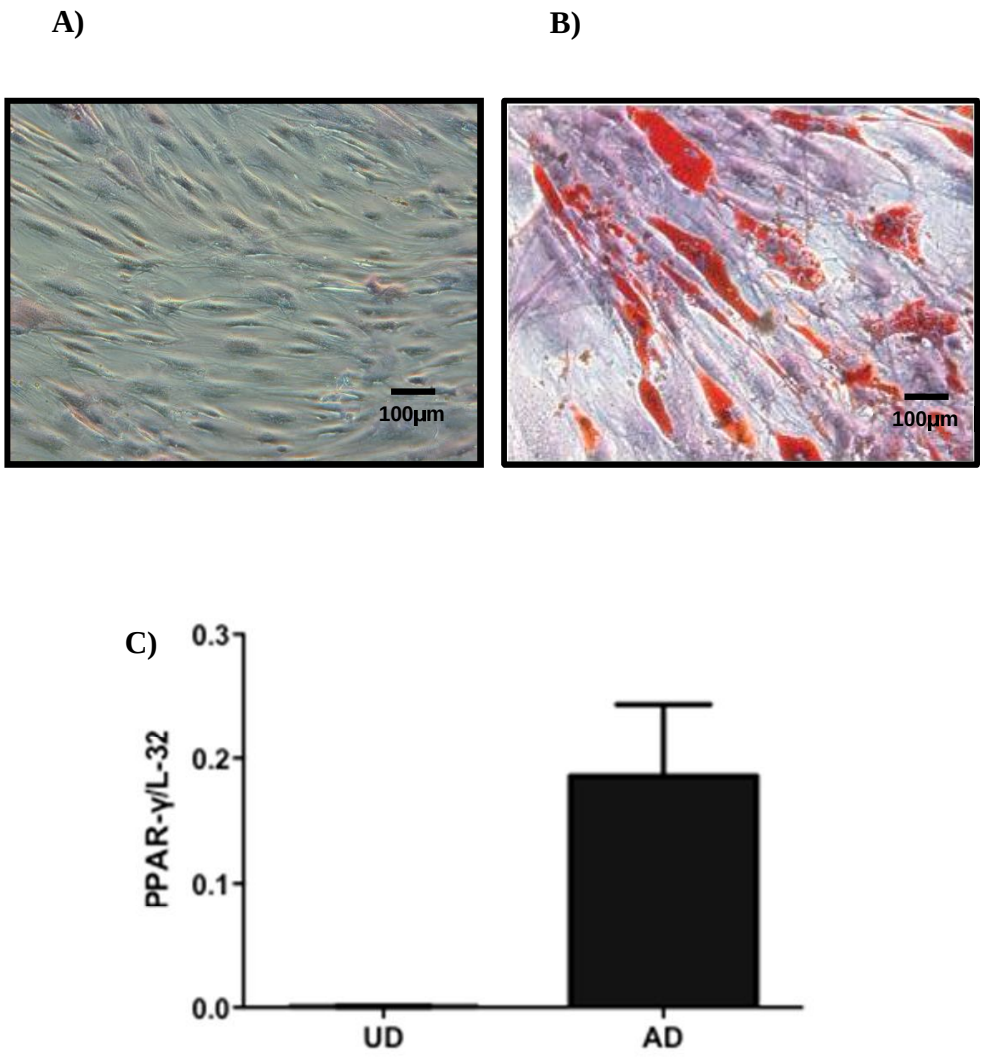


Fig 3.3: hMSC cultures show signs of adipogenic differentiation

Figure A and B are representative examples of undifferentiated hMSC and hMSC exposed to adipogenic differentiation supplements and stained for Oil Red O. Figure C shows relative gene expression of adipogenic marker PPAR-γ normalised to L-32 at day 14 of adipogenic differentiation hMSC cultures (AD) compared to undifferentiated hMSC culture (UD) assessed by RT-PCR (n=3). Data presented as mean ± SEM and analysed by student's t test, p-non-significant. Images were taken using a light microscope Leica Leits DMRB at 10x magnification.

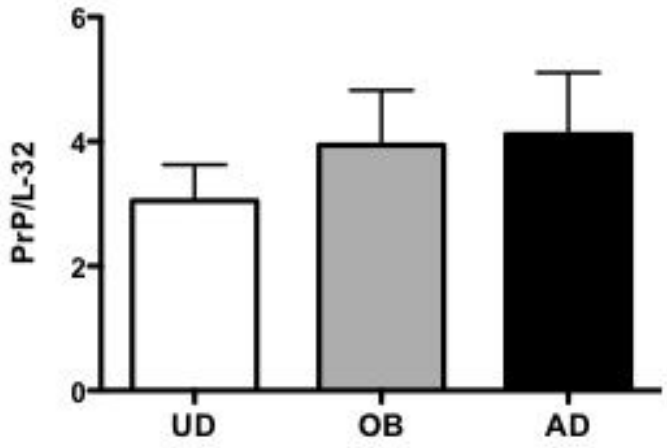


Fig 3.4: Detection of PrP expression in undifferentiated hMSC and hMSC induced to differentiate to osteoblast and adipocyte lineage by RT-PCR

The graph shows quantification of PrP expression normalised to L-32 in undifferentiated hMSC (UD, n=3) and hMSC differentiated to the osteogenic (OB) and adipogenic (AD) lineage and assessed by RT-PCR followed by analysis using standard curve method. Data presented as mean ± SEM and analysed by one way ANOVA with Bonferroni multiple comparison post-test, p-non-significant.

3.4.2 Detection of PrP expression by western blotting

To determine whether the level of PrP changed with differentiation at protein levels, the same hMSC cultures (n=3) were assayed for the expression of PrP by western blotting. The expression levels of PrP were normalised to the house-keeping gene GAPDH and the band density was evaluated by using ImageJ software. Finally the ratio between PrP and GAPDH was obtained. The undifferentiated and the differentiated MSC cultures showed only one distinct band in the range of 30-33kilo Dalton (kDa) (Fig 3.5A). All the hMSC cultures expressed PrP regardless of the differentiation status and if anything, an increase of 30%-61% in level of PrP expression was seen with osteogenic differentiation although it did not reach statistical significance. A significant increase of 61%-84% was observed with adipogenic differentiation (Fig 3.5B). These data suggest that PrP expression is not a unique property of undifferentiated hMSC.

3.5 Detection of PrP expression with cellular ageing

3.5.1 Changes in properties of hMSC with cellular ageing

To verify that hMSC in culture underwent changes as previously described with cellular ageing, hMSCs were expanded in culture and analysed for their proliferation and differentiation ability. All the hMSC cultures exhibited spindle-shaped fibroblast like morphology in culture in the early passages (Fig 3.6A). Subsequently, they appeared to become more flattened and larger (Fig 3.6B). All the three hMSC cultures showed an initial phase of rapid growth followed by slower phase of growth (Fig 3.7A). As expected the time taken to undergo PD increased from an average 5 days at p3

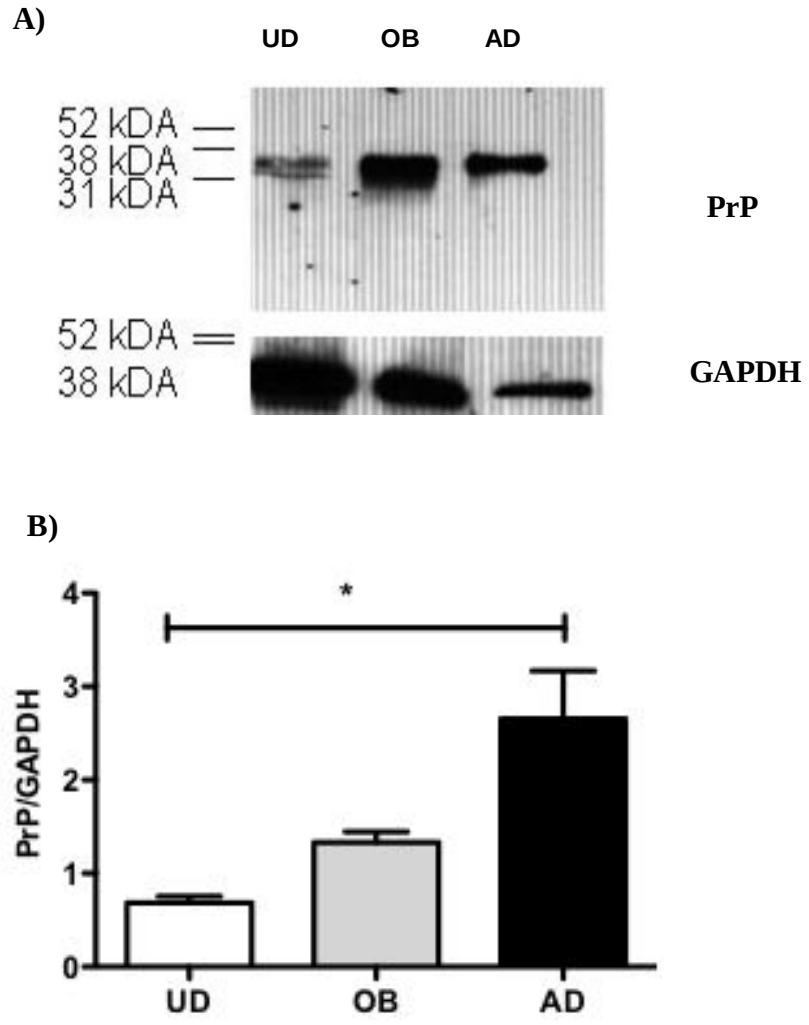


Fig 3.5: Detection of PrP expression in undifferentiated hMSC, osteoblast and adipocyte by western blotting

*Figure A shows a representative example of western blot of hMSC culture labelled with antibodies specific for cellular prion protein (PrP, top panel) and glyceraldehydes-3 phosphate dehydrogenase (GAPDH, bottom panel). Figure B shows quantification of PrP expression normalised to GAPDH in undifferentiated hMSC (UD, n=3) and hMSC differentiated to the osteogenic (OB) and adipogenic (AD) lineage and assessed by western blot followed by analysis with ImageJ software. Data presented as mean ± SEM and analysed by one way ANOVA with Bonferroni multiple comparison post-test, *p<0.05.*

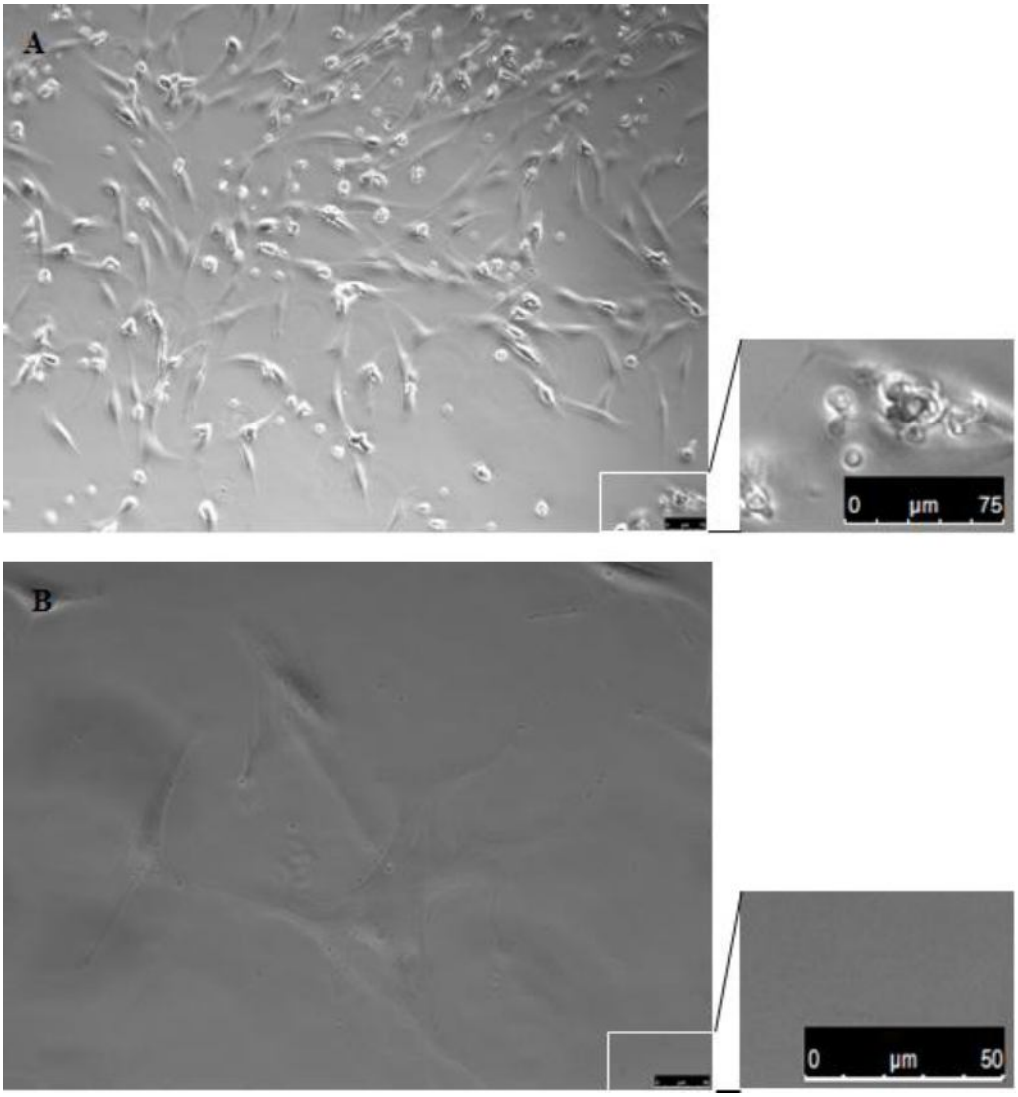


Fig 3.6: Morphology of MSC at early and late passage in culture

Figure A and B shows a representative example of undifferentiated hMSC culture at an early passage 3 (p3) and late passage 8 (p8) respectively. Images were taken using a light microscope Leica Leits DMRB at 10x magnification for MSC at early passage and at 20x magnification for MSC at late passage.

(equivalent to 13-16 PD) to 10 days at p8 (equivalent to 19-23 PD) (Fig 3.7B).

As hMSC cultures are heterogeneous and contain a considerable number of highly proliferative progenitors, I enumerated the number of CFU-F and the number of clonogenic progenitors with osteogenic (CFU-O) and adipogenic (CFU-A) potential at p3 and p8 passage in culture. As expected, all the three MSC cultures showed a significant reduction of 68.82%±8.80% in the number of CFU-F when comparing hMSC cultures at p8 and p3 (Fig 3.8A). The hMSC cultures also showed a significant decrease of 85.86%±4.00% in the number of CFU-O (Fig 3.8B) and 29.6%±13.06% in the number of CFU-A (Fig 3.8C) at p8 when compared to p3.

3.5.2 Detection of PrP expression in hMSC cultures with time in culture

To determine whether loss of hMSC proliferation and differentiation potential with serial passage in culture co-related with changes in PrP expression, the level of PrP expression was measured at early p3 and late p8 passages by RT-PCR and western blotting. RNA (section 2.8.1) and protein lysates (section 2.9) were collected and the level of PrP expression was normalised to the levels of L-32 and GAPDH expression by RT-qPCR and western blotting respectively. In all the three MSC cultures, there was no statistical significance in the level of expression of PrP normalised to L-32 by RT-PCR (Fig 3.9).

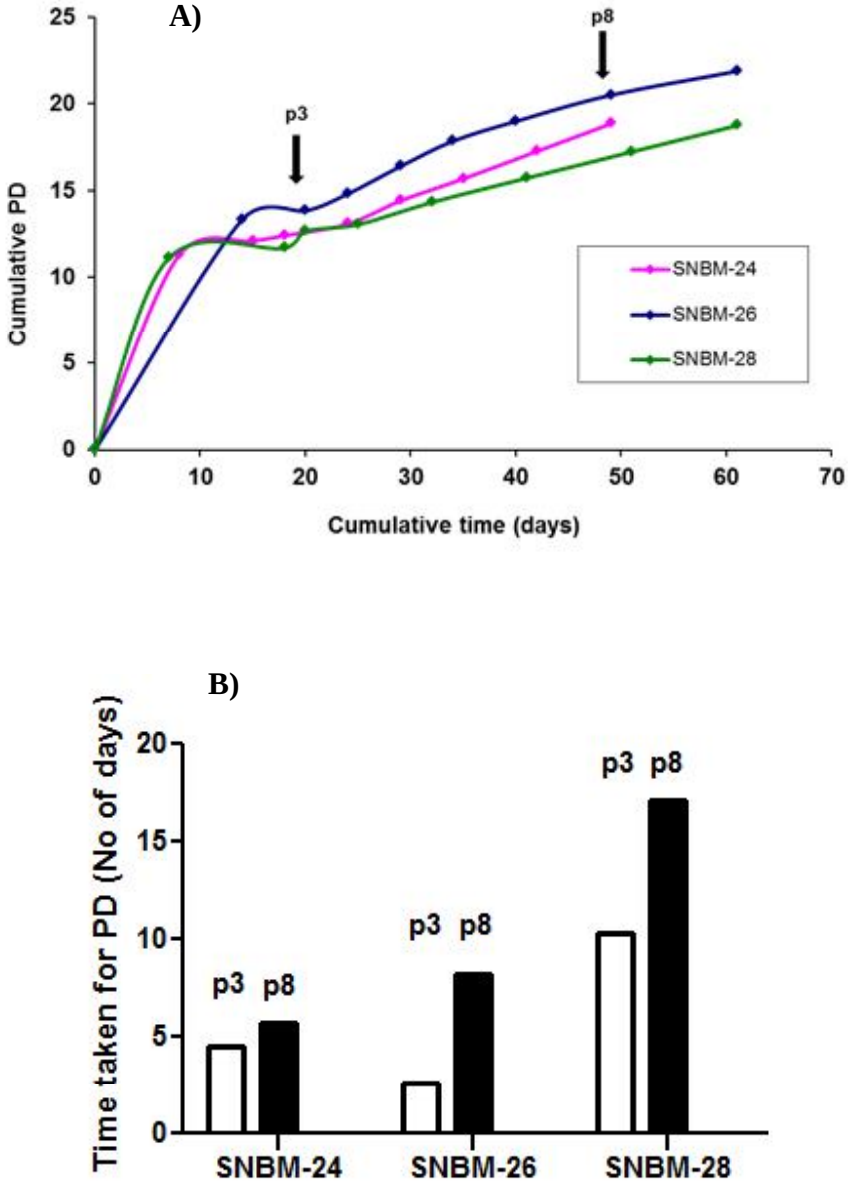


Fig 3.7: Growth kinetics and comparison of population doubling time between p3 and p8 in hMSC cultures

Figure A shows three hMSC cultures in an initial phase of rapid growth followed by slower phase of growth. Figure B show the time taken for hMSC cultures (n=3) to undergo a population doubling (PD) at passage 3 (p3) and passage 8 (p8).

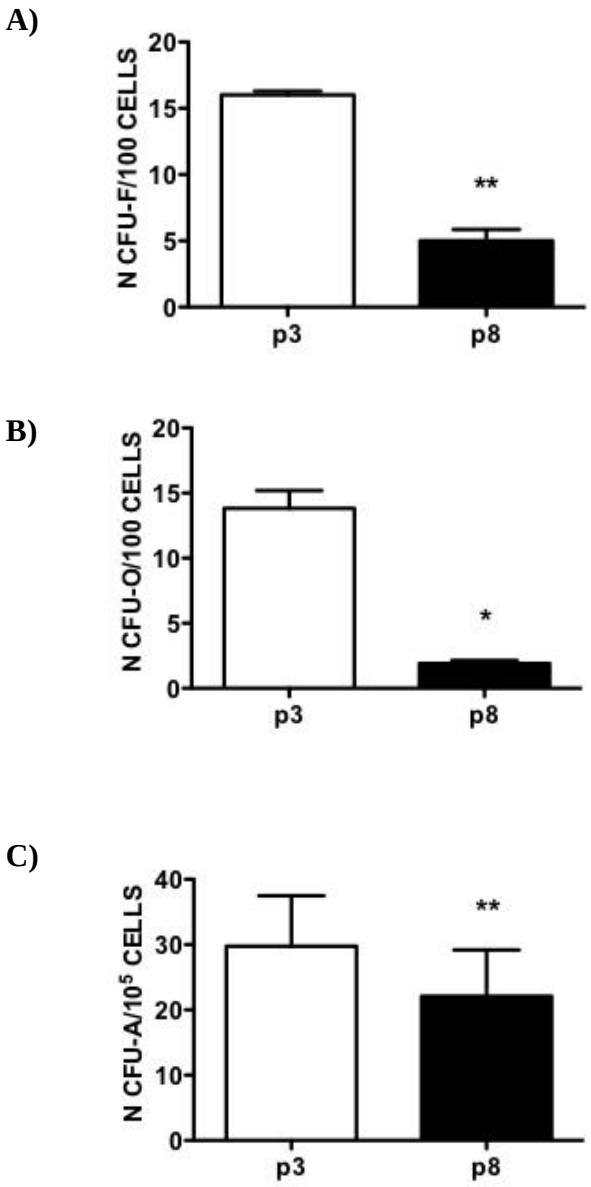


Fig 3.8: The clonogenic potential of hMSC decreases with time in culture

*Figure A, B and C shows decreased frequency of CFU-F, CFU-O and CFU-A respectively at passage 8 (p8) compared to passage 3 (p3) in hMSC cultures (n=3). Data presented as mean ± SEM and analysed by paired student's t test, *p<0.05, **p<0.01.*

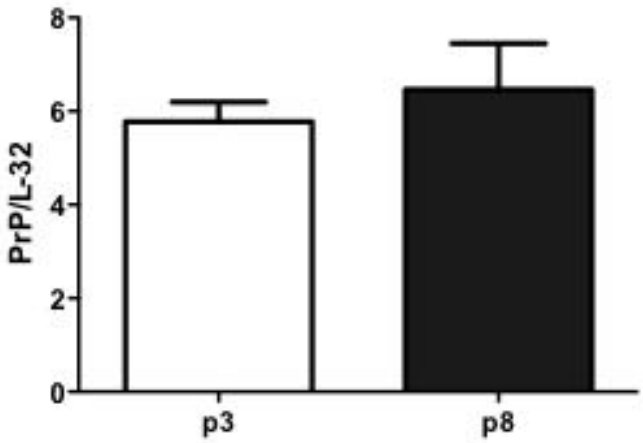


Fig 3.9: PrP is expressed in hMSC culture and its expression does not change with time in culture by RT-PCR

The graph shows quantification of PrP expression normalised to L-32 in hMSC cultures at early passage 3 (p3) and late [passage 8 (p8) by RT-PCR (n=3). Data presented as mean ± SEM and analysed by paired student's t test, p-non-significant.

However, at the protein level, all the three MSC cultures at p3 showed two distinct bands. One band was of low molecular weight and was in the expected range of 30-33kDa and the other band was of high molecular weight and was in the range of 58-60kDa for all the three cultures, compatible with dimmer formation (Fig 3.10A). In all three cultures at p8, a unique band of 32-36kDa was observed. Densitometry was performed to detect the levels of GAPDH and PrP expression using the ImageJ software. A significant reduction of 61%-95% was observed in the level of PrP expression normalised to GAPDH at p8 when compared to p3 (n=3) (Fig 3.10B).

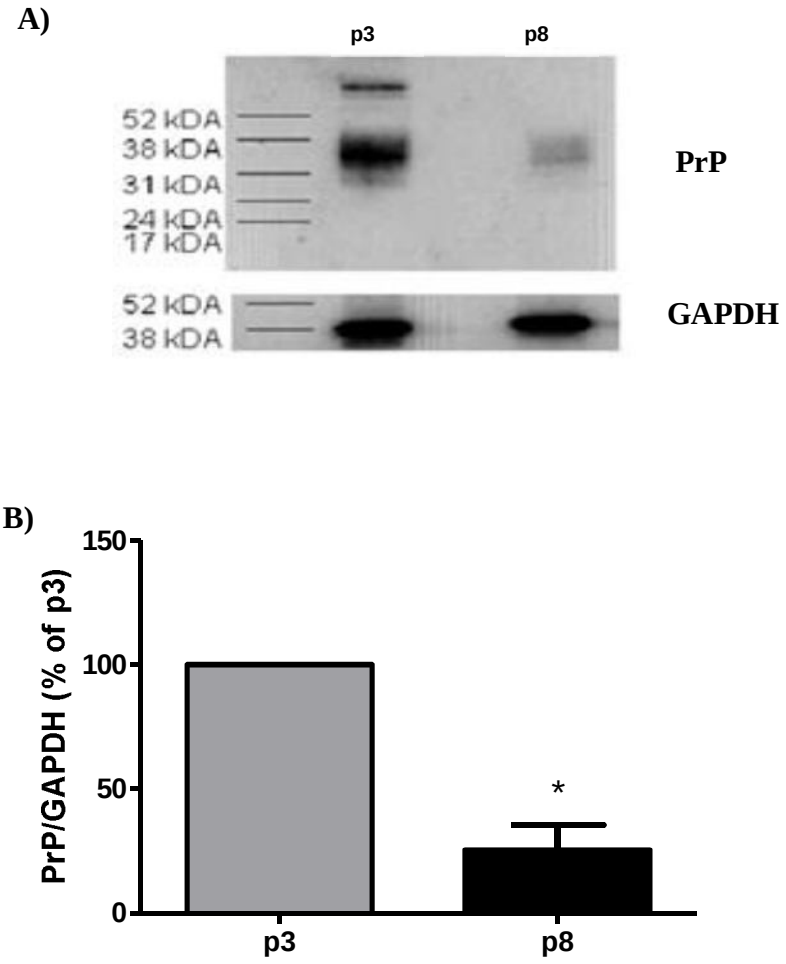


Fig 3.10: PrP is expressed in hMSC culture and its expression significantly decreases with time in culture by western blotting

*Figure A shows a representative example of western blot of hMSC culture at passage 3 and at passage 8 labelled with antibodies specific for cellular prion protein (top panel) and glyceraldehydes-3 phosphate dehydrogenase (GAPDH, bottom panel). Figure B shows quantification of PrP expression normalised to GAPDH in hMSC cultures (n=3) at p3 and p8 and expressed as a percentage of p3, assessed by western blot and analysed using ImageJ software. Data presented as mean ± SEM and analysed by paired student's t test, *p<0.05.*

3.6 Discussion

The aim of this chapter was to study the expression of PrP in hMSC culture with cellular ageing. To achieve this, I differentiated hMSC to osteogenic and adipogenic lineage; characterised hMSC, their morphology and growth kinetics following serial passage in culture. The hMSC cultures were derived by plastic adherence (Friedenstein, Chailakhjan et al. 1970; Friedenstein, Gorskaja et al. 1976; Bruder, Jaiswal et al. 1997; Pittenger, Mackay et al. 1999; Dominici, Le Blanc et al. 2006) and as expected, they were devoid of haematopoietic and endothelial contamination and positive for SH2- a monoclonal antibody raised against hMSC that recognises the TGF-β receptor endoglin (CD105) and CD29, similar to what was reported by several authors (Barry, Boynton et al. 1999; Pittenger, Mackay et al. 1999; Deans and Moseley 2000; Zhou, Ma et al. 2003).

As PrP has been shown to be important for the identification and function of freshly isolated mammary gland stem cells and HSC (Zhang, Steele et al. 2006), I wanted to determine whether hMSCs expressed PrP and whether differentiation affected its expression. To do that I measured the levels of PrP expression at mRNA and protein levels by RT-PCR and western blotting, in hMSC cultures differentiated to osteogenic and adipogenic lineage. All hMSC cultures expressed PrP, regardless of the differentiation status and if anything, the expression of PrP was upregulated at mRNA and protein level in differentiating hMSCs, suggesting that PrP expression is not a unique property of stem cells. If PrP expression was a unique property of stem cells, one would have expected low or no expression of PrP in differentiated cells. Up-regulation

of PrP with differentiation has been seen before in different mature cell types. While investigating role of PrP in differentiation of neural stem cells by using differentiating murine embryonic stem cell (ES) as a model, Parelta O A et al., (2011) showed that ES cells differentiated over 21 days showed increased levels of PrP expression both at mRNA and protein levels by day 9 and continued to increase through day 18 (Peralta, Huckle et al. 2011). Indeed, a four-fold increase of PrP mRNA was observed at day 7 in rat pheochromocytoma PC12 cells induced with neural growth factor to differentiate into cholinergic cells (Lazarini, Castelnau et al. 1994) compared to undifferentiated cells Likewise, an *in vitro* model of human neurogenesis, fetal human GFAP-positive astrocytic cells showed low levels of PrP expression when undifferentiated compared to differentiated neuronal cells (Witusik, Gresner et al. 2007).

As PrP has been shown to be important in stem cell function, I wanted to investigate whether PrP changed expression with time in culture in hMSC and whether this correlated with loss of their proliferation and differentiation potential. To verify hMSC underwent cellular ageing, I measured changes in their proliferation and differentiation capacity with time by CFU-F, CFU-O and CFU-A assays at two time points: early passage 3 (p3) and late passage 8 (p8). An early p3 was chosen because it was the earliest possible time point when sufficient number of MSC were available to carry out all the assays and p8 was chosen as the latest time point when cells started slowing down substantially in growth but still had sufficient number of cells to carry out all the assays. As expected the number of progenitors in both undifferentiated hMSC cultures and with osteogenic and adipogenic potential showed a decrease in their proliferation and differentiation ability with time in culture. This was indeed observed in several studies

where the number of CFU-F (Digirolamo, Stokes et al. 1999) and the potential to undergo osteogenic and adipogenic differentiation had declined in MSC not only with time in culture but also from older donors compared to young donors (Digirolamo, Stokes et al. 1999; Muraglia, Cancedda et al. 2000; Baxter, Wynn et al. 2004; Stolzing, Jones et al. 2008; Zhou, Greenberger et al. 2008).

To determine whether there was a correlation in the level of PrP expression with loss of proliferation and differentiation ability, protein lysates at p3 and p8 passage was obtained from the three hMSC cultures and western blotting was carried out. When quantifying both the bands, all the three cultures showed a reduction of 68%-94% in the level of PrP expression between p3 and p8 passages. Our data is similar to Liao M et al , (2005) who showed that 10,000 freshly isolated mammary epithelial cells in which sphere initiating cells are enriched in the PrP^{med} fraction generated an average of 6.1 spheres *in vitro*, in contrast cultured mammospheres lost PrP expression and generated only 4.1 mammospheres from 10,000 pre-cultured mammary gland cells (Liao, Zhang et al. 2007). Likewise, during serial expansion of HSCs in culture have shown that the expressions of PrP from freshly isolated stem cells are either partially or completely lost with time in culture. However, this did not correlate with the marrow repopulating ability (Zhang and Lodish 2005). These studies suggest that decrease in PrP expression in stem cell upon long term culture may be a common phenomenon. However, this may not be always linked to loss of stem cell function and careful studies needs to be conducted to verify this in each stem cell type.

There are studies which have shown that PrP mRNA levels were similar in young and old animals, despite changes in protein levels. Avrahami D et al., (2009) demonstrated the detection of PrP in the brains of aging mice and that these levels were similar in young and old mice at the mRNA level (Avrahami and Gabizon 2009). A study in a bovine model showed no correlation between age and the amount of PrP expressed at mRNA levels indicated by RT-PCR (Didier, Dietrich et al. 2006). All these studies indicate that there are differences in PrP protein expression with age and at mRNA PrP levels, they remain unchanged. The reason for such age-related discrepancy at protein and mRNA levels was suggested to be due to the fact that PrP is post-transcriptionally regulated possibly by differences in their protein trafficking or degradation (Ford, Burton et al. 2002). However no clear evidences have been found so far with regard to any of those claims.

