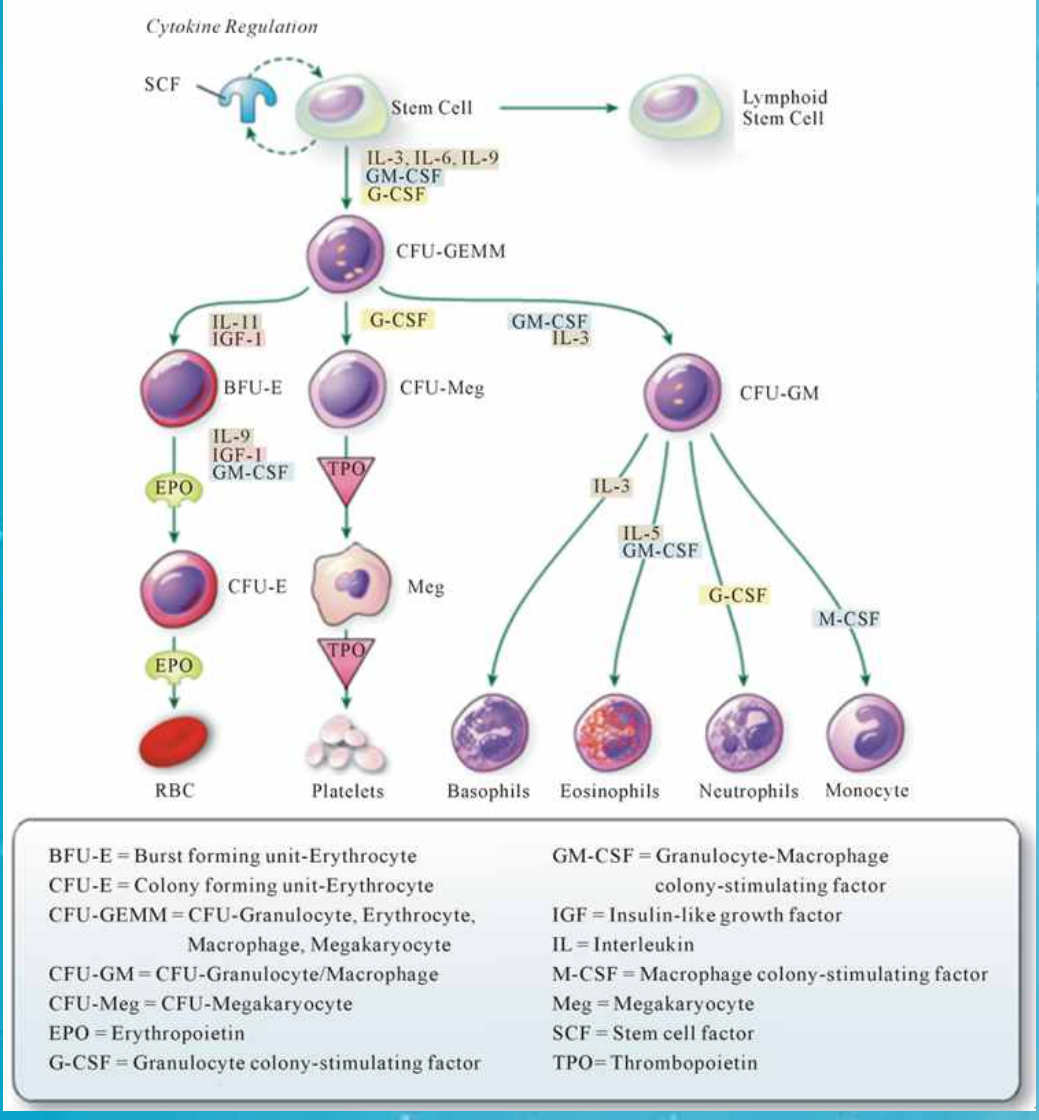
ISSN: 2161-6760 Vol. 6, No. 1, January 2016

Scientific Research Publishing

Stem Cell Discovery



ISSN: 2161-6760
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ISSN: 2161-6760 (Print) ISSN: 2161-6787 (Online)

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Stem Cell Discovery, 2016, 6, 1-48
Published Online January 2016 in SciRes. http://www.scirp.org/journal/scd

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The figure on the front cover is from the article published in Stem Cell Discovery, 2016, Vol. 6, No. 1, pp. 39-48 by Pierre Luc Pace, Renald Blundell.

Stem Cell Discovery (SCD)

Journal Information

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Stem Cell Discovery, 2016, 6, 1-12
Published Online January 2016 in SciRes. http://www.scirp.org/journal/scd
http://dx.doi.org/10.4236/scd.2016.61001

Production of Neural Progenitors from Bone Marrow Mesenchymal Stem Cells

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Received 1 August 2015; accepted 25 December 2015; published 29 December 2015

Abstract

In the brain, there are hundreds of types of specialized neurons and to generate one type of them we need to have neural progenitors for differentiation to specific neuron type. Mesenchymal stem cells (MSCs) are easily isolated, cultured, manipulated ex vivo, showing great potential for therapeutic applications. The adult MSCs have the potential to produce progeny that differentiate into a variety of cell types such as neurons. This fact suggests that MSCs derived neurons are an important cell type and a deep understanding of the molecular characteristics of it would significantly enhance the advancement of cell therapy for neurological disorders. Therefore, in this study, we isolated, identified, and studied neural progenitors by measuring expression levels through neurogenesis pathway of three neural differentiation markers nestin (NES), neurofilament (NF-L), and microtubule association protein (MAP-2) from mouse bone marrow MSCs (mouse bmMSCs) by using butylated hydroxyanisole (BHA) and diethyl sulfoxide (DMSO) as neural inducers agents. The results of immunocytochemistry and Real Time-PCR showed that in contrast to MSCs, neural differentiated cells showed neural progenitor pattern by showing stable increase in NES gene expression through differentiation process with increasing the protein expression through different exposures times, while NF-L gene and protein expression start to increased after 48 h but not replaced the NES expression completely even when its expression passed NES levels. The maturation marker Map-2 expression was low during the duration of differentiation period in protein and gene expression, which prove that these cells are still progenitors and can be redirected into specific type of neurons by further treatments.

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How to cite this paper: Mohammad, M., Yaseen, N., Al-Joubory, A., Abdullah, R., Mahmood, N., Ahmed, A.A. and Al-Shammari, A. (2016) Production of Neural Progenitors from Bone Marrow Mesenchymal Stem Cells. *Stem Cell Discovery*, **6**, 1-12. http://dx.doi.org/10.4236/scd.2016.61001

Keywords

BHA, Nestin, Neural Progenitors Cells, MSCs, MAP-2

1. Introduction

Mesenchymal stromal cells or MSCs are a type of widely distributed adult stem cell in connective tissues [1] [2]. They were easy to isolate and propagate and possess the ability to differentiate into many different cell lineages, including osteoblasts, chondrocytes, adipocytes, hepatocytes, and neuron-like cells since the nineteenth of the past century [3]-[6]. With these properties, MSCs have been widely applied in regenerative medicine research and experimental cell therapy for a wide range of disorders such parkinson's disease (PD) [7], huntington's disease [8], amyotrophic lateral sclerosis (ALS) [9], and alzheimer's disease (AD) [10].

Mesenchymal stem cells are induced to differentiate into neural cells under the appropriate differentiation media by several researches [11]-[14]. The neural development of MSCs could provide a source to treat specific neurological deficits because the central nervous system (CNS) has limited capacity for self-repair and the loss of its cells generally results in permanent tissue damage [15]-[17].

There have been several studies showing that sulfhydryl groups (-SH) in compounds such as β-mercaptoethanol (BME), BHA, DMSO, cysteine, and glutathione (GSH) are essential for neural induction of bone marrow MSCs [18]-[21]. Exposure of MSCs to agents such as BHA, induces neural morphological features along with the expression of neural-specific genes such as NEFL, TUBB3 (β III-tubulin), and neuron-specific enolase (NSE). In addition, protein expression levels of nestin, β III-tubulin, and tyrosine hydroxylase remarkably increased in differentiated MSCs [22].

The intermediate filament protein (IMF) which is the major stricture of cytoskeletons is classified into several types, which expressed mostly in nerve cells and brain, such as NES as a type IV which are implicated in the radial growth of the axon, the nestin-expressing cells are found frequently (though not necessarily exclusively) in areas of regeneration, where they might function as a reservoir of stem/progenitor cells capable of proliferation and differentiation [23]. Neurofilaments (such as NF-L) are another type of IMF group, which expressed predominantly in axonal neurons, peripherin by a subset of neurons [24], also expressed during neurogenesis process, and play as a biomarker for axonal injury, because of his higher expression in axon [25]. Another neural marker was MAP-2 that belongs to the microtubule-associated protein family which involved in microtubule assembly, which is an essential step in neuritogenesis and serves to stabilize microtubules growth by cross linking with intermediate filaments. It expressed in mature neurons such as dendrite cells and perikarya cells, and transiently expressed in axonal neurites during early neural cytogenesis [26] [27].

The objective of this study was to produce neural progenitor cells from MSCs that can be directed later to produce specific neuron type for cellular therapy of the nervous system.

2. Materials and Methods

This study was carried out on the Iraqi center of cancer and medical genetic research (ICCMGR)/experimental therapy department between 2012-2014.

2.1. Isolation of MSCs from Mouse Bone Marrow

The bone marrow culture was prepared as described by Freshney [28] by Killing the donor male Swiss Albino mice by cervical dislocation (3 - 6 weeks old, provided by ICCMGR animal house unite, all the work approved by the ICCMGR animal care and use committee). Under sterilize conditions, the fur washed with 70% alcohol femurs and tibias were removed and both collected in a petri dish containing transport media MEM (Minimum Essential medium) (US Biological, Massachusetts USA) supplemented with five fold antibiotics streptomycin (Cox Pharmaceutical CO., LTD, UK) and ampicillin (Kontam Pharmaceuticals CO., LTD, China) as 500 mg/ml. In the laboratory and under sterilize conditions, the femurs and tibias were clean off from remaining muscle tissues with sterile surgical tools and washed few time with normal saline solution phosphate buffer saline (PBS), holed the femur with forceps and cute off the knee end. The 27G needle which contained MEM media supplemented with 20% fetal bovine serum (FBS) (Cellgro Mediatech, USA) and 100 mg/ml for each ampicillin and

streptomycin as culturing media should fit snugly into the bone cavity, then end of the femur was cut off as close to the end. The tip of the bone was inserted into a test tube (15 ml) and aspirated and depressed the syringe plunger several times until all the bone marrow is flushed out of the femur and tibias. The marrow was dispersed to a suspension by pipetting the large marrow cores through pipetting. Finally the 10-mL aliquots of the cell suspension were dispensed into 25-cm^2 tissue culture flasks and the cultures were maintained at 37˚C in humidified 95% air and 5% CO_2 incubator.

2.2. Culturing and Propagation of MSCs

According to Freshney [28], cells where allowed to adhere overnight and non adherent cells were washed out with medium changes after 24 h on MEM media with 20% FBS. Then cultures were maintained, and the remaining non adherent cells was removed by exchanging of culture medium each 2 - 3 day until the cultures were get hold of developing colonies of adherent cells (about 5 - 7 days) to form monolayer's cells. After that cells were recovered (sub cultured) after getting monolayer cells using 0.25% trypsin-EDTA (US Biological, Massachusetts USA). The first passage (P1) cells began to proliferate and formed a monolayer of cells for the next 3 - 5 days. the culturing of P1 of MSCs were ready to used for immunophenotypic analysis.

2.3. Immunophenotypic Analysis of MSCs

The MSCs were re-cultured (P1) in 8-well tissue culture chamber slide (IWKA, Japan) in MEM media supplemented with 20% FBS. The cells were allowed to developing a monolayer of adherent cells within 3 - 5 days, then the medium was aspirated and the multi-well plates washed two times with PBS, fixed with 4% paraformaldehyde (diluted in PBS) for 10 minutes, washed with PBS and leaved to dry, these slide were used in immunocytochemistry assay as triplicate. Four specific markers for immunocytochemistry analysis were used: CD90 (1:100; Mouse anti-human antibody, US biological, USA), CD105 (1:100; Mouse anti-human antibody, US biological, USA), and CD44 (1:100; Rat anti-mouse antibody, US biological, USA) as positive markers and CD34 (1:100; Goat anti-human antibody, Santa Cruz Biotechnology) as negative markers.

The fixed slides were incubated in a humidified chamber with 1% hydrogen peroxide (H_2O_2) for 10 minutes, washed two to three times with PBS, incubated with 1.5% blocking serum for 30 - 40 minute at room temperature (the kit was ImmunoCruz mouse ABC Staining System from Santa Cruz Biotechnology, Europe). Then the ICC done according to the manufacture protocol with triplicate replication for each CDs marker. The slides were stained with DAP (provided in the kit) and Hematoxlin stain (SYRBIO, Syria) mounted with DPX (CDH, India) then were inspected by using light microscope and photographed by using digital camera.

2.4. Neural Differentiation Induction of MSCs

Subconfluent cultures of P1 MSCs were pretreated for 24 h in MEM media with 20% FBS and 10 ng/ml bFGF (US biological, USA) as preinduction media. Then neural differentiation (as post induction media) was induced with MEM media without FBS (serum free media), 2% DMSO (Santa Cruz, USA), and 200 µM BHA (Santa Cruz Biotechnology, USA) as modified from Woodbury *et al.* [29]. Then cells were fixed for immunocytochemistry for MSCs and seven different time (25, 27, 29, 48, 96, and 144 h) for each NES, NF-L, and MAP-2 with triplicate replication for each marker. The cells were fixed for immunocytochemistry study (the kit from Immuno Cruz mouse ABC Staining System, Santa Cruz Biotechnology, Europe) at all these different times postinduction, and stained for NES (1:50; Mouse anti-rat, Santa Cruz Biotechnology, Europe), and NF-L (1:100; Mouse anti-porcine, US biological, USA) as immature neural cells markers, and for MAP-2 (1:50; Mouse anti-human, Santa Cruz Biotechnology) as mature neural markers, and the ICC assay was carried out as described above.

2.5. RNA Isolation

Total RNAs were collected using the absolutely RNA miniprep kit (Stratagen Aglient, Germany). Cells before (MSCs) and after differentiation (neural cells) from different exposure times (25, 27, 29, 48, 96, and 144 h) were collected by trypsinization of adherent cells. The quality and quantity of total extracted RNA samples were then examined using minodrop UV-Vis. Spectrophotometer measurements (Quawell, Vietnam), and then the extracted RNAs were storage at −80˚C on deep freezer (Nüve, Turkey) until used.

2.6. Real Time-PCR

The isolated RNAs reversed transcribed to produce double stranded cDNA via reverse transcriptase polymerase enzyme (KAPA SYBR FAST One-Step qRT-PCR Kit Universal, KAPA BIOSYSTEMS, South Africa, USA) to quantify expression of specific 3 genes nestin (forward: 5'-GCACT GGGAAG AGTAGAAGATG-3', length 22 and the reverse: 5'-GGAGTAG AGTCAGGGAGAGTTT-3', length 22) with fragment size 131bp, NF-L (forward: 5'-TGATGTCT GCTCGCTCTTTC-3', length 20 and the reverse: 5'-CTCAGCTTTCGTA GCCTCAAT-3', length 21) with fragment size 95 bp, MAP-2 (forward: 5'-CACAGGGCACCTAT TCAGATAC-3', length 22 and the reverse: 5'-CAGATACCTCCTCTGCTG TTTC-3', length 22) with fragment size 87 bp, GAPDH (forward: 5'-GGAGAGT GTTTCCTCGTCCC-3', length 20 and the reverse: 5'-TTTGC CGTGAGTGGAGTCAT-3', length 20) with fragment size 188 bp. These primers were optimized for use with SYBR green and normalization with housekeeping gene GAPDH with 50 ng/µ concentration. The Real Time-PCR done according to the manufacturer's protocol with annealing temperature 62˚C and 40 cycle. All primers were designed in house with the National Center for Biotechnology Information (NCBI) Designer software (http://www.ncbi.nlm.nihgov/) and synthesized by BIO-Synthesis (Lewisville, TX, USA) for all 3 primers except GAPDH primer from Bio Corp (North America, USA). Once suitable reference genes (GAPDH) were identified, the mean CT values of the three candidate genes were calculated for each individual sample (as duplicate replication for each sample) and used to normalize expression levels using the ΔΔCT method described previously [30]-[32].

2.7. cDNA Electrophoresis

All RNAs were running by gel electrophoresis method for MSCs and all different exposure time for each four primers, and with DNA Ladder (KAPA Express DNA Ladder Kit, KAPA Biosystems, USA) to show the quantity of genes expression studied. The preparations were done according to Maniatis and co-workers [33] and all reagents and marker were freshly prepared [1]. The gel afterward, removed from the tank and visualized by gel documentation system specialized with ethidium bromide/UV filter (SCIE-PLAS) and photographed.

2.8. Statistical Analysis

All ICC (as average percentage means) and Real Time-PCR data (as average means after finding the ΔΔCT values) was statistically analyzed using One Way ANOVA and LSD test in IBM SPSS Statistics Software (version 20) and the difference of means was considered significant at $p < 0.05$.

3. Results

3.1. Culturing and Propagation of MSCs

The MSCs from mouse bone marrow was cultured in tissue culture flasks in MEM supplemented with 20% FBS. After 24 h, only a few cells attached to the plastic culture flasks sparsely, and formed adherent cells while the non-adherent cells were discarded by the first medium change usually after 24 or 48 h (**Figure 1(A)**). The adherent cells began to proliferate, as soon as 2 - 3 days after cultivation numerous fibroblast like-cells could be observed, and gradually grow to form small individual colonies displaying fibroblast-like morphology with short and long processes as well as, a small round cells with a high nuclear to cytoplasmic ratio can also seen (**Figure 1(B)**). Mesenchymal stem cells are characterized by their ability to form colonies comprising spindle-shaped cells deriving from a single cell. The number of cellular colonies with different size has obviously increased.

Cells in large colonies were more densely distributed and showed a spindle like shape, as growth of cells continued, colonies gradually expanded in size with adjacent ones interconnected with each other. When the cells grow to 80% confluence (which observed through microscopic examination) after 5 - 6 days so they were ready to passaged (**Figure 1(C)**). Subculture done by through the media, treating with 0.25% trypsin-EDTA and cell suspension passed into new tissue culture flasks under the same conditions at a split ratio 1:2 for the first passage culture. The MSCs began to grow and formed colonies then expanded, and after few days from P1, a homogeneous layer of fibroblastoid-like cells occupied the whole plastic surface (**Figure 1(D)**). The cells can re-seeded in same conditions for the second passage culture, and these adherent cells could be readily expanded in vitro by successive cycles of trypsinization, seeding and culture every 5 - 7 days without visible morphologic alteration.

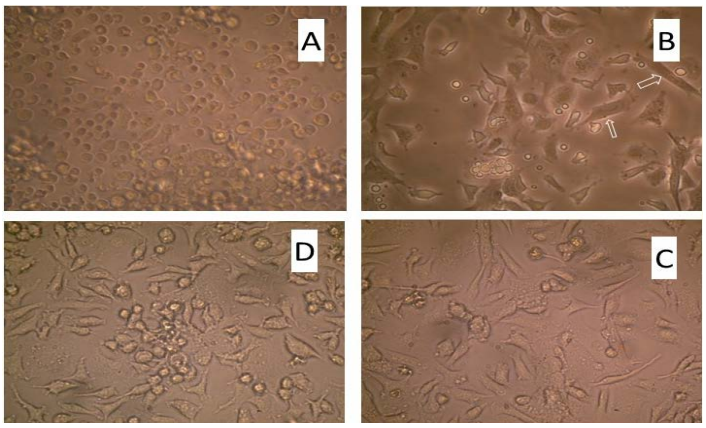


Figure 1. (A): Morphology characteristics of mouse MSCs cultured in MEM +20% FBS after 24 h 20×. (B) After 72 h of culturing mouse MSCs in MEM +20% FBS, note that some of cells adherent and began to elongated 20×. (C) After 5 - 6 days of culturing (monolayer cells), note that the colonies interconnected with each other and reaching a confluent stage 20×. (D): MSCs morphology after the first passage 20×.

3.2. Immunophenotypic Characterization of MSCs

The Immunophenotypic characterization of the cultured MSCs expanded adherent cells revealed that the cells were stained with blue color and were negative for CD34 (**Figure 2(G)**). Cells with brown color were positive for each of CD44, CD90 and CD105 (**Figures 2(A)-(F)**).

These results demonstrated that the cells turned out to be positive for CD105, CD90 and CD44,which take the brown color (from DAP stain) indicating that they retain the phenotype of MSCs. And the results showed that MSCs were negative for CD34 which take the blue color (from Hematoxlin stain), which indicating that these cells are not from hematopoietic origin but they were MSCs.

