SPANDIDOS PUBLICATIONS

ONCOLOGY REPORTS 26: 135-143, 2011

Maintenance of retinal cancer stem cell-like properties through long-term serum-free culture from human retinoblastoma

BO MA[1], XIA LEI[2], YUAN GUAN[2], LI-SHA MOU[2], YI-FEI YUAN[1], HAN YUE[1], YI LU[1], GUO-TONG XU[2,3] and JIANG QIAN[1]

[1]Department of Ophthalmology, Eye and ENT Hospital of Fudan University; [2]The Laboratory of Clinical Visual Sciences, Institute of Health Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and Shanghai Jiao Tong University School of Medicine; [3]The Tongji Eye Institute and Department of Regenerative Medicine, Tongji University School of Medicine, Shanghai, P.R. China

Received February 22, 2011; Accepted March 29, 2011

DOI: 10.3892/or.2011.1291

Abstract. Previous studies have demonstrated that a small population of cancer stem cell-like cells exists in retinoblastoma. To provide a model for studying this population, we sought to establish a long-term culture from human retinoblastoma that have cancer stem cell-like properties. Fresh tumor tissue was digested and cultured in serum-free medium. Tumor spheres formed and were passaged continuously. Stem cell properties were examined through immunostaining, real-time quantitative RT-PCR and chemoresistance assay. Tumorigenicity of the tumor sphere-forming cells was confirmed by xenograft experiments. Furthermore, we examined the expression of cell surface markers CD44 and CD133. Tumor cells expanded as floating spheres for more than 30 passages. Sphere-forming cells overexpressed stem cell genes *Oct-4*, *Nestin* and *Pax6*. Immunostaining of spheres showed positivity for Nestin, Pax6 and also ABCG2. In contrast, differentiated cells derived from these spheres expressed high levels of mature retinal cell markers MAP2, GFAP, recoverin, Opsin B and Nrl, and showed immunoreactivity for NF200, GFAP, recoverin and PKCα. Furthermore, both CD44 and CD133 were highly expressed in sphere-forming cells vs. differentiated cells. Sphere-forming cells displayed higher chemoresistance to carboplatin as opposed to differentiated cells. Moreover, intraocular injection of as few as $2x10^3$ sphere-forming cells into NOD/SCID mice gave rise to new tumors similar to the original patient tumors. These results revealed that the sphere-forming cells preserved their stem cell properties and tumorigenicity, even after long-term culture. This would be a suitable *in vitro* model to study cancer stem-like cells in retinoblastoma and to develop chemotherapeutic drugs and strategies.

Correspondence to: Dr Jiang Qian, Department of Ophthalmology, Eye and ENT Hospital of Fudan University, Shanghai 200031, P.R. China
E-mail: qianjiang58@hotmail.com

Key words: retinoblastoma, cell culture, tumor stem cell

Introduction

Retinoblastoma (Rb) is a rare type of eye cancer that accounts for only 4% of pediatric malignancies (1). Nonetheless, it develops rapidly in the retina, and can be fatal if not treated promptly. Although survival rates in patients with Rb have been exceptionally high up to 95% in developed countries, the management of retinoblastoma is still a challenge (2). According to the International Classification of Retinoblastoma, globe salvage is achieved in only 47% of group D cases; most eyes with group E retinoblastoma are managed with enucleation (2). Chemotherapeutic drug resistance is common in retinoblastoma (3), resulting in the increased incidence of unsuccessful treatments. Therefore, chemoreduction failure defined as unresponsive or recurrent retinoblastoma (4) plays a very important role in the particularly difficult management of advanced cases. Previous studies have shown that P-glycoprotein and multidrug resistance proteins (MRPs) may contribute to drug resistance (5-7). However, the mechanism involved in resistance and recurrence is still not clear, and improved targeted therapies are essential for alleviating this devastating malignancy.

In past years, one emerging hypothesis postulates that the development of drug-resistant tumors is sustained by a self-renewing cell subpopulation termed putative cancer stem cells (CSCs) (8). Current therapies target rapidly dividing cells that comprise the bulk of the tumor while failing to eradicate the CSCs which subsequently re-initiate the entire malignancy. It is likely that these residual CSCs are able to survive in a dormant state for many years after remission due to their resistant ability (9-11). This has considerable implications in the way cancer treatment should be conceived.

CSCs have been identified in hematopoietic malignancies as well as in many solid tumors including brain, breast, colon, pancreas, prostate and ovarian cancers (9,10,12-16). In retinoblastoma, Seigel and colleagues (17) demonstrated that a small subpopulation of cancer stem-like cells existed in mouse retinoblastoma cells and human Y79, WERI-Rb27 cell lines, which express human embryonic and neuronal stem cell markers. Another study identified tumorigenic retinal stem-like cells in

human retinoblastoma, which is direct evidence of CSC presence in Rb (18). However, compared to other solid tumors, cancer stem cell research is impeded in this intraocular cancer because of the shortage of fresh samples and the difficulty of isolation from varying Rb lesions, a mixture of tumor cells with necrosis and calcification. The other major obstacle is the absence of candidate markers for identifying CSCs in Rb. Therefore, a proper *in vitro* model is required to study retinoblastoma cancer stem-like cells and to design future therapeutic approaches. Here, we report that a long-term culture of sphere-forming cells from human retinoblastoma was established; these cells maintain their cancer stem-like cell properties including the ability of chemoresistance. Moreover, we found that the percentage of $CD44^+/CD133^+$ cells increased with continuous passaging, which may indicate their roles as putative cancer stem-like cell surface markers in human retinoblastoma.

Materials and methods

Tissue collection and primary tumor sphere formation. Our study was approved by the Institutional Review Boards of Eye and ENT Hospital (EENT), Fudan University in accordance with the Declaration of Helsinki. Tumor samples were obtained from a 3-year-old female patient with grade group D retinoblastoma according to the International Classification of Retinoblastoma (19). Tumor tissues were collected as previously reported (18,20), then washed, minced and digested with 0.25% trypsin. Enzymatically dissociated suspensions were filtered (45-μm cell strainer), washed and resuspended in serum-free DMEM/F12 medium (SFM) supplemented with 20 ng/ml human recombinant epidermal growth factor (EGF; PeproTech, Rocky Hill, NJ, USA), 20 ng/ml human basic fibroblast growth factor (bFGF; PeproTech), B27 supplement (Invitrogen, Carlsbad, CA, USA) and 20 ng/ml recombinant human leukemia inhibitor factor (LIF; Chemicon-Millipore, Billerica, MA, USA) at a density of 10^5 cells/ml. RBCs were removed by Histopaque-1077 (Sigma, St. Louis, MO, USA).

