Ann Natl Acad Med Sci (India), **51(3):**105-124, 2015

Biological Basis and Molecular Mechanism of Regeneration

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ABSTRACT

Stem cells are responsible for regenerating the lost and damaged cells and tissue. Because of this known fact, regenerative medicine has emerged as a promising field to explore. As a result of exhaustive series of research, in 2012, Shinya Yamanaka and John B Gurdon were awarded the Nobel Prize for their remarkable contribution in the field of Physiology and Medicine. Stem Cell Facility (SCF) at AIIMS was established in 2005 and was the first centre in North India to start stem cell trial. Department of Biotechnology has granted Centre of Excellence in stem cell research to SCF, AIIMS to carry out basic, pre- clinical and clinical research. The main purpose of exploring all these various aspects of research is to study the molecular biology involved in the process of stem cell differentiation, mode of action of stem cells and their pre-clinical and clinical implications in terms of homing of stem cells at the site of damage, differentiation into desired cells and interaction with the tissue *in vivo* and finally, in disease prognosis

Keywords: Regeneration, Stem Cells, Regenerative Medicine, Embryonic Stem Cells, Adult Stem Cells.

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INTRODUCTION

What is Regeneration?

Regeneration is the process of renewal, restoration, and growth that makes genomes, cells, organs, organisms and ecosystems resilient to natural fluctuations or events that cause disturbance or damage. The regeneration of lost body parts and injured organs has captured the human imagination since the time of the ancient Greeks. Every living organism from bacteria to human has the capability to regenerate (Fig. 1). Regeneration can either be complete (where new tissue is same as the lost tissue) or incomplete (where after the necrotic tissue comes the fibrosis) (1, 2).

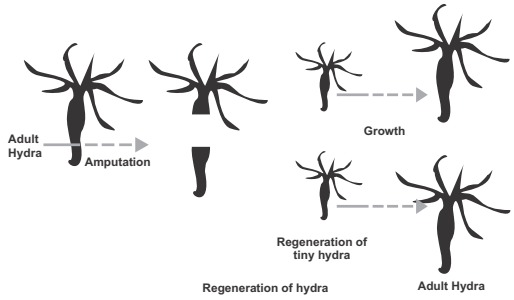

Fig 1: Process of regeneration in Hydra (http://www.tutorvista.com/content/biology/biology-iv/growth-regeneration ageing/regeneration.php)

Stem Cells and types:

In multicellular organisms, stem cells play a very important role in the tissue regeneration *in vivo*. These are defined as the biological cells, found in multi- cellular organisms, which are capable of both self renewal and differentiating into various diversified cell types. There are various types of stem cells present in the human body. Embryonic stem cells (ESC) are the pluripotent cells, obtained from the inner cell mass of blastocyst stage of embryo. Adult stem cells (ASC) are the multipotent cells, present at the local niche of every tissue. ASC can be obtained from tissues like bone marrow, umbilical cord, umbilical cord blood, adipose tissue, dental pulp, skin, hair follicle, parotid glands, etc. (Fig. 2) There are several pros and cons of these stem cell sources.

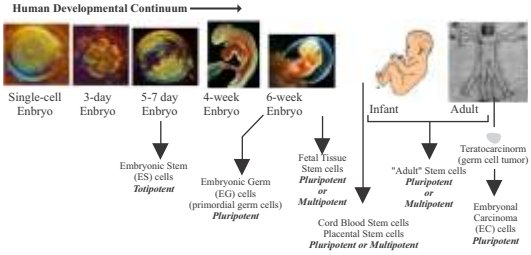

Fig 2: Different sources to obtain Stem Cells of varied potencies (cyhsanatomy1.wikispaces.com)

Ethical Issues and other issues:

Although ESCs are pluripotent and can be used to study the embryological development processes, yet they have limitations in not being immunologically naive and having ethical issues as well. Moreover, it is very cumbersome to isolate culture and maintain ESCs. These issues can be overcome with ASCs but they lack in their potency to differentiate into cells of any lineage. Moreover, ASCs have the trans-differentiation potential to differentiate cells of other lineages also. As they are

immunologically naive, these cells are good for translational purposes.

Mode of Action of Stem Cells:

On transplantation *in vivo*, stem cells interact with the native tissue via various modes of action like differentiation, fusion, paracrine factors (immunomodulation, releasing angiogenic factors, anti- apoptotic factors and anti- oxidative factors) and by releasing microvesicles and miRNA (3). Differentiation of stem cells require various factors like FGF2, FGF8, SHH, BDNF, TGFβ, etc. depending upon the cell lineage into which they are to be differentiated. This fact has been fully explored by various research groups of the world. These growth factors are being used *in vitro* to study the differentiation pattern of stem cells into cells of different types. *In vitro*, stem cells can be differentiated by the process of reprogramming also (4, 5). Cell fusion is a process that has an important biological role in the development, physiology and disease of multicellular organisms. For example, we have the zygote formation and organogenesis of various tissues, such as placenta, bone and skeletal muscle. Cell fusion is triggered by inflammation. It is important to note that lipid bilayer membranes do not spontaneously fuse, and that fusion between membranes involves a highly intricate choreography of lipids and proteins (6).

