2.1 Introduction:

Bone marrow is a complex tissue containing stem cells with hematopoietic properties. The hematopoietic stem cells, which are the primary source of blood cells in the adult body, are regulated within a microenvironment of stromal cells in the bone marrow (Hunt P et al., 1987; Aubin JE et al., 1999; Chen ZZ et al., 1991, Colter DC et al., 2001, Deans RJ et al., 2000). The stromal cells exert their effects on the hematopoietic cells through direct cell-cell interactions as well as by the release of soluble factors (Yanai N et al., 1994; Ryan DH et al., 1991; Dittel BN et al., 1993). Stromal cells isolated from bone marrow (BMSC) are heterogeneous and fibroblastic in appearance (Prockop et al., 1997). In 1974, Friedenstein et al., isolated fibroblastoid cells in bone marrow by plastic adherence. Fibroblastoid cells make up 0.001-0.01% of bone marrow cells and display a colony forming unit (CFU-F). They were initially named plastic-adherent cells or colony-forming-unit fibroblasts and subsequently referred to as either marrow stromal cells or mesenchymal stem cells (MSC), due to their potency to differentiate into various connective tissue lineages including adipocytes, osteoblasts, chondrocytes or myoblast (Pittenger MF et al., 1999; Jiang et al., 2002).

Bone marrow derived MSCs have been isolated from a variety of species, including mouse (Peister A et al., 2004), rat (Javazon EH et al., 2001), rabbit (Johnstone B et al., 1998) and human subjects (Colter DC et al., 2001). Although MSCs from different species have similar characteristics in part, some data
suggest that variations occur among species. MSCs from human bone marrow are relatively easy to harvest and to expand in culture (Sekiya et al., 2002a), whereas rodent MSCs have proven more difficult (Friedenstein AJ et al., 1974; Simmons DJ et al., 1991; Rennick D et al., 1987), although this is not without controversy (Javazon EH et al., 2001). The technical difficulties in preparing MSCs from rodent bone marrow have limited the number of experiments, because animal transplantation models are required for preclinical studies. The selection of suitable cell populations is apparently crucial for the outcome of in vivo experiments with MSCs.

Although there are many methods to isolate MSCs from the bone marrow, no optimal method is available. The methods include plastic adherence (Dexter et al., 1981), gradient density centrifugation (Chen ZZ et al., 1991) and immunomagnetic selection (Dezawa M et al., 2004; Jia L et al., 2002). Different methods have different defects and virtues. Plastic adherence is an easy method of obtaining such cells on the basis of their plastic adherence characteristics, but it is difficult to get pure stromal cells. Gradient density centrifugation depends on the relative density of MNCs to separate MSCs. Immunomagnetic selection uses the principle of separating the MSC based on the immune recognition of the surface antigens by the use of appropriate antibodies. Extensive experimentation has defined the conditions for the isolation, propagation, and differentiation of MSCs in vitro and in vivo. In our study we have isolated and established bone marrow stromal cells by the simple and reliable method of combining density
gradient centrifugation with plastic adherence and differentiated them to adipocytes, osteocytes and neuronal like cells.

2.2. Hypothesis:

We hypothesize that rat bone marrow mesenchymal stem cells can be isolated by simple and reliable method

2.3. Aim

1. Isolation and characterization of rat bone marrow mononuclear cells
2. Establishment and characterization of stromal of cell cultures
3. Differentiation of stromal cells into adipocytes, osteocytes and neural lineage

2.4. Material and Methods

The protocol was approved by the Institutional Review Board (IRB) at L.V.Prasad Eye Institute.

2.4.1. Preparation of Chemicals

All the chemicals and culture media were prepared as described in the appendix I.

2.4.2. Sterility Check for Chemicals & Media:

Following the filter sterilization the media and chemicals were kept for sterility check. A few drops of media/chemicals were inoculated on chocolate agar and in thioglycolate broth to screen for both aerobic and anaerobic microorganisms. Inoculated media were then incubated in a bacterial incubator at 37\(^{0}\)C for about 7 days, before the media/chemicals are approved for tissue culture use.
2.4.3. Source of Animals

Wistar rats (12 weeks old) were used. All protocols followed for the use of animals were approved by the Institutional ethical committee and Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA).