3.3. Neural Differentiation Induction of MSCs

In the preinduction media (for 24 h exposure), the MSCs was showed to be more elongated (**Figures 3(A)-(G)**) without increased the protein expression (Immunocytochemistry assay ICC) for each NES, NF-L, and MAP-2 (**Figure 4(A)** and **Figure 4(B)**, **Figures 4(A)-(G)** for each marker). After exposure to BHA for different times as postinduction differentiation media, the MSCs started to be more elongated and it increased in cells size, then finally it formed cells branches as the neuron cells with the increased of the NES and NF-L protein expression with significant difference level at $p < 0.05$ with the higher proteins expressions in the 144 h compared with the control (undifferentiating MSCs). Whereas the MAP-2 proteins showed the less expression levels compared with NES and NF-L with significant difference between different time at level at $p < 0.05$ compared with control (undifferentiating MSCs) (as showed in **Figure 5**). These results indicated the activity of BHA in the neurogenesis stages of MSCs toward the neural differentiation cells.

3.4. Real Time-PCR

The results of RealTime-PCR revealed that the expression of NES gene (as immature differentiation gene) was considerable in the BHA treated cells and gave rise to a detectable band, which increased with exposure time started with 29, 48, 96 and 144 h While no gene expression was observed in the control cells (undifferentiated MSCs). In the first hours 25 and 27 h, the NF-L gene was showed higher expression levels after treated and give rise to detectable band (as showed in nestin gene) which increased with exposure time started from 29 - 144 h, compared with the MAP-2 gene (as post maturation gene) which showed low expression levels in all time exposure after treatment compared with control (MSCs). Also the results showed the stability of gene expression levels of GAPDH gene (as showed in **Figure 6**). These results indicated that the immature neural markers NES and NF-L were started to increase their gene expression through the neurogenesis stages of MSCs toward neural cell differentiation compared with low gene expression levels in the mature neural marker MAP-2 gene which indicated the affectivity of BHA towards neural cells differentiation.

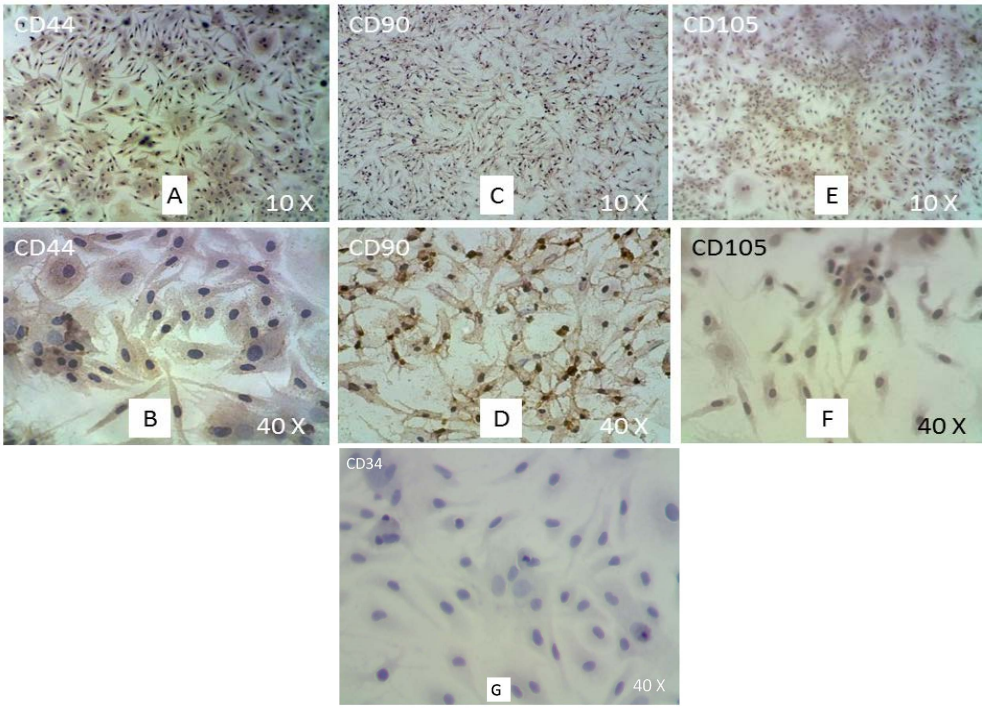


Figure 2. Immunophenotypic analysis of MSCs at the first passage revealed under light microscope show that the MSCs were positive cells which stained with brown color. (A & B): CD44, (C & D): CD90, (E & F) CD105, note that all CDs showed with 10× and 40× respectively. (G): Immunophenotypic analysis of MSCs at the first passage showing that the MSCs were negative for CD34 by cell stained only with blue color 40×.

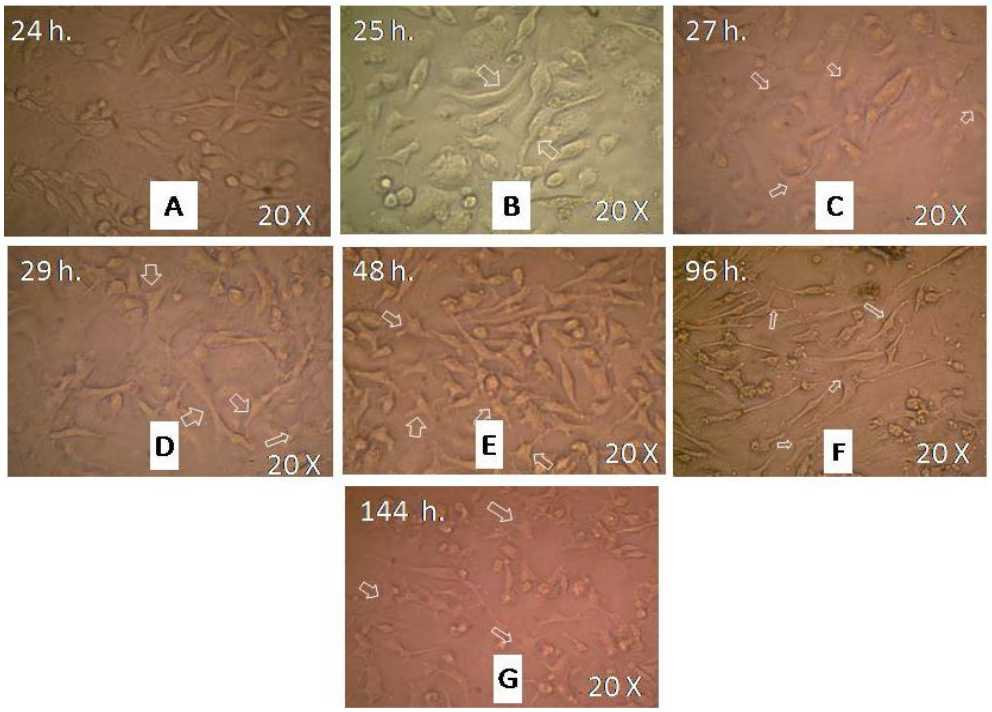


Figure 3. MSCs after induced differentiation by BHA, which showed the elongation of cells and branched form towards neural cells as revealed under inverted microscope, all figure showed in 20×. the **Figures 3(A)-(G)** were presented (24 - 144 h) exposure time to differentiation media.

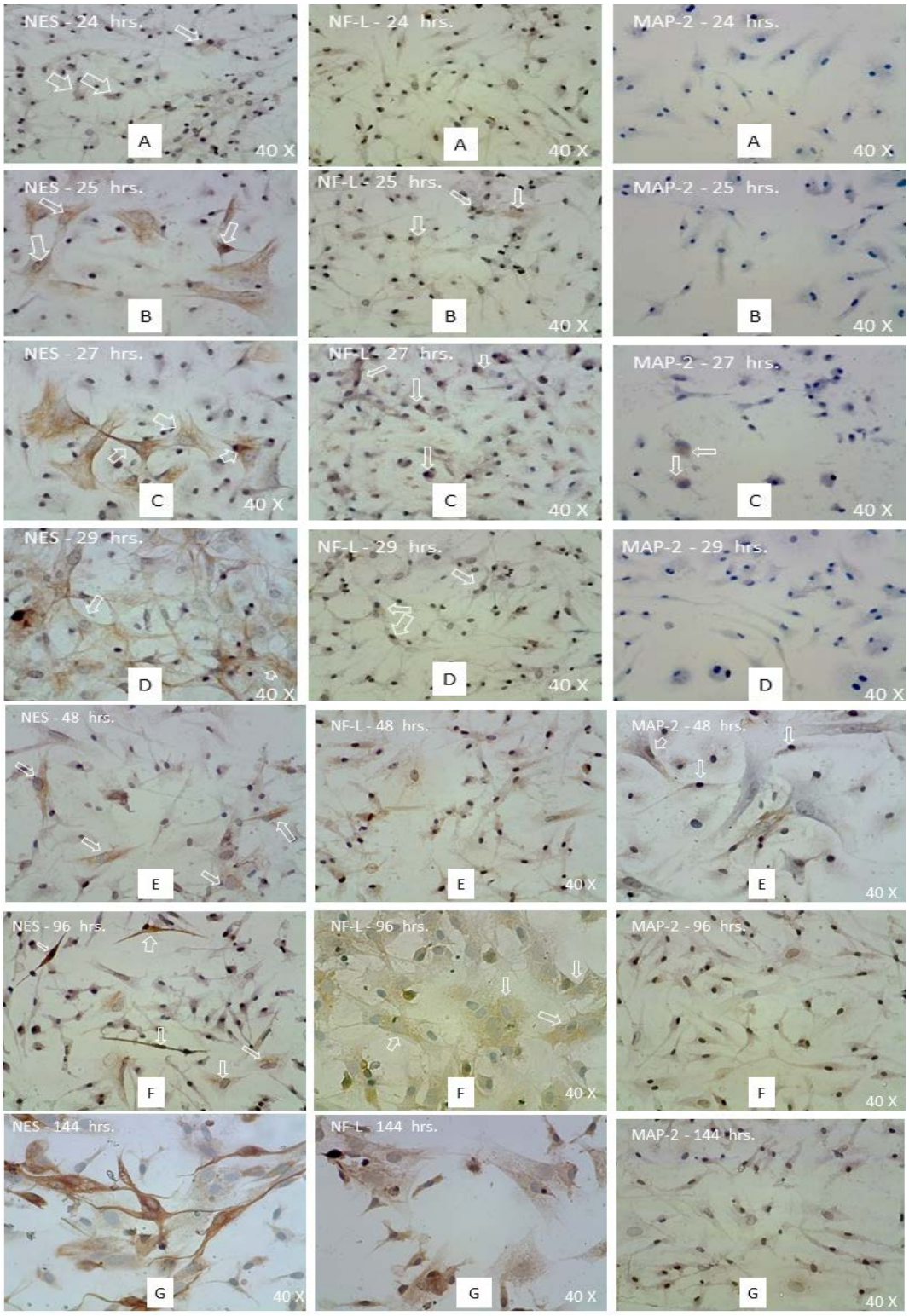
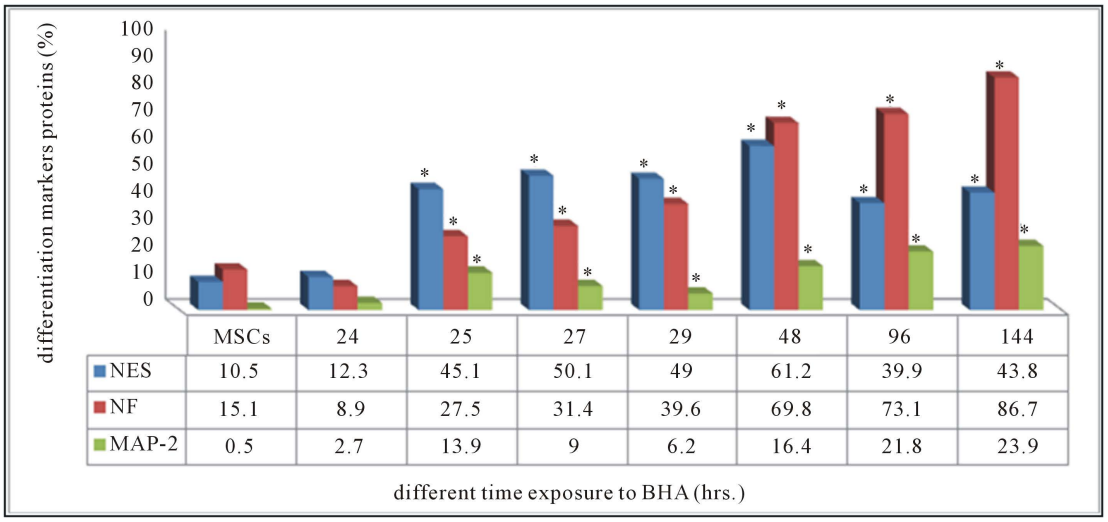
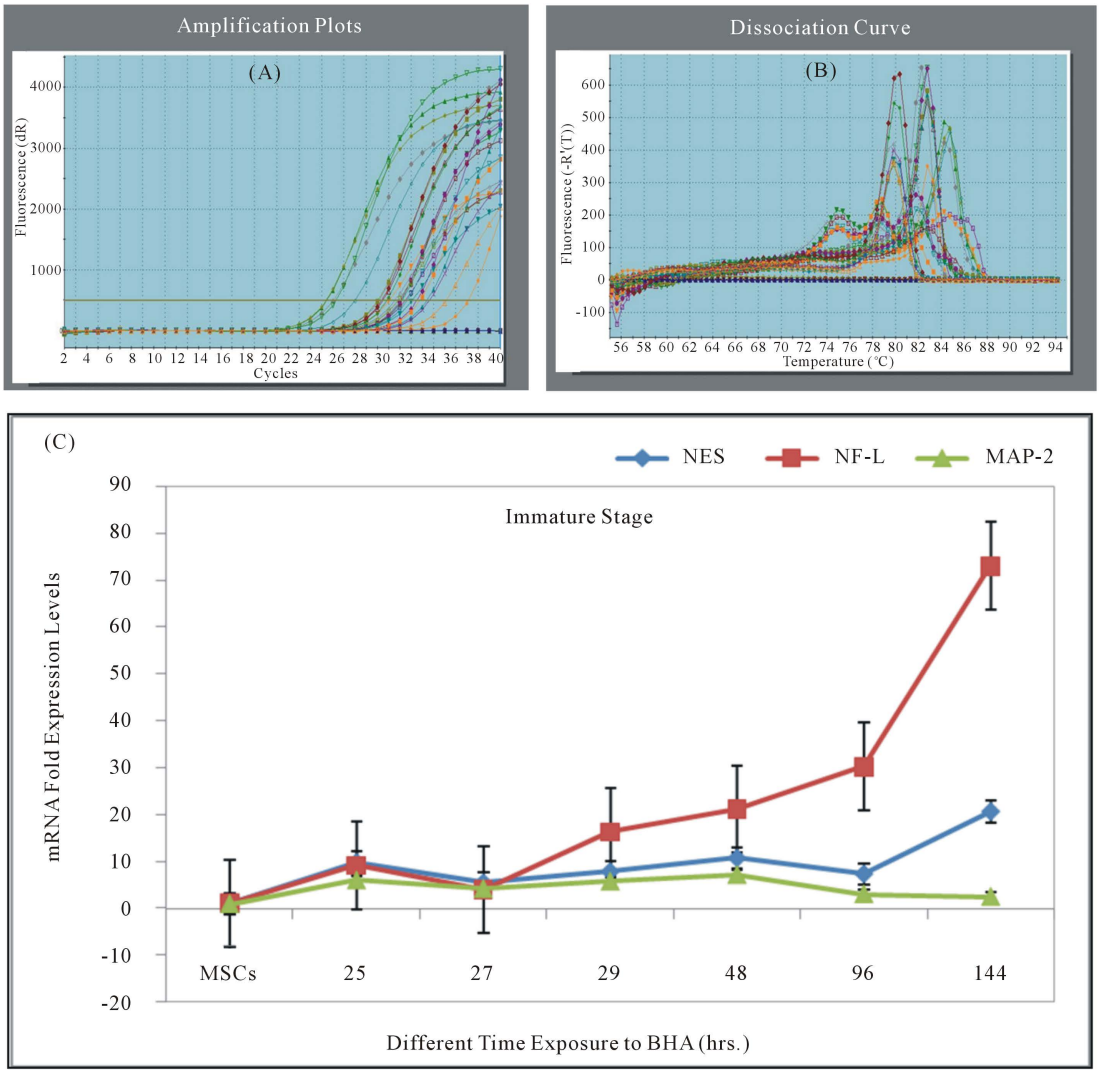


Figure 4. (A, B): Immunocytochemistry detection of neural progenitor markers after MSCs differentiation induction by BHA. The figure showed increased expression of NES, NF-L proteins through the different exposure times as presented by increase the intensity of staining compared with no increasing of MAP-2 protein as revealed under Light microscope, all figure showed in 40×, the **Figures 4(A)-(D)** were for (24 - 29 h) and figures (E-G) were for (48 - 144 h) exposure times.



	MSCs	24	25	27	29	48	96	144
NES	10.5	12.3	45.1	50.1	49	61.2	39.9	43.8
NF	15.1	8.9	27.5	31.4	39.6	69.8	73.1	86.7
MAP-2	0.5	2.7	13.9	9	6.2	16.4	21.8	23.9

Figure 5. The proteins expression levels of Immunocytochemistry assay for NES, NF-L, and MAP-2 antibodies after differentiation by BHA. *Refer to significant effect at $p < 0.05$.



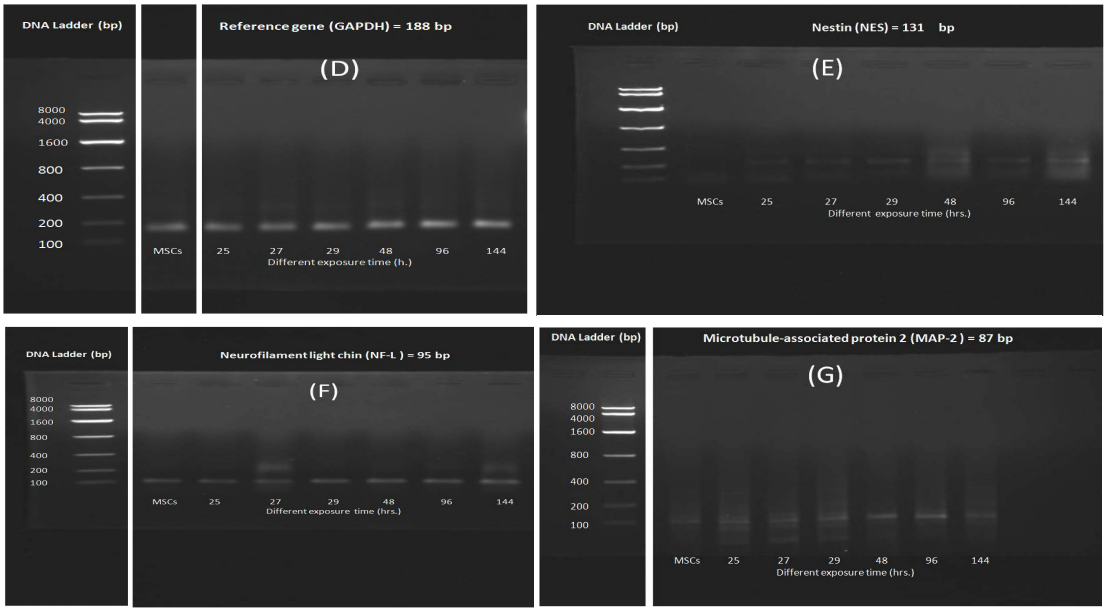


Figure 6. mRNA expression levels of Real Time-PCR analysis of NES, NF-L, and MAP-2 genes in mouse MSCs treated with BHA, there was a high expression levels with significant difference between each NES and NF-L primer compared with control (MSCs) ($p < 0.05$) for different exposure times, and low expression levels on MAP-2 with significant difference for different exposure times ($p > 0.05$). Note that all these 3 primers was normalized with GAPDH gene (A): Amplification Plot of all primers, (B): Dissociation curve of all primers, (C) Results of Real Time-PCR, note that these data represents the ΔCT of mean ± SEM. (D-G): Gel electrophoresis analysis of Real Time-PCR of NES, NF-L, and MAP-2 genes respectively.

4. Discussion

This study showed that after exposure to BHA started from 1 - 5 day can lead to morphological changes in mouse bone marrow MSCs featuring a neuron-like morphology such as rounded cell bodies and long branching processes along with the growth of cone-like terminal structures. Many studies demonstrated the differentiation of human and mice MSCs to neuron-like cells following induction with BME, DMSO and BHA [34]-[40], which exhibited a neuron-like morphology for few hours after induction which is due to a breakdown of the actin cytoskeleton and a retraction of the cell edge [40]. Since BHA also contains the sulfhydryl group, it may be deduced that the neuron-like morphology adopted by the exposed mouse MSCs has the same mechanism and could be due to the disruption of F-actin and cytoskeleton reorganization.

