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Generation of transgenic mesenchymal stem cells expressing green fluorescent protein as reporter gene using no viral vector in caprine

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Mesenchymal stromal cells (MSC) are multipotent cells that can be derived from many different organs and tissues. While there are many ways to label and track cells each with strengths and weakness, the green fluorescent protein (GFP) is a reporter gene commonly employed. In the present study, caprine MSC were collected from bone marrow and cells were characterised with MSC specific markers. Passage 10 (P10) MSC cells were transfected using plasmid vector containing GFP as reporter gene with different concentrations of DNA and lipofectamine. Six different concentrations of DNA and lipofectamine as 1 µg DNA: 2 µL lipofectamine, 1 µg DNA: 2.5 µL lipofectamine, 1.2 µg DNA: 2.2 µL lipofectamine, 1.2 µg DNA: 2.5 µL lipofectamine, 1.5 µg DNA: 2.5 µL lipofectamine, 1.5 µg DNA: 3 µL lipofectamine were used. After 24 h and 48 h of transfection, caprine MSC were observed under florescent microscope. Highest transfection rate indicating green flourecscent MSC were found when the cells were transfected with 1.2 µg DNA: 2.2 µL lipofectamine and 1.5 µg DNA: 2.5 µL lipofectamine than other combinations. These cells have been propagated beyond 4th passage maintaining GFP expression. The results indicated that stable GFP positive MSC cells can be generated using the above protocol. These cells are being used for transplantation studies.

Keywords: Caprine, green fluorescent protein, Lipofectamine, Mesenchymal stromal cells, Transfection

Stem cells are defined by their ability to self-renew and to form one or more differentiated cell types[1,2]. A well-known type of adult stem cells is bone marrow mesenchymal (stromal) stem cells (MSCs), first described by Friedenstein *et al*.[3] MSC are multipotent cells that can be derived from many different organs and tissues. MSCs maintain an undifferentiated and stable phenotype over many generations *in vitro* and are progenitors for different types of somatic cells, such as osteocytes, chondrocytes and adipocytes[4-6]. MSCs have been demonstrated to play a role in tissue repair and regeneration in both preclinical and clinical studies to improve myocardial and cerebral function (after cerebral infarction), repair of liver damage[7], bone fracture[8], healing of wound[9,10], repair of damaged ligaments and joint damage[11,12], repair of spinal cord injury[13,14]. There are several mechanisms proposed for beneficial effect of MSC on tissue regeneration based mostly on laboratory animals. In domestic animal the validation of these hypotheses also needs to be checked for proper application of MSC. But one of the challenges lies in tracking these cells *in vivo* following MSC transplantation. While there are many ways to label and track cells each with strengths and weaknesses, the transgenic MSC expressing green fluorescent protein (GFP) is employed because it can be detected with high sensitivity and specificity, combined with its relative ease of insertion, expression, and detection[15,16].

The generation of transgenic MSC with reporter gene has been attempted in different species using viral and non viral based vectors[17]. In most of the studies it has been reported that plasmid vector is very less efficient[17] and stable transgenic MSC could not be generated. Among viral based transgenesis, lentivirus has been able to produce comparatively stable transgenic MSC[18] in different species including human being. But lentivirus based transgenesis has several limitation particularly due to HIV virus. Further, in domestic animal, very less attempt has been made to prepare transgenic MSC for transplantation studies. Therefore, in the present study, plasmid vector carrying GFP as reporter gene

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has been attempted in caprine MSC for generating transgenic MSC their use in transplantation studies.

Materials and Methods

Collection of bone marrow—The adult goats was anesthetized by the intramuscular injection of xylazine @ 0.3 mg/kg body weight followed by ketamine @ 30 mg/kg body weight about ten minutes later, in the thigh muscles. The area of iliac crest on either side of the animal was prepared in an aseptic manner for bone marrow aspiration. The bone marrow aspirate was collected with the help of an 18 gauge bone marrow biopsy needle from the lateral aspect of superior iliac crest.

