CE-CAM model for evaluating CD133^{lo} Cancer Stem Cells in Retinoblastoma

Rohini M Nair

University of Pennsylvania

Narayana VL Revu

University of Hyderabad

Sucharita Gali

University of Hyderabad

Prathap Reddy Kallamadi

National Institute of Nutrition

Varsha Prabhu

University of Hyderabad

Radhika Manukonda

University of Hyderabad

Harishankar Nemani

National Institute of Nutrition

Swathi Kaliki

LVPEI: LV Prasad Eye Institute

Geeta K Vemuganti (✉ gkvemuganti@gmail.com)

University of Hyderabad <https://orcid.org/0000-0002-0432-2109>

Research

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Abstract

Background

Cancer Stem Cells (CSCs) reported in various tumors, play a crucial role in tumorigenesis and metastasis. Following the efforts to reduce, replace and refine the use of mammalian models, we aimed to establish a short-term xenograft for Retinoblastoma (Rb) to evaluate the tumorigenic and metastatic potential of CD133^{lo} CSCs in Rb Y79 cells, using the well-established chick embryo (CE) model.

Methods

Total and CD133 sorted Rb Y79 cells, labelled with eGFP/CM-Dil tracking dye, were transplanted onto the chorioallantoic membrane (CAM) of day-7 chick embryos and incubated for 7 days. The tumor formation on CAM and metastasis to the embryos were evaluated by confocal microscopy, *in-vivo* imaging, and histopathology.

Results

Y79 cells formed pink-white raised perivascular nodules on the CAM with CD133^{lo} CSCs exhibiting larger nodules when compared to CD133^{hi} cells and total Y79 ($p < 0.05$). *In-vivo* imaging revealed that the labeled cells metastasized to the embryos with the fluorescent signals visible in the abdominal area, cephalus and the limbs. Histopathologic studies confirmed the presence of tumor cells on the CAM, organs of embryos transplanted with Y79 cells, more so with CD133^{lo} CSCs.

Conclusions

This study highlights that the CE-CAM is a feasible alternative non-mammalian model for evaluating tumorigenicity and metastatic potential of Rb CSCs. The study also provides preliminary evidence that Rb Y79 CD133^{lo} CSCs show higher propensity to form tumor nodules on the CAM and are more invasive than non CSCs, thus, supporting our earlier evidence that they are endowed with CSC properties.

Background

Several *in-vivo* model systems have been developed to study tumor biology and metastasis(1)(2). Among them, the Chick Embryo-Chorioallantoic Membrane (CE-CAM) model has been the model of choice for more than a century, owing to its naturally immunodeficient state, ease of manipulation, visualization and short growth period(3). One of the earliest reports of tumor transplantation onto the CAM was from Murphy and his group(4), who demonstrated that rat sarcoma grew on the CAM and retained its histological properties after several days of inoculation. The CAM is a specialized structure that comprises of fully developed ectoderm capillary plexus representing a network of tiny capillaries connecting the arterial and venous blood vessel networks within 10 days of embryonation, therefore making it a suitable model to explore the progression and metastasis of tumor cells(3)(5)(6). The CE-

CAM model has been used extensively over several decades as an *in-vivo* growth system for studying various attributes of cancer varying from angiogenesis to targeted therapy(7)(8)(9).

Retinoblastoma (Rb) is the most common intraocular paediatric tumor that affects the developing retina, caused predominantly due to mutations in the *RB1* gene(10). Survival with the precise and timely diagnosis with treatment is as high as 95%, but when left untreated, the disease can be fatal. Rb can occur in one or both the eyes and advanced bilateral Rb is very difficult to treat owing to tumor cell invasion into the vitreous, sub-retinal space and aqueous chamber with high chances of extraocular metastasis(11). Therefore, it is imperative to understand the tumorigenesis, invasion and assess the effectiveness of different agents in order to treat these advanced cases. Translation of *in-vitro* studies to *in-vivo* model systems has become an integral part of functional studies in tumor biology and clinically relevant model systems, greatly help in developing better treatment strategies(12). Several genetic and xenograft animal models have been established to explore Rb tumorigenesis, metastasis and targeted therapies(13). The genetic models have been developed based on knockout strategies of key *Rb*-related genes such as *p107*, *p130*, *p53* and over expression of oncogenes such as *Chx10* and *Pax6*(14). Orthotropic and xenograft rodent models have also been developed; however, the major disadvantage of these models is the lag time of tumor development and resemblance to the developmental nature of Rb tumorigenesis. The CE-CAM model has been explored for ocular tumors; however, its potential to validate the CSC properties has not been evaluated(15)(16)(17). In our attempt to understand the role of CSCs in Rb, which would help to develop better diagnostic and therapeutic strategies, we demonstrated that the CSC properties within the Y79 Rb cell line are endowed in the CD133^{lo} population as evident by the stem cell-like properties, such as colony forming ability, differentiation, chemoresistance, invasion, and stem cell-specific gene expression signature(18). Interestingly, in other tumors, for e.g. neural tumors, the CSC properties were observed in CD133 expressing cells(19). We believe that the phenotypic markers may be specific to tumor type and tissue of origin with different tumors exhibiting CSC markers, such as CD133, CD44, EpCAM, and ABCB1(20)(21)(22).