Apart from these, stem cells mend the injury by paracrine and endocrine effects, i.e., by immunomodulation by regulating macrophages, dendritic cells, B and T lymphocytes, NK cells, etc. (7), angiogenic factors like vascular endothelial growth factor (VEGF), stromal cell derived factor- 1 (SDF1), fibroblast growth factor- 2 (FGF2), etc. these factors function mainly by CXCR4 pathway (8). Anti- apoptotic factors involved in this procedure are brain derived growth factor (BDNF), hepatocyte growth factor (HGF), insulin growth factor (IGF), etc. involved in the interaction of stem cells in *in vivo* conditions (9). Besides making inflammatory response, stem cells interact by releasing anti- oxidative factors/ mediators like IGF, platelet derived growth factor (PDGF), superoxide dismutase (SOD), HGF and IL-6 (10). Despite the bioactive molecules secreted by MSC, Bruno et al have showed that inside the conditioned medium also there are some micro vesicles. These micro vesicles (MVs) are circular membranes fragments that shed from the cell surface membrane carrying protein and lipids from the membranes of the cells from which they originate. It has been shown that stem cells are able to shed off these MVs and they contain mRNA and micro RNA for the amelioration of injured site (11) (Fig. 3).

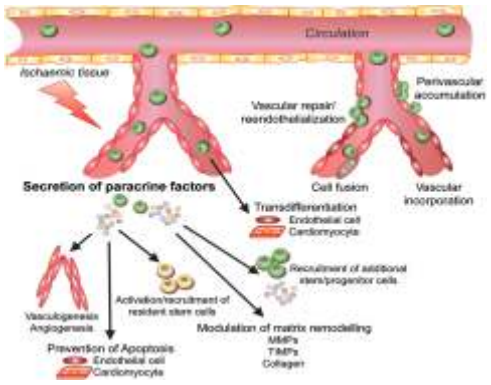

Fig. 3: Proposed mechanisms of ischaemic tissue repair via stem and progenitor cell- based therapies (Ref: European Heart Journal, 2011: doi:10.1093/eurheartj/ehr018)

Significance of mitochondria in Repair and Regeneration of damaged tissue:

Studies have shown the role of mitochondria in maintenance of pluripotency, differentiation and reprogramming of iPSCs. While glycolytic energy is observed at pluripotent state, mitochondrial oxidative phosphorylation (mtOXPHOS) is necessary for cell differentiation. It is hypothesized that reprogramming of somatic cells towards a pluripotent state, by somatic cell nuclear transfer (SCNT), transcription-induced pluripotency or creation of pluripotent cell hybrids, requires acquisition of mitochondrial properties characteristic of pluripotent blastomeres and ESCs. In a recent study, it was reported that cells and tissues created through nuclear transfer can be rejected by the body because of an immune response to the cell's mitochondria. In addition, differentiated stem cells and pluripotent stem cells differ in mtDNA copy number, ATP production, mtOXPHOS and total cell (12). Mitochondria are also known to mediate stress-induced apoptosis in embryonic stem cells (13). However, the mechanisms and exact associations of mitochondrial activities associated with stemness and cell differentiation remains elusive. In addition, there is evidence that suggest that MSCs can rejuvenate damages cells by mitochondrial transfer (Fig. 4). Miro1/Rho1 mitochondrial transport proteins positively regulate the transfer of mitochondria from MSCs to the damages cells with dysfunctional mitochondria (14, 15). It has been shown that Miro1 over expression can lead to enhanced therapeutic efficacy and reversed inflammation by releasing anti-inflammatory products (16). The presence of oxidative stress and imbalance in potassium efflux and membrane potential is associated with inflammatory responses (17, 18). The role of mitochondria in immunomodulatory function of MSCs remains widely unknown.

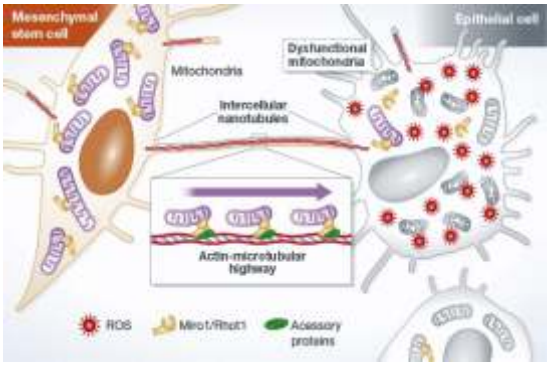

Fig. 4: Mesenchymal Stem Cells (MSC) act as mitochondrial donors during attenuation of lung inflammation and injury. Mitochondrial donation is an essential part of the MSC therapeutic effect in these models and is positively regulated by Miro1 / Rhot1 mitochondrial transport proteins. (The EMBO Journal. 2014;2;33(9):994-1010)

Role of Exosomes secreted by Mesenchymal Stem Cells in Tissue Repair & Regeneration:

Exosomes are small membrane vesicles (between 30 and 100 nm in diameter) of endocytotic origin that are secreted by most cells in culture. They seem to form by invagination and budding from the limiting membrane of late endosomes, resulting in vesicles limited by a lipid bilayer containing cytosol from the producing cells. Fusion of the exosomes with the plasma membrane of the recipient cell, allows transfer of the internal components to the target cell and thus, the transfer of information (Fig. 5). It has been long understood that MSCs can lead to repair of damaged tissue by differentiating to the cell type, for example to cardiomyocyte in case of myocardial infarction. However, several studies suggest that contrary to the previously known belief, new hypothesis secretion of complex paracrine factors for tissue repair and regeneration (19, 20). Several growth factors including vascular endothelial growth factors (VEGF) and basic fibroblast growth factor (bFGF) (21).