The biopsy needle was inserted through the skin, the muscle and through bone by rotating it slowly until the bone cortex is penetrated. The stylet of the needle was removed and about 4-5 mL bone marrow aspirate was drawn into a hypodermic needle containing heparin. The needle was removed and the same procedure was followed in the contralateral bone and another 5 mL bone marrow aspirate was collected in the same syringe. Thus a total quantity of 10 mL of bone aspirate was collected. Tincture iodine was applied at the biopsy site after withdrawal of biopsy needle.

Culture of mesenchymal stem cells (MSC)—The bone marrow sample was mixed with an equal amount of Dulbecco's phosphate buffered saline (DPBS) and layered onto 10 mL of Ficol-Histopaque. The sample was subjected to centrifugation at 2000 rpm for 30 min and the nucleated cells were collected from the interface. The cells were washed twice with DPBS centrifugation (2000 rpm for 10 min each) and the cell pellet was resuspended with small volume of DPBS. The cells were counted and plated at the rate of 2×10^5 cells/cm^2 in 25 mm^2 tissue culture flasks. The cells were maintained in the culture medium with antibiotics (100 units/mL of penicillin and 100 µg/mL of streptomycin) in an atmosphere of 5% CO_2 with 95% humidity at 37 °C for 48 h. The culture medium used for cell propagations was DMEM (low glucose) + 10% serum.

After 2 days of primary culture, all the non adherent cells were removed by changing the medium. The medium was changed every third days and the experiment was repeated for three times under each culture conditions.

Expansion and characterization of mesenchymal stem cell—Once the cells attain 80-90% confluence, the cells (MSC) were passaged several times to increase cell population. The culture medium from normal and transgenic MSC was aspirated and cells layer was washed twice with DPBS without calcium and magnesium. Later, 2 mL trypsin/EDTA (0.25%) was added into the flask and incubated at 37 °C for 5 min or until the cells separated from the bottom of the flask. The equal volume of culture medium was added to the flask and mixed gently with strokes, transferred into 15 mL tube and centrifuged at 1000 rpm for 5 min at room temperature. The cell pellet was resuspended in 5-6 mL DPBS containing calcium and magnesium and immediately the cells number were counted following the standard methods. The cell suspension containing approximately 500,000 MSC were transferred into a culture medium for further passage. Cells were examined regularly for its viability, morphological features and confluence under microscope. After 4th passages, the MSC were characterized for expression of MSC specific markers.

Chromosome analysis—Fourth passage cBM-MSCs were used to perform the chromosome analysis using conventional Giemsa staining. In early growing (48 h of seeding) cBM-MSCs, culture media replaced by colcemid (0.05 µg/mL) containing culture media & incubated for 15 h at 37 °C, 5%CO_2 in CO_2 incubator. The cells were trypsinize and treated with 0.56% KCl hypotonic solution. After this, cells pellet were treated with 3:1 methanol: acetic acid and slides were prepared. These slides were stained with Giemsa stain and observed under microscope at 100×.

RNA extraction and reverse transcription—For gene expression analysis, MSCs were splited into single cells and washed with 1× PBS. Then, transfer the cells in 2 mL centrifuge tube for RNA isolation. The tatal RNA was isolated by Quick-RNA™ MicroPrep, Zymo Research, Catalog No. R1050. cDNA was synthesised using iScript select cDNA Synthesis kit (catalog # 170-8897). After preparing cDNA, O.D of cDNA was maintained at 800 ng/µL and used for real time PCR study.