Tumor sphere passage and limiting dilution assay. After primary culture for 11 days, tumor spheres formed and were harvested by sedimentation for 10 min. Sphere cells were treated with 0.05% trypsin-EDTA (Gibco-Invitrogen), and digestion was terminated with egg trypsin inhibitor (Sigma). Dissociated cells ($2x10^5$ cells/ml) were transferred to low attachment dishes containing the same medium. Media were changed twice a week, and the same procedure was performed during serial passage. For limiting dilution assay, the analysis was performed as described previously (21,22). Briefly, dissociated sphere cells were plated in 96-well microplates in 0.2 ml volumes of SFM. Final cell dilutions ranged from 800 to 1 cell/well, and cultures were fed 0.05 ml of SFM every 3 days until Day 10 after which time the fraction of wells not containing tumor spheres for each cell plating density was calculated and plotted against the cells plated per well. The number of cells required to form at least one sphere was determined based on the regression line. The growth curve of tumor cells was also studied as described previously with some minor modifications (23).

Differentiation assay. To examine the multipotent differentiation of tumor sphere cells, spheres were plated on poly-D-lysine-coated (0.1 mg/ml, Chemicon) and laminin-coated (20 μg/ml, Invitrogen) glass coverslips under differentiating conditions including DMEM/F12 medium, 10% fetal bovine serum (FBS, Hyclone-Thermo Fisher Scientific, Waltham, MA, USA) and B27, without growth factors. Culture media were fed every 3 days. Cell morphology was assessed 11 days after plating using immunochemistry.

Table I. Primary antibodies with their dilutions and suppliers.

Antibody	Company	Species	Dilution
Pax6	Santa Cruz Biotechnology	Mouse monoclonal	1:500
Nestin	Chemicon	Mouse monoclonal	1:200
ABCG2	Abcam	Mouse monoclonal	1:50
NF200	Sigma	Rabbit polyclonal	1:200
NSE	Neomarker	Mouse monoclonal	1:400
GFAP	Chemicon	Mouse monoclonal	1:400
Recoverin	Abcam	Mouse monoclonal	1:400
PKCα	Santa Cruz Biotechnology	Rabbit polyclonal	1:400

Immunocytochemical staining. Immunostaining of undifferentiated tumor spheres was performed as previously reported (18,22). Briefly, spheres were collected and plated onto poly-D-lysine-coated and laminin-coated glass coverslips in SFM for 8 h. Cells were then fixed with 4% paraformaldehyde and stained with antibodies against Nestin, Pax6 and ABCG2 (Abcam, Cambridge, MA, USA). For differentiated tumor cells, immunochemistry was performed against NF200 (Sigma), GFAP (Chemicon), recoverin (Abcam) and PKCα (Santa Cruz Biotechnology, CA, USA). A list of primary antibodies with their dilutions and suppliers is provided in Table I. For immunofluorescence studies, anti-mouse-FITC, anti-rabbit-Cy3 and anti-mouse-Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were used as secondary antibodies. Nuclei were counterstained with DAPI (Invitrogen). Staining was observed by inverted fluorescence microscopy (Nikon).

Real-time quantitative RT-PCR. Gene expression was quantified by real-time quantitative RT-PCR using SYBR Green Master Mix (Toyobo, Japan). Total RNA was extracted from sphere-forming cells, differentiated cells or parental tumor tissue, and was reverse transcribed into cDNA. All the primer sets for amplification are provided in Table II. Real-time RT-PCR was performed in 384-well plates on a sequence-detection system (ABI PRISM 7900; Applied Biosystems, Inc., Foster City, CA). The relative expression of the sphere-forming and differentiated group was presented as fold change compared to that of the parental tumor tissue, and was normalized to hGAPDH expression in each group. The comparative Ct ($\Delta\Delta$Ct) method was used to obtain quantitative data of relative gene expression, according to the manufacturer's instructions.

Chemotherapy sensitivity assay. Chemoresistance of both sphere cells and differentiated cells to carboplatin was

Table II. Primers and product size for real-time quantitative RT-PCR.

Gene	Accession no. (GenBank)	Primer sequences	Product size (bp)
Oct-4	NM_203289	F: CAGCGACTATGCACAACGAGA	196
		R: GCCCAGAGTGGTGACGGA	
Nestin	NM_006617	F: CGGTGGCTCCAAGACTTCC	156
		R: GGCACAGGTGTCTCAAGGGTA	
Pax6	NM_001127612.1	F: CTGCTGGCTGGCTTACTTCTT	165
		R: CCACGTCACTCCTTTTCTCCC	
CD133	NM_001145847.1	F: GGTCTGGCGAGCTAAGGGAA	217
		R: GGGGAAGGCAAGCGTGTT	
CD44	NM_001001390.1	F: CCAATGCCTTTGATGGACC	248
		R: TCTGTCTGTGCTGTCGGTGAT	
GFAP	NM_001131019.1	F: AGCCCAGGACGTGGTACAGA	176
		R: TCCAGGCACAGCGAGACC	
Map2	NM_031845.2	F: GAAGCAGCAGGTGGGGAATC	136
		R: GCCGAGGAGGGAGAATGGA	
Recoverin	NM_002903	F: CCAGCAGCAGTTCCAGAGC	217
		R: GATGGTCCCGTTACCGTCC	
Nrl	NM_006177.3	F: GCACCTTGTGGTGTAGTGGG	114
		R: GGTCATACAGGGCGTGGC	
Opsin B	NM_001708.2	F: GCGGCAGCCCCTCAACTA	143
		R: GGAAGCCCTCCAAAGCACA	
hGAPDH	NM_002046	F: CACTCCTCCACCTTTGACGC	180
		R: TCTTCCTCTTGTGCTCTTGCTG	

assessed using methods previously described with some modifications (9). Briefly, spheres were trypsinized, dissociated and seeded at 10^4 cells per well into 96-well plates. In parallel, differentiated cells were trypsinized and replated at the same density. Carboplatin at various concentrations was added 48 h after plating. Drug cytotoxicities were determined 48 h after treatment by Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Rockville, MD, USA). The same cells (sphere and differentiated cells) without carboplatin treatment were used as control following the kit protocol. All results were based on at least four parallel measurements for each time, and each measurement was repeated in up to three independent experiments.