In this study, we aimed to establish a developmental xenograft model system for investigating the role of CSCs in Rb tumorigenesis and metastasis using the CE-CAM model; specifically, to validate the tumor-initiating properties and invasive properties of CD133^{lo} CSCs by gross, confocal, *in-vivo* imaging and histological techniques.

Methods

Cell Culture

Rb Y79 cell line (Riken: RCB1645) was a kind gift from Dr. S. Krishnakumar, Sankara Nethralaya, India and eGFP Y79 cells was a generous gift from Prof Sarah E Coupland, Liverpool Ocular Oncology Research Group, University of Liverpool, UK. The cells were cultured in RPMI-1640 (Gibco™, Thermo Fisher Scientific, USA) medium supplemented with 10% Fetal Bovine Serum (Gibco™, Thermo Fisher Scientific, USA) and 1X antibiotic-antimycotic solution (Gibco™, Thermo Fisher Scientific, USA). They

were cultured at 5% CO₂ and 37⁰C until they reached over 80% confluence following which they were sub-cultured for the experiments.

Magnetic Activated Cell Sorting (MACS) and Flow cytometry

The Y79 cultured cells were sorted using CD133 micro bead kit according to manufacturer's protocol (Miltenyi Biotec Inc., auburn, CA) as described previously(18). Briefly, Cultured Rb Y79 cells were washed with MACS buffer (2mM EDTA and 0.5% FBS containing PBS; pH-7.2), and re-suspended with 300µl of MACS buffer. 100µl of FCR blocking reagent and 100µl of CD133 micro beads were added and incubated for 30 minutes at 4°C for CD133 magnetic labelling. The cell mixture was passed through the LS mini MACS columns followed by the repeated washes with MACS buffer, CD133^{lo} cells were eluted first and collected in tube with media. The columns were removed from MACS magnet and CD133^{hi} cell fractions were eluted out using MACS buffer. Both the cell populations were concentrated and re-suspended in 1 ml of serum free media. The cell count and cell viability of post MACS were assessed by haemocytometer. The purity of the sorted cells was analysed by flow cytometry (BD LSRFortessa™).

Chick Embryo CAM assay

Embryonated white leghorn Gramapriya eggs (*G. gallus*) were procured from ICAR-Directorate of Poultry Research, Hyderabad, India. Ethics approval was obtained by the Institutional Ethics Committee at the University of Hyderabad and all the experiments were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The eggs were sterilized with 70% alcohol and incubated at 38⁰C and 68% relative humidity in an egg incubator (Sun Engineering, India). Growth, viability and vascularization of eggs were monitored by candling every alternate day post E3 stage. On E7-8 stage, a small circular window was made into the air sac of the egg using sterile micro-scissors. The CAM was identified by lifting the inner shell membrane and a small capillary was gently abraded. Two million cells of CM-Dil Y79 total (n=38), CD133^{lo} (n=41) and CD133^{hi} (n=53) and eGFP Y79 total (10), CD133^{lo} (n=10) and CD133^{hi} (n=10) cells in media containing Matrigel (BD Biosciences™) were transplanted on the CAM. The window was sealed back with sterile tape and was placed in the incubator undisturbed for 7 days until dissection.