Real time PCR—Expression of MSC specific genes were done using real time polymerase chain reaction method. Briefly, a Biorad CFXManager™ Software and EvaGreen supermix (catalog # 172-5200), as a double stranded DNA-specific fluorescent dye were used to determine the DNA copy number. For Amplification of genes, primers were designed in beacon software. Primers for real time PCR were GAPDH, THY1 and ENG. The real time PCR

thermocycling conditions were: an initial denaturation step at 95 °C for 30 sec followed by 45 cycles of 95 °C for 3 sec, annealing for 10 sec. Transcript level of all three genes were quantified using the relative quantification method based on comparative threshold cycles values (Ct). The abundance of gene was determined relative to the abundance of the housekeeping gene GAPDH. The expressions of different MSC specific genes were studied in three independent samples. Details of primers (GAPDH, THY1 and ENG) used is presented in Table 1.

Transfection procedure—The following procedures were used to transfect mesenchymal stem cells in a 24-well format with P10 MSCs. The plasmid vector (pMCs-GFP) carrying GFP as reporter gene was used for transfection.

1. Adherent cells: The P10 cells were cultured in 24 wells plate and once the cells were 90-95% confluent, transfection was done.

2. For each sample, vector DNA-Lipofectamine-2000 complexes were prepared as follows:

a. Diluted vector DNA (0.8-1.5 µg) (pGFP-290 ng/µL) in 50 µL of Opti-MEM®, gently mixed.

b. Mixed Lipofectamine gently before use and then diluted the appropriate amount in 50 µL of Opti-MEM® I Medium. Mixed gently and incubated for 5 min at room temperature.

c. After the 5 min incubation, the diluted vector DNA was combined with the diluted Lipofectamine-2000 (total volume is 100 µL). Mixed the content gently and incubated for 20 min at room temperature to allow the vector DNA-Lipofectamine 2000 complexes to form.

3. The medium were removed from well and added 100 µL of DNA-Lipofectamine 2000 complexes to each well containing cells and 150 µL fresh medium were also added. The content was mixed gently by rocking the plate back and forth.

Six different concentrations of vector DNA and lipofectamine were used as 1 µg DNA: 2 µL lipofectamine, 1 µg DNA: 2.5 µL lipofectamine, 1.2 µg DNA: 2.2 µL lipofectamine, 1.2 µg DNA: 2.5 µL lipofectamine, 1.5 µg DNA: 2.5 µL lipofectamine, 1.5 µg DNA: 3 µL lipofectamine. After transfection, the cells were observed under florescent microscope. The transfected cells were passaged by trypsinisation and fluorescent cells were observed in each passage.

Results

The isolated bone marrow was cultured in DMEM with low glucose with 15% FBS and it took around 12-15 days to get confluent monolayer of cells. The confluent cells were passaged every 6-7 days in the same medium. The confluent culture of caprine MSC at passage 10 has been presented in Fig. 1A. Normal karyotyping patterns were found in the cultured cells (Fig. 1B). These cells were characterised using MSC specific markers including ENG and THY1, by real time PCR (Fig. 2). The ct values of GAPDH (20.14), ENG (22.04) and THY1 (24.73). The PCR products were used for gel electrophoresis in 2% agarose to conform the results (Fig. 3).

P10 cells were cultured in 24 wells plate and incubated in CO_2 incubator till cells were confluent up to 85-90%. Confluent P10 MSC cells were transfected using plasmid vector containing GFP as reporter gene with different concentrations of DNA and lipofectamine. Following transfection, the caprine MSC started expressing GFP after 24 h of transfection (Data not shown). It was observed that all concentrations of DNA and lipofectamine combination, used in the present study, transfection was achieved, but the efficiency of transfection varied among different transfection protocol (Fig. 4A-F). It was observed that out of six combinations used, more number of GFP positive cells were observed when transfection was done using 1.2 µg vector DNA: 2.2 µL lipofectamine and 1.5 µg vector DNA: 2.5 µL lipofectamine Fig. 4 (C and E). When the transfected