2.4.4 Isolation of mononuclear cells:

Wistar rats were sacrificed by cervical dislocation, and then placed in 70% alcohol for 10 min. Both femurs from one rat were taken and stripped of adherent muscles of the knee end. A needle was inserted into the bone and cells were aspirated followed by several flushes through the bone using a 1 ml syringe filled with culture medium, until all the bone marrow was flushed out of the bone. A similar procedure was performed from other end of the bone as close to the tip as possible. The marrow thus obtained was suspended by pipetting the large marrow cores through a 1 ml pipette. The medium containing the cells was layered on HISTOPAQUE – 1077 (Sigma) and centrifuged on 400xg for 30min. Mononuclear cells were removed from the gradient interface and washed with PBS. The suspension was then centrifuged at 200xg for 5min. The pellet thus obtained was dissolved in 1ml of PBS; the cell count was done in a Neubauer chamber and tested for viability by the Trypan Blue elution test. The mononuclear cells were resuspended in growth medium (see below), and plated in 25cm² tissue-culture flasks made of polystyrene plastic (Nunclone) at a density of 1x10⁶cells/ml. Nonadherent cells were removed after 48h, replacing the media every 2-3 days.
2.4.5 Cell culture conditions:
The adherent cells were cultured in the growth medium containing Dulbecco’s modified Eagle’s medium (DMEM; Sigma) supplemented with 10% Fetal bovine serum (FBS; SIGMA) 250KU/L Penicillin, 1.25mg/L Amphotericin-B, 100mg/L Streptomycin, 50µl/L Gentamycin and 1.2g/L Sodium bicarbonate. The cultures were maintained at 37°C in a humidified 5% CO₂ incubator. When the cells reached 80-90% confluency, cultures were harvested with Trypsin-EDTA solution (0.25% trypsin, 1mM EDTA; Sigma).

2.4.6 Colony-forming assays:
For these assays, 2 cells per cm² at passage 0 were plated and cultured for 14 days in 75 cm² tissue culture flasks. The cells were fixed with methanol and stained with Giemsa. Colonies less than 2 mm in diameter and those that were only faintly stained were ignored.

2.4.7 Characterization of mononuclear and stromal cells
2.4.7.1 Immunophenotyping (Immunocytochemistry)
Immunophenotyping is a technique for identifying cellular or tissue constituents (antigens) by means of Ag-Ab (Antibody) reactions, the site of Ab binding being identified either by direct labelling of the Ab or by use of a secondary labelling method.

MSC’s (3rd passage) were seeded into 24 well plates and cultured up to confluency. The cells were fixed with 4% Paraformaldehyde in 0.1 M phosphate buffer (PH 7.2) for 20 min. and then processed for immunocytochemistry. Non-specific reactions were blocked with 5% fetal calf serum for 30 min at room
temperature. The fixed cells were then incubated for one hour with primary antibodies (Table 2.1). After three washes cells were incubated with FITC-conjugated secondary antibody for an hour. They were washed three times and counter-stained with Propidium Iodide (PI) to detect the cell nuclei. Cells were photographed by confocal Laser Scanning microscopy (LSM510; Carl Zeiss) with a fluorescent light source (excitation wavelength 480 and 540 nm).

**Table 2.1: Antibodies used for immunocytochemistry**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Antibody</th>
<th>Dilutions</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CD90</td>
<td>1:200</td>
<td>Millipore</td>
</tr>
<tr>
<td>2</td>
<td>CD45</td>
<td>1:200</td>
<td>Millipore</td>
</tr>
<tr>
<td>3</td>
<td>CD11a</td>
<td>1:200</td>
<td>Millipore</td>
</tr>
<tr>
<td>4</td>
<td>CD18</td>
<td>1:200</td>
<td>Millipore</td>
</tr>
<tr>
<td>5</td>
<td>CD34</td>
<td>1:200</td>
<td>Millipore</td>
</tr>
<tr>
<td>6</td>
<td>CD31</td>
<td>1:200</td>
<td>Millipore</td>
</tr>
<tr>
<td>7</td>
<td>FITC- Conjugated 2nd</td>
<td>1:250</td>
<td>Millipore</td>
</tr>
<tr>
<td></td>
<td>Antibody</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Vimentin</td>
<td>1:250</td>
<td>Dako Cyomation</td>
</tr>
</tbody>
</table>

2.4.7.2 Flowcytometry:

Flow cytometry is a useful technique due to the fact that the cells can be monitored, providing sensitive and specific information about each single cell. In relation to the optics of flow cytometry, when the light source hits a cell, amount of light scattered to the side is detected by the size and shape of the cell. Flow cytometers use lasers as their source to excite cells. The excitation from the lasers must be equivalent to the absorption wavelengths of fluochrome used. The argon laser is the most commonly used since it produces several lines in the
UV, and can excite fluorescein, which is a common fluorochrome. The other parameter detected is forward scatter and it provides information about the surface properties, complexity of the cells and can determine how granulated the cells are. Various population of cells can be distinguished from the information provided by side and forward scatter following acquisition of samples. In addition, antibodies have fluorescent attached enabling the surface expression of specific cell markers.