CAM Tumor Volume measurement

The tumor volume measurements were performed for the eGFP Y79 cells transplanted tumor nodules. Briefly, the window was reopened to identify tumor nodules on day 14, the eGFP Y79 transplanted cells tumor volumes were measured using a Vernier caliper and volume was estimated by the following formula(23):

$$\text{Tumor Volume} = \frac{4}{3} \times \pi \times r^3 \quad (r = \frac{1}{2} \times \text{diameter}_1 \times \text{diameter}_2)$$

CAM whole mount and Confocal Imaging

The CAM tissues with the tumor nodules were gently rinsed with cold Phosphate buffered saline (PBS), placed on a glass slide and mounted with 50% Glycerol for Confocal analysis. The CAM was scanned on Laser Scanning Confocal microscope (Carl Zeiss NLO-710) at 550/570 excitation and emission spectrum for CM-Dil Y79 transplanted cells and 488/509 excitation and emission spectrum for eGFP Y79 transplanted cells. The 3D images were taken at different magnifications and the CAM was also scanned at different range of depths by using Z-stack method. Localized depths having strong fluorescent signals were selected for image analysis by Image J software and parameters were adjusted using Zen 2010 software.

***In-vivo* imaging of embryos**

The whole embryos were analysed using IVIS Spectrum *in-vivo* optical imaging system (PerkinElmer) to track the presence of CM-Dil Y79 cells and eGFP Y79 cells. The embryos transplanted with CM-DilY79 cells and eGFP Y79 cells were exposed to excitation/emission spectrum of 550/570 and 488/509 for 30 seconds respectively. Embryos injected with PBS solution were used as controls. Image display analyses were performed using Living Image software (version 4.3.1, Xenogen, Alameda, USA). Data was obtained from the fluorescent images by selecting the region of interest (ROI) and the number of photons emitted was measured as average radiance (photons/sec/cm²/sr).

Histological analysis

The nodules with the surrounding CAM tissues were rinsed with PBS and fixed in 10% buffered formalin for 24 hours. The embryos were lifted from the egg and cleaned with PBS to remove the yolk and extra embryonic membranes. The embryos were decapitated and the embryonic organs (brain, eye, femur-bone marrow, and liver) were dissected and placed in formalin fixative for 24 hours. Following fixation, they were processed for routine histological analysis. Immunohistochemistry was performed with KI67 antibody (Roche Life Sciences) to detect Rb Y79 proliferating tumor cells using automated benchmark ultra IHC diagnostic system (Roche Life Sciences). The slides were then analysed under a light microscope and the staining was independently assessed by an experienced ocular pathologist (GKV).

Statistical Analysis

The quantitative data were stated as Mean±SEM and GraphPad Prism (GraphPad Software, La Jolla, CA) was used for unpaired Student's t-test and ANOVA with Tukey's Post-hoc multiple comparison tests. The representative images were analysed using ImageJ software. The experiments were repeated at least thrice with biological replicates and p<0.05 was considered as statistically significant difference between the groups.

Results

Analysis of CSCs in Rb Y79 cell line

Using CD133 surface marker, flow cytometry analysis showed that $15.5 \pm 0.32\%$ of Y79 cells were CD133^{lo} (Fig. 1). The percentage of CD133^{lo} CSCs within the Y79 cell line is concordant with our earlier study. The cells were sorted for both the populations using magnetic activated cell sorting (MACS) with a purity of $\geq 90\%$. Cell viability was found to be over 85% for all the cell groups.

In-vitro CM-Dil fluorescent labelling and GFP transduction efficiency

For xenograft studies, two approaches of fluorescent cell tracking were utilized and their staining intensities were analysed *in-vitro* prior transplantation. Y79 cells, labeled with fluorescent cell tracker CM-Dil dye were assessed for staining intensity and were observed to retain the dye for 17 days in culture (Fig. 2a-c). However, uniformity of staining within individual cells were noted to reduce as the number of days *in-vitro* increased, possibly owing to cell proliferation.

The enhanced green fluorescent protein (eGFP) labeled Y79 cells were tracked and assessed for GFP expression up to 2 weeks in culture by fluorescence microscopy. The intensity of GFP fluorescence varied cell to cell and majority of cells expressed GFP *in-vitro* (Fig. 2d-f).