Table 1—Primers and conditions used for gene specific real time PCR analysis

S. No	Target Gene	Primer sequence, 5'-3'	Annealing temperature (°C)	Product (bp)	Accession no.
1.	GAPDH	f 5´ggagaaacctgccaagtatg3´ r 5´tgagtgtcgctgttgaagtc3´	65	126	DQ152956.1
2.	THY1 (CD90)	f 5´cctcctgctaacagtcttac 3´ r 5´atccttggtggtgaagttg 3´	60	271	BC104530
3.	ENG (CD105)	f 5´agcgatggcatgactctg 3´ r 5´aggctgtccgtgttgatg 3´	65	251	NM_001076397

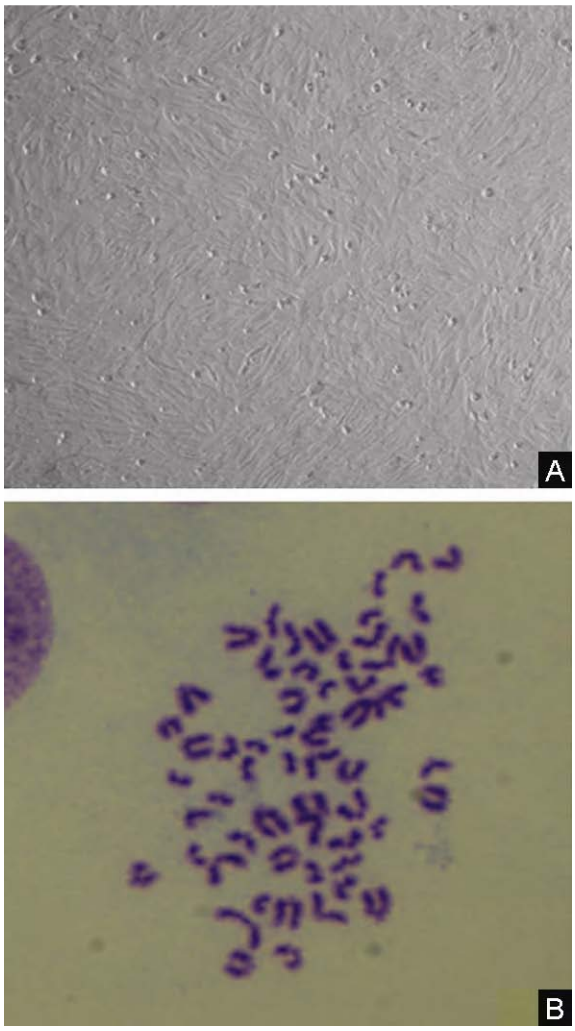

Fig. 1—A: Development of caprine MSCs in FBS supplemented Low glucose DMEM medium. Bone marrow were collected from caprine pelvic bone and cultured in tissue culture flask and it was further passaged subsequently. Figure showing fourth passaged confluent mesenchymal stem cells (4×). B: Chromosome analysis-normal karyotypic pattern were found.

cells were further passaged, the cells lost GFP expression at subsequent passages (Fig. 5). At 3rd passage following transfection (P3), comparatively less number of cells were showing the green fluorescence in all the combination of transfection (and the intensity of green fluorescence were also decreased subsequently (Fig. 5).

Discussion

The possibility of transplanting autologous adult stem cells into damaged organs has opened prospects for treating severe diseases such as acute myocardial infarction[19-21], many neurological disorders including spinal cord injury, traumatic brain injury[14], Parkinson disease[22], stroke[23] etc. MSCs can be explored for their use in both preclinical and clinical studies. Mesenchymal stem cells have wide range of applications in regenerative medicine because they have a strong immunosuppressive effect on the host immune system and alter the relative level of pro- and anti-inflammatory cytokine expression by T cells[24]. Among the various possible sources of such cells, MSCs have been studied extensively because of their ability to self-renew and to give rise to a variety of differentiated cell types[25,5,6,2] and because of the relative ease with which they can be obtained from bone biopsies and cultured.

