Isolation and Characterization of a New Cell Line of Retinoblastoma

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ABSTRACT

BACKGROUND: This article describes the characterization of a new cell line (IRB-1), which was established from an Iranian child with retinoblastoma.

METHODS: Using cell culture techniques, we isolated a clonal population from primary Cultures of retinoblastoma tumor samples and after several passages an established cell line, IRB-1, was obtained. The following techniques were performed: cell proliferation studies by MTT assays, scanning electron microscopy, cell surface analyses by flowcytometry, creation of neurospheres in soft agar and the expression of ABCG2, RAR-α, GFAP, and ACTB genes by real time PCR.

RESULTS: Of total 6 received samples, only a single cell line was successfully isolated froma group E sporadic unilateral retinoblastoma. The primarily enucleated left eye had a differentiated retinoblastoma, with a slight degree of necrosis in pathology report. The adherent cultured cells were in spindle-shaped morphology with similar appendages and pulled neurons. Doubling time is 36 ± 5 hours. After 30 days, the effect of retinoic acid on

isolated cells and expression of RAR-α receptor was appeared. Chromosomal abnormalities in chromosomes 3, 9, 10, 11, 14, 16, 17, and 22 were observed. Specific gene expression of ABCG2, RAR-α, and ACTB gene expression of GFAP positive expression of these genes in the control cells were observed. This cell line passaged 132 consecutive times.

Discussion: IRB-1 is a novel human adherent RB cell line, which with interesting characteristics of adherent and flocculent growth and more chromosomal imbalances different from other RB cell lines. A novel human RB cell line, IRB-1, was established from a 1-year old patient with a unilateral sporadic RB at Farabi Hospital- Tehran University Medical Sciences. The cultural characteristics of IRB-1 cell line were comparable to those of Y79 in characteristic.

Key words: Retinoblastoma, Cell line, Real Time Polymerase Chain Reaction, Gene Expression Profilings

Introduction

Retinoblastoma (RB) is the most common intraocular childhood cancer, presenting as hereditary and non-hereditary forms. [1] It arises from undifferentiated photoreceptors [2-4] with acquired defects in both alleles of the retinoblastoma susceptibility gene (RB1). The majority of cells, however, undergo cell death. [5] During the evolution of the untreated disease, the RB cell can become more aggressive with a potency of extension and cells immortality. [6] According to this idea, several immortalized human retinoblastomas cell lines have been generated. [7-10] the majority of in vitro and in vivo investigations for RB have been carried out using human RB cell lines.

Herein, we report the establishment and characterization of a new, spontaneously immortalized human retinoblastoma cell line, designated "IRB-1". This newly established cell line could be a domestic in vitro model to provide the means for the study of human RB and response to treatment, according to the ethnic.

Materials and methods

Retinoblastoma tumors were obtained under human subject research protocols approved by the Institutional Review Boards (The Tehran University of medical sciences granted Ethical approval to carry out the study within its facilities (Ethical Application Ref: IR-.TUMS.REC.1390.13523). Written informed consent was obtained and the study followed the tenets of the Declaration of Helsinki. A 1-year-old Iranian male patient with no family history of retinoblastoma was referred to Farabi Hospital for more evaluation for leukocoria. On October 2012, examination revealed a big group E RB tumor in the left eye and the affected eye was primarily enucleated. The globe was incised and a portion of the tumor was immediately transferred to the Stem Cell Unit (SPU) of Farabi Hospital for tissue culture

studies.

Isolation and Culture Methods

Tumor tissue was placed in 2 ml cell preserving medium (50:50, v/v), washed five times in Hanks' balanced saline and minced with a scalpel into 1– 2 mm^3 pieces. After digestion by collagenase (sigma USA), explants were cultured in Dulbecco minimum essential medium (DMEM, Gibco) with 15% fetal bovine serum (FBS, Gibco), penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37°C in a humidified incubator containing 5% carbon dioxide. After 2 weeks, fibroblast-like colonies were formed and the explants was moved to a new container in which outgrowths of epithelial cells were evident. Tissues were removed and the media was supplemented with selective media containing DMEM/F12, 10% FBS, 5µg/ml insulin, 1µg/ml hydrocortisone and 10 ng/ml EGF. Cell growth was monitored and gradually, the EGF, hydrocortisone and insulin were removed from the culture media within a week. Tumor explants remained in suspension or were adherent to the bottom of the culture flask. The ability to grow in serial passages was evaluated by trypsinization at 80% confluence or bi-weekly intervals and retrieval after cryopreservation.

Three weeks after initial cultivation, cell migration from the attached explants was observed and by the 6th weeks a confluent monolayer was evident. This flask was placed in the normal horizontal position and incubated.

Preparation of scanning electronic microscopy:

For SEM study, the cultured IRB-1 were washed by phosphate -buffered saline (PBS) and then fixed in glutaraldehyde (2.5%) at 4 °C for 4 hr. The samples were dehydrated by ethanol and then kept with tetroxide osmium vapors at 4 °C for 2 hr. The samples were kept in a desiccator, then coated with gold (Bal-tec, Switzerland), and investigated by SEM (Philips, XL30, Nederland).

CHARACTERIZATION:

Population-Doubling Times

Population-Doubling Times (PDT) is a measure of tumor aggressiveness, which serves to quantify growth ratio. Moreover, at the passage 7 adherent RB cells (IRB-1) were counted and then cultured at the starting number of 5000 cells in a24 well plates. At 80% confluency, the cells were trypsinized, counted and then re-plated. From passage 7 of cells, the PDT evaluation was three times repeated and finally the average cell number was used for the final calculations. This process was continued up to ten passages (30 days) and the in vitro doubling time was calculated using the *exponential curve equation*.

Surface markers assay:

Flowcytometry analysis was used for evaluation of cell surface markers. The cell mixture was passed through a nylon mesh; 100 µl of the mixture was added to each tube in vicinity of anti-CD31-PE (Platelet endothelial cell adhesion molecule), anti-CD166-FITC (Activated leukocyte cell adhesion molecule), anti-CD90-FITC (Cluster of Differentiation 90), and anti-CD34-FITC (Hematopoietic progenitor cell antigen), anti-CD44-PE (cell-surface glycoprotein involved in cell–cell interactions), and anexin V-FITC (Annexin A5; a marker of apoptosis) (all products from Abcam, Uk) antibodies. The tubes were incubated at 4 °C in a dark condition for 46 minutes. Then the cells were fixed in 100 µl of 1% paraformaldehyde diluted in PBS before flowcytometric analysis. The Becton Dickenson device was utilized and analysis was performed using flowing software 2.5.1 (BD Company, USA).

Proliferation test by MTT assay

IRB-1 cells at a plating density of 5×10^3 cells/well were seeded on 96 well pleat cell cultures. For the analysis of proliferation rate and viability, after 24, 48, 72 hours of culture, 20µL of MTT (sigma, UK) substrate (of a 2.5 mg/ml stock solution in phosphate-buffered saline) was added to the each well, and finally all the plates were returned to standard tissue incubator conditions for an additional 4 hours. After this step, medium was then removed,

the cells were solubilized in 100 µL of dimethyl sulfoxide (DMSO) (sigma, UK), and the colorimetric analysis was performed (wavelength, 570 nm, PHOMO micropleat reader).

Karyotyping Analysis

IRB-1 cells were collected at 80% confluence at 10 passages and resuspended in 10 µl/ml of colcemid in DMEM. Cells were incubated at 37°C for 4 hours then cells were re-suspended in 0.5 ml medium and mixed with 0.075 M KCl to a volume of 10 ml. After incubation of 10 min at 37°C in a water bath, cells were resuspended to a total of 10 ml fixative agent (methanol: acetic acid, 3:1). G banding was performed by equilibrating the slides in 0.3 M sodium citrate, containing 3M NaCl for five minutes and subsequent addition of two drops of Antifade per slide prior to visualization (LabPro CETI, OXFORD).

Soft agar tumorigenesis assay

Soft agar assays were performed as described previously. [11] Briefly, 1% agar (MERK) was melted by microwave and kept warm in 56°C water bath. The melted agar was diluted with 2X DMEM and 0.5 ml of solution was dispensed into24 well plates. The bottom agar was left to cool under sterile conditions. IRB-1 cells were harvested and pipette well into single-cell suspension in the complete culture media (10% FBS DMEM) at 2500 cells/ml for making the upper layer. Then the equal amounts of melted 0.7% agarose (Fermentase) and 10% 2X DMEM was mixed and the prepared cells were added tto the mixture. Gently, 0.5 ml of the upper layer was added onto the bottom layer and was left to set under cell culture safety cabinet. After a while, 0.25 ml media was added and plates incubated at 37°C. The media was exchanged every other day for 3 weeks. Subsequently, the colonies were fixed and stained with 0.005% crystal violet. [11]

Mycoplasma Contamination

The cell lines were normally confirmed for the presence of mycoplasma contamination by conventional PCR. Momentarily, cells were trypsinized and 10^6 cells were washed twice

with PBS. The pellet was suspended in 1 ml Trizol (TAKARA, japan) lysis buffer and moved into a 1.5 ml Eppendorf tube. 250-µl of chloroform (sigma, Germany) have been added and mixed vigorously. After 15 minutes incubation at room temperature (RT), cell lysate was centrifuged at 12,000 g for 15 min at 4°C. The Nucleic acid layer was transferred to a new 1.5 ml Eppendorf tube and precipitated with 600 µl isopropanol, kept at -20°C for 30 min and centrifuged for 15 min at 4°C. Pellet was washed with 70% ethanol, air dried and resuspended in 20 µl Distil Water. RNA content was determined by ScanDrop spectrophotometer (analytica, Germany) and cDNA was synthesized as described previously. 100 ng of sample cDNA and 1 ng of positive control (genomic DNA from Mycoplasma oral) were used for PCR amplification at an annealing temperature of 55°C. The PCR primers used were as follows: FW: 5′-GCGTAGATATWWGGAAGAACAC-3′; RV: 5′-RGATGTCAAGAGTGGGTAAGG-3′. (Figure 5)

Quantitative relative Real-time PCR

Column RNA extraction kit (Fermentas International, Burlington, Canada) used fortotal RNA isolation from the cells DNase I reatment was done for the extracted RNA from the cells. (Fermentas International, Burlington, Canada) in order to avoid the genomic DNA contamination. RNA quantity was assessed by spectrophotometry (NanoDrop; Thermo, Wilmington, USA). 2 µg of total RNA was used for reverse transcription with the Revert Aid-first strand cDNA synthesis kit (Fermentas International, Burlington, Canada). The limbus tissue RNA was subjected as a positive control. Real Time-PCR (Rotor-Gene Q Real-Time PCR System, Qiagen, USA) was performed by SYBR® Premix Ex Taq™ (TAKARA BIO, INK, Japan) which uses Taq Fast DNA Polymerase and SYBR Green I dye to detect double-stranded DNA. The reaction was performed with the following program; 5 min of 95°C for enzyme activation, initial denaturation for 20 sec at 95°C, annealing temperature for 40 sec, and extension at 72°C for 1 min, followed by 40 cycles with a final

extension at 72°C. The final stage comprises the melting analysis. The Levels of mRNA for tested genes were quantified using ΔΔCT method and normalized against human β-actin. All data were expressed as Log 10 mean. Moreover, statistical analysis was performed using ANOVA and P value of 0.05 was considered as significant. The level of candidate genes in different sample types was compared by the Fisher LSD test **(Table 1.** Primer Sequences).

Trans-retinoic acid induction

Trans-retinoic acid was dissolved in dimethyl sulfoxide to make 10 mM stock solution. After 2 days of culture, the medium was replaced with fresh medium containing 10 μ molar retinoic acids. The culture medium was changed every three days. After ten days, cells harvested, their RNA extracted and to confirm differentiation induced by the retinoic acid the RT-PCR reaction for retinoic acid receptor α (RARα) has been done.

Neurospheres formation

IRB-1 was plated in a 96-well dish at densities from50 cells up to 1,000 cells per well . The cells were plated at a low density (50 cells per well) to prevent the probability of the cells encountering one another in the well (Figure 1). After 5 days, resulted neurospheres in the wells were counted,then dissociated into single-cell suspensions and re-plated. For the differentiation study, the IRB-1cells were cultured separately in complete RPMI medium for 2 weeks. Then, the cells were subjected to Real Time PCR analysis to determine the specific gene evaluation.

Results

Of total 6 received samples from six patients, only a single cell line was successfully passaged and isolated. The sample belongs to a one-year-old male with a group E sporadic unilateral RB. The primarily enucleated left eye had a differentiated RB, with a slight degree of necrosis, and pre-laminar and laminar optic nerve involvement in pathology report (figure 1).

Tumor cell morphology and growth in vitro

Tumor tissues from six RB patients have been placed in cell culture. Only a single continuously proliferating cell line, named IRB-1, has been established. All tumor cells showed growing as a monolayer of fibroblast or ganglion cell-like exhibiting identical growth in vitro. The IRB-1 initially cultured in adherent has been maintained in continuous culture since October 2012. The cells characteristically grew in loose plate-like or clusters at the bottom of the culture flask. (Figure 2)

Population-Doubling Times

The first cellular passage of the IRB-1 cells was done at 6^{th} week. For evaluation of cellular proliferation and cellular permanence, MTT method was used. Figure 2 the average population doubling time of the IRB-1 was 36 ± 4.6 hours. The IRB-1 cell line was checked regularly for alterations in morphology and growth shape. The IRB-1 cells characteristic when they grew as adherent monolayer are fibroblastic-like morphology. The cultured cells preserved consistent morphology from the primary culture to the subsequent subculture passages. IRB-1cells have shown continuous growth for over16 months and have undergone

more than 132 passages. They performed to be permanent cell lines since growth continued after recovery from multiple cryopreservation's**.** Although IRB-l attach to the culture flasks, high capacity for growth as a monolayer was expressed. (Figure 3)

Growth and Cloning in Agar: anchorage independent growth

Attempts to clone IRB-1 in soft agar proved to be successful. Figure 3 Anchorage-independent growth is one of the characteristics of cancer cells and a hallmark of cancer. Soft agar growth was assessed in 3% agarose (Grand Island Biological Co.). The cultured cells were able to form colonies in the agarose layer. Colonies of IRB-1 were evident at the end of the first week and at the end of the third week; the colonies were fixed with 4% para-formaldehyde and stained with 0.005% crystal violet. Colonies were counted manually using an inverted microscope and the percent rate of colony formation was 9.24. (180±9.7 colonies from 2000 seeded cells). (Figure 4)

Evaluation of cell growth and proliferation after adding retinoic acid

Adding retinoic acid to cell culture affected the cellular morphology and 5000 cell /well were evaluated at 24, 48 and 72 hours after adding retinoic acid (10 micromoles) with the tetrazolium-based colorimetric assay (MTT technique). It was shown that retinoic acid slowed down the rate of proliferation resulting from more differentiation. (Figure 3)

IRB-1 was evaluated in 3 groups.

A. The cells exposed to 10 micromoles retinoic acid for 10 days.

B. The cells without exposure to retinoic acid.

C. The cells 15 days of induction.

The expression of CRX, Recoverin, MAP2, Tuj1, PKC- α, ABCG2, ACTB, RAR- α, GFAP

were evaluated by real-time PCR. The differentiation markers were detected more prominently in the exposure group and neurospheres group, as opposed with ABCG2, which had comparable results in all three groups (Figure 5).

Cell surface marker on RB cell line

The immunohistochemical immune-phenotyping staining showed positive CD166, CD90 , CD44, CD31 CD29, CD105 and Annexin V markers. The marker for CD34 was totally negative and for CD73 partially positive (Figure 6).

Chromosome Studies

Karyotype analysis was performed on the IRB-1 cell for evaluation chromosomal abnormality. Characterization was made on the basis of 50 conventionally stained metaphase cells and 12 Giemsa-banded cells. In nearly all metaphases 51 chromatids were visible including a single chromosome. IRB-1 has significant chromosomal imbalances on chromosome 3, 9, 10, 11, 14, 16, 17, and 22. In all metaphases, diploid set of chromosomes 7,11,18, and 20, and two additional chromosomes 17 were shown. Two marker chromosomes were detected. In one of the chromosome 1, a deletion at p34 and duplication in the bands q21 and q32 were present. Homolog chromosome showed a translocation [t(3;1;7)(p12;q10;q36)]. Another translocation between chromosome 2 and 6 was noted [t(2;2;6)(p25;q13;p21.3)]. (Figure 7)

Chromosome 4 showed another deletion from band q21 to the end of chromosome arm [del (4)(q21)]. On chromosome 7 in the band q11.2 an additional band with unknown origin was noted [ins (7;?)(q11.2);?)]. A translocation between chromosomes 11 and 13 was visible [t (11; 13)(p11.2; q11)].

The end of q arm of chromosome 9 from band q 13 was added to the end of q arm of chromosome 12 [der (12) t (9; 12)(q13; q24)]. A Robertsonian translocation was formed between 14 and 15 and 21 and 22 [t(14;15)(q10;q10),t(21;21)(q10;q10)]. The short arm of

chromosome 22 in the band q 21.2 has a deletion [del (22)(q11.2)]. (Figure 4)

Discussion

A novel human RB cell line, IRB-1, was established from a 1-year old patient with a unilateral sporadic RB at Farabi Hospital- Tehran University Medical Sciences. The cultural characteristics of IRB-1 cell line were comparable to those of Y79 in characteristic. The malignancy of this cell line was determined by measuring the doubling time of cells. The doubling time of IRB-1 is shorter than Y79 and WERI-Rb1 in adherent or floating growth, [7,8] that means active proliferation of IRB-1 maximal growth between 30 to 40 days. IRB-1 has morphological and biochemical characteristics similar to previous human RB cell lines: morphological features of fibroblast- or ganglion-like cells. [12-15] In vitro growth of WERI-Rbl and Y79 in rosettes and chains was unique. [13] Pseudorosettes have been described in hetero-transplanted neuroblastoma tumors in nude mice.[13] In IRB-1 cells no rosette formation was noted during the multiple passages.

Unlike fibroblastic cells the present cells had tumorigenic characteristic and immortalized characteristics during 132 continuous passages.

In karyotyping analysis, we found that IRB-1 has significant chromosomal imbalances on chromosome 2, 6, 7, 9, 11, 12, 13, 14, 15, 17, 18, 21 and 22. SNUOT-Rb1 has significant chromosomal imbalances on chromosome 3, 9, 10, 11, 14, 16, 17, and 22. [12] Our finding is different from SNUOT-Rb1. [12] Different patterns of deletions, insertion and translocation was found in reported RB cell lines. Unlike IRB-1, Kim et al identified a minimal 16q genomic loss (16q23.2 and 16q24.2) that was previously described for primary RB tumors. [9] Unlike WERI-Rbl, which showed a stable modal chromosome number of 46, with at least 5 markers present in every cell, [8] IRB-1 showed 51 chromosomes with two markers. With the

exception of chronic myelogenous leukemia, [17] no human tumor has exhibited a consistent karyotype, although some correlations have been shown between specific cancers and a specific chromosome abnormality. [18]

Aneuploidy has frequently been noted in many solid tumors, [2, 19-21] and in cell lines derived from them [2, 19]; large marker chromosomes often occur in these karyotypes.[10]

Despite the presence of a high frequency of aneuploidy in cancer, the exact role of aneuploidy in carcinogenesis is poorly understood. [22] The evolution of cancer cells from benign tumor to invasive metastasis appears to correlate with increased aneuploidy and karyotype complexity. [23] It has been shown that in tumor cells containing less than 46 chromosomes, the genome contains more than the diploid amount of DNA. [24]

The importance of all the markers of IRB-1 is not known yet. IRB-1 cells showed CD166, CD90, CD44, CD31, CD105, CD34, and Anexin V markers. CD133 (Promini1) was already reported in Y79 and WERI- Rb1. [25] CD133 (Prominin-1) is a cell surface marker of cancer stem cells. [26]

Histochemically, IRB-1 cells, compared with Y79, [25-27] showed different neuronal and astroglial markers. Rb gene controls the human CD34 promoter region by antagonizing the CD34 promoter factor nucleolin to provide a mechanism that links expression of endogenous CD34 to cell cycle progression. [28] The severe expression of CD34 on the IRB-1 cells could follow the underaction of RB gene in the cells.

There are evidences for the presence of cells with photoreceptors characteristics in RB cells that were detectable mainly in Flexner-Wintersteiner rosettes. [28-31] As shown in other studies, CD90, a differentiated retinal cell marker, is positive in IRB-1 cells. [31-34]

Neurosphere in RB cells is an indicator of self-renewal and cycling in the stem cells, especially Y79 and WERI- Rb1. [35] IRB-1 showed the capacity to form neurospheres, an indicator of stem cell self-renewal. [35]

IRB-1 Cells derived from primary RB tumor could be used as a model for cancer research including for genomic and transcription analysis and evaluation of different treatment protocols for RB. The main limitation of this study is the inability to make genetic study over IRB-1 cells at the time.

Conclusions

In conclusion, we have developed a RB cell line capable of being propagated in tissue culture. IRB-1 is a novel human RB cell line, which with interesting characteristics of adherent and flocculent growth and more chromosomal imbalances different from other RB cell lines. We will have the opportunity for more evaluating the biological and genetic characteristics of IRB-1 cells.

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Table I . Sequences of primers used for Real-time PCR

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Figure 1. Light microscopy of eye tissue. (A) Posterior part of eye globe with show scleral section of optic nerve, optic disc and a neoplasm (retinoblastoma) in to the vitreous cavity scale bar: 200 µm, magnification: x100. (B) Retinoblastoma, composed of sheets small blue cells scale bar: 200 µm magnification: x100. (C and D) retinoblastoma and section of retina scale bar: 200 µm, magnification: x100. (E) Retinoblastoma with section of culinary body scale bar: 300 µm, magnification: x100. (F) Well differentiated retinoblastoma composed of small blue cells with scan cytoplasm, hyperchromatic nuclei and scanty strome, Flexner-winterSteiner rosettes and homer-wright rosettes are also seen scale bar: 50 µm, magnification: x400.

Figure 2. Morphology of isolated IRB-1 cells from explants and the subsequent cultures by invert and scanning electron microscopy (SEM). Cells possessed spindle shape morphology and formed adherent monolayer in culture. A: 10x; Confluent cells with ganglion like morphology is evident; scale bar: 20 µm. B: 40x; Sub confluent culture; scale bar: 10 µm. C: 10x; Neurospheres culture; scale bar: 100 µm. D: SEM image 250x; scale bar: 100 µm. E: SEM image 1.50 kx; scale bar: 10 µm.

Figure 3. Investigation of doubling time and proliferation rate. Passage 10 cells cautiously were counted and plated. After 80% confluent cells were trypsinized, counted and plated. After 10 passages, cell count was plotted and population-doubling time was calculated. Doubling time was calculated as 36±7 hours (A). Proliferation test for IRB-1 cell line with and without treated by retinoic acid (RA) at 24h, 48h and 72h (B). Mycoplasma

contamination were evaluated using RT-PCR. Genomic DNA from Mycoplasma orale was used as positive control. A reaction without DNA template was used as negative control. PCR results of IRB-1, showed no evidence of mycoplasma contamination (C).

Fig 4. Soft agar tumorigenicity assay: IRB-1 cells were cultured in 3% agarose and the ability to form a colony was evaluated. Three weeks later, colonies were stained by DAPI. A, D: 4x, scale bar: 100 µm. B, E: 20x, scale bar: 20µm, C, F: 40x, scale bar: 20µm.

Figure 5. Relative gene expression for *GFAP, ABCG2, RAR-a, ACT-b, PKC, TUJ, MAP2, Recoverin and CRX* genes was evaluated by real-time PCR method in (A) retinoblastoma cell line, (B) Neurospheres induced formation of retinoblastoma cell line, (D) retinoblastoma cell line after induction by retinoic acid.

Figure 6. Flowcytometry analysis for cell surface markers consist of CD105, CD31, CD34, CD166, CD44, CD90 and Anexin. M1: negative area and M2 positive area.

Figure 7. Distribution of chromosome numbers in IRB-1 cell line. cells in their logarithmic growth phase were treated with colcemid, fixed and stained. Chromosomes were counted in 1000 cells and were analyzed for chromosomal abnormalities.

Figure1:

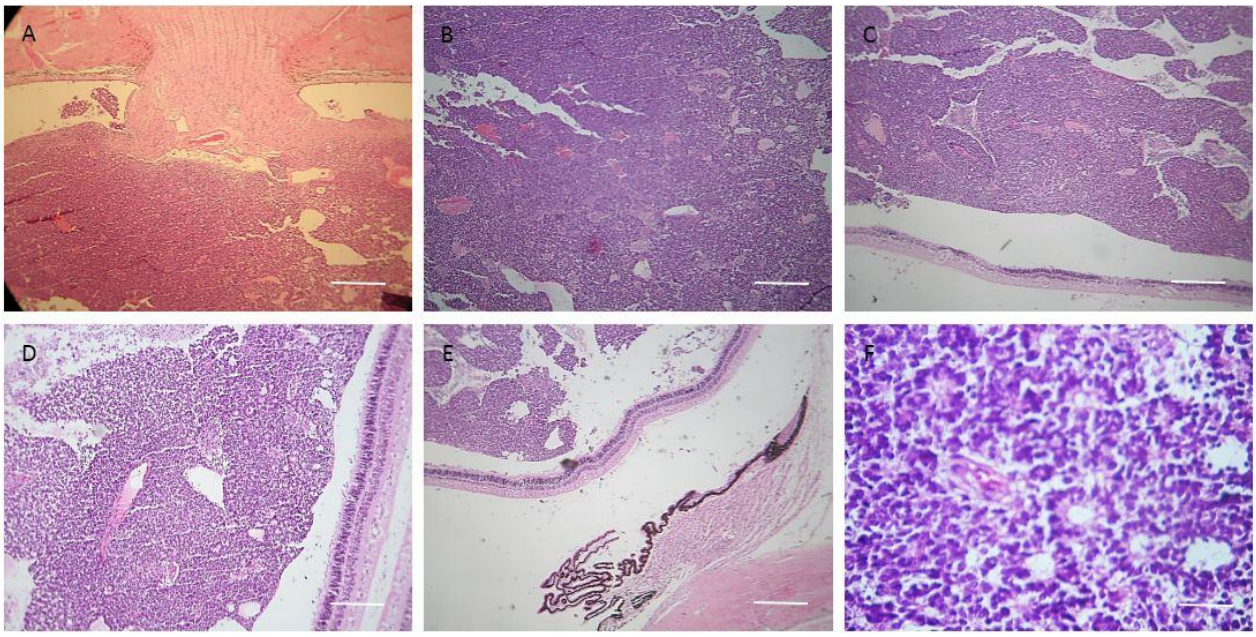


Figure 2:

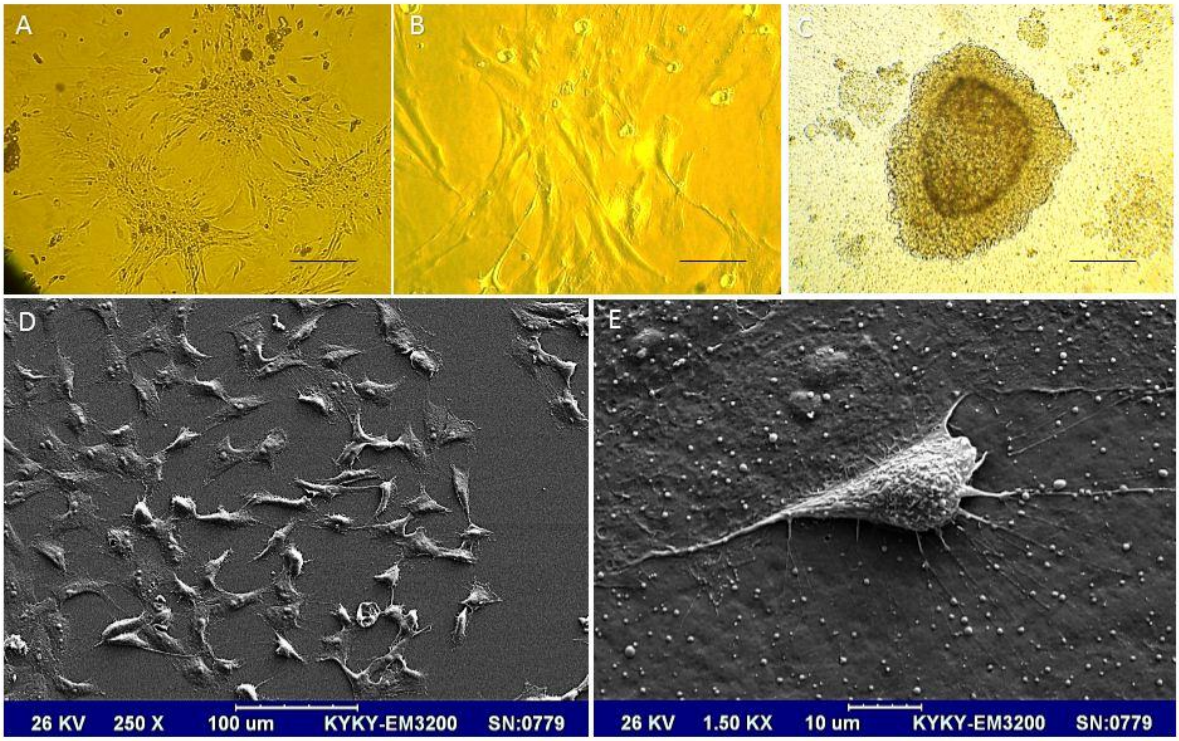


Figure3:

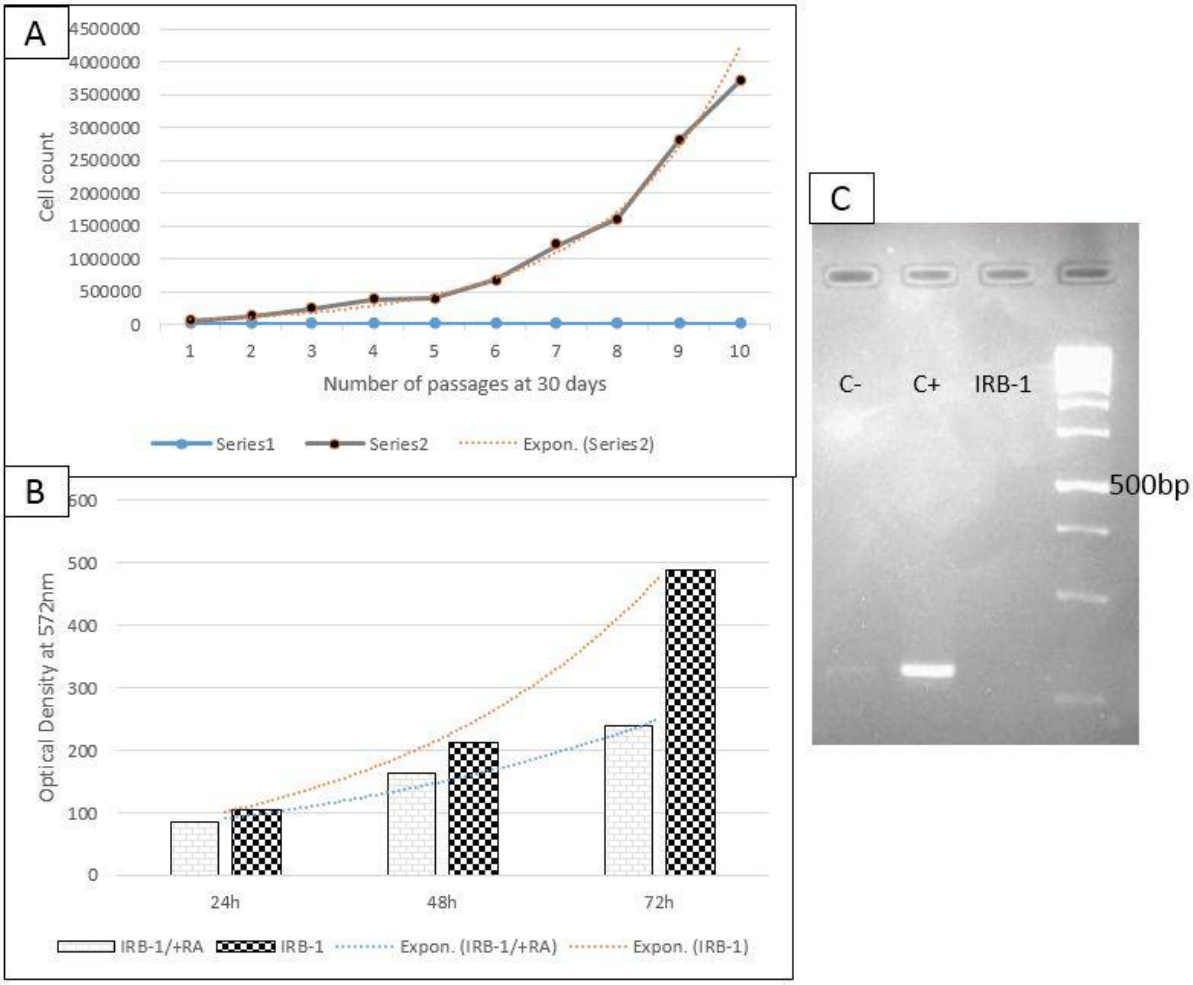

A
4500000
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
Cell count
1
2
3
4
5
6
7
8
9
10
Number of passages at 30 days
Series1
Series2
Expon. (Series2)
B
600
500
400
300
200
100
0
Optical Density at 572nm
24h
48h
72h
IRB-1/+RA
IRB-1
Expon. (IRB-1/+RA)
Expon. (IRB-1)
C
C-
C+
IRB-1
500bp

Figure 4:

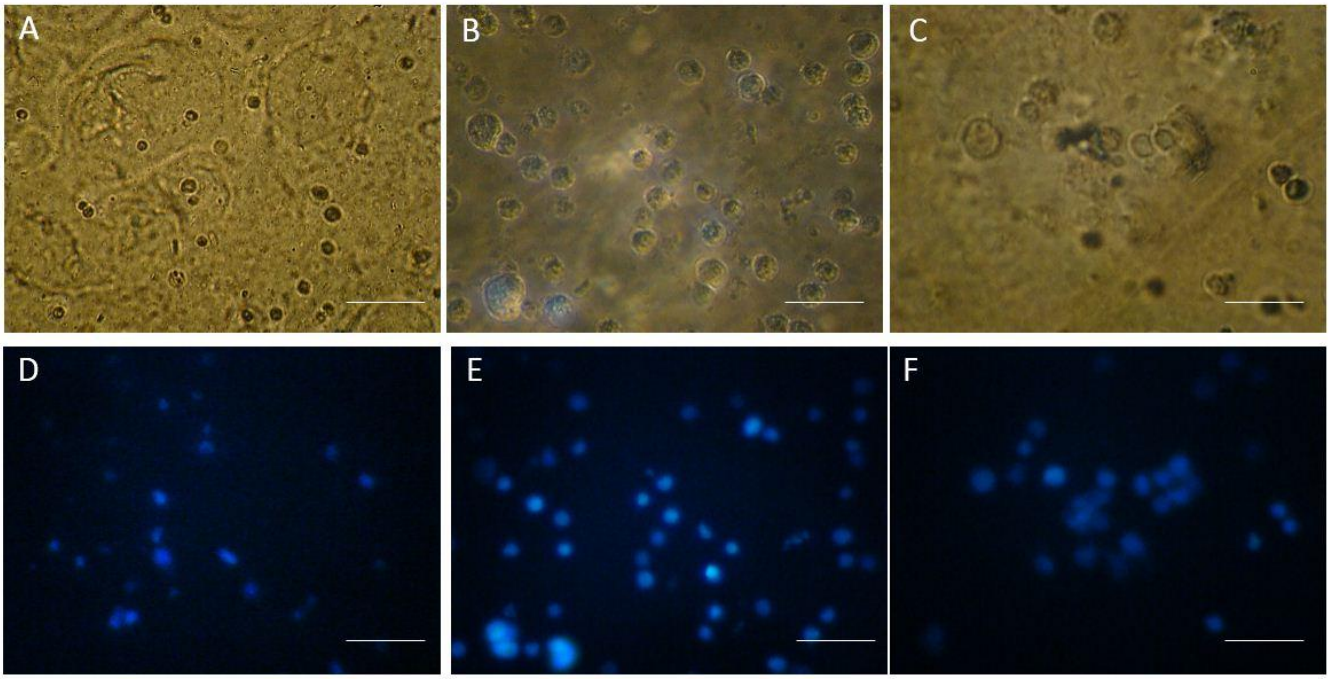


Figure 5:

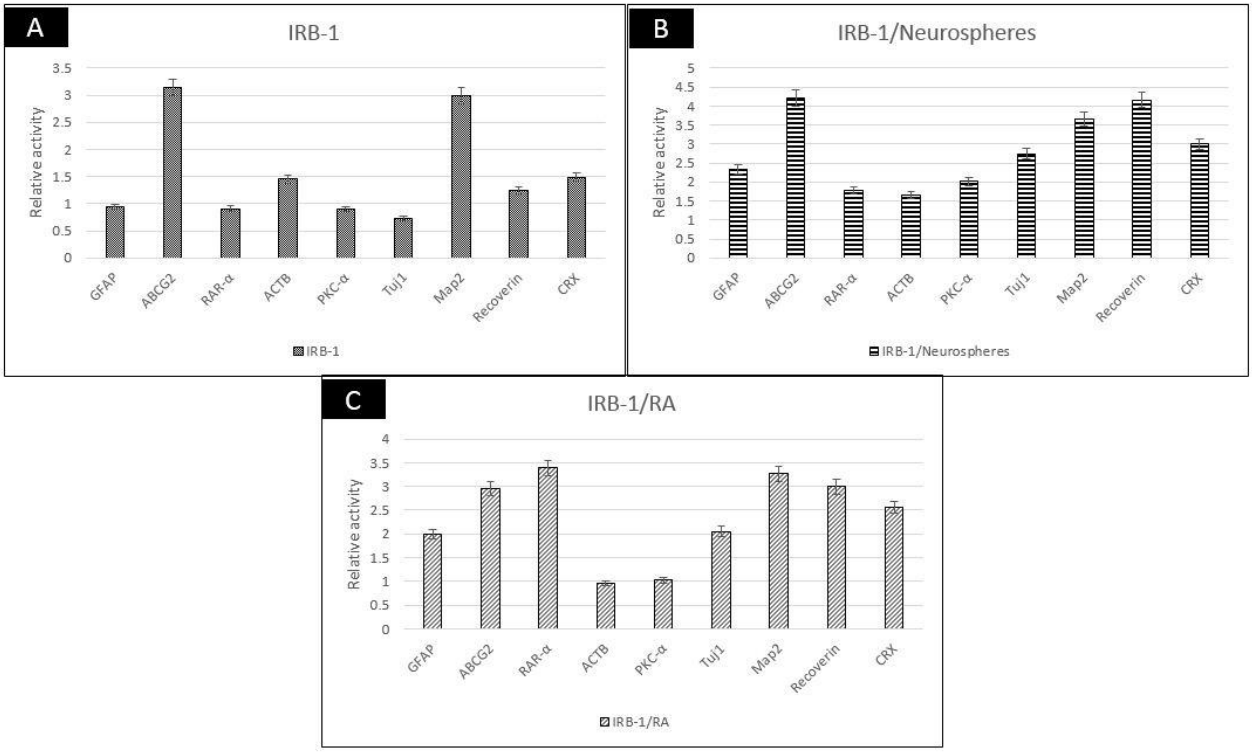
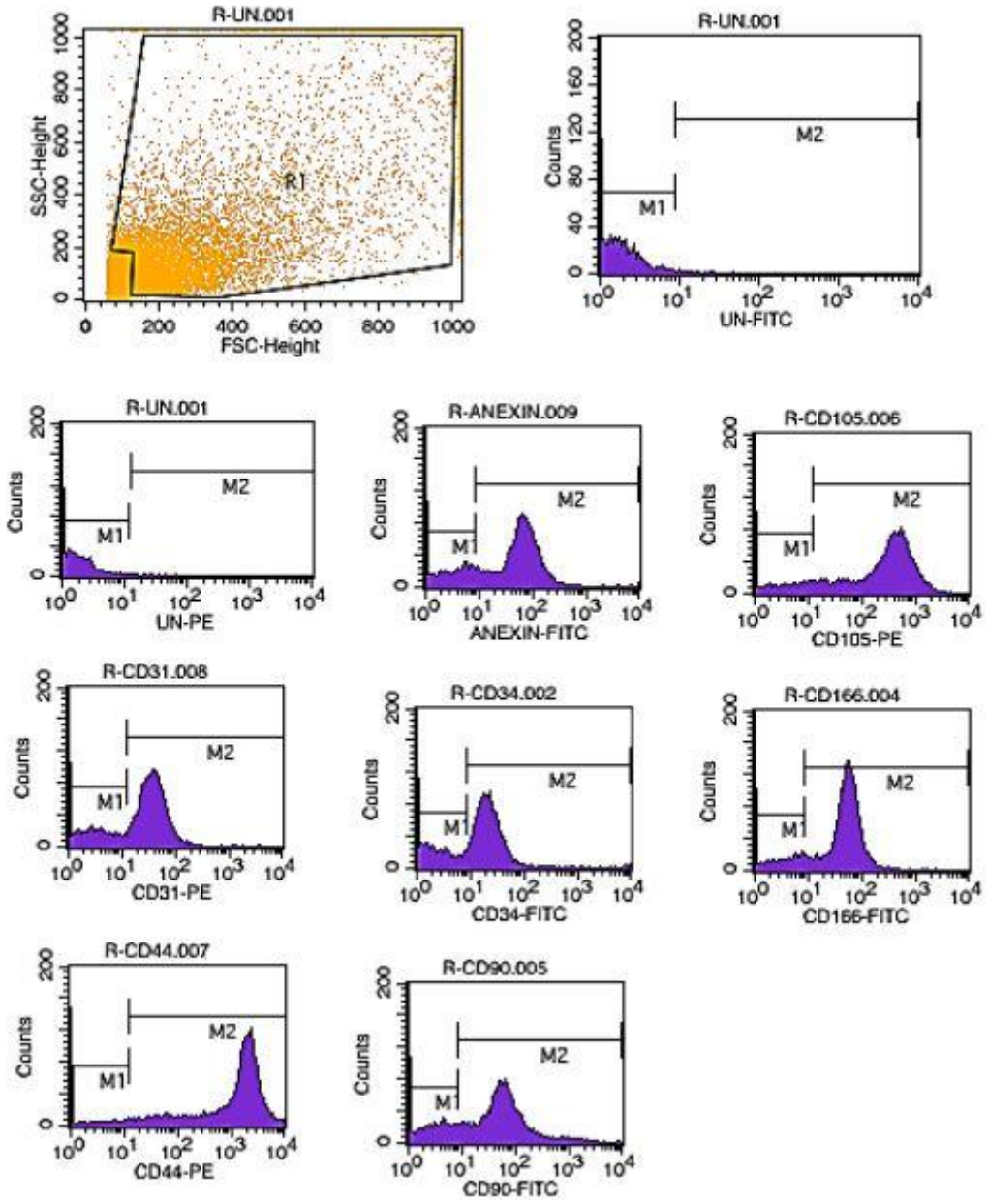


Figure 6:


R-UN.001
SSC-Height
FSC-Height
R1
R-UN.001
Counts
M1
M2
UN-FITC
R-UN.001
UN-PE
R-ANEXIN.009
ANEXIN-FITC
R-CD105.006
CD105-PE
R-CD31.008
CD31-PE
R-CD34.002
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R-CD166.004
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CD44-PE
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CD90-FITC

Figure 7:

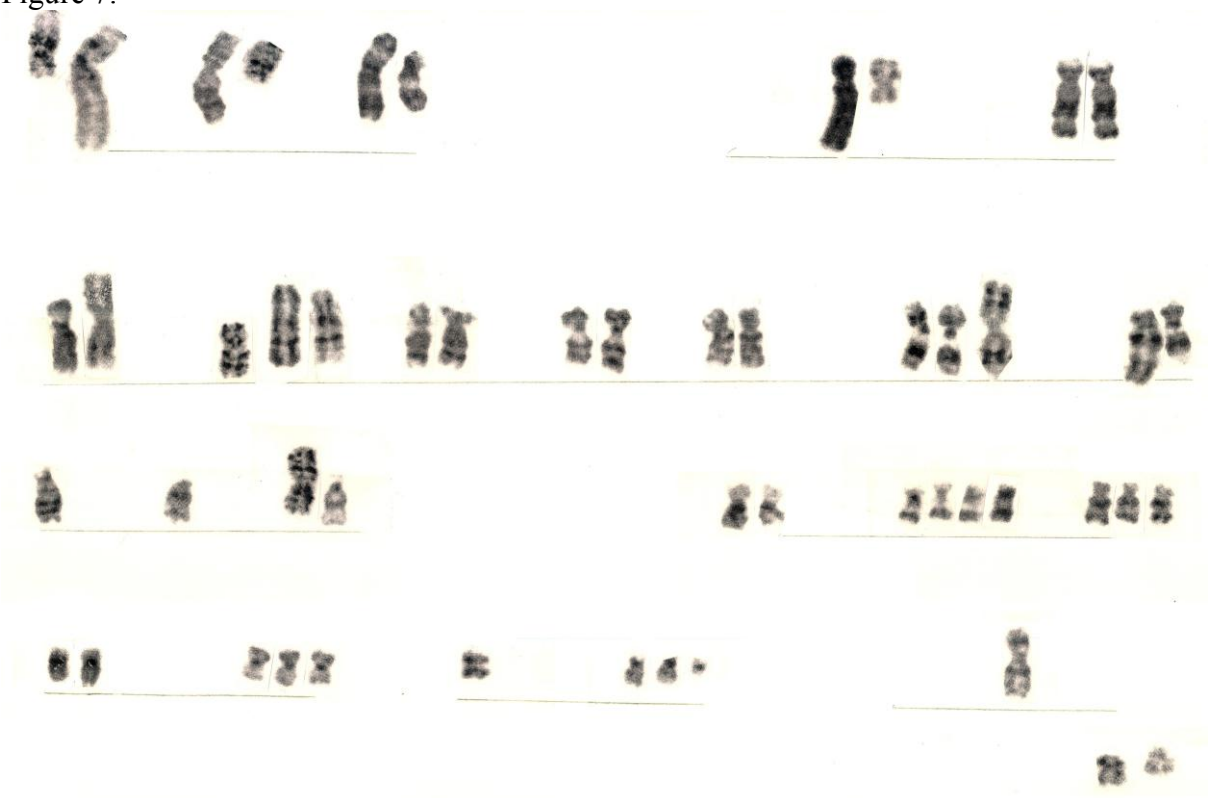

Table 2:

Table 1: Primers used for RT-PCR.

Primers	Sequence (5′->3′)	Tm **(ºC)**	Length (bp)
Glial fibrillary acidic protein (GFAP)	F: *AGGAAGCAGATGAAGCCACC* *R: CATACTGCGTGCGGATCTCT*	60	**207**
Retinoic acid receptor alpha (RARA)	F: *AGATCACCCTCCTCAAGGCT* *R: CATCATCCATCTCCAGGGGC*	59	**201**
ATP-Binding Cassette, Sub-Family G, Member 2 (ABCG2)	F: *CCTGGGGCTTGTGGAAGAAT* *R: GCAACAGTGTGATGGCAAGG*	59	**213**
Actin beta (ACTB)	F: *GTGGATCAGCAAGCAGGAGT* *R: TGTCACCTTCACCGTTCCAG*	60	**230**
Neuron-specific class III beta-tubulin (*Tuj-1*)	F: GGCCAAGGGTCACTACACG R: GCAGTCGCAGTTTTCACACTC	60	**321**
Microtubule associated protein 2 (MAP2)	F: *CTCTGCAACAAACCAGTGGC* *R: TCTGCAGGTTTTGGGGGTTT*	60	**346**
Recoverin (RCVRN)	F: *CACTCACATGACACCCGTGA* *R: CTTACAGCGGGGTGACACTT*	59	**298**
Cone rod Homeobox protein (CRX)	F: *AGCAGCTAGACAGACTCCCA* *R: CACAAGTGCTTGCCAGTGTC*	60	**247**