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HIGHLIGHTS

The present study showed that caprine mesenchymal stem cells could be successfully isolated, cultured and characterized from umbilical cord tissue cells. Their morphology, immunophenotype and differentiation potential are comparable with MSCs from other source. The results of the present study demonstrated the proliferative and differentiation potential of cUCTs which could serve as a potent source of mesenchymal stem cells. With future research and standardization they could serve as valuable resource for various clinical applications.

Isolation and characterization of mesenchymal stem cells from caprine umbilical cord tissue matrix

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ABSTRACT

Cord tissue fills the umbilical cord around the blood vessels and contains types of stem cells (mesenchymal stem cells or MSCs) that are not generally found in cord blood. MSCs are the stem cells that give rise to many of the "support tissues" in the body, including bone, cartilage, fat and muscle. Umbilical Cord Tissue cells (UCTs) possessing the capacity to differentiate into various cell types such as osteoblasts, chondrocytes and adipocytes have been previously isolated from different species including human, canine, murine, avian species etc. The present study documents the existence of similar multipotential stem cells in caprine UCTs having similar growth and morphological characteristics. The cells were isolated from caprine umbilical cord and cultivated in DMEM (low glucose) supplemented with 15% FBS, L-glutamine and antibiotics. Primary culture achieved confluence in 5-7 days having spindle shaped morphology. The cells were morphologically homogeneous, showed robust proliferation ability with a population doubled time of 92.07 h as well as normal karyotype. In vitro self-renewal capacity was demonstrated by colony-forming unit assay (CFU). The cells expressed MSC specific markers and showed multi-differentiation capability into adipogenic and osteogeneic. The results indicated that caprine UCTs (cUCTs) were isolated and characterized from umbilical cord tissue which can be used for tissue regeneration.

Keywords: Caprime, umbilical cord tissue, mesenchymal stem cells.

1. Introduction

Since the discovery of embryonic stem cells (ESC) in mice (Evan and Kaufman 1981) and human being (Thomson et al., 1998), adult stem cells were isolated from various tissues as an alternative to ESC. These adult stem cells are mostly multipotent and undifferentiated cells (Cai *et al*., 2004) and now a preferred cell types for therapeutic application because of several inhereant advantages as well as no ethical issues are involved. These postnatal sources of stem cells can be achieved from any tissue type including brain (Uchida *et al.*, 2000), bone marrow (Minguell *et al*., 2001), adipose tissue (Zuk *et al.*, 2001), amniotic fluid (De Coppi *et al.*, 2007), umbilical cord blood (Lee *et al*., 2004) etc. The umbilical cord is an extraembryonic structure essential to provide feeding for the fetus during the intrauterine development. The umbilical cord is formed early during gestation and encloses the yolk sac, which is the embryonic source of two different populations of mesenchymal stem cells. This structure contains mesenchymal stem cells or unrestricted somatic stem cells isolated from fresh umbilical cord blood at the time of birth (Fuchs *et al.*, 2005) and fibroblastoid mesenchymal stem cells isolated from umbilical cord matrix (Mitchell *et al.*, 2003) that can be collected and stored after birth for therapeutic uses.

Previous studies have shown that cells derived from human (Mitchell *et al.*, 2003) or porcine umbilical cord matrix are capable of expressing a variety of stem cell characteristics (Carlin *et al*., 2006). The stem cells isolated from the umbilical cord have properties that make them of interest. For example, they are simple to harvest through non-invasive methods, provide large numbers of cells without risk to the donor, the stem cell population may be expanded *in vitro*, cryogenically stored, thawed, expanded, genetically manipulated and differentiated *in vitro* (Mitchell *et al*., 2003; Fu *et al*., 2004; Wang *et al*., 2004; Carlin *et al*., 2006).

In the present study, stem cells were isolated from caprine umbilical cord tissue, studied their ex vivo proliferation rate and differentiation ability into adipogeneic, chondrogenic cells, transdifferentiation ability into neural cells etc. These cUCT can be used for therapeutic purposes including spinal regeneration.

2. Material and Methods

2.1. Primary isolation and expansion of cUCTs

In the present study, we have isolated and differentiated Caprine Umbilical Cord Tissue cells (GUCTs). Uteri of pregnant goats were collected from nearby slaughter house and were transported within 2 h to the lab. For isolation of cUCTs from umbilical cord tissue, cord were obtained from the late-gestation fetuses and placed in sterile phosphate buffer saline supplemented with amphotericin B, penicillin and streptomycin. Umbilical cord segments, 5 cm in length, were cut longitudinally and then the umbilical cord artery and veins were cleared off. The remaining umbilical cord tissue was cut into 2 × 2 mm^2 segments by using small scissors. The umbilical cord tissue was chopped in a plate containing Dulbecos Modified Eagle Media (DMEM) supplemented with fetal bovine serum (15%) and antibiotics (Streptopenicillin, In vitrogen, cat no15140–122) @10 000 units/ml. The cells were separated by in-out pipetting and the cell suspension was centrifuged at 500 rpm for 2–3 min. The supernatant containing single cells were collected in a separate centrifuge tube. This tube was centrifuged at 1000 rpm for 5–10 min and the supernatant was discarded. The washing was repeated at least three times. The cells were then resuspended in cUCTs medium (DMEM+ 15%FBS and antibiotic) and further cultured in T25 cm^2 tissue culture plate in a CO_2 incubator at 37˚C, 5% CO_2 and maximum humidity. The cells were observed for the time taken for attachment, initial colony formation and time required for 70–80% confluence. Once the cells achieved this confluence, they were continuously propagated by trypsinization method in the same cUCTs medium supplemented with 15% FBS.

2.2. Growth kinetic studies of cUCTs

The growth kinetic study was done by counting the trypsinized cells manually. cUCTs were cultured in 12 well plate at a density of 50,000 cells/well. The numbers of cells of each dish were counted every day. The population doubling time of cUCTs was estimated on logarithmic growth phase based of the cell growth curve.

2.3. Chromosome Analysis

Karyotyping was done with as per the already standardized method in our laboratory (Kumar et al., 2014). The actively growing cUCTs were taken and incubated with colchicine (0.1 µg/ml) for 4 h at 37◦C in CO_2 incubator. The treated cells were washed twice with DPBS and subjected to trypsinize to get single cell. Individual cell was incubated in a hypotonic solution (68 mM KCl) for 20 min at 37˚C. Then, the cells were dropped about 2 feet height onto ice cold

glass slides in methanol: acetic acid solution (1:1) and fixed in chilled fixative comprising of methanol and glacial acetic acid (3:1) for overnight at 4°C. Glass slides were washed to remove fixative and then the air-dried cells were stained with Giemsa stain (5%) for 30 min and observed under oil immersion (100X) with a compound microscope.

2.4. Cell viability study during propagation

For viability, cells were trypsinized into single cell and stained with propidium iodide (GalBiochem, Cat. No. 537059) stain to check live and dead cells by FACS (Becton Dickinson, San Jose, CA, USA). The data obtained was analyzed by using Cell Quest program and plotted as single parameter histogram.

2.5. Immunofluorescence staining of cUCTs

The immunocytochemistry of cUCTs was done by MSC specific markers, Thy, Endoglin and CD73 as per the methods followed by the National Institutes of Health (NIH), 2009 resources for stem cell research, using fluorescent isothyocynate (FITC) conjugated antibody. The cUCTs were grown over cover slip in six well tissue culture plates till it reached 60–70% confluence. The cells were fixed with 4% paraformaldehyde in 1X PBS for 20 min followed by permeabilization with 0.3% Triton X-100 in PBS for 20 min for detection of intracellular markers. The cells were washed thrice with PBS at room temperature (RT) and nonspecific binding sites were blocked with 10% normal goat serum in PBS for 40 min at RT. The cells were then incubated with primary antibodies viz Thy (Santa Cruiz # sc-9162), Endo (Santa Cruiz # sc-19793) and CD73 (Santa Cruiz # sc-19793) for overnight at 4◦C. After thorough washing, the cells were further incubated with appropriate secondary antibodies (1:500 dilution) for 1 h followed by counter staining with DAPI for 5 min. Slides were mounted with pro-Long gold antifade agent (Invitrogen, Catalog no-P36930) and the cells were examined under ZEISS fluorescent microscope. For control, cUCTs were stained simultaneously without addition of primary antibody.

2.6. Osteagenic and adipogeneic differentiation of cUCTs in vitro

To induce osteogenic differentiation, cUCTs were plated on gelatin coated coverslip in a six well plate tissue culture dish in MSC medium. After 48 h of culture, the media was replaced with osteogenic differentiation medium (DMEM containing 10% FBS and 10 nmol-Dexamethasone, 10 mmol –β-glycerophosphate, 0.3 mM –L-ascorbic acid) for additional 7 d. The osteogenic differentiation was assessed by Alizarin red staining. For adipogenic differentiation, the cells were cultured in adipogenic induction medium consisting of DMEM containing 10% FBS, 100 nmol-Dexamethasone, 50 mg/ml indomethacin and 10 µg/ml insulin. The induction medium was changed after every 3rd day and after reaching confluence, cells were fixed with 4% paraformaldehyde for 10 min and were stained for the lipid droplets by Oil Red O staining.

2.7. Molecular analysis on cUCTs

For gene expression analysis, cells of different passages were taken from monolayer culture separately and washed with 1X PBS. The total RNA was isolated by Quick-RNATM MicroPrep (Zymo Research, Catalog No. R1050). The quality of RNA was assessed by running the agarose gel electrophoresis. The cDNA was synthesized using iScript select cDNA Synthesis kit (Biorad, catalog # 170–8897). The expression of MSC specific genes viz Thy, Endo and CD73 was done by real time polymerase chain reaction method using EvaGreen supermix (Biorad, catalog # 172–5200). The primers used in the study have been presented in Table 1.

2.8. Colony forming unit assays

To assess the capacity and efficiency for self renewal, cells (P2) were seeded at low dencity and new fibroblast colonies derived from single cells were counted. Following expansion cells were seeded in 6-well culture plate (50 cells/cm^2). Day 15 cultures were fixed and stained with 1% crystal-violet in 100% methanol (Mensing *et al*., 2011).

Result

3.1. Primary isolation and expansion of cUCTs

cUCT cells from each fraction were morphologically homogeneous populations at the early stage and looked as fibroblast-like cells in of the primary culture. However, on reaching confluence, these cells spindle shaped morphology that looks like as three-dimensional structures

and this behavior was consistently observed when the culture reached the confluence at every subsequent passage (Fig. 1).

3.2. The growth curve studies of cUCTs

Growth curve of cUCTs was obtained by counting the cells manually and the average of population doubling time was calculated based on the logarithmic growth phase (Fig. 2).The average population doubling time was about 92.07 h.

3.3. Chromosome Analysis

All the cells maintained normal karyotype (Fig. 3) during in vitro propagation.

3.4. Cell viability study during propagation

Cell viability during passages was analyzed by PI staining and the cells counted by FACS, showed 93% live (Fig. 4) cells.

3.5. MSC identity of cUCTs

For characterizing the cUCTs, specific markers of MSC were tested. Immunofluorescent staining showed that cUCTs expressed Thy (Fig. 5 A-C) Endoglin (Fig. 5 D-F) and CD73 (Fig. 5 G-I). As shown by RT-PCR, the isolated cUCTs cells expressed MSC-marker genes (Thy, Endo and CD73) and lacked hemato-poietic ones (CD34 and CD45). PCR products also run on gel electrophoresis. (Fig. 5J)

3.6. Osteogenic and adipogeneic differentiation of cUCTs in vitro

After 10 days of induction, osteogenic differentiation was confirmed by Alizarin red staining, which was more intense in intervascular than perivascular cells. The control (non induced cells) was negative for Alizarin red staining (Fig. 6 A-B). The isolated cells were also able to undergo adipogenic differentiation, as demonstrated by the development of positive staining for Oil Red O after 10 days of culture in adipogenic induction medium. Control cells, maintained in regular control medium, showed no lipid deposits (Fig. 6 C-D).

3.6. Self renewal capacity CFU assays

CFU assays demonstrated that cultures contained a subpopulation of cells capable of generation new fibroblast colonies from single cells (Fig.7).

Discussion

From past few years, parallel to the great efforts for exploring the novel and alternative sources of stem cells in the human and animal body, the umbilical cord appeared to be a promising reservoir of fetal cells that could be easily used as multipotent stem cells. There are many reports of the characterization and stem cell potency of cells isolated from the umbilical cord (Can and Karahuseyinoglu, 2007,Majore et al., 2011, Puranic et al., 2012), but only a few reports were observed in animals (Zucconi *et al*.--, 2010; Raoufi *et al*., 2011). In present study we report characteristics of the isolated cUCTs and its differentiation.

In the present study, caprine umbilical cord matrix isolated cells displayed spindle-form cells and confluent cells were arranged in parallel arrays (Fig. 1). Similar reports exist from Babei et al., 2008 and later by Azari et al., 2011 and Pratheesh et al., 2014 for caprine species. The ability to generate clones, e.g., cellular colony derived from a single cell, is a formal demonstration of the self-renewal ability, a characteristic of stem cell populations (La Rocca *et al.*, 2009).

In the present investigation, the doubling time of GUCTs was shorter than neural stem cells (Kumar et al., 2014). Shorter doubling time is a common feature for mesenchymal stromal cells derived from fetal blood (Campagnoli *et al.*, 2001). Also, from the previous reports of our laboratory, it can be concluded that doubling time of umbilical cord blood mesenchymal stromal cells is shorter than adult bone marrow-derived mesenchymal stromal cells (Manish Kumar, 2013). This feature was thought to reflect the relatively primitive nature of mesenchymal stromal cells compared to the adult stromal cells (Troyer and Weiss, 2008).

For characterization, we carried out immunocytochemistry assays. cUCTs exhibited specific mesenchymal stem cell marker expression (CD73, Thy-1 and CD105) based on RT-PCR and immunocytochemistry results. The general strategy for identifying in vitro cultivated mesenchymal stem cells as per ISCT (International Society for Cytotherapy) is to analyze the expressions of cell-surface markers such as CD-73, Thy-1 and CD-105. We demonstrated that the cUCTs are positive for CD-73, Thy-1, and CD-105 whereas, negative for CD-34, a cell-surface marker associated with lympho-hematopoietic cells. The real time data also demonstrated the expression of Thy-1, Endoglin, CD-73. The present study also demonstrated that they

successfully differentiated into osteogenic and adipogenic lineages. This is at par with similar reports from human, equine, and bovine umbilical cord tissue and explanted as primary culture (Wang et al., 2014, Raufi et al., 2011).

The present study showed that caprine mesenchymal stem cells could be successfully isolated, cultured and characterized from umbilical cord tissue cells. Their morphology, immunophenotype and differentiation potential are comparable with MSCs from other source. The results of the present study demonstrated the proliferative and differentiation potential of cUCTs which could serve as a potent source of mesenchymal stem cells. With future research and standardization they could serve as valuable resource for various clinical applications.

Acknowledgments

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Table 1: List of primers used in the study

S. No	Genes	Primer sequence	Product (bp)
1	Thy (CD90)	F: 5'-CCTCCTGCTAACAGTCTTAC-3' R: 5'-ATCCTTGGTGGTGAAGTTG-3'	271
2	Endo (CD105)	F: 5'-AGCGATGGCATGACTCTG-3' R: 5'-AGGCTGTCCGTGTTGATG-3'	251
3	CD 73	F: 5'-AACACACAGTGGTGCTCTCTTCC-3' R: 5'-TGTTGTCTTGGGTGTGTGTGCCTAGA-3'	401
4	CD 34	F: 5'-CAGCCTCTACGATGTCTC-3' R: 5'-CAGCCTCTACGATGTCTC-3'	276
5	CD 45	F: 5'-AACCGCTCTCTCAACCATAG-3' R: 5'-TCATCTTCCACGCAGTCTAC-3'	288

Legends

Figure-1. Morphology of GUCTs at different passage (A) P0 (B) P3 (C) P6

Figure 2. The growth curve of GUCTs was obtained by cell count manually and average doubling time was counted from the logarithmic growth phase.

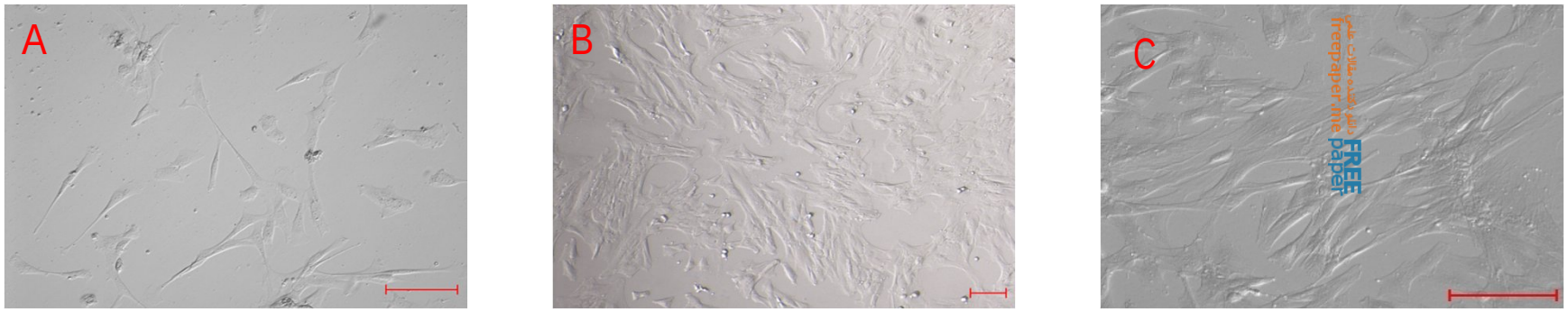
Figure 3. GUCTs maintained the normal caprine karyotype throughout the *in vitro culture period*

Figure 4. The cell viability was analyzed by PI stained cell with FACS.

Figure 5. Charracterization of GUCTs by Thy (A-C), Endog (D-F) and CD73 (G-I) MSC specific markers. RT-PCR results in this figure indicated that cUCTs expressed Thy, Endo and CD 73 marker (J) Scale bar = 100 μm

Figure 6. Transdifferentiation of GUCTs. (A.) showing differentiated cells into adipogenic cells from GUCTs and (B) showing control without differentiation media. Osteogeniccells differentiation (C) and control D) without differentiation media. Scale bar = 100 μm.

Figure 7. CFU assay of GUCTs. Cell colonies (P2) were stained with 1% crystal-violet in methanol at day 15 of culture



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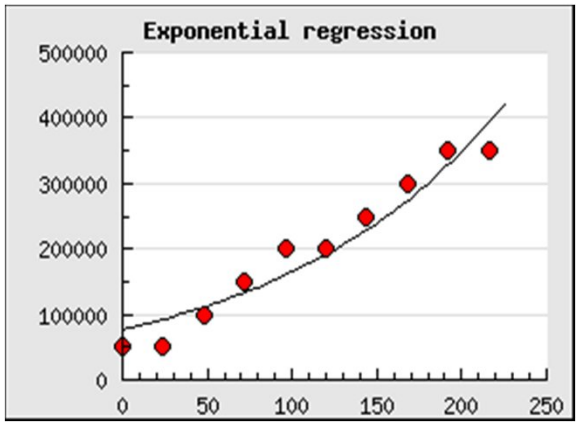

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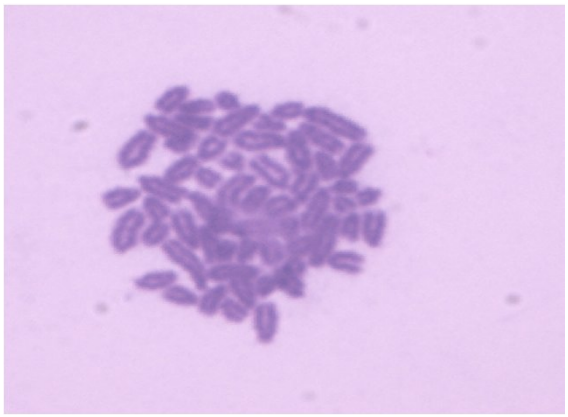

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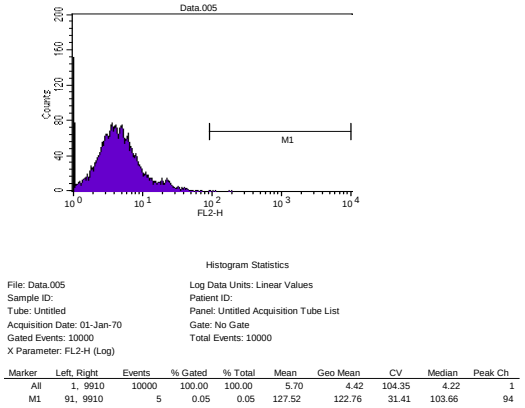

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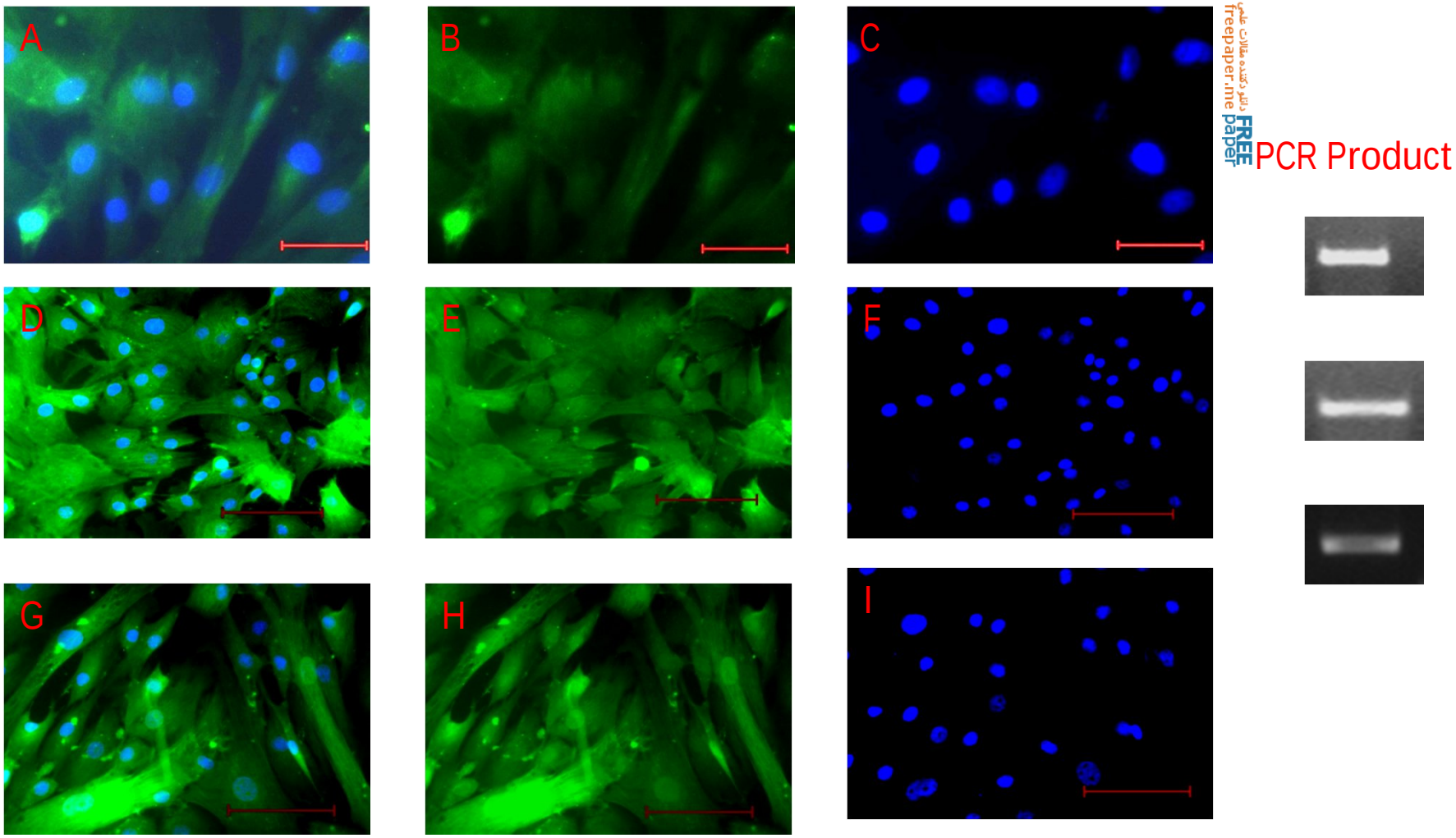

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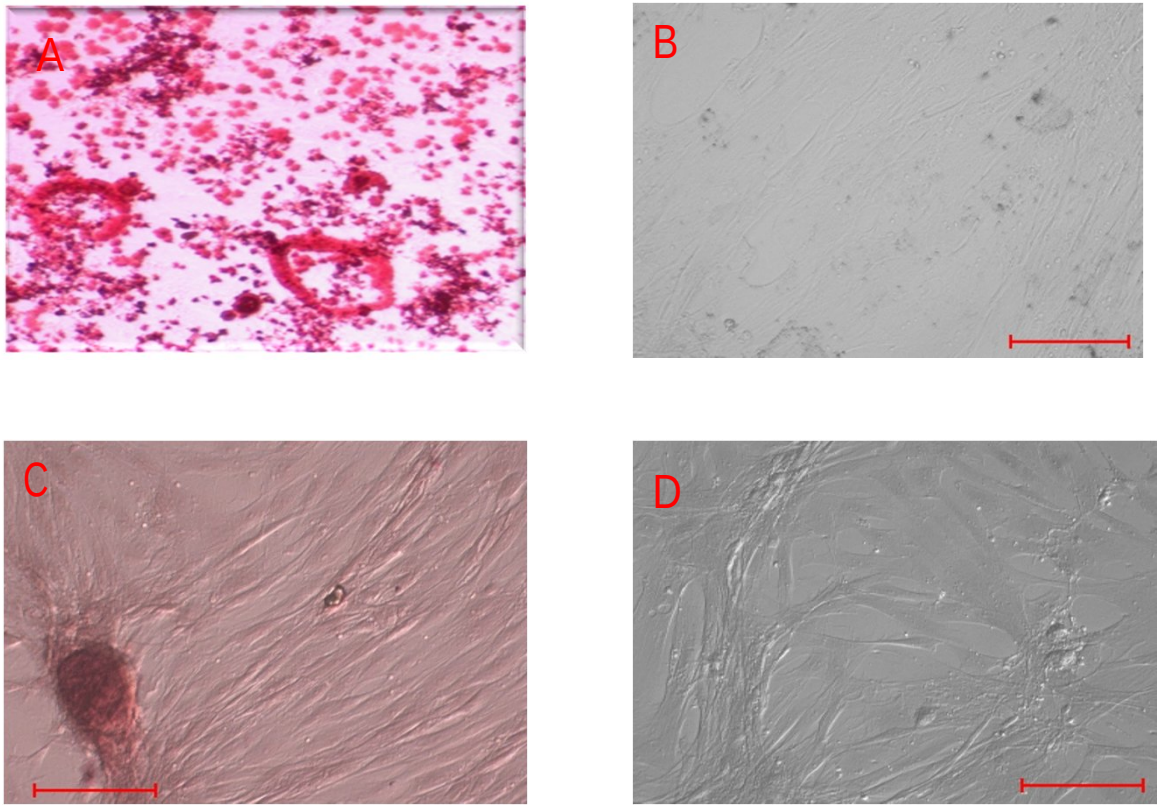

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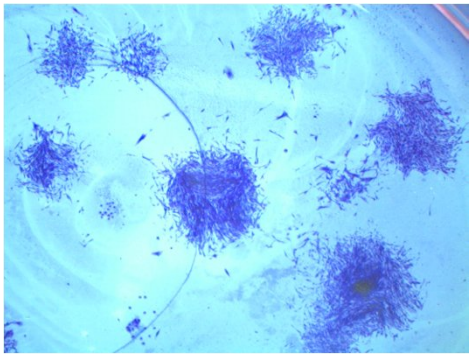

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