To be certain that this particular cell population, was separated from an aspirate of bone marrow harvested from the superior iliac crest of the pelvis[26,25], an isolation technique that is considered to be specific for separating MSCs[27,5,6,28] was used.

In addition, we have shown that all cells expressed MSC specific markers as observed by different groups in different species[25,29].

GFP is widely applied for stem cell tracking because of the possibility of its stable expression, high specificity, and its easy *in vivo* tracing[30]. Lee *et al*[31] transduced human MSCs by a GFP-retroviral vector. Viral vectors have been also used in other similar studies[32,33]. In the present study, the non-viral plasmid vector was used for transfection of bone marrow derived caprine MSCs and expression of GFP was observed in these cells albeit the number of cells varied with different transfection protocol (Fig. 4). It was observed that when transfection was done using 1.2 µg DNA: 2.2µl lipofectamine and 1.5µg DNA: 2.5µl lipofectamine (Fig. 4C and E), the number of MSC cells showing GFP was higher than other combination. But unfortunately, the number of cells showing GFP expression in subsequent passage per field was decreased (Fig. 5). The reasons for loss of GFP expression could not be ascertained as similar studies in domestic animal are scanty. It may appear that initial higher passage cells used in this study or loss instability of chromosome numbers following transfection may be responsible. But we have observed that cMSC expressed MSC specific markers as well as sowed the capacity to differentiate into mesodermal lineages all the way upto 21 passages. The maintenance of MSC stemness during prolong culture has been supported by Peters *et al*[34] who claimed that MSC displayed a fibroblast-like morphology, expressed several stromal/MSC-related

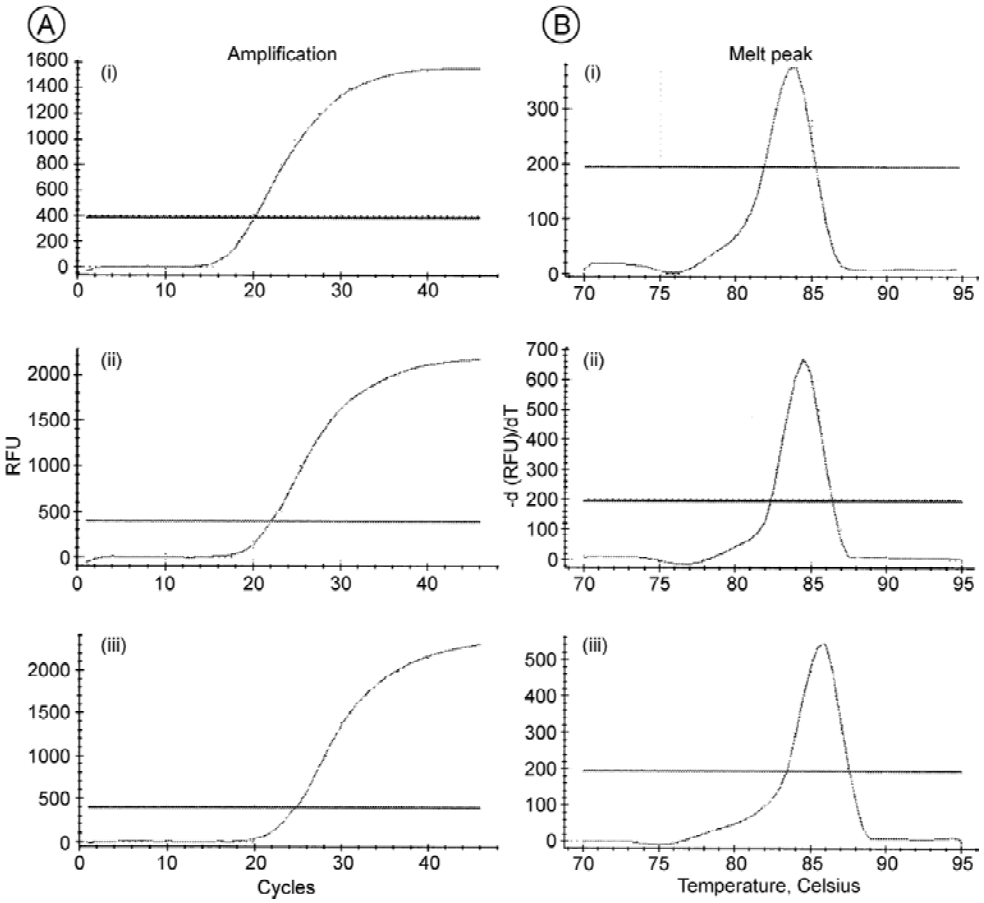

Fig. 2—Amplification (A) and Melting (B) curves of GAPDH (i), ENG (ii) and THY1 (iii).

antigens (CD105, CD73, CD29, CD44, CD133 and Nestin) but were negative for haematopoietic cell markers (CD45, CD34 and CD14). They reported that MSC stemness phenotype and their differentiation capacity in vitro before and after high dilution were preserved throughout long-term culture. Even at passage 24, cells remained Nestin+, CD133+ and >95% were positive for CD105, CD73, CD29 and CD44 with the capacity to differentiate into mesodermal lineages[34]. In another report, cultured amniotic fluid-MSCs and BM-MSCs from the 3rd-17th passages were characterized by flow cytometry analysis (FACS) for MSC specific markers suggesting that bone marrow derived MSCs maintain stamnenss even at 17th passage[35]. The human MSCs from first trimester placenta underwent up to 20 passages and displayed no visible changes with regard to their morphology by light microscopy, forward and side scatter properties on flow cytometry, or growth patterns[36]. These results supported mesenchymal stem cells can be propagated continuously without losing MSC characteristics.

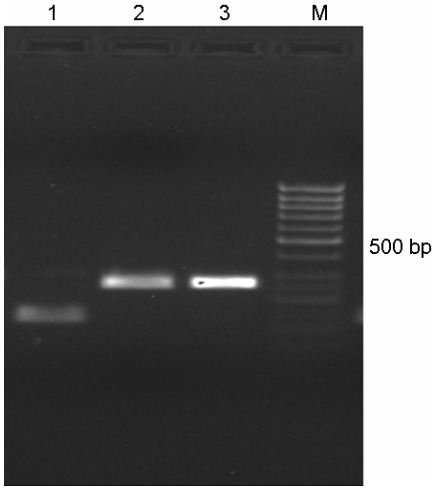

Fig. 3—Caprine MSCs were cultured and propagated up to P10 in DMEM low glucose supplemented with 15% FBS. Once the cells are confluent, It was splitted into single cells and washed with 1× PBS. Molecular characterization was done using RT-PCR of MSCs specific markers THY1 and Endoglin (ENG). The amplified products of mesenchymal stem cells was used for gel electrophoresis in 2% agarose gel [Lane M: 50 bp DNA ladder; Lane 1: GAPDH-126; Lane 2: THY1-271; Lane 3: ENG-251].