2.4.7.2.1 Preparation and staining of MNC’s and MSC’s

Mononuclear cells were obtained as described above. MSC’s were harvested from the tissue culture flasks after passage 3 in vitro and centrifuged at 200xg for 5 min at room temperature. The cells were washed and counted in a Neubauer Chamber. A single cell suspension of 0.5 to 1x $10^6$ cells were placed in 50µL of buffer (PBS, 0.1% sodium azide, 2% FBS). The cells were incubated with primary antibody for 40 min with saturating concentrations of monoclonal antibodies (Table 2.2). After the cells were washed three times in buffer and centrifuged at 200xg for 5 min, they were resuspended in ice cold PBS and incubated with the FITC-labelled or TRITC-labelled secondary antibody for 30 minutes in the dark at 4°C. Cell fluorescence was evaluated by flow cytometry in an FACS Calibur instrument (Becton Dickinson) and the data were analyzed using Cell Quest software (Becton Dickinson). An isotype control was included in each experiment, and specific staining was measured from the cross point of the isotype with a specific antibody graph.
Table 2.2: Antibodies used for flow cytometry

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Antibody</th>
<th>Dilutions</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CD90</td>
<td>1:50 (10µl)</td>
<td>Millipore</td>
</tr>
<tr>
<td>2</td>
<td>CD45</td>
<td>1:50 (10µl)</td>
<td>Millipore</td>
</tr>
<tr>
<td>3</td>
<td>CD11a</td>
<td>1:50 (10µl)</td>
<td>Millipore</td>
</tr>
<tr>
<td>4</td>
<td>CD18</td>
<td>1:50 (10µl)</td>
<td>Millipore</td>
</tr>
<tr>
<td>5</td>
<td>Fibronectin</td>
<td>1:50 (10µl)</td>
<td>Millipore</td>
</tr>
<tr>
<td>6</td>
<td>CD31</td>
<td>1:50 (10µl)</td>
<td>Millipore</td>
</tr>
<tr>
<td>7</td>
<td>FITC or TRITC-Conjugated 2° Antibody</td>
<td>1:50 (10µl)</td>
<td>Millipore</td>
</tr>
<tr>
<td>8</td>
<td>Vimentin</td>
<td>1:25</td>
<td>Dako Cyomation</td>
</tr>
<tr>
<td>9</td>
<td>IgG1</td>
<td>1:25 (10µl)</td>
<td>Millipore</td>
</tr>
<tr>
<td>10</td>
<td>IgG2a</td>
<td>1:25 (10µl)</td>
<td>Millipore</td>
</tr>
</tbody>
</table>

2.4.7.3 Reverse Transcription PCR (RT PCR)

2.4.7.3.1 Isolation of Total Cellular RNA (Trizol-Method):

The Isolation of RNA from cultured cells involves the following steps:

I) Homogenization, II) Phase Separation, III) RNA precipitation, IV) RNA wash

V) Resuspension of the RNA pellet

I) Homogenization: Rat bone marrow stromal cells were dislodged using trypsin. Cells were counted on Neubaur’s counting chamber. One ml TRIzol was added to the cells and passed several times through a pipette for homogenization.

II) Phase Separation: Homogenized samples were incubated for 5mins at 15-30°C. 0.2ml of chloroform was added for each 1 ml TRIzol reagent added. Tubes
were shaken vigorously for 15-20 seconds, incubated at 15-30°C for 2-3mins. Centrifuged at 12,000xg for 15mins at 2-8°C. After centrifugation the lower red phenol-chloroform phase, an interphase, and an upper aqueous phase is formed.

III) RNA Precipitation: The aqueous phase containing (organic phase - for DNA, Protein isolation) the RNA was transferred to a fresh tube. RNA was then precipitated by isopropyl alcohol (0.5 ml/1ml TRIzol) and incubated at 15-30°C for 10mins. The sample was then centrifuged at 12,000xg for 10mins at 2-8°C. The RNA gets precipitated, and forms a gel like pellet at the bottom of the tube.