Of interest is that hMSC cells at p3 showed presence of two bands detected by the PrP antibody. One of the bands observed was in the range of 30-38kDa and another in the range of 58-60 kDa. Studies show that the expected molecular masses of PrP ranges from 25-40 kDa which includes the non- (25-28p kDa), mono- (28-31 kDa) and di-glycosylated (33-40 kDa) forms of PrP (Mouillet-Richard, Ermonval et al. 2000; Riley, Leucht et al. 2002; Segarra, Lehmann et al. 2009), with molecular masses 25-40 kDa are most commonly described isoforms (Peralta, Huckle et al. 2012). A predominant band at 31kDa and a less predominant band at 29kDa was observed, suggesting the presence of mono- and non- glycosylated isoforms of PrP. In adult tissues, variable glycosylation of the two PrP asparagine linked oligosaccharide sites by N-glycans resulted in three PrP bands, representing di-glycosylated, mono-glycosylated and non-glycosylated PrP

glycoforms (Stimson, Hope et al. 1999). The less common is the 60 kDa form of PrP, which I observed and was also reported by Priola et al., (1995) who identified 60 kDa PrP derived from hamster PrP (Priola, Caughey et al. 1995). Peptide mapping studies indicated that dimer formation of two 30kDa PrP monomers that were covalently linked resulted in this form. The function of the 60 kDa protein in unknown but could be a precursor of the infectious form of PrP (Priola, Caughey et al. 1995).

To summarise, my data from this chapter showed that hMSC have a limited life span and that their proliferative and differentiation ability is restricted with time in culture. Prion protein expression was shown to decrease with time in culture and was not a property unique to hMSC. It will now be interesting to address if PrP has a functional role in preserving the proliferation and differentiation ability of hMSC with time in culture by knocking down its gene expression.

CHAPTER-4

4.1 Introduction

In the previous chapter, I have shown how expression of PrP is associated with hMSC cellular ageing. In this chapter, I want to address the question whether down-regulation of PrP is responsible for the functional changes that hMSC undergoes with time in culture. To test this I chose lentiviral vectors (LV). They serve as efficient tools for gene transfer as they can infect both dividing and non-dividing cells and can also integrate stably into the host genome to provide long-term stable gene expression (Zhang, La Russa et al. 2002; Van Damme, Thorrez et al. 2006). Since they are human immunodeficiency virus (HIV)-1 based vectors, it is important to develop a vector system which minimizes the risk of generating replication-competent lentiviruses (RCL). In this chapter, I initially tested four-plasmid vector systems (otherwise called as 3rd generation LV system) but resolved to use the three-plasmid vector system (otherwise called as 2nd generation LV system). The 3rd generation LV is generated by reducing the lentiviral sequences and splitting the essential genes required for replication in four packaging constructs. One construct contains the *rev* gene responsible for the synthesis of viral proteins and another plasmid construct contains gag/pol genes. Moreover, the envelope gene is contained in a third plasmid and a 4^{rth} plasmid called as the transfer plasmid contains the lentiviral backbone with the packaging signal and the gene of interest (Dull, Zufferey et al. 1998). This increases the biosafety as four homologous recombinational events are required for the formation of RCL. However, the downside of this vector system is that the viral titres are lower than 2nd generation packaging system. The 2nd generation LV is generated by incorporating the *rev/tat* gene and gag/pol genes in one single packaging plasmid deleting the accessory genes such as

vip, vpr, vpu and nef (that are not essential for viral replication *in vitro*), and maintaining the envelope and the transfer vectors in separate plasmid (Gibbs, Regier et al. 1994). Although the level of safety is not as high because only three homologous recombinational events are required for RCL formation, the viral titres are higher. Therefore to achieve a yield of high transduction efficacy and high viral titres, I tested both 3rd and 2nd generation LV packaging system and resolved to use 2nd generation LV system for my experiments.

In this chapter, four vectors containing different shRNAs (shRNA-1, shRNA-2, shRNA-3, shRNA-4) targeting PrP were either tested alone or in combination to determine the best condition for an effective knockdown. I then selected the best construct and tested its effect on the properties of hMSCs in culture. The vector I have chosen contained not only shRNA sequences specific for PrP knock-down but also a sequence expressing enhanced green fluorescence protein (eGFP). This allowed assessing the transduction efficiency and selecting hMSC expressing the hair-pin loop.

4.2 Production of Lentiviral particles using 3rd generation packaging cells

4.2.1 Lentiviral transduction using 3rd generation packaging in HEK293t cells

To optimise the production of lentiviral particles, first, I used the vector shRNA-1 to knockdown the expression of PrP and the control vector pCL-10.1 which encodes for the expression of eGFP (Buckle, De Leenheer et al. 2012). I have used a 3rd generation packaging system according to the protocol detailed in section 2.10.1 of material and

methods. To determine the viral supernatant produced, I initially transduced HEK293t cells with different dilutions (250ul, 500ul and 1000ul) of primary viral supernatant. Forty eight hours post transduction, the transduced cells were harvested and analysed for eGFP expression by FACS analysis. The HEK293t population transduced with pCL-10.1MSCV ires GFP (n=3, Figure 4.1 C-E) and shRNA-1 (n=3, Figure 4.1 F-H) was selected by gating the R1 region on a FSC (forward scatter) Vs. SSC (side scatter) plot (n=3, Fig 4.1 A-B) to analyse GFP expression. No eGFP positive HEK293t cells were obtained suggesting the viral production was below detection.

4.2.2 Lentiviral transduction using 2nd generation packaging cells

As 3rd generation LV packaging systems are known to be less efficient in producing viral titre, I produced viral particles using a three-plasmid lentiviral system (otherwise called 2rd generation lentiviral system) by co-transfection of HEK293t cells with the envelope and packaging plasmids pMD.G2 and pCMVΔR8.91, (produced by L. Naldini and kindly donated by A Thrasher) along with either one transfer plasmids containing the hairpin sequence that specifically knock down the expression of PrP (shRNA-1/shRNA-2/shRNA-3/shRNA-4) or with a vector known to produce high titre virus and have high transduction efficiency, p'HRsincpptSEW, which expresses eGFP. A similar shRNA construct containing a non-silencing hairpin sequence (shRNA-ns) was used as a control. The primary viral supernatant of the four shRNA constructs along with p'HRsincpptSEW and shRNA-ns were harvested and HEK293t cells were initially transduced at dilutions of 1:500, 1:5000 and 1:50,000 to determine number of virus particles contained in the viral supernatant (viral titre). Forty eight hours post transduction, cells were analysed for eGFP expression by FACS analysis.

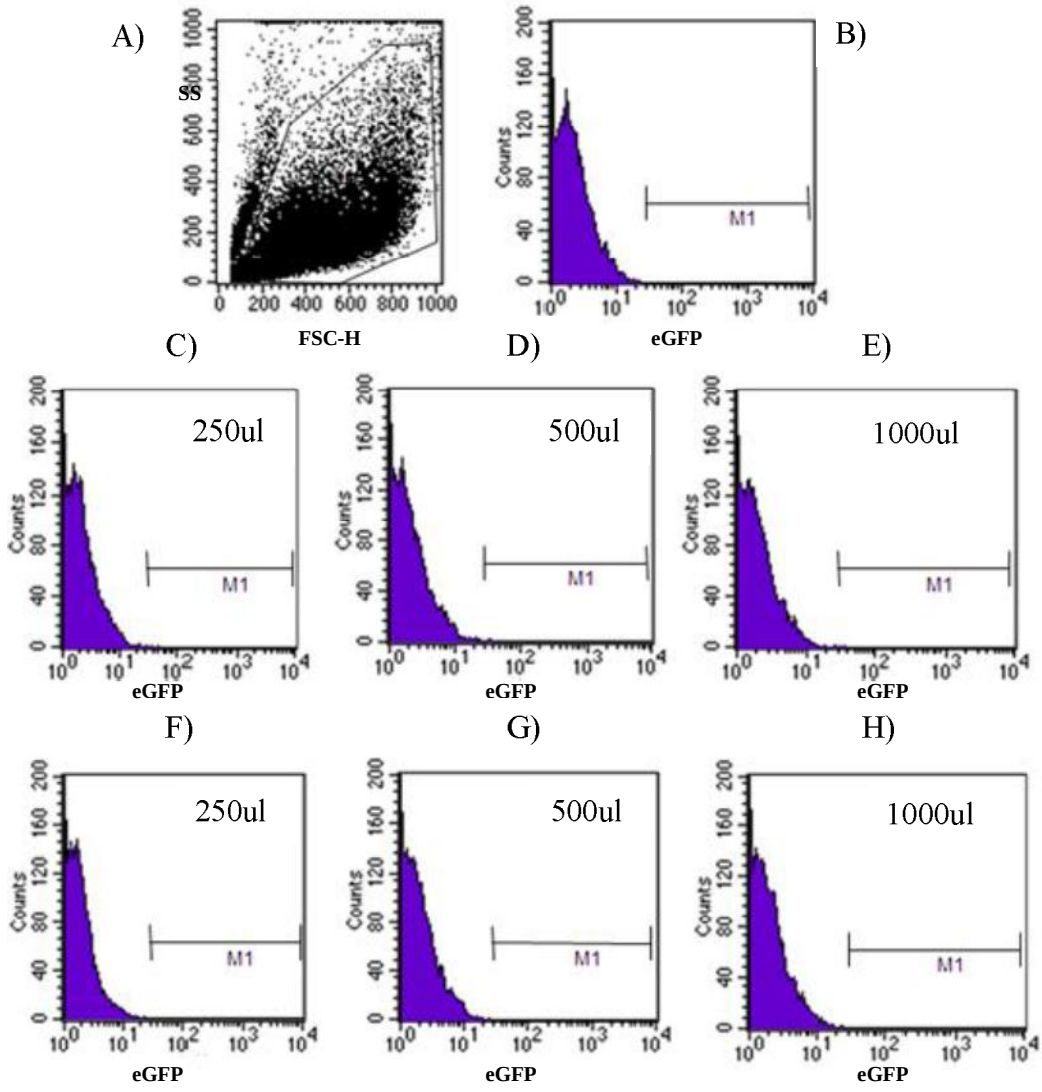


Fig 4.1: A representative example of 293t cell line transduced with pcl10.1 and shRNA-1 vector and analysed by FACS

Figure A shows a representative example of FSC Vs SSC plot of 293t cell line – cells gated with region R1 were analysed for level of eGFP expression. Figure B shows a purple histogram which represents 293t cells not exposed to viral supernatant. Region M1 was set to determine the number of eGFP positive cells. The remaining figures represent 293t cells transduced with 250ul, 500ul and 1000ul of primary viral supernatant of pCL-10.1 (C-E) and shRNA-1 (F-H) respectively and analysed for the level of fluorescence by FACS (n=3).

Table 4.1 show the number of eGFP positive cells measured at each dilution of virus. The dilutions showing between 5-30% positive cells were considered for calculation of the viral titre to maximise the chances that one positive cell corresponded to one viral particle. The viral titre was calculated using the following formula detailed in section 2.10.4. Table 4.2 shows the viral titre of primary viral supernatant in the four shRNA constructs, shRNA-ns and p'HRsincpptSEW.

4.3 Determination of expression of PrP following knock-down

4.3.1 Optimization of lentiviral transduction in hMSC

Following determination of viral titre, transductions in hMSCs were carried out for all shRNA constructs and p'HRsincpptSEW at a primary viral dilution of 1:50 and 1:500. The highest transduction efficiency was found in 1:50 dilution (n=8) (Table 4.3). The transduction efficiency was high with p'HRsincpptSEW, shRNA-2 and shRNA-ns constructs in comparison with shRNA-1, shRNA-3 and shRNA-4 constructs. In an attempt to increase the transduction efficiency, the effect of polybrene was tested. It was carried out using the test shRNA-2 construct and the non-silencing shRNA construct (shRNA-ns) at 1:50 dilution. Forty-eight hours post transduction, the percentage of eGFP positive hMSCs were analysed by FACS. The experiment was conducted in eight samples and the average percentage of eGFP positive hMSCs from eight samples is summarised in Table 4.4 (n=8). The result indicates that polybrene did not influence transduction efficiency and if anything an increase in the percentage of eGFP positive cells was seen in absence of polybrene. Therefore, the following transductions in hMSCs

Dilution	Vector transduced	Percentage of eGFP positive cell (%)
1:500	p'HRsincpptSEW	87.32
1:5000	p'HRsincpptSEW	29.63
1:50,000	p'HRsincpptSEW	5.04
1:500	shRNA-1	30.20
1:5000	shRNA-1	7.59
1:50,000	shRNA-1	2.23
1:500	shRNA -2	40.02
1:5000	shRNA -2	24.44
1:50,000	shRNA -2	5.48
1:500	shRNA -3	55.15
1:5000	shRNA -3	7.11
1:50,000	shRNA -3	1.02
1:500	shRNA -4	54.44
1:5000	shRNA -4	8.32
1:50,000	shRNA -4	1.88
1:500	shRNA -ns	44.62
1:5000	shRNA -ns	22.36
1:50,000	shRNA -ns	5.43

Table 4.1: Percentage of eGFP positive 293t cells following transduction with primary viral supernatant of p'HRsincpptSEW, shRNA-1, shRNA -2, shRNA -3, shRNA -4 and shRNA -ns vector , using three-plasmid lentiviral system and analysed by FACS

Vector transduced	Viral titre (Transducing units (TU)/ml)
p'HRsincpptSEW	10^8
shRNA-1	$1.326 * 10^7$
shRNA -2	$9.905 * 10^7$
shRNA -3	$1.775 * 10^7$
shRNA -4	$2.08 * 10^7$
shRNA -ns	$9.58 * 10^7$

Table 4.2: The viral titre of primary viral supernatant of p'HRsincpptSEW, shRNA-1, shRNA -2, shRNA -3, shRNA -4 and shRNA -ns vector in 293t cells

A)

Dilution of VSN	MOI	Percentage of eGFP +ve cells %
1:50	40	88.63
1:500	4	60.26

B)

Dilution of VSN	MOI	Percentage of eGFP +ve cells %
1:50	5.34	21.22
1:500	0.5304	5.22

C)

Dilution of VSN	MOI	Percentage of eGFP +ve cells %
1:50	39.62	54.46
1:500	3.962	16.22

D)

Dilution of VSN	MOI	Percentage of eGFP +ve cells %
1:50	39.62	14.56
1:500	3.962	5.66

E)

Dilution of VSN	MOI	Percentage of eGFP +ve cells %
1:50	39.62	13.35
1:500	3.962	3.54

F)

Dilution of VSN	MOI	Percentage of eGFP +ve cells %
1:50	38.32	73.50
1:500	3.832	32.33

Table 4.3: Percentage of eGFP positive hMSCs following transduction with primary viral supernatant of p'HRsincpptSEW (A), shRNA-1 (B), shRNA -2 (C), shRNA -3 (D), shRNA -4 (E) and shRNA-ns (F) at the dilution of 1:50 and 1:500

n=8

Dilution of VSN	Vector transduced	MOI	Percentage of eGFP +ve cells with polybrene (%)	Percentage of eGFP +ve cells without polybrene (%)
1:50	p'HRsincpptSEW	40	78.25	88.63
1:50	shRNA -ns	38.32	69.33	73.50

Table 4.4: Percentage of eGFP positive hMSCs following transduction with primary viral supernatant of p'HRsincpptSEW and shRNA-ns in presence or absence of polybrene.

n=8

were carried out without polybrene.

4.4 Knock down of the expression of PrP in hMSCs

To determine which of the four vectors (shRNA-1, shRNA-2, shRNA-3 or shRNA-4) was able to knockdown the highest amount of PrP expression, hMSC culture SNBM-17 was transduced at a similar MOI 40 with either shRNA-1; shRNA-2; shRNA-3; shRNA-4; or in combination shRNA-1,2,3,4 and shRNA-ns as control. As the transduction efficiency was indicated low at MOI 40 (Table 4.3), forty-eight hour post transduction, hMSCs were further sorted to obtain an enriched population of eGFP positive cells using FACS Aria and were expanded in culture. Post sorting, hMSC cultures were expanded in culture for another one passage and analysed for the number of eGFP cells (Table 4.5). Protein lysates was then collected to perform western blotting and level of PrP expression was determined. The test construct shRNA-2 showed the highest inhibition of PrP expression with expression levels at 3% when normalised to GAPDH and expressed as percentage of the same ratio in the shRNA-ns transduced MSC culture (Fig 4.2). ShRNA-2 primary viral supernatant was therefore selected for further experiments.

To further confirm that shRNA-2 was knocking down PrP expression efficiently hMSC cultures (n=8) were then divided into 3 subcultures: a) not exposed to the lentivirus (untransduced, UT), b) exposed to lentivirus containing shRNA-2, c) exposed to lentivirus containing shRNA-ns. The cultures were transduced with primary viral supernatant of shRNA-2 and shRNA-ns at MOI equivalent to 40. Forty-eight hour post

Vector transduced	Percentage of eGFP positive cell (%)
shRNA-1	84.32
shRNA-2	90.22
shRNA-3	86.45
shRNA-4	85.84
shRNA-1,2,3,4	85.11
shRNA-ns	94.52

Table 4.5: Percentage of eGFP positive hMSCs post sort following transduction with shRNA-1, shRNA -2, shRNA -3, shRNA -4, shRNA-1, 2, 3, 4 and shRNA -ns vector, using 2nd generation lentiviral system and analysed by FACS

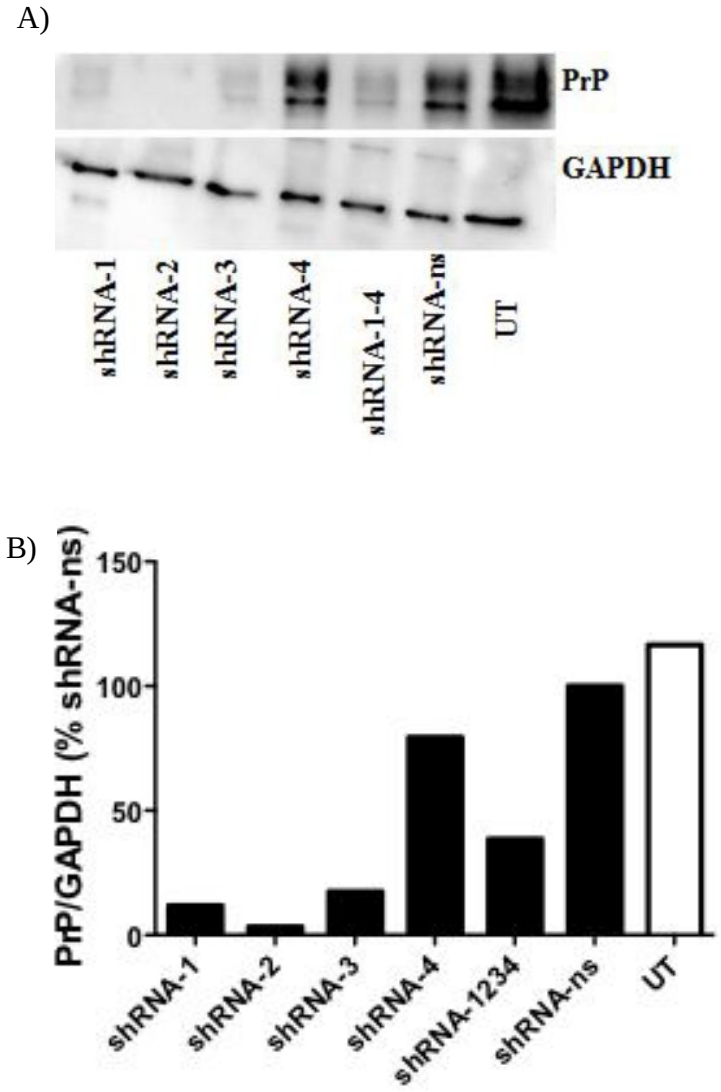


Fig 4.2: sh-RNA2 viral supernatant shows maximum efficacy in knocking down the expression of PrP in MSC by western blot.

Figure A shows western blot of hMSC culture transduced with primary viral supernatant of shRNA-1; shRNA-2; shRNA-3; shRNA-4; or in combination shRNA-1,2,3,4 and shRNA-ns and labelled with antibodies specific for cellular prion protein (top panel) and glyceraldehydes-3 phosphate dehydrogenase (GAPDH, bottom panel). Figure B shows quantification of PrP expression normalised to GAPDH and expressed as percentage of non-silencing vector shRNA-ns in hMSC culture transduced with shRNA-1; shRNA-2; shRNA-3; shRNA-4; or in combination shRNA-1,2,3,4 and shRNA-ns. Human MSC not exposed to viral supernatant served as untransduced control (UT).

transduction, hMSCs were further sorted using FACS Aria. Human MSC UT cultures were used as a negative control. By gating the R1 region on a FSC vs. SSC plot, no eGFP positive cells were present in hMSC cultures not exposed to viral supernatant (Fig 4.3A-B).In contrast selecting the same region in shRNA-ns hMSC, (Fig 4.3C-D) and shRNA-2 hMSC (Fig 4.3E-F) showed presence of eGFP positive cells. Post sorting hMSC cultures were expanded in culture for another one passage and analysed for the number of eGFP cells which was found to be 93.26%±3 eGFP+ when transduced with shRNA-ns and 86%±9% with shRNA-2 (Fig 4.4).

To confirm whether there was a reduction in the levels of PrP expression following transduction with eGFP positive hMSCs containing the shRNA-2 vector (shRNA-2 hMSC), protein lysates were collected at the first passage and western blotting was carried out. When assessed for the levels of expression of PrP expression in shRNA-2 hMSC cultures, they expressed significantly reduced levels of PrP (93.5%±5.7%) compared to shRNA-ns transduced cultures (Fig 4.5A and B, n=4). No significant difference in PrP levels was seen when comparing untransduced and shRNA-ns hMSC cultures (n=4).

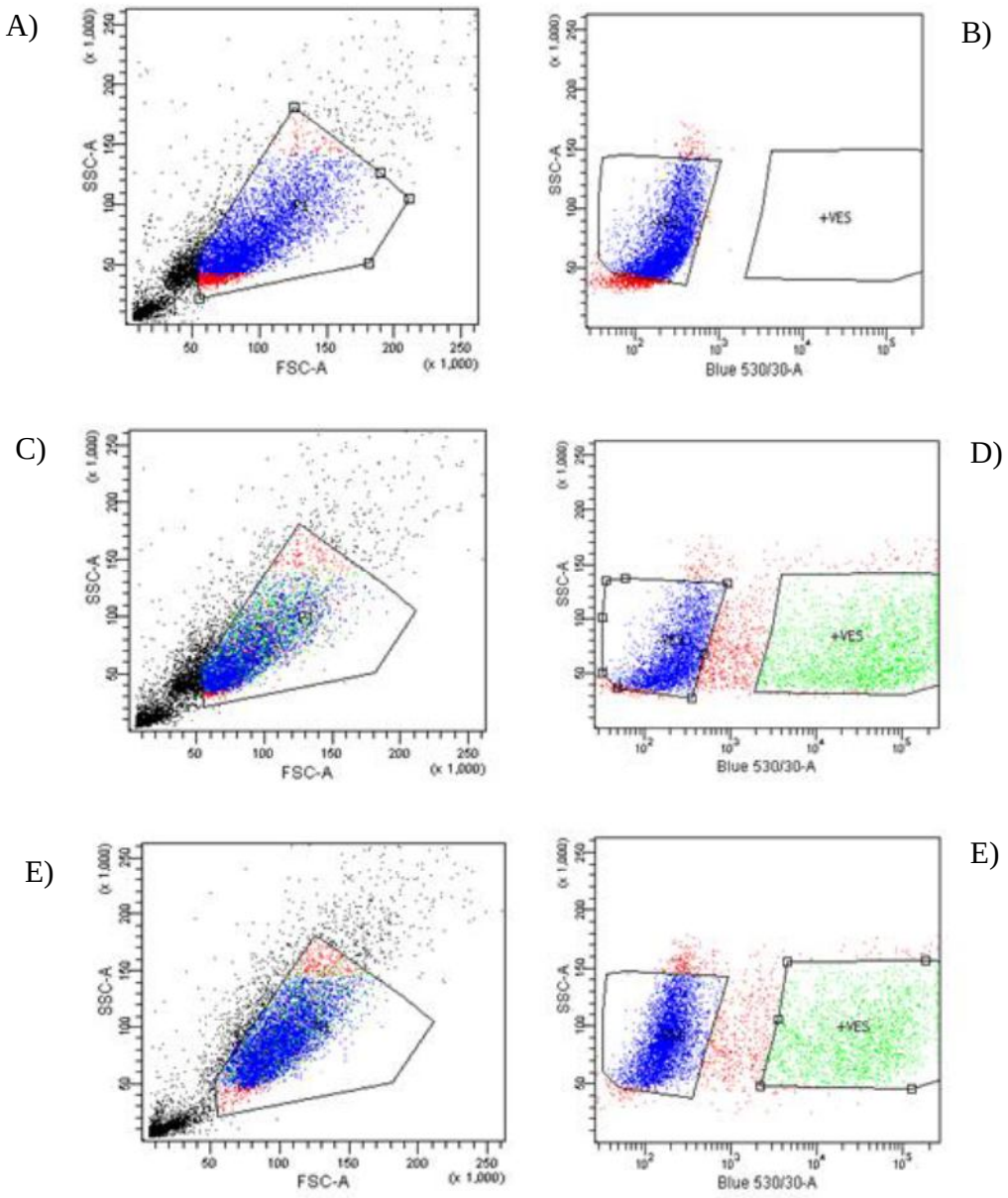
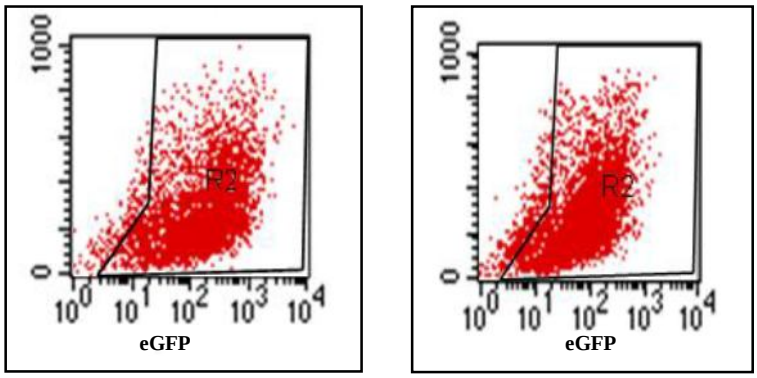


Fig 4.3: A representation of selection of eGFP positive hMSCs transduced with primary viral supernatant of shRNA-ns and shRNA-2, post sorting by FACS Aria

Figure A, C and E represent FSC Vs SSC plot of hMSC culture – cells gated with region R1 were analysed for level of eGFP expression. Figure B shows a scatter plot of hMSC culture not exposed to viral supernatant. Scatter plot in green represents the number of sorted green cells transduced with primary viral supernatant of shRNA-ns (D) and shRNA-2 (E) at MOI 40 (n=8).

A)



B)

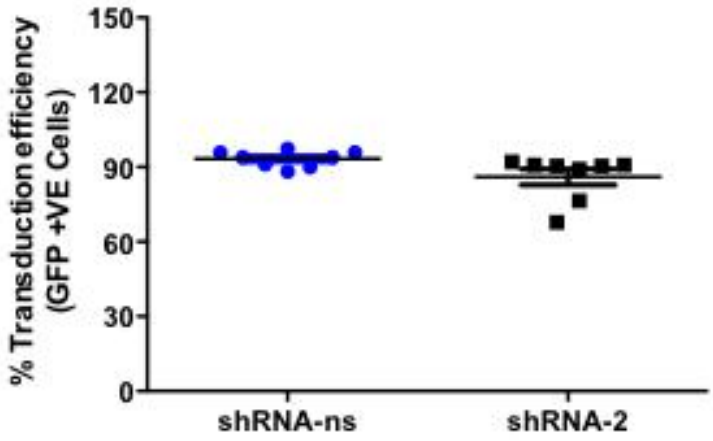
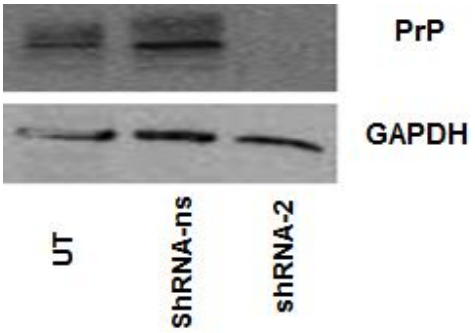


Fig 4.4: Transduction efficiency of eGFP positive hMSC transduced with primary viral supernatant of shRNA-ns and shRNA-2 virus post selection

Figure A is a representative example of scatter plot. In red represents the number of eGFP positive hMSC cells post sort and previously transduced with primary viral supernatant of shRNA-ns and shRNA-2 at MOI 40 (n=8). Figure B shows percentage of eGFP positive hMSCs in the cultures used in subsequent experiments (n=8).

A)



B)

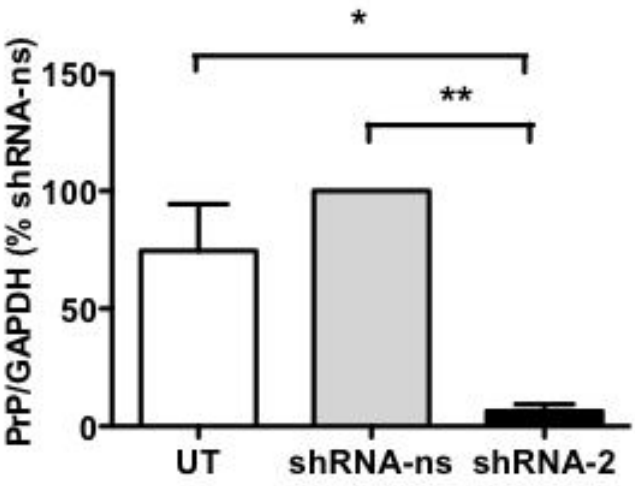


Fig 4.5: Detection of decreased level of PrP expression in hMSC cultures transduced with shRNA-2 virus

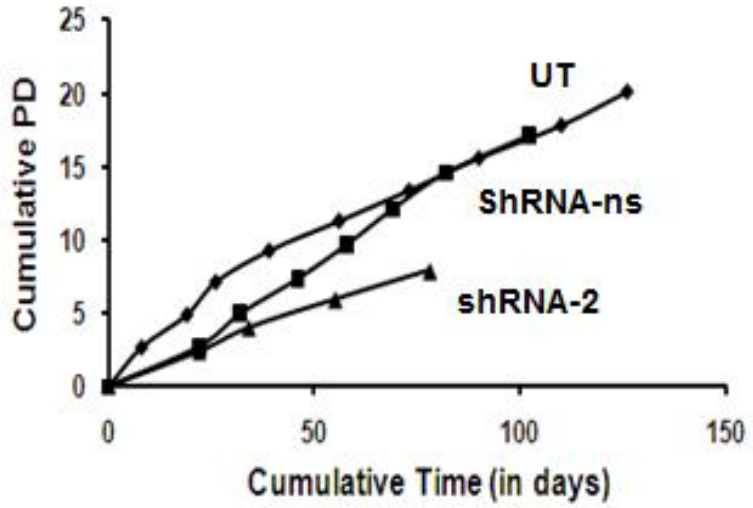
*Figure A shows western blot of hMSC culture transduced with shRNA-2and shRNA-ns and labelled with antibodies specific for cellular prion protein (PrP, top panel) and glyceraldehydes-3 phosphate dehydrogenase (GAPDH, bottom panel). Figure B shows quantification of PrP expression normalised to GAPDH and expressed as percentage of non-silencing vector shRNA-ns in hMSC cultures transduced with shRNA-2 and shRNA-ns. Human MSC not exposed to viral supernatant served as untransduced control (UT) (n=4). Data presented as mean ± SEM and analysed by one way ANOVA with Bonferroni multiple comparison post-test, *p<0.05, **p<0.01.*

4.5 Assessment of growth kinetics and clonogenic potential in PrP knockdown hMSC cultures

Following transduction and selection of eGFP positive cells, hMSC cultures were expanded in culture and growth kinetics was examined until proliferation stopped for 3 weeks, at which point the cultures were considered senescent. ShRNA-2 hMSC cultures underwent an average 8.5±1.7 less total number of PDs compared to shRNA-ns hMSC cultures (Fig 4.6A-B, n=5).