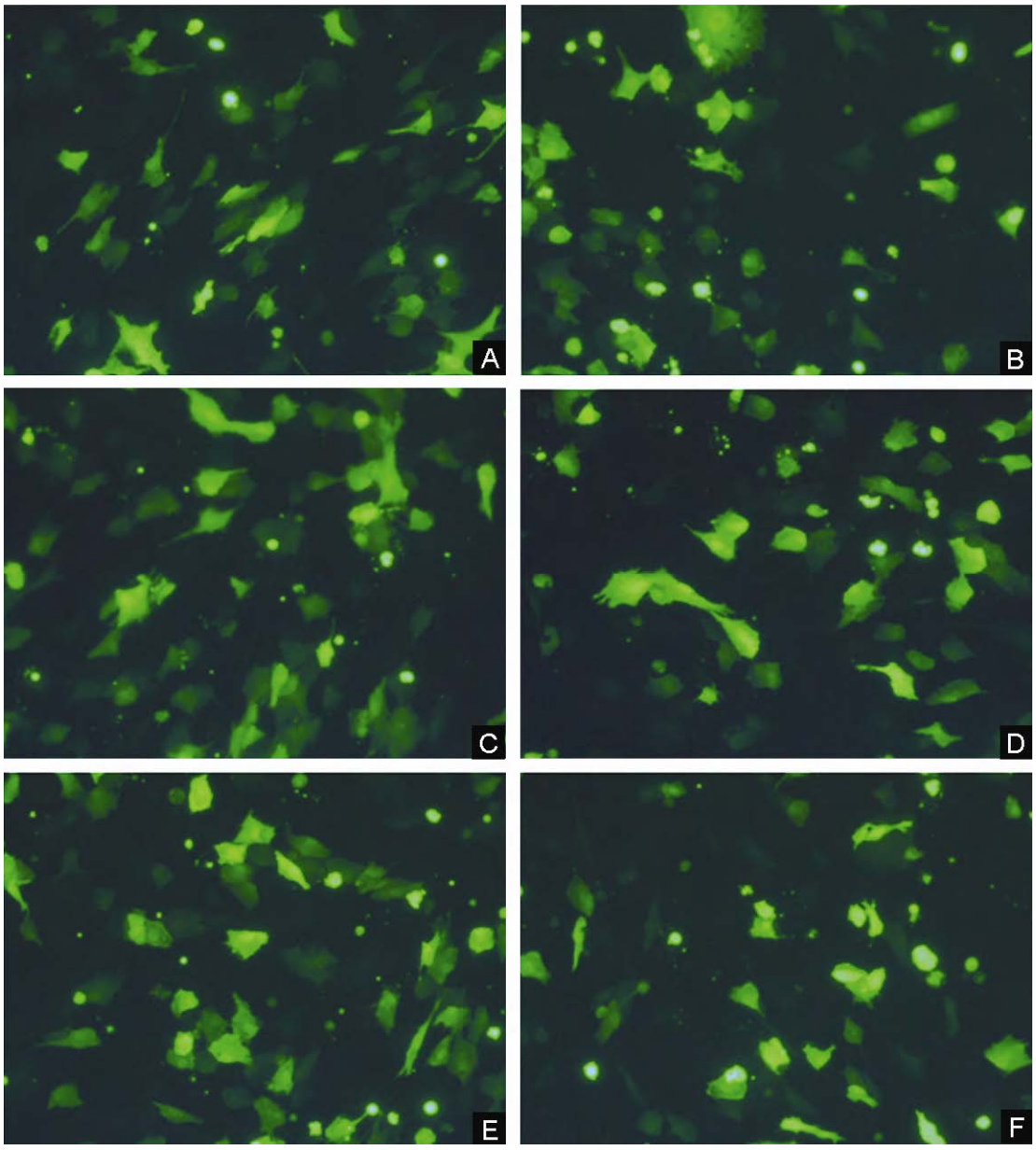

Fig. 4—Effect of different concentration of DNA and lipofectamine in P10 Caprine MSCs on transfection. Different combinations of DNA and lipofectamine were used to transfect the Caprine MSCs and found that combinations of 1.2 µg DNA: 2.2 µL lipofectamine and 1.5 µg DNA: 2.5 µL lipofectamine showed the highest transfection than other combinations used. A: 1 µg DNA: 2 µL lipofectamine, B: 1 µg DNA: 2.5 µL lipofectamine, C: 1.2 µg DNA: 2.2 µL lipofectamine, D: 1.2 µg DNA: 2.5 µL lipofectamin, E: 1.5 µg DNA: 2.5 µL lipofectamine, F: 1.5 µg DNA: 3 µL lipofectamine. (100×).