IV) RNA Wash: The supernatant was removed and RNA pellet was washed in 75% ethanol (1ml/1ml of TRIzol). The sample was mixed by vortexing and centrifuged at 7.500xg for 5mins at 2-8°C.

V) Resuspension of the RNA pellet: RNA pellet was then dried (air dried) and dissolved in RNase-free water and stored at -70°C.

2.4.7.3.2 Reverse Transcription [Synthesis of cDNA (1st Strand) using Oligo-d (T) primers]:

CDNA strand was synthesized using reverse transcriptase from Moloney-Murine Leukemia Virus (M-MLV). The total cellular RNA (1-2µg) was used for cDNA synthesis. For the first strand synthesis, two master mix were prepared

Master Mix 1: 1 µg of Total RNA + 500ng of oligo - (dT) primer

Master Mix 2: 1X RT buffer (10X stock), 200U of RT enzyme (200U/µl) were added for a 50 µl reaction, RNase free water.
Master mix 1 was denatured at 65°C for 10 minutes, followed by immediate chilling on ice to avoid renaturation. Master mix 2 was then added to master mix 1 and a PCR amplification reaction was carried out under following conditions.

PCR Conditions:
- 25°C for 10 mins (Primer Annealing)
- 42°C for 1 hour (Amplification)
- 70°C for 30 mins

Synthesis of 2nd strand using gene specific primers:
The second strand was synthesized using gene specific primers (Table 2.3) for the following genes – Collagen type 1alpha 1 and Vimentin. The Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was used as internal control.

### Table 2.3: Rat primer sequences used for RT PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Fragment Length</th>
<th>Accession Number</th>
<th>Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vimentin</td>
<td>F:AATTGCAGGAGCTGAATGAC</td>
<td>301bp</td>
<td>NM_031140</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>R:AATGACTGCAGGGTGCTCTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen type 1 alpha 1</td>
<td>F:ACAGACCAACAAACCCAACACTC</td>
<td>379bp</td>
<td>XM_213440</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>R:GTAAGGTTGAATGCACTTTTGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: CCCACGGCAAGTTCAACGGGCA</td>
<td>606bp</td>
<td>NM_017008</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>R: TGGCAGGTTTCTCCAGGC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All the primers were obtained from published literature (Chen et al 2004).
PCR conditions

PCR for all the three sets of primers was performed for 35 cycles with the reaction mixtures and conditions shown in table 2.4

Table 2.4: Reaction mixture for various primer sets

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Vimentin</th>
<th>Collagen type alpha 1</th>
<th>GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA</td>
<td>2 µl</td>
<td>2 µl</td>
<td>2 µl</td>
</tr>
<tr>
<td>1X PCR buffer</td>
<td>2.5 mM</td>
<td>2.5 mM</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>1.5 mM</td>
<td>1.5 mM</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>dNTPs</td>
<td>200 µM</td>
<td>200 µM</td>
<td>200 µM</td>
</tr>
<tr>
<td>Forward primer</td>
<td>5.0 pm</td>
<td>5.0 pm</td>
<td>5.0 pm</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>5.0 pm</td>
<td>5.0 pm</td>
<td>5.0 pm</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>1U</td>
<td>1U</td>
<td>1U</td>
</tr>
<tr>
<td>Total volume</td>
<td>25 µl</td>
<td>25 µl</td>
<td>25 µl</td>
</tr>
</tbody>
</table>

PCR conditions for Vimentin, collagen type 1 alpha 1 and GAPDH primers

1. Initial denaturation at 94°C for 3 minutes
2. Denaturation at 94°C for 30 seconds
3. Annealing of primers at 55.5°C for 30 seconds
4. Extension at 72°C for 45 seconds. The steps 2-4 were repeated for 35 cycles.
5. Final extension step at 72°C for 5 minutes.

2.4.7.3.3 Quality of PCR products

PCR products were run on 1.5% agarose gel to determine their quantity and quality.
Gel Preparation

1. Required amount of agarose was added to electrophoresis buffer (1X Tris Borate EDTA- TAE) in a glass flask.
2. Agarose was boiled in microwave and the flask was swirled to ensure even mixing.
3. Melted agarose was cooled to a tolerable temperature (~55°C).
4. Ethidium bromide (EtBr-5µg/mL) was added and the flask was swirled for even mixing of EtBr.
5. The melted gel was poured in the casting apparatus with an inserted comb.
6. The gel was allowed to stand till it got solidified.
7. The comb was removed gently from the gel plate after solidification.