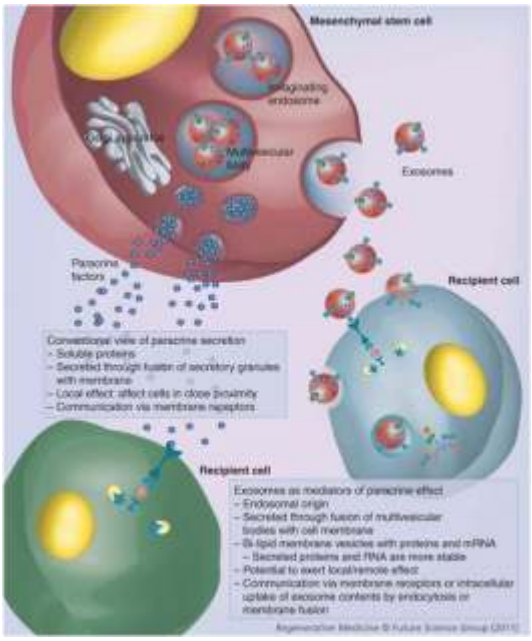

Fig. 5: Proposed mode of action of exosomes released by Mesenchymal Stem Cells (beyondthedish.wordpress.com)

Stem Cells in regenerative medicine:

Regenerative medicine is the "process of replacing or regenerating human cells, tissues or organs to restore or establish normal function". Stem cells play a very indispensable role here. Now a days, scientists all over the world are trying to prepare biocompatible scaffolds that support the growth and differentiation of stem cells and can also act as a mode of transport of these stem cells at the localised site. Various research groups all over the world have reported scaffolds that are differentially compatible to neurons, cardiomyocytes, osteocytes, chondrocytes, etc. Generation of disease specific Induced Pluripotent Stem Cells (iPSC) represents a clear breakthrough in regenerative medicine. iPSCs were first developed in mouse cells in 2006, followed by development of human iPSCs in 2007 by the group headed by Prof. Shinya Yamanaka at Kyoto University. Four important genes involved in the development of iPSCs are Oct3/4, c-myc, Sox2 and Klf-4 (22). Hence, it may be inferred from this point that regenerative medicine holds a potential scope in cell based therapy for the most dreadful

degenerative diseases.

At the Stem Cell Facility, AIIMS, we are carrying on research at all the three stages, i.e., basic, pre- clinical and clinical. Basic research is going on in the field of differentiation of mesenchymal stem cells from various sources like bone marrow, umbilical cord, umbilical cord blood, skin, hair follicle, etc. into neuronal cells, cardiomyocytes, chondrocytes, osteocytes and adipocytes, etc. The pre-clinical research includes the transplantation of cell- loaded biocompatible scaffolds into the rat model to study the bone formation (unpublished data). We have also studied the effect of transplantation of mononuclear cells in the brain infarction, nerve injury and spinal cord injury in rat model having damaged sciatic nerve. Under the clinical research, patient trials for myocardial infarction, vitiligo, stroke, corneal defects, etc. are going on. Limbal stem cells have been established to be an effective therapy in case of eye disorders.

In the current write up, the process of regeneration and role of stem cells in the process of regeneration will be elaborated. Work done and the research going on in AIIMS, with their future prospective will also be discussed.

Materials and Methods:

All the studies were initiated after the approval from Institute Ethics Committee (IEC) & Institute Committee-Stem Cell Research and Therapy (IC-SCRT).

1. Clinical Research:

With the bone marrow mononuclear (MNC) cells various clinical trials are going on at various levels. We have three multi-centric trials in collaboration with various multispecialty tertiary hospitals in India. Various double blinded randomized controlled trial studies are also going on. Stem cell clinical trials in AIIMS are going on for dilated cardiomyopathy, coronary artery disease, paediatric diseases, limb ischemia, macular hole, stroke and spinal cord injury.

Apart from these Bone Marrow MNC involving Stem Cell Trials we have been able to translate the bench side research into translational research with significant success in the following instances:

1. 1 Stem Cell Clinical Trials in Heart Diseases:

a. Application of Stem Cell Technology for Dilated Cardiomyopathy:

The ABCD (Autologous Bone Marrow Cells in Dilated Cardiomyopathy) Trial was the first clinical trial involving stem cells in Dilated Cardiomyopathy in India. The study included patients between 15 and 70 years of age with idiopathic dilated Cardiomyopathy with normal coronary arteries, an ejection fraction (EF) of< 40%, and no other severe co morbidities (e.g., chronic renal failure). The study design was an open-label, randomized

trial in which 85 patients were enrolled (23).

b. Application of Stem Cell Technology for Coronary Artery Disease:

Although the myocytes that are lost during Myocardial Infarction (MI) cannot be regenerated, a small population of muscle cells in the region of viable myocardium may replicate and prevent heart failure. At our institute, 43 patients underwent combined CABG (coronary artery bypass grafting) and stem cell transplantation between February 2003 and October 2006. Their mean age was 51.6 ± 6.5 years (range, 42- 62 years). Eighty-six percent had a documented previous MI, 43% had hypertension, 36% were chronic smokers, and 14% had diabetes. The basal New York Heart Association class ranged from 2 to 4 (mean, 2.9 ± 0.7). All patients had akinetic areas with 86% of the akinetic areas occurring in the anterior wall. The basal LV ejection fraction was 33% ± 16%. These patients received 2 to 4 grafts (mean, 2.8 ± 0.6). Additional procedures were Dor's procedure (n = 2), LV clot removal (n = 1), and post-MI ventricular septal defect closure (n = 1) (24).