In the present study, the differentiation process of mouse bone marrow MSCs was followed by monitoring the mRNA expression (protein expression) of nestin and NF-L genes as the two important neuron-specific markers (REF). The expression of nestin and NF-L genes increased in MSCs started from 5 hours of exposure to BHA (29 h treated cells), which could be a sign of differentiation in the treated cells.

Other investigations have studied the expression of a wide range of mRNAs and proteins, including those normally reported in terminally differentiated neural cells [41]-[43]. Then many studies demonstrated that neural cells derived from bone marrow MSCs expressed mRNA species encoding many type of genes such as β-tubulin III (an early neural marker), nestin, neurofilaments (NFs), glial fibrillary acidic protein (GFAP) (as neural cell markers), and the specific neural markers such as choline acetyltransferase (ChAT), and MAP-2 and many other markers [22] [42] [44]-[46].

The results showed that nestin protein existed in high levels from first 24 h Until the end of the differentiation time at 144 h, Nestin expression is transient and does not persist into adulthood and nestin become down regulated and replaced by tissue specific intermediate filament proteins [34]. In our results neurofilament protein expression start to increase after 48 h but not replaced the nestin expression completely even when its expression passed nestin levels where the nestin still existed in good levels. Lalonde *et al.* [46] showed that neurofilament is necessary to function primarily to provide structural support for the axon and to regulate axon diameter. The maturation marker Map-2 expression was low during the duration of differentiation period, which prove that

cells are still progenitors and can be redirected into specific neurons by further treatments [36] [47] [48].

5. Conclusion

The current work spotted the light on the specific expression timing for the differentiation markers (NES, NF-L and Map-2) to help identify the progenitor status of the induced neurogenesis of the mouse bone marrow mesenchymal stem cells and the possibility of using them for further maturation into highly specialized specific functional neuron of many types of neurons present in the nervous system.

Acknowledgements

This work was supported from the Iraqi Center of Cancer and Medical Genetic Research (ICCMGR) and Biology Department/College of Science for Women/University of Baghdad.

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Stem Cell Discovery, 2016, 6, 13-23
Published Online January 2016 in SciRes. http://www.scirp.org/journal/scd
http://dx.doi.org/10.4236/scd.2016.61002

Use of Cryopreserved Osteogenic Matrix Cell Sheets for Bone Reconstruction

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Received 14 July 2015; accepted 5 January 2016; published 8 January 2016

Abstract

Skeletal diseases, such as nonunion and osteonecrosis, are now treatable with tissue engineering techniques. Single cell sheets called osteogenic matrix cell sheets (OMCSs) grown from cultured bone marrow-derived mesenchymal stem cells show high osteogenic potential; however, long preparation times currently limit their clinical application. Here, we report a cryopreservation OMCS transplantation method that shortens OMCS preparation time. Cryopreserved rat OMCSs were prepared using slow- and rapid-freezing methods, thawed, and subsequently injected scaffold-free into subcutaneous sites. Rapid- and slow-frozen OMCSs were also transplanted directly to the femur bone at sites of injury. Slow-freezing resulted in higher cell viability than rapid freezing, yet all two cryopreservation methods yielded OMCSs that survived and formed bone tissue. In the rapid- and slow-freezing groups, cortical gaps were repaired and bone continuity was observed within 6 weeks of OMCS transplantation. Moreover, while no significant difference was found in osteocalcin expression between the three experimental groups, the biomechanical strength of femurs treated with slow-frozen OMCSs was significantly greater than those of non-transplant at 6 weeks post-injury. Collectively, these data suggest that slow-frozen OMCSs have superior osteogenic potential and are better suited to produce a mineralized matrix and repair sites of bone injury.

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How to cite this paper: Kura, T., Akahane, M., Shimizu, T., Uchihara, Y., Tohma, Y., Morita, Y., Koizumi, M., Kawate, K. and Tanaka, Y. (2016) Use of Cryopreserved Osteogenic Matrix Cell Sheets for Bone Reconstruction. *Stem Cell Discovery*, **6**, 13-23. http://dx.doi.org/10.4236/scd.2016.61002

Keywords

Bone Marrow Stromal Cell, Cryopreservation, Cell Sheet, Injectable Bone, Bone Reconstruction

1. Introduction

With rapid advancements in tissue engineering, various skeletal diseases and complications—such as osteonecrosis and nonunion—can be now treated using tissue-engineered bone derived from bone marrow-derived mesenchymal stem cells (BMSCs) [1]-[4]. In the fields of orthopedic and plastic surgery, open surgery for conventional bone transplantation is still the gold standard for nonunion and osteonecrosis. However, a less invasive approach for bone reconstruction is clinically preferable since it is likely to result in reduced scarring and an earlier start to rehabilitation. To accomplish this, further technical advances and investigations are required, including minimally invasive surgery using tissue engineering.

Recently, the use of cell sheets has been widely investigated as a promising technology in regenerative medicine research [5] [6]. Cell sheets have been created from the skin [7], myocardial cells [8] [9], periodontal ligaments [10], corneal epithelia [11], bladder epithelia [12], and mucosal epithelia [13]. A cell sheet transplantation technique for bone regenerative medicine was recently developed using BMSCs [14]-[16]. BMSCs are cultured in media containing dexamethasone (Dex) and ascorbic acid phosphate (AscP), and then lifted as single cell sheets using a scraper. The resulting sheets are referred to as osteogenic matrix cell sheets (OMCSs). OMCSs show high osteogenic potential *in vitro* and *in vivo* after subcutaneous scaffold-free transplantation [14] and in combination with artificial bone, such as beta-tricalcium phosphate [16]. Moreover, successful *in vivo* bone formation was observed after scaffold-free OMCS injection [14]. Experimental animal models have successfully demonstrated the ability for OMCSs to treat fracture nonunion [15] and ligament reconstruction [17] by enhancing bone union and callus formation between bones, as well as ligamentous surfaces and bone tunnels. Tissue invasion by injectable bone may be small, as transplantation is conducted only by injection; however, this method can be used to treat delayed union and fracture nonunion with repeated cell sheet transplantation.

One disadvantage of this technique is that the process of OMCS preparation, including BMSC culture, takes approximately 4 weeks. This may limit its clinical application. To expand the applications of OMCS transplantation in orthopedic and plastic surgery, new approaches that shorten the preparation time for cell sheets are required.

To reduce the cell preparation time required prior to transplantation, we developed a method of OMCS cryopreservation and transplantation that can be used for skeletal reconstruction. In this study, OMCSs were cryopreserved by rapid- and slow-freezing methods, thawed, and either injected scaffold-free as injectable bone to a subcutaneous site or transplanted directly onto sites of bone defects in rat femurs. The capacity of grafts to repair bone was then monitored over the course of 6 weeks.

2. Methods

2.1. Ethics Statement

The care and handling of the rats used in this study were approved by our institute's Animal Care Committee, and met the standards of the National Institutes of Health. Male Fischer 344 (F344) rats were purchased from Japan SLC (Shizuoka, Japan), and used as donors and recipients.

2.2. Bone Marrow Cell Preparation

Bone marrow cell preparation was conducted according to previous reports [14]-[18]. Briefly, bone marrow cells were obtained from the femur shafts of 7-week-old male F344 rats. Both femur ends were cut and the bone marrow was flushed out using 10 mL of standard culture medium [minimal essential medium] (Nacalai Tesque Inc.; Kyoto, Japan) with 15% fetal bovine serum (Gibco Life Technologies; Carlsbad, CA, USA) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin; Nacalai Tesque Inc.). Released cells were collected into two T-75 flasks (BD Falcon; BD Biosciences; Franklin Lakes, NJ, USA) containing 15 mL of standard culture medium. Cells were cultured in an incubator under 5% CO_2 at 37˚C. After reaching confluence, primary cultured

cells were trypsinized from T-75 flasks using trypsin/ethylenediaminetetraacetic acid (Nacalai Tesque Inc.).

2.3. OMCS Preparation

OMCSs were prepared according to previously reported methods [14]-[18]. Briefly, primary cultured BMSCs were seeded at 1×10^4 cells/cm^2 in 10-cm dishes (100 × 20 mm; BD Falcon) containing 10 nM Dex (Sigma; St. Louis, MO, USA) and AscP (L-ascorbic acid phosphate magnesium salt n-hydrate, 82 μg/mL; Wako Pure Chemical Industries; Kyoto, Japan), and grown until confluent (approximately 14 days). Cells were rinsed twice with phosphate-buffered saline (PBS; Gibco) and then OMCSs were lifted using a scraper.

2.4. Cryopreserved OMCS Preparation

Cryopreserved OMCSs were prepared using rapid- and slow-freezing methods. For the rapid- and slow-freezing groups, tweezers were used to transfer OMCSs grown in 10-cm dishes to 2-mL cryovials (cryogenic vial; BD Falcon) containing 500 μL cryopreservation medium (Cell Banker 1; Juji Field, Inc.; Tokyo, Japan).The OMCSs in the slow-freezing group were then cryopreserved in a controlled-rate freezing chamber (Bicell; Nihon Freezer, Tokyo, Japan) placed in a −80˚C freezer. The rate of cryopreservation was set at −1˚C/min from +4˚C to −80˚C. After overnight storage at −80˚C, OMCSs were stored in a liquid nitrogen tank. For the rapid-freezing group, cryovials were placed directly into a −80˚C freezer with no controlled-rate freezing chamber. After overnight storage at −80˚C, cryovials were transferred and stored in a liquid nitrogen tank. In both groups, temperature changes in the cryopreservation medium were measured in cryovials using a thermometer sensor (CENTER370 RTD thermometer; Center Technology Group; New Taipei, Taiwan),which was inserted into the cryopreservation medium through a hole in the cryovial cap.

After cryopreservation, samples were placed in a water bath at 37˚C until completely thawed. Thawed OMCSs were rinsed twice with PBS prior to use in subsequent experiments.

2.5. Cell Viability Assay

Viability of OMCSs in both the rapid- and slow-freezing groups was determined using a previously reported method based on tetrazolium reductase activity (Cell Counting Kit-8 [WST-8]; Dojindo; Kumamoto, Japan) [18] [19]. Briefly, standard curves were generated using OMCSs cultured in 6-, 12-, 24-, and 48-well culture plates (BD Falcon; n = 5 per plate). The differently sized OMCSs were harvested from each culture plate using a scraper, and incubated in 1 mL culture medium in a 95% humidified atmosphere with 5% CO_2 at 37°C for 24 h. Next, samples were placed in WST-8 solution (100 μL in 1 mL culture medium) in culture wells. After 2h incubation, the solution obtained from each culture well was analyzed using a spectrophotometer set at 450 nm. A linear relationship (correlation $R^2 = 0.969$) was observed between the average optical density and number of seeded cells per unit volume of cultured medium (cells/mL).

OMCSs cultured in 12-well plates (n = 5) were cryopreserved using the same protocol for the rapid- and slow-freezing groups, and after thawing at 37˚C were incubated in the same manner as for standard curve preparation. Using the standard curve, the number of viable cells in OMCSs from each group was analyzed before freezing and after thawing. Measurement of cell viability was the same as for standardization. For all samples, cell viability was calculated at 24 h after initiation of thawing as a percentage relative to the fresh group.

2.6. Osteogenesis of Injected Cryopreserved OMCSs at Ectopic Sites

Osteogenesis of rapid- and slow-freezing OMCSs was compared to that of fresh OMCSs. OMCSs obtained from anentire 10-cm dish were collected into 500 μL standard culture medium in a 1-mL syringe (JMS Co. Ltd.; Tokyo, Japan), and then injected into a subcutaneous site on the backs of F344 rats (n = 12) using a 16-G needle (Terumo; Tokyo, Japan). Thawed OMCSs were transplanted immediately without culturing. Four weeks after injection, all samples were harvested and fixed in 10% neutral buffer formalin for 2 days. X-ray images of each sample were then obtained. Next, samples were decalcified in K-CX solution (Falma Co.; Tokyo, Japan), embedded in paraffin, and cut in parallel down the middle prior to hematoxylin and eosin (H&E) and Sirius red staining.

2.7. RNA Isolation and Real-Time Quantitative Polymerase Chain Reaction (PCR)

To confirm osteogenesis in the harvested OMCSs, levels of osteocalcingene expression was measured. RNA was isolated from five samples from each group using an Isogen RNA extraction kit (Nippon Gene Co. Ltd.; Toyama, Japan). Harvested samples were placed in matrix bead vials with Isogen solution and disrupted using a Fast Prep FP120 cell disrupter (Qbiogene, Inc.; Carlsbad, CA, USA). The remaining steps of RNA isolation were performed according to the manufacturer's instructions.

To measure mRNA expression levels, real-time quantitative PCR (ABI PRISM 7700 Sequence Detection System; Applied Biosystems; Norwalk, CT, USA) was performed using appropriate primers and specific fluorogenic probes for rat cDNA sequences of osteocalcin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as described previously [14] [20] [21]. Target osteocalcin mRNA levels in injected OMCS samples collected from each group (n = 5) were compared after correcting to GAPDH levels. We used GAPDH as an internal standard to adjust for sample differences in reverse transcription efficiency. Osteocalcin (Rn01455285 g1), GAPDH (Rn99999916 s1) primer and probe sets were purchased from Applied Biosystems (Foster City, CA, USA). Thermal cycling conditions were 10 min at 95˚C for activation of universal mixture Ampli Taq Gold Polymerase, followed by 35 cycles of 15 s at 95˚C for denaturing and 1 min at 60˚C for annealing and extension. PCR experiments were conducted in duplicate.

2.8. OMCS Osteogenesis at Bone Defect Sites

An experimental rat femur bone defect model was generated under isoflurane anesthesia. Briefly, a lateral incision was made on the hind limb, and the vastus muscle was divided longitudinally to expose the right femur. After making a triangle osteotomy of the femoral shaft using an oscillating mini saw, a 1.2-mm K-wire was inserted into the femoral shaft intramedullary from the distal femoral condyle in a retrograde fashion, resulting in rigid fixation to maintain the bone defect. The femur bone defect was wrapped using two pieces of OMCSs from the rapid- and slow-freezing groups. In the non-transplant group, the right femur was treated in the same manner but without OMCS transplantation. Unprotected weight bearing was allowed immediately after the operation.

Post-operative femur X-ray photographs were taken under anesthesia at 3 and 6 weeks to evaluate callus and bridging bone formation at the bone defect site. Two femurs from each group were harvested at 3 and 6 weeks post-transplant. After removing the intramedullary pins from the femur, the surrounding muscle was dissected, and harvested femurs were fixed in 10% neutral buffer formalin, decalcified using ethylene diamine tetraacetic acid solution, and embedded in paraffin. Femurs were cut longitudinally, H&E stained, and histologically evaluated.

2.9. Biomechanical Analysis

Fifteen rats were used for biomechanical evaluation. Femurs were harvested at 6 weeks post-operatively, and three-point bending tests were performed in a vertical direction using a universal testing machine (EZgraph, Shimadzu; Kyoto, Japan). After removing the intramedullary pins, the harvested femurs were fixed in a clamping jig. All 15 femurs (five from each group) were bent at a constant rate of 10mm/min, and the maximum force at failure was recorded.

2.10. Statistical Analysis

Cell viability, real-time PCR, and biomechanical test values are represented by means± standard deviation. Multiple comparisons among groups were evaluated by one-way analysis of variance with post-hoc multiple comparisons using Tukey's test. $P < 0.05$ was considered statistically significant. All statistical analysis were conducted by using Ekuseru Toukei 2010 (Social Survey Research Information Co., Ltd; Tokyo Japan).

3. Results

3.1. Temperature Change during Cryopreservation

Representative temperature changes in the rapid- and slow-freezing groups are shown in **Figure 1**. In the slow-freezing group, almost 3 h was required for cryopreservation from 0˚C to −80˚C. In contrast, in the rapid-

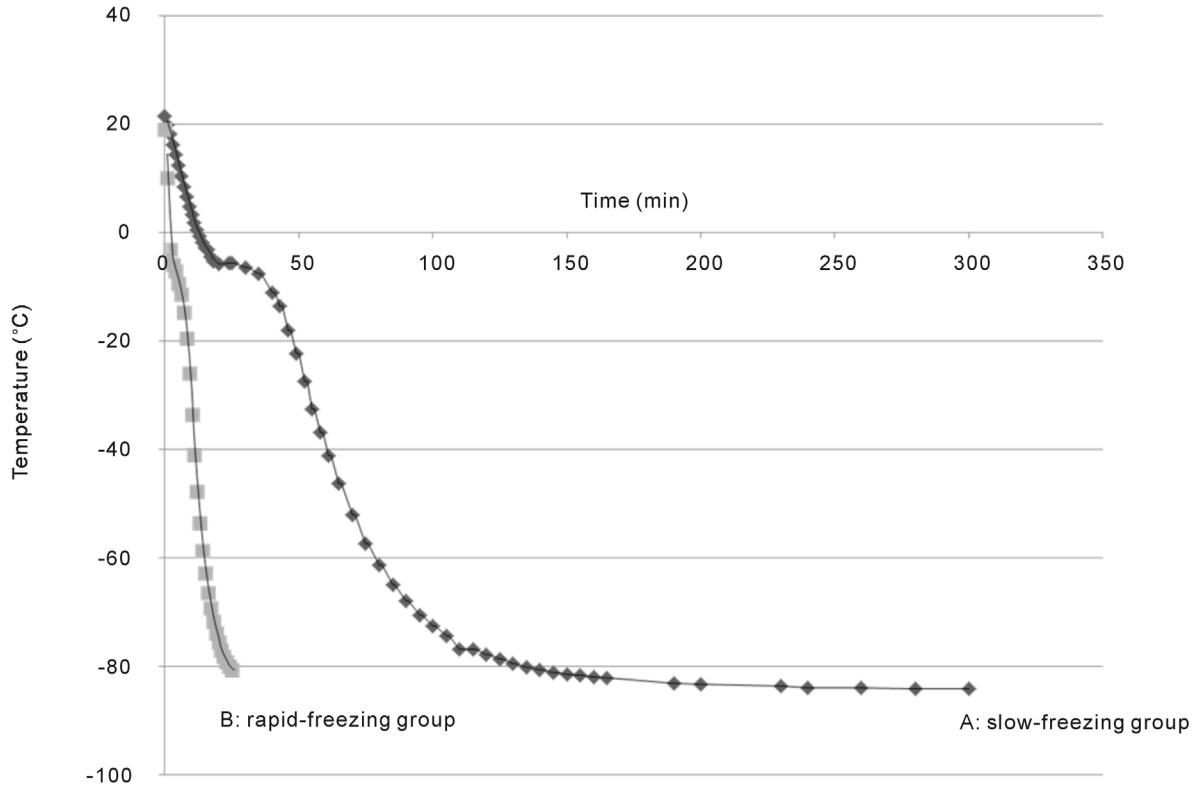

Figure 1. Temperature change during cryopreservation of osteogenic matrix cell sheets by slow-freezing (A) and rapid-freezing (B). A sensor was inserted into the cryopreservation medium through a hole in the cryovial cap. Measurements (n = 1) were repeated three times, with similar results.

freezing group, only 25 min was needed for cryopreservation from 0˚C to −80˚C. Measurements (n = 1) were repeated three times, with all recordings showing a similar trend.

3.2. Cell Viability of OMCSs

The cell viability of cryopreserved and thawed OMCSs is shown in **Figure 2**. There was no significant difference in cell viability between the fresh and slow-freezing groups, whereas cell viability in the rapid-freezing group was significantly lower than that of the fresh group. Average cell viability in the slow-freezing group was approximately 70% that of the fresh group.

3.3. Osteogenesis of Injected Cryopreserved OMCSs at Ectopic Sites

The macroscopic appearances of harvested specimens obtained from the fresh, rapid-, slow-freezing groups are shown in **Figure 3(A)**, **Figure 3(G)** and **Figure 3(M)**. Specimens appeared as hard masses and showed calcification, as determined by X-ray photography (**Figure 3(B)**, **Figure 3(H)**, **Figure 3(N)**).H&E staining showed a bone matrix with osteocytes in each groups (**Figure 3(C)**, **Figure 3(D)**, **Figure 3(I)**, **Figure 3(J)**, **Figure 3(O)**, **Figure 3(P)**). Sirius red staining was used to assess the extent of collagen deposition (predominantly collagen type I and III fibers), and positive staining was observed on newly formed bone (**Figure 3(E)**, **Figure 3(F)**, **Figure 3(K)**, **Figure 3(L)**, **Figure 3(Q)**, **Figure 3(R)**). These observations indicate that after injection, OMCSs in the fresh, rapid-freezing, and slow-freezing groups were viable in subcutaneous sites and form bone tissue in a scaffold-free manner.