Embryo viability and assessment of tumor formation

Y79 cells sorted using CD133 marker was examined for their tumorigenicity and metastasis *in-vivo*. Briefly, 2 million total Y79, CD133^{lo} and CD133^{hi} cells were transplanted separately onto the abraded CAM and the embryos were sacrificed after 7 days. The survival percentages of CM-Dil Y79 transplanted embryos were 69.32% (n = 27), 69.14% (n = 28) and 79.71% (n = 41), and eGFPY79 cells transplanted embryo viability were 50% (n = 5), 90% (n = 9) and 90% (n = 9) for total Y79, CD133^{lo} and CD133^{hi} cells respectively. Both the labeled cells of total Y79 and CD133^{lo} groups formed pinkish-white raised wet perivascular nodules with feeder vessels on the CAM (Fig. 3a, c, e and g), whereas CD133^{hi} formed smaller plaque-like growths on the CAM (Fig. 3i and k), as observed upon gross dissection. The tumor volume of eGFP Y79 CD133^{lo} nodules were significantly higher ($40.44 \pm 7.74\text{mm}^3$) when compared to total ($p = 0.02$, $5.3 \pm 1.01\text{mm}^3$) and CD133^{hi} ($p = 0.005$, $2.56 \pm 0.66\text{mm}^3$) nodules (Fig. 5a). Confocal imaging confirmed the presence of growing tumor nodules within the CAM (Fig. 3). The fluorescence intensity from the region of interest (ROI) analysis of CM-Dil Y79 cells transplanted CAM tissues showed that CD133^{lo} group ($\text{AUF} = 6.37 \times 10^7 \pm 7.7 \times 10^6$) (Supplementary Material: Video I) had higher localization of cells when compared to total Y79 group ($\text{AUF} = 3.33 \times 10^7 \pm 0.2 \times 10^6$) and CD133^{hi} group ($p < 0.0001$, $\text{AUF} = 1.08 \times 10^7 \pm 1.6 \times 10^6$) (Supplementary Material: Video II) (Fig. 5b). Similarly, the eGFP Y79 cells transplanted CAM tissues showed that CD133^{lo} group ($\text{AUF} = 13.94 \times 10^4 \pm 2.54 \times 10^4$) (Supplementary Material: Video III) had higher localization of cells when compared to total Y79 group ($p = 0.0018$, $\text{AUF} = 3.75 \times 10^4 \pm 0.9 \times 10^4$) and CD133^{hi} group ($p = 0.0003$, $\text{AUF} = 1.39 \times 10^4 \pm 0.4 \times 10^4$) (Supplementary Material: Video IV) (Fig. 5c).

In-vivo imaging analysis of spontaneous metastasis to the embryo

The IVIS-spectral images of the chick embryos transplanted with tumor cells on the CAM revealed fluorescence signals in the cephalic, abdominal areas and within the bones of hind limbs. The control group (injected with PBS) did not show any fluorescence signals within the embryo (Fig. 4a and e). The embryos transplanted with CM-Dil Y79 CD133^{lo} cells showed intense fluorescence and more spread out signal compared to total Y79 and CD133^{hi} group ($p = 0.0277$) (Fig. 4b-d and 5d). Similarly, eGFP Y79 CD133^{lo} cells showed intense fluorescence and more spread out signal compared to total Y79 ($p = 0.0168$) and CD133^{hi} group ($p = 0.0049$) (Fig. 4f-h and 5e). Further analyses of the three areas of the embryos showed that abdominal region had higher fluorescence followed by the cephalic region and the limbs. The embryos transplanted with CD133^{lo} CSCs had increased metastases to the abdominal, cephalic and limbs when compared to CD133^{hi} non-CSCs of both the labeled cell groups.

Histological analysis of the xenograft formation and metastases

Histological examination of the tumor nodules showed areas of viable cells as well as necrotic tumor areas within the CAM along with surface ulceration (Fig. 6a-c). In contrast to the host cells within the CAM, the tumor cells were larger with high nuclear-cytoplasmic ratio and mitotic figures (Fig. 6b and c). The surrounding CAM also revealed focus areas of inflammatory cells and granulation tissue with giant cells (Fig. 6a). Metastatic tumor deposits were seen in the embryos transplanted with CD133^{lo} cells as pockets of infiltrating round cells next to the blood vessels within the embryonic liver tissues, brain and the eye (Fig. 6d-f). The metastatic Y79 cells within the liver, brain and eye showed immunoreactivity for Ki-67 indicating proliferation in the distant sites (Fig. 6g-i).