Moreover, to determine whether knocking down the expression of PrP affected the clonogenic potential of hMSCs, I expanded the UT, shRNA-2 and shRNA-ns hMSC cultures for 15.4±0.5 days and all the three subcultures were re-plated at low density to assess the number of clonogenic progenitors. The result indicated that shRNA-2 hMSC cultures contained significantly reduced number of clonogenic progenitors as indicated by the number of CFU-F (Fig 4.7A, n=8), CFU-O (Fig 4.7B, n=8) and CFU-A (Fig 4.7C, n=8) compared to shRNA-ns or UT cultures, suggesting that expression of PrP is important to maintain the proliferative capacity of hMSCs.

A)



B)

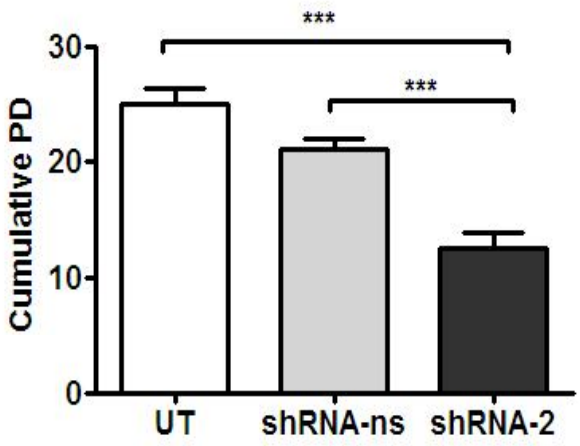


Fig 4.6: Human MSC cultures transduced with shRNA-2 virus show decreased expansion capacity

*Figure A shows a representative example of growth kinetic of hMSC culture, not exposed to viral supernatant (untransduced, UT, filled diamonds) transduced with shRNA-ns (filled squares) or shRNA-2 (filled triangle). Figure B shows total number of population doublings accomplished by hMSC cultures transduced with shRNA-2 compared to the same cultures transduced with the shRNA-ns or not transduced (n=5). Data presented as mean ± SEM and analysed by one way ANOVA with Bonferroni multiple comparison post-test, *p<0.05, **p<0.01, ***p<0.001.*

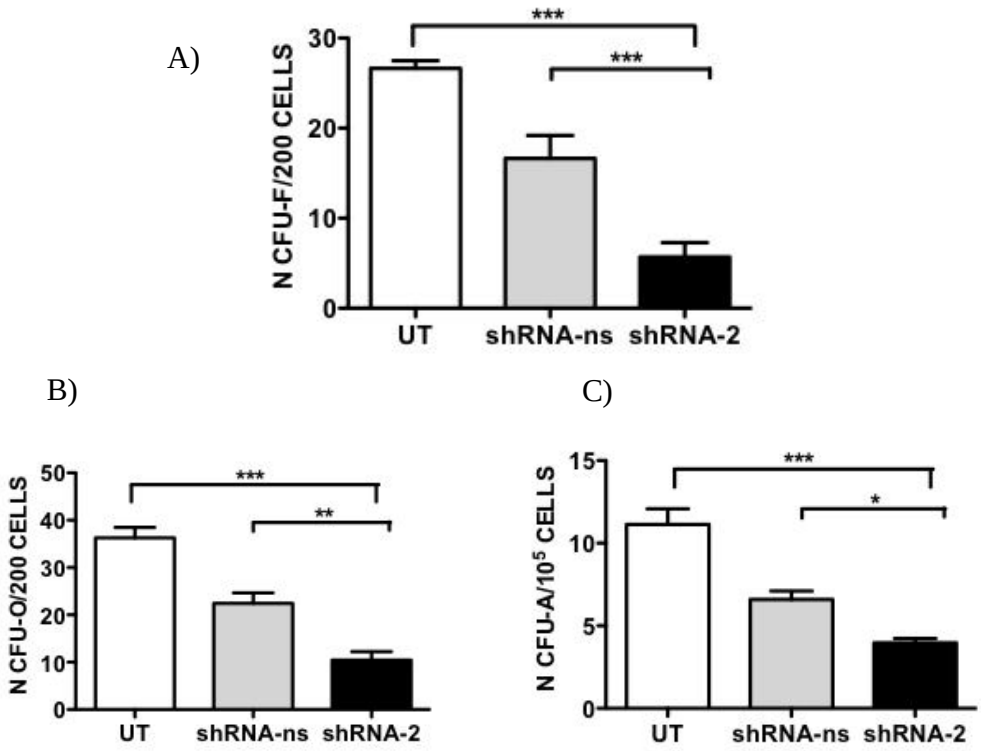


Fig 4.7: The number of clonogenic progenitors decreases in hMSC cultures transduced with shRNA-2 virus

*Figure A, B and C shows the quantification of the number of CFU-F, CFU-O and CFU-A respectively, obtained upon re-plating at low density of shRNA-2, shRNA-ns and untransduced hMSC cultures (UT). (n=8). Data presented as mean ± SEM and analysed by one way ANOVA with Bonferroni multiple comparison post-test, *p<0.05, **p<0.01, ***p<0.001.*

4.6 Assessment of differentiation potential in PrP knockdown hMSC cultures

To determine whether reduced PrP expression affected differentiation of hMSCs, cultures were also exposed to osteogenic and adipogenic differentiation supplements for 2 weeks and assessed for markers of osteogenic and adipogenic differentiation respectively by quantitative RT-PCR. As expected an increase in all markers of differentiation was observed after two weeks exposure to differentiation stimuli in UT cultures or shRNA-ns hMSC cultures (Fig. 4.8A-F n=4). In contrast a significant decrease in all markers of differentiation was seen in cultures exposed to differentiation supplements and transduced with shRNA-2 compared to the undifferentiated controls (Fig 4.8A-F, n=4), suggesting that low PrP expression reduces hMSC differentiation.

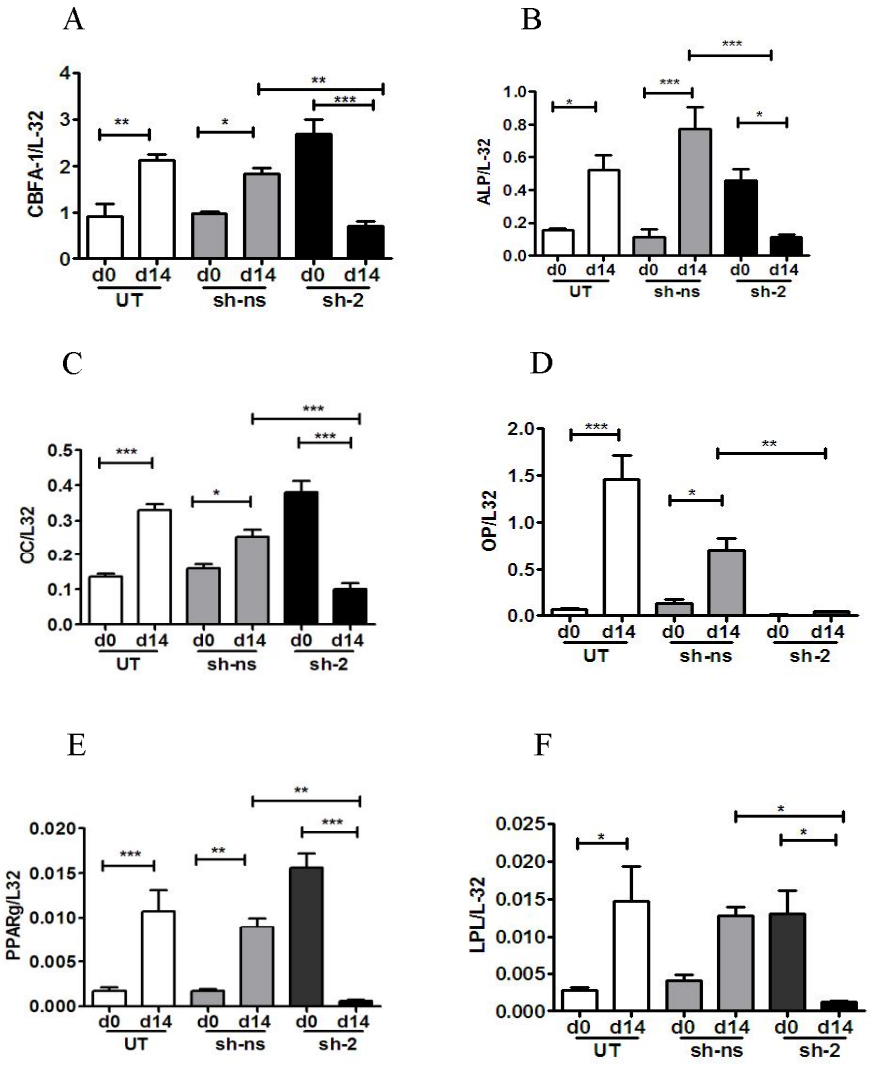


Fig 4.8: Human MSC cultures transduced with shRNA-2 inhibits differentiation of hMSC cultures

*The graphs shows real-time qPCR of shRNA-2, ShRNA-ns and untransduced hMSC cultures exposed to osteogenic and adipogenic differentiation supplements for 14 days and assessed for the expression of osteogenic differentiation marker (A) core-binding factor subunit alpha-1 (CBFA-1), (B) alkaline phosphatase (ALP), (C) osteocalcin (OC) and (D) osteopontin (OP) and adipogenic differentiation marker (E) peroxisome proliferator-activated receptor γ (PPAR-γ) and (F) lipoprotein lipase (LPL) (n=4). All markers were normalised to ribosomal protein L-32 and assessed by RT-PCR. Data presented as mean ± SEM and analysed by one way ANOVA with Bonferroni multiple comparison post-test,*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*

4.7 Discussion

This study was designed to investigate the functional role of PrP in maintaining the proliferation and differentiation ability of hMSCs following expansion in culture. To achieve this, firstly I have identified a shRNA sequence which knockdowns PrP expression effectively using lentiviral vectors. Secondly, I determined the growth kinetics, clonogenic ability and ability to differentiate to osteogenic and adipogenic lineage in hMSC cultures where the PrP expression was knocked down.

Genetically modified hMSCs have been produced by incorporation of various viral vectors such adenovirus (Lou, Xu et al. 1999; Kumar, Mahendra et al. 2004), lentivirus and even non-viral vectors (Kyriakou, Yong et al. 2006) for the delivery of shRNAs. However lentiviral vectors stand an advantage owing to their ability to stably transduce a wide range of different cell types and especially promote transduction of non-dividing cells (Blomer, Naldini et al. 1997; Kafri, Blomer et al. 1997; Naldini 1998; Gasmi, Glynn et al. 1999), including stem cells (Zhang, La Russa et al. 2004; Van Damme, Thorrez et al. 2006). Most lentiviral supernatants are produced by a 3rd or 2nd generation lentiviral vector system. In this study, I initially used the 3rd generation lentiviral vector system because it was considered safer and was available in a collaborating group in the department. However, no transduction of HEK293t cells, usually easier to transduce than hMSC was seen. The reasons for the absence of transduction are not clear but most likely are to do with a very low viral titre. In a very recent study McGinley L et al, (2011) showed that using a three-plasmid lentiviral system, at MOI 100, transduction efficiency in rat MSCs over a span of 7-28 days were in the range of 90%-98%

compared to four-plasmid system which generated only 1-5% GFP positive MSCs (McGinley, McMahon et al. 2011), suggesting that a three-plasmid lentiviral system could generate high transduction efficacy compared to four-plasmid lentiviral system. I therefore decided to switch to 2nd generation lentiviral vector system where I found that the transduction efficiency in HEK293t cells were higher.

Of interest, while the transduction of hMSCs with p'HRsincpptSEW (expressing only eGFP) showed a transduction efficiency of approximately 88% with single exposure of virus for 8 hours at MOI 40, transduction of hMSCs with shRNA-2 and shRNA-ns was much lower. One possibility is that the transfer vector shRNA-2 and shRNA-ns were not compatible with the packaging system (materials and methods section 2.10.1) as the vectors were produced by the company, Autogen bioclear UK Ltd and they advised the use of their own packaging system. Alternatively, this may have been to do with the specific sequence used to knockdown PrP. Indeed I have shown that different construct showed different levels of transduction**.** The use of same control vector p'HRsincpptSEW in murine MSCs at MOI 50 reported by Anjoso-Afonso et al, 2004 lead to similar transduction efficiency as I have obtained (Anjos-Afonso, Siapati et al. 2004) suggesting that the protocol I was using was appropriate and that the particular vectors I was using to knockdown may be the cause of lower transduction efficiency.

In an attempt to increase the transduction efficiency, I tested the use of a cationic polymer, polybrene (4µg/ml) as this has been shown to increase transduction efficiency in some studies (Zhang, La Russa et al. 2004). If anything, the transduction efficiency was lower in the presence of polybrene. This is not surprising as some reports have

suggested no difference in the outcome of transduction efficiency in presence or absence of polybrene (Zielske and Gerson 2002; Mostoslavsky, Kotton et al. 2005). To ensure that the knock-down occurred in a high number of hMSC, selection of eGPF positive hMSCs was performed by fluorescent activated cell sorting. Following selection of eGFP positive hMSCs, the cells were expanded in culture in order to obtain sufficient numbers to determine whether the clonogenic and differentiation potential of shRNA-2 transduced hMSC cultures was affected in absence of PrP in those cultures. I showed a reduction in both proliferation and differentiation of hMSC, suggesting an impairment in stem cell properties similar to what was observed in the study by Zhang C C et al, 2006 which demonstrated that PrP-null bone marrow cells showed less engraftment and impaired self-renewal ability in HSC (Zhang, Steele et al. 2006).

To determine whether the proliferation ability of hMSCs was compromised when expression of PrP was knocked down, growth kinetics was carried out in hMSC cultures in presence or absence of PrP. I showed that shRNA-2 transduced hMSC cultures stopped proliferating much earlier than UT and shRNA-ns hMSC cultures. It is possible that proliferation in shRNA-2 hMSC cultures was disrupted because the cells either underwent more apoptosis or they became more senescent. The former occurrence is unlikely to happen as I did not observe any dead cells floating in the culture medium; rather the cells would have become senescent. However, to confirm whether proliferation ability was indeed compromised or enhanced, future studies involving a Brdu (bromoxyuridine) staining assay could be conducted to determine the number of proliferating cells in hMSCs cultured in presence or absence of PrP, as Brdu is incorporated into the newly synthesized DNA of only proliferating cells. Similarly, cell

viability assay such as an apoptosis assay involving caspases could be carried out to determine the number apoptotic cells in hMSCs cultured in in presence or absence of PrP. Also, to determine whether cells cultured in absence or presence of PrP were senescent or not, senescent marker such as β-galactosidase could be used.

The mechanism for reduced proliferation and differentiation ability in absence of PrP may be multiple and studies on several actions of PrP are available but data are not very strong. One possibility is that like any GPI-anchored protein, as PrP localises in lipid rafts in the plasma membrane, it is possible that PrP might interact with certain signalling proteins in these domains to facilitate cell-surface signalling and cell adhesion (Naslavsky, Stein et al. 1997). For example PrP has been reported to interact with several adhesion molecules including laminin (LM) and its receptor (Graner, Mercadante et al. 2000). PrP specifically binds to the γ-1carboxyterminal domain of LM. Laminins are known to play an important role in promoting adhesion and regulating osteogenic differentiation in MSCs (Klees, Salasznyk et al. 2005; Hashimoto, Kariya et al. 2006). For example LM-5 stimulated osteogenic differentiation of hMSCs by interacting with LM receptor, α3β1 integrin and mediated activation of extra-cellular signal related kinase (ERK) pathway (Klees, Salasznyk et al. 2005; Hashimoto, Kariya et al. 2006). ERK is a member of mitogen-activated protein (MAP) kinase family which stimulates differentiation of hMSC into osteoblasts via phosphorylation of osteogenic transcription factor RUNX-2/CBFA-1 (Jaiswal, Jaiswal et al. 2000). Indeed, hMSCs coated on LM-5 stimulated differentiation of hMSCs to osteoblasts by phosphorylation of RUNX-2/CBFA-1 by 2.5 times more and expressed osteogenic markers such as alkaline phosphatase, osteopontin and osteocalcin compared to hMSCs plated on poly-L-

lysine control surfaces. Addition of ERK inhibitor PD98059 reduced phosphorylation by nearly 50% and also reduced expression of osteogenic markers significantly (Klees, Salasznyk et al. 2005). Recently, Mruthyunjaya S et al., (2010) reported that LM-1 promoted induction of neurite outgrowth in hMSCs in serum-deprived and differentiation free conditions by interaction with LM-1 specific integrin receptor-α6β1. This interaction subsequently activated the MAPK pathways which were confirmed by the elevated levels of phosphorylated-MEK and –ERK from cells plated on LN-1 coated surfaces compared to poly-L-lysine coated control surfaces (Mruthyunjaya, Manchanda et al. 2010). All these studies suggests that PrP could promote differentiation by enhancing LM signalling which is important in regulating differentiation of stem cells to osteoblasts by activating MAP kinase pathway. This might explain the block in differentiation that I see in my study.

Another possibility is that PrP has been reported to interact with glycosaminoglycans (GAG) (Pan, Wong et al. 2002) which is a major constituent of ECM. One particular member of GAG family that seems to associate stronger was heparin. The binding of PrP with heparin was specific to N-terminal region of PrP and was shown to be enhanced in presence of Cu^{+2} and Zn^{+2} (Pan, Wong et al. 2002, (Gonzalez-Iglesias, Pajares et al. 2002). Heparin sulphate (HS) is an important GAG family member whose function is to control the activity of several growth factors; primarily fibroblast-growth factor-2 (FGF-2) produced by osteoblast and modulates MSC and osteoblast proliferation through autocrine/paracrine mechanisms (Jackson, Nurcombe et al. 2006). MSCs supplemented with HS-2 triggered significant production of FGF-2 and increased the cell number by stimulating a population of quiescent cells to enter into cells cycle

and sustain their proliferation (Helledie, Dombrowski et al. 2012). Moreover hMSCs expanded for over 15 population doublings had significantly longer telomeres in presence of HS-2 compared to control media (Helledie, Dombrowski et al. 2012). Also GAG mimetic compound such as OTR_{4120} stimulated the proliferation, migration and osteogenic phenotype of MSC *in vitro* and such effects were FGF-2-dependant interactions as the effects were mitigated in presence of a FGF receptor-1 signalling pathway blocker (Frescaline, Bouderlique et al. 2012). All these studies suggest that PrP could promote proliferation by enhancing GAG signals which were important to maintain the proliferation and telomere length of MSC in an autocrine loop.

A third possibility is that PrP has been also proposed to act against reactive oxygen species (ROS)-mediated DNA damage. Indeed following exposure of ROS by either hydroxyl radicals following exposure to Cu^{2+} or Fe^{2+} or singlet oxygen following exposure to photosensitizer methylene blue and white light, human neuroblastoma SH-SY5Y cells transfected with PrP showed significantly lesser DNA damage compared to untransfected SH-SY5Y cells (Watt, Routledge et al. 2007). Mice brains devoid of PrP also showed increased levels of oxidative stress markers compared to wild type mice (Wong, Liu et al. 2001). Moreover lack of PrP expressions resulted in a phenotype that was more sensitive to oxidative stress (Brown, Schulz-Schaeffer et al. 1997). ROS levels may act in two ways by increasing DNA damage and reducing stem cell function both proliferation and differentiation or as signalling molecule mediator. In a very recent study it was shown how ROS levels may modulate the regeneration ability of stem cells by acting as a signalling mediator and directing the self-renewal or differentiation of stem and progenitor cells. In drosophila multipotent hematopoietic progenitors displayed

increased levels of ROS under *in vivo* physiological conditions, which were down regulated on differentiation. However, scavenging ROS from these progenitors by increasing the expression of catalase retarded their differentiation into mature blood cells. Conversely increasing hematopoietic progenitors ROS beyond the basal levels by mutating SOD2 gene triggered precocious differentiation into all 3 mature blood cell types which was mediated through a signalling pathway involving JNK and FoxO activation (Owusu-Ansah and Banerjee 2009). This suggested that an optimal level of ROS may be necessary to maintain stem/progenitor cells and any further ROS increase or decrease away from the wild type basal levels may enhance or suppress differentiation. There is evidence that this may happen in hMSC too and lend an explanation to the reduction in differentiation to the osteogenic and adipogenic lineage which I have observed when PrP expression was knocked down. Indeed ROS have been seen to increase during osteogenic differentiation with concomitant increase of SOD2 but excess ROS levels by exogenous addition of H_2O_2 inhibited osteogenic differentiation (Chen, Shih et al. 2008). From my data, it is also probable that the differentiation of hMSCs transduced with shRNA-2 could have been more differentiated to osteogenic and adipogenic lineage at the start of differentiation (day 0). Since ROS levels are crucial to the shift between self-renewal and differentiation and that there is an optimal level of ROS required to promote differentiation (Chen, Shih et al. 2008), it could be speculated that when PrP is knocked down in hMSC cultures, there is less buffering of ROS and the cells start undergoing more differentiation at the start of differentiation itself. Future studies are warranted in attempt of this to measure ROS levels at day 0 and day 14 of hMSC cultures differentiated to osteogenic and adipogenic lineage in absence or presence of PrP.

To summarise, my data showed that lack of PrP expression resulted in dysfunctional stem cell pool with signs of accelerated ageing. It will be now interesting to understand how this occurs and to determine whether chemical intervention using a small molecule that targets PrP will delay cellular ageing. This was extensively studied in the next chapter.

CHAPTER-5

5.1 Introduction

As knocking down the expression of PrP in hMSC cultures showed reduced clonogenic and differentiation ability, I wanted to determine whether stabilizing the expression of PrP levels using a small molecule modulator of PrP would preserve their clonogenic and differentiation ability following expansion in culture. Small molecules can modulate stem cell fate and could be used to identify new mechanisms that regulate stem cell behaviour (Gambardella, Nagaraju et al. 2010). A number of small molecules have been identified which modulate the proliferation and differentiation ability of stem cells by interfering with signalling pathways such as the Wnt signalling pathway (Ding, Wu et al. 2003; Gambardella, Nagaraju et al. 2010), hedgehog mediated signalling (Wichterle, Lieberam et al. 2002) and BMP/SMAD signalling pathway (Park, Waki et al. 2009). Administration of small molecule inhibitors of glycogen synthase kinase (GSK-3) have shown to augment haematopoietic repopulation into recipient NOD/SCID mice, which have been transplanted with human HSCs, suggesting that the small molecule acts as regulator in HSC repopulation *in vivo* (Trowbridge, Xenocostas et al. 2006). Indeed, in our previous studies we have shown that another small molecule inhibitor of GSK-3α/β was able to increase the number of endogenous MSC progenitor and induce their differentiation to the osteogenic lineage at the expense of adipogenesis in vitro and in vivo (Gambardella, Nagaraju et al. 2010). Similarly, small molecule modulators of PrP are also available (Thompson, Borsenberger et al. 2009; Thompson, Louth et al. 2011). However, use of those small molecules to modulate hMSC proliferation and differentiation *in vitro* and *in vivo* has not yet been identified.

The small molecule modulator of PrP, I have selected was obtained from a virtual high throughput screening (VHTS) carried out to select binders of PrP from a library of 1.3 million compounds designed to target PrP. A sensor-based PrP binding assay (SPR, Biacore) was validated and used as a primary screening tool to identify affinity ligands to PrP (Touil, Pratt et al. 2006). The Biacore screening had revealed 100 binders. Four hundred analogues were synthesized and screened using embryonic carcinoma stem cells. The screening was carried out on embryonic carcinoma stem cells because they were easier to culture in large numbers. The small molecules were tested on these cells for their ability to proliferate by fluorescence imaging. Two potential hits were obtained (3/165 and 3/689). In this chapter, I have tested both molecules and chose to study the effects of 3/689 in more detail. I have tested whether 3/689 was able to enhance the proliferation of hMSCs and extend their lifespan in culture, while retaining their ability to differentiate and engraft to bone marrow. I have also investigated the mechanism of action of the PrP binder 3/689 by whole genome expression arrays and functional assays.

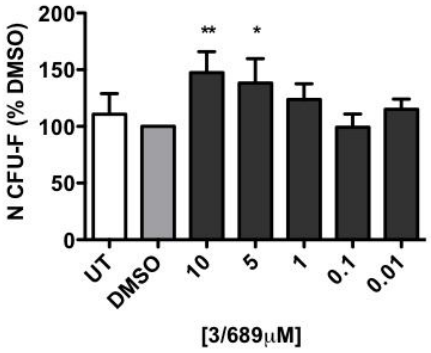
5.2 Small molecule modulators of PrP (3/165 and 3/689) increase the number of clonogenic progenitors

To determine the best dose at which small molecule modulators of PrP showed activity on mesenchymal progenitors, previously established hMSC cultures were seeded at low density and exposed to 3/689 and 3/165 at varying concentrations. The hMSC cultures exposed to 3/689 and 3/165 were fed twice a week in culture for 14 days and assessed for the number of CFU-F. A significant increase in the number of CFU-F was observed

when cells were exposed to 3/689 at 10µM and 5µM (Fig 5.1A, n=7). But no significant effect was observed with 3/165. However, when I extended the dose of 3/165 further to concentrations of 0.001uM and 0.0001uM, the number of CFU-F was found to be significantly increased at 0.001 µM (Fig 5.1B and C, n=6). As the effect with 3/689 was more pronounced, I decided to carry out the rest of the work with this compound.

To determine whether 3/689 showed activity on the clonogenic progenitors with osteogenic and adipogenic potential, previously established hMSC cultures were exposed to 3/689 at concentration from 10µM to 1µM in presence of osteogenic and adipogenic supplements and the number of CFU-O and CFU-A were assessed. A significant increase in the number of CFU-O was observed when cells were exposed to 3/689 at 10µM, 5 µM and 1 µM (Fig 5.2A, n=6). A similar response was also seen when measuring the effects on mesenchymal progenitors with adipogenic potential at 10µM (Fig 5.2B, n=6).

A)



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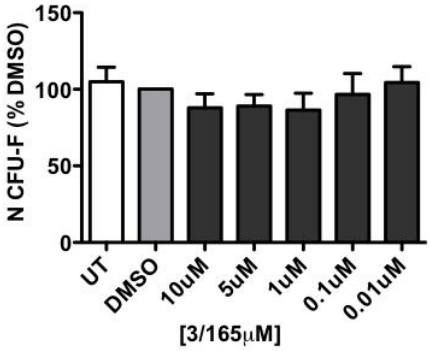

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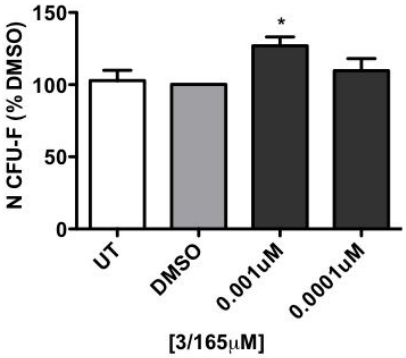
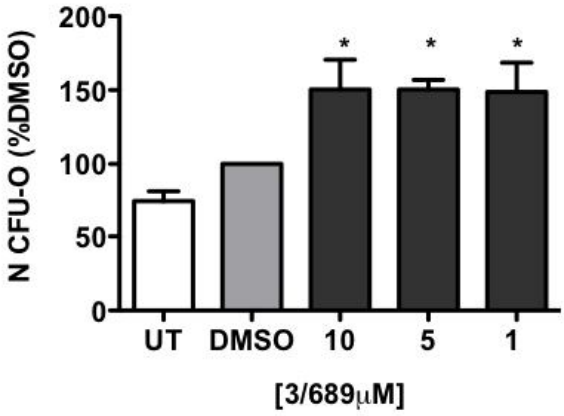


Fig 5.1: Small molecule modulators of PrP increases the number of CFU-F in hMSC cultures

*Figure A shows the effect of 3/689 on the number of colony forming unit-fibroblast (CFU-F, n=7). Figure B and C show the effect of 3/165 on the number of CFU-F (n=6). The CFU-F was obtained by plating hMSC from established cultures at low density. Data presented as mean ± SEM and analysed by one way ANOVA with Bonferroni multiple comparison post-test, *p<0.05, **p<0.01, ***p<0.001.*

A)



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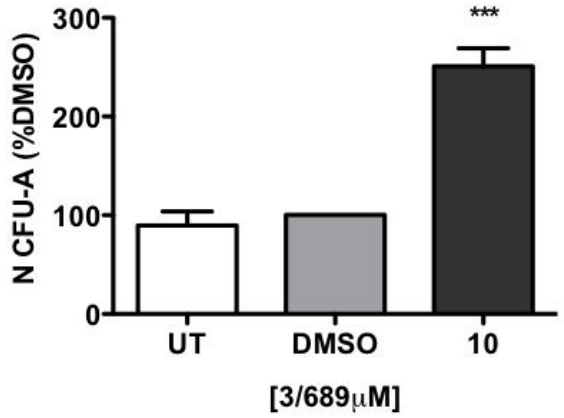


Fig 5.2: 3/689 increases the number of CFU-O and CFU-A in hMSC cultures

*Figure A and B show the effect of 3/689 on the number of colony forming unit-osteoblast (CFU-O) and -adipocyte (CFU-A) obtained by plating hMSC from established cultures (n=6) at low density. Data presented as mean ± SEM and analysed by one way ANOVA with Bonferroni multiple comparison post-test, *p<0.05, **p<0.01, ***p<0.001.*

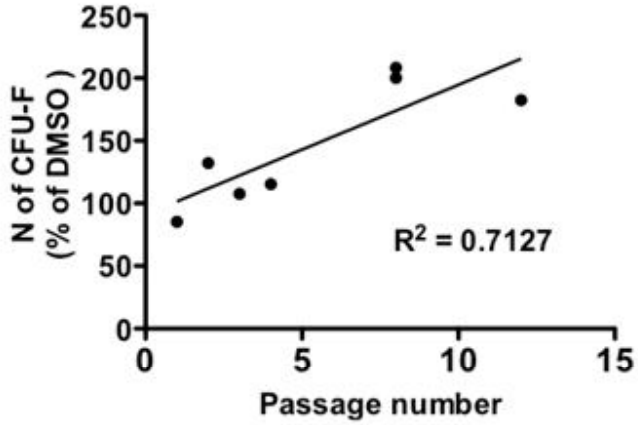
5.3 3/689 has higher activity with cellular ageing

During the analysis of the effect of 3/689 on the clonogenic progenitors, I noticed that there was a significantly positive strong correlation between the increase in the number of CFU-F obtained following exposure to 3/689 at 10μM and the time hMSC had been in culture prior to clonogenic assay (Fig 5.3A, n=7, r^2=0.7127, p=0.02) compared to untreated hMSC (Fig 5.3A, n=7, r^2=0.1428, p=0.40). The hMSCs that had been expanded for longer periods of time had an enhanced response. This prompted us to test whether this was also true when hMSCs were derived from older donors compared to younger donors. Human MSC cultures were isolated from the bone marrow of donors aged 9-14 years old (n=6) and 55-80 years old (n=5) and allowed to proliferate for similar numbers of PD (passage 2) prior to exposure to 3/689. A significant increase in the number of CFU-F was seen in cultures derived from older donors in presence of 3/689 compared to younger donors (Fig 5.3B, n=5, p=0.03). These data suggests that 3/689 has more potent effect with cellular ageing.