Gel loading and running

1. The gel plate was placed in the electrophoresis tank
2. 1X TAE buffer was poured to cover the wells
3. PCR products were mixed with 1µl of loading buffer (6X) (MBI Fermentas) on parafilm and were loaded with a micropipette into the wells along with the marker (100-bp ladder) (MBI Fermentas) to determine their size
4. The gel was run for approximately half an hour at a voltage supply of 10V/cm till bromophenol migrated atleast half the distance through the gel
5. The gel was removed from the tank and was placed on UV transilluminator (UV tec) and the amplification was documented in a gel doc system
2.4.8 Differentiation potential

2.4.8.1 Adipogenic differentiation

Passage 2 cells were seeded on cover slips in 6 well plates and cultured in complete medium up to confluency. At confluency, the cells were switched to an adipogenic induction medium (Appendix 1) and further cultured up to 21 days with the medium being changed on every alternate day.

2.4.8.1.1 Oil red O staining

After 21 days, the adipogenic cultures were fixed in 4% paraformaldehyde for at least 1 hr and fixative was carefully aspirated and cultures were rinsed three times with PBS. Then washed twice with water. Three ml of fresh 0.3% oil Red-O solution was added and incubated for 2 hr at room temperature. After incubation, the oil red O solution was removed and washed thrice with water. Then counterstained with haematoxylin for 5 to 15 minutes.

2.4.8.2 Osteogenic differentiation

Passage 2 cells were seeded on cover slips in 6 well plates and cultured in complete medium up to confluency. The medium was then replaced with a calcification medium (Appendix 1) and incubated for 21 days. After incubation these cover slips were stained with fresh 0.5% alizarin red solution.

2.4.8.2.1 Alizarin Red staining

Alizarin Red S, an anthraquinone derivative, may be used to identify calcium in tissue sections. The reaction is not strictly specific for calcium, since magnesium, manganese, barium, strontium, and iron may interfere, but these elements usually do not occur in sufficient concentration to interfere with the staining.
Calcium forms an Alizarin Red S-calcium complex in a chelation process, and the end product is birefringent.

1. Coverslips were fixed with methanol for 5 min.
2. Coverslips were stained with alizarin red solution (2%) for 30 seconds to 5 minutes
3. Shakeoff excess dye and blot sections
4. Dehydrated in acetone (20 dips). Then in acetone-xylene (1:1) solution (20 dips)
5. Clear in xylene and mount in a mounting media

2.4.8.3 Neural differentiation: Passage 2 cells were used for neural differentiation. At confluency, the cells were switched to DMEM+ ITS for 24 hrs. After 24 hrs, the neurogenic induction medium (Appendix 1) was added. After 6-7 days the cultures were terminated and processed for immunocytochemistry and RT-PCR analysis.

2.5 Results:

2.5.1 Isolation and culturing of MSCs:

2.5.1.1 By plastic adherence: The cell suspension containing both stromal and hematopoietic were seeded in tissue culture flasks using DMEM with 10% FCS. At the end of two days, many of the round and spindle shaped cells had attached to the base of the tissue culture flask. The rounded cells remained adherent even after subsequent media change (Figure 2.1a).
Figure 2.1: Bone marrow stromal cells by plastic adherence (a) and combined density gradient and plastic adherence (b-h). The bone marrow stromal cells (BMSCs) showing spindle like shape in morphology (c) by adherence to the plastic. The BMSC showed diverse morphologies including spindle shaped cells (indicated by arrow) and broad flattened cells (indicated by arrowheads) (d). These cells are forming colonies on day 4 (e) and growing the colonies more densely distributed (f). Cells formed colonies after 14 days of culture, when seeding on low density stained with methylene blue. g – Single colony; h – T75 flask.

