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Comparative analysis of ABCG2+ and ABCG2- retinoblastoma cells in three-dimensional culture

Linda Cassidy, Robert Diaz, Ruby Yanru Chen-Tsai, Gail M Seigel

ABSTRACT

Aims: Retinoblastoma (Rb) is the most common intraocular malignancy of early childhood. Subpopulations of Rb cells express the stem cell marker ABCG2; yet little is known regarding the differences between ABCG2+ and ABCG2- subpopulations, particularly in three-dimensional culture. The aim of this study was to compare ABCG2+ (stem-like) populations with ABCG2- cells in retinoblastoma in three-dimensional, tumor-like populations. Methods: We compared aggregates of ABCG2+ and ABCG2- Rb cells to test the hypothesis that ABCG2+ cells would preferentially exhibit stem cell markers and less mature retinal marker expression as compared with ABCG2- cells. Retinoblastma cells (Y79 and WERI-Rb27) were immunomagnetically enriched into ABCG2+ and ABCG2- populations and grown as three-dimensional aggregates. Enriched populations were examined as aggregates, along with embryoid bodies formed by iPSCs. Results: Immunostaining revealed that both ABCG2+ and ABCG2- cell aggregates were immunoreactive to tubulin III (ectoderm), but immunonegative for smooth muscle actin (mesoderm) and alpha-fetoprotein (endoderm), typical human embryonic markers. ABCG2+ aggregates also exhibited greater immunoreactivity to stem cell markers ABCG2, ALDH1A1 and CD164, but less immunoreactivity to mature markers (MAP-2 and S-Antigen) as compared with ABCG2- cells. Conclusion: ABCG2+ aggregates express more stem cell markers as compared with ABCG2- Rb cells, but both appear to be restricted to an ectodermal lineage. These results aid in our understanding of ABCG2+ subpopulations of cells in Rb, with possible implications for disease development and chemoresistance.

Keywords: ABCG2, In vitro tumor assay, Stem cells, Retinoblastoma

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INTRODUCTION

Retinoblastoma (Rb) is an ocular malignancy of early childhood. Previous studies from our own group [1–3] and others [4–7] established expression of stem cell markers, including the chemoresistance marker ABCG2

in retinoblastoma. The ATP-binding cassette sub-family G member 2 (ABCG2, BCRP) is a multi-drug resistance transporter [8] and neural stem cell marker [9]. In cancer, ABCG2 is reported to play a role in the resistance of cancer cells to chemotherapeutic agents [10]. The expression of ABCG2 has been described in hematologic and lymphoid malignancies [11], as well as solid tumors, including malignancies of the breast, lung, pancreas, colon, liver, stomach and brain [12–18]. Its expression is also frequently correlated with chemotherapy-resistant disease and shortened survival [19, 20]. Therefore, comparisons between ABCG2+ and ABCG2- populations may have applications for potential treatment approaches and therapeutic targets for Rb and other malignancies.

Approximately, 4% of retinoblastoma cells express ABCG2, with some co-localization with other stem cell markers, such as Oct3/4 and Nanog [1, 2]. Although ABCG2 is a stem cell marker expressed in retinoblastoma, little is known about the characteristics of these ABCG2+ subpopulations, particularly in three-dimensional culture. The degree of potency for ABCG2+ and ABCG2- is not clear. The purpose of this study was to make direct comparisons of ABCG2+ and ABCG2- enriched Rb populations in three dimension, without the confounding host influences of tumor xenografts or human tumors *in situ*. To this end, we enriched Rb cells into ABCG2+ and ABCG2- populations and grew them as three-dimensional aggregates in an embryoid body assay. We compared these Rb cells with iPSC-induced embryoid bodies in order to test the hypothesis that ABCG2+ Rb aggregates contain pluripotent cells [21]. We examined aggregates to determine the degree of immunoreactivity for pluripotent stem cell markers and mature retinal markers.

MATERIALS AND METHODS

Cell culture: Retinoblastoma cell lines Y79 (ATCC, Manassas, VA) and WERI-Rb27 (a generous gift from Dr. John Ludlow) were cultured in Dulbecco's modified eagle medium (DMEM) containing 10% calf serum. Both cell lines have been maintained in the Seigel laboratory for over 20 years and are periodically thawed from earlier frozen stocks, tested for the presence of mycoplasma as well as confirmation of human origin (qPCR and immunohistochemistry). The cells are far removed from their original tumors, but are commonly used cell lines for retinoblastoma research. Strict separation is always maintained between cell lines. The cells were incubated at 37°C with 5% CO_2. Induced pluripotent stem cells (iPSC) for the embryoid body formation were prepared from human skin cells that were obtained from patients according to the Declaration of Helsinki.