5.4 3/689 requires PrP expression for its activity

To determine whether 3/689 required PrP expression for its action, hMSC cultures untransduced, transduced with shRNA-2 vector (expressing low levels of PrP) or transduced with a shRNA-ns vector (non-silencing vector) were seeded at low density in presence or absence of 3/689 and assessed for the number of CFU-F and CFU-O. As expected untransduced (Untrans) and shRNA-ns hMSC cultures showed a significant increase in the number of CFU-F (Fig 5.4A, n=8) and CFU-O (Fig 5.4B, n=8). In

A)



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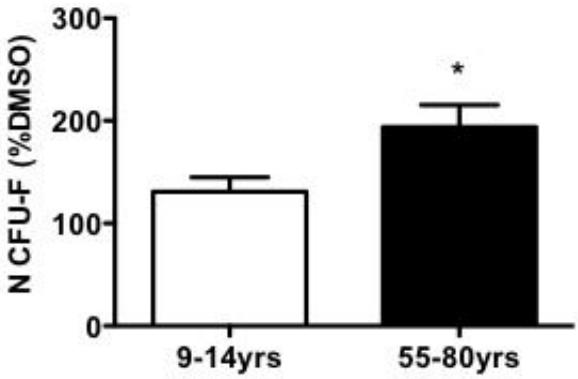


Fig 5.3: 3/689 has higher activity on hMSC cultures with cellular ageing

Figure A shows the percentage increase in the number of colony forming unit-fibroblast (CFU-F) in presence (black circle) or absence (black square) of 3/689 (compared to DMSO control) with time in culture (n=7). Figure B show the percentage increase in the number of CFU-F in hMSC cultures derived from older donors (n=5) compared to younger donors (n=6) in presence of 3/689 (Data represented as percentage of DMSO control). Data presented as mean ± SEM and analysed by unpaired student's t test, p-0.03.

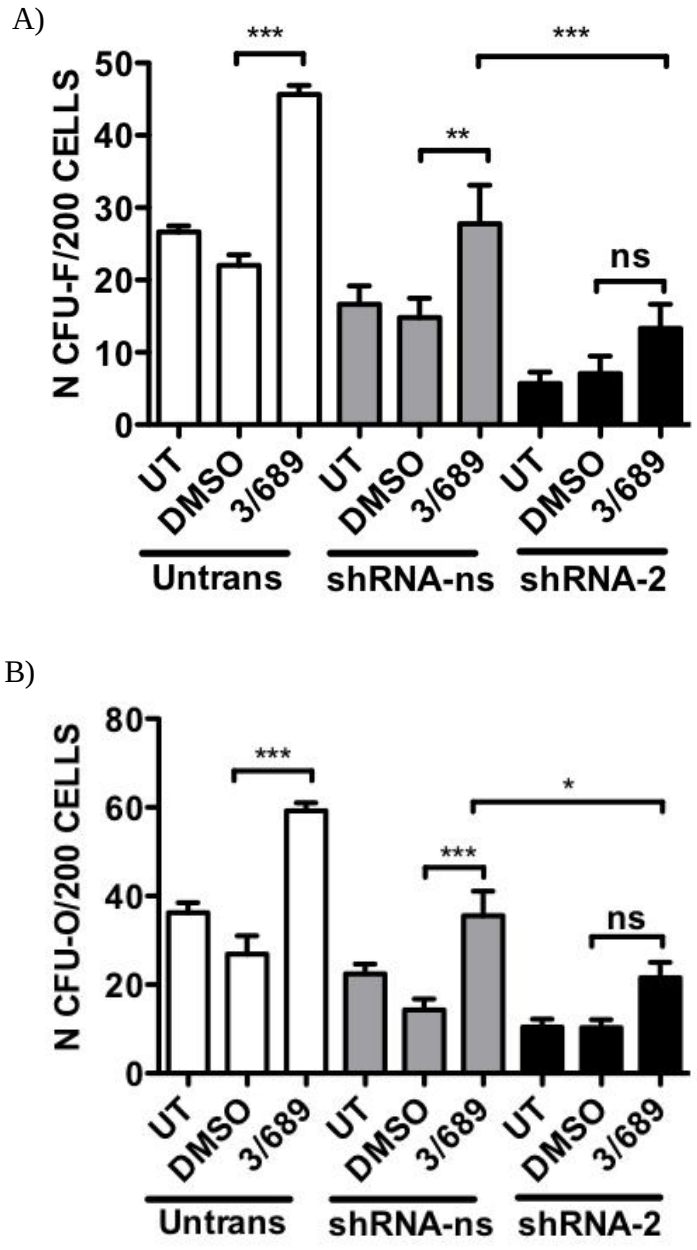


Fig 5.4: 3/689 requires PrP expression for its activity

*Figure A and figure B show hMSC cultures untransduced, transduced with shRNA-ns and shRNA-2 in presence or absence of 3/689 and assessed for the number of colony forming unit-fibroblast (CFU-F) and –osteoblast (CFU-O) respectively. (N=8). UT- hMSC cultures expanded in medium alone. Data presented as mean ± SEM and analysed by one way ANOVA with Bonferroni multiple comparison post-test, *p<0.05, **p<0.01, ***p<0.001, ns-non-significant.*

contrast, no significant increase was seen when 3/689 was administered to shRNA-2 hMSC cultures, suggesting that 3/689 requires PrP for its activity.

5.5 3/689 extends the lifespan of hMSC cultures

To determine whether 3/689 extended the lifespan of hMSC following *in vitro* expansion and delayed the loss of proliferative and differentiation ability, cultures were isolated and maintained in presence or absence of 3/689. Briefly, human mononuclear cells from each bone marrow sample were divided into three equal parts: one part was used to derive hMSC cultures in presence of 3/689 at 10uM, one part in presence of DMSO and one part which was cultured with medium alone. The cultures were maintained and fed twice a week with or without 3/689 until they stopped proliferating for at least 3 weeks, when they were considered to have reached senescence. There was no difference in the number of population doublings in the first 19-24 PD. However, after this, cultures exposed to 3/689 underwent an average 7±3 PD more than hMSC cultures exposed to DMSO (Fig 5.5A, n=5). This was equivalent to a significant 300-fold increase in total cell numbers generated from each culture (Fig 5.5B, n=5).

To determine whether hMSC cultures treated with 3/689 contained higher number clonogenic progenitors, at 90.4±17 days in culture, I replated the cells at low density and assessed the number of CFU-F, CFU-O and CFU-A. Human MSC cultures grown in presence of 3/689 contained significantly higher numbers of CFU-F, (Fig 5.6A, n=5), CFU-O (Fig 5.6B, n=5) and CFU-A, (Fig 5.6C, n=4) compared to DMSO control cultures.

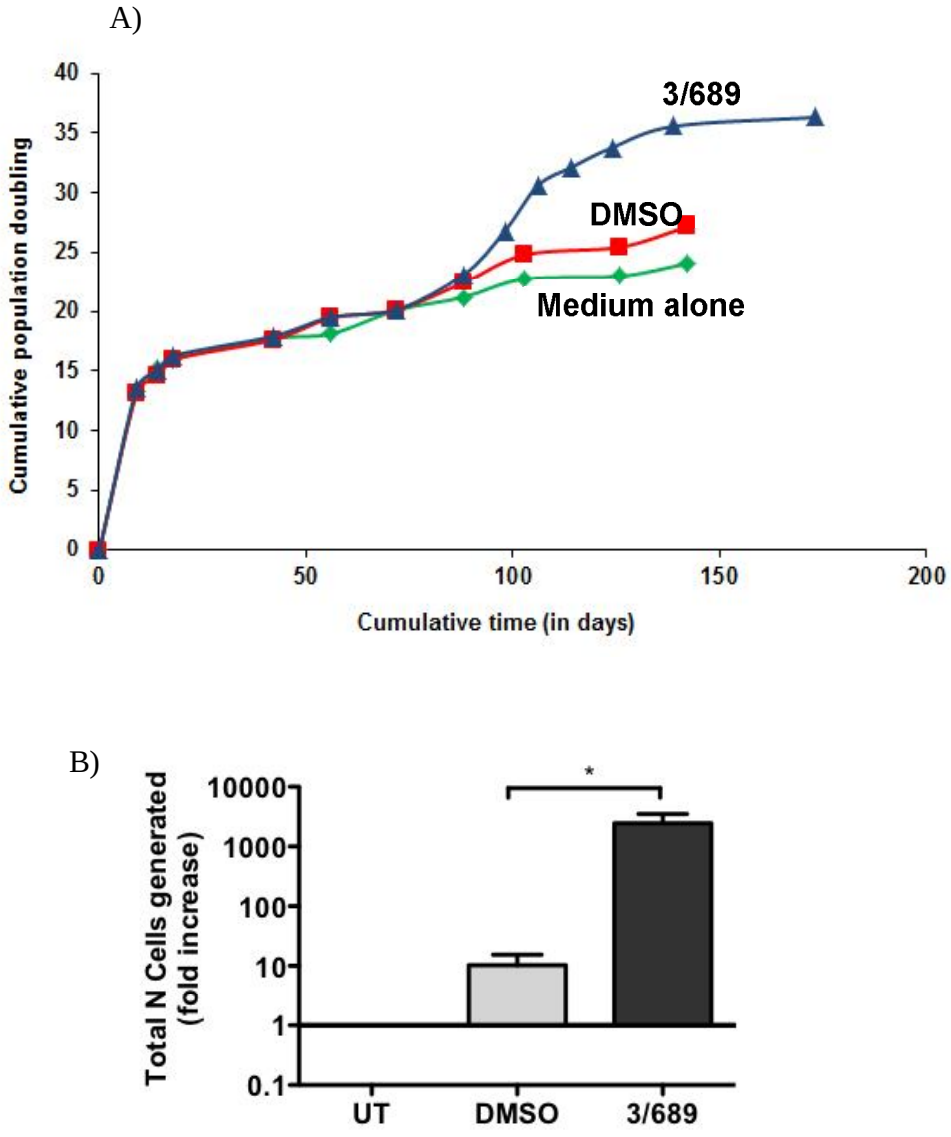


Fig 5.5: 3/689 extends hMSC lifespan in culture

*Figure A shows a representative example of growth kinetic of hMSC cultures (n=5) expanded in : medium only (green diamonds), in presence of DMSO (red squares) or in presence of 3/689 at 10µM (blue triangles). Figure B shows total number of cells generated from the expansion of hMSC cultures (n=5) isolated and maintained in medium only (UT), DMSO or in presence of 3/689. Data presented as mean ± SEM and analysed by one way ANOVA with Bonferroni multiple comparison post-test, *p<0.05.*

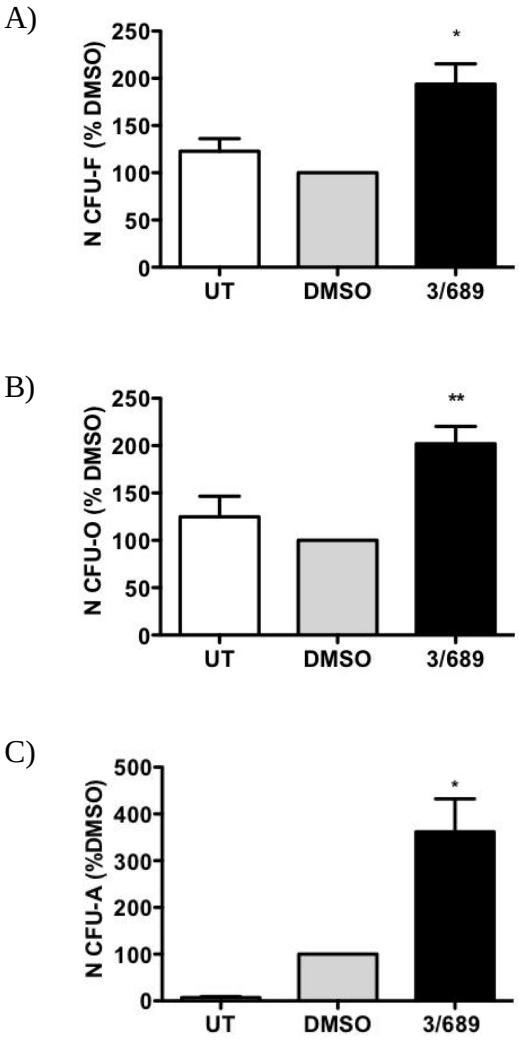


Fig 5.6: 3/689 increases the content of highly proliferative clonogenic progenitors of hMSCs in culture.

*Figure A, B and C show the percentage increase in the number of colony forming unit-fibroblast (CFU-F, n=5), -osteoblast (CFU-O, n=5) and –adipocyte (CFU-A, n=4) respectively (compared to DMSO control) obtained by replating hMSC cultures expanded in medium alone (UT), in presence of DMSO or 3/689 at 10μM after 90.4±17 days in culture. Data presented as mean ± SEM and analysed by one way ANOVA with Bonferroni multiple comparison post-test, *p<0.05, **p<0.01.*

5.6 3/689 enhances differentiation of hMSC cultures

To determine whether hMSC cultures expanded in presence of 3/689 retained their differentiation ability, hMSC cultures previously expanded in presence of medium alone, DMSO or 3/689 were exposed to osteogenic and adipogenic differentiation supplements for 2 weeks in absence of the small molecule. RNA was isolated and assessed for the expression of markers of osteogenic and adipogenic differentiation by RT-PCR. 3/689 hMSCs showed a significant increase in the expression of late osteogenic markers, osteopontin and osteocalcin (Fig 5.7 A-D, n=4) compared to DMSO controls. Similarly, an increase in expression of adipogenic markers PPARγ and lipoprotein lipase (LPL) was also seen in cultures previously expanded in presence of 3/689 and exposed to adipogenic differentiation supplements although it did not reach significance compared to DMSO treated cultures (Fig 5.7E-F, n=4).

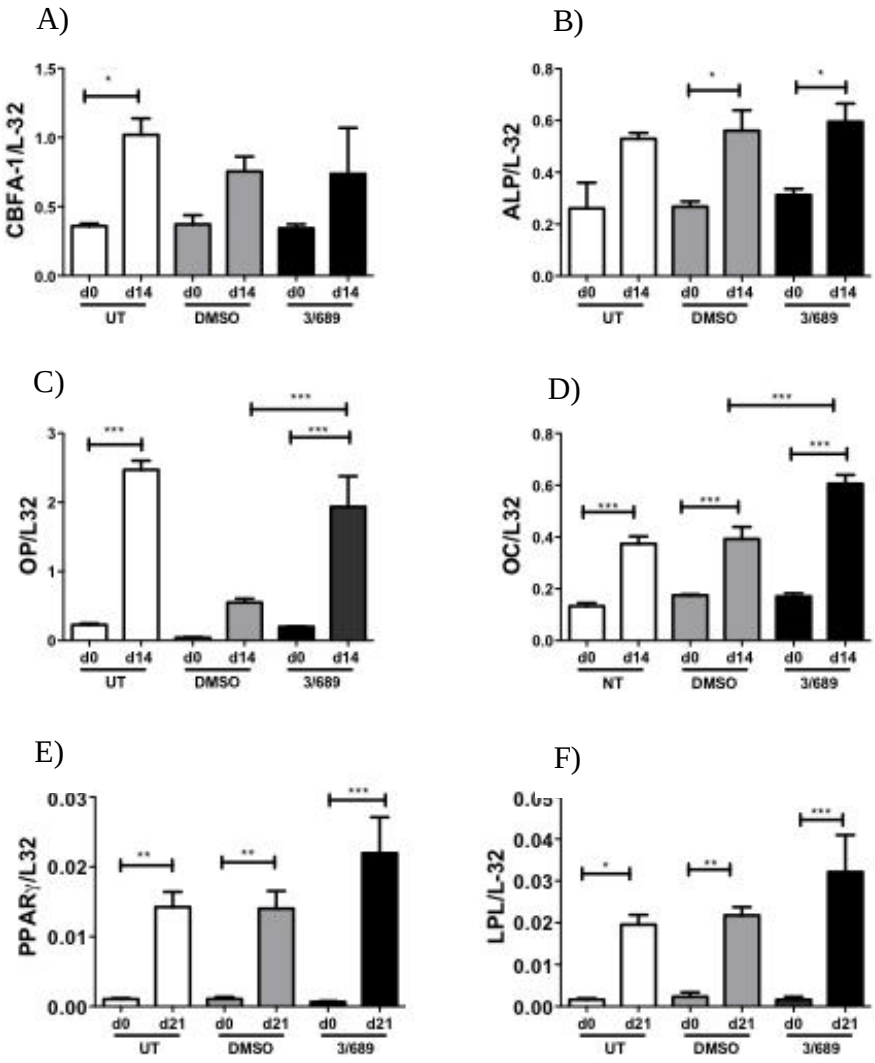


Fig 5.7: 3/689 enhances differentiation of hMSC cultures towards osteogenic and adipogenic lineage

*The graphs shows real-time qPCR of hMSC cultures (n=4) expanded in medium alone (UT), in presence of DMSO or 3/689 and exposed to osteogenic and adipogenic differentiation supplements for 14 days and assessed for the expression of osteogenic differentiation marker (A) core-binding factor subunit alpha-1 (CBFA-1), (B) alkaline phosphatase (ALP), (C) osteopontin (OP) and (D) osteocalcin (OC) and adipogenic differentiation marker (E) peroxisome proliferator-activated receptor γ (PPAR-γ) and (F) lipoprotein lipase (LPL). All the markers were normalised to ribosomal protein L-32. Data presented as mean ± SEM and analysed by one way ANOVA with Bonferroni multiple comparison post-test, *p<0.05, **p<0.01, ***p<0.001.*

5.7 3/689 stabilizes the expression of PrP in MSC cultures with time in culture

To confirm that exposure of hMSC cultures to 3/689 resulted in retention of PrP expression with time in culture, cultures were analysed for PrP expression at passage 2 and 8. As expected, western blotting data showed that hMSC cultures exposed to DMSO showed lower levels of PrP expression with serial passage in culture (Fig 5.8A, n=3). In contrast 3/689 hMSC cultures retained similar levels of PrP expression (Fig 5.8B, n=3), suggesting that exposure of hMSCs to 3/689 led to retention of the levels of PrP expression with time in culture.

5.8 hMSC cultures derived in presence of 3/689 showed increased engraftment ability

It has been shown that the ability of hMSCs to engraft to bone marrow is severely affected following serial passage in culture (Kyriakou, Rabin et al. 2008). To determine whether hMSCs expanded in presence of 3/689 showed enhanced engraftment potential, hMSC cultures were transduced with a lentiviral vector expressing eGFP (93.26%±3 eGFP+) and expanded in presence of 3/689 or DMSO for 5 passages, prior to transplantation in NOD/SCID mice by intra-femoral injection (Fig 5.9). During this time they showed an average two fold expansion in cell number in presence of 3/689 compared to cultures exposed to DMSO (Fig 5.10A, n=3). Prior to transplantation studies, I wanted to confirm whether 3/689-derived hMSCs were karyotypically normal and did not show any genetic abnormalities. All the hMSC cultures showed a normal

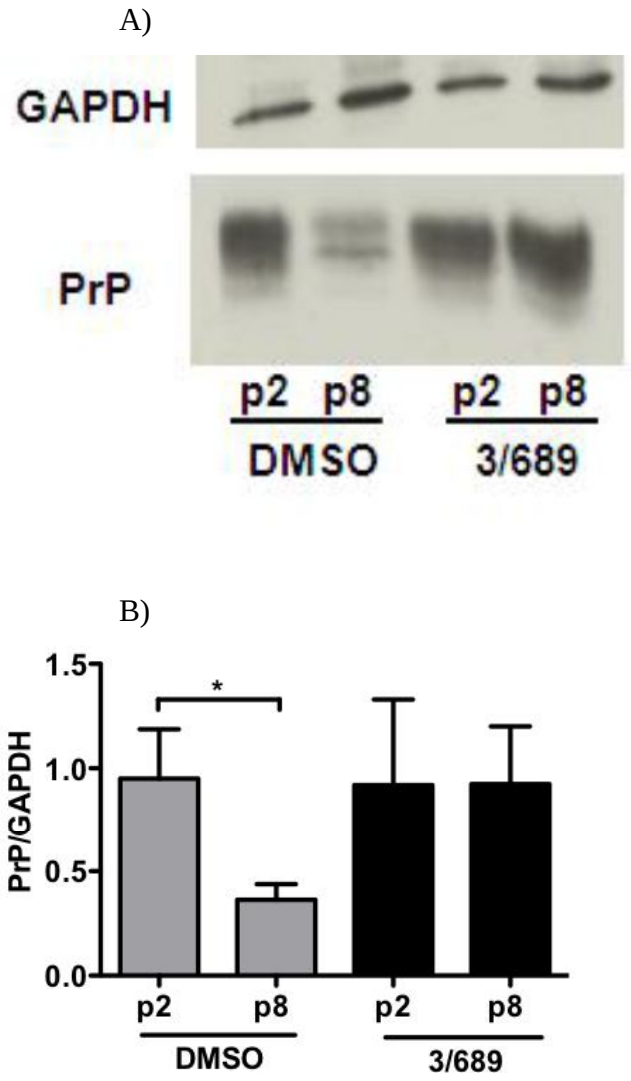


Fig 5.8: hMSCs retains PrP expression in presence of 3/689 with time in culture

*Figure A shows a representative example of western blot of hMSCs (n=3) cultured in presence or absence of 3/689 at passage 2 (p2) and passage 8 (p8) labelled with antibodies specific for glyceraldehydes-3 phosphate dehydrogenase (GAPDH, top panel) or cellular prion protein (PrP, bottom panel). Figure B shows quantification of PrP expression normalised to GAPDH of hMSCs (n=3) cultured in presence or absence of 3/689 at p2 and p8, assessed by western blot and analysed by ImageJ software. Data presented as mean ± SEM and analysed by one way ANOVA with Bonferroni multiple comparison post-test, *p<0.05.*

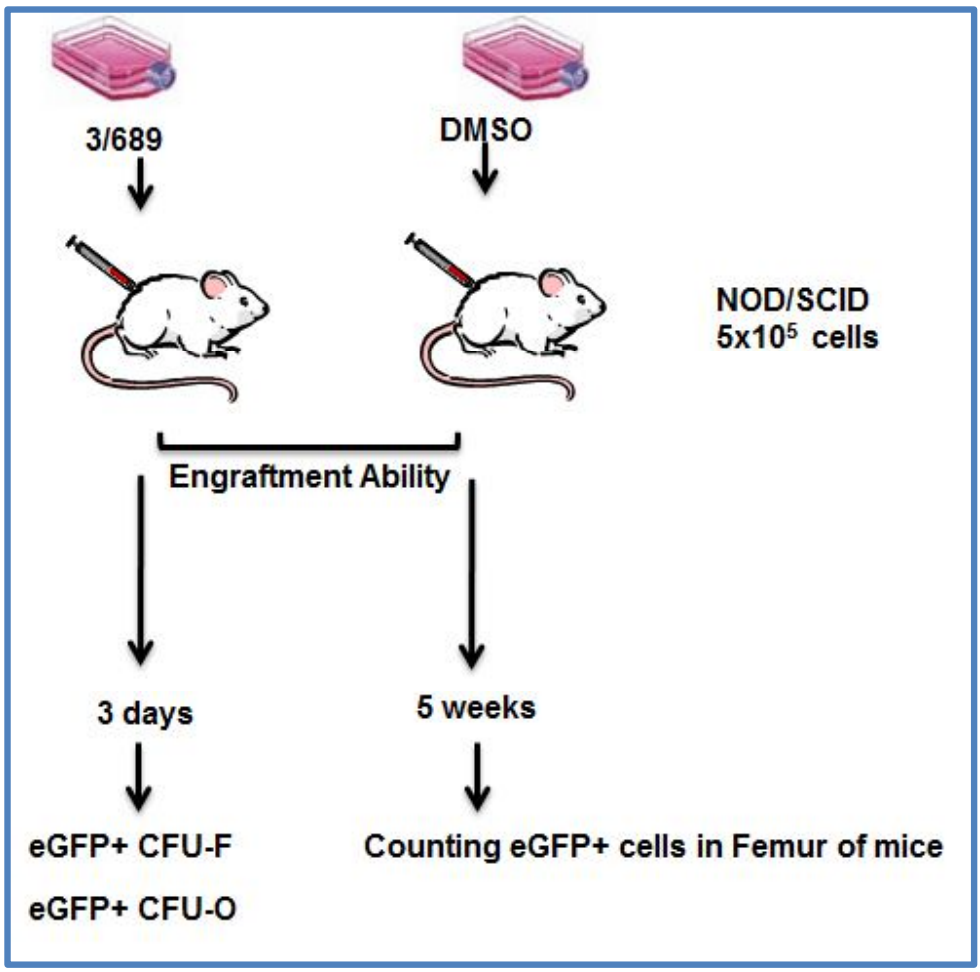
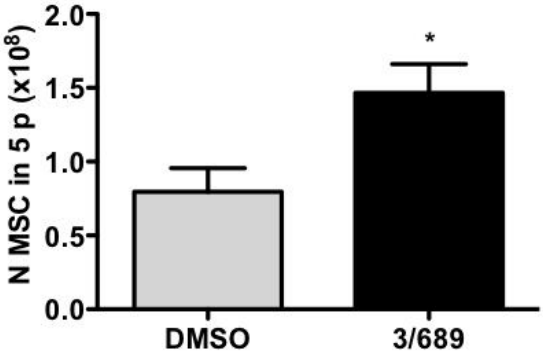


Fig 5.9: Schematic representation of experimental plan for assessing the engraftment ability of hMSCs cultured in presence of 3/689 in NOD/SCID mice

A)



B)

Fig 5.10: 3/689 enhances expansion ability in vitro and exhibits a normal karyogram

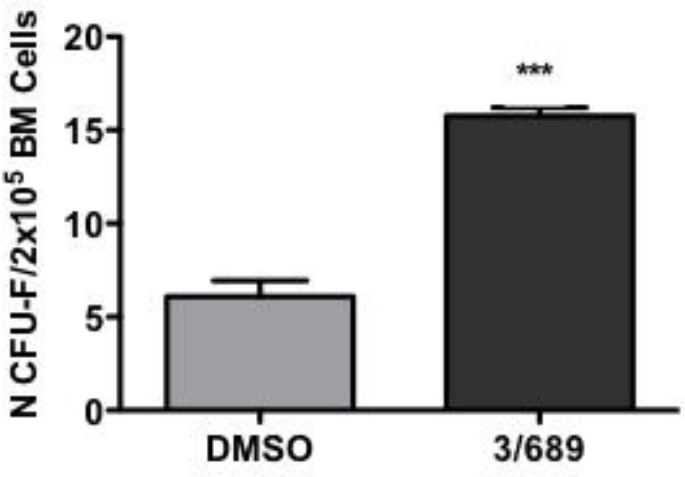
*Figure A shows the total number of hMSCs (n=3) expanded for 5 passages in presence of 3/689 or DMSO. Figure B shows a representative example of karyogram of hMSC culture derived and grown in presence of 3/689 at 10uM at the time of transplant. Data presented as mean ± SEM and analysed by unpaired student's t test, *p<0.05.*

karyotype at the time of transplantation (Fig 5.10B).

Human MSCs expanded in presence of 3/689 or DMSO was injected into NOD/SCID mice by intra-femoral injections and engraftment ability was assessed at 3 days and 5 weeks post-transplant. Three days post-transplant, engraftment ability was assessed by measuring the number of eGFP+ CFU-F and CFU-O and 5 weeks post-transplant was assessed by counting the number of eGFP+ cells detected by immuno-staining. A significant average 3 fold increase in the number of eGFP+ CFU-F (Fig 5.11A, n=6) and CFU-O (Fig 5.11B, n=6) was observed in the bone marrow of NOD/SCID mice transplanted with 3/689 treated hMSC cultures compared to DMSO controls, 3 days post injection.

Interestingly, even five weeks post injection an average 10 fold increase in the number of eGFP+ hMSC was seen in the bone marrow of NOD/SCID mice by immuno-histochemistry (Fig 5.12, n=9). In mice transplanted with 3/689 expanded hMSC cultures, cells with morphology and locations resembling pericyte, stromal cells, osteocyte and adipocyte were seen (Fig 5.13A, n=9 and Fig 5.13B). In contrast mice injected with hMSCs in absence of 3/689 showed only morphology of pericytes and stromal cells. These data suggest that hMSC cultured in presence of 3/689 are able to engraft to bone marrow and are superior in quality both *in vitro* and *in vivo*.

A)



B)

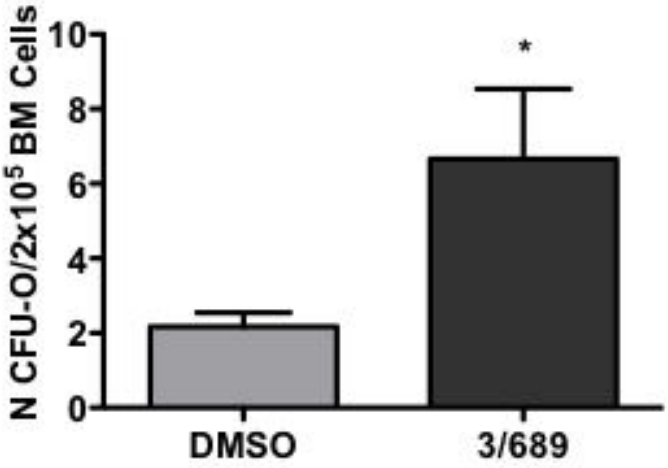


Fig 5.11: Human MSC cultures derived in presence of 3/689 showed increased engraftment ability, 3 days post injection

*Figure A and B shows the number of human eGFP+ colony forming unit-fibroblast (CFU-F) and –osteoblast (CFU-O) respectively recovered from the bone marrow of NOD/SCID mice (n=6) transplanted with hMSC cultures derived in presence of 3/689 at 10uM or DMSO, 3 days post intra-femoral injection. Data presented as mean ± SEM and analysed by unpaired student's t test, *p<0.05, **p<0.01, ***p<0.001.*

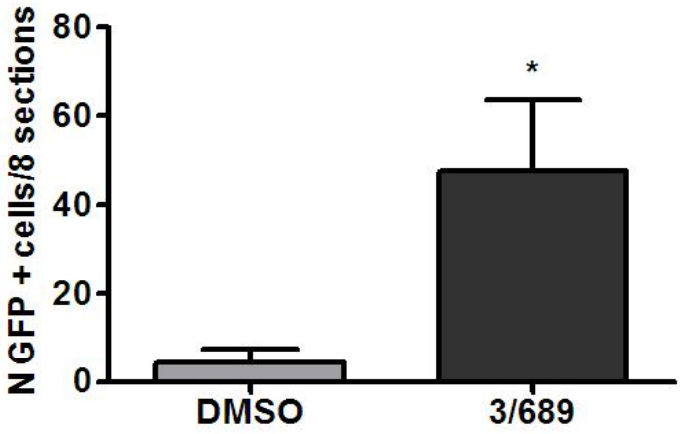
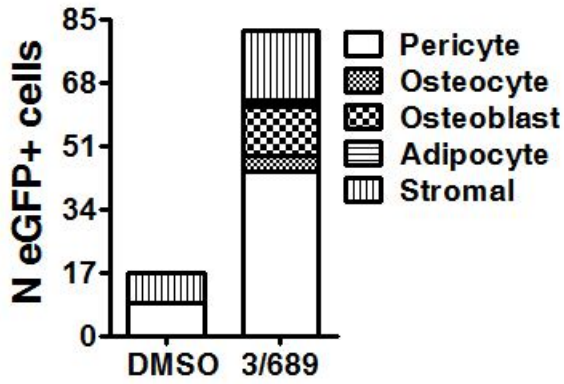


Fig 5.12: Human MSC cultures derived in presence of 3/689 showed increased engraftment ability, 5 weeks post injection

*Figure shows the number of eGFP+ hMSCs in the femur of NOD/SCID mice (n=9) transplanted with hMSC cultures derived in presence of 3/689 at 10uM concentration or DMSO, 5 weeks post intra-femoral injections. Data presented as mean ± SEM and analysed by unpaired student's t test, *p<0.05.*

A)



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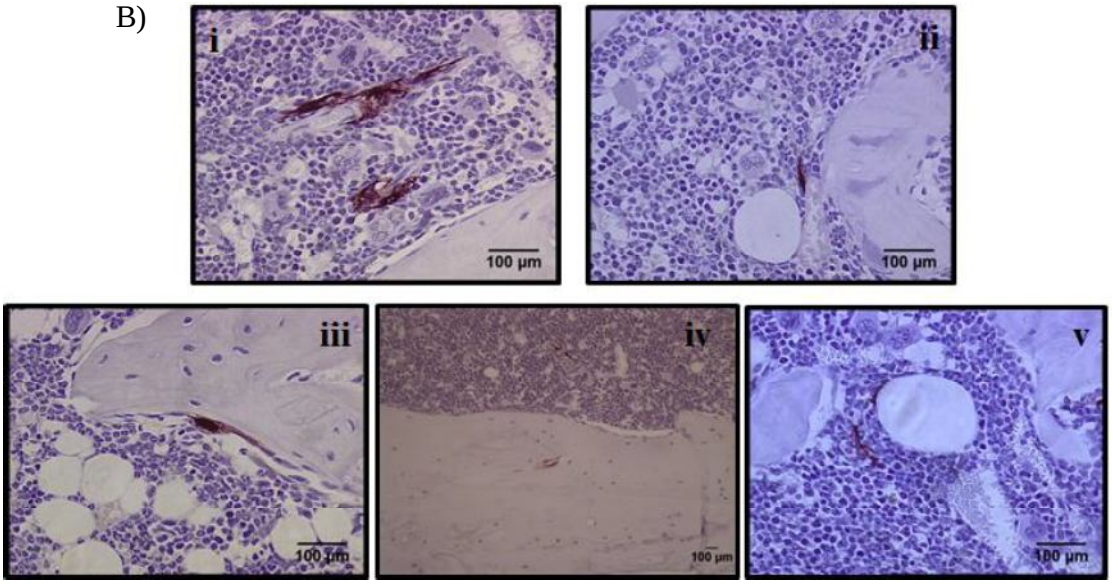


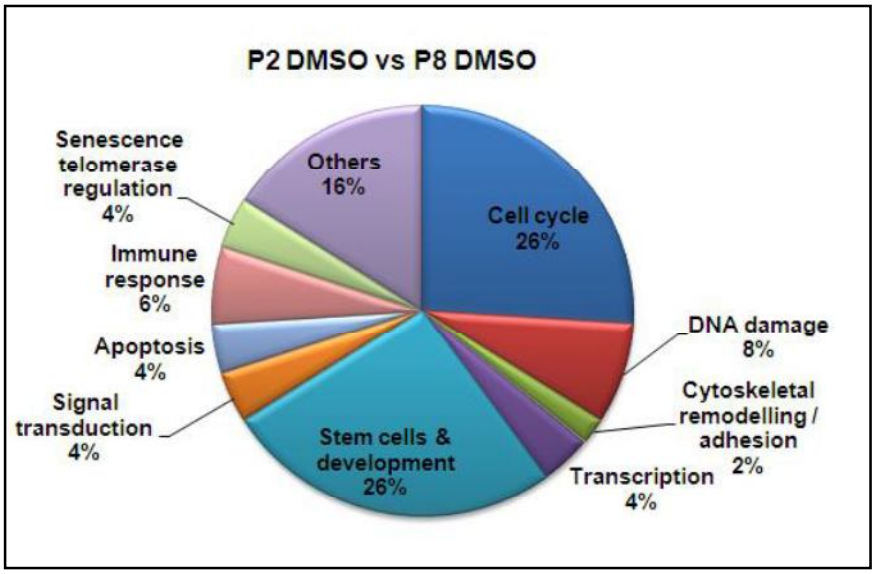
Fig 5.13: Morphology and anatomical locations of eGFP positive hMSC in the bone marrow of NOD/SCID mice, 5 weeks post injection

Figure A shows a wide distribution of several cell types in the femur of NOD/SCID mice transplanted with hMSC cultures expanded in presence of absence of 3/689 at 10uM concentration (n=9). Figure B shows a representative example of eGFP+ hMSC transplanted in NOD/SCID mice and immuno-stained for eGFP expression. The figure shows example of (i) pericyte, (ii) stromal cells, (iii) bone lining cells (iv) osteocyte and (v) adipocyte based on morphology and anatomical location. Images were taken using a light microscope Leica Leits DMRB at 10x magnification for pericytes, stromal cells, bone line cells and adipocytes; and 5x magnification for osteocyte.

5.9 Mechanism of action of 3/689

To determine the mechanism of action of 3/689, changes in gene expression profiling of hMSC cultures exposed to 3/689 or DMSO at passage 2 (p2) and passage 8 (p8) were examined by whole genome expression arrays using the Agilent whole genome chip. A list of differentially expressed genes was obtained using Gene Spring GX by paired t-test at $p<0.05$ significance. These differentially expressed genes were mapped to existing networks using the software GeneGo Metacore and revealed statistically over-represented networks in several processes (Fig 5.14). Of interest were the processes related to cell cycle (26%) and DNA damage (8%) which were significantly dysregulated in hMSCs with serial passage in culture in the absence of 3/689, consistent with cellular ageing (Fig 5.14A). These processes were no longer significantly overrepresented with time in culture when hMSC were cultured in the presence of 3/689 (Fig 5.14B). Moreover there were more networks, which were related to stem cell and development, immune responses and cytoskeletal development in hMSC exposed to 3/689 with time in culture compared to DMSO controls (Fig 5.14A-B).

A)



B)

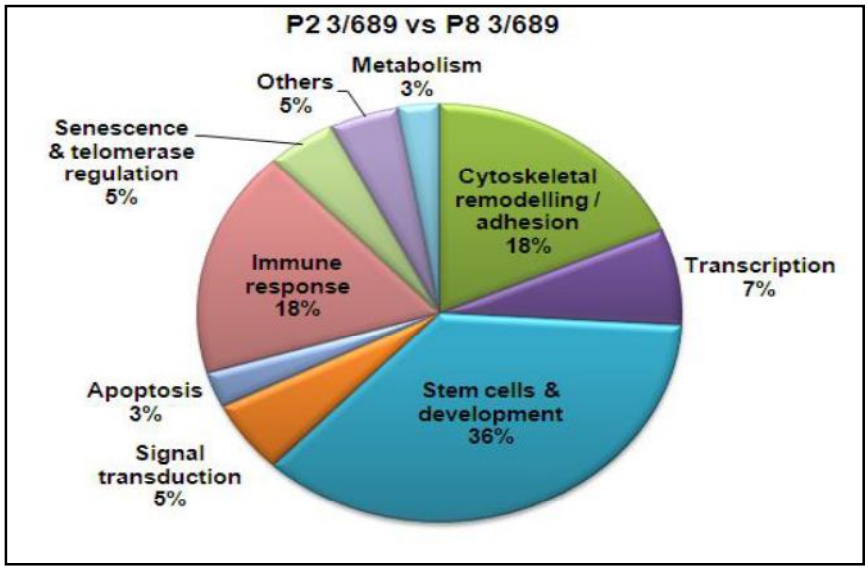


Fig 5.14: 3/689 prevents dysregulation of gene expression of networks related to DNA damage and cell cycle

Graphical representation of the categories of networks containing a significant number of differentially expressed genes, obtained by comparing gene expression profiling of hMSC cultures at passage 2 and 8 cultured in absence (A) or presence of 3/689 (B).

As processes related to DNA damage and cell cycle are important in stem cells and cellular aging, I wanted to explore in more details the list of differentially expressed genes in those processes (Fig. 5.15). The DNA damage response gene ATM was up regulated and the DNA repair genes *Bard1*, *Brca1* and *Bloom* were down regulated in hMSCs with time in culture. Genes such as *Cdc25a*, *Cyclin E* and *Cyclin A* which are involved in cell cycle progression were significantly down regulated in hMSC cultures with increased passage number. These were no longer differentially expressed in hMSCs cultured in presence of 3/689. While Cdc25a was up regulated, other cell cycle regulators such as *Cyclin E* and *A* were within the normal range and only *Cyclin D* was down regulated. The data suggests that 3/689 prevents accumulation of DNA damage with time in culture and lead to slower cell cycle progression with expansion. The up regulation of SOD2 gene in the presence of 3/689 was of utmost interest. This gene acts as an important defence against oxidative damage. SOD2 has been associated with PrP as it requires Mn^{+2} for its function. PrP is known to bind and transport Mn^{+2} and have SOD-like activity (Brown, Hafiz et al. 2000). These data led to the hypothesis that 3/689 may prevent DNA damage and promote cell cycle progression by enhancing SOD2 reactive oxygen species scavenging activity.

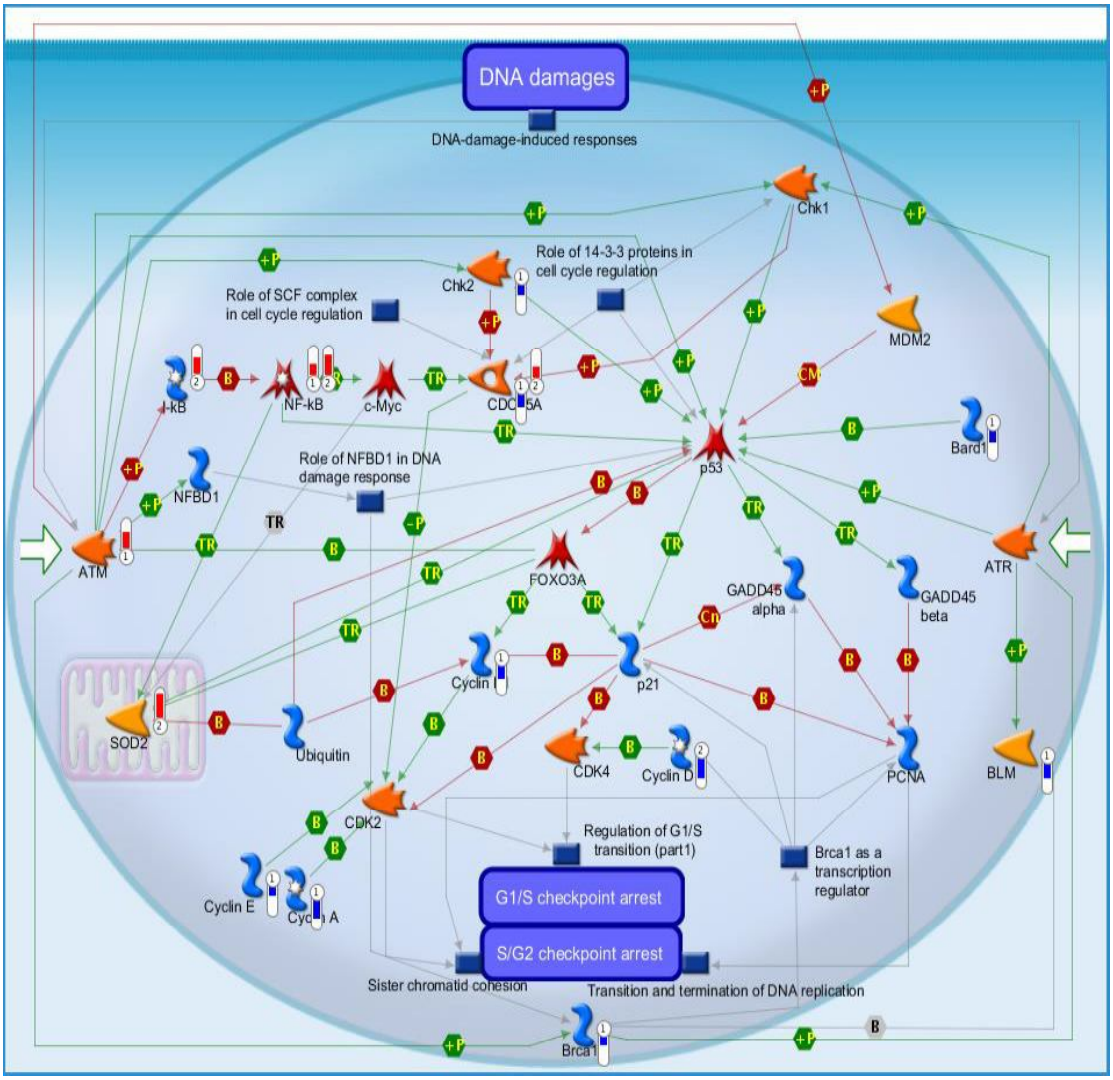


Fig 5.15 Network representation containing a significant number of differentially expressed genes related to DNA damage and cell cycles in hMSC cultures with cellular aging

Figure shows representation of the network containing differentially expressed genes related to Cell cycle – DNA damage obtained using the software GeneGo Metacore. The genes represented with a red thermometer were found to be upregulated while those with a blue thermometer were down regulated with serial passage in culture. Thermometer labelled 1 and 2 represent differentially expressed genes in hMSC cultures expanded in absence or presence of 3/689 respectively.

5.9.1 Validation of microarray data

To test whether 3/689 prevented occurrence of DNA damage and promoted cell cycle progression, first, I determined whether exposure of hMSC cultures to 3/689 in presence of DNA damaging agent H_2O_2 for 1 hour prevented damage. This was achieved by determining the levels of phosphorylated γH2AX as a measure of double strand breaks, an hour later. Cultures exposed to H_2O_2 in the presence of 3/689 showed reduced levels of phosphorylated γH2AX compared to cultures exposed to H_2O_2 in presence of DMSO (Fig 5.16Ai-iv, 5.16B, n=3). Secondly, to confirm whether hMSC cultures exposed to 3/689 showed an increase in cell cycle progression, cells were labelled with CFSE and the levels of fluorescence were tested 1 and 5 days after labelling. CFSE is a colourless dye that passively diffuses into cells and is equally divided between the daughter cells during cell division and as a result high resolution tracking of cell division can be monitored by the reduction in fluorescence seen using flow cytometry. Human MSC treated with 3/689 underwent a significant reduction in levels of CFSE compared to DMSO cultures (Fig 5.17Ai-ii, 5.17B, n=3), suggesting that exposure to 3/689 resulted in enhanced cell cycle progression.

To determine whether SOD2 was important for the action of 3/689, real time qPCR expression was carried out in the presence or absence of PrP expression. A significant up-regulation in SOD2 expression was seen in the presence of 3/689 (Fig 5.18A, n=3). More importantly SOD2 expression was not increased in cultures exposed to 3/689 when PrP expression was knocked down (Fig 5.18B), suggesting that 3/689 requires PrP to up regulate SOD2.

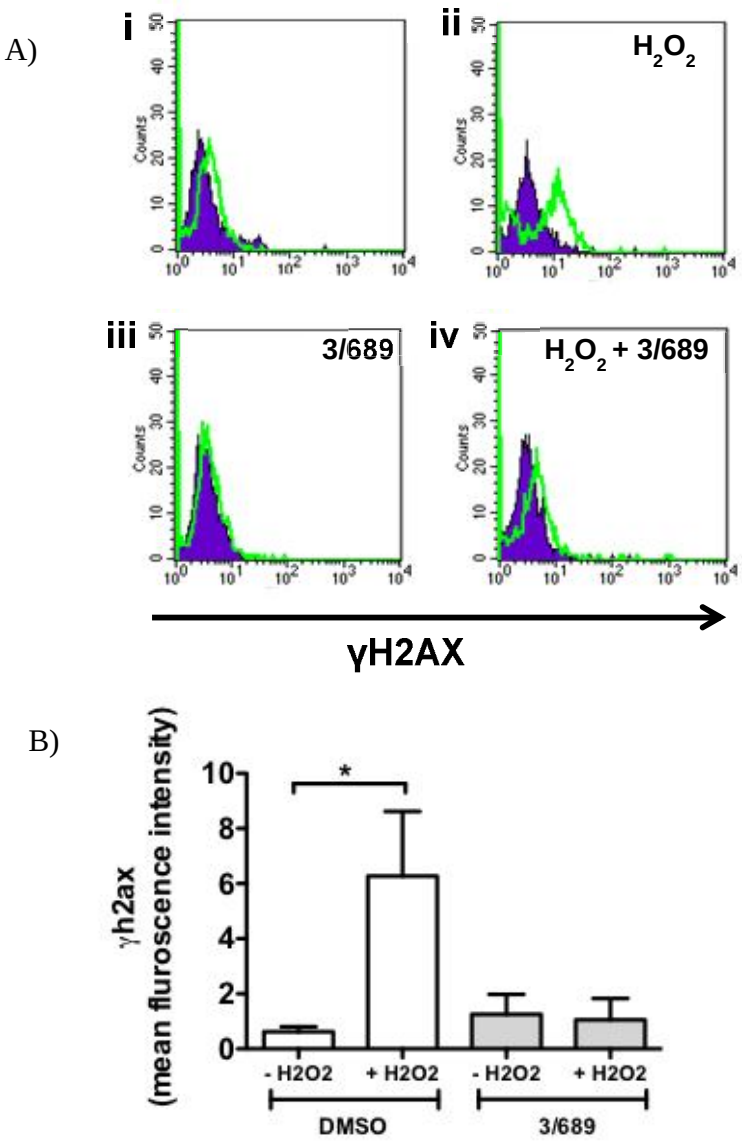


Fig 5.16 3/689 protects hMSCs from DNA damage

*Figure A is a representative example of flow cytometry profile of hMSC culture stained for the presence of phosphorylated γH2AX following exposure to H_2O_2 (75uM) for 1h. Human MSC exposed to (i) medium only (ii) H_2O_2 (iii) 3/689 and (iv) to H_2O_2 plus 3/689. Purple and green histogram represent hMSC culture stained with the isotype control and phosphorylated γH2AX specific antibody respectively. Figure B shows quantification of the mean fluorescent intensity of hMSCs (n=3) stained with phosphorylated γH2AX following exposure to H_2O_2 for 1h in presence or absence of 3/689. Data presented as mean ± SEM and analysed by one way ANOVA with Bonferroni multiple comparison post-test, *p<0.05.*

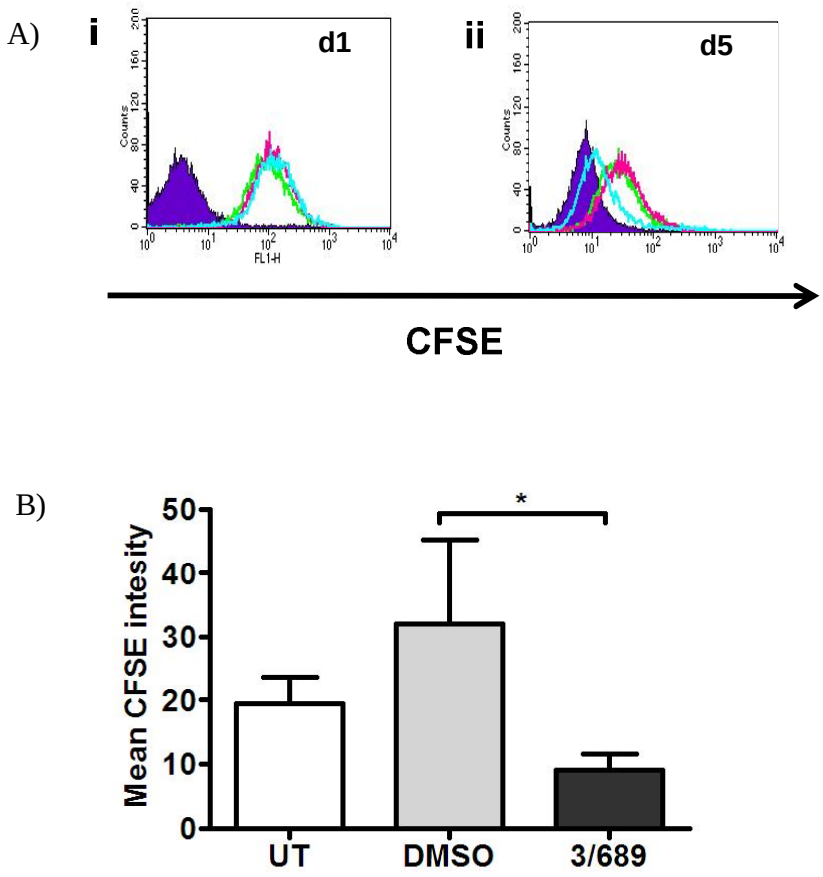


Fig 5.17 3/689 enhances cell cycle progression in hMSC cultures

*Figure A is a representative example of hMSC labelled with CFSE and cultured in presence of 3/689 (blue histogram), DMSO (red histogram) or medium alone (green histogram) for 1 day (i) and 5 days (ii). The purple histogram represents unlabelled hMSC cultures. Figure B shows quantification of the mean fluorescent intensity of MSCs (n=3) stained with CFSE at day 5 in presence or absence of 3/689.UT represents untreated hMSC cultures stained with CFSE. Data presented as mean ± SEM and analysed by one way ANOVA with Bonferroni multiple comparison post-test, *p<0.05.*

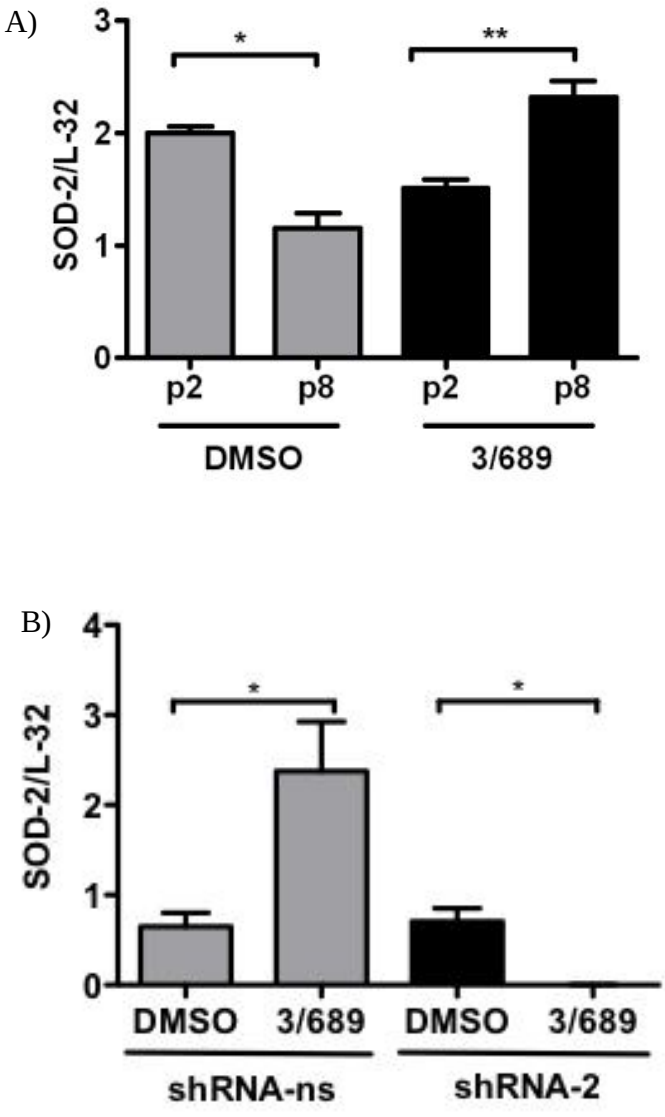


Fig 5.18 Small molecule 3/689 acts by up regulating SOD2 expression

*Figure A shows the expression of SOD-2 in hMSC cultures maintained in culture for 2 and 8 passages in presence or absence of 3/689 by real time PCR. Figure B shows the expression of SOD-2 in hMSC cultures (n=3) transduced with a non-silencing vector (shRNA-ns) or a vector to knock down the expression of PrP (shRNA-2) and exposed to DMSO control or 3/689. Data presented as mean ± SEM and analysed by one way ANOVA with Bonferroni multiple comparison post-test, *p<0.05, **p<0.01.*

5.10 Discussion

Previously I showed that the lack of PrP expression in hMSC reduced their proliferative capacity with time in culture (Chapter-4). Hence, I wanted to test whether a small molecule capable of modulating PrP activity was able extend their proliferation following expansion *in vitro*. The exposure of 3/689 at 10uM concentration to previously established hMSC cultures increased the number of mesenchymal progenitors. Cultures isolated and maintained in presence of 3/689 underwent an extra 6-10PD, showed an average two fold expansion over five passages, contained 40-175% more highly proliferative progenitors, including those capable of differentiation to osteoblasts and enhanced differentiation ability to the osteogenic lineage in presence of 3/689. Integral to the definition of stem cells is their ability to regenerate the tissue in which they reside through their ability to self-renew and differentiate. The best *in vivo* assay known to assess the regenerative capacity of MSC is the ectotopic bone formation assay (Daga, Muraglia et al. 2002). However, this assay does not test the ability of hMSC to regenerate and contribute to tissue maintenance and repair in the appropriate environment. For this reason I have transplanted eGFP labelled hMSC exposed 3/689 directly in the femur of immuno-deficient mice and assessed their engraftment potential 5 weeks post-injection. 3/689 derived MSC cultures showed a 10-fold increase in the number of donor cells in bone marrow 5 weeks post-transplant. Infact transplanted cells showed morphology and anatomical location of pericytes, stromal cells, bone lining cells and osteocytes in contrast to their DMSO controls, suggesting that the cultures were not only superior in quality *in vitro* but also enhanced the preservation of their *stemness in vivo*. These findings are in agreement with studies that have demonstrated that the

ancestral origin of hMSC is associated with blood vessel wall and belongs to a subset of perivascular cells, pericytes (Hirschi and D'Amore 1996; Crisan, Yap et al. 2008). In fact cultured human perivascular cells from bone marrow expressed markers of hMSC and in presence of suitable differentiation supplements gave rise to chondrocytes, osteocytes and adipocytes (Crisan, Yap et al. 2008; (Sacchetti, Funari et al. 2007; Mendez-Ferrer, Michurina et al. 2010).

As much as this chapter demonstrated that the lifespan and function of hMSC can be manipulated by modulating PrP expression and provide new strategies for using stem cells for clinical practise in regenerative medicine, the safety of this small molecule requires thorough evaluation. In this study, although, I have not seen changes in chromosomal integrity and tumorigenic potential following hMSC expansion with 3/689; routine karyotyping that has been used in the study is the most basic and is relatively a crude and quick assay to measure tumorigenic potential. However, if one has to thoroughly monitor and measure long term tumorigenic potential, more in depth studies are required. For example, time-point karyotype analysis is a widely recommended technique to monitor quality control chromosomal integrity (Catalina, Cobo et al. 2007). This method allows for careful monitoring of cells cultured with drug for signs of any disrupted chromosomal integrity which are subjected to extensive 'passaging'. However this technique is time-point driven and is more labour intensive. Increasing interests to monitor chromosomal instability in human ESC and human IPSCs *in vitro* is developing microarray-based techniques such as comparative genomic hybridization (CGH), single nucleotide polymorphism analysis and transcriptional profiling (Moralli, Yusuf et al. 2011). For rapid chromosomal assessment even

fluorescence in situ hybridisation (FISH) based protocols for karyotyping such as multiplex-FISH (M-FISH) and spectral karyotyping (SKY) are used to detect recurring aneuploidies by means of chromosome specific probes (Moralli, Yusuf et al. 2011). While most of these techniques including M-FISH, SKY and CGH can refine complex karyotypes, they cannot detect chromosomal balanced translocation, small intrachromosomal re-arrangements such as deletions, duplications or inversions. Moreover, they can be performed mostly on metaphasic spreads of cells and are highly expensive techniques. The most robust cost-effective technique that can provide quick results would be to conduct a FISH experiment as it is a very sensitive technique and can detect both numerical and structural chromosomal abnormalities in both interphase and metaphase nuclei. FISH can be even performed in frozen tissue samples (Catalina, Cobo et al. 2007) unlike other techniques.

Understanding that 3/689 enhances the proliferative and self-renewal ability of hMSC both *in vitro* and *in vivo*, it was now important to address the mechanism by which 3/689 confers its activity on hMSC cultures. To determine the molecular pathways leading to ageing of hMSC in presence or absence of 3/689, gene expression profiling was carried out. From the microarray studies four major areas seemed to be affected with cellular aging including cytoskeletal remodelling/ adhesion, inflammation, DNA damage/cell cycle and processes related to stemness. Adhesion molecules play a very important role in maintaining the proliferation and differentiation ability of stem cells. Differentially expressed genes that are essential to cytoskeletal remodelling/adhesion processes included the extra-cellular matrix (ECM) proteins laminin-1, epithelial (E)-cadherin; growth factors such as fibroblast growth factor (FGF); cell-adhesion molecules such as

melanoma cell adhesion molecule (MCAM) were upregulated in hMSC cultures with cellular ageing in presence of 3/689 and were no longer differentially expressed in hMSCs cultured in absence of 3/689. The possible role of PrP in promoting proliferation of MSCs by enhancing glycosaminoglycans signalling, another major constituent of ECM, by regulating the activity of FGF is discussed in chapter-4. Another cell adhesion molecule that was upregulated in presence of 3/689 was MCAM. Expression of MCAM, otherwise called as CD146 in hMSCs has been shown to regulate proliferation, differentiation and maintenance of haematopoietic stem and progenitor cell (HSPC) through direct cell-cell contact (Stopp, Bornhauser et al. 2013). In the study, knocking down of MCAM expression in MSCs not only impaired proliferation, osteogenic differentiation and migration of hMSC but also stimulated HSPC proliferation and reduced the formation of long-term culture initiating cell formation significantly. Moreover, MCAM-overexpressing hMSCs resulted in providing a microenvironment conducive for HSPC and increased adhesion of HSPC to hMSCs. It is not known how PrP may interact with MCAM and aid in the maintaining the stemness of hMSCs and further studies are warranted to attempt this.

Dysregulation of processes related to inflammation is not surprising when looking at cellular ageing (Freund, Orjalo et al. 2010; Campisi 2011). The senescence associated secretory phenotype (SASP) with pro-inflammatory phenotype has also been described (Behrens, van Deursen et al. 2014); (Parrinello, Coppe et al. 2005); (Coppe, Patil et al. 2008). However, surprisingly, the microarray data in my study showed the secretion of pro-inflammatory cytokines including IL-6, tumor necrosis factor-α (TNF-α), IL-1β and IL-8 to be upregulated with age in presence of 3/689. Apart from their pro-inflammatory

role, these cytokines have recently been shown to be involved indirectly in MSC-mediated immunosuppression by the secretion of soluble factors including inducible nitric oxide synthase (iNOS), indoleamine 2,3-deoxygenase (IDO), prostaglandin E_2 (PEG2) (Ghannam, Bouffi et al. 2010). For example in murine MSCs, activation of pro-inflammatory cytokines such as interferon-γ (IFN-γ), TNF-α, IL-1α or IL-β induced secretion of iNOS in MSCs and inhibited T-cell proliferation (Ghannam, Bouffi et al. 2010). Moreover, MSCs from iNOS-/- and IFN-γ-/- mice were unable to supress T-cell proliferation (Ren, Zhang et al. 2008). Similarly, IL-6 dependent PEG2 secretion by murine MSCs inhibited local inflammation in collagen-induced arthritis (CIA) model of auto-immune disease (Bouffi, Bony et al. 2010). The study showed that injection of IL-6-/- MSCs in the allogeneic CIA model was less efficacious in decreasing paw swelling in arthritic mice compared to wild-type MSCs due to PGE2 secretion (Bouffi, Bony et al. 2010). The study suggested that MSC-mediated immuno-suppression was regulated by PEG2 secretion that inhibited proliferation of immune cells at the vicinity of MSCs. Also PEG2 secreted by MSCs as a result of stimulation of TNF-α during sepsis has shown to reprogramme monocytes and macrophages to produce IL-10 which is an anti-inflammatory cytokine (Nemeth, Leelahavanichkul et al. 2009). The production of IL-10 prevented neutrophils to migrate to tissues and cause oxidative damage, thereby alleviating damages to multiple organs. Moreover PGE2 secretion by MSCs has reported to block differentiation of monocytes towards dendritic cells (Spaggiari, Abdelrazik et al. 2009), resulting in accumulation of immature dendritic cells and providing immune tolerance (Djouad, Charbonnier et al. 2007). Prion disease has been linked to an enhance inflammatory state (Crespo, Roomp et al. 2012), however the exact mechanisms by which cellular prion protein induce a dysregulated immune function is unknown.

The most striking effect in the microarray study was related to cell cycle, protection from DNA damage and how PrP may play a role in scavenging ROS through upregulation of SOD-2. DNA damage has been shown to have a role in the loss of function of stem cell with age (Vaziri, Schachter et al. 1993) There are several other studies, which suggest that cells lacking PrP or PrP knockout cells are more sensitive to DNA damage via oxidative stress (Kim, Lee et al. 2004; (Brown, Nicholas et al. 2002; Senator, Rachidi et al. 2004). Exposure of 100 μM H_2O_2 over a period of 12 hours induced DNA fragmentation in mouse neural cells line expressing PrP by 152% and 224% not expressing PrP (PrPKO) (Anantharam, Kanthasamy et al. 2008). To understand whether prevention of DNA damage by 3/689 enhanced cell cycle progression of MSC cultures exposed to 3/689, an increase in the loss of CFSE was seen in hMSC cultures treated with 3/689 suggesting that exposure to 3/689 results in enhanced cycling and expansion of hMSC. PrP has shown to promote proliferation in gastric cancer cells by activating genes such as Cyclin D1 and E2F that are related to regulation of cell cycle G1/S transition via the p13K/Akt pathway (Liang, Pan et al. 2007). Skin fibroblast cell lines from PrP knock-out mice showed reduced levels of Cyclin D1 by northern blotting (Satoh, Kuroda et al. 2000).

PrP has been reported to confer enhanced resistance to stress by contributing to cellular SOD activity (Brown, Schulz-Schaeffer et al. 1997; Brown and Besinger 1998). Indeed my data shows that in presence of 3/689, there is up-regulation of SOD-2 and this up-regulation is annulled in the absence of PrP expression. Although the role of SOD2 in controlling aging and extending lifespan through its scavenging activity of ROS is debatable, the effect observed in the data is in agreement with the data reported on the

effect of SOD2 in connective tissues. Heterozygous SOD2 knock-out mice showed reduction in SOD activity with increased DNA damage and no signs of accelerated ageing and extension in lifespan compared to the wild type controls (Van Remmen, Ikeno et al. 2003). To the contrary, a conditional SOD-2 knock-out in connective tissues (from which MSCs originate) conferred selective loss of redox balance in the fibroblast cells which facilitated accelerated ageing phenotype in mice and shorten their life-span (Treiber, Maity et al. 2011). Modulation of ROS may explain also why the effect was prominent with passage number or in cells from older donors. It is quite possible that PrP is useful only at times of proliferative stress. Indeed PrP gene was up-regulated in CD8+ lymphocytes only when they underwent intense proliferation upon transferring into lymphopaenic mice (Goldrath, Luckey et al. 2004). Alternatively with age there an increase in ROS (Ito, Hirao et al. 2004; Ito, Hirao et al. 2006);(Schubert, Erker et al. 2004) and when ROS levels are increasing beyond a certain threshold it is possible that PrP is there to buffer any excess.

There are several reasons why ROS levels may require tight regulation. As mentioned earlier, ROS has been associated with modulating the regeneration ability of stem cells and directing the self-renewal or differentiation of stem and progenitor cells, which suggests that an optimal level of ROS is required to maintain stem/progenitor cells (Chen, Shih et al. 2008; Owusu-Ansah and Banerjee 2009).

Another most obvious reason is the ability to cause genetic damage. Although stem cells are equipped with properties that minimise acquisition of damage (Bunting 2002; Scharenberg, Harkey et al. 2002; Sung, Cho et al. 2008), adult stem cells remain at risk

of acquiring mutations that could lead to cancer or to a robust DNA damage response leading to apoptosis or senescence (Mandal, Blanpain et al. 2011). This has been shown to diminish their regenerative capacity and lead to tissue aging (Rossi, Bryder et al. 2007). To what extent stem cells undergo DNA damage as a consequence of ROS exposure remains unclear, but there is evidence that increased ROS affects stem cell regenerative capacity. Increased ROS, even at low levels led to a depletion of the hematopoietic stem cell pool and inhibition of their repopulation capacity (Ito, Hirao et al. 2006). Elevation of ROS due to loss of ATM led to depletion of HSC pool and bone marrow failure in old mice in a ROS dependent manner (Ito, Hirao et al. 2004). Activation of p38MAPK has been seen to increase in response to increasing levels of ROS and this has been shown to limit HSC lifespan *in vivo* (Ito, Hirao et al. 2006). In contrast prolonged treatment with an inhibitor of p38MAPK extended their lifespan in serial transplantation (Ito, Hirao et al. 2006).

In conclusion, in this chapter I have shown that expansion of hMSCs in presence of PrP led to delay in cellular ageing. This molecule needs further characterization for safety and refinement of structure. However, it does open up new opportunity for their use *in vitro* and *in vivo* to expand the stem cell pool.

CHAPTER-6

Human mesenchymal stem cells have been shown to have potential in regenerative approaches in bone and blood. In principle, hMSC could be isolated from the bone marrow**,** expanded in culture until sufficient numbers are obtained to be used for clinical application. Most protocols rely on their *in vitro* expansion prior to clinical use. However, generating sufficient numbers of hMSC *in vitro* is associated with cellular ageing with loss of proliferation and differentiation capacity (Stenderup, Justesen et al. 2001); (Mendes, Tibbe et al. 2002). Therefore, it is necessary to study how hMSC undergo a process of ageing and identify target molecules which would facilitate their long-term self-renewal and differentiation ability. In my project, I have identified a small molecule, 3/689 which delayed hMSC cellular ageing by targeting PrP. However, there a number of issues to be addressed before this molecule can be used clinically.

As much as the current study demonstrated that the lifespan and function of hMSC can be manipulated by modulating PrP expression and provide new strategies for using stem cells for clinical practice in regenerative medicine, it is now important to consider how 3/689 can be used as a potential drug both *in vitro* and *in vivo* for it to be considered for clinical purposes. For *in vitro* application, first, the safety of the drug is of utmost importance and requires thorough evaluation which has been discussed in chapter 5. Secondly, the possible involvement of PrP in cancer cannot be completely ruled out. Although there is no direct evidence confirming the role of prion in establishment or progression of cancer, yet, PrP has been shown to be over-expressed in many cancers including gastric, colorectal, breast cancers. Studies indicate that over-expression of PrP promoted metastasis in colon cancer lines (McEwan, Windsor et al. 2009) and gastric cancer lines (Pan, Zhao et al. 2006) through the activation of MEK/ERK pathway and

subsequent transactivation of matrix metalloproteinase (MMP)-11 which is associated with extra-cellular matrix degradation, a key step involved in tumor invasion and metastasis (Pan, Zhao et al. 2006). Also increased PrP expression in gastric adenocarcinomas co-related with histopathological differentiation and tumor progression. Loss of increased PrP expression inhibited cancer cell growth (Li, Cao et al. 2009) and restored the sensitivity of cells towards chemotherapeutic drugs (Du, Pan et al. 2005) and suggested a requirement for PrP over-expression in rapid-cell proliferation and/or enhanced cell survival. In contrast mice where PrP has been over-expressed (tg20, tg35), they have not been reported to have higher incidence of cancer (Fischer, Rulicke et al. 1996). Such studies provoke question whether cancer phenotype is a consequence of PrP expression or whether PrP can stimulate cancer formation only in cells that are already predisposed to develop cancer. It is also possible that the short exposure of 3/689 required during expansion and the prevention of DNA damage offset the possibility of developing cancer. However, this needs to be carefully evaluated involving large *in vivo* studies, where cells cultured with PrP are transplanted and the animals are observed for longer periods of time for signs of any cancer phenotype in syngeneic animal models.

From a clinical perspective, 3/689 may also have potential *in vivo* by protecting directly the cells *in vivo*. It is known that the normal haematopoietic system and the gastrointestinal tract (GI) are the most affected tissues in patients undergoing radiation/chemotherapy (Mauch, Constine et al. 1995; Yu 2013). As 3/689 is shown to protect hMSCs from DNA damage, one possible application could be to administer 3/689 to cancer patients prior to undergoing radiation or chemotherapy. This approach

might prove to be strictly selective in rendering radio-/chemo-protection of normal and healthy hMSCs and indirectly play a role in generating a better functional cell type and rejuvenating the haematopoietic compartment and GI in those cancer patients. Of course this is subject to tests that 3/689 does not improve cancer development.

In this study, 3/689 was shown to enhance SOD2 ROS scavenging activity and suggested a possible role in preventing DNA damage. However, there are other mechanisms which have not been looked into in detail. For example, DNA repair genes were down-regulated with age which were no-more differentially expressed in presence of 3/689, suggested that the small molecule may also be involved in DNA repair mechanisms. Provided 3/689 is also involved in DNA repair mechanism, this molecule can also be of potential use in patients with myelodysplastic syndrome (MDS). MDS occurs mostly in elderly people or in more than 15% of cases post radiation/chemotherapy in cancer patients. MDS patients suffer from bone marrow disorders characterized by initial impaired haematopoiesis followed by acute myelopoisis and ultimately leading to increased risk of leukemia (Mufti 2004). Most of these patients require red blood cell transfusions for anaemia and develop iron overload. The iron overload in cells can catalyse the generation of ROS leading to DNA damage in cells (Greenberg, Koller et al. 2010; Kikuchi, Kobune et al. 2012). In this regard, 3/689 could be administered in early stages of MDS to help prevent DNA damage and delay the development of leukemia in the patients. Of course this is subject to tests that 3/689 also mediates repair mechanisms. With so many possible applications, there are major drawbacks before 3/689 can be considered for clinical applications. It is not known if the administration of 3/689 could promote cancer *in vivo* as there is dearth of evidences

suggesting that PrP may play a role on cancer development. Although 3/689 is a lead molecule in the study, however it shows a functional effect at a very high concentration of 10µM with high degree of aspecificity. It can pose to be highly toxic when administered *in vivo* and is highly unlikely to work effectively. Therefore, designing of analogues of 3/689 needs to be warranted.

In order to design better analogues of 3/689, it is important to understand the pathway leading the DNA damage which would possibly target the increase DNA repair function of MSCs or render protection from DNA damage with age without the risk of cancer. This could be achieved by knocking in and out each element of the pathway and check whether the anti-ageing activity of hMSCs still exists or not without signs of cancer. The pathway studies would also aid in designing better assays to screen more functionally effective analogues. This will be needed to reduce the side effects due to toxicity and reduce aspecificity of the molecule. The initial screening carried out in my study was a very crude screening test conducted on embryonic carcinoma stem (EC) cells which was based on ability of EC cells to proliferate by fluorescence imaging. For example from my data, the pathway involving protection from DNA damage by upregulation of SOD2 gene can be a more specific and stringent assay to consider for effective screening of analogues. To this end, a reporter MSC line could be constructed which could fluoresce when SOD2 gene is upregulated. Also intensive structure activity-relationship (SAR) studies involving techniques such as molecular modelling, x-ray crystallography and NMR would be conducted to discover new and specific binding sites on PrP based on the information available about the structure of PrP. Once this is established, a dose-response curve is mandatory to fine-tune the dose at which the

activity of molecule is at its peak functional ability both *in vitro* and *in vivo*. This way a much better and efficient screening of analogues could be performed which could be more specific in its function and less toxic. Once a lead molecule is obtained, the pharmacokinetics (PK) properties of 3/689 analogue such as ADME (adsorption, distribution, metabolism and excretion) properties, human ether-a-go-go-related gene (hERG) inhibition cannot be ignored. Also pharmacodynamics of the molecule is essential to understand how the molecule interacts with PrP *in vivo*. A bio-marker to PrP will enable to study how this interaction occurs *in vivo*. This is quite a lot of work to achieve. Achieving all of the above parameters would eventually lead to clinical trials.

In conclusion, my study has investigated the potential role of a small molecule, 3/689 by interacting with prion protein. It essentially amplifies the stem cell pool and its differentiated progenies suggesting that the cells are better in quality and highlights the novel idea of promoting long term self-renewal of culture expanded hMSC, which can be implicated for clinical applications in regenerative medicine. However, a lot more in-depth studies are required prior to its clinical application.

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APPENDIX

This study was supported by **Biotechnology and Biological Sciences Research Council** (BBSRC). My contributions towards the publication of my PhD thesis involved largely the entire research work. I have collected human bone marrow samples from hospitals following informed consent in accordance with the protocol approved by the South Sheffield local ethical research committee. I have planned and designed the experimental plan of most experiments which I eventually carried out in the laboratory with approval from my supervisor **Professor. Ilaria Bellantuono**. Those experiments that have not been performed by me are mentioned in the 'Declaration' at the beginning of my thesis. I have also analysed all the data and have written the initial draft of the manuscripts.

APPENDIX-I

STEM CELLS®

REGENERATIVE MEDICINE

A Small Molecule Modulator of Prion Protein Increases Human Mesenchymal Stem Cell Lifespan, Ex Vivo Expansion, and Engraftment to Bone Marrow in NOD/SCID Mice

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Key Words. Marrow stromal cells • Aging • Osteoblast • Mesenchymal stem cell • Stem cell transplantation • Adult stem cells

ABSTRACT

Human mesenchymal stem cells (hMSCs) have been shown to have potential in regenerative approaches in bone and blood. Most protocols rely on their in vitro expansion prior to clinical use. However, several groups including our own have shown that hMSCs lose proliferation and differentiation ability with serial passage in culture, limiting their clinical applications. Cellular prion protein (PrP) has been shown to enhance proliferation and promote self-renewal of hematopoietic, mammary gland, and neural stem cells. Here we show, for the first time, that expression of PrP decreased in hMSC following ex vivo expansion. When PrP expression was knocked down, hMSC showed significant reduction in proliferation and differentiation. In contrast, hMSC expanded in the presence of small molecule 3/689, a modulator of PrP expression, showed retention of PrP expression with ex vivo expansion and extended lifespan up to 10 population doublings. Moreover, cultures produced a 300-fold increase in the number of cells generated. These cells showed a 10-fold increase in engraftment levels in bone marrow 5 weeks post-transplant. hMSC treated with 3/689 showed enhanced protection from DNA damage and enhanced cell cycle progression, in line with data obtained by gene expression profiling. Moreover, upregulation of superoxide dismutase-2 (SOD2) was also observed in hMSC expanded in the presence of 3/689. The increase in SOD2 was dependent on PrP expression and suggests increased scavenging of reactive oxygen species as mechanism of action. These data point to PrP as a good target for chemical intervention in stem cell regenerative medicine. STEM CELLS *2012;30:1134–1143*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Bone marrow (BM) mesenchymal stem cells (MSCs) are thought to have clinical potential in the regeneration of tissues, such as bone, cartilage, and BM stroma, thanks to their ability to proliferate and differentiate to osteoblasts, chondrocytes, and hematopoietic supporting stroma [1]. However, MSCs require expansion in culture prior to clinical use [2]. Regardless of the protocol used during this process, they lose proliferative and differentiation ability with serial passage in culture and undergo replicative senescence, limiting their clinical applications [3–5].

Cellular prion protein (PrP) has been shown to enhance proliferation and promote self-renewal of stem cells [6–8]. Hematopoietic stem cells (HSCs) from PrP-null mice showed diminished engraftment in successive serial transplants. The defect was rescued when PrP-null HSCs were genetically

Author contributions: S.T.M.: collection and assembly of data, data analysis, writing of the manuscript and final approval; C.J.C.: collection and assembly of data, data analysis and interpretation, final approval of manuscript; A.D.C., S.M., and J.F.S.J.H.: conception and design, provision of study material, final approval, H.D.M.: conception and design of the project, M.J.T.: synthesis of compound 3000689(3/689), B.C.: conception, design of the project, design of indole-3-glyoxylamide family of antiprion compounds, A.T.: conception and design, provision of study material, final approval; W.N.K.: conception and design, data analysis 2 and interpretation, final approval of manuscript; I.B.: conception and design, data analysis and interpretation, manuscript writing and final approval of manuscript.

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modified to express PrP [6]. Similarly in the mammary gland, only those cells expressing PrP were able to form mammospheres and regenerate the mammary gland when implanted into the mammary fat pad [8]. In adult neurogenic regions, cellular proliferation was increased in mice overexpressing PrP compared to wild-type and PrP knockout mice [7]. It is unknown whether human MSCs (hMSCs) express PrP and whether PrP enhances their proliferation ability.

Small molecules are attractive tools to manipulate stem cells and understand the signaling pathways regulating their self-renewal and differentiation. Canonical Wnt signaling is a prime example. Progress is well underway for the manipulation of both ESCs and adult stem/progenitor cells using inhibitors of glycogen synthase kinase 3 (GSK-3) [9–11]. Our group has shown that a novel selective inhibitor of GSK-3 was able to amplify mesenchymal progenitors and induce their differentiation to the osteogenic lineage in vitro and in vivo [12]. Administration of another GSK-3 inhibitor was shown to enhance the self-renewal ability of human HSC following transplantation [10]. Small molecules targeting PrP are available [13, 14], but their effect on MSC is unknown. In this study, we hypothesize that PrP is required for proliferation of hMSC and that a small molecule modulator of PrP can be used to enhance their proliferation and extend their lifespan in culture, while still retaining their ability to differentiate and engraft to BM.

Materials and Methods

Isolation and Culture of hMSC from Human BM

hMSCs were derived from BM harvested from the iliac crest of patients undergoing osteotomy for reasons other than metabolic disorders, or patients undergoing a diagnostic BM, which were subsequently reported normal. BM was obtained following informed written consent in accordance with the protocol approved by the South Sheffield local ethical research committee, and hMSCs were isolated and cultured as previously described [15]. "Briefly bone marrow mononuclear cells (MNC) were isolated by density gradient separation medium (1.077 g/l, PAA laboratories, Pasching, Austria; http://www.paa.com/the_cell_culture_company.html) and plated at 8,000 MNC/cm^2 in hMSC medium composed of Dulbecco's modified Eagle medium (DMEM) and 10% fetal calf serum (Hyclone, Fisher, Scientific, Loughborough, UK, http://www.fisher.co.uk/) and incubated at 37°C in 5% carbon dioxide in air. Nonadherent cells were removed 48 hours later. Thereafter the medium was changed weekly until the cells were confluent. At confluence hMSC cultures were detached by incubation with 0.5% trypsin/1 mM EDTA (Gibco, Paisley, U.K., http://www.lifetechnologies.com/uk/en/home.html) and plated at 1000/cm^2." The number of progenitor MSCs was determined by the colony-forming unit fibroblast (CFU-F) assay as previously described [3]. For assessment of growth kinetics, the number of CFU-F at the start of the culture was used to determine the number of population doublings (PDs) that cells have undergone to reach primary confluence. Thereafter, the number of PD was calculated as log N/log 2, where N is the number of cells at confluence divided by the number of cells at the start of the culture. Small molecule 3000689 (3/689) is a member of the indole-3-glyoxylnaphthylamide family of antiprion compounds, which was selected for this study from an early screening programme (a separate publication is in preparation). It was synthesized and characterized as previously described [13,16]. 3/689 was dissolved in dimethylsulfoxide (DMSO) at 3.89 mg/ml and then diluted further in MSC medium for in vitro and in vivo studies. Cultures were fed twice weekly.

Clonogenic and Differentiation Assays

CFU-F. The number of clonogenic progenitors contained in hMSC cultures was obtained by replating hMSC at 10 and 20 cells per square centimeter in DMEM plus 10% fetal calf serum (FCS) (Hyclone, Fisher) (hMSC medium) for 14 days at 37°C. Colonies were visualized by Wright's Giemsa staining (VWR, Leicestershire, U.K., https://uk.vwr.com/app/Home). Colonies with a minimum of 50 cells by visual inspection were considered as one CFU-F. Colonies were scored from plates seeded with 10 cells per square centimeter, unless growth was not observed. In this case, wells seeded with 20 cells per square centimeter were scored and frequency adjusted accordingly.

CFU Osteoblasts. The number of clonogenic progenitors with osteogenic potential was obtained by seeding 10 and 20 hMSCs per square centimeter from established cultures in hMSC medium supplemented with osteogenic supplements 0.05 mM L-ascorbic acid (Sigma-Aldrich, St. Louis, USA, www.sigmaaldrich.com), 10 mM β-glycerophosphate (Sigma-Aldrich), and 100 nM dexamethasone (Sigma-Aldrich). Cells were maintained for 14 days at 37°C and fed twice a week. Colonies were stained for alkaline phosphatase (ALP) activity using the 86R alkaline phosphatase kit (Sigma-Aldrich) according to manufacturer's instructions. Colonies with a minimum of 40 cells positive for ALP were considered as one CFU osteoblasts (CFU-O).

CFU Adipocyte. The number of clonogenic progenitors with adipogenic potential was determined by plating hMSC at limiting dilutions (range 10^5–6.25×10^3 cells, eight wells per dilution in 96-well plate) in hMSC medium and cultured at 37°C as previously described [17]. After 2 days in culture, the cells were induced to differentiate by adding adipogenic supplements 100 nM dexamethasone and 1 μg/ml 3-isobutyl-1-methylxanthine (Sigma-Aldrich) for 2 weeks and then stained with Oil red O to detect lipid vacuoles. A well was considered positive if it contained more than 20 cells with red lipid vacuoles. The number of CFU adipocyte (CFU-A) was obtained following the Poisson distribution and using the formula $F_o = e^{-x}$, where F_o is the fraction of colony-negative wells, e is the constant whose value is 2.71, and x is the number of CFUs per well.

Differentiation of hMSC Cultures. Osteogenic differentiation was induced by plating 1.2×10^3 hMSCs per square centimeter in hMSC medium supplemented with osteogenic supplements. Adipogenic differentiation was induced by plating 2.8×10^3 hMSCs per square centimeter in hMSC medium supplemented with adipogenic supplements. Cells were maintained at 37°C and fed twice a week. After 2 weeks, total RNA was extracted using RNAqueous 4PCR Kit (Ambion, Warrington, U.K., http://www.invitrogen.com/site/us/en/home/brands/ambion.html) according to manufacturer's instructions. Two micrograms of total RNA was used for reverse transcription using the first Strand cDNA Synthesis Kit (GE Healthcare, Amersham, U.K., http://www.gehealthcare.com/uken/about/about_amersham.html). Quantitative real-time polymerase chain reaction (PCR) was performed using SYBR green PCR Master Mix (Eurogentec, Romsey, U.K., http://www.eurogentec.com/eu-home.html) and the primers (Supporting Information Table S1) were used at 0.1 μM. PCR amplification was carried out according to the following conditions: 1 cycle of 50°C for 2 minutes, 95°C for 10 minutes; 40 cycles of 95°C for 15 seconds, 60°C for 1 minute. The data were analyzed using SDS 2.0 software.

Western Blotting

Protein lysates (20 μg) were separated using 12% tris-glycine gels. Membranes were blocked by incubation for 2.5 hours in 5% bovine serum albumin in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBS-T) for the detection of glyceraldehyde-3 phosphate dehydrogenase (GAPDH) and 5% casein in PBS-T for the detection of PrP. Membranes were then incubated overnight at 4°C with either the primary antibody mouse anti-human prion protein SAF32 (Spibio, Massy, France, http://www.bertinpharma.com/about.aspx) at 1:400 or mouse anti-human GAPDH (Abcam, Cambridge, U.K.,

http://www.abcam.com/) at 1:1,000. Goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (DAKO, immunoglobulin A/S, Copenhagen, Denmark, http://www.dako.com/uk/index.htm) was then used at 1:3,000 for 1 hour at room temperature. Detection was carried out using enhanced chemiluminescence plus ECL reaction kit (GE Healthcare, Little Chalfont, U.K., http://www.gehealthcare.com/uken). Quantification of protein expression was carried out using Image J Software.

Lentiviral Vectors

Lentiviral particles were generated by cotransfection of HEK293T cells with the envelope and packaging plasmids pMD.G2 and pCMVΔR8.91 (produced by L. Naldini and kindly donated by A. Thrasher), and plasmid containing the vector of interest as previously described [18]. p'HRsincpptSEW (kindly donated by A. Thrasher, University College London) was used to express enhanced green fluorescent protein (eGFP) to label hMSC for transplantation experiments. pGPIZ shRNAmir contained a hairpin sequence to specifically knock down expression of PrP (shRNA-2, TGCTGTTGACAGTGAGCG**CGCACTGAATCGTTTCATGTAA**TAGTGAAGCCACAGATGTA**TTACATGAAACGATTCAGTGCA**TGCCTACTGCCTCGGA), an internal ribosome entry site and eGFP (Thermofisher, Epsom, U.K., https://www.openbiosystems.com/default.aspx). A similar pGPIZ shRNAmir containing a nonsilencing hairpin sequence (shRNA-ns TGCTGTTGACAGTGAGCGATCTCGCTTGGGCGA GAGTAAGTAGTGAAGCCACAGATGTACTTACTCTCGCCCA AGCGAGAGTGCCTACTGCCTCGGA) was used as control. hMSCs were transduced at a multiplicity of infection of 40 based on 3T3 transducing units by fluorescent-activated cell sorting (FACS).

Transplantation of hMSC

NOD/LtSz-Prkdc^{scid} (nonobese diabetic severely combined immunodeficient [NOD/SCID]) mice aged 5–6 weeks were injected with 5×10^5 hMSC-expressing eGFP by intrafemoral injection according to the approved home office license. Mice were sacrificed 3 days and 5 weeks later and femurs were fixed with 10% formalin. Following rapid decalcification in Surgipath decalcifier II (Leica Microsystems, Milton Keynes, U.K., http://www.leica-microsystems.com/) for 2 hours, tissues were embedded in wax and 3-μm sections were cut using a Leica Microsystems microtome (Leica Microsystems) and stained for the detection of eGFP. Briefly, sections were fixed with 99% ethanol for 5 minutes and blocked with 3% H_2O_2 for 10 minutes. They were blocked with 10% goat serum (DAKO, Ely, U.K., http://www.dako.com/uk/index.htm). The primary rabbit anti-GFP antibody (Invitrogen, Paisley, U.K., http://www.lifetechnologies.com/uk/en/home.html) was used at 1:600 overnight at 4°C and the goat anti-rabbit HRP secondary antibody at 1:400 (Insight Biotech, Wembley, U.K., http://www.insightbio.com/) for 45 minutes at room temperature. Detection was carried out using Vector NovaRED substrate kit (Vector Laboratories Ltd., Peterborough, U.K., http://www.vectorlabs.com/uk/default.aspx) and counterstained by Gill's hematoxylin stain (VWR International, Lutterworth, U.K., https://uk.vwr.com/app/Home). Eight sections per animal were scored for the presence of eGFP+ cells.

Cell Cycle Analysis and DNA Damage Detection

For cell cycle analysis, 10^5 hMSCs cultured in the presence or absence of 3/689 were seeded at 4,000/cm^2. The following day, cells were incubated with carboxyfluorescein succinimidyl ester (CFSE) at 1 μM using the Cell Trace CFSE Cell proliferation kit (Invitrogen) for 15 minutes, after which they were incubated in fresh MSC medium and analyzed after 1 and 5 days in culture by FACS using Cell Quest software. Cells are labeled with the fluorescent dye and cell division results in sequential halving of fluorescence. For the induction of DNA damage, hMSCs (2,000 cells per square centimeter) grown in the presence or absence of 3/689 were exposed to H_2O_2 at 75 μM for 1 hour. Phosphorylation of histone H2AX on Ser 139 (γH2AX) was carried out using the γH2AX phosphorylation assay kit (Millipore, Watford, U.K., http://www.millipore.com/) according to manufacturer's instruction. Cells were analyzed by FACS using Cell Quest software.

Microarray

Total RNA was extracted from hMSC cultures ($n = 3$ per group) expanded in the presence or absence of 3/689 at 10 μM and harvested after two passages or eight passages using the Nucleospin II RNA extraction kit (Macherey-Nagel, Duren, Germany) following manufacturer's instructions. RNA was amplified and labeled using the Agilent Low RNA Input Linear Amplification Kit PLUS, One-Color and hybridized to Agilent whole human genome 4 × 44 K gene expression arrays as per manufacturer's instructions. Raw data were extracted from scanned images using Agilent feature extraction software (Agilent Technologies, Santa Clara, CA). All array data were then imported into GeneSpring GX (version 11, Agilent Technologies) and normalized to the 75th percentile. Differentially expressed genes were obtained using GeneSpring GX by paired t test with $p < .05$. The data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE31205 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31205). The network analysis was performed using Metacore software (GeneGo Inc.).

Statistical Analysis

All experiments were analyzed using t tests or one-way ANOVA—Bonferroni's for multiple comparisons. All results are expressed as the mean ± SEM. Significant p values were less than .5 with *, $p < .05$; **, $p < .01$; ***, $p < .001$.

Results

PrP Is Expressed in hMSC and Its Expression Decreases with Serial Passage in Culture

To determine whether hMSC expressed cellular PrP and whether differentiation affected its expression, three cultures were established from the BM of donors aged 14–32 years and hMSCs were assayed for the expression of PrP after 19–23 PD and following exposure to adipogenic and osteogenic supplements. As expected, hMSC exposed to osteogenic differentiation supplements showed upregulation of osteocalcin and ALP expression (Supporting Information Fig. S1A, S1B, $n = 3$) compared to undifferentiated cultures, and hMSC cultures exposed to adipogenic supplements showed increased expression of peroxisome proliferator-activated receptor γ (PPARγ) and formation of Red oil O lipid vacuoles (Supporting Information Fig. S1C, S1D, $n = 3$), confirming they had undergone differentiation. All cultures expressed PrP regardless of the differentiation status and, if anything, an increase was seen (Fig. 1A, 1B). These data suggest that PrP expression is not a unique property of undifferentiated hMSC.

To determine whether loss of hMSC proliferation and differentiation capacity with serial passage in culture was associated with decreased PrP expression, the same cultures were expanded and replated at low density to assess the content of clonogenic progenitors together with PrP expression at passage 3 (equivalent to 13–16 PD) and after a further 6–7 PD (passage 8). As expected, a significant decrease in the number of CFU-F (Fig. 1C-a, $n = 3$, $p = .004$), CFU-O (Fig. 1C-b, $n = 3$, $p = .013$), and CFU-A (Fig. 1C-c, $n = 3$, $p = .008$) was observed in hMSC cultures at passage 8 compared to passage 3. This correlated with a significant 61–95% decrease in PrP expression (Fig. 1D, 1E, $n = 3$, $p = .018$), suggesting that PrP expression is downregulated upon serial passage.

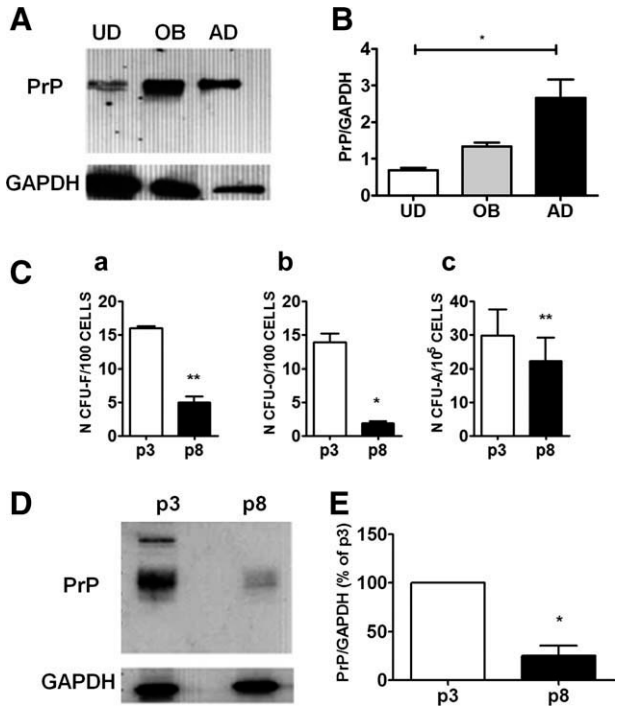

Figure 1. Cellular PrP is expressed in undifferentiated human mesenchymal stem cells (hMSC), osteoblasts, and adipocytes and significantly decreases with time in culture. **(A):** A representative example of Western blot of hMSC culture hybridized with antibodies specific for cellular prion protein (top panel) and GAPDH (bottom panel). **(B):** Quantification of PrP expression normalized to GAPDH in undifferentiated hMSC (UD, $n = 3$) and hMSC differentiated to the OB and AD lineage and assessed by Western blot followed by analysis with imageJ software. **(C):** Analysis of the number of clonogenic mesenchymal progenitors **(a)** CFU-F, **(b)** CFU-O, and **(c)** CFU-A obtained by replating hMSC from cultures at passage 3 (p3) and passage 8 (p8) at low density. **(D):** A representative example of Western blot of hMSC culture at passage 3 and at passage 8 detecting prion protein expression (top panel) and GAPDH (bottom panel). **(E):** Quantification of PrP expression normalized to GAPDH in hMSC at p3 and p8 assessed by Western blot and analyzed using ImageJ software. All data are presented as mean ± SEM and analyzed by paired *t* test or one-way ANOVA and Bonferroni for multiple comparison post-test with *, $p < .05$; **, $p < .01$. Abbreviations: AD, adipogenic; CFU-A, colony-forming unit fibroblast adipocyte; CFU-F, colony-forming unit fibroblast; CFU-O, colony-forming unit osteoblast; GAPDH, glyceraldehyde-3 phosphate dehydrogenase; PrP, prion protein; OB, osteogenic; UD, undifferentiated.