2.5.1.2 Ficoll hypaque separation and plastic adherence (Combination Method): The cell suspension was layered on hypaque and was subsequently seeded in tissue culture flask. The spindle shaped cells attached to the bottom of
the flask while the round cells remain suspended in the medium and were mostly eliminated from the culture with subsequent media changes. (Figure 2.1b). Majority of adherent cells displayed a spindle like shape (Figure 2.1c). These cells began to proliferate at about day 4, and gradually grew to form small colonies (Figure 2.1d). By day 7, the number of cellular colonies of different sizes had obviously increased. In large colonies, cells were more densely distributed and showed a spindle shape (Figure 2.1e). As cells continued to grow, colonies gradually expanded in size and reached confluency by day 10. Passaged MSCs behaved similarly to those in primary cultures. However, the cells were larger and more heterogeneous in morphology and growth properties. Grossly, the MSCs in subcultures could be divided into two types, spindle shaped and broad flattened cells (Figure 2.1f). The flattened cells seldom proliferated and were gradually surrounded by the spindle shaped cells, which replicated faster. It seemed that the spindle-like MSCs gradually transformed into broad flattened cells with further passages. When seeding on low density the cells form colonies. The colony forming efficiency was counted as 10%.

2.5.2 Characterization

2.5.2.1 Flow Cytometry: The mononuclear cells expressed CD45, CD11a, CD18 and CD31 (Figure 2.2) suggesting hematopoietic lineage. The adherent marrow stromal cells expressed Vimentin, Fibronectin and CD90 (Figure 2.3). They expressed neither hematopoietic lineage markers such as CD45, CD11a, CD18 nor an endothelial related antigen CD31 (Figure 2.3). The lack of expression of CD45, CD11a, CD18 and CD31 suggests that cell cultures were
depleted of hematopoietic cells during sub cultivation. Table 2.5 summarizes the expression of markers by stromal cells isolated by solo density gradient centrifugation, solo plastic adherence, and combination of both.

Table 2.5: Flow cytometry analysis of rat marrow stromal cells were isolated by different techniques and the percentage of expression of each marker was measured by flow cytometry analysis.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Ficoll density gradient centrifugation (1x10^6 cells)</th>
<th>Plastic Adherence (1x10^6 cells)</th>
<th>Ficoll density gradient centrifugation and plastic adherence (1x10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD90</td>
<td>1.2%</td>
<td>24.4%</td>
<td>84%</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>0.9%</td>
<td>19.3%</td>
<td>77%</td>
</tr>
<tr>
<td>CD45</td>
<td>85.0%</td>
<td>15.4%</td>
<td>6.7%</td>
</tr>
</tbody>
</table>

Figure 2.2: Flow cytometry analysis of rat bone marrow mononuclear cells: Bone marrow mononuclear cells expressed the markers of CD45, CD31, CD11a and CD18 and the percentage of positivity was mentioned in brackets. The purple line indicates the isotype matched antibody serving as a control, x-axes intensity log values, y-axes cell counts.
Figure 2.3: Flow cytometry analysis of bone marrow stromal cells expressed the markers of CD90, fibronectin and vimentin and negative for CD31, CD45, CD11a, CD18 and the percentage of positivity was mentioned in the brackets. The purple line indicates the isotype matched antibody serving as a control, x-axes intensity log values, y-axes cell counts.

### Table 2.3

<table>
<thead>
<tr>
<th>Marker</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11a</td>
<td>11.5%</td>
</tr>
<tr>
<td>CD31</td>
<td>10.0%</td>
</tr>
<tr>
<td>CD18</td>
<td>9.8%</td>
</tr>
<tr>
<td>Vimentin</td>
<td>1.4%</td>
</tr>
<tr>
<td>CD90</td>
<td>97.98%</td>
</tr>
<tr>
<td>CD34</td>
<td>0.7%</td>
</tr>
<tr>
<td>CD45</td>
<td>0.0%</td>
</tr>
<tr>
<td>vimentin</td>
<td>90.33%</td>
</tr>
</tbody>
</table>

2.5.2.2 Immunocytochemistry: The mononuclear cells showed a high nucleus to cytoplasmic ratio on giemsa staining (Figure 2.4). Immunocytochemistry examination clearly detected the localization of CD34, CD45, CD11a, CD18 and
CD31 on bone marrow mononuclear cells (Figure 2.4). Rat bone marrow stromal cells were expressing the markers of Vimentin and CD90 but negative for CD45, CD11a, CD18, and CD31 (Figure 2.5).

Figure 2.4: Characterization of rat bone marrow nuclear cells by immunocytochemistry: Bone marrow mononuclear cells showing high nucleus to cytoplasmic ratio by giemsa staining. Immunofluorescence analysis showing the cells positive (green fluorescence) for CD11a, CD34, CD18, CD31 and CD45. Nucleus was counterstained with propidium iodide (red).
Figure 2.5: Immunofluorescence analysis of bone marrow stromal cells showing positive (green fluorescence) for vimentin, CD90 and negative for the CD45, CD11a, CD18 and CD31. Nucleus was counterstained with propidium iodide.