Discussion

Retinoblastoma, a childhood ocular cancer, accounts for 3% of all childhood tumors and can be fatal if left untreated owing to the rapidly growing tumor cells within the developing mutated retina(24). Although several *in-vitro* and *in-vivo* animal model systems have been explored to study the underlying mechanisms of Rb tumorigenesis and metastasis, there is a challenge in investigating the tumor in a developmental microenvironment. In this study, we demonstrate the establishment of CE-CAM xenograft model for Rb Y79 cell line with formation of tumor nodules on CAM, that exhibit spontaneous metastasis to the embryo. Our study also provides preliminary evidence for CD133^{lo}, being the putative stem cells in Rb with higher potential to form tumor and metastases as documented by the *in-vivo* study using CM-Dil labeled and eGFP transduced Rb Y79 cells on chick embryo.

This novel work demonstrates that the CE model is a cost effective, time saving (procurement and maintenance), and a suitable model for visualization and examining Rb tumorigenesis and metastasis

that could pave the way for exploring targeted therapy. This study provides *in-vivo* evidence and validates the results of our *in-vitro* studies that cancer stem cells properties are evident in CD133^{lo} population of RbY79 cells(18).

Animal models using transgenic and xenograft approaches have been well-established for Rb(13)(25). Most of them include the mammalian system in order to resemble the tumor microenvironment. However, a developmental model is difficult to recapitulate in this system due to many technical difficulties(13). There is a dire need to simulate Rb tumorigenesis and metastasis within a developmental microenvironment in order to develop strategies for better therapeutic targeting. The CE-CAM, therefore, offers not only a temporal investigation but also provides a reliable tool for screening therapeutics in a short duration.

Our study demonstrated the formation of tumor nodules on CAM and spontaneous metastatic spread to the embryo using CM-Dil labeled and eGFP Y79 cells. The potential of CE-CAM model in Rb studies has been explored by Busch et al, who demonstrated the tumor forming ability of different cell lines (normal and chemoresistant) in soft agar as well as CAM and also evaluated the tumor suppressor effect of the Trefoil factor family (TFF) peptides in Rb cells using the CE- CAM model(15)(16)(25). Though the purpose of their study was towards cell line characterization and chemoresistance, the data does not provide any evidence for metastasis within the embryo. One of the possible reasons could be that the authors checked for invasion on E17/18, by which time the immune system of the embryo is observed to be fully functional and reactive(3). We concur with their observation of Y79 cells being capable of forming nodules on CAM and having the potential of spontaneous invasion within the CAM tissue. This observation is also similar to studies done on other solid tumors, such as glioblastoma, ovarian cancer, prostate cancer and uveal melanoma(7)(17).

The tumor nodules formed by the Y79 cells had presence of feeder vessels developing around indicative of tumor angiogenesis. Ribatti et al., demonstrated the presence of multiple host vessels around and within the tumor nodule in a neuroblastoma CAM xenograft model suggesting that the tumor cells were capable of inducing angiogenesis using the chorioallantoic vasculature(26). This could also serve as an important model to enhance or suppress Rb angiogenesis in tumor models due to its ease of visualization.

The spontaneous metastasis was observed in the cephalic, abdominal and limb regions of the embryo using the whole embryo *in-vivo* imaging and confirmed by histology in the sections of embryonic organs such as the brain, eye and liver. Rb is clinically known to metastasize to the brain by invading through optic nerve and to the bone and liver via haematogenous spread and, therefore, involvement of optic nerve, uvea and sclera are considered as one of the important prognostic factors to predict metastasis(10)(27). Our findings also concur with the study done by Palmer and co-workers who investigated spontaneous metastasis of Human Epidermoid Carcinoma cell line (HEp3) to the chick liver and lung(28). Though the imaging of CAM and embryo was possible after dissection, there was an observed signal in the beak area which did not reveal any presence of tumor cells upon histological

analysis. This could be due to autofluorescence due to the density of the bone and keratin present on the beak.