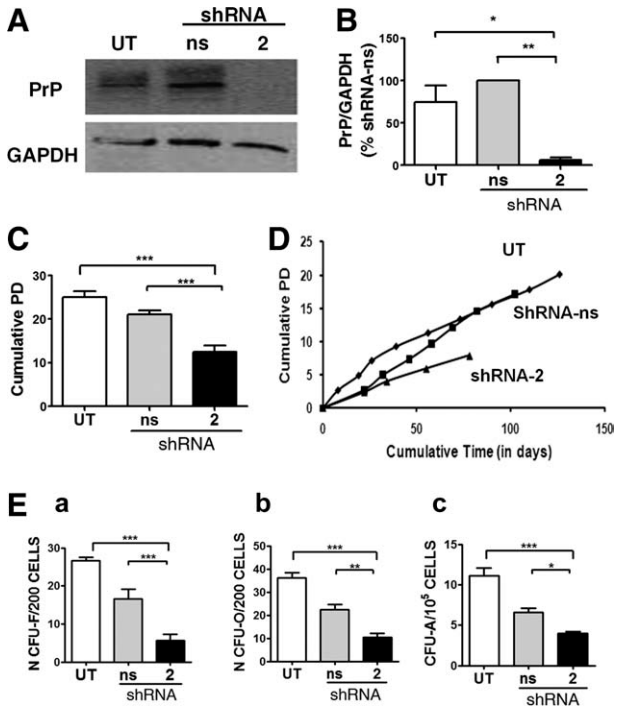

Figure 2. Knockdown of PrP expression decreases human mesenchymal stem cell (hMSC) expansion. **(A):** A representative example of Western blot of hMSC culture UT, transduced with the lentiviral vector pGIPZ shRNAmir containing the short hairpin sequence to specifically knock down the expression of PrP (shRNA-2) or containing a short hairpin nonsilencing sequence (shRNA-ns) and hybridized with a rabbit polyclonal anti-human PrP (top panel) or mouse anti-human GAPDH (bottom panel) antibody. **(B):** Quantification of PrP expression normalized to GAPDH of UT hMSC cultures, transduced with shRNA-2 or shRNA-ns and assessed by Western blot followed by analysis with imageJ software. **(C):** Total number of population doublings performed by hMSC cultures transduced with shRNA-2 compared to the same cultures transduced with the shRNA-ns or not transduced. **(D):** A representative example of growth kinetic of hMSC culture, UT (filled diamonds) transduced with shRNA-ns (filled squares) or shRNA-2 (filled triangle). **(E):** Quantification of the number of **(a)** colony-forming unit fibroblast, **(b)** colony-forming unit osteoblast, and **(c)** colony-forming unit adipocyte obtained upon replating at low density of shRNA-2, shRNA-ns, and UT hMSC cultures after 15.4 ± 0.5 days of culture. All data are presented as mean ± SEM and analyzed by one-way ANOVA and Bonferroni multiple comparison post-test with *, $p < .05$; **, $p < .01$; ***, $p < .001$. Abbreviations: GAPDH, glyceraldehyde-3 phosphate dehydrogenase; PrP, prion protein; UT, untransduced.

PrP Expression Is Required for hMSC Proliferation and Differentiation

To verify whether PrP was required for the proliferation and differentiation of hMSC, PrP expression was knocked down using pGIPZ lentiviral vector shRNA-2. A similar vector containing the nonsilencing shRNA-ns sequence was used as control. Following isolation, each hMSC culture was divided into three subcultures: (a) not exposed to the lentivirus (untransduced), (b) exposed to lentivirus containing shRNA-2, and (c) exposed to lentivirus containing shRNA-ns. Cultures ($n = 8$) were found to be 93.26%±3 eGFP+ when transduced with pGIPZ shRNA-ns and 86%±9% with pGIPZ shRNA-2. hMSC transduced with pGIPZ shRNA-2 expressed significantly reduced levels of PrP (93.5%±5.7%) compared to pGIPZ shRNA-ns transduced cultures (Fig. 2A, 2B, $p < .01$, $n = 4$). No significant difference in PrP levels was seen when comparing untransduced and pGIPZ shRNA-ns transduced cultures ($n = 4$).

Following transduction, hMSCs were expanded in culture and growth kinetics examined until proliferation stopped for 3 weeks, at which point the cultures were considered senescent. pGIPZ shRNA-2 hMSC cultures underwent an average 8.5 ± 1.7 less total number of PDs compared to pGIPZ shRNA-ns cultures (Fig. 2C, 2D, $p < .005$, $n = 5$). Moreover, pGIPZ shRNA-2 hMSC cultures were replated at low density after 15.4 ± 0.5 days from the time of transduction and contained a significantly reduced number of clonogenic progenitors as shown by the number of CFU-F (Fig. 2E-a, $p < .01$, $n = 8$), CFU-O (Fig. 2E-b, $p < .01$, $n = 8$) and CFU-A (Fig. 2E-c, $p < .05$, $n = 8$) compared to pGIPZ shRNA-ns or untransduced cultures, a loss which was similar to that seen in cultures with serial passage.

To determine whether reduced PrP expression affected differentiation of hMSC, cultures were also exposed to osteogenic and adipogenic differentiation supplements for 2 weeks and assessed for markers of osteogenic and adipogenic differentiation, respectively. As expected, an increase in all markers

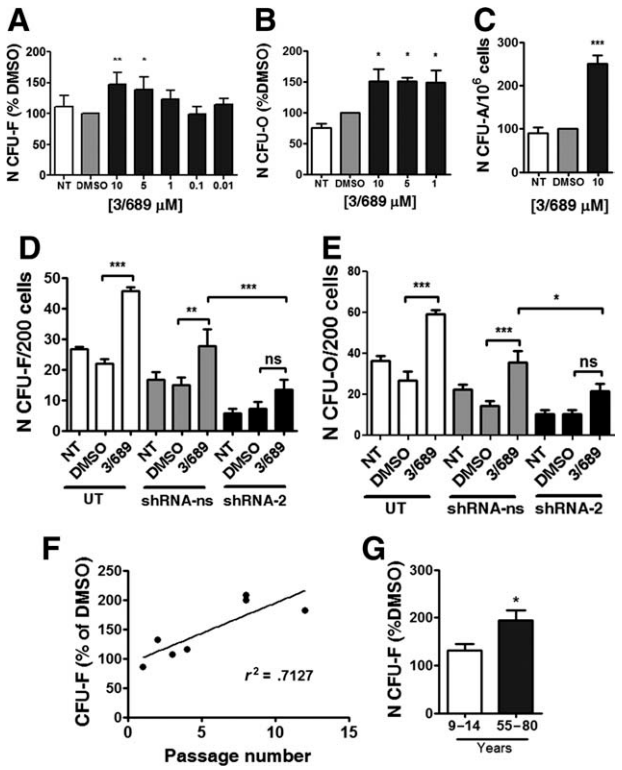

Figure 3. Small molecule 3/689 enhances the number of clonogenic mesenchymal progenitors and requires prion protein (PrP) expression for its action. **(A):** Dose-response analysis of the ability of 3/689 to increase the number of CFU-F upon replating of human mesenchymal stem cell (hMSC) cultures at low density. **(B):** Dose-response analysis of the ability of 3/689 to increase the number of CFU-O upon replating of hMSC cultures at low density. **(C):** Graph showing the number of CFU-A obtained upon replating of hMSC cultures at limiting dilution in the presence of 3/689 at 10 μM. **(D, E):** Untransduced hMSC cultures, transduced with shRNA-ns and shRNA-2 were replated at low density in the presence or absence of 3/689 and assessed for the number of CFU-F and CFU-O. **(F):** A significant correlation was found between the percentage increase in the number of CFU-F in the presence of 3/689 at 10 μM (compared to DMSO control) and the time in culture at which hMSCs were replated at low density for this assay. **(G):** Number of CFU-F obtained upon replating of hMSC cultures at low density in the presence or absence of 3/689. Cultures were established from the bone marrow of donors aged 1–14 years and 55–80 years and expanded in culture for two passages prior to the assay. All data were analyzed by one-way ANOVA and Bonferroni multiple comparison post-test with *, $p < .05$; **, $p < .01$; ***, $p < .001$. Abbreviations: CFU-F, colony-forming unit fibroblast; CFU-O, colony-forming unit osteoblast; CFU-A, colony-forming unit adipocyte; DMSO, dimethylsulfoxide.

of differentiation was observed after 2 weeks exposure to differentiation stimuli in untransduced cultures or cultures transduced with pGIPZ shRNA-ns (Supporting Information Fig. S2A–S2F, $n = 4$). In contrast, a significant decrease in all markers of differentiation was seen in cultures exposed to differentiation supplements and transduced with pGIPZ shRNA-2 (Supporting Information Fig. S2A–S2F, $n = 4$), suggesting that reduced PrP expression blocks hMSC differentiation.

Small Molecule Modulator of PrP Enhances Proliferation of Mesenchymal Progenitors and Has Increased Activity with Cellular Ageing

To ascertain whether targeting PrP delayed loss of hMSC proliferation and differentiation ability with serial passage in culture, we exposed hMSC cultures to the small molecule 3/689. This was obtained from a library of compounds with antiprion activity and it was previously shown to enhance embryonic carcinoma cell proliferation [13, 16]. Previously established hMSC cultures were seeded at low density and exposed to 3/689 at concentrations ranging from 10 to 0.01 μM to assess the effects on the number of the clonogenic progenitors CFU-F and CFU-O. A significant increase in the number of CFU-F (Fig. 3A, $p < .001$, $n = 7$) and CFU-O (Fig. 3B, $p < .05$, $n = 6$) was observed when cells were exposed to 3/689 with maximum response at 10 μM. A similar response was also seen when measuring the effects on mesenchymal progenitors with adipogenic potential at 10 μM (Fig. 3C, $p < .0001$, $n = 6$). To verify that 3/689 required PrP expression for its action, hMSC cultures transduced with pGIPZ shRNA-2 and expressing low levels of PrP were also exposed to the compound. As expected, a significant increase in the number of CFU-F (Fig. 3D, $p < .001$, $n = 8$) and CFU-O (Fig. 3E, $p < .001$, $n = 8$) was observed when untransduced and pGIPZ shRNA-ns transduced hMSC cultures were exposed to 3/689. In contrast, no significant increase was seen when 3/689 was administered to hMSC cultures transduced with pGIPZ shRNA-2, suggesting that 3/689 requires PrP for its activity.

While carrying out those experiments, we noticed a significant positive strong correlation between the increase in the number of CFU-F obtained following exposure of hMSC to 3/689 at 10 μM and the time hMSC had been in culture prior to the clonogenic assay (Fig. 3F, $n = 7$, $r^2 = .7127$, $p = .02$). hMSC that had been expanded for longer periods of time had an enhanced response. This prompted us to verify whether this was also true for hMSC derived from older donors compared to younger donors. hMSC cultures were isolated from the BM of donors aged 9–14 years old ($n = 6$) and 55–80 years old ($n = 5$) and allowed to proliferate for similar number of PD (passage 2) prior to exposure to 3/689 at 10 μM. A significantly higher increase in the number of CFU-F was seen in cultures derived from older donors in the presence of 3/689 compared to cultures obtained from younger donors (Fig. 3G, $p = .03$, $n = 5$). These data suggest that 3/689 has a more potent effect with cellular aging.

To establish whether 3/689 extended the lifespan of hMSC following in vitro expansion, each human BM sample was divided in three equal parts; one part cultured in the presence of 3/689 at 10 μM, one part in the presence of an equivalent amount of DMSO, and one part in standard culture conditions. Cultures were maintained until they stopped proliferating for at least 3 weeks, when they were considered to have reached senescence. No difference in proliferation rates was seen in the first 19–24 PD ($n = 5$). However after this, cultures isolated and maintained in the presence of 3/689 showed a significantly decreased PD/t (data not shown) and underwent an average 7 $\pm$ 3 PD more than hMSC cultures exposed to DMSO ($n = 5$, Fig. 4A). This was equivalent to more than 300-fold increase in the total number of cells generated from each culture (Fig. 4B, $p = .03$, $n = 5$). More importantly, hMSC cultures expanded in the presence of 3/689 were assayed for their content of clonogenic progenitors by replating the cells at low density after 90.4 $\pm$ 17 days in culture. hMSC cultures grown in the presence of 3/689 contained significantly higher number of CFU-F (Fig. 4C-a, $p < .05$, $n = 5$), CFU-O (Fig. 4C-b, $p < .001$, $n = 5$), and CFU-A (Fig. 4C-c, $p < .05$, $n = 4$) compared to DMSO control cultures. Moreover, their differentiation ability to the osteogenic lineage was enhanced as shown by the increased expression levels of osteopontin and osteocalcin (Supporting Information Fig. S3A–S3D, $n = 4$), used as late markers of osteogenic differentiation. These levels were significantly increased in 3/689 treated cultures compared to DMSO. An increase in the expression of markers of adipogenic

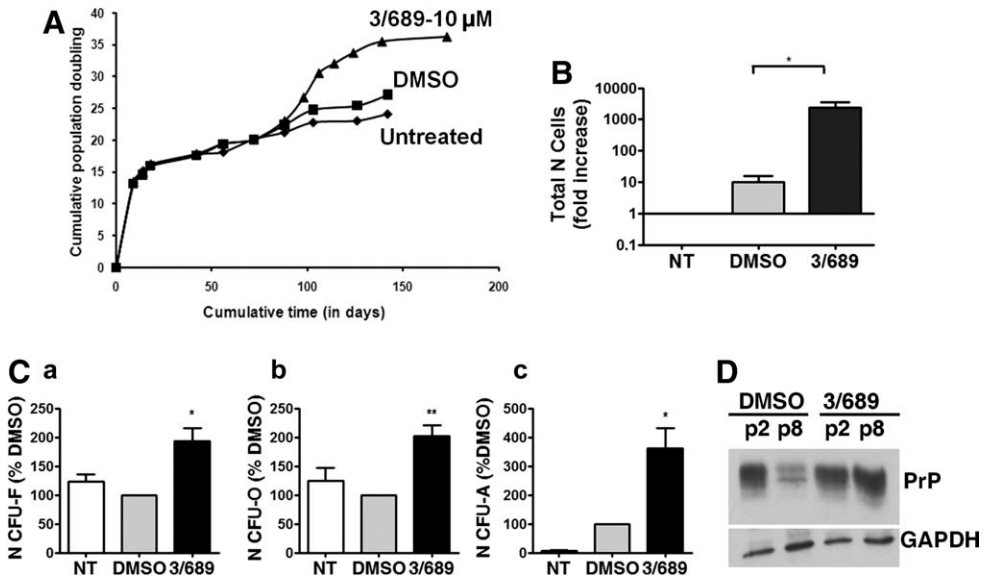

Figure 4. 3/689 extends human mesenchymal stem cell (hMSC) lifespan and retains PrP expression with time in culture. **(A):** A representative example of growth kinetic of hMSC cultures expanded in medium only (filled diamonds), hMSC cultures expanded in the presence of DMSO (filled squares) or in the presence of 3/689 at 10 μM (filled triangle). **(B):** Total number of cells generated from the expansion of hMSC cultures isolated and maintained in medium only (NT), medium plus DMSO, or in the presence of 3/689 ($n = 5$). **(C):** The number of CFU-F **(a)**, CFU-O **(b)**, and CFU-A **(c)** obtained by replating hMSC cultures expanded in medium alone, in the presence of DMSO or 3/689 at 10 μM for 90.4 $\pm$ 17 days. **(D):** A representative example of Western blot of hMSC cultured in the presence or absence of 3/689 at passage 2 (p2) and passage 8 (p8) hybridized with an antibody specific for GAPDH (bottom panel) or cellular prion protein (top panel). Data are presented as mean $\pm$ SEM and analyzed by one-way ANOVA and Bonferroni multiple comparison post-test with *, $p < .05$; **, $p < .01$. Abbreviations: CFU-F, colony-forming unit fibroblast; CFU-O, colony-forming unit osteoblast; CFU-A, colony-forming unit adipocyte; DMSO, dimethylsulfoxide; GAPDH, glyceraldehyde-3 phosphate dehydrogenase.

differentiation PPARγ and lipoprotein lipase was also seen in cultures previously expanded in the presence of 3/689 and exposed to adipogenic differentiation supplements but was not significantly different from DMSO-treated cultures (Supporting Information Fig. S3E, S3F, $n = 4$). To confirm that cultures exposed to 3/689 retained PrP expression with serial passage, cultures were analyzed for PrP expression at passage 2 (equivalent to 14–16PD) and passage 8 (equivalent to 21–30PD). As expected, cultures exposed to DMSO lost PrP expression with time in culture. In contrast, no difference in levels of PrP expression was seen in cultures exposed to 3/689 following expansion (Fig. 4D, $n = 3$). These data suggest that enhanced expression of PrP using the small molecule 3/689 is associated with increased hMSC proliferation and delayed loss of mesenchymal clonogenic progenitors with serial passage in culture.

hMSC Expanded in the Presence of 3/689 Showed Increased Engraftment Ability

hMSC engraftment to BM has been difficult and very low at best. It has been shown that serial passage in culture affects hMSC ability to engraft to BM [19]. To determine whether expansion of hMSC in the presence of 3/689 enhanced engraftment ability, hMSC cultures were transduced with a lentiviral vector expressing eGFP and grown in the presence of 3/689 or DMSO for five passages prior to transplantation in NOD/SCID mice by intrafemoral injection. During this time, cultures expanded in the presence of 3/689 showed an average twofold increase in cell number compared to control cultures and a normal karyotype (Fig. 5A). Engraftment was assessed 3 days after transplantation by measuring the number of eGFP+ CFU-F and eGFP+ CFU-O and 5 weeks after by counting the number of eGFP+ cells present in the femurs. A significant increase in the number of eGFP+ CFU-F (Fig. 5B, $p < .0001$, $n = 6$) and eGFP+ CFU-O (Fig. 5C, $p = .040$, $n = 6$) was observed in the BM of NOD/SCID mice transplanted with hMSC cultures expanded in the presence of 3/689 compared to DMSO controls, 3 days postinjection. More importantly, 5 weeks postinjection, an average 10-fold increase in the number of eGFP+ cells was seen in the BM of NOD/SCID mice by immunohistochemistry (Fig. 5D, $p = .025$, $n = 9$). Cells with morphology and locations resembling pericyte, stromal cells, osteoblast, and osteocyte were seen in mice transplanted with 3/689 expanded cultures (Supporting Information Fig. S4 and Fig. 5E, $n = 9$). In contrast, mice injected with DMSO-expanded cultures showed only morphology of pericytes and stromal cells. These data suggest that hMSCs cultured in the presence of 3/689 have enhanced engraftment ability.

hMSC Treated with 3/689 Showed Increased Superoxide Dismutase-2 Expression and Are Protected from DNA Damage

To determine the mechanisms of action of 3/689, changes in gene expression profile of hMSC cultures exposed to 3/689 or DMSO at passage 2 (p2) and passage 8 (p8) were examined by whole genome expression arrays. Analysis of the shortlist of differentially expressed genes in terms of biological function using the manually curated pathway analysis software Metacore revealed statistically over-represented networks in processes related to cell cycle (26%) and DNA damage (8%) occurring in hMSC with serial passage in culture in the absence of 3/689 (Supporting Information Fig. S5A). These processes were no longer significantly overrepresented when hMSCs were cultured in the presence of 3/689 with time in culture (Supporting Information Fig. S5B). Moreover, there were more overrepresented networks related to stem cell and development, immune responses, and cytoskeletal development in hMSC exposed to 3/689 compared to DMSO controls (Supporting Information Fig. S5A, S5B).

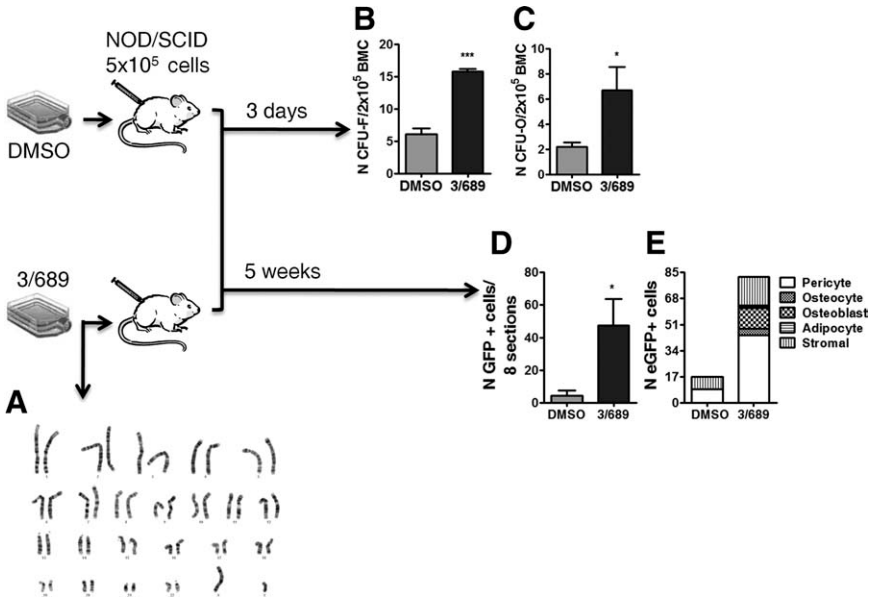

Figure 5. Human mesenchymal stem cell (hMSC) expanded in the presence of 3/689 showed increased engraftment ability. **(A):** A representative example of karyogram of hMSC culture derived and expanded in the presence of 3/689 at 10 μM performed at the time of transplant. **(B):** The number of human eGFP+ CFU-F and **(C)** eGFP+ CFU-O recovered from the bone marrow (BM) of NOD/SCID mice transplanted with hMSC cultured in the presence of 3/689 at 10 μM concentration or DMSO 3 days postinjection. **(D):** The number of eGFP+ hMSC detected in the BM of NOD/SCID mice 5 weeks postinjection following transplantation of hMSC cultures expanded in the presence or absence of 3/689. Number of eGFP+ cells detected in the BM of NOD/SCID mice transplanted with hMSC cultures expanded in the presence or absence of 3/689 and classified based on their morphology and anatomical location (iv). All data are presented as mean ± SEM and analyzed by unpaired *t* test with *, $p < .05$; ***, $p < .001$. Abbreviations: CFU-F, colony-forming unit fibroblast; CFU-O, colony-forming unit osteoblast; DMSO, dimethylsulfoxide; eGFP, enhanced green fluorescent protein; NOD/SCID, nonobese diabetic severely combined immunodeficient.

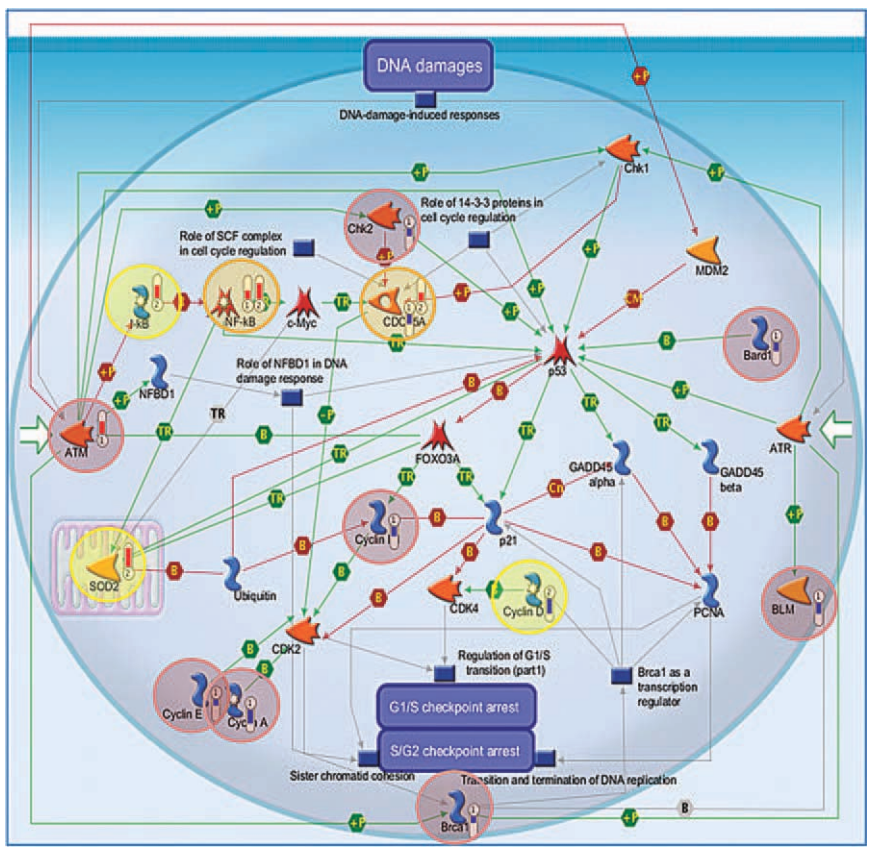

Figure 6. Network representing enrichment in differentially expressed genes related to DNA damage and cell cycle is important in stem cells and aging. A representation of the network containing a significant number of differentially expressed genes related to cell cycle—DNA damage as obtained from the analysis using GeneGo Metacore software. The genes represented with a red thermometer were found to be upregulated with serial passage in culture while those with a blue thermometer were downregulated. Thermometer labeled 1 and highlighted in pink represent differentially expressed genes in human mesenchymal stem cell (hMSC) cultures expanded in absence of 3/689, whereas thermometer labeled as 2 and highlighted in yellow represents differentially expressed genes in hMSC cultures expanded in the presence of 3/689 relative to controls. Abbreviation: SOD2, superoxide dismutase-2.

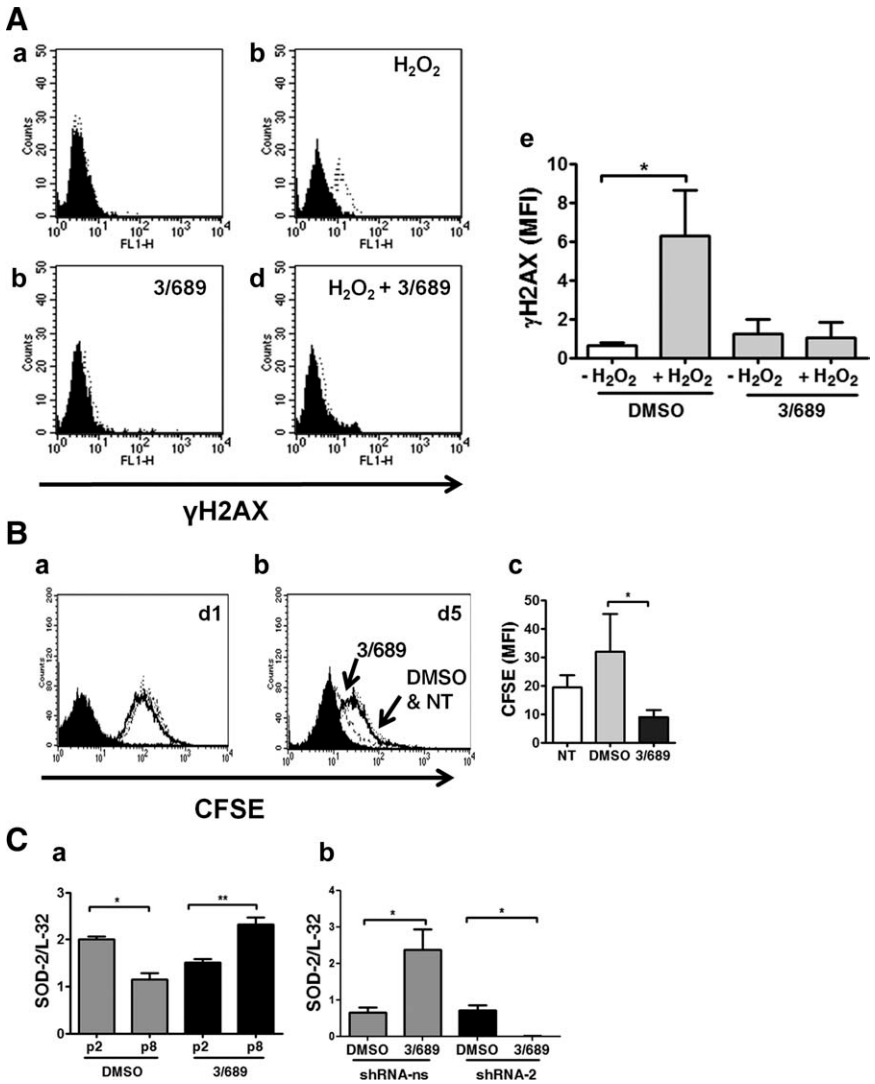

Figure 7. Small molecule 3/689 acts by upregulating SOD2, protects from DNA damage, and enhances cell cycle progression. **(A):** A representative example of flow cytometry profile of human mesenchymal stem cell (hMSC) culture stained for the presence of phosphorylated γH2AX following exposure to H_2O_2 for 1 hour. hMSC exposed to **(a)** medium only, **(b)** H_2O_2, **(c)** 3/689, and **(d)** to H_2O_2 plus 3/689. Filled histogram represents hMSC culture stained with the isotype control and open histogram hMSCs stained with phosphorylated γH2AX specific antibody. **(e)** Quantification of the MFI of hMSC stained with phosphorylated γH2AX following exposure to H_2O_2 for 1 hour in the presence or absence of 3/689 ($n = 3$). **(B):** A representative example of hMSC labeled with CFSE and cultured in the presence of 3/689 (open dotted histogram) DMSO (open single line histogram) or medium alone (open single lined histogram) for 1 day **(a)** and 5 days **(b)**. The filled histogram represents unlabeled hMSC cultures and open histograms cultures labeled with CFSE and treated with 3/689 (gray large dotted line), DMSO (black line), and medium alone (gray small dotted line). **(C):** Real-time quantitative PCR to detect the expression of **(a)** SOD-2 in hMSC cultures maintained in culture for two passages and eight passages in the presence or absence of 3/689. **(b):** SOD-2 in hMSC cultures transduced with shRNA-ns or shRNA-2 and exposed to DMSO or 3/689. Column data are expressed as mean ± SEM and analyzed by one-way ANOVA and Bonferroni for multiple comparisons post-test *, $p < .05$; **, $p < .01$. Abbreviations: CFSE, carboxyfluorescein succinimidyl ester; DMSO, dimethylsulfoxide; H_2O_2, hydrogen peroxide; MFI, mean fluorescent intensity; SOD2, superoxide dismutase-2.