2.5.2.3 RT-PCR: As the RT-PCR results showed expression of Vimentin and collagen type 1 alpha 1 in isolated BMSCs (Figure 2.6). This shows that the isolated cells are genuine marrow stromal cells with little or no contamination from other bone marrow cells such as hematopoietic cells.

Figure 2.6: Gene expression during culture of marrow stromal cells by reverse transcription/polymerase chain reaction (RT-PCR). Samples were run in duplicates. Lane 1&2: Vimentin; Lane 3&4: GAPDH (D-glyceraldehyde-3-phosphate dehydrogenase used as a internal control); Lane 5: 100bp ladder; Lane 6 and 7: Collagen type 1 alpha 1.
2.5.3 Differentiation

2.5.3.1 Adipocytic and Osteocytic differentiation: MSCs were differentiated in vitro using adipogenic, osteogenic and chondrogenic induction media. Following 3 weeks of adipogenic induction, the cells stained Oil red ‘O’ positive showing lipid laden adipocyte phenotype (Figure 2.7). Similarly, when induced with osteogenic induction medium for 2-3 weeks, these cells showed osteogenesis upon staining with alizarin red for calcium deposits (Figure 2.8).

Figure 2.7: Adipocyte differentiation of rat marrow stromal cells. Upon induction with adipocyte induction media cells showed adipocyte globules on oil redo staining. a- before differentiation b- after differentiation with oil red o stain c- after differentiation with oil red o and counter stain with giemsa d- negative control (without induction media). (Magnification, 20X)
2.5.3.2 Neural Differentiation: MSCs when induced with neural differentiation media for 8 days under serum-free conditions started showing neuron like morphology by day 4 with slender dendritic processes and characteristic aura around soma. Under the induction conditions provided in our lab, different types of neuron like cells were observed based on their morphology and axonal polarity like unipolar, bipolar, bipolar pyramidal etc (Figure 2.9). Apart from these, flat glial like cells were also seen extensively. Cultures induced beyond day 10 however gradually lost affinity to polystyrene and floated off the flask surface.
Figure 2.9: Neural Differentiation of Rat MSC. Neural differentiation of bone marrow stromal cells showed expressing the neural progenitor marker nestin and differentiated markers synaptophysin and neurofilament-H. (Magnification, 20x)

Immunocytochemistry was done on stromal cells differentiated into neuronal lineage. The cells were stained for neural specific markers using monoclonal antibodies. The differentiated neural cells stained positive for neural markers like nestin, Neurofilament, Synaptophysin (Figure 2.9).

2.6 Discussion
There are many methods to isolate stromal cells from bone marrow, including plastic adherence (Dexter et al., 1981), gradient density centrifugation (Chen et al., 1991) and Immunomagnetic selection (Dezawa et al., 2004; Jia et al., 2002).
Different methods have their own limitations and advantages. For example, plastic adherence is an easy method of obtaining such cells on the basis of their plastic adherence characteristics, but it is difficult to get pure stromal cells. Gradient density centrifugation depends on the relative density of the cells to separate MSCs. Immunomagnetic selection uses MSC receptors and antigens. Other methods have also been used to isolate MSCs (Silva et al., 2003; Korkko et al., 2001), but none of these has been found to be optimal. In this study we used the Ficoll (1.077g/ml) method to isolate MSCs from bone marrow aspirate. After centrifugation, we found many suspended cells in the medium for 72 hrs. This could be due to the density of cells, which was changed slightly in DMEM. Therefore we combined the density gradient centrifugation with plastic adherence and changed the medium three times to obtain a purer isolate of MSCs after the density gradient centrifugation. According to the results, this method is relatively simple and can easily be used to obtain pure MSCs.

MSCs were first described in 1968 by Friedenstein et al., who discovered that MSCs adhered to tissue culture plates, resembled fibroblast in their morphology, and formed colonies (Friedenstein et al., 1976). These characteristics have been identified in MSCs from numerous species including human, rat, mouse, rabbit and monkey. However, the expandability of MSCs in vitro varied dramatically among different species and different methodologies for isolation and plating of the cells. In our study, the MSCs adhered to the plate and had a fibroblast spindle-shaped morphology, forming colonies when grown in the low plating density. A small number of MSCs have a broad flattened shape.
The in vitro cultures of bone marrow stromal cells serve as a useful system for the investigation of various aspects of these cells, including a) establishing reproducible ways of culturing them in different labs by different techniques and sorting out the different population of cells from the same source 
b) exploring the stem cell like characteristics of these cells c) identifying the common characteristics of all mesenchymal stromal cells obtained from different parts of adult tissue e.g., fat, muscle, limbus, etc. d) exploring the potential of plasticity in vivo and in vitro and e) investigating their ability to reduce immunological rejection when given along with solid organ transplantation.

Several groups have illustrated the multipotentiality of rat bone marrow MSCs and their usefulness as sources for cell therapy. For example, Woodbury et al., (2000) stimulated rat MSCs to differentiate into neurons by plating rat MSCs at 8,000cells/cm$^2$ and growing them to confluency. Passage 6 cells were then used for neuron differentiation (Woodbury et al., 2000). Hofstetter et al. (2002) implanted rat MSCs into the spinal cord. They plated rat MSCs at 5,000cells/cm$^2$ and grew them to confluency. Passage 5 cells were used for implantation (Hofstetter et al., 2002). Dezawa et al. (2001) induced rat MSCs to differentiated into Schwann cells in vitro and implanted into the sciatic nerve. Rat MSCs were subcultured four times and used (Dezawa et al., 2001). None of the authors have noted the quantum of increase of rat bone marrow MSCs; however, the cells had to be replated more than 4 times to harvest enough cells for their purposes. In one report (Yoshimura et al., 2007) obtained $10^8$ cells at passage 4 with the initial density of 6000 cells at passage 2. In our study, we started with
10,000 cells in 75 cm$^2$ flasks at passage 1 and obtained $10^8$ cells at passage 3. These cells seem to be sufficient for in vitro or transplantation analyses. These data indicate that the proliferation ability of our rat bone marrow MSCs compares favorably with those in previous reports. However it needs to be seen whether these cells consist of a single of mixed population of stromal cells.

The stromal cells are non-hematopoietic in lineage (Dexter et al., 1981, Chen et al., 1991). To prove this concept we have performed Flow cytometric analysis for bone marrow mononuclear and stromal cells. Bone marrow mononuclear cells showed positive results for the CD45, CD11a, CD31 and CD18, indicating they are of haematopoietic lineage. Dezawa et al., (2004) have stated that rat mesenchymal stem cells were positive for CD29, CD90 and negative for the CD11b/c, CD31, CD34 and CD45. After culturing, the mononuclear cells, the stromal cells that were adherent at passage 3 were negative for CD45, CD11a, CD31 and CD18, indicating the absence of any hematopoietic contamination in the culture. Additionally, these cultures have shown positive result for CD90 and Vimentin, and Fibronectin, indicating they are pure non-hematopoietic stromal cells.

Prior gene expression profile studies of BMSCs, including micro array analysis, have shown that certain genes such as Vimentin and collagen type 1 alpha 1 are selectively enriched in these cells (Jia et al., 2002; Silva et al., 2003; Korkko et al., 2001). Therefore we selected these genes that confirm the identity of isolated cells. As the RT-PCR results show, these transcripts are expressed in
isolated BMSCs. This indicates that the isolated cells are genuine, non-hematopoietic marrow stromal cells.

The phenotypic analysis shows that the marrow stromal cells do not express markers of hematopoietic lineage but do express markers of non-hematopoietic cells. Based on the phenotypic analysis by IF and FACS and RT-PCR analysis, particularly the presence of CD90, fibronectin and Vimentin antibodies, there is a strong suggestion these mesenchymal cells have stem cell like characteristics. The self-renewal capacity up to an average of 20 passages also points towards the increased potential for proliferation, and some of the cells so obtained may prove to possess stem cell properties.

As for many other adult stem cells, MSCs are traditionally considered to be capable of differentiating into cell types of their own original lineage, i.e. mesenchymal derivatives. This study supports the findings of many other groups (Muraglia A, 2000) in showing that MSCs are capable of forming osteoblasts, chondrocytes and adipocytes in vitro. The ability of clonally expanded cells to form these three distinct cell types remains the only reliable functional criterion available to identify genuine MSCs and distinguish it from preosteoblast, preadipocyte or prechondreocytic cells which each only give rise to one cell type (Halleux C et al., 2001).

In summary, by the simple principle of adhesion, it is possible to establish an efficient method of harvesting a fairly homogenous population of bone marrow stromal cells the phenotypic characteristics of which point towards the stem cell like features.