When karyotypig was done, a normal chromosome number was observed during propagation of these caprine MSC. A normal karyotype, as assessed by GTG banding, was confirmed in cultured AF-MSCs samples in different passages (passages 2-30). The normal karyotype of high-passaged (passage 30) cultured AF-MSCs implies that the extensive proliferation potential is associated with the fetal stage and is not related to any transformation status[35].

MSC has been a difficult cell for making a stable transgenic cells using plasmid base non viral vector[17]. The higher efficiency of transfection and stable expression has been achieved by using lentiviral vectors in different species[17]. But it should always be considered that the safety of viral vectors is a major concern in application of MSCs in transplantation. The probable loss of GFP expression may not be due to higher passage of MSC. So far no stable transgenic

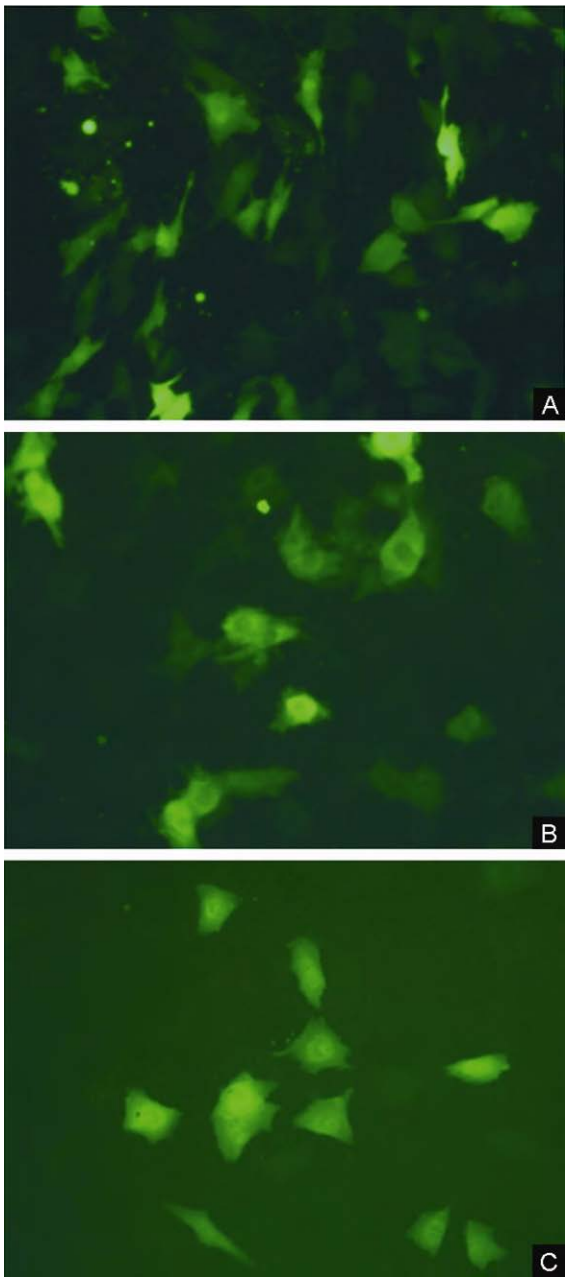

Fig. 5—The caprine MSCs transfected with the combination of 1.2 µg DNA: 2.2 µL lipofectamine were further passaged after 4-5th days of transfection and cultured subsequently. Cells were maintained more than 3 passage. It was observed that cells were loosing the GFP expression in subsequent passages as shown in figure above, A: GFP positive cells in Passage1 showing more number of GFP positive cells then Passage, 2 (B) and Passage 3 (C). (100×).

MSC could be derived in any domestic animal using plasmid vector. It needs to some kind of modifications in protocol to generate stable transgenic MSC for the transplantation studies.

In conclusion, in the present study, transgenic MSC were generated from bone marrow derived caprine MSCs which expressed green fluorescence protein as a reporter gene and recombinant non viral plasmid vector can be used for this in caprine. However, further work is needed to make a stable transgenic MSC line in caprine.

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