Osteocalcin expression levels evaluated by real-time PCR are shown in **Figure 4**. Osteocalcin expression level was 0.97 ± 0.44, 1.33 ± 0.57 and 1.08 ± 0.34 in fresh, rapid-freezing and slow-freezing groups, respectively. There was no significant difference in osteocalcin expression among the fresh, rapid-freezing, and slow-freezing groups.

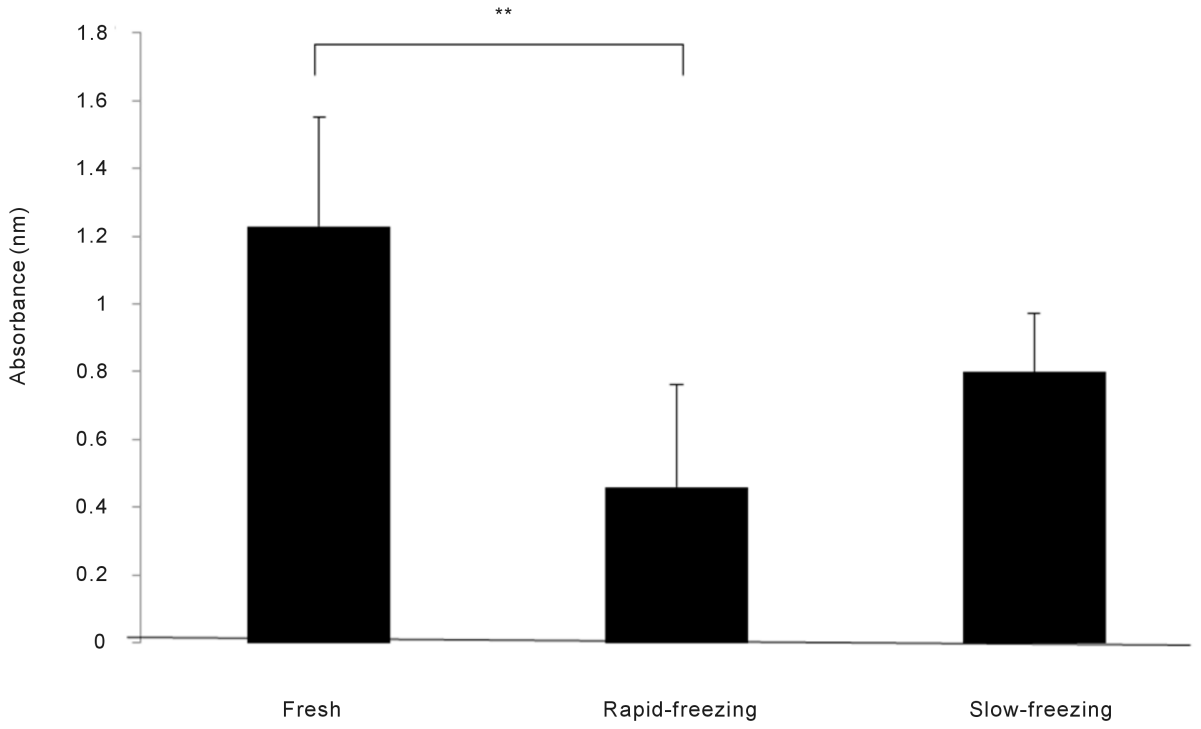

Figure 2. Cell viability in fresh, slow-, and rapid-frozen osteogenic matrix cell sheets. Viability was measured using tetrazolium activity, and compared to standard curves (n = 5). Cell viability differed significantly between the fresh and rapid-freezing groups. $^{*}P < 0.05$. $^{**}P < 0.01$.

3.4. Osteogenesis of OMCSs at Bone Defect Sites

In the rapid- and slow-freezing groups, radio graphs taken 3 weeks after transplantation of cryopreserved/thawed OMCSs showed callus formation around bone defect sites (**Figure 5(A)**). The cortical gap at the defect site disappeared by 6 weeks post-transplantation. In the non-transplant group, only faint callus formation was seen at 3 weeks, and the cortical gap was still present even at 6 weeks. At 6 weeks, cortical bone continuity was observed in the rapid- and slow-freezing group but not in the non-transplant group (**Figure 5(B)**).

The maximum forces applied to femurs in the non-transplant, rapid-, and slow-freezing groups are shown in **Figure 6**. While no significant difference in the maximum force at defect sites was observed between the non-transplant and rapid-freezing groups at 6 weeks, femurs from the slow-freezing group were able to withstand much greater force than those from the non-transplant group.

4. Discussion

The results of our study demonstrate that cryopreserved OMCSs have osteogenesis. Rapid freezing decreased OMCS cell viability to a greater degree compared to slow freezing. Following injection of rapid- and slow-frozen OMCSs into subcutaneous sites or transplantation to bone defect sites, we observed abundant bone formation or bone union. Osteocalcin was used as a marker of bone maturation, since its expression increases continuously throughout this process; however, no significant difference in osteocalcin expression among three groups. Moreover, in femurs transplanted with slow-frozen OMCSs, our biomechanical examination demonstrated significantly higher bone strength against bending stress than non-transplant group, indicating that slow-frozen OMCSs are useful for skeletal reconstruction. Our group has previously reported on the usefulness of fresh sheets [15]. In cases of delayed union, fracture bone formation is generally promoted using low-intensity pulsed ultrasound [22] [23] or pulsed electromagnetic fields [24]. If the fracture develops into a nonunion, an invasive operation, such as bone transplantation with autologous (including vascularized) bone grafts, is commonly performed. However, such procedures require sacrificing intact bone such as that of the pelvis or fibula, and there is limited bone available for autologous transplantation. Therefore, a less invasive technique is required.

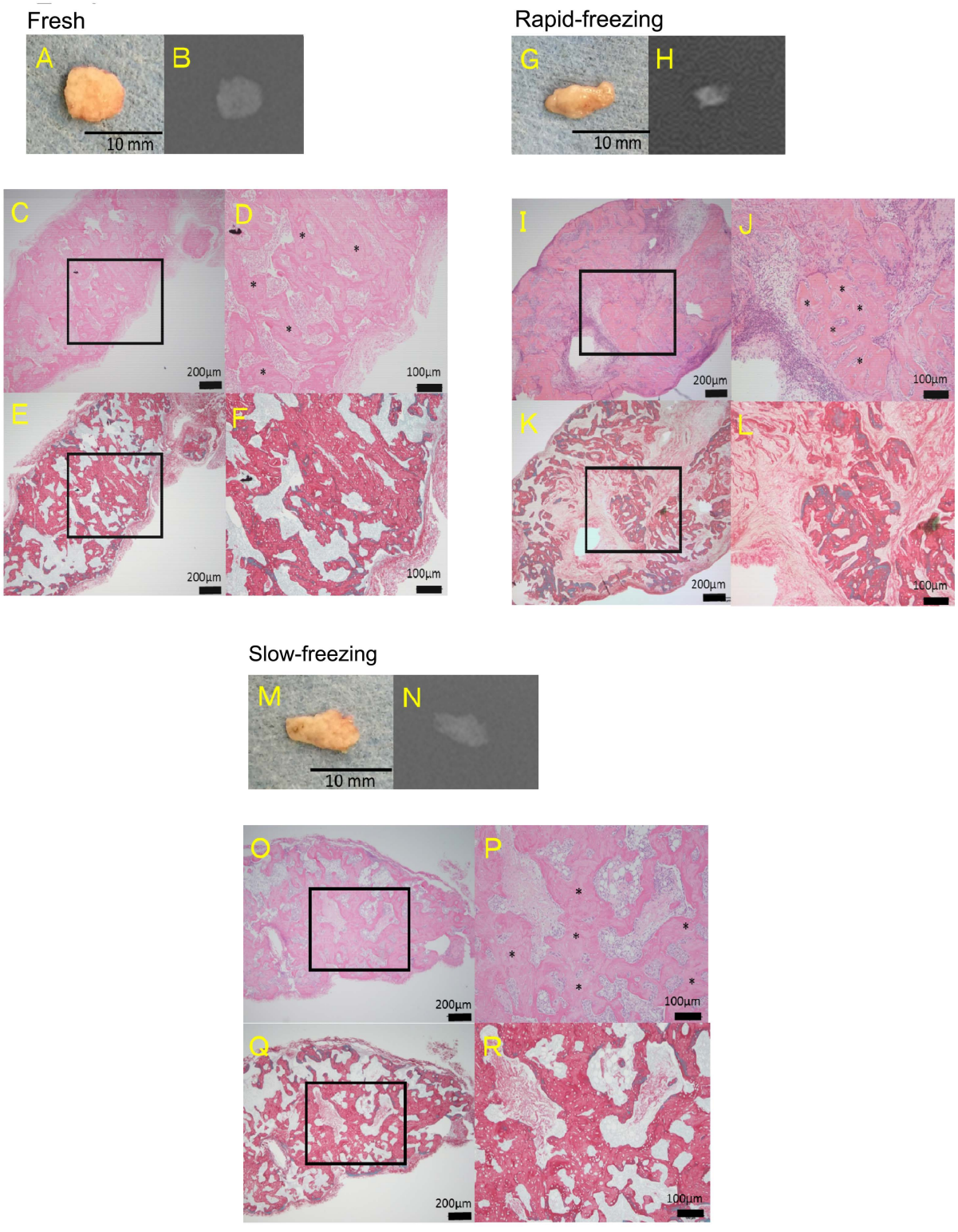

Figure 3. Osteogenesis after subcutaneous injection of fresh (A)-(F), rapid-frozen (G)-(L), and slow-frozen (M)-(R) osteogenic matrix cell sheets. (A), (G), (M): macroscopic appearance. (B), (H), (N): X-ray photography. (C), (D), (I), (J), (O), (P): hematoxylin and eosinstaining. Asterisks indicate newly formed bone. (E), (F), (K), (L), (Q), (R): Sirius red staining. (D), (F), (J), (L), (P), (R): high-magnification images of each rectangular area (C)-(D), (E)-(F), (I)-(J), (K)-(L), (O)-(P), (Q)-(R).

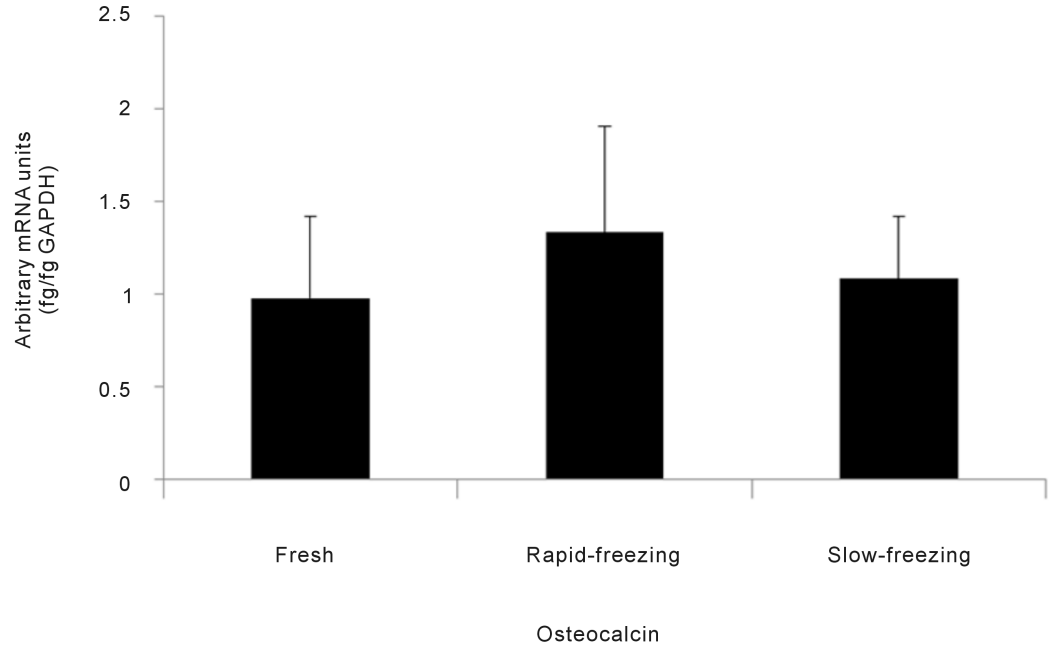

Figure 4. Osteocalcin mRNA expression after subcutaneous injection of the fresh, rapid-, and slow-freezing groups. There was no significant difference in osteocalcin expression. n = 5; $^{*}P < 0.05$.

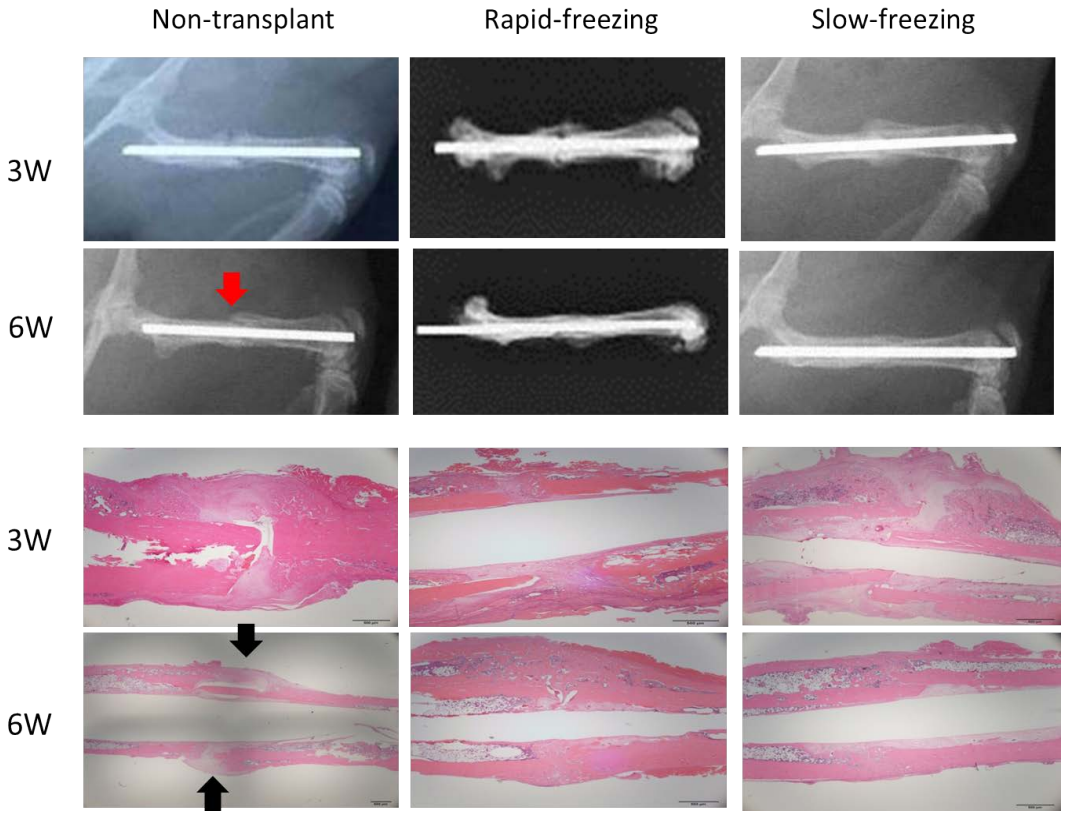

Figure 5. Osteogenesis at bone defect sites after injection with rapid- or slow-frozen osteogenic matrix cell sheets as compared to non-transplant. A: radiographs at 3 and 6 weeks (3W, 6W). B: representative histological sections at 3 and 6 weeks. Red arrow, cortical gap; black arrows, soft tissue.

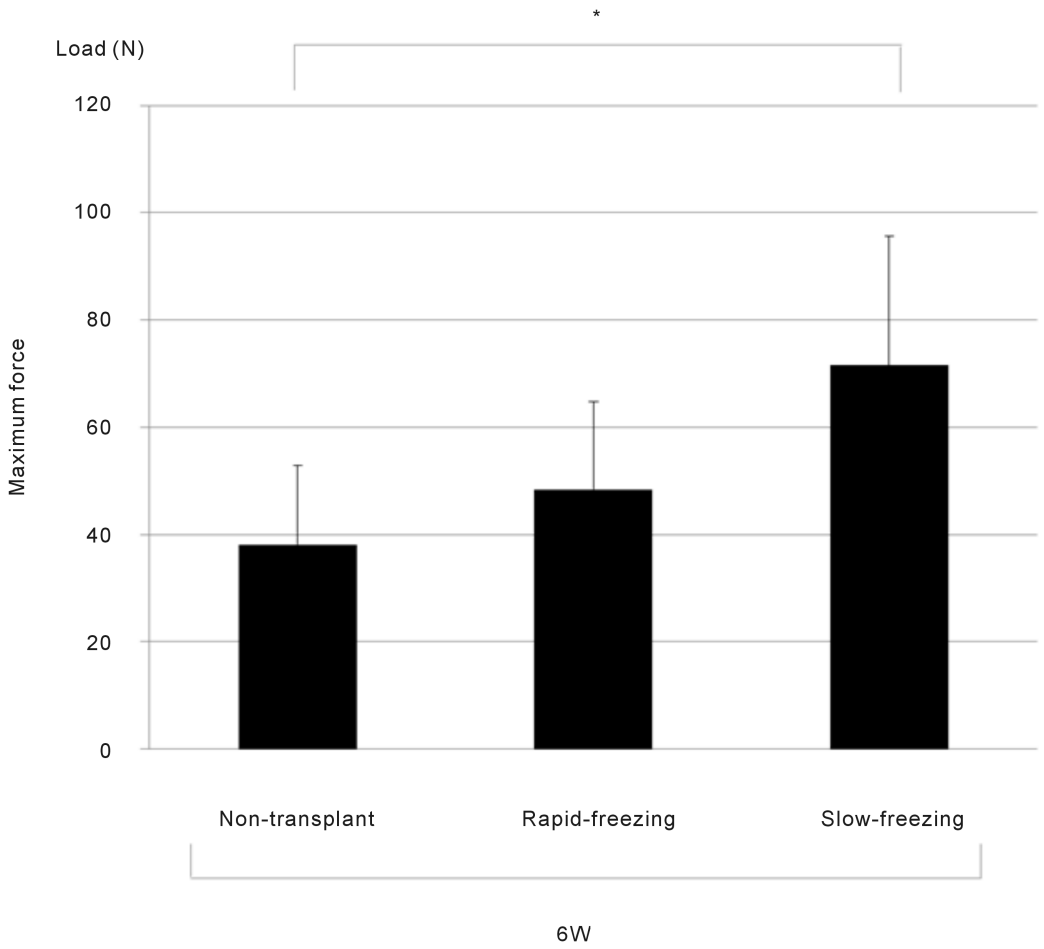

Figure 6. Biomechanical analysis. Results of three-point bending tests of femurs at 6-weeks post-transplant (n = 5 per group). $^{*}P < 0.05$.

In the orthopedic and plastic surgery fields, distraction osteogenesis techniques are commonly applied to the lower extremities and craniofacial skeleton. Robiony *et al*. [25] and Kitoh *et al*. [26] reported that distraction can be accelerated by mesenchymal stem cells and platelet-rich plasma transplantation, resulting in a shortened treatment period. Our results suggest that injectable bone using slow-frozen OMCSs may also enhance or speed up bone formation in cases of delayed union and distraction osteogenesis, and would enable repeated treatments.

Successful clinical application of regenerative treatment for skeletal reconstruction based on cell transplantation requires precise timing, so that cell preparation coincides with the time of the operation. Decreasing cell preparation time is currently an unmet clinical need. Although prior BMSC cryopreservation is a technique that may shorten the process, cultivation time is nonetheless required before transplantation to prepare the cell/scaffold constructs for thawed BMSCs [27]. An OMCS cryopreservation method was developed to overcome this disadvantage [18]. The previously reported cryopreservation method used a rapid-freezing technique that resulted in deterioration of OMCS cell viability during the cryopreservation/thawing process. Here we have demonstrated that a slow-freezing method for cryopreserved OMCSs maintains cell viability and promotes osteogenesis. Both nonunion and distraction bone reconstructions tend to require long treatment periods; thus, OMCS cryopreservation will enable the rapid preparation and immediate use of cell sheets that may expedite this process.

Our study had a few limitations. First, we used rat BMSCs, and further experiments using human BMSCs must be performed to evaluate clinical applications of this technique. Second, the experimental bone defect we investigated was relatively small, and further study using a critical-sized bone defect model is needed. Third, we showed bone formation after cryopreserved OMCS injection to subcutaneous sites; however, it is also necessary to examine the bone union capacity of OMCSs at defect sites to fully evaluate the effects of repeated OMCS in-

jection. Thus, additional research is needed to clearly determine the clinical significance of cryopreserved OMCSs, and we are preparing further studies to address these points.