In vivo xenograft experiments. Xenograft experiments were performed on 4- to 6-week-old female NOD/SCID (non-obese diabetic, severe combined immunodeficient) mice. All mice were maintained in the Animal Facility of Shanghai Jiao Tong University School of Medicine, and our experimental studies conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. To evaluate the tumorigenicity of the tumor sphere-forming cells, dissociated tumor cells were counted and resuspended in PBS with various cell concentrations. Under a binocular surgical microscope, a 5-μl cell suspension was transsclerally injected into the vitreous cavity through a 5-μl microsyringe, whereas the contralateral eyeball was injected with 5 μl PBS as control. Engrafted mice were inspected biweekly and sacrificed after 10-12 weeks. Eyeballs were enucleated, fixed in 4% paraformaldehyde and embedded for paraffin or cryostat sections. Hematoxylin and eosin (H&E) staining was performed on paraffin sections, and cryostat sections were used for detection of Nestin, NSE (Neomarker-Thermo Fisher Scientific), GFAP and recoverin. Immunodetection was performed using an ImmunoPure ABC staining kit (Santa Cruz Biotechnology).

Flow cytometry. Sphere-forming cells from 7-10 days of different passages were dissociated by 0.05% trypsin and then resuspended in PBS with 1% BSA and 0.1% sodium azide. Aliquots of single cells were evaluated by flow cytometry with a FACS Calibur machine (BD Bioscience, San Jose, CA, USA) using PE-conjugated anti-human CD133 (Miltenyi Biotec, Germany) and APC-conjugated anti-human CD44 (eBioscience, San Diego, CA, USA) according to the manufacturer's recommendations.

Statistics. Values are presented as the mean ± SD. Statistical analyses were performed by the Student's t-test. A level of $p<0.05$ was considered to be statistically significant.

Results

Maintenance of sphere-forming, proliferation and differentiation capacity of original CSCs after long-term culture. Sphere formation is considered an ideal *in vitro* assay to identify cancer stem cells (24). A previous study showed that

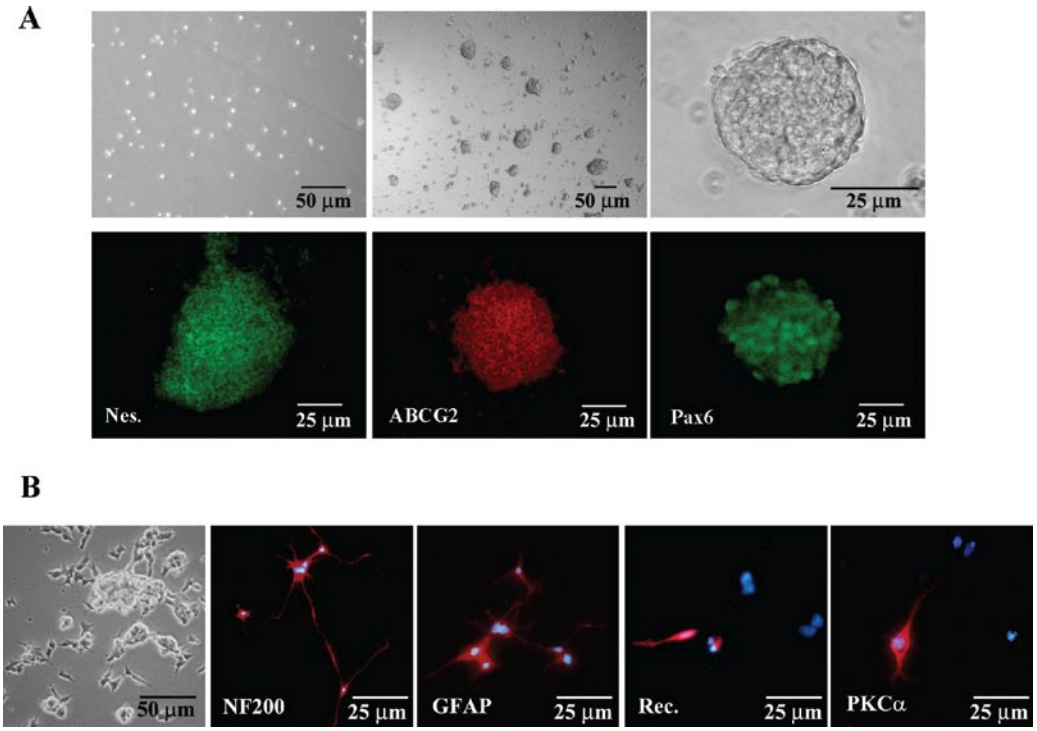

Figure 1. Maintenance of sphere-forming and differentiation capacity of original CSCs after long-term culture. (A) After 160 days, enzymatically dissociated single cells continued to form tumor spheres with positive immunostaining for retinal stem cell marker Nestin (Nes), Pax6 and ABCG2. (B) When these spheres were transferred to differentiating condition, cells migrated out of the core of spheres and extended processes were noted. Immunocytochemistry showed that differentiated cells expressed mature retinal cell marker neurofilament 200 (NF200, neurons), glial fibrillary acidic protein (GFAP, glia cells), recoverin (Rec., photoreceptors) and protein kinase Cα (PKCα, bipolar cells). Nuclei were counterstained with DAPI.

cancer stem-like cells exist in retinoblastoma capable of proliferation as neurospheres for up to 8 weeks (18). In our study, long-term primary cultures were established which were able to be expanded as floating spheres for >30 passages. Primary tumor specimens were dissociated and incubated in serum-free medium containing growth factors. After primary spheres were observed 11 days later, these floating spheres were enzymatically dissociated as single cells and replated two times every three weeks to generate secondary spheres. Instead of fetal bovine serum, egg trypsin inhibitor was used to neutralize without inducing differentiation of potential self-renewing cells. After 160 days, sustainable tumor spheres were obtained (Fig. 1A) and passaged weekly, indicating that self-renewing cells were maintained in these floating spheres. The population doubling time of the sphere-forming cells was ~60 h as determined from the growth curve of passage 20 spheres (Fig. 2A). Limiting dilution assay (Fig. 2B) revealed that at least 179.88 cells were required to generate one tumor sphere. Based on Poisson distribution and intersection at the 37% level, the minimal frequency of cancer stem-like cells in the sphere population was estimated to be 0.6%. Immunocytochemical staining of spheres from passage 25 showed positivity for retinal stem cell markers Nestin, Pax6 and also ABCG2 which has an important role in maintaining retinal stem cell properties (25) (Fig. 1A).