A further novel aspect of our study is the use of this model to study the tumorigenic and metastatic potential of CD133^{lo} CSCs within the Y79 cell line. This is an important validating point as CD133^{hi} subset is projected as CSC in tumors of the brain(29), while the negative cells seem to harbour CSC properties in Rb(18). It would be logical to propose that while many CSC markers could be common to most tumors, some of them could vary in each tumor specifically due to the lineage of the tumor or specific aspects of differentiation of cells in that target tissue. For instance, CD133 is a marker of photoreceptor differentiation(30), hence their precursor cells are expected to be negative for this marker, which we have documented both in primary as well as Y79 cell line(18)(31).

The CE-CAM model is an ideal short-term *in-vivo* system to study different subsets of tumors and identify tumor initiating cells. We also demonstrate the use of a cell tracker dye CM-Dil, with comparison to eGFP labeled cells, in order to track Rb cells *in-vivo*. CM-Dil dye has been successfully used earlier in Rb zebrafish orthotopic xenograft model by Chen et al. who tracked the tumor cell invasion locally and along the optic nerve(32). However, considering that CM-Dil dye intensity could reduce with proliferation of cells(33), we also assessed the same using stable eGFP labeled Y79 cells. GFP labeled tumor cells were successfully used by several investigators to track *in-vivo* metastases within the chick embryos in several tumors(17)(34)(35). Our analyses showed that both the cell labeling strategies can be utilized for *in-vivo* tracking. To the best of our knowledge, this is the first study that explores the use of *in-vivo* fluorescence imaging of whole embryo for identifying spontaneous metastasis in a CE-CAM Rb xenograft model.

Use of other alternate imaging technologies such as *in-vivo* PET/CT has been demonstrated using radiotracers by Warnock et al in a glioblastoma xenograft model for studying tumor metastasis(36). Their studies showed the PET tracer uptake over time by the tumor within the embryo and were able to measure the tumor volume with improved accuracy using CT imaging. The CE-CAM, owing to the ease of visualization through the window from the eggshell makes it extremely suitable and convenient for tracking tumor cells *in-vivo*.

The clinical relevance of this study is the use of an inexpensive, easy to establish *in-vivo* model using cell lines and patient derived xenografts for exploring existing and novel therapeutics. However, there are certain limitations of the CE model and further studies are required to understand the cellular adaptations to avian microenvironment. The incubation period of about a week may not be suitable for slow growing tumor cells and it may be difficult to observe visible metastases in such cases. We believe that validating these results with early passage Rb primary cells and other Rb cell lines- WERI-Rb1, etc., in the future, would add more value to this *in-vivo* model. Use of CM-Dil dye for tracking rapidly growing cells may be a challenge owing to its loss with each successive tumor generations, which could possibly under represent the tumor load of the tissue. Parallel confirmation with GFP labeled Y79 cells helped overcome this limitation by demonstrating similar results in tumor formation and metastasis. Quantitative studies using human specific gene expression assays could compliment these assays in future(28). Though the

imaging of CAM and embryo was possible after dissection, the serial imaging of the entire live egg including the shell would be ideal for which different kinds of fluorescent dyes and standardization of the software settings may be required, which we hope to address in our future studies.

Conclusions

In this study, we have successfully established an *in-vivo* tumor xenograft model for Rb CSCs using chick embryo, which simulates the developmental nature of Rb tumorigenesis. We showed evidence for tumor formation within the CAM and spontaneous metastasis to the embryo. CD133^{lo} cells showed higher tumor forming ability and metastatic potential when compared to total, CD133^{hi} Rb Y79 cells. Therefore, this model system can be used as a valuable *in-vivo* platform to understand Rb tumorigenesis, metastasis and pave the way for CSC specific targeted therapies.

Abbreviations

CE (Chick Embryo), CSCs (Cancer Stem Cells), CE-CAM (Chick Embryo- Chorioallantoic Membrane), CD133 (Prominin-1), CD44 (Hyaluronic acid receptor), EpCAM (Epithelial cell adhesion molecule), ABCB1 (ATP-binding cassette sub-family B member 1), CM-Dil- Chloromethylbenzamido-Dil, eGFP- enhanced Green Fluorescent Protein, ROI (Region of Interest), SEM (Standard Error of Mean), ANOVA (Analysis of Variance), AUF (Arbitrary unit of Fluorescence), TFF (Trefoil factor family), PET (Positron Emission Tomography), CT (Computed Tomography), RPMI-1640 (Roswell Park Memorial Institute-1640), USA (United States of America), CA (California), MACS (Magnetic Activated Cell Sorting), BD (Becton, Dickinson and Company).

Declarations

Ethics approval and consent to participate:

Ethics approval was obtained by the Institutional Ethics Committee of the University of Hyderabad, India.

Consent for publication:

Not applicable

Availability of data and material:

Not applicable

Competing interests:

The authors declare no competing financial interests.

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Authors' contributions:

The study was conceptualized by GKV, experiments were designed by RMN, NVLR and GKV. CE-CAM assays and experiments post dissection were performed by RMN, NVLR, SG, VP and RM. *In-vivo* image acquisition and ROI analyses were done by NVLR, PRK and HN. GKV, RMN, NVLR, SG, and VP contributed to histological analyses. Validation of experiments, data interpretation and manuscript preparation were done by RMN, NVLR, and GKV. All the authors contributed to the discussion of the results and approval of the final manuscript.

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Figures

Figure 1

Flow cytometry analysis of CD133 expression in Rb Y79 cell line. a) Scatter plot of Y79 cells with gating around the live population b and c) Doublet discrimination plots d) CD133-APC expression e) Analysis of the sub-populations

Figure 2

Tracking of fluorescent labeled (CM-Dil and GFP) Y79 cells in-vitro. CM-Dil labeled Y79 cells in culture on day a) one, b) five, and c) 17 days under TRITC filter (40X, 10X, 20X). eGFP Y79 cells in culture on day d) one e) ten and f) 15 days under FITC filter (10X).

Figure 3

Y79 Rb cells form visible tumor nodules (marked with dotted lines) with vascularization within the CAM tissue following a week of transplantation. a, b) Gross and Confocal images of CM-Dil Y79 total cells c, d) Gross and Confocal images of eGFP Y79 total cells e, f) Gross and Confocal images of CM-Dil Y79 CD133lo cells g, h) Gross and Confocal images of eGFP Y79 CD133lo cells i, j) Gross and Confocal images of CM-Dil Y79 CD133hi cells k, l) Gross and Confocal images of eGFP Y79 CD133hi cells. (*Area of tumor nodule imaged using Confocal microscopy).

Figure 4

Evidence of Rb spontaneous metastasis in different parts of chick embryo analysed using in-vivo IVIS spectral imaging. a) Chick embryo injected with PBS solution imaged using 550/570 excitation/emission spectrum (negative control), b) Chick embryo transplanted with CM-Dil Y79 total cells, c) CM-Dil Y79 CD133lo cells, d) CM-Dil Y79 CD133hi cells, and e) Chick embryo injected with PBS solution at 488/509 excitation/emission spectrum (negative control), f) Chick embryo transplanted with total eGFP Y79 cells, g) eGFP Y79 CD133lo cells and h) eGFP Y79 CD133hi cells.

Figure 5

Imaging analysis of tumor nodules formed by total Y79 cells, CD133^{lo} and CD133^{hi} cells on the CAM and within the embryo (metastatic foci). a) CD133^{lo} cells formed larger nodules on CAM when compared to total and CD133^{hi} cells (*p=0.02, **p=0.005). Corresponding confocal analysis of tumor nodules on CAM showed that CD133^{lo} cells displayed highest fluorescence intensity when compared to the nodules formed by total and CD133^{hi} cells labeled with b) CM-Dil (**p<0.0001) c) eGFP (**p=0.0018, ***p=0.0003). CD133^{lo} cells also showed increased spontaneous metastasis to the embryo when compared to CD133^{hi} cells labeled with d) CM-Dil (*p=0.0277) e) eGFP (*p=0.0168, **p=0.0049).

Figure 6

Histopathology of CE-CAM and organs (Hematoxylin and Eosin staining) shows evidence of proliferating tumor cells. Microphotographs of the CE showing a,b,c) thickened CAM with multiple foci of tumor infiltrates, edema and inflammation and tumor nodules infiltrating the CAM stroma. CD133lo cells transplanted cells show tumor cells in d) blood vessels of the chick liver (vascular emboli), e) brain and f) eye. Corresponding immunohistochemical images of KI-67 stained CD133lo Rb Y79 tumor cells within the g) liver h) brain and i) eye.

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