Enrichment of ABCG2+/- retinoblastoma cells: Human Rb cells were magnetically separated using an ABCG2-FITC antibody (Stem Cell Technologies Inc., Vancouver, British Columbia) and the EASYSep Human FITC Positive Selection Kit (Stem Cell Technologies Inc. Vancouver British Columbia). For each enrichment experiment, we collected approximately 4% of the cells as ABCG2+ and 96% of the cells as ABCG2-. Enriched cell populations were used in amodified embryoid body assay to examine cells in three-dimensional aggregates.

Embryoid Body Assay/In vitro tumor assay: Although both the WERI-Rb27 cells and Y79 cells normally grow in suspension, each cell line was subjected to the same culture conditions used for embryoid body (EB) formation applied to adherent iPSC lines. WERI-Rb27 cells and Y79 cells were incubated on Matrigel (Gibco/Life Technologies) for ten days while the cell lines were transitioned to mTeSR-1 media (Stem Cell Technologies Inc., Vancouver British Columbia) with 10% daily increments. Afterward, the size of the aggregates was visually scored. Aggregates judged either too small or large, when compared to the average size of iPSC colony aggregates used for EB formation were discarded. The aggregate mix was then transferred to six well Ultra Low Adhesion plates (Corning) and allowed to grow for five days in mTeSR-1 media. The plates were manually shaken twice daily. The medium was changed every two days. The aggregates were then fixed in 10% formalin, embedded in paraffin, and cut into 5-µm serial sections for immunohistochemistry.

Immunohistochemistry: Serial sections were processed for staining. The sections were first permeabilized for 30 minutes at room temperature in a PBS-Tween-20 solution, followed by two washes with 1X PBS. The sections were then incubated in blocking solution (Applied Stem Cell) for one hour at room temperature, washed with 1X PBS, incubated in an Avidin/Biotin blocking solution (IHC Tekcat# IW-1301) for one hour at room temperature and washed three time with 1X PBS. For the EB analysis, the following primary antibodies were used (dilution factors are available, but not concentrations): anti-α-fetoprotein (endoderm) (1:400, DAKO, Glostrup, Denmark), anti-smooth muscle actin (mesoderm) (1:100 DAKO) and anti-tubulin III (ectoderm) (1:200, Abcam, Cambridge, MA, USA). After the application of the primary antibody, the samples were incubated at room temperature for two hours, washed three times with 1X PBS-Tween-20, incubated in peroxidase blocking solution (IHC-Tek, cat# IW-1300) for ten minutes at room temperature and then washed three times with 1X PBS-Tween-20. The biotinylated secondary antibody was applied and the samples were incubated at room temperature for two hours and then washed in 1X PBS-Tween-20. The staining procedure consisted of a 30 minute incubation in a streptavidin-HRP solution (IHC-Tek, cat# D03-110) for 30 minutes at room temperature, three washes of two minutes each in 1X PBS, a 30 minutes incubation in DAB solution (IHC, cat# C02-100) and three washes of five minutes each in 1X PBS-Tween-20. Lastly, the samples were rinsed in ddH20, dehydrated in 95% and 100% ethanol, cleared in

xylene twice (five minutes) and mounted. The images of the stained aggregates were captured using a Leica DFC-500 camera attached to LeicaDM200 LED microscope.

Immunofluorescence staining: Paraffin slides were baked for 60 minutes at 60°C, then incubated stepwise in xylene, alcohol and boiling sodium citrate (pH 6.0). Slides were blocked with commercial blocking solution (Syd Laboratories, Malden, MA, USA) and permeabilized in PBS with Tween-20 (0.05%). Slides were examined by immunofluorescence for markers ALDH1A1 (Abcam), Nestin (Sigma, St. Louis, MO), ABCG2 (Sigma), CD164 (Sigma), S-Antigen (Abcam country), MAP-2 (Abcam) and PAX6 (Abcam). The slides were incubated in primary antibodies (or isotype control) diluted to 1:100 for one hour. Secondary antibody conjugated with TRITC and/or FITC (Sigma, stock concentration ranging from 3–6.5 mg/mL), diluted to 1:400 in PBS was added to the slides for one hour after washing three times with PBS. Slides were incubated in DAPI (Life Technologies, Grand Island, NY, USA) and rinsed in PBS. Fluorescent mounting medium (KPL Gaithersburg, MD, USA) was used to cover the slides. Immunofluorescence was photographed using Spot Advanced software (Spot Imaging Solutions, Sterling MI, USA).