To determine whether tumor spheres maintain their differentiation potential during long-term culture, spheres were plated on poly-D-lysine- and laminin-coated dishes under differentiating conditions. During the first 4 h after plating, floating spheres attached to the culture dishes rapidly. The outgrowth of cells and the extension of cell processes were observed. After 5-6 days, the majority of sphere cells migrated out of the sphere edge, growing as flat, neuron-like or fibroblast-like cell shapes. Expression of mature retinal cell markers was examined during the following 3-4 days; immunoreactivity for neurofilament 200 (NF200, neurons), glial fibrillary acidic protein (GFAP, glial cells), recoverin (photoreceptors) and protein kinase Cα (PKCα, bipolar cells) was demonstrated (Fig. 1B).

Differences in gene expression between sphere-forming cells and differentiated cells derived from the spheres. We next examined expression of genes specific to embryonic or retinal stem cells and mature retinal cells in both undifferentiated spheres and differentiated cells. RNA extracted from passage 25-27 spheres and differentiated cells derived from those spheres was analyzed by real-time quantitative RT-PCR (Fig. 3). The expression level in sphere-forming or differentiated cells was relative to that of parental tumor tissue. Sphere-forming cells overexpressed stem cell markers for *Oct-4*, *Nestin* and *Pax6*, which are essential for embryonic, neural and retinal development respectively (26-29). In contrast, differentiated cells had greatly reduced expression of these genes while they expressed markedly elevated levels of transcripts encoding mature retinal cell markers, such as microtubule-associated protein-2 (MAP2, neurons), GFAP, recoverin (photoreceptors), Opsin B (blue cone photoreceptors) and Nrl (a subtype decision transcription factor for rod photoreceptors). These findings

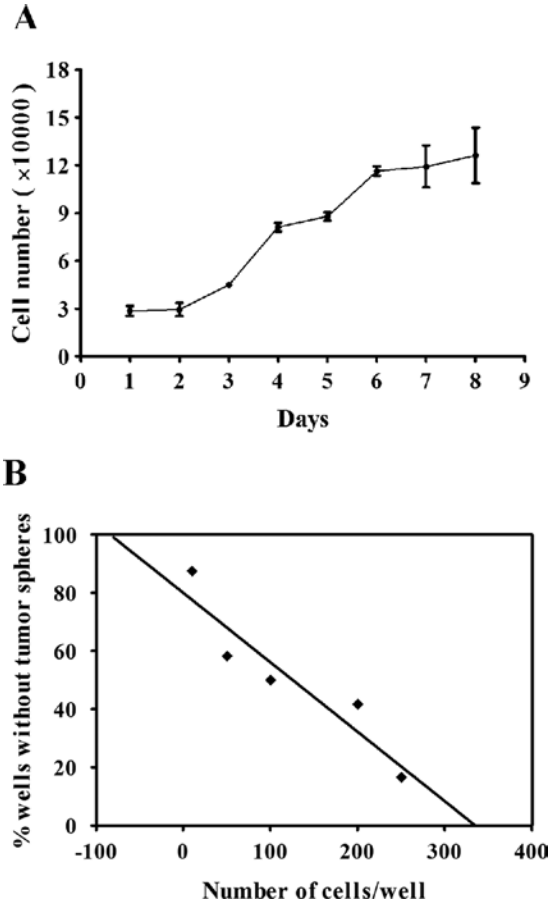

Figure 2. Self-renewal and proliferation properties of long-term sphere-forming cells. (A) The growth curve of sphere-forming cells from passage 20 was plotted, and the doubling time was calculated from the exponential growth phase of the growth curve. (B) Cells plated at limiting dilution (200 μl of media) revealed that the minimal number of cells required to form one tumor sphere was 179.88 cells, and the frequency of cancer stem-like cells in the sphere population was estimated to be 0.6%.

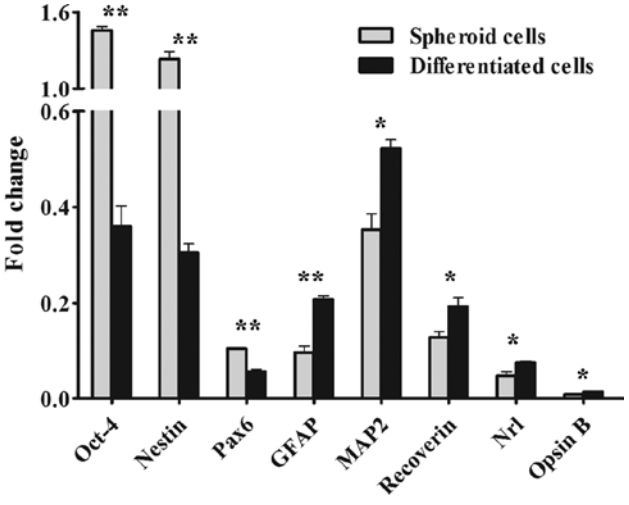

Figure 3. Gene expression analyses of sphere-forming and differentiated cells by real-time quantitative RT-PCR. Sphere-forming cells overexpressed stem cell marker genes *Oct-4*, *Nestin* and *Pax6*, while differentiated cells had a significantly elevated expression of mature retinal cell marker microtubule-associated protein-2 (MAP2, neurons), GFAP (glia cells), recoverin (photoreceptors), Opsin B (blue cone photoreceptors) and Nrl (a subtype decision transcription factor for rod photoreceptors). Data presented were derived from three independent experiments. (*p<0.05, **p<0.01).

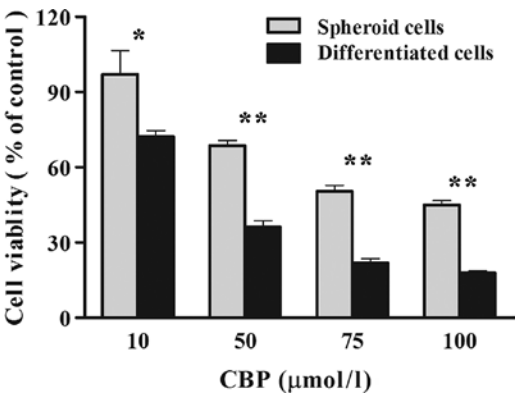

Figure 4. Sphere-forming cells showed increased resistance to conventional chemotherapeutic agent carboplatin (CBP). Cell viability was determined by CCK-8. Data presented were derived from three independent experiments. (*p<0.05, **p<0.01).

provide evidence that sphere-forming cells maintain the ability to differentiate, and differentiated cells retain a different gene expression signature from sphere-forming cells after long-term culture, which is consistent with their different phenotypes.

Sphere-forming cells display chemoresistance to carboplatin. To investigate whether the retinal cancer stem-like cells display resistance to conventional chemotherapies, sphere-forming cells and differentiated cells were exposed to carboplatin, a basic chemotherapeutic agent for retinoblastoma chemoreduction (1,30). Cell viable assays showed that sphere-forming cells were dramatically resistant to carboplatin at different concentrations compared to differentiated cells (Fig. 4). Meanwhile, carboplatin IC_{50} values were greater (p<0.01) for sphere cells under stem cell conditions (87.94 μmol/l) vs. differentiating conditions (25.05 μmol/l). These results indicate that cancer stem-like cells may contribute to the common drug resistance in retinoblastoma.

Maintenance of tumorigenicity of sphere-forming cells after long-term culture. Another essential criterion for cancer stem cells is their *in vivo* capacity for self-renewal and recapitulation of the original tumor. To examine whether the sphere-forming cells perpetuate tumorigenicity through long-term culture, various amounts of sphere-forming cells from passage 21 were injected into the vitreous cavity of NOD/SCID mice. After 10-12 weeks of latency, one of two mice with a minimum injection of $2x10^3$ cells was found to have formed a visible gross tumor, and the other was observed through a microscope to present tumor formation. Correspondingly, injections of $2x10^4$, $2x10^5$ and $2x10^6$ sphere-forming cells were also markedly tumorigenic in both of two mice of three groups within 10 weeks (Fig. 5A and B). The orthotopic xenograft tumors (Fig. 5C and D) presented dense monotonous proliferation with atypical nuclei and scanty cytoplasm, accompanied with eosinophilic necrotic substance, all of which resembled histological features of the original patient tumors (Fig. 5E and F). Many cells in the xenografts expressed Nestin, neuron specific enolase (NSE), while fewer cells showed positive staining for recoverin. Consistent with previous reports (18), we did not observe the expression of GFAP (Fig. 5G) in the

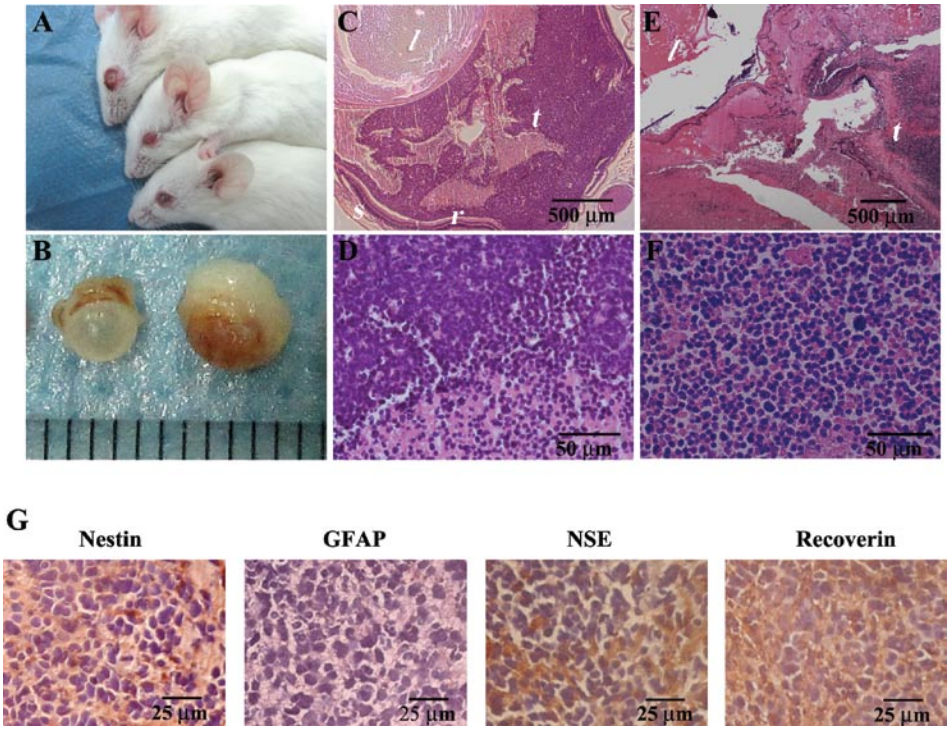

Figure 5. Sphere-forming cells from passage 21 generated tumors in NOD/SCID mice after orthotopic transplantation. Injection of at least $2x10^3$ dissociated sphere-forming cells generated tumors within 10-12 weeks (A). Representative mouse eyeball with a xenograft tumor exhibited corneal necrosis and was abnormally larger than normal control eye with injection of PBS (B). Hematoxylin and eosin (H&E) staining showed that the transplanted tumor (C and D) exhibited typical histological features of human Rb (E and F). Magnification, x40 (C and E); x400 (D and F). At a lower magnification, the invasive original tumor (t) damaged the retina, spread to the vitreous cavity and reached the posterior of the lens (l). Similarly, the xenograft tumor bulk (t) was visible behind the lens (l) and infiltrated the retina (r), choloid and sclera (s). At a higher magnification, the xenograft tumors (D) had atypical small rounded nuclei with scanty cytoplasm, and eosinophilic necrotic substance, all of which resembled the histological features of the original patient tumors (F). Immunochemical staining indicated that the xenograft tumor expressed nestin, neuron specific enolase (NSE) and recoverin, but not GFAP (G).

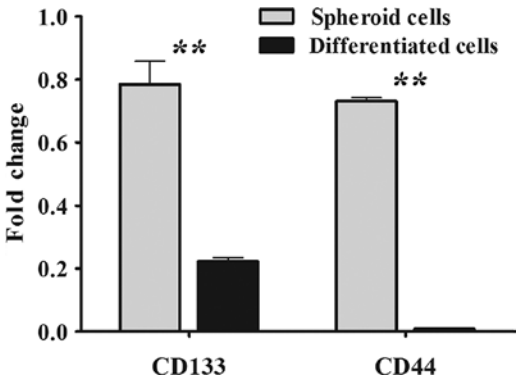

Figure 6. Gene expression of CD44 and CD133 by real-time quantitative RT-PCR. Sphere-forming cells had a significantly higher expression of both CD44 and CD133 compared to differentiated cells. Data presented were derived from three independent experiments. $^{**}p<0.01$.

xenograft tumors which is present in the immunoreactivity of *in vitro* tumor cells. As a previous study reported that tumor neural stem-like cells in glioblastomas were multipotent *in vitro* but monopotent or bipotent *in vivo* (31), the difference between xenograft tumor cells and *in vitro* tumor cells might indicate that cancer stem-like cells are affected by the cell microenviroment during the course of *in vivo* assay (24). Taken together, the *in vivo* tumorigenicity was stably maintained even after a long period of cultivation.

Overexpression of cell surface markers CD44 and CD133 in sphere-forming cells during long-term culture. Based on both our biological and functional characterization of the long-term cultures of retinal tumorigenic cells with stem cell properties, we further examined the expression of cell surface proteins CD44 and CD133 in our culture cells, each of which have been used to identify cancer stem cells from different types of tumors (9,10,14,15,22,32,33). Strikingly, both CD44 and CD133 have a similar expression pattern with stem cell markers. Compared with cells under stem cell conditions, gene expression of CD44 and CD133 was significantly reduced under differentiating conditions (Fig. 6). Furthermore, flow cytometric analysis was performed to evaluate the percentage of these two markers in various passages. With sphere-forming cells propagated from passage 1 to passage 25, the percentage of $CD44^+CD133^-$ population decreased while that of the $CD44^+CD133^+$ population increased under stem cell conditions. In contrast, $CD44^+CD133^+$ cells were present in a much lower percentage when cells were transferred to differentiating conditions (Fig. 7; Table III). These results indicate that CD44 combined with CD133 could be a potential candidate cell surface marker for retinal cancer stem-like cells.

Discussion

There is increasing evidence for the presence of cancer stem cells in malignant tumors. This subpopulation may play a

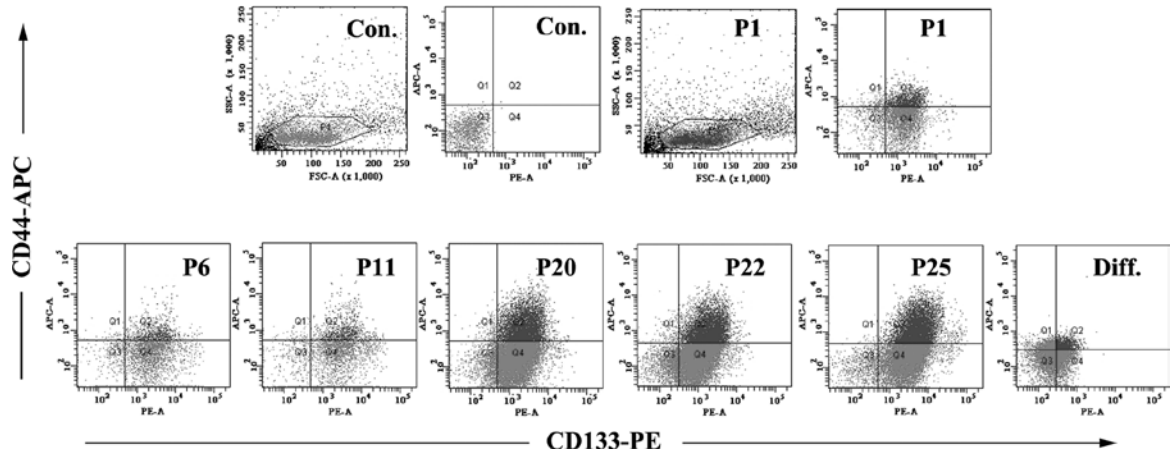

Figure 7. Flow cytometric analysis of CD44 and the CD133 in sphere-forming cells from different passages (P1, P6, P11, P20, P22 and P25) as well as differentiated cells (Diff.). As shown by dot plots, the $CD44^+CD133^+$ population (Q4 quadrant) was increased in the sphere-forming cells, whereas they were greatly reduced when transferred to differentiating conditions.

Table III. Expression of cell surface markers CD44 and CD133 in different passages.

Passage	$CD133^+$ Q2+Q4 (%)	$CD44^+$ Q2+Q1 (%)	$CD44^-CD133^+$ Q4 (%)	$CD44^+CD133^-$ Q1 (%)	$CD44^+CD133^+$ Q2 (%)
P0	84.8	21.6	65.4	2.2	19.4
P6	88.6	17.9	71.9	1.2	16.7
P11	86.7	25.6	62.7	1.6	24.0
P20	91.1	26.9	64.9	0.7	26.2
P22	89.2	28.2	61.6	0.6	27.6
P25	94.2	35.8	58.5	0.1	35.7
Diff.	44.5	27.1	29.4	12.0	15.1

pivotal role in tumor initiation, growth, chemoresistance and recurrence (34,35). To establish a more detailed understanding, functional assays of this subpopulation have been evaluated, and consequently long-term cultures of cancer stem-like cells have been established recently in several types of malignancies (9,32,36-38).

In the present study, serum-free culture conditions were applied as previously described (18,22) for the culturing of retinal cancer stem-like cells obtained from human Rb lesions. Serum-free medium allows for the maintenance of an undifferentiated stem cell state, and addition of EGF and bFGF induces the proliferation of multipotent, self-renewing neural stem cells (22). Similar serum-free medium has also been used for long-term culture of human retinal progenitor cells; the majority of cells continue to express progenitor markers as well as the ability to express markers of neuronal and glial lineage *in vitro*. (Yang J, *et al*, IOVS 51: ARVO E-abs. 2649, 2010). Fortunately, we established long-term cultures of retinal cancer stem-like cells as non-adherent tumor spheres, which can be derived to differentiate as adherent neuron-like or fibroblast-like cells. The ability of cells to generate spheres after serial passages demonstrates their self-renewal potential. In addition, immunocytochemical staining of stem cell markers and the limiting dilution assay both confirmed this long-lasting capacity. For the multipotent differentiation criterion for distinguishing stem cells, sphere-forming cells were transferred to differentiating conditions and produced a similar phenotype with Rb tumor containing neurons, glial cells and photoreceptors. To confirm that the sphere-forming cells possess the capacity to produce the heterogeneous lineages of tumor cells that comprise the tumor, at least $2x10^3$ cells were injected into the vitreous cavity of mice, which resulted in grafted tumors that were histologically identical to the original Rb tumors. Moreover, previous reports demonstrated that cancer stem cells are responsible for the natural resistance to chemotherapeutic drugs (9,11,39). To the best of our knowledge, our study is the first to report that sphere-forming cells in retinoblastoma are markedly chemoresistant to carboplatin as opposed to differentiated cells. According to these findings, established long-term cultures can be used as an *in vitro* model with which to study cancer stem-like cell properties in human retinoblastoma. These cultured cells can be frozen and thawed and still retain the same cancer stem cell properties including capacities of tumor sphere-forming, differentiation and tumorigenicity. Compared to the retinoblastoma cell lines available to date, these cells have a unique characteristic of growing in non-adherent spheres. The doubling time is approximately 60 h, which is longer than Y79 and SNUOT-Rb1 cells (23,40).

They also produced a tumor with as few as $2x10^3$ cells, which is much lower than previously reported. To provide further evidence, karyological examination will be performed in future studies.

As mentioned above, another currently accepted characterization of cancer stem cells is isolation using distinct cell surface antigens. To identify any alterations in cells under stem cell and differentiating conditions, real-time quantitative RT-PCR was used to analyze the gene expression patterns. It is notable that sphere-forming cells overexpressed stem cell genes such as *Oct-4*, *Nestin* and *Pax6*, while differentiated cells showed lower expression of these genes but greater expression of differentiated state genes including MAP2, GFAP, recoverin, Nrl and Opsin B. Moreover, it is interesting that CD44 and CD133 showed a similar expression pattern to stem cell genes. CD44 is a receptor for hyaluronic acid and has been described as a breast, ovarian, gastric, prostate and pancreatic cancer stem cell marker (9,10,15,32,33). The CD133 antigen is a penta-span membrane glycoprotein, which has been proposed as both a neural stem cell marker and a CSC marker in brain, colon and other solid tumors (14,22). Conversely, some studies imply that CD133 appears to be a component in photoreceptor maintenance and/or function (17). In retinoblastoma, Balla *et al* studied the phenotypic characterization for the presence of putative cancer stem-like cell markers using flow cytometric analysis of freshly isolated tumor cells. The authors revealed that the FSC^{lo}/SSC^{lo} subpopulations, which appeared to be more primitive, were positive for CD44 but negative for CD133, CXCR4 and CD90 (41). In our study, CD44 combined with CD133 indicated the existence of putative cancer stem-like cells in Rb, since there was a significant difference in expression in sphere-forming cells vs. differentiated cells. To verify this possibility, long-term cultures were used as a tool to detect the percentage of CD44- and CD133-positive cells in various passages. As expected, the $CD44^+CD133^+$ population increased in the sphere-forming cells, which may have been enriched and perpetuated in the serum-free medium over a prolonged culture; however, this population was greatly reduced when transferred to differentiating conditions. Therefore, CD44 and CD133 could be potential candidate cell surface markers for identifying retinal cancer stem-like cells. However, whether $CD44^+CD133^+$ cells are actually enriched vs. any other cell surface marker combination needs to be further assessed through *in vivo* experiments isolating and identifying the tumorigenicity of this small subset of tumor cells.

To conclude, long-term cultures of retinal cancer stem-like cells represent a suitable *in vitro* model with which to study cancer stem-like cells in retinoblastoma and to develop more sensitive chemotherapeutic drugs and therapeutic strategies aimed at eradicating this tumorigenic subpopulation within retinoblastoma.

Acknowledgements

The authors thank Jihong Wu, Department of Ophthalmology, Eye and ENT Hospital, Fudan University for providing technical support and providing the Nestin and NSE antibodies, and also thank Rongjia Chen, Yinwen Bi, Xiaping Li, Department of Pathology, Eye and ENT Hospital, Fudan University for support in acquiring fresh samples and paraffin sections. This project was supported by Shanghai Science and Technology Committee Grant 074119636.