5. Conclusion

Cryopreserved OMCSs prepared using slow-freezing and rapid-thawing methods have osteogenic potential. Cryopreserved/thawed OMCSs are capable of producing a mineralized matrix at bone defect sites that results in bone union. Slow-frozen OMCSs are useful for skeletal reconstruction such as in cases of bone defects, nonunion, and osteonecrosis. This method shows good potential for reducing the cell preparation time needed for OMCS treatment.

Acknowledgements

We thank F. Kunda, M. Yoshimura, and M. Matsumura (Nara Medical University School of Medicine, Japan) for technical assistance. Y. Katoh and T. Inoue (Department of Biomedical Engineering, Doshisha University) for biomechanical analysis. This study was conducted with the support of a Grant-in-Aid for Young Scientists.

Competing Interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Stem Cell Discovery, 2016, 6, 24-38
Published Online January 2016 in SciRes. http://www.scirp.org/journal/scd
http://dx.doi.org/10.4236/scd.2016.61003

Rat Mesenchymal Stem Cells from Adipose Tissue Reduce Bleomycin-Induced Lung Remodeling in Late Stage

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Received 4 November 2015; accepted 9 January 2016; published 12 January 2016

Abstract

Idiopathic pulmonary fibrosis (IPF) is progressive fibrosing interstitial pneumonia of unknown cause, chronic and incurable interstitial lung disease, associated with high mortality rates and unresponsive to treatments currently available. The prevalence of IPF is estimated at approximately 20/100,000 in men and 13/100,000 in women, and the mean age at the time of diagnosis is 67 years and the median survival is 2 to 5 years. Therapies available to date, proved, therefore, only palliative measures with doubtful or unsatisfactory result. Many experimental models of pulmonary fibrosis are described. Bleomycin-induced pulmonary fibrosis is a widely used experimental model to identify and validate new therapeutic targets. We have induced pulmonary fibrosis by intratracheal bleomycin and late instillation of mesenchymal stem cells (MSC) from adipose tissue as a therapeutic proposal was used. MSC have the capacity to modulate inflammatory and immune response. Furthermore, the long-term effect of MSCs could also regulate and control to collagen deposition of the myofibroblasts, a final and pivo cell of pulmonary fibrosis. MSC from adipose tissue is an effective therapy to decrease collagen synthesis and expression in late stage of bleomycin-induced pulmonary fibrosis model, which may contribute to new therapeutic targets.

How to cite this paper: Felix, R.G., Fabro, A.T., Vicentini-Oliveira, J.C., Bianchi, E.H., de Assis Golim, M., Cotrim, O.S., Ribeiro-Paes, J.T. and Deffune, E. (2016) Rat Mesenchymal Stem Cells from Adipose Tissue Reduce Bleomycin-Induced Lung Remodeling in Late Stage. *Stem Cell Discovery*, **6**, 24-38. http://dx.doi.org/10.4236/scd.2016.61003

Keywords

Mesenchymal Stem Cell, Idiopatic Lung Fibrosis, Animal Models, Treatments

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a non-neoplastic lung disease characterized by the formation of scar tissue in the lungs, in the absence of any known provocation. It is a rare disease that affects about 5 million people worldwide [1].

The estimated prevalence of the IPF is approximately 20/100,000 13/100,000 in men and women [2]. The mean age at diagnosis is 66 years and the median survival is 2 to 5 years [3]. The IPF presents with clinical picture of gradual onset of dyspnea with or without dry cough, and patients may present digital clubbing and crackles during inspiration. Although some environmental factors (smoking, exposure to silica and livestock) are associated with the disease, the etiology remains unknown [1].

Typical radiological images show basal and peripheral reticular opacities associated with bronchiectasis and standard on "honey-comb". The diagnosis of IPF requires correlation of the clinical picture, changing radiographic images and a lung biopsy [4].

The evaluation by pulmonary function tests, in most cases, shows a restrictive ventilatory defect associated with the reduction of carbon monoxide diffusion and hypoxemia exacerbated or caused by exercise [5].

The clinical treatment of IPF includes anti-inflammatory drugs (steroids), anti-fibrotic agents, immunomodulatory and cytotoxic drugs (cyclophosphamide, azathioprine) associated with partial and temporary improvements. Other support measures include: treatment of complications, such as heart failure and infections, rehabilitation and oxygen therapy [5] [6]. The surgical approach to FPI basically comprises lung transplantation [7]. The different clinical therapeutic approaches, associated with pulmonary rehabilitation techniques, have undeniably contributed to the extension and improvement in quality of life of patients with IPF [8] [9]. Despite significant advances resulting from the introduction of new therapeutic approaches and rehabilitation not managed to present a form of effective treatment, other than just palliative. Lung transplantation, in turn, is rare due to the shortage of donors [10]. Considering these aspects, the social and family impact due to this pathological condition, several experimental models have been proposed, aiming to advance the knowledge on the pathophysiological processes and new therapeutic approaches to IPF [11] [12]. Cell therapy (CT) stem cells (SC) in this context are presented as a therapeutic alternative with great potential applicability in pulmonary fibrosis. There is an extensive literature reporting promising results and therapeutic perspectives with adult stem cells (ASC) and embryonic stem cells (ESC) in the regeneration and repair of organs, including the lung tissue [13]-[18]. Based on this evidence, it is proposed in this project, the use of allogeneic stem cells obtained from adipose tissue for the treatment of fibrosis in rat experimental model.

1.1. Relevance

Cell therapy is one of the most interesting topics of biomedical research today and offers support for the so-called regenerative medicine [19]-[21]. The highlight for research on stem cells derived from information that challenge well-established biological concepts and hope that research results can be applied to repair lesions in human tissues. Considering the importance of these issues, this study proposes the use of stem cells from adipose tissue for the treatment of pulmonary fibrosis [22]-[24].

1.2. Goals

- To assess the contribution of cell therapy in an animal model of pulmonary fibrosis
- Specific
- Establish animal model of pulmonary fibrosis in albino Wistar rats
- Expand and characterize stem cells from adipose tissue autologous
- To infuse intravenously (caudal vein) and monitor the recovery of the lung tissue

2. Material and Methods

2.1. Animals

Will be used 56 male albino Wistar rats (average weight 250 g, 8 weeks old) coming from the Central Animal Laboratory of the Faculty of Medicine of Botucatu, UNESP and approved by the Institutional Laboratory Animal Care and Use Committee (Protocols CEEA 852/2010). The animals will be kept in polypropylene boxes coated with sawdust, in rooms with controlled temperature and light (22˚C and 12 h-12 h light and dark, respectively). During the period of the experiment, the rats receive solid diet and water supplemented with vitamin *ad libitum*.

2.2. Induction of Experimental Lung Fibrosis

For induction of pulmonary fibrosis in rats, will be used the experimental model proposed by Punithavathi *et al.*, 2000 [25]. Bleomycin is an antineoplastic antibiotic extracted as fermentation product of the fungus "Streptomyces verticillus", with important clinical use in the treatment of various cancer lymphomas and sarcomas. The experimental use of bleomycin lies in the fact that during chemotherapy was realized that between 4% - 10% of patients had pulmonary toxicity drugs and this resulted in pulmonary fibrosis process [26]. The use of bleomycin by intratracheal instillation in rats was performed pioneering way in 1979 [27] and generated a reliable model is often used, in view of the development of pulmonary fibrosis capability of rapid and predictable manner.

After single dose intratracheal instillation of bleomycin, one can see three different stages of evolution: the first stage (acute stage of inflammation) corresponding to the first seven days after instillation when there is migration of inflammatory cells, interstitial edema and alveoli in addition activation of mediators of inflammation. In the second stage (stage subacute) that lasts from the 7th to 15th day after installation, realizes an intense inflammation with increased cuboidal epithelial cells in order to repair the attacked epithelium and is noted also the presence of pulmonary fibrosis. In the third stage (resolution stage) which lasts from 15 to 30 days after instillation, inflammation decreases and is noticeable alveolar epithelialization and collagen deposition in the granulation tissue with the formation of fibrosis in the lung. It is understood to 14 days after instillation as the best to measure the process of fibrosis in view of the extensive fibrosis above, but with less variability in fibrotic response [28]. Crutoneo *et al.* (2006) describe the use of bleomycin to produce the IPF stimulates the pro-fibrotic growth factor TGF-B1, which is considered one of the most important in fibrogenesis. This factor is produced by fibroblast and myofibroblasts [29] [30].

2.3. Collection, Processing and Characterization of Adipose Tissue Stem Cells

Study design—intravenous injections of MSC were given 14 d after bleomycin instillation, in collagen production peak, and rats were sacrified after 2 weeks. To obtain allogenic MSCs from adipose tissue blocks of 5 different animals were used. After removing a sample of 3.35 g of adipose tissue as preceded dissociation with collagenase type I. We obtained 5.3×10^4 lymphomononuclear cells/g of adipose tissue. These cells were expanded in culture for 21 days Knockout DMEM-F12 medium added with 10% fetal bovine serum. Three criteria were considered proof of MSC: adhesion to plastic, expression of CD90 by flow cytometry and the ability to differentiate into three lineages of mesodermal origin [18]-[21]. It performed a pool of 5 samples at a final concentration 10^6 cells for intravenous injection. A total of 60 male rats, albino Wistar rats, weighing 250 g aged 8 weeks. Wistar mice were treated with (BLM-W, n = 15) intratracheal bleomycin (1.5 U/kg) or (CTR-W, n = 15) placebo. At 14 day 1×10^6 MSCs were infused into half of placebo (CTR-MSC, n = 15) and half of bleomycin-treated mice (BLM-MSC, n = 15). Four experimental groups being monitored: weight and saturation index in 3 stages: D0, D14 and D28. In D0 was performed instillation of bleomycin at a dose of 1.5 U/Kg. In D14 infusion of mesenchymal stem cells quantified and phenotyped by flow cytometry. In D28 was the sacrifice of animals for macro and microscopic analyzes. The lung was fixed and 3-mm sections were stained with Hematoxylin & Eosine and Picro-sirius Red staining. Quantification and morphometric analysis was performed by two double-blind pathologists and by image analysis following a conventional stereological method, as recommended by ATS/ERS standards for quantitative assessment of lung structure [23] [24].

Taking into consideration the leaflet involved in embryonic lung origin of the cells it was decided to obtain adipose tissue abdominal wall for processing and characterization of stem cells. The adipose tissue (AT) emerges in the last 5 years as a promising source for cell therapy CT. The AT collection site was set according to prelim-

inary results of Cell Engineering Laboratory of the Blood Center of Botucatu (CELab) which shows that the tissue obtained from the abdomen of AT present growth curve above the AT obtained from other parts of the body.

After anesthetizing the animals with 30 mg/kg of thiopental sodium 2.5%, intraperitoneal (IP), they are placed in a laminar flow HEPA filter to make the removal of the AT fragment. Proceeds to antisepsis of the hair, opening 2 - 3 cm skin, AT identification and removal fragment with an average weight of 4 g. The AT fragment is washed thoroughly with 0.9% saline (100 - 200 ml) and then placed in a sterile conical tube containing HEPES medium (Life Technologies®) transport with antibiotics type penicillin and streptomycin (Life Technologies®). It will be packaged in polystyrene boxes and transported to CELab. Upon receiving the material, the adipose tissue is heavy undergoing washing in PBS buffer suffered further incubation with collagenase type I (Sigma Aldrich®) at rest for 12 hours in oven at 37˚C. It blocks the reaction with fetal bovine serum for 5 minutes, and transferred to the material 15 ml conical tube. It was centrifuged at 700 g for 10 minutes, the supernatant aspirated, the pellet dissolved in PBS and centrifuged again for complete removal of the enzyme. In a Neubauer chamber determines the viability and cell count by exclusion method of trypan blue dye (Invitrogen®). An aliquot of cells is taken to characterization by flow cytometry using the markers: CD90, CD44 (positive marker) and CD34, CD14 or 45 as negative markers. The cells were seeded in in T-flasks 25 cm^2 (10^5 cells/cm^2). After 5 days (establishment of the adherent mesenchymal stem cell colonies) return to the DMEM-Ham F12-based medium supplemented with Knockout Serum Replacement, nonessential amino acids, 2-mercaptoethanol, and Glutamax (life Technologies®) (5 ml) every two days. At this time, the cells will again be subjected to enzymatic processing and new rate will be processed by flow cytometry using the same markers. Comet test and micronucleus will monitor any DNA damage determined by cell culture. The analysis was performed on a FACSCalibur cytometer of BD® flow through reading in Cell Quest Software Pro®. Importantly, MSC were plated amplified by the 4th passage to obtain the appropriate number of cells for the experiment, with the same stored frozen in liquid nitrogen.

2.4. Groups

The animals were divided into 4 groups of 15 animals, kept under strict control by veterinarian during the 28 days of observation from the date of tracheal instillation of bleomycin or saline, **Table 1**.

The 4 groups were formed as:

Group 1 BLM-W = fibrosis control. Wistar mice instilled with bleomycin solution and "treated" with physiological serum, **Table 1**.

Group 2 CTR – W = animals controls: Wistar mice instilled with saline and "treated" with saline (placebo).

Group 3 BLM-MSC = study group: Wistar mice instilled with bleomycin solution and treated with MSC obtained from adipose tissue.

Group 4 CTR-MSC = MSC control action: Wistar mice control group instilled with saline and treated with MSC stem cells obtained from adipose tissue.

All animals were subjected to the same stress level in handling and receiving in the 14˚ day after bleomycin intravenous infusion procedure with MSC or saline.

2.5. Mesenchymal Stem Cell Infusion (MISI)

Cell transplantation was performed on the 30th day after intratracheal instillation of bleomycin solution. The infusion of MSC was performed by intravenous (tail vein). The group treated with MSC was infused with 0.4 ml of the diluted cell solution in DMEM at a concentration of 1×10^6/ml.

Table 1. Constitution of study groups, induction of fibrosis and treatment.

Group	Instillation Day	Instilled Solution	Infusion Day	Infused Solution Treatment IV	Sacrifice Day
G1 BLM-W	0	Bleomycin	14˚	Saline	28˚
G2 CTR-W	0	Saline	14˚	Saline	28˚
G3 BLM-MSC	0	Bleomycin	14˚	MSC	28˚
G4 CTR-MSC	0	Saline	14˚	MSC	28˚

2.6. Macroscopic and Microscopic Analysis

Once the animals sacrificed on D + 28, after determining the saturation measurement and weighing the same, the lungs were transported in labeled tubes in sequential numerical order to the Pathology Service. The material was analyzed by two different pathologists double blind study. After processing the lungs, always having been processed the same lobe, namely, right middle lobe, the remainder was kept preserved for possible analysis. After proceedings the material was proceeded the hematoxylin-eosin and Picrosirius. For the evaluation was established by pathologists semi-quantitative classification Singh (2005) for perivascular inflammation: 0: no inflammation; 1: one or two concentric rows of inflammatory cells; two, three or more concentric rows of inflammatory cells and 3: accumulation of inflammatory cells between vessels and bronchioles, while the quantification of pulmonary fibrosis is worth Ashcroft scale modified Hübner (2008) [30].

2.7. Statistical Analysis

Statistical analyzes were performed using the Stat software. For the variables, saturation, weight and degree of fibrosis calculations were made using the Kruskal-Wallis test, non-parametric data (ANOVA) with post-test for multiple comparisons by Dunn's method.

3. Results and Discussion

After expansion, the control flow cytometry showed 98% of cells expressing CD90. In D14, there was a pool of these MSC were infused intravenously into the tail vein at a concentration of 1×10^6 cells/animal in a volume of 200µl saline. No adverse reactions or deaths were identified during the infusion or within 14 days. Histological analyzes were performed by two different experts double-blind study. Intrataqueal instillation of bleomycin at a dose of 1.5 U/Kg PF determined in 100% of animals whereas animals had 57.14% of PF in grade IV. The instillation of intratracheal bleomycin compromised the saturation index of animals and histological analyzes showed an UIP-pattern similar with evidence of marked fibrosis and architectural distortion in a patchy-in- volvement manner. The parameters of weight gain, improved saturation index, recovery macroscopic and microscopic lung tissue were fully achieved in the group treated with MSC-AT. BLM-MSC animals showed a significant reduction in lung fibrosis compared with BLM-W animals, according to histology and morphometry. Remodeled lung structure with contiguous fibrotic areas in BLM-W mice was recovered after MSC therapy by focal fibrotic walls or single fibrotic area. Weight gain, saturation index and macroscopic aspect were improved BLM-MSC group ($p < 0.05$). Collagen fibers per septal area were decreased in BLM-MSC mice in compared to BLM-W mice ($1.5 \pm 0.1 \times 5.9 \pm 0.4$; $p = 0.0005$). Surprisingly, collagen fibers in CTR-MSC were slightly reduced compared to CTR-W ($1.2 \pm 0.1 \times 1.6 \pm 0.1$; $p = 0.04$).

The mesenchymal stem cells were obtained from abdominal adipose tissue of 4 Wistar male animals weighing between 250 and 300 g at 8 weeks of age, who were not part of the 60 animals were divided into 4 experimental groups. The adipose tissue removed medium was 3.35 g and that after dissociation with collagenase type I use, the lymphomononuclear cells count was 5.3×10^4 cells/g of AT, a value considered sufficient to the needs of the experiment, in view of the subsequent amplification stages in culture. The seeded cells were expanded until the 4th passage in the Knockout DMEM, kept in conditions of sterile cultivation.

To prove the phenotypic profile of MSC obtained from adipose tissue in this study were used 3 confirmatory method as indicated by the International Society for Stem Cell: the criteria for adhesion and formation of fibroblast colonies; analysis profile of the cells by flow cytometry and differentiation in 3 tissue strains as Standard Operating Procedure of CELab. For this purpose we used specific culture media conditioned to chondrogenic differentiation, adipogenic and osteogenic (StemPro®). The criterion of confirmation for stem cells based on adhesion to plastic in the presence of fibroblast colony forming units was successfully achieved, as photomicrographs inverted phase contrast microscopy (**Figure 1**).

The second criterion was the analysis of the profile of the cells by flow cytometry, using specific surface markers. The best analyzes are those which occur in the range between 5000 and 10,000 events (cells). The mean of 4 samples were calculated. The CD45, CD34 and CD90. CD44 markers specific for mouse, were executed in two stages: after dissociation, before plating, when the cells were considered lymphomononuclear, and after cultivation. The minimum number of events (cells) analyzed in this experiment was of 8436 and up to 10,000 cells. The maximum number is determined by the equipment itself. **Table 2** shows the numerical results of the analysis made.

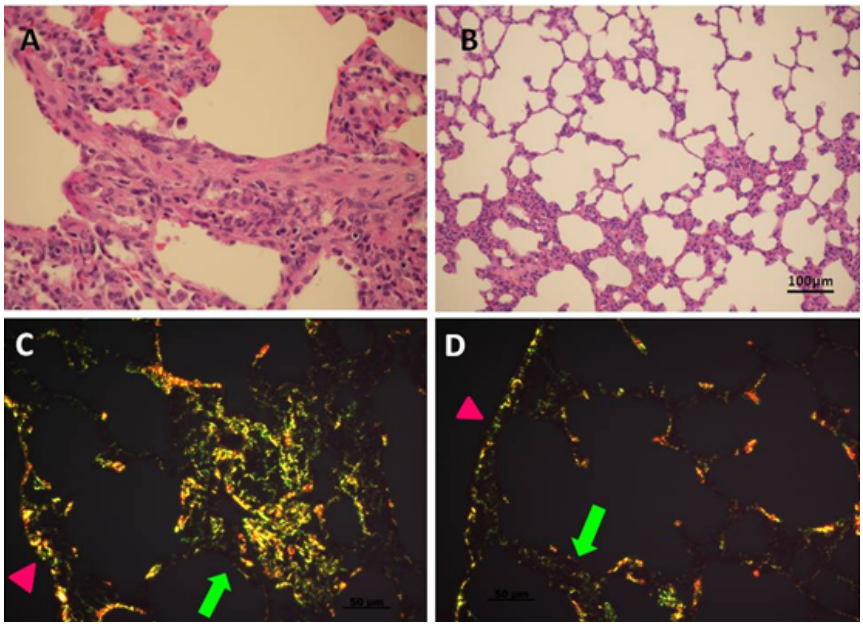

Figure 1. Inverted microscopy Contrast the first criterion of proof of MSC phase: the plastic adherence. (A) = Aspect culture 4 days after seeding (magnification 5×); (B) = Presence of fibroblast colony forming units 15 days after the start of culture (10× magnification) and (C) = Presence of fibroblast colony forming units 15 days after the start of culture (magnification 20×).

Table 2. Phenotypic profile by Flow Cytometry using markers CD34, CD44, CD45 and CD90.

Markers	Negatif control	CD34	CD44	CD45	CD90
Average number of events (cells) analyzed	9013	8857	10.000	8436	10.000
Mean Flurescence Intensity (%)	1.83	1.70	18.59	53.38* 1.72**	17.35* 98.97**

*After enzymatic cleavage, before plating; **After 21 days in culture specific culture medium (expansion) for MSC.

The distribution of lymphomononuclear cells (LMNC) after enzymatic dissociation by size and granularity scatter plot is available in **Figure 2(A)**. In **Figure 2(B)**, the cell population without specific marker, which is considered as negative control. The bar with the designation M1 defines the area corresponding to the peak of cells expressing markers for positive MSC. In C, the result of which CD34 expression was observed reactivity of 1.70% on average, a result expected for this sample.

Te third profile verification criteria of the MSC-AT used was the ability to differentiate into three different tissues. It was proved the capabilities of amplified cells to differentiate with conditioned media into chondrocytes, osteoblasts and adipocytes. The verification was performed by immunohistochemistry method using specific markers.

3.1. Weight Variation of Animals

The literature indicates the weight loss of the animals submitted to intratracheal instillation with bleomycin. To verify this finding, the animals, kept under identical conditions, were weighed in three times: D0, D14 and D28. The working hypothesis was that the bleomycin-induced pulmonary fibrosis decrease the weight of the animals which could be re-established with the MSC therapy. Statistical analysis showed significant differences, $p < 0.0001$ in D0, since there was no decision to pair the animals by weight. Compared the weight of the animal G1BLM-W groups and G2 CTR-W, there was no statistical difference, $p > 0.05$, as well as between G3 BlM-MSC and G4 CTR-MSC. Comparisons between different groups on D + 28 also did not identify statistically significant difference.

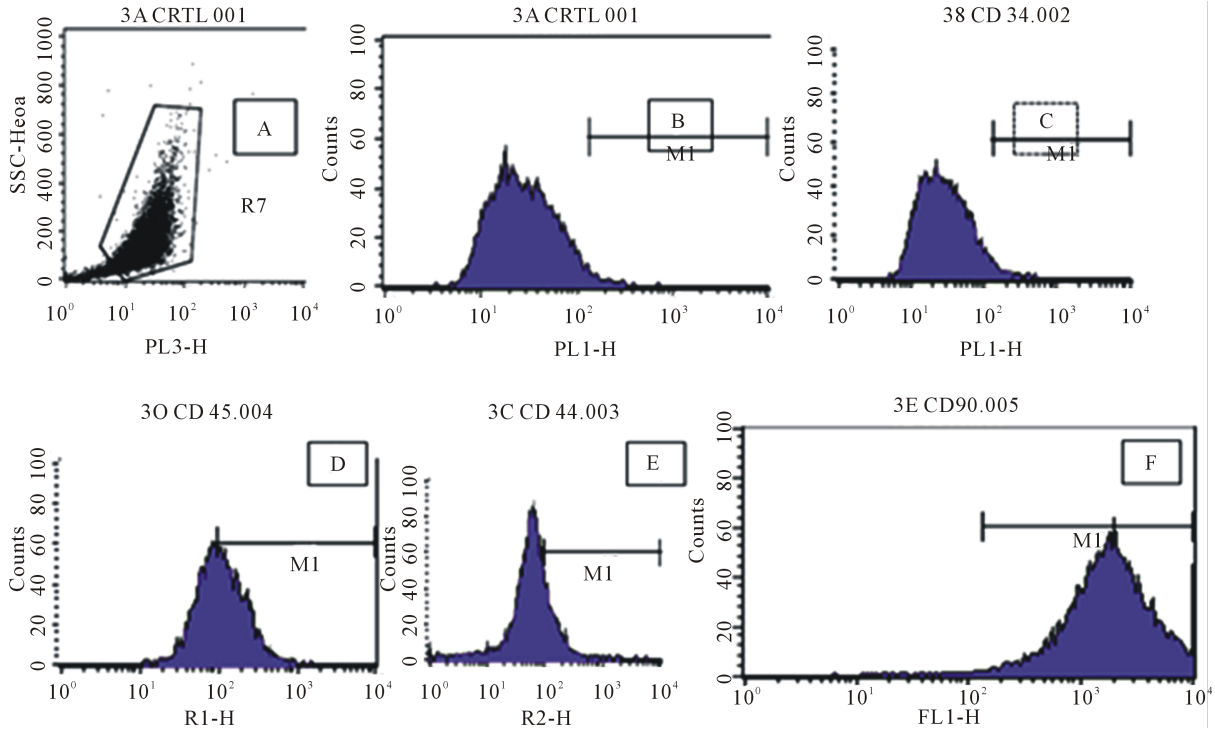

Figure 2. Analysis by flow cytometry with fluorescent markers. (A) = Dispersion graph of LMNC; (B) = negative control; (C) = CD34; (D) = CD45 pre-plating; (E) = (F) = CD44 and CD90 post amplification in culture.

Martinez (2008) publishes the isolated use of bleomycin is associated with significant loss of total body weight and a high mortality rate (40%) compared to control groups [31]. Data from this study do not corroborate the results of that author, because the mortality rate in this study was low and there was no statistically significant difference with respect to weight. During the 28 days of monitoring were registered two losses in different groups (2/60 or 3.33%) at the time of instillation of bleomycin, can be related to the process of anesthesia or chemotherapy itself. No deaths among the animals in the days following the injection of bleomycin, or systemic infectious process was not registered.

3.2. Variation Saturation

To monitor the induced fibrosis was used the saturation measurement with pediatric equipment setting the sense in the proximal portion of the tail. The formulated hypothesis was that it would decrease with the installation of the fibrosis process and that there would be a recovery of saturation indexes with use of the MSC-AT. This fact would be an indicator of clinical and anatomical and pathological improvement. The data confirmed the fall of the saturation index in animals in which was instilled bleomycin (G1BLM-W and G3 BLM-MSC). In D0, statistically significant differences were recorded between groups, as expected ($p = 0.6313$). Statistical analyzes of the saturation measurement for D14 between G2/G2 and G3/G4 show significant differences with $p < 0.05$. D + 28 $p < 0.049$, very significant.

The data indicating the supremacy of the control group G2 CTR-W on the other, including on the G4 CTR-MSC. This can be explained by the fact that the animals were handled in conventional animal facility without filtered air. However, the recovery of saturation measurement indexes can be observed in the groups, according to the moment to be analyzed (14 + D or D + 28) even in G1 BLM-W. This result is expected because King (2011) indicates that one of the limitations of pulmonary fibrosis model with the use of bleomycin is their potential spontaneous recovery, however, it is the most used model [32] [33]. The decision, in this experiment, to sacrifice 28 days later, was basically made on this fact. That is, to the MSC intervention before the onset of spontaneous regression. In fact, many other authors critical to IPF animal models, which do not reflect faithfully the intricate mechanism that occurs in humans [34]-[36].

3.3. Macroscopic Examination

Macroscopic analysis of the lung took into account the following characteristics: architecture, proportion, and surface staining and confirmed the expected changes and described in the literature [30].

For group 1 (BLM-W), which was induced fibrosis and no infusion of MSC-AT, from the original 15 animals occurred in D0 loss of them because the animal was at death, two minutes after instillation of bleomycin. Of the 14 animals alive at the end of the experiment (D28) analysis showed macroscopic changes in 100% of the lungs, and the same could be divided into 4 groups according to the degree of progression of macroscopic lesions identified, **Figure 3**.

It was identified only 1rat/14 (7.14%) showing few signs on surface and kept the other macroscopic characteristics of a healthy lung, **Figure 3(A)**.

Two animals showed a moderate or intermediate II (N = 2/14) corresponding to 14.28% of the group with the entire surface plates, color change, but maintained the proportionality and lung architecture. Touch the lung tissue showed friable, **Figure 3(B)**.

In the classification degree III or advanced (N = 3/14, or 21.42%) the lungs showed plates by entire surface, obvious change of color, observing loss of proportion and of architecture pulmonary, beyond the tissue be quite friable, **Figure 3(C)**.

The most animals (57.14%) developed a fibrosis grade IV, advanced with complications (8/14). Watchlisted up plates per entire surface, with alteration staining, occurring loss of proportion and architecture, tissue quite crispy being that in 6 animals were detected various points of necrosis, **Figure 3(D)**.

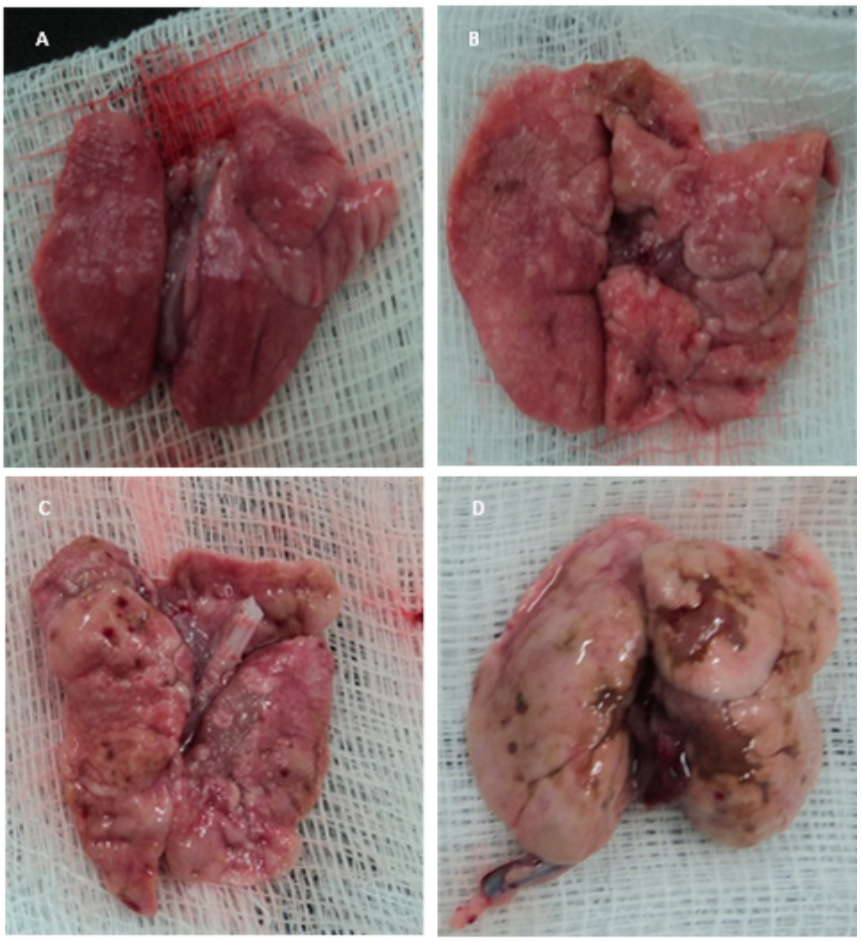

Figure 3. Classification of degrees of fibrosis according to the macroscopic examination of the lungs of animals in the G1 (BLM-W in D28. A = grade I (mild); B = moderate (intermediate); C = advanced degree III and D = advanced degree with complications.

For the Group 2 (CTR-W), therefore for these pets not there was the induction of fibrosis and neither the use of MSC-AT. This group corresponds to the group control, not having been recorded any intercurrence. They were evaluated the 15 animals of group control being that the lungs presented preservation of architecture, proportion, coloration and surface, **Figure 4**.

The experimental group G3 (BLM-MSC, the animals suffer PF induced tracheal instillation of bleomycin, they had on Day + 14 infusion 1×10^6 MSC-AT infused by tail vein. This group had the loss of one animal in D0, 1 minutes after instillation of bleomycin, a fact similar to that in the G1.

In the lungs 14 of the live animals until the end of the experiment (day + 28) were observed in 100% of specimens: no surface boards, preservation architecture and proportion and a non-friable parenchyma. The brightly pink color with brighter surface drew attention. In 50% of cases, besides the aforementioned characteristics it was noticeable with some small necrotic spots surrounding apparent recovery, **Figure 5**.

In group 4 (CTR-MSC) in which the animals were exposed to the same stress handling in D0, the saline intratracheal instillation and D + 14 received similar amounts of MSC relative to the G3 group was also not observed any death among the 15 in the group. The lungs of animals in this group showed preservation of architecture and proportion and more intense pink color, brighter and as expected surface, the tisssue was not friable, **Figure 6**.

3.4. Pulmonary Histological Analysis

This analysis was conducted in a double-blind study by interpreting two expert pathologists in lung histopathology. The analyzes involved classical hematoxylin-eosin and cytochemistry of Picrosirius. The perivascular

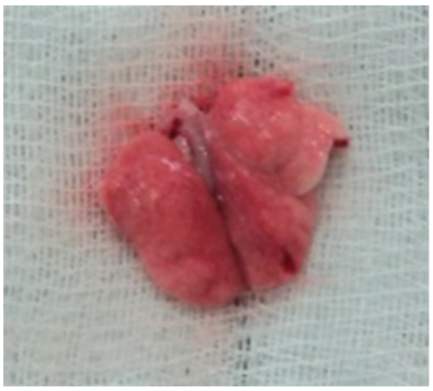

Figure 4. Animal lung macroscopic appearance of a member of the G1 Group (CTR-W). Normal control.

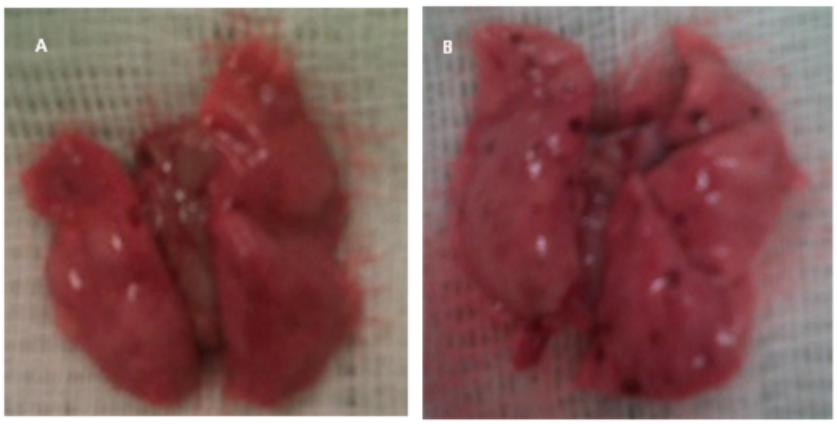

Figure 5. Macroscopic analysis of the lungs of animals in group G3 (BLM-MSC) in D28 with architecture, proportion and preserved color. (A) and (B) represent dif- ferents animals. B is observed necrosis points with surrounding tissue recovered.

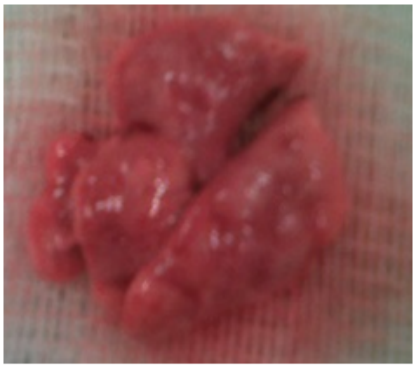

Figure 6. Macroscopic analysis of the lungs of the animals of the G4 group (CTR-MSC) with normal tissue architecture, proportion and normal coloring.

inflammation assessment used the semi-quantitative histopathologic evaluation system for perivascular inflammation as described by Singh [35] described in materials and methods, while quantification of pulmonary fibrosis is worth Ashcroft scale modified Hübner [30].

In G1 (BLM-W) notices a pattern of diffuse pulmonary fibrosis, tending to subpleural region, but not outright mass formation (except case 15), but a feature presentation sacular parenchymal remodeling, where the alveoli show large septal thickening forming aberrant nonfunctional sac-like structure. Inflammation is sharp and primarily affects the peripheral vessels, which show large medial hypertrophy. There frank bronchitis and bronchiolitis with epithelial destruction intense (**Figure 7**).

Group 2: it is observed a more diffuse pattern with dense fibrosis, but with peribronchial and bronchiolar focus, beyond subpleural. The parenchymal remodeling affects almost all septa and is observed on the border between the fibrotic mass areas and slightly thickened fibrotic septa-like structures fibroblastic foci, in which occurs the myofibroblast activation and progression of lung fibrogenesis. Inflammation is mild to moderate and eventually accompanying vessels, which are often with medial hypertrophy. It is noticed Frank bronchitis and bronchiolitis with intense epithelial destruction (**Figure 8**).

Group 3: In this group there was a significant reduction of fibrosis, but in most cases it is noticed even isolated fibrous masses (**Figure 9**). The septa sometimes present with thin and delicate usual structural sometimes show different degrees of thickening to form true dense fibrous masses. Not observed abnormal alveolar septa or abnormal septal architecture in preserved areas. It is noted macrophages with finely granular cytoplasm of bluish-between some fibrotic and perivascular regions, may represent some exogenous pigment and/or bacteria or other. Inflammation is centered on the axis-bronchial vascular characterized in bronchoalveolar mononuclear cells and epithelial-tropism. Bronchopneumonia was identified in some cases by inflammatory infiltrate intra-bronchial neutrophilic intraepithelial abscess and its consequent organizing pneumonia. It is noted medial hypertrophy of the pulmonary arteries and arterioles, perhaps less so than the other groups. Interestingly there was "hyperplasia" of its endothelial cells, which show very well defined and "healthy". There focal mesothelial hyperplasia, usually associated with fibrotic areas.

Group 4: we can see a significant attenuation of fibrosis, without the formation of fibrous masses except the case 11. Most of the septa has his usual delicate look or with minimal changes. Yet other areas there is a greater thickening and eventually with the presence of small fibrotic nodules. Inflammation is moderate with variable perivascular involvement. Note to intense chronic bronchitis and bronchiolitis in activity, indicating major antigenic stimulus. It is observed for signs of pulmonary hypertension, but appear to a lesser degree (**Figure 10**).

3.5. Histological Analysis in Masson's Picrosirius

The method of staining by Masson's Picrosirius is the color red by Sirius Red collagen protein found in collagen fibers, reticular, cartilage and in basement membranes (**Figure 11**). When combined with polarizing microscopy, allows collagen viewing by the parallel organization of tropocollagen molecules. Type I collagen fibers present color ranging from red to bright yellow with intense birefringence. Collagen type II presents less intense birefringence and pale yellow color. Type III collagen appears as delicate fibers of greenish. After statical analysis, all the results are represented in **Figure 12**.

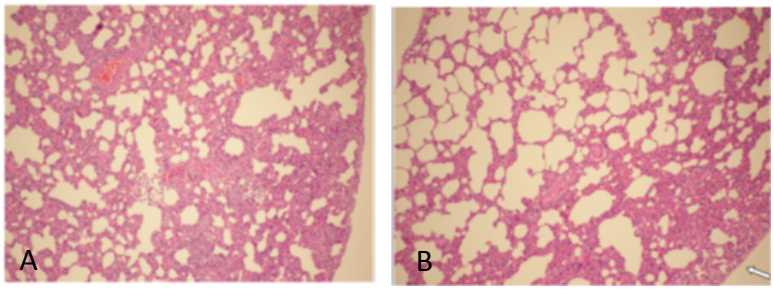

Figure 7. Pulmonary fibrosis pattern—histological appearance with Histological, representative of the G1 (BLM-W). A = Septal thickening diffuse untrained fibrotic masses, 10×; B = Septtal thickening diffuse affecting more subpleural region (arrow). 10×.

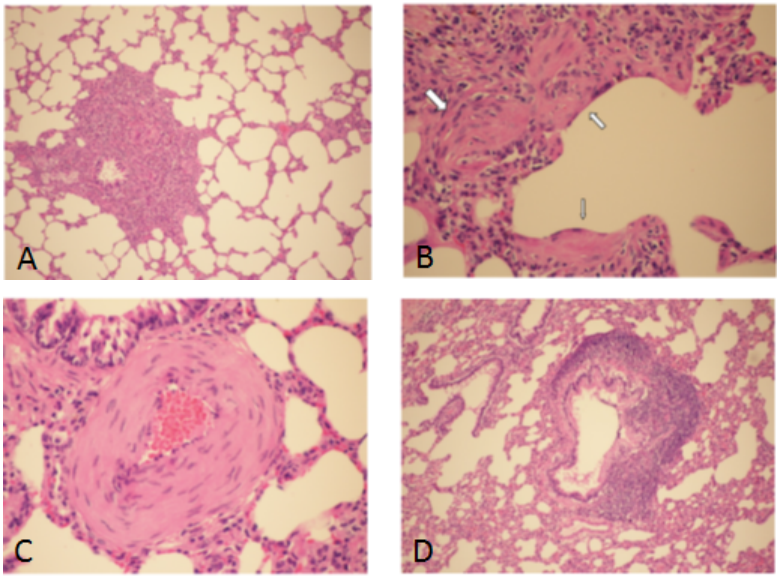

Figure 8. Diffuse pattern with dense fibrosis em Group 2. (A) = Most peribronchial fibrotic involvement, however note the alveolar septa are abnormally thickened. 10×; (B) = Matrix structure in which myofibroblasts are active, remembering fibroplastic focus of Idiopathic Pulmonary Fibrosis (arrow). 40×; (C) = Hypertrophy of the middle layer of peripheral vessels. 40×; (D) = Active chronic bronchitis with destruction of the epithelium layer and muscle. 10×.

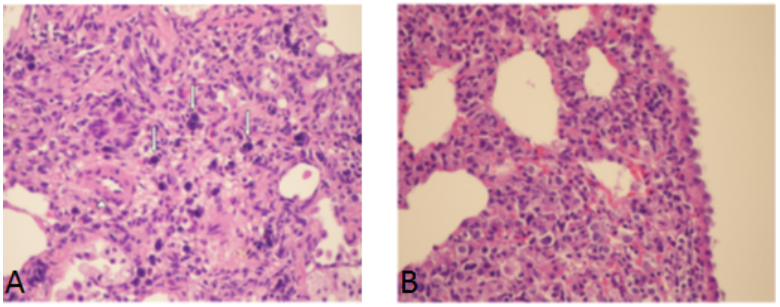

Figure 9. Fibrous mass consists of (myo) fibroblasts, extracellular matrix and vessels (group 3). (A) = Note the vessel with moderate hypertrophy of the middle and preserved endothelial cells (arrowhead). Macrophages microvacuolized basophilic are distributed by mass (arrow). 20×; (B) = Mesothelial hyperplasia adjacent the fibrotic area. 20×.

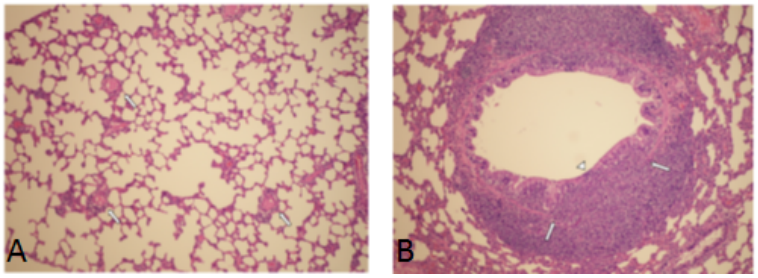

Figure 10. Histological aspects Group 4. (A) = Alveolar septa of normal fine delicate aspect or with minimal changes the interstitial component. Note the intense perivascular inflammatory infiltrate (arrows). 10×; (B) = Terminal bronchioles and respiratory bronchioles usual appearance. 10×.

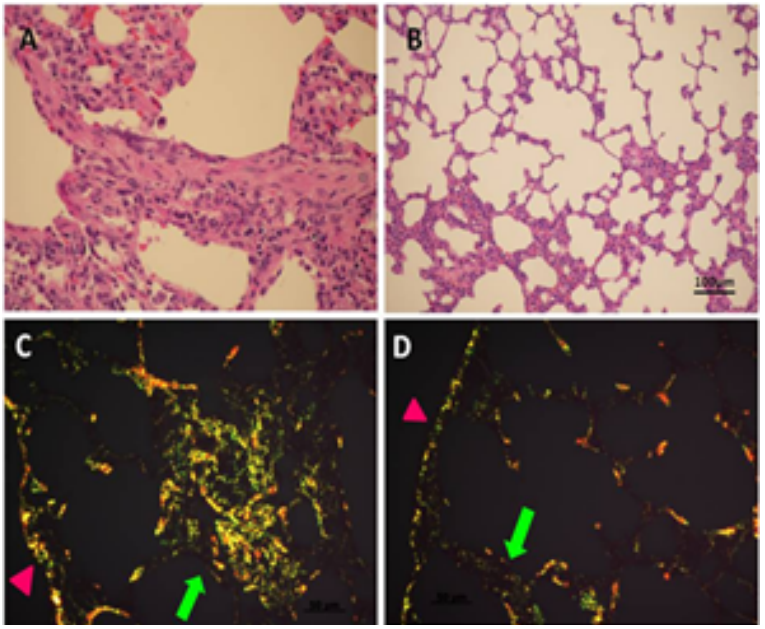

Figure 11. H&E (A), (B) and Pricrosirius Red (C), (D) stained sections and morphometric analysis of BLM-W (A), (C) and BLM-MSC (B), (D). Note the fibrotic mass (C, arrow) and pleural thickening (C, arrowhead) in BLM-W group in contrast to the fibrotic wall (D, arrow) and normal pleura (D, arrowhead) em BLM-MSC group.

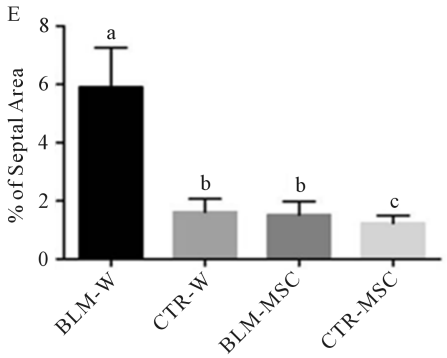

Figure 12. All the results are presented as mean and associated standard errors and different small letters (a-c) denote a significant difference between groups ($p < 0.05$).

4. Conclusion

MSC from adipose tissue is an effective therapy to decrease collagen synthesis and expression in late stage of bleomycin-induced pulmonary fibrosis model, which may contribute to new therapeutic targets.

Acknowledgements

Carlos Roberto Gonçalves Lima for their support in animal care and Cell Engineering Laboratory staff.

Competing Interests

No competing interests.

Authors' Contributions

Renato Gonçalves Felix: pulmonologist founder and conductor of the proposal;
Alexandre Todorovic Fabro: senior thoracic pathologist responsible for the audit and review of histomorphometric analysis;
Josy Campanhã Vicentini-Oliveira: veterinarian who advised and conducted experiments on animals and cell culture;
Ednelson Henrique Bianchi: zootecnist assist the technical procedures with animals;
Marjorie de Assis Golim: expert for flow cytometry;
Ondina Silvia Cotrim: academic support assistant in cell culture procedures;
João Tadeu Ribeiro-Paes: Data analysis and discussion;
Elenice Deffune: coordinator of cell engineering laboratory and mentor project.

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Abbreviations

IPF: Idiopathic pulmonary fibrosis
MSC: Mesenchymal Stem Cells
DMEM: Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
BLM-W: Wistar mice instilled intratracheal bleomycin
CTR-W: Wistar mice Control group (placebo)
CTR-MSC: Wistar mice control group treated with Mesenchymal Stem Cells
BLM-MSC: Wistar mice instilled intratracheal bleomycin and treated with mesenchymal stem cells
ATS/ERS: American Thoracic Society/European Respiratory Society

Stem Cell Discovery, 2016, 6, 39-48
Published Online January 2016 in SciRes. http://www.scirp.org/journal/scd
http://dx.doi.org/10.4236/scd.2016.61004

Stem Cells: Daddy or Chips?

—An Up-to-Date Review on Ground-Breaking Discoveries in Stem Cell Research, with Special Attention to iPSC Applications in Osteoarthritis

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Received 16 September 2015; accepted 18 January 2016; published 21 January 2016

Abstract

"***Stem Cells is what stem cells does***"

not Forrest Gump

In the present day Stem Cells are increasingly becoming popularized as the potential "ultimate" cure for the most challenging maladies… the "Daddy of medical intervention". Forefront SC research on human induced pluripotent stem cells (iPSCs) and other sub-disciplines, is quickly revolutionizing healthcare towards "Regenerative Medicine", as beautifully exemplified by the use of iPSCs in treating and possibly curing osteoarthritis, discussed at the end of this publication. This review documents and reflects on the most topical discoveries in SC research, and the challenges researchers in this field nowadays face. Major Findings: 1) In 2006 Yamanaka *et al.* generated the first iPSCs from *mouse* fibroblasts, using retroviral transmission of c-Myc, Oct3/4, Klf4 and SOX2 transcription factors. Later, they successfully generated iPSCs from *human* fibroblasts (2007). 2) Contemporary cultivation methods carry high risks of iPSC genome disruption, possibly leading to tumorigenesis, teratoma formation and reducing iPSC induction efficacy. 3) Many studies on preserving genome integrity and decreasing malignancy in iPSCs, suggest using valiproic acid and protecting tumour suppressor genes. 4) In many *malignant* tumours only a small minority of cells, called Cancer Stem Cells, metastasise and hyper-proliferate. 5) Not all mature cell sources yield the same [undifferentiated iPSCs: lineage-committed] ratio as others. Feb 2014: Obokata *et al.* claimed to have generated iPSCs by exposing mature cells to a 25 min, pH 5.7 bath. These iPSCs were termed "Stimulus-triggered Acquisition Pluripotency Cells" (STAP). However by July 2014 this study had been revoked, as the results could not be replicated. Conclusion: Stem cells have enormous potential to offer, especially iPSCs. Although currently not a viable treatment option on their own, for many daunting diseases they will definitely be at the core of multi-disciplined therapies within the near-future, including multi-factorial diseases like osteoarthritis.

How to cite this paper: Pace, P.L. and Blundell, R. (2016) Stem Cells: Daddy or Chips? *Stem Cell Discovery*, **6**, 39-48.
http://dx.doi.org/10.4236/scd.2016.61004

Keywords

Cancer Stem Cell, iPSC, Yamanaka, STAP, Arthritis

1. Introduction: What Are Stem Cells?

Stem cells are simply, complicated little things. In the last few years they have been causing many a hullabaloo and a to-do, than ever before. And rightly so.

Recent breakthroughs in stem cell technology have led to a better understanding of how they proliferate, grow, and then differentiate into functional tissues and organs. Yet more astonishingly is how much we've learned regarding how these natural, physiological processes can be conditioned to do the reverse, *i.e.*: to go from a highly specialized cell to a pluripotent stem cell.

Stem cells are every doctor's, and hence every patient's, hope for a far better future of standard care. This is because stem cell technology holds a possible cure for the most daunting and most heart-breaking of diseases, including cancer, auto-immune disease, neurological disorder, diabetes, cardiovascular disease and arthritis, to mention a mere few.

Stem cells differ from other cell types, being defined as:

"Clonogenic, self-renewing progenitor cells that have the ability to divide for an indefinite period and can give rise to one or more differentiated cell types" [1].

The uniqueness behind stem cells lies in **two** main capacities [2] [3]:

1) The capacity to **self-renewal**, *i.e.* proliferating into a line of cells which retain the same degree of potency as the original mother cell.

2) To give rise to a progeny with a more **specialized** function, where potency decreases from one generation to the next, as differentiation increases.

As the progeny from a single stem cell line grows and progresses from one generation to the next via mitosis, the cells of each generation become even more committed to a particular cell line of specialized cells, at the expense of becoming less able to differentiate into other cell types. These "transit amplifier cells" present in the intermediate stages, between the undifferentiated parent cells and the end-line specialized progeny, also exhibit a decreasing ability for self-renewal together with the increasing possibility for differentiation [4].

Selecting the specialty of the mature, end-lineage daughter cell basically depends on the *potency* of the original 1st generation stem cell, as well as which specific chemicals (growth factors, cytokines, hormones etc.) the cell line was exposed to during its journey to differentiation. Moreover some chemical components have to be present only at certain specific times, in order to contribute to specialization of a progeny [3].

This is brilliantly demonstrated by the near-magical complexity responsible for the precise interplay between countless growth factors and cytokines, all necessary for the growth and development of a foetus. And it all starts off from just a single cell. It must be noted that under different ambient conditions, which include culture medium, ECM composition and temperature, various cell types can be derived from each kind of stem cell both *in vitro* and *in vivo* [1].

1.1. Stem Cell Classification

Stem cells can be classified by **Origin**, thus belonging to one of the 3 classical groups:

1) **Embryonic Stem cells (ESCs)**, derived from the embryonic cell pool at any stage of development during gestation.

2) **Umbilical stem cells (USCs)**, sampled from cord blood

3) **Adult stem cells (ASCs)**, or more correctly *mature* stem cells, found in all post-natal humans

Now, recent paradigms are pushing on with the addition of two other, newly-discoveredclasses of origin, namely **Cancer stem cells** and "reprogrammed" adult stem cells or **Induced Pluripotent Stem Cells (iPSCs)**, which shall be discussed further ahead [1].

Stem cells are also classified by their **Degree of Potency**, determined by the number of diverse cell types a single parent cell can potentially give rise to. Different classes of stem cells possess different degrees of this so-called "developmental plasticity" [1]. The highest level of potency lies with the fertilized ovum (zygote),

which is thus called **totipotent**, since not only is iTable to give rise to *any* type of cell, but can develop into a separate, individual, multicellular organism: a foetus. Totipotency also belongs to early embryonic blastomeres, but potency decreases rapidly with each generation of cells. In fact between day 3, when the morula forms, and day 14 (in humans) the blastomeres are termed **pluripotent**. Pluripotent SCs, like totipotent ESCs, can still produce specialized cells from any one of the 3 embryonic germ layers: endoderm, mesoderm and ectoderm.

However a single pluripotent cell on its own cannot give rise to an individual, multi-cellular foetus. Such a wonderful capacity, as head-scratching in origin as the legendary "life spark" that blazed into all life on Earth, *only* belongs to *totipotent* ESCs. Post-morular ESCs and USCs are examples of similarly powerful, pluripotent SCs [1].

Totipotent and many pluripotent cells are also considered biologically immortal, capable of proliferating endlessly if nourished constantly, where the capacity to self-renewal diminishes little. This immortality is thanks to the high activity of telomerase and DNA repair mechanisms which keep the SCs' karyotype healthy and fully intact, throughout their actively reproductive life span [1].

After day 14 many embryonic cells are termed **multipotent**. They have now lost much of the capacity to self-renewal and only differentiate into a limited number of cell lines. For example, Hematopoietic stem cells (HSCs) mainly generate white blood cells, erythrocytes and platelets. Embryonic mesenchymal stem cells (MSCs) give rise to muscle, bone and ligamentous tissue. Although both HSCs and MSCs do quite often demonstrate pluripotency. HSCs and MSCs are present both in embryos and post-natal humans, and hence are termed Adult/mature/somatic Stem cells (ASCs) [1] [5].

ASCs also include the pluripotent-to-multipotent stem cells in the Gastro intestinal mucosal epithelium, skin epidermis, liver stem cells etc. [5].

Other stem cells include cancer stem cells (CSCs), which are the cause of malignant tumorigenesis. It has very recently been reported in numerous studies, that the "majority of cells within a population of cancer cells are non-replicating *i.e.* non-malignant" [1] [6]. It is only the small minority of CSCs that are actively tumorigenic and can colonize a secondary site, hence resulting in the metastatic spread of malignant cancers from one tissue to another.

1.2. The Biochemistry of Stem Cells

Apart from their highly reproductive nature and high telomerase activity, exhibiting varying degrees of potency, stem cells often require various cytokines and growth factors, as well as other specific conditions to promote proliferation of one cell line and not the other [7].

For example, progenitor HSCs give rise to various cell lineages in the bone marrow. However in order to specifically produce erythrocytes (RBCs) as opposed to white blood cells or platelets, HSCs must undergo 3 phases: **1) non-specific proliferation; 2) lineage specific proliferation; 3) maturation**. All 3 processes require interactions with various chemicals [7]:

1) Non-specific proliferation: (see **Figure 1**) HSCs normally remain "dormant" within the G_o phase of the cell cycle. To initiate proliferation, Stem Cell factor is required to (SCF) bind its specific SCF receptor (tyrosine kinase receptor) on the HSC. This ligand-receptor complex undergoes autophosphorylation.

This autophosphorylation then activates the Grb2/Sos adaptor complex and the PI3-kinase pathway, to stimulate the Ras/MAPk pathway. MAPk ultimately activates early response genes, which activate late response genes coding for cyclins and Cyclin-dependent kinases (CDKs). The end-result: HSC proliferation [7].

(Follow **Figure 2**) Lineage non-specific cytokines IL-3, IL-6, IL-9, Granulocyte-Colony Stimulating Factor and Granulocyte Macrophage-CSF, then stimulate the transformation of activated HSCs into **CFU-GEMM**[1] cells. These CFU-GEMMs have decreased self-renewal, yet later give rise to the more differentiated CFU-GM (Granulocyte/Macrophage), CFU-Megakaryocyte, Blast Forming Unit-Erythroid (**BFU-E**) colonies [7].

2) Lineage specific proliferation: Interaction with IL-11 and Insulin-like growth Factor (IGF-1) commits CFU-GEMMs to transform into BFU-Es, which can only generate RBCs. Other specific molecules are required to commit CFU-GEMMs to CFU-Meg or CFU-GM families, as shown in **Figure 2** [7].

3) Maturation: BFU-Es become **CFU-Es**, under control of erythropoietin (EPO), IGF-1, IL-9 and GM-CSF. EPO then stimulates the last steps of CFU-E maturation into fully functional, highly-specialized RBCs, which bear almost none of the self-renewal and potency of the original HSC [7].

[1]Colony Forming Unit—Granulocyte, Erythrocyte, Macrophage, Megakaryocyte.

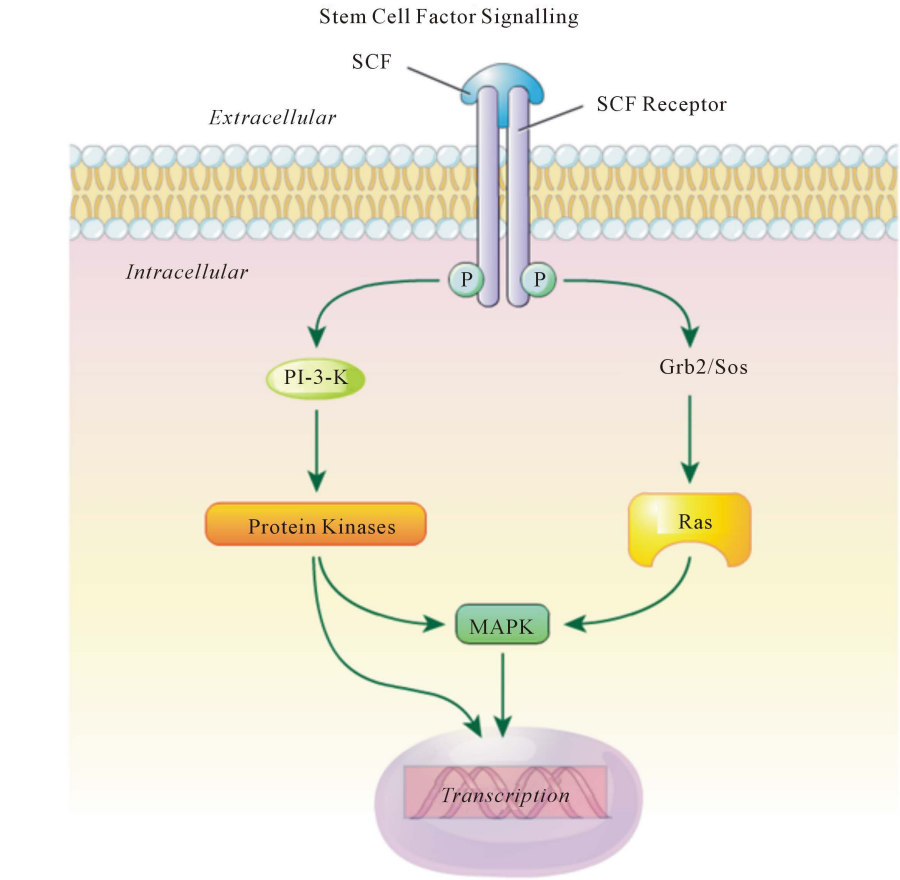

Figure 1. SCF/SCFr mediated chemicalpathway inducing non-specific HSC proliferation [7].

Apart from growth factors Stem cells may require other forms of conditioning. For example, proliferating HSCs must be "nursed" by stromal cells such as fibroblasts, osteoblasts and macrophages etc. which actually supply the necessary cytokines for development [7]. The bone marrow ECM also plays a vital role. Inactive HSCs adhere to the ECM via integrins and selectins expressed on the cell surface, which then detach to set the activated HSCs free to expand and differentiate.

In conclusion, understanding the interactions of SCs with various growth factors, ECM modulation and the use of helper-nurse cells where required, is thus clearly a major priority for developing novel Stem cell therapies, whatever the disease.

2. Before We Had iPSCs

In 1998, Thomson et al had become the first to extract pluripotent ESCs from human blastocysts, yet this had required the consequent termination of many human embryos [8] [9]. Klimanskaya (2006) however managed to extract human pluripotent ESCs from the Inner Cell Mass, without causing any significant harm to the embryo [10]. This thus may have proven a better alternative, as regards alleviating the many obvious ethical controversies in Thomson's and others' methods. Nevertheless, Klimanskaya's method is still a very invasive procedure carrying a number of risks. Hence it is certainly not the ideal way of extracting pluripotent human stem cells [1].

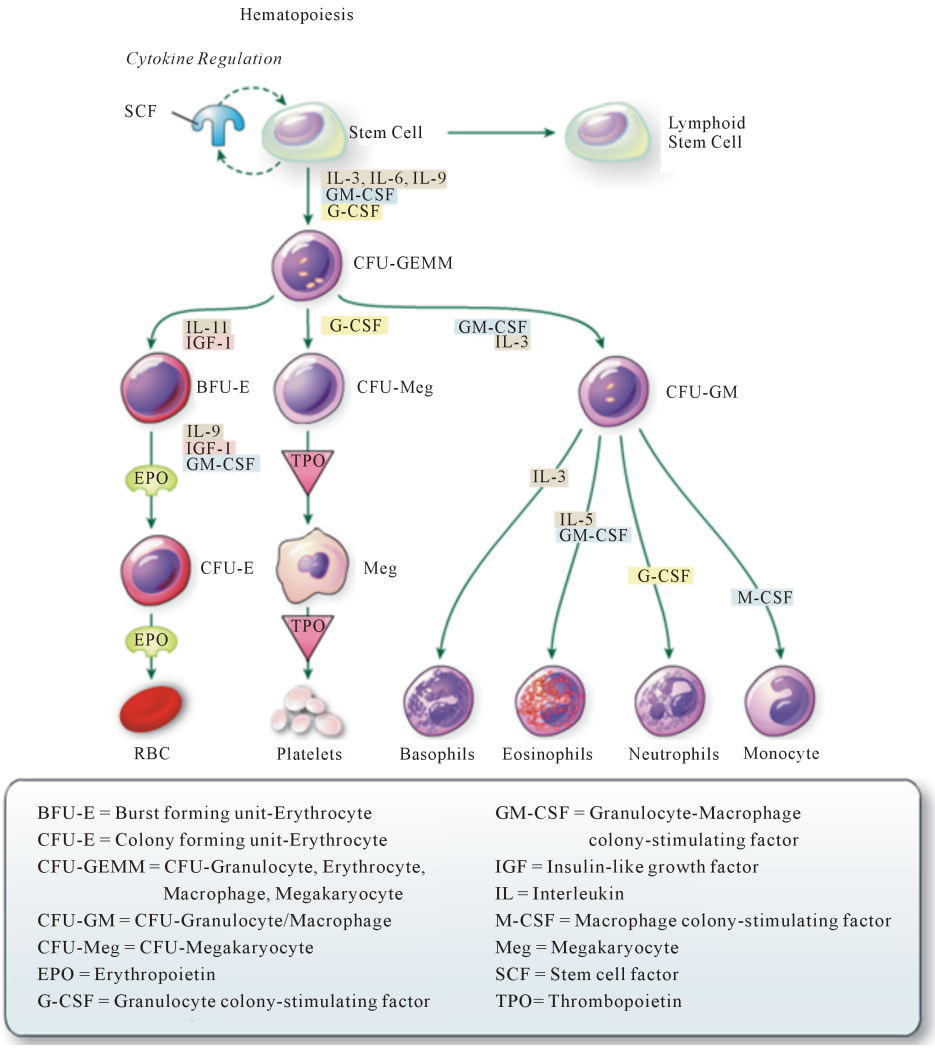

Figure 2. Haematopoietic pathway as controlled by interactions with lineage specific and non-lineage specific factors [7].

As regards USCs, harvesting umbilical cord blood simply requires a needle and a specially designed bag for collection. They can then be concentrated and stored in liquid nitrogen and still remain stable for years. When needed they are then "thawed out" [1]. The main advantage of USCs is that they do not present with the moral headaches tied with ESCs, as one is actually using a rapidly available stem cell source that does not put the donor's life at risk. In fact, as is well-known, the umbilical cord is usually discarded post-partum without a second thought.

Moreover, as regards patients requiring bone marrow transplants, USC transplants to HLA-matched siblings [11], relatives [12], as well as non HLA-matched recipients [13], markedly demonstrate superiorhost acceptance and reduced graft-versus-host disease, compared to conventional bone marrow transplants in similar donor-recipient scenarios. This hints that pluripotent USCs have a stronger immunological naiveté than the collectively less potent bone marrow cells and probably other lineages of similarly inferior potency, even when USCs are donated from one person to another who is not fully HLA-matched [14]. Could this mean that with finer tuning in USC transplantation, even the headaches of cautiously administering immuno-suppressive therapy in post-transplant patients, may one day become nothing more than a fading memory?

Despite these very plausible hypotheses, the current applications of USCs in treating disease are still limited. Although USCs may even at times match the pluripotency of ESCs, USCs do not always produce all cell types effectively. Rather, they frequently differentiate into the more-multipotent-than-pluripotent HSCs and MSCs,

which generate quite a limited number of specialized cells in total [1]. Furthermore cord blood samples have a fixed, small volume. "Cord blood" grafts yield only a mean total nucleated cell dosage (nucleated cells/kg of patient's mass) of less than circa 1/10th of the average bone marrow graft, making graft-host integration much slower than conventional transplants [15].

Therefore despite successful use in even curing haematological disease, including Fanconi's anaemia and breast cancer, USCs are not sufficiently effective in regenerating complex tissues like hyaline cartilage for example [1]. Therefore having said that, USCs are currently an unlikely treatment option for arthritis, a family of diseases marked by cartilage degeneration in synovial joints.

3. iPSCs: Their Discovery and the Possibilities they Promise

The issues currently causing greatest excitement in the world of stem cell research regard induced Pluripotent Stem Cells (iPSCs) [8]. In 2006, Dr Shinya Yamanaka and Takahashi became the first to stimulate iPSC formation *in vitro*, by using **retroviral transduction** of the 4 transcription factors c-Myc, Oct3/4, Klf 4 and SOX2 into mature rodent fibroblasts.

In vitro, these "reprogrammed" cells formed ESC-like aggregates, and when injected into mice they generated teratomas *in vivo*. Cells from all 3 embryonic layers were produced in either case, which provided strong evidence of the high degree of potency belonging to these iPSCs (refer to **Figure 3**).

Yamanaka then established *human* iPSCs in 2007 using the same 4 transcription factors, whilst in the same time period Thomson succeeded similarly with Oct3/4, Nanog, Lin28 and SOX2 [8]. Thus far human iPSCs have been derived from keratinocytes, skin fibroblasts and blood cells to mention a few. It is clear what potential iPSCs have to offer in treating ailments like myocardial infarcts, arthritis and neurological disease.

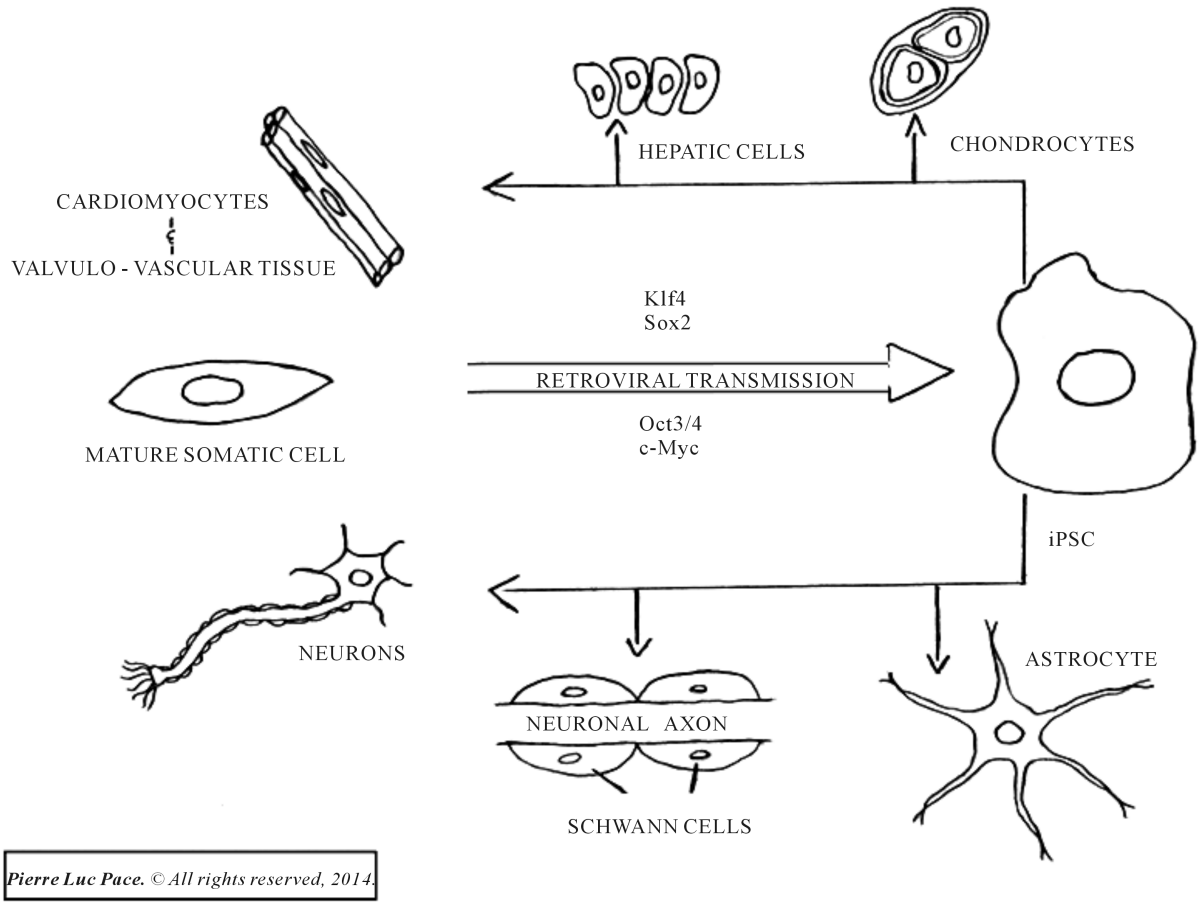

Figure 3. Illustrating the generation of induced Pluripotent Stem cells (iPSCs) from somatic cells by *in vitro* transfection of the 4 Transcription Factors Oct3/4, SOX2, Klf 4 and c-Myc. iPSCs are then able to differentiate into various lineages.

"Now one needn't worry much about finding HLA-matched donors, as required in bone marrow transplants or heterologous USC infusions. Neither do we need trouble with the ethical concerns of having to use human ESCs. They are no longer the only powerful pluripotent cell. Autologous iPSCs can now be made from virtually any mature cell from one's own body. Moreover, each patient would be his/her own SC donor, thus avoiding tedious HLA matching and immunological rejection reactions." [8]

However despite the "miracle" of iPSCs, Yamanaka himself admits that retroviral transgene invasion can disrupt the host cell genome, although much of transcription factor activity is silenced during reprogramming [8].

Some residual transcription factor hyperactivity may still persist: If oncogenic c-Myc remains expressed *after* induction of a pluripotent state, it can cause tumorigenesis in transplanted iPSC-derived cells. Okita *et al.* also reported tumours caused by c-Myc reactivation, together with a low reprogramming success rate of somatic cells into iPSCs [16]. Other studies reported epigenetic changes, which coupled with particular genomic errors, may even cause serious teratoma formation [17].

Reducing the risk of tumorigenesis and increasing induction efficiency, are challenges crucial to making iPSC therapy truly effective *and* safe [8]. Numerous approaches to preserving iPSC genome integrity are being investigated, including growth factors and chemicals like valiproic acid (histone deacetylase inhibitor). Suppression of TSGs like p53 may improve the induction efficiency, however may increase risk of tumorigenesis. One must also note that not all mature cells yield iPSCs equivalently. Miura *et al.* (2009) attempted neural regeneration in mice [8]. They found that with iPSCs derived from tail-tip fibroblasts, the secondary neurospheres had significantly yielded more *undifferentiated* iPSCs than mouse embryonic fibroblasts. Hence different tissues in humans may also yield various ratios of [Undifferentiated: Lineage-committed] iPSCs.

Yet overall the most unbelievable of all approaches to iPSC generation, is that of February 2014 when Haruko Obokata *et al.* claimed to have simply exposed differentiated T-cells to an acid bath of pH 5.7 for circa 25 mins, and iPSCs were produced [18].

This simple technique is termed 'Stimulus-triggered Acquisition of Pluripotency Cells' (STAP cells) "which requires neither nuclear transfer nor the introduction of transcription factors" [18]. The basis is that under certain forms of stress (Ex: physical stricture, toxoid exposure or mildly low pH), specialized cells can revert back to a pluripotent progenitor state.

However, just recently new doubts cropped up amongst researchers including Obokata herself, as attempts to replicate her results with the same method proved unsuccessful. Thoughts of re-writing the paper with more accurate results and photographs were to be implemented in March 2014 [19]. Yet, by July 2014 most STAP papers published by Obokata were revoked [20].

Despite this very short-lived paradigm shift and the disappointing, even sorrowful events that followed[2], further investigation into the matter is still of the utmost importance. A series of slip-ups should not falter our hope in digging deeper into whether STAP cells are indeed a reality waiting to be discovered, even if under a completely different set of laboratory conditions. It is in this reviewer's opinion: "Let's not be stupid by hesitating to try and try again".

4. iPSCs in Osteoarthritis: Direct SC Therapy & Our Tools for Further Study

Osteoarthritis (OA) is one of the most common musculoskeletal problems causing pain, disability, and a significant economic burden on the patient, his/her family, and society itself for that matter. As life expectancy increases in today's ageing population, the prevalence of OA will also increase. Increasing the number of highly invasive and highly expensive, joint replacement surgeries per year for end-stage, severe OA is not an option. Economic pressure on patients and government funds would have to rise disproportionately, since relatively little can be done to resolve these patients' disabling predicament [21].

Therefore the quest for more effective, less invasive treatment has become ever more crucial.

There is already growing evidence that bone marrow and adipose-derived Mesenchymal Stem Cells, aka bm-MSCs and ADSCs respectively, have a great role to play in cartilage repair stratagems, against the chondro-degenerative pathogenesis of OA. However, they do show decreased differentiation potential in elderly and/or obese individuals [22], which are the main groups vulnerable to OA, and which make the majority of OA cases. Yet if the autologous source is a problem, **allogeneic** MSC sources may sometimes be a safe alternative [23].

However, an autologous source is always optimal, and at times vital. IPSCs permit efficient **autologous** tis-

[2]Tragically, one of the scientists involved in Obokata'sSTAP team, YoshikiSasai, committedsuicide [20].

sue-engineering even for elderly and/or obese OA patients [22]. Moreover they are relatively easy to obtain since even fibroblasts from skin scrapings may be stimulated pluripotentally.

Still, the persistent challenge is to commit all the iPSCs in a single culture toward effective chondrogenesis, without resulting in teratoma formation [22]. As had been done by others with human embryonic SCs, Diekman et al initiated chondrogenesis in a simple fashion by short-term exposure of rodent iPSCs to BMP-4 (bone morphogenetic protein 4). About **10%** of the cells expressed hyaline-specific, Collagen Type II (Col2 gene) and aggrecan (Acan). These were marked by green fluorescent protein expression (GFP+ cells).

The small GFP+ population was then "**purified**" from GFP- cells by separating the two populations through flow cytometry.

After expansion both groups were cultured with six passages of TGF-β3. The successfully differentiated GFP+ culture yielded **larger** cartilage pellets, with a **higher concentration** of GAGs and hyaline type II collagen. Purification of GFP+ cells also helped *eliminate* unspecialized cells that could form teratomas, *only allowing chondrogenic cells* to develop [22].

However after passage 3, GFP+ cells became *de-differentiated* due to excessive expansion. In fact pellets became less homogenous and hyaline-specific since type II collagen decreased, whilst synthesis of ***fibrocartilage*** marker, collagen I increased.

In conclusion passage 2 GFP+ cells offered the best results, synthesizing cartilage similar to ***immature*** (almost embryonic) cartilaginous tissue, since collagen VI seemed scattered throughout the matrix, rather than peripherally zoned as in mature tissue. What is remarkable is that GFP+ cartilage had a **stronger elastic modulus** at the periphery than the centre, thus strongly resembling **native** hyaline cartilage which has similar zonal variations in mechanical function. Moreover when introduced into femoral pig FCLs, the iPSC derived cells showed good defect filling and integration with native cartilage [22].

If not directly used for in-vivo reconstruction of FCLs and OA, iPSC-engineered cartilage may be used as an important model for conducting new pathophysiological or pharmacological research on defect repair. This is an advantage over using animal models, which allow a limited number of tests each [22]. In fact, through in-vitro studies on the effects of chondrogenic factors on MSCs, the molecule Kartogenin was recently discovered. It promotes chondrocyte differentiation *and* chondroprotective pathways. When tested as a pharmaceutical, Kartogenin proved "efficacious in two OA animal models" [24].

Furthermore, using iPSC expansion, samples from animals or humans with increased genetic susceptibility or resistance to OA may be replicated multiple times for any number of desired studies.

So far iPSCs are painting a bright future for OA patients. However there is more to OA than one may want to imagine. As is so with many multi-factorial diseases, several studies emphasize OA as being at least partially heritable. Various twin and family studies estimate that OA has a 40% - 65% genetic factor [25]. iPSCs and MSC therapies alone may not be enough to cure OA completely. Gene modification therapy is clearly needed, and ideally should be tailor-made to the individual patient's particular alleles.

Combined therapy is probably the key. More accurate, cost-effective diagnosis of early cartilage lesions, and a standardized treatment protocol, involving (1) Intra-articular Platelet-Rich Plasma Injections (PRPIs), Autologous Conditioned Serum (ACS) or Autologous Protein Solution (APS) with (2) Stem Cells, ex: autologous iPSCs and MSCs, together with (3) Gene therapy [21]. That's where we are headed, although there's no clear-cut yellow, brick road to get there. But hopefully this gold-standard protocol might eventually one day, blow the need for tedious joint replacement to Betsy.

5. Conclusions

After this very brief overview of the latest advancements in Stem Cell research, it may be now easier to paint a clearer, more hopeful, yet more realistic picture of the future of Modern Medicine. Most tissues lack the ability to regenerate, be it the precious cartilage lining our arthritic joints, the transmural infarcts in our fragile hearts or the deep-seated glial scars in our brains.

And this is the promise of Stem Cells: that degenerative conditions like osteoarthritis, cardiovascular and neurological disease, assaulting our most vulnerable structures, may be forgotten… for good [1]. This may well mark one large step forward in the journey of modern medicine. And the best part, for many, is that thanks to Yamanaka and numerous other research teams, the vast potential of Pluripotent SCs can be exploited without sacrificing our precious moral standings, serving as the very bedrock for the beliefs of millions of people.

The question yet still remains: "Are SCs Daddy or chips?"

It is easy to imagine stem cells as the "Daddy of all cures and medical interventions". Still, we know so little about the true aetiologies and pathogeneses of multi-factorial diseases like cardiovascular disease, that saying that stem cells alone can replace conventional treatment is illogical. For now stem cells are more like the "chips" (fries) next to your quarter-pounder and salad; a side dish that can complement wonderfully to the whole meal, but still not the focus of the main course.

However the future is definitely looking brighter. It is impossible to imagine stem cells side-lined out of either standard care or complex treatment. It is in this reviewer's opinion that to combat multi-factorial disease, one needs multi-disciplined treatment. Stem cells would definitely be at the core of *combination therapy*, for example working together with gene therapy and surgical interventions, as is being researched regarding the treatment of OA. They simply won't be working alone.

"*Chips for now… but not for too long mind you lad*"

Acknowledgements

My sincerest gratitude goes to Dr Renald Blundell for being my mentor and for providing sound guidance throughout this project.

Further regards go to Dr Pierre Schembri-Wismayer and Dr Isabelle Borg for their very helpful advice.

I would also like to thank Dr Christopher Vidal, a leading researcher in genetic studies of the Maltese population, for giving me support in this field despite being retired from Medical Science.

Best thanks must also go to Stephanie Searle and Prof Israels for helping me obtain legal permission to use Figures 5-1 and 5-2 from "*Mechanisms in Hematology*", as **Figure 1** and **Figure 2** in this review. Both of these figures were reproduced with permission from Core Health Services Inc. © Core Health Services Inc. All rights reserved.

Above all I am grateful for my kind family for whom the title was inspired. My parents, Jacqueline and Edmund Pace for their love and scientific wit, as well as my sisters Nicole, Raquel and Gyllain who teach me so much, often without them realizing it.

For all the mentioned above and everything else, I thank God for His endless love despite my weaknesses, and for putting me on the right track in spite of my many detours.

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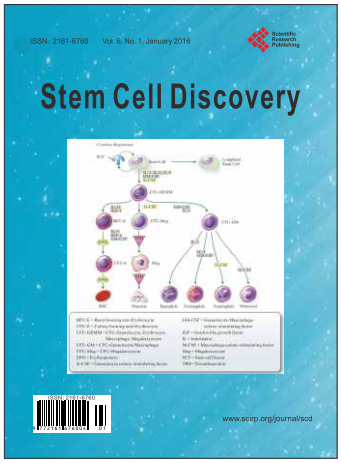

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