1.2 Stem Cell Clinical Trials in Pediatric Diseases:

Stem Cell Clinical trial was done in challenges faced in the management of bilateral multicystic kidney disease (MCKD). The tibial bone marrow tap was dry. Repeated blood transfusions and erythropoietin injections were given. At the age of 1 year, the bone marrow tap was attempted again. Ten milliliters of bone marrow was collected under local anesthesia, from the patient's tibia (autologous). Autologous bone marrow mononuclear stem cells were injected into the aorta at the level of the renal arteries, applying pressure over the aorta below the renal arteries during injection, to direct the stem cells into the renal arteries. Repeated renal biopsies at the time of stem cell transplant were taken (25).

1.3 Stem Cell Clinical Trials in corneal defects:

a. Limbal stem cell transplantation:

Stem Cell clinical trial has been done with cultivated epithelium was transplanted in patients with total or partial limbal stem cell deficiency. Limbal tissue specimens were from cadaveric corneo- scleral rims, live related donors or contra lateral eye of the patients. Harvested tissue was cultured on denuded human amniotic membrane (dHAM) using various techniques to stabilize the dHAM (26).

b. Assessment of Central Retinal Function after Autologous Bone Marrow Derived Intravitreal Stem Cells Injection in Patients with Retinitis Pigmentosa using Multifocal ERG :

Patients with RP with visual acuity (VA) =1.90 were included. All patients underwent mf-ERG testing (61 hexagons) prior to intravitreal stem cells injection. Mf-ERG was repeated at end of 1st month,

3rd month and 6th month post injection. First order kernel mf-ERGs were analyzed (amplitude and implicit time of n1 and p1) (27).

1.4 Hair Follicle Outer Root Sheath Cells in the Treatment of Vitiligo:

We have also undertaken the first of its kind in the world- translational research by using the extracted non cultured hair follicle outer root sheath cell suspension (EHF-ORS-CS) in the treatment of vitiligo (28).

2. Pre- clinical Research:

2.1 Effect of bone marrow-derived mononuclear cells on nerve regeneration in the transection model of the rat sciatic nerve:

Materials and Methods:

Bone marrow from 24 adult male Wistar rats was aspirated and MNCs were isolated by Ficoll- Paque gradient separation method. For transplantation, 4 million to 8 million BM-MNCs were suspended per millilitre of normal saline. MNCs were transplanted between the approximated ends of the sciatic nerve. In the control group, the transected nerve ends were repaired with two epineural microsutures using 10-0 monofilament nylon and fibrin sealant only. On day 30 and day 60, Phases I and II respectively, the right sciatic nerve was re-explored and transected and the distal end was labelled with a thread. It was fixed in 3% gluteraldehyde. Nerve segments were processed for histopathological analyses for neurofilament, CD34, S100 and leukocyte common antigen markers (29).

2.2 Dose-dependent facilitation of peripheral nerve regeneration by bone marrow–derived mononuclear cells: a randomized controlled study:

Materials and Methods:

The right sciatic nerve of 60 adult female Wistar rats (randomized into 2 test groups and 1 control group, 20 rats in each group) underwent transection under an operating microscope. The cut ends of the nerve were approximated using 2 epineural microsutures. The gap was filled with low-dose (5 million BM-MNCs/100 ml phosphate-buffered saline [PBS]) rat BM-MNCs in one group, high-dose (10 million BM-MNCs/100 ml PBS) rat BM-MNCs in another group, and only PBS in the control group, and the approximated nerve ends were sealed using fibrin glue. Histological assessment was performed after 30 days by using semi quantitative and morphometric analyses and was done to assess axonal regeneration, percentage of myelinated fibres, axonal diameter, fiber diameter, and myelin thickness at distal-most sites (10 mm from site of repair), intermediate distal sites (5 mm distal to the repair site), and site of repair (30).

2.3 MSC differentiation onto 3D biocomposite scaffolds and transplantation in rat model:

Materials and Methods:

3D biocomposite scaffolds of chitosan by freeze drying method and the bone formation was studied in three study groups, i.e. only scaffold, scaffold+ undifferentiated MSCs and scaffold+ osteoinduced MSCs, when transplanted in the subcutaneous pockets in Wistar rats. Except the only scaffold's group, other two groups were seeded with $1X10^5$ cells/20µL of solution were seeded and finally, cells were induced for osteocytes. On the completion of 14 days of incubation, scaffolds were transplanted in the subcutaneous pockets in Wistar rats. X-ray and histological studies were carried out at 2nd, 4th, 6th, 8th and 12th week of transplantation of scaffolds. All surgical procedures were performed under aseptic conditions using dual access animal handling station [Unpublished data].

3. Basic Research:

3.1 Differentiation of bone marrow stem cells into cells of neuronal lineage:

Materials and Methods:

Bone marrow from 5 patients (Age range- 13-58 yrs) was aspirated and isolated by plating bone marrow directly onto the culture vessel and expanded in LG-DMEM supplemented with 10% FBS at 37ºC/5%CO_2. Cells were characterized by the presence of various surface proteins (CD105, CD90, CD29, CD73, HLA I & II and CD 34/45) using flow cytometry. Cells of 3rd passage were used for the experimental purpose. Different induction cocktails were used to differentiate BMSCs into neuronal cells. 12 days differentiation protocol was followed for four different differentiation media (NIM1: FGF2, NIM2: FGF8+ SHH, NIM3: ATRA, NIM4: SHH+FGF8+ATRA) with neurobasal media, B27 supplement, EGF, L-glutamine and antibiotics in common. The results were evaluated on the basis of RT-PCR, q-PCR, immunoflorescence and flow cytometric analysis of neuron specific markers NF, NSE, β- tubullin III, astrocytic marker GFAP, etc. Dopamine level secreted in the media by the DA cells was quantified by ELISA. The functionality assay of the neurons was done by calcium ion signaling, using confocal microscopy (31).

3.2 Differentiation of bone marrow stem cells into cardiomyocytes:

Materials and Methods:

BM-MSCs from bone marrow were isolated by direct plating and expanded in LG-DMEM supplemented with 10% FBS at 37ºC/5%CO_2. All the experiments were performed using 3rd passage cells after their characterisation. They were checked for the presence of vimentin and Fibroblast Specific Protein (FSP) (Immuno fluorescence (IF) assay); Surface markers -CD105, CD90, CD29, CD73, HLA I & II and CD 34/45 by flowcytometry. After characterization, the cells were differentiated *in vitro* into cardiomyocytes using 2 induction media (1) 5-Azacytidine, 6uM for 24 hours and then kept in expansion media for 30 days (2) TGFβ1, 10ng/ml for 14 days.

Morphological changes were observed and documented. After differentiation, cells were characterized by Reverse Transcriptase-PCR (RT-PCR), Immunofluorescence (IF), Flow cytometry and qPCR studies for cardiac specific markers –Myosin light chain -2v (Mlc-2v), Cardiac Actin (CA), Connexin 43 (Cx43), GATA4, Cardiac Troponin I (cTnI).These cells were positive for vimentin, CD105, CD90, CD29, CD73, HLA I and negative for FSP, HLAII and CD34/45 (32).

3.3 Differentiation of Epidermal Stem Cells (EpiSCs) into Melanocytes, Keratinocytes and Neurons:

Materials and Methods:

Healthy pigmented skin tissues were collected from the patients undergoing surgery for vitiligo treatment in the Department of Dermatology, AIIMS and from children coming for circumcision in the Department of Pediatric Surgery, AIIMS. Healthy pigmented skin tissues were harvested by two different methods (i) skin blister and (ii) foreskin. Healthy pigmented hair tissues were harvested by the following three methods of biopsies (i) Follicular Unit Extraction (FUE), (ii) Scalp Tissue and (iii) Follicular plucking. Hairs were trimmed (shaft removed) and the root was treated with 0.25% trypsin-0.05% EDTA thrice for an incubation of 30 min each. Cells obtained from each digestion were pooled and filtered with 70µm cell strainer. The final cell pellet was suspended in DMEM-HG medium supplemented with 10% FBS, added with choleratoxin, glutamine, FGF2, insulin and adenine and plated on the culture vessel and expanded further. Skin biopsies were treated with dispase with overnight incubation at 4°C. After the incubation period is over, epidermis is peeled off and dermis was discarded. Epidermis was chopped and trypsinized for 15- 20 min using 0.25% trypsin-0.05% EDTA. Final cell suspension was passed through a 70µm cell strainer and the resulting cells were suspended in Epidermal stem cell culture medium. Cells from both the sources were characterized using flowcytometry for CK15, CK19, and β1-Integrin markers. Undifferentiated cells were assessed for doubling time and proliferation assay (MTT Assay).

EpiSCs were differentiated into melanocytes using differentiation induction media consisting of 50% MCDB 201 media, 40% Ham's F12, nutrient mix, supplemented with 10% fetal calf serum, 2mM/ml L-glutamine 10-4 mol/L L-ascorbic acid, 10 nM/ml phorbol 12-myristate 13-acetate, 10ng/ml cholera toxin, 20ng/ml fibroblast growth factor, 100 IU/ml penicillin and 100 mg/ml streptomycin. Differentiated cells were characterized by Fontana Masson staining, IF staining for HMB45 and S100, RT- PC for TYR and Melan A genes, qRT-PCR for MITF and TYR genes. Functional assessment of the melanocytes was done by L-DOPA staining.

The media used for the keratinocyte differentiation was of following composition- DMEM, and

Ham's F12 medium in a 3:1 ratio supplemented with 10% FBS, 10 ng/ml Epidermal Growth Factor, Ca Cl2, Dihydroxyvitamin D, hydrocortisone, Adenine, Insulin, 5µg/ml Transferrin, Triiodothyronine, Penicillin and Streptomycin. Keratinocytes were characterized by IF staining for K1/K10, RT-PCR for K1/K10 and Involucrin genes and qRT-PCR for K1/K10 gene (33).

Neuronal differentiation induction media consisted of neurobasal media containing penicillin and streptomycin, supplemented with bFGF, EGF, B-27 supplement and L-glutamine. Post induction, differentiated cells were characterized by IF staining for Neurofilament, Nestin and Tyrosine hydroxylase and qRT-PCR for TH and NF genes.

3.4 Standardizing the technique of *ex vivo* culture of limbal epithelial stem cells (LESCs):

Materials and Methods:

Limbal tissue specimens were isolated from cadaveric corneoscleral rims, living related donors, or contralateral eye of the patients. Harvested tissue was cultured on denuded human amniotic membrane (dHAM) using various techniques to stabilize dHAM. The optimization of *in vitro* culture conditions was achieved by modifications in culture media. The LESCs were cultured in both types of media for 2 weeks, and growth patterns were observed. Expanded cells were further characterized by Immunocytochemistry (K3/12, K19, and ABCG2) and reverse transcriptase polymerase chain reaction (K12, Cx43, Pax6, ABCG2, p63, and K19) (34).

3.5 Improved reprogramming efficiency of disease specific iPSC using immortalized human foreskin fibroblast feeder cells:

Materials and Methods:

In vitro-culture and expansion of fibroblasts cells were established using explant method. $1x10^5$ Fib cells were infected with hSTEMCCA (Human Stem Cell Cassette) lentiviral vector in the presence of 5ug/ml Polybrene. Next day, cells were plated onto mouse and I-HFF feeder at a density of $1x10^4$ cells onto the 6- well plate. After 24 hours, the medium was switched to reprogramming medium (ESC media and I-HFF conditioned media in 1:1 ratio).

Induced colonies were picked up based on human ES cell colony morphology and live staining for TRA-1-60 marker at days 16-24 post infection. The iPSC lines were assessed on the basis of morphology, expression of pluripotent makers by Immunoflorescence (Oct4, Sox2 and Klf4) & RT-PCR (Oct4, Sox2, c-myc, Nanog and Klf4). The in –vitro pluripotency and ability to differentiate into three germ layers was assessed by embryoid body formation. The experiments were performed using fibroblast cells from two different patients in triplicates [Unpublished data].

3.6 Maintenance of human Embryonic Stem Cells in feeder and bFGF free culture system using conditioned media from immortalized human Foreskin Fibroblast Cells:

Materials and Methods:

The secretion of TGF- β and IGF-II from the mitomycin-C treated I-HFF supplemented with various exogenous bFGF concentration (0, 2, 5 and 10ng/ml) was assessed by ELISA. The KIND -1 hES cell lines were gradually adapted to grow in feeder free system on geltrex coated culture dishes using CM at varying concentration of exogenous bFGF supplementation (0,2,5 and 10ng/ml). The hESC line grown in feeder free culture with CM was assessed on the basis of morphology, expression of pluripotent makers at 1st, 3rd, 5th and 10th passage by Immunoflorescence and flow cytometry. Any karyotypic abnormalities were also assessed at 20th passage [Unpublished data].

Results and Conclusion:

1. Clinical Research:

1.1 Stem Cell Clinical Trials in Heart Diseases:

a. Application of Stem Cell Technology for Dilated Cardiomyopathy:

In the initial 6 months of the ABCD (Autologous Bone Marrow Cells in Dilated Cardiomyopathy) trial, we found that 76% of our patients who were NYHA functional class III showed an improvement in EF by 5.4%. This improvement manifested after 1 month, which is too early to be explained by formation of fresh myocytes. This benefit was predominantly due to improvement in end-systolic volumes while the end diastolic volumes remained the same. This is similar to a number of other studies that also showed an improvement in EF of about 4% to 6%, and also with no change in end-diastolic volumes. This would suggest that stem cells do not cause any change in the remodelling process but improve myocardial cell function.

b. Stem Cell Clinical Trials in Pediatric Diseases:

An improvement seen in urinary parameters, with fall in urea from 260 to 163 mg/dl, fall in creatinine from 1.9 to 1.5 mg/dl and fall in serum K from 6.6 to 3.6 was noted at first month following stem cell transplant. A marked reduction was seen in the doses of medicines needed to keep the child metabolically stable. The baby kept struggling but succumbed at the age of 17 months and 15 days due to end stage renal failure. Post mortem bilateral trucut renal biopsies demonstrated presence of glomerular sclerosis, primitive appearing renal tubules, thick walled blood vessels and scattered blastemal cells that were not demonstrated earlier. However, though the tissue obtained was little so it was difficult to confirm regressive changes, there was an improvement in the fibrosis as appreciated histologically.

1.2 a. Stem Cell Clinical Trials in corneal defects:

After transplantation, epithelial transparency increased in all patients. Reduction or absence in superficial corneal vascularization was observed in 80% patients. Conjunctivilizaion of the cornea seen preoperatively was found to improve in all patients after the ex-vivo cultured limbal stem cell transplant.

b. Assessment of Central Retinal Function after Autologous Bone Marrow Derived Intravitreal Stem Cells Injection in Patients with Retinitis Pigmentosa using Multifocal ERG :

Thirty patients (26 male and 4 female) aged 18 to 58 years (mean 35.9) were included in the study. Visual acuity pre- injection ranged from 0.48 to 1.9 log MAR (mean 1.25289 ± 0.5324). At 6 months of follow-up there was no statistically significant change in the best corrected visual acuity after stem cells injection (p = 0.785 Friedman test). At 6 months follow-up period mf-ERG p1 wave amplitude within 2° from fovea (ring 1) showed improvement (p-value 0.014). The p1 wave latencies also showed reduction in the implicit times (p-value 0.03). The maximum mean value of p1 wave amplitude was observed at 3 months of injection. The increase in P1 wave amplitude was maximal in ring 1. The change observed was statistically significant in ring 1 (p-value 0.014).

1.3 Hair Follicle Outer Root Sheath Cells in the Treatment of Vitiligo:

The mean ± SD repigmentation was 65.7 ± 36.7%. Overall, nine of 14 patients achieved > 75% repigmentation. Mean percentage repigmentation was significantly higher in patients with ± 1 year stability than those with < 1 year stability (P = 0.02).

2. Pre- clinical Research:

2.1 Effect of bone marrow-derived mononuclear cells on nerve regeneration in the transection model of the rat sciatic nerve:

Histological assessment of the nerve was performed 30 days and 60 days after the operation and regenerative changes were compared between the two groups. The recovery after nerve anastomosis was far better in the test group at both 30 days and 60 days. There was a statistically significant difference in axonal regeneration, remyelination and myelin thickness at sites 5 mm and 10 mm from the site of repair of the nerve. Schwann cell proliferation and degenerative changes were more prevalent in the controls.

2.2 Dose-dependent facilitation of peripheral nerve regeneration by bone marrow–derived mononuclear cells: a randomized controlled study:

The recovery of nerve cell architecture after nerve anastomosis was far better in the high-dose BM-MNC group than in the low-dose BM-MNC and control groups, and it was most evident (p < 0.02 in the majority of the parameters [3

of 4]) at the distal-most site. Overall, the improvement in myelin thickness was most significant with incremental dosage of BM-MNCs, and was evident at the repair, intermediate distal, and distal-most sites ($p = 0.001$).

2.3 MSC differentiation onto 3D biocomposite scaffolds and transplantation in rat model:

Scaffolds removed from back of the rats for 2, 4, 6, 8 and 12 weeks after implantation were immediately photographed and assessed for opacity and formation of any bone like tissue radiographically. Retrieved implants were fixed in 10% formalin and taken to G.B.Pant Hospital for histopathology. Tissue blocks were sectioned at 5um thickness, positioned on glass slide and stained by hematoxylin and eosin (H&E). The stained sections were observed under microscope and analysed to evaluate host tissue response in terms of inflammation, fibrosis, necrosis, vascularisation, scaffold degradation and tissue ingrowths in scaffolds.

3. Basic Research:

3.1 Differentiation of bone marrow stem cells into cells of neuronal lineage:

Induced BM-MSCs revealed neuron like morphology and expressed cellular markers suggesting neuronal differentiation with all the inducing agents. However, upon quantitative analysis through q- PCR, cells induced with FGF2 were found to show maximum expression of tyrosine hydroxylase (TH). ELISA revealed the highest level of dopamine secreted by the cells in the culture media, induced with FGF2 alone. The presence of TH was observed in the cells when induced with other inducers but with significantly lesser expression as compared to that observed in FGF2 alone. We conclude that BM-MSCs can be coaxed to differentiate efficiently into dopaminergic neurons in the presence of a very simple media cocktail containing only one main inducer and thus contribute towards cellular therapy in Parkinson's and other related disorders.

3.2 Differentiation of bone marrow stem cells into cardiomyocytes:

Differentiation studies revealed the expression of Mlc-2v, CA, Cx43, GATA4, cTnI by RT-PCR and Mlc-2v and cTnI by IF in both treatment groups. The cells after completion of the differentiation protocol were checked for (Transcription factors) TFs TBX5 apart from GATA4 and NKX 2-5. Also, these were checked for BAF chromatin remodelling complex, BAF60C through RT-PCR. It was found that all these were expressed in the BM-MSC derived cardiac cells. However, qPCR results revealed that the TFs NKX 2-5, GATA4, TBX5 and chromatin remodelling complex, BAF60C are expressed at significantly lower levels when compared to adult beating cardiomyocytes.

3.3 Differentiation of Epidermal Stem Cells (EpiSCs) into Melanocytes, Keratinocytes and Neurons:

The new method of extracting the stem cells from outer root sheath of the hair follicle is a very effective one. The EpiSCs isolated by this method can be differentiated successfully into melanocytes and keratinocytes, as shown by IF, RT-PCR and qRT-PCR results. These cells also have the tendency to differentiate into neuronal like cells, supported by the results of IF, RT-PCR and qRT-PCR.

3.4 Standardizing the technique of ex vivo culture of limbal epithelial stem cells (LESCs):

Stabilization of dHAM was successfully achieved using coverslips. Histopathological analysis showed multilayer formation and immunostaining, and reverse transcriptase polymerase chain reaction data confirmed the expression of both stem cell markers (K19, p63, and ABCG2) and differentiation markers (K3, K12, and Cx43). Patients who had undergone limbal stem cell transplantation showed a stable ocular surface with improved visual acuity over a long-term follow-up period.

3.5 Improved reprogramming efficiency of disease specific iPSC using immortalized human foreskin fibroblast feeder cells:

We found that the average days of appearance of colonies was 16 on human feeder in comparison to 24 on mouse feeder. The total number of colonies obtained from two independent experiments at the end of reprogramming period was 41 and 23 on human and mouse feeders respectively. The average reprogramming efficiency of iPSC on human vs. mouse feeders were 0.1 % and 0.05 % respectively as demonstrated by TRA 1-60 Live staining. Disease specific DMD-iPSC generated in this manner displayed ES cell like morphology, expressed stem cell markers TRA 1-60 and TRA 1-81. These iPSC lines exhibited endogenous expression of pluripotency markers like OCT-4, Sox2, Klf-4, cMYC and Nanog. The iPSC lines derived using both the feeder cells were able to spontaneously differentiate into cells of all three germ layers as characterized by Immnunofluorescence and RT-PCR assay.

3.6 Maintenance of human Embryonic Stem Cells in feeder and bFGF free culture system using conditioned media from immortalized human Foreskin Fibroblast Cells :

ELISA results confirmed that the level of both TGF- β and IGF-II secretion was comparable at all bFGF treated group versus no exogenous bFGF added. The cells cultured in the CM in the feeder free conditions even after 20 passages, showed typical hESC morphology and expression of pluripotency-related proteins, SSEA-4, TRA-1-60, OCT4, alkaline phosphatase and normal karyotypes in all groups compared to positive control. Flow cytometric analysis for TRA1-60 and SSEA-4 surface marker expression shows the increasing trend but the difference was negligible among different groups. (From 0-, 2, 5,10ng/ml and positive control

TRA1-60: 0- 75.6% ± 3.86, 2- 76.87% ± 5.64, 5- 77.28% ± 5.21, 10- 78.1% ± 5.83 and 81.6% ± 3.53, SSEA-4: 0- 81.47%±4.27, 2- 82.9%±3.86, 5- 82.73%±3.80, 10- 84.07%±5.72 and 82.73%±3.80 respectively). There was no difference in the expression of pluripotency-related genes (OCT4, SOX-2, c-MYC, Klf-4 and NANOG) in test groups as compared to positive control as revealed by semi quantitative RT-PCR.

Discussion :

Stem cells, the foundation of all life forms has remarkable potential which gives rise to different cell type right from the early life to the adult form. These stem cells are termed as the embryonic stem cells. These embryonic stem cells have the highest plasticity. The major drawback of embryonic stem cells is that they generate the tumor of all the three germ layers; teratoma, when injected into nude mice. This limits their potential to be exploited into the clinical application. Tissue derived stem cells are there in almost every organ right from hair follicle to the linings of the intestine. These stem cells reside in the discrete pockets inside the tissue/ organs called as stem cell niche. Stem cell niche provides a sort of protective environment for stem cells which helps maintain the homeostasis of the cellular damage to the tissue/ organs due to wear and tear. These tissue- based or adult stem cells are the oldest known stem cells in the clinical practice. The autologous bone marrow derived stem cells are into clinical trials at different centres in India and abroad.

AIIMS is one of the major centres in India to have involved in clinical trials of autologous bone marrow derived stem cells. The stem cell clinical trials in which AIIMS is involved include myocardial infract, dilated cardiomyopathy, critical limb ischemia, cerebral palsy, stroke and macular hole. Apart from this we are also involved in clinical trials involving tissue derived stem cell in ocular surface reconstruction and vitiligo treatment. Clinical trials at AIIMS are still at infancy. Limbal stem cell therapy has been done routinely at AIIMS for various kinds of ocular diseases. We are at par with the clinical trials that are being carried out all over the world.

As far as the pre- clinical studies are concerned, we are working on bone regeneration, using biocompatible scaffolds. Cell based therapy of peripheral nerve injury, using stem cells is also being studied at pre- clinical level at AIIMS. Our study reveals that local delivery of BM-MNCs (which can be isolated easily from bone marrow aspirates) into injured peripheral nerve increases the rate and degree of nerve regeneration, but in a dose dependant manner.

Moving further to the basic research, at this level, we are working on the differentiation aspect of stem cells from various sources and the molecular mechanisms involved in the same. An in-depth study of the factors responsible for and affecting the differentiation of stem cells is required to develop any kind of cell based therapy regime for the degenerative diseases. This knowledge helps in better

understanding the cell behaviour in- vitro. The pre- clinical studies give an outlook to the cellular and molecular mechanisms that may be occurring in the *in vivo* conditions. A balance between the *in vitro* and *in vivo* behaviour of stem cells is mandatory to initiate any kind of clinical trial.

AIIMS has taken the lead in all the aspects of stem cell research, starting from basic research to the clinical trials. We, at AIIMS, are aimed to develop the best cell based therapy that can be taken from bench top to bed side to the patients. The stem cell technology is advancing at a very high pace from the humble beginning of autologous bone marrow stem cells into clinical practice to the arena of iPSCs. We need to move step by step and with great caution. Potential is huge and great, we may have touched the tip of the iceberg but true story remains to unfold.

Future Scenario:

Stem Cells and tissue engineering hold a great potential in the field of regenerative medicine. Scientists, now days, are trying to develop organs by using stem cells on organ prints or moulds made up of biodegradable composites. One such example is the Vacanti mouse, developed by Charles Vacanti, who injected cow cartilage stem cells, seeded on a biodegradable biopolymer and implanted under the skin. Scientists are focusing more on tissue engineering as the biodegradable polymers hold a lot of benefits like, better stem cell delivery and homing, added drugs can be transplanted together, act as a matrix and support for stem cells to grow and lastly, as they are biodegradable, there is no issue of them being hazardous to the body. With the help of tissue engineering, scientists have also been successful in creating trachea lumen model. Apart from the use of stem cells in tissue engineering, these are also being explored for the treatment of deafness, blindness, diabetes type I, bone regeneration, blood supply, Parkinson's disease, Alzheimer's disease, Huntington's disease, myocardial infarction, etc.

Stem cells will be available as off-the-shelf product in the near future for treating various unmet medical needs. The stem cell technology is advancing at a very high pace from the humble beginning of autologous bone marrow stem cells into clinical practice to the arena of iPSCs and tissue engineering. Generation of induced pluripotent stem cell (iPSC) lines and their differentiated derivatives will promote patient specific & disease specific drug development. Tissue engineering will omit the need of donors for organ transplantation, hence reducing the risk of organ rejection and HLA matching. The major concerns of teratoma formation by these cells have to be overcome before their clinical application. Due to their pluripotent nature, iPSCs might change the entire scenario in drug development and will open avenues for personalized medicine using stem cells. We need to move step by step and with great caution. Potential is huge and great, we may have touched the tip of the iceberg but true story remains to unfold.

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