References

1. Schefler AC and Abramson DH: Retinoblastoma: what is new in 2007-2008. Curr Opin Ophthalmol 19: 526-534, 2008.
2. Shields CL, Ramasubramanian A, Thangappan A, *et al*: Chemoreduction for group E retinoblastoma: comparison of chemoreduction alone versus chemoreduction plus low-dose external radiotherapy in 76 eyes. Ophthalmology 116: 544-551 e541, 2009.
3. Chan HS, Gallie BL, Munier FL and Beck Popovic M: Chemotherapy for retinoblastoma. Ophthalmol Clin North Am 18: 55-63, viii, 2005.
4. Gunduz K, Gunalp I, Yalcindag N, *et al*: Causes of chemoreduction failure in retinoblastoma and analysis of associated factors leading to eventual treatment with external beam radiotherapy and enucleation. Ophthalmology 111: 1917-1924, 2004.
5. Chan HS, Lu Y, Grogan TM, *et al*: Multidrug resistance protein (MRP) expression in retinoblastoma correlates with the rare failure of chemotherapy despite cyclosporine for reversal of P-glycoprotein. Cancer Res 57: 2325-2330, 1997.
6. Chan HS, Thorner PS, Haddad G and Gallie BL: Multidrug-resistant phenotype in retinoblastoma correlates with P-glycoprotein expression. Ophthalmology 98: 1425-1431, 1991.
7. Filho JP, Correa ZM, Odashiro AN, *et al*: Histopathological features and P-glycoprotein expression in retinoblastoma. Invest Ophthalmol Vis Sci 46: 3478-3483, 2005.
8. Reya T, Morrison SJ, Clarke MF and Weissman IL: Stem cells, cancer, and cancer stem cells. Nature 414: 105-111, 2001.
9. Zhang S, Balch C, Chan MW, *et al*: Identification and characterization of ovarian cancer-initiating cells from primary human tumors. Cancer Res 68: 4311-4320, 2008.
10. Collins AT, Berry PA, Hyde C, Stower MJ and Maitland NJ: Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res 65: 10946-10951, 2005.
11. Ma S, Lee TK, Zheng BJ, Chan KW and Guan XY: $CD133^+$ HCC cancer stem cells confer chemoresistance by preferential expression of the Akt/PKB survival pathway. Oncogene 27: 1749-1758, 2008.
12. Singh SK, Hawkins C, Clarke ID, *et al*: Identification of human brain tumour initiating cells. Nature 432: 396-401, 2004.
13. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ and Clarke MF: Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 100: 3983-3988, 2003.
14. Ricci-Vitiani L, Lombardi DG, Pilozzi E, *et al*: Identification and expansion of human colon-cancer-initiating cells. Nature 445: 111-115, 2007.
15. Li C, Heidt DG, Dalerba P, *et al*: Identification of pancreatic cancer stem cells. Cancer Res 67: 1030-1037, 2007.
16. Bonnet D and Dick JE: Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 3: 730-737, 1997.
17. Seigel GM, Hackam AS, Ganguly A, Mandell LM and Gonzalez-Fernandez F: Human embryonic and neuronal stem cell markers in retinoblastoma. Mol Vis 13: 823-832, 2007.
18. Zhong X, Li Y, Peng F, *et al*: Identification of tumorigenic retinal stem-like cells in human solid retinoblastomas. Int J Cancer 121: 2125-2131, 2007.
19. Shields CL, Mashayekhi A, Au AK, *et al*: The International Classification of Retinoblastoma predicts chemoreduction success. Ophthalmology 113: 2276-2280, 2006.
20. Shields JA, Shields CL, Lally SE and Eagle RC Jr: Harvesting fresh tumor tissue from enucleated eyes: the 2008 Jack S. Guyton lecture. Arch Ophthalmol 128: 241-243, 2010.
21. Tropepe V, Sibilia M, Ciruna BG, Rossant J, Wagner EF and van der Kooy D: Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon. Dev Biol 208: 166-188, 1999.
22. Singh SK, Clarke ID, Terasaki M, *et al*: Identification of a cancer stem cell in human brain tumors. Cancer Res 63: 5821-5828, 2003.
23. Kim JH, Yu YS, Kim DH, Kim CJ and Kim KW: Establishment and characterization of a novel, spontaneously immortalized retinoblastoma cell line with adherent growth. Int J Oncol 31: 585-592, 2007.

24. Clarke MF, Dick JE, Dirks PB, *et al*: Cancer stem cells - perspectives on current status and future directions: AACR Workshop on Cancer Stem Cells. Cancer Res 66: 9339-9344, 2006.
25. Bhattacharya S, Das A, Mallya K and Ahmad I: Maintenance of retinal stem cells by Abcg2 is regulated by notch signaling. J Cell Sci 120: 2652-2662, 2007.
26. Loh YH, Wu Q, Chew JL, *et al*: The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nat Genet 38: 431-440, 2006.
27. Wiese C, Rolletschek A, Kania G, *et al*: Nestin expression - a property of multi-lineage progenitor cells? Cell Mol Life Sci 61: 2510-2522, 2004.
28. Ashery-Padan R and Gruss P: Pax6 lights-up the way for eye development. Curr Opin Cell Biol 13: 706-714, 2001.
29. Mathers PH and Jamrich M: Regulation of eye formation by the Rx and pax6 homeobox genes. Cell Mol Life Sci 57: 186-194, 2000.
30. Carcaboso AM, Bramuglia GF, Chantada GL, *et al*: Topotecan vitreous levels after periocular or intravenous delivery in rabbits: an alternative for retinoblastoma chemotherapy. Invest Ophthalmol Vis Sci 48: 3761-3767, 2007.
31. Galli R, Binda E, Orfanelli U, *et al*: Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. Cancer Res 64: 7011-7021, 2004.
32. Ponti D, Costa A, Zaffaroni N, *et al*: Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. Cancer Res 65: 5506-5511, 2005.
33. Takaishi S, Okumura T, Tu S, *et al*: Identification of gastric cancer stem cells using the cell surface marker CD44. Stem Cells 27: 1006-1020, 2009.
34. Pardal R, Clarke MF and Morrison SJ: Applying the principles of stem-cell biology to cancer. Nat Rev Cancer 3: 895-902, 2003.
35. Jordan CT: Cancer stem cell biology: from leukemia to solid tumors. Curr Opin Cell Biol 16: 708-712, 2004.
36. Inagaki A, Soeda A, Oka N, *et al*: Long-term maintenance of brain tumor stem cell properties under non-adherent and adherent culture conditions. Biochem Biophys Res Commun 361: 586-592, 2007.
37. Wang J, Wang X, Jiang S, *et al*: Partial biological characterization of cancer stem-like cell line (WJ(2)) of human glioblastoma multiforme. Cell Mol Neurobiol 28: 991-1003, 2008.
38. Attard G, Rizzo S, Ledaki I, *et al*: A novel, spontaneously immortalized, human prostate cancer cell line, Bob, offers a unique model for pre-clinical prostate cancer studies. Prostate 69: 1507-1520, 2009.
39. Rich JN and Bao S: Chemotherapy and cancer stem cells. Cell Stem Cell 1: 353-355, 2007.
40. Reid TW, Albert DM, Rabson AS, *et al*: Characteristics of an established cell line of retinoblastoma. J Natl Cancer Inst 53: 347-360, 1974.
41. Balla MM, Vemuganti GK, Kannabiran C, Honavar SG and Murthy R: Phenotypic characterization of retinoblastoma for the presence of putative cancer stem-like cell markers by flow cytometry. Invest Ophthalmol Vis Sci 50: 1506-1514, 2009.