As processes related to DNA damage and cell cycle are important in cellular aging, networks with enrichment in differentially expressed genes related to these processes were examined in more detail (Fig. 6). The DNA repair genes *Bard1*, *Brca1*, and *Bloom* were downregulated in hMSC with time in culture and the DNA damage response gene *Atm* was upregulated. Moreover, genes involved in cell cycle progression such as *Cdc25a*, *Cyclin E*, and *Cyclin A* were significantly downregulated in hMSC cultures with increased passage number. These data suggest that accumulation of DNA damage may lead to slower cell cycle progression with expansion. In contrast, in hMSC expanded in the presence of 3/689, genes related to DNA damage response or repair were no longer differentially expressed. Cell cycle regulators such as *Cyclin E* and *A* were within the normal range, whereas *Cdc25a* was upregulated and only *Cyclin D* was downregulated, suggesting an overall enhanced cell cycle progression compared to untreated cultures. Of interest was the upregulation of *Sod2* in the presence of 3/689, an important defense against oxidative damage. Superoxide dismutase-2 (SOD2) has been associated with PrP as it requires Mn^{2+} for its function. PrP is known to bind and transport Mn^{2+} and have SOD-like activity [20]. These data suggest that 3/689 may prevent DNA damage by enhancing SOD2 reactive oxygen species (ROS) scavenging activity and, by doing this, prevent DNA damage and allows faster cell cycle progression.

To verify whether 3/689 prevented occurrence of DNA damage, hMSC cultures were exposed to H_2O_2 for 1 hour in the presence or absence of 3/689 and levels of phosphorylated γH2AX were determined as a measure of double-stranded breaks an hour later. hMSC cultures exposed to H_2O_2 in the presence of 3/689 showed reduced levels of phosphorylated γH2AX compared to control cultures exposed to H_2O_2 (Fig. 7A-a–e, $n = 3$). To confirm that hMSC cultures exposed to 3/689 showed an increase in cell cycle progression, they were labeled with CFSE and the levels of fluorescence were tested 1 and 5 days after labeling. hMSC treated with 3/689 underwent a significant reduction in levels of CFSE after 5 days compared to control cultures (Fig. 7B-a–c, $p < .001$, $n = 3$), suggesting that exposure to 3/689 results in enhanced cell cycle progression.

To determine whether *Sod2* was important for the action of 3/689, real-time quantitative PCR was carried out. A significant upregulation in *Sod2* expression was seen in the presence of 3/689 (Fig. 7C-a, $n = 3$, $p < .05$). More importantly *Sod2* expression was not increased in cultures exposed to 3/689 when PrP expression was knocked down (Fig. 7C-b, $p < .001$), suggesting that 3/689 requires PrP to upregulate *Sod2*.

Discussion

MSCs play an important role in maintenance and repair of bone and blood tissue. In principle, hMSC can be isolated from the patient and expanded in culture until the large numbers required for clinical application are obtained. However, generating sufficient numbers without loss of proliferation and differentiation capacity has been challenging and prolonged expansion is one of the causes of low engraftment [19]. Here, for the first time, we identify the importance of PrP expression for the expansion of hMSC and how hMSC lose its expression with serial passage in culture. We also demonstrate how addition to the cultures of a small molecule able to prevent loss of PrP expression enhances hMSC proliferative capacity and lifespan with an average 300-fold increase in the total number of cells generated. In addition, engraftment to BM increased an average 10-fold compared to the untreated counterpart.

While there is a consensus that the expression of cellular PrP enhances the proliferative of stem cells in vitro and in vivo, no data are available as to how this may be achieved. Our data go some way to support the notion that PrP is involved in cellular protection from oxidative stress, especially at times of intense proliferative stress. Although the specificity of the small molecule we have used to modulate PrP expression requires further investigation, our data suggest that, when PrP expression is retained, cells are protected from DNA damage and expression of DNA repair genes such as *Brca1* remains within normal range. Moreover, DNA damage is prevented and this seems to occur through the enhancement of ROS scavenging activity mediated by SOD2. Our data are in agreement with studies showing that lack of PrP expression results in a phenotype that is more sensitive to oxidative stress [21]. PrP has been reported to confer enhanced resistance to stress by contributing to cellular SOD activity [22]. Our data show upregulation of *Sod2* in the presence of 3/689 and this upregulation is abrogated in the absence of PrP expression, suggesting a link between SOD2 and PrP.

Although the role of SOD2 in controlling aging and extending lifespan through its scavenging activity of ROS is controversial, the effect we have observed is well in line with data reported on the effect of SOD2 in connective tissues. Indeed, while heterozygous SOD2 knockout mice did not show an accelerated ageing phenotype and extension in lifespan [23], specific deletion of SOD2 in connective tissues showed selective loss of redox balance in tissue resident fibroblasts cells, and this was sufficient to drive an accelerated ageing phenotype in different organs and shorten lifespan [24].

Of interest is that the benefit of administering 3/689 to the cultures is seen only in cells that have undergone several cell divisions in vitro or in cells derived from older donors. This may be due to the fact that PrP is active only in situations of cellular stress due to extensive proliferative demands as a fine tuning mechanism to ensure ROS levels remain tightly controlled. Indeed PrP gene was upregulated in CD8+ lymphocytes only when they underwent intense proliferation upon transferring into lymphopaenic mice [25]. Indeed, MSCs have been shown to be particularly sensitive to oxidative stress compared to other cell types such as fibroblasts and undergo DNA damage [26, 27].

There are several reasons why ROS levels may require tight regulation. The most obvious is the ability to cause genetic damage. Although stem cells are equipped with properties that minimize acquisition of damage, adult stem cells remain at risk for acquiring mutations that could lead to cancer or to a robust DNA damage response leading to apoptosis or senescence [28]. This has been shown to attenuate their regenerative capacity and lead to tissue aging [29]. To what extent stem cells undergo DNA damage as a consequence of ROS exposure remains unclear, but there is evidence that increased ROS affects stem cell regenerative capacity. Increased ROS, even at low levels, led to a depletion of the HSC pool and inhibition of their repopulation capacity [30]. Elevation of ROS due to loss of ATM led to depletion of HSC pool and BM failure in old mice in a ROS-dependent manner [31]. Activation of p38MAPK has been seen to increase in response to increasing levels of ROS and this has been shown to limit HSC lifespan in vivo [30]. In contrast, prolonged treatment with an inhibitor of p38MAPK extended their lifespan in serial transplantation [30]. An alternative reason why ROS requires tight control may be found in more recent data that show how ROS levels modulate the regeneration ability of stem cells by acting as signaling mediator and directing the self-renewal or differentiation of stem and progenitor cells. In drosophila, multipotent hematopoietic progenitors displayed increased levels of ROS under in vivo physiological conditions, which were downregulated on differentiation [32]. Scavenging ROS by increasing the expression of catalase retarded their differentiation into mature blood cells. Conversely, increasing hematopoietic progenitors ROS beyond the basal levels by mutating SOD2 gene triggered precocious differentiation into all three mature blood cell types [32] suggesting that there is an optimal level of ROS to maintain stem/progenitor cells and any further ROS increase or decrease away from the wild-type levels enhances or suppresses differentiation. There is evidence that this may happen in hMSC too and lend an explanation to the block in differentiation to the osteogenic and adipogenic lineage we have observed when PrP expression was knocked down. Indeed, ROSs have been seen to increase during osteogenic differentiation with concomitant increase of SOD2, but excess ROS levels by exogenous addition of H_2O_2 inhibited osteogenic differentiation [33].

Conclusions

Enhancing stem cell proliferation and differentiation is important for tissue maintenance and regeneration. The current results demonstrate that the lifespan and function of hMSC can be manipulated by modulating PrP expression. It is important to further understand the mechanism of action by which PrP mediates its effects, especially in relation to ROS production and induction of cellular senescence. Moreover, the safety of this target requires thorough evaluation. In this study, we have not seen changes in chromosomal integrity and tumorigenic potential following hMSC expansion with 3/689. However, this is a

relatively crude measure of long-term tumorigenic potential and more in depth studies are required. Nevertheless, these studies provide promising evidence that modulation of PrP expression enhances stem cell proliferation and differentiation. These findings have substantial potential clinical utility to enhance stem cell therapy and regenerative medicine.

Acknowledgments

This study was supported by Biotechnology and Biological Sciences Research Council (BBSRC, BB/E014119/1). The gene expression profiling was funded by Cancer Research UK. We are grateful to Orla Gallagher, Daren Lough and Julia Hough (Department of Human Metabolism, University of Sheffield) for their expert technical assistance. We thank Peter Andrews, Paul Gokhale and Ramya Udayashankar (Centre for Stem Cell Biology, University of Sheffield) for helpful discussion and Sandra Knight (Department of Chemistry, University of Sheffield) for technical assistance.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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APPENDIX-II

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Chapter Title	Intra-femoral Injection of Human Mesenchymal Stem Cells	
Chapter SubTitle		
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Abstract	In vivo transplantation of putative populations of hematopoietic stem cells (HSC) and assessment of their engraftment is considered the golden standard to assess their quality and degree of stemness. Transplantation is usually carried out by intravenous injection in murine models and assessment of engraftment is performed by monitoring the number and type of mature blood cells produced by the donor cells in time. In contrast intravenous injection of mesenchymal stem cells (MSC), the multipotent stem cells present in bone marrow and capable of differentiating to osteoblasts, chondrocytes and adipocytes, has not been successful. This is due to limited or absent engraftment levels. Here, we describe the use of intra-femoral injection as an improved method to assess MSC engraftment to bone and bone marrow and their quality.

Key words (separated by '-')	Mesenchymal stem cells - Marrow stromal cells - Intra-femoral injection - Gene marking - Lentiviral transduction - Enhanced green fluorescent protein

Chapter 10

Intra-femoral Injection of Human Mesenchymal Stem Cells

Sindhu T. Mohanty and Ilaria Bellantuono

Abstract

In vivo transplantation of putative populations of hematopoietic stem cells (HSC) and assessment of their engraftment is considered the golden standard to assess their quality and degree of stemness. Transplantation is usually carried out by intravenous injection in murine models and assessment of engraftment is performed by monitoring the number and type of mature blood cells produced by the donor cells in time. In contrast intravenous injection of mesenchymal stem cells (MSC), the multipotent stem cells present in bone marrow and capable of differentiating to osteoblasts, chondrocytes and adipocytes, has not been successful. This is due to limited or absent engraftment levels. Here, we describe the use of intra-femoral injection as an improved method to assess MSC engraftment to bone and bone marrow and their quality.

Key words Mesenchymal stem cells, Marrow stromal cells, Intra-femoral injection, Gene marking, Lentiviral transduction, Enhanced green fluorescent protein

1 Introduction

Integral to the definition of stem cells is their ability to regenerate the tissue in which they reside through their ability to self-renew and differentiate. Stem cells are thought to play a role in maintenance and repair of tissues. Studies in murine models have highlighted the importance of testing the regenerative capacity of stem cells by transplantation in the context of ageing to determine the changes they undergo with in vivo or during expansion in culture and how this impact on tissue homeostasis. For example HSC from 22–24-month-old mice have been shown decreased engraftment ability following transplantation compared to younger mice and a skewed regeneration of the myeloid lineage at the expense of the lymphoid lineage (1).

Mesenchymal stem cells (MSC) reside in bone marrow and are able to differentiate to osteoblasts, adipocytes, chondrocytes and hematopoietic supporting stroma (2, 3). They have an important role in repair and maintenance of bone and the bone marrow microenvironment. Moreover, loss of proliferation and differentiation ability has been reported with age in vitro (4). However, robust

Kursad Turksen (ed.), *Stem Cells and Aging: Methods and Protocols*, Methods in Molecular Biology, vol. 976,
DOI 10.1007/978-1-62703-317-6_10, © Springer Science+Business Media, LLC 2013

evidence of reduced regenerative capacity in vivo is scant. This is due to the limitation of the current transplantation methodologies available. The best in vivo assay to assess MSC regenerative capacity and quality is the ectotopic bone formation assay. This consists of seeding MSC in an appropriate porous scaffold and implanting this under the skin of a mouse. Five weeks later an ossicle is formed with areas of chondrogenesis and host hematopoiesis (5), allowing assessment of their ability to undergo osteogenic, adipogenic, and chondrogenic differentiation. However, this assay does not test the ability of MSC to regenerate and contribute to tissue maintenance and repair in the appropriate environment. To test this we describe a method where labelled human MSC are injected directly in the femur of immunodeficient mice and their engraftment is assessed 5 weeks later.

2 Materials

The culture of human MSC and the preparation of all reagents require sterile conditions and are carried out in a class II biological safety cabinet. The intra-femoral injection is a regulated procedure and requires a licence according to the regulations for animal procedures imposed by the country where the procedure is taking place. Lentiviral work will require appropriate genetic modified organism risk assessment and approval according to the regulations of the country where the work takes place. Follow waste disposal regulations when disposing of materials.

2.1 Cell Preparation

1. Human mesenchymal stem cell medium: Dulbecco's modified eagle medium (DMEM, Life technologies, Paisley, UK) containing 10% fetal calf serum (Hyclone, Fisher Scientific, Loughborough, UK). Store at 4°C.
2. MSC isolated from human bone marrow and expanded in culture (see Note 1).
3. Lentiviral particle containing a vector expressing enhanced green fluorescent protein (eGFP) at a concentration of 10^6 vp/ml minimum (see Note 2).
4. 0.05% Trypsin–0.53 mM EDTA (Ethylenediaminetetraacetic acid) (Life technologies) store at 4°C.
5. Sterile phosphate buffered saline (PBS).
6. Sterile Dulbecco's modified eagle medium (DMEM). Store at 4°C.

2.2 Surgical Components and Reagents

1. A pair of sterile scissors, forceps, artery forceps, scalpel blade, scalpel holder, and surgical drapes (see Note 3).
2. Cork sheet.

3. Surgical electric shaver.
4. Three 1 ml 27 g × ½ inch syringes with needles (see Note 4).
5. Hamilton syringe and removable needles (RN) (Hamilton, Bonaduz, Switzerland) (see Note 5).
6. Needles 25 g to position the mouse and keep it steady.
7. Blue monofilament adsorbable suture 45 cm (Ethicon, Edinburgh, UK).
8. Ketaset and Rompun (see Note 6).
9. Bone wax (Ethicon).
10. Sterile ethanol wipes.
11. Sterile distilled water and PBS.
12. Temperature controlled incubators set at 37°C.
13. NOD/LtSz-Prkdc*scid* (NOD/SCID) mice 5–6 weeks old.

2.3 Immuno-Staining Reagents

1. 10% neutral buffered formalin: Weigh 8 g Sodium dihydrogen orthophosphate dehydrate, 13 g Disodium hydrogen orthophosphate dehydrate. Add 200 ml concentrated formaldehyde (i.e., 37–41%) and 200 ml warm tap water (helps to dissolve buffers). Mix to dissolve buffers and top up with tap or distilled water to a final volume of 2 l. Store at room temperature (see Note 7).
2. Neutral EDTA: Weigh 250 g Ethylenediaminetetraacetic acid (disodium salt), 25 g Sodium hydroxide and mix with 1,750 ml Distilled water. The solution will be cloudy until the addition of sodium hydroxide which will also neutralize it to pH 7. Mix well by stirring and store at room temperature.
3. SuperFrost Plus slides (VWR International Ltd, Lutterworth, UK).
4. Xylene (VWR International Ltd).
5. 99% absolute industrial methylated spirit (ethanol) (Thermo Fisher UK Ltd, Loughborough, UK).
6. Leica decalcifier II (Leica Microsystems, Milton Keynes, UK).
7. Leica RM2265 rotary microtome (Leica Microsystems).
8. Leica TP1020 carousel tissue processor (Leica Microsystems).
9. Histology wax (Leica Microsystems).
10. 3% hydrogen peroxide: add 3 ml of hydrogen peroxide (VWR International Ltd) in 97 ml of PBS.
11. PBS.
12. Normal Goat serum (Dako UK Ltd, Ely, UK).
13. Anti-GFP, rabbit IgG fraction (polyclonal) (Life Technologies).
14. Goat anti-rabbit horseradish peroxidase (HRP) (Insight Biotechnology Ltd, Wembley, UK).

15. Vector staining kit (Vector laboratories Ltd, Peterborough, UK).
16. Gill's hematoxylin stain (Merck chemicals Ltd, Nottingham, UK).
17. Di-*n*-Butyl Phthalate in Xylene (DPX) (VWR International Ltd).

3 Methods

3.1 Labelling of hMSC to Express eGFP by Lentiviral Transduction

All procedures are carried out in a Class II biological safety cabinet.

1. Human MSCs previously isolated and expanded in MSC medium and devoid of any hematopoietic contamination are plated at a density of 10,000/cm^2 in MSC medium
2. The day after, MSC are incubated with MSC medium containing viral particles at a multiplicity of infection of 40–60 and left at 37°C in 5% carbon dioxide (CO_2) in air for 8 h
3. The media is then removed and fresh MSC medium is added to the cells which are further incubated for 5 days at 37°C in 5% CO_2
4. Cells are washed once with PBS (10 ml/T25) and incubated with Trypsin/EDTA (1 ml/25 cm^2) for 2 min at 37°C in 5% CO_2 in air (see Note 8)
5. Cells are then detached from the flask and harvested using MSC medium (10 ml/T25 flask).
6. An aliquot of the cells is removed to determine the transduction efficiency by assessing the expression of eGFP by fluorescent activated cell sorting (FACS).
7. Human MSC showing greater than 90% eGFP expression are suitable for transplantation and are replated in MSC medium for expansion until the correct number of cells required for transplantation is obtained.

3.2 Preparation of hMSC for Injection

1. Use human MSC culture which are 80–90% confluent for transplant
2. Detach the cells from the flask as described in Subheading 3.1, steps 4 and 5
3. Centrifuge at 800 × *g* for 5 min.
4. Discard the supernatant and resuspend the cell pellet in 1 ml of DMEM
5. Count the cells using hemocytometer and trypan blue to exclude dead cells

6. Transfer the 1 ml of cell suspension into an eppendorf and centrifuge at $800 \times g$ for 5 min.
7. Discard the supernatant (see Note 9) and resuspend the cells in DMEM at a final concentration of 5×10^5 cells/5 µl (see Note 10). Keep cells on ice until injection.

3.3 Surgical Procedure

Wear lab coats, surgical gloves and masks. Clean all the working areas with ethanol wipes. Maintain aseptic conditions throughout the procedure.

1. Place the cork sheet and cover it with a sterile drape.
2. Open sterile distilled water, PBS and fill 1 ml syringe with each and label the syringes.
3. Open 2×25 g needles. These will be used to pin the mice leg and to drill a hole in the femur.
4. Open the sterile suture material, the sterile scissors, artery forceps, blades and scalpel holder from sterile pouches.
5. Weigh the mouse and intraperitoneally inject it with the ketamine–Rompun mix (see Note 6). Use 100 µl/10 g in weight of mouse
6. Leave the mouse in the 37°C incubator covered with a sterile drape and allow 5 min for the anesthetic to act (see Note 11)
7. Pin the leg of the mice where injection is intended to the cork sheet with the 25 g needle and shave off the hairs using the electrical shaver (see Note 12) (Fig. 1a)
8. Make a small deep incision using the sterile scalpel blade above the knee joint and expose the kneecap slowly using sterile forceps by separating the tissue around without damaging any blood vessels (see Note 13) (Fig. 1b)
9. While holding the top part of the femur with sterile forceps, drill a hole gently through the groove of the kneecap in the femur about half a centimeter deep (see Note 14) (Fig. 1c)
10. Resuspend the cells gently (avoiding bubble formation) and aspirate 5 µl using the Hamilton syringe. Gently, place the needle of the Hamilton syringe in the hole and slowly inject 5 µl pushing the piston very slowly and gently (see Note 15)
11. While removing the needle, immediately close the holes with bone wax using a sterile scalpel blade
12. Finally, wash the surgical area with sterile distilled water. Wipe the excess water using ethanol wipes and close the wound using the appropriate suture
13. Leave the mice in 37°C incubator and monitor for recovery. Once the mice have recovered from the anesthesia, transfer them to sterile cages and monitor for infection until the date of sacrifice (5 weeks or longer)

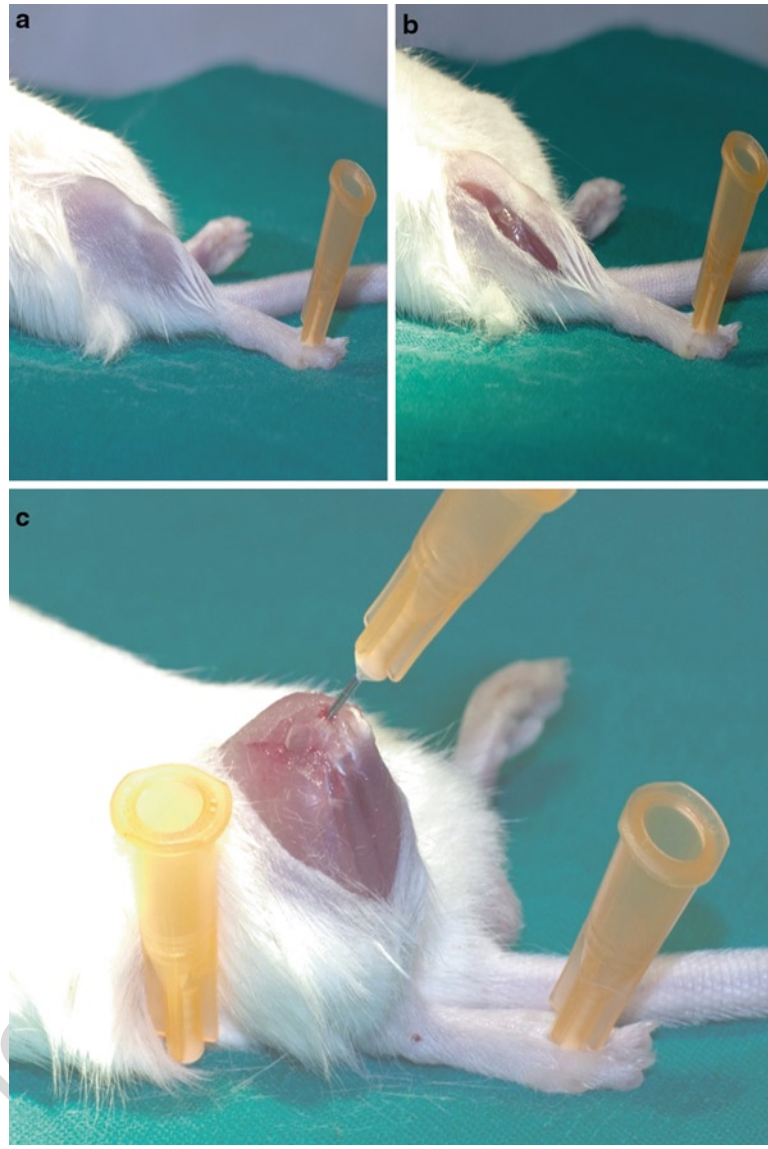

Fig. 1 Surgical procedure involving intra-femoral injections of eGFP labelled hMSC cells. (**a**) A representative image of area where the fur of the mouse has been shaved at the knee joint using an electrical shaver. (**b**) shows a 1 cm deep incision using a sterile scalpel blade aside of the knee joint and (**c**) shows the position where the hole is drilled gently through the groove of the kneecap to allow injection of hMSC cells

3.4 Immuno-Staining

To detect the levels of engraftment animals are sacrificed and bones are processed to assess the number of cells expressing eGFP by immuno-staining.

1. The femurs of mice are collected in 10% neutral buffered formalin and kept for 16 h on shaker at 4°C

Table 1
Processing of tissue section on the Leica TP1020 carousel tissue processor

Station	Solution	Time	Vacuum
2	70% Ethanol	2 h	No
3	70% Ethanol	2 h	No
4	70% Ethanol	2 h	No
5	95% Ethanol	2 h	No
6	95% Ethanol	2 h	No
7	100% Ethanol	2 h	No
8	100% Ethanol	2 h	No
9	Xylene	2 h	No
10	Xylene	2 h	No
11	Histology wax melting point 56°C	2 h	Yes
12	Histology Wax (as above)	2 h	Yes
		Total 22 h plus 15 min drain time	

2. Following 16 h bones are decalcified in Leica decalcifier II at room temperature on a shaker for 2 h. Add 10–20 times volume of decalcifier to volume of bone
3. On completion of decalcification, bones are transferred to labelled tissue cassettes in 70% ethanol. The tissues are immediately processed on the Leica TP1020 carousel tissue processor for paraffin embedding (Table 1)
4. After 22 h of processing, the femur is orientated longitudinally and embedded in molten wax and trimmed very slowly on the Leica RM2265 rotary microtome at 3 μm until the full head of the femur is exposed
5. The exposed paraffin block surface is cooled for 30–60 min on an ice block stored at 4°C
6. 25 serial sections of 3 μm thickness are cut and transferred to a 45°C distilled water bath and allowed to float for up to 30 min to avoid contraction of the bone marrow and endocortical bone
7. The serial sections are mounted on SuperFrost Plus slides
8. Slides are placed on a tray and dried on a hotplate at 45°C for 30 min before further drying at 37°C overnight

9. Cooled slides are stored at 4°C before performing anti-GFP staining (no longer than 2 weeks)
10. The slides containing tissue sections are first dewaxed in xylene two times for 5 min each
11. The tissue sections are rehydrated in 99% ethanol two times for 5 min each following which endogenous peroxidase is blocked using 3% hydrogen peroxide for 10 min (see Note 16)
12. The slides are then washed in distilled water for three times for 1 min each
13. Wash the slides in PBS for three times for 2 min each
14. Prepare 10% normal goat serum in PBS and incubate the tissue sections by covering the surface for 30 min at room temperature
15. Incubate the tissue section with 200 µl primary antibody (anti-GFP, rabbit IgG fraction) overnight (see Note 17) at 4°C
16. Wash the slides with PBS twice for 5 min each
17. Incubate the tissue section with 200 µl secondary antibody (Goat anti-rabbit horseradish peroxidase) for 45 min at room temperature (see Note 18)
18. Wash the slides with PBS twice for 5 min each
19. Prepare the reagents in the vector kit solution (see Note 19), add the solution (enough to cover the tissue surface) and incubate for 15 min
20. Rinse the slides in distilled water and wash for 5 min under running tap water
21. Counterstain nuclei in Gill's Hematoxylin for 20 s and then wash the stain with running tap water for 4 min
22. Dehydrate the tissue sections in the order of 70, 95 and 99% industrial methylated spirit for 1 min each
23. Finally to remove any excess trace of water the tissue sections are washed in xylene for 1 min each
24. Clean any remaining xylene surrounding the slide using a soft tissue paper. Place a drop of DPX on a coverslip and gently invert the slides on top of it and press it gently to remove any trapped air bubble and allow the slides to dry at room temperature
25. The eGFP stained cells will appear brown in color (see Fig. 2) and can be evaluated microscopically

4 Notes

1. This procedure can also be carried out with freshly isolated human MSC (e.g., Lin– CD45– LNGFR+ bone marrow cells), murine MSC or HSC when numbers are small. The strain of mice used will depend on the type of cells injected

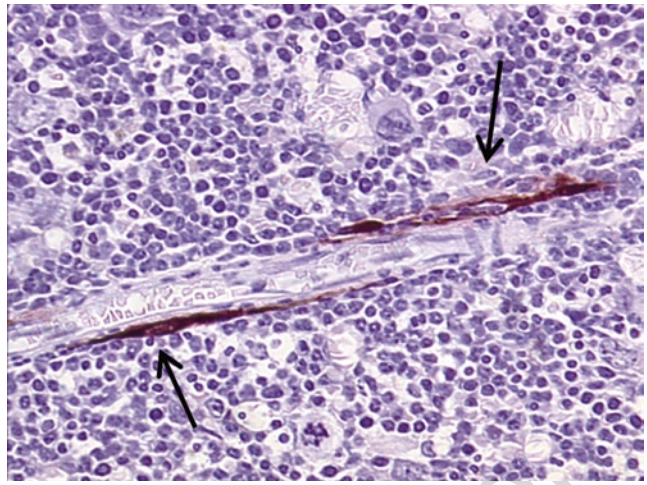

Fig. 2 eGFP+ cells in the femur of mice 5 weeks after transplantation. Representative examples of eGFP positive cells located around the vessels (resembling pericytes)

2. If an in-house system for producing lentiviral particle is not available, lentiviral vectors expression eGFP can be purchased from a number of vendors including Thermo Scientific, Sigma-Aldrich, CellBio Labs and viral particle produced according to manufacturer instructions. Lentiviral vectors are recommended over other systems due to the higher efficiency of transduction and integration of the expression vector in the genome of the cells for long term monitoring. Alternatively, other ways to detect transplanted cells can be used depending on the type of cells transplanted. For example to detect human MSC transplanted in mice an antibody to human nuclei can be used
3. All the surgical equipments should be autoclaved at 121°C for 20 min
4. Those needles are used to administer the anesthetic to the animals, and to clean the surgical field with PBS.
5. Syringes needs to be clean and sterilized by aspirating water first and discarding it. Repeat the operation 3 times. Then wash with ethanol 95% three times and then with PBS three times
6. Ketaset and Rompun are anesthetics which are delivered by intraperitoneal injection. Ketaset is a regulated drug and therefore should be kept out of reach when not in use. 1 ml of Ketaset contains 100 mg/ml (equivalent to ketamine hydrochloride 115.36 mg/ml) with 0.01% benzethonium chloride as a preservative. Rompun is a concentration of 2% w/v and each ml contains xylazine hydrochloride (equivalent to 20 mg xylene) as active substance and 1.5 mg methyl-4-hydroxy-benzoate as preservative. The Ketaset and Rompun mix is prepared

at a volume of 0.5 ml of Ketaset, 0.25 ml of Rompun, and 4.3 ml of sterile distilled water.

7. Wear gloves and mask and prepare in containment hood.
8. Do not incubate the cells in trypsin/EDTA for longer time. After 2 min observe the cells under the microscope and if MSC are circular in morphology gently brisk the flask and collect the cells
9. Ensure that when discarding the supernatant, the cell pellet is devoid of any medium. Any excess medium is removed using a sterile pipette. Avoid bubble formation while gently mixing the cell suspension in the small volume. Transfer the contents into a sterile eppendorf and store it on ice to reduce clumping of cells.
10. Prepare cells in excess of the number of mice to inject, at least 10^6 hMSC in excess to account of loss while pipetting. Moreover, if injecting cells from different donors, take into consideration that engraftment levels decrease with increasing numbers of population doublings.
11. To ensure the mouse is under the effect of the anesthetic, pinch the tip of one of the legs. If the mouse twitches, leave the mouse for few more minutes before starting the surgical procedure. Only when the mouse becomes unresponsive to the pinching, the procedure can be initiated
12. Shave half a centimeter above and below the knee joint
13. While making incision, if the blood vessels are cut pour sterile PBS and wipe the excessive blood using ethanol wipes and wait for a while for the blood flow to stop and then continue with the procedure
14. Once the hole has been drilled, let pressure to ease and bone marrow to ooze out. Use sterile PBS to drain off excessive blood/marrow and use ethanol wipes to clean the blood. Always maintain aseptic conditions and keep as clean as possible to avoid infection.
15. Injecting the cells will create high pressure in the bone marrow cavity. To avoid cells leaking out, and therefore introducing greater variability in engraftment rates, cells need to be injected very slowly over 30 s. At the end of the injection leave the syringe and needle in position for 5 s before withdrawing it to stop cells exiting the bone marrow cavity.
16. Place the microscopic slides in an immuno-tray and use a Pasteur pipette to add hydrogen peroxide enough to cover the surface of the tissue section in the microscopic slide.
17. Primary antibody is prepared at a dilution of 1:600 in 5% normal goat serum. 5% normal goat serum is prepared by dissolving 1 ml of goat serum in 4 ml of PBS.

18. Secondary antibody is prepared at a dilution of 1:400 in PBS.
19. Vector kit solution is prepared for immediate use: to 5 ml of distilled water add three drops of reagent 1 (mix well), add two drops of reagent 2 (mix well), add two drops of reagent 3 (mix well), and then add two drops of hydrogen peroxide and mix well.

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