RESULTS

Aggregates of ABCG2+ and ABCG2- retinoblastoma cells do not resemble iPSC-derived embryoid bodies

Since retinoblastoma is a tumor of infancy/early childhood and expresses some human embryonic stem cell markers [1–3], we tested the hypothesis that ABCG2+ Rb cells would express markers characteristic of human embryonic ectoderm, mesoderm and endoderm as an indicator of embryonic-like pluripotency. Retinoblastoma cells were enriched for ABCG2+ or ABCG2- and grown in an embryoid body assay to avoid complications with host xenograft tissues. We chose immuno staining as our endpoint, as this would allow results on a cell-by-cell basis, in contrast to molecular methods that would involve nucleic acid extraction from multiple aggregates. Retinoblastoma cell aggregates were compared with embryoid bodies formed by induced pluripotent stem cells (iPSCs). Figure 1 illustrates the morphology of WERI-Rb27 and Y79 cells grown as ABCG2+ and ABCG2- cells as compared with standard iPSCs. Note the ragged appearance of the Rb aggregates created by both WERI-Rb27 and Y79 cells, as compared with the rounder, smoother embryoid bodies formed by iPSCs (Figure 1).

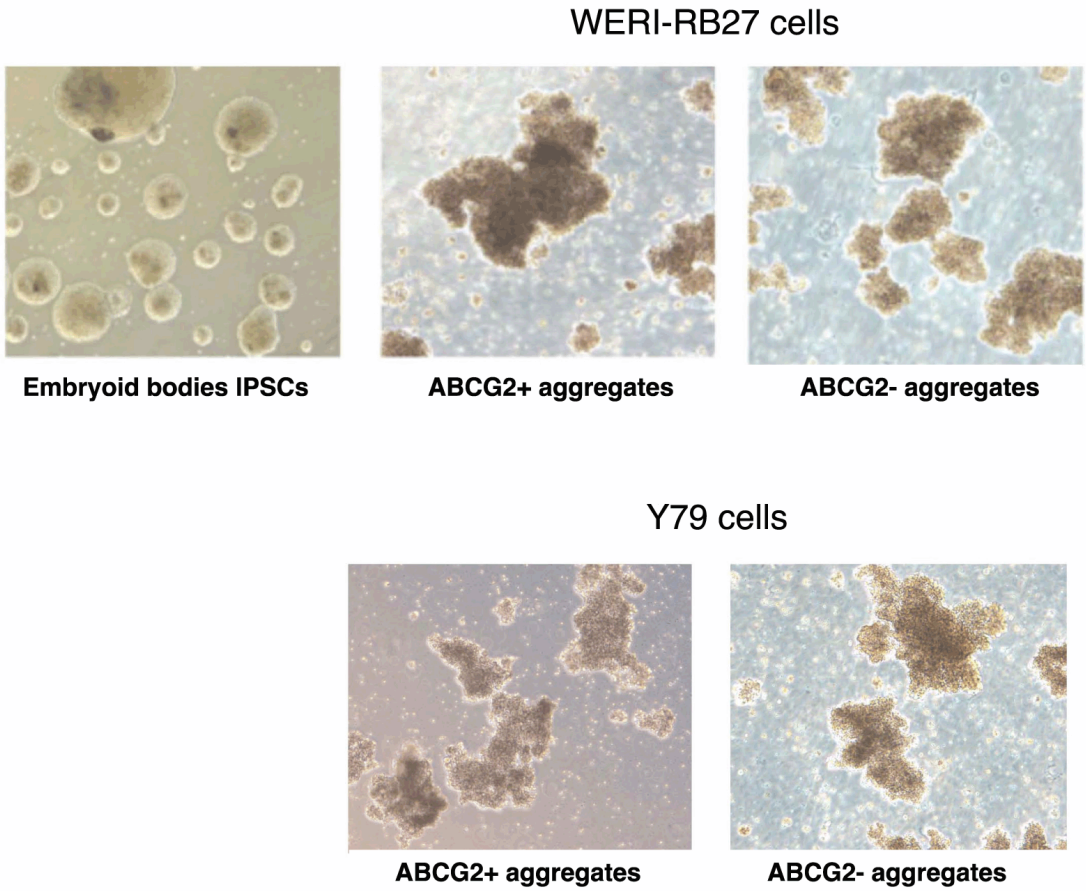

Figure 1: Aggregates of ABCG2+ and ABCG2- retinoblastoma cells grown in an in vitro tumor assay do not resemble iPSC-derived embryoid bodies. ABCG2+ and ABCG2- enriched Y79 and WERI-Rb27 cells were grown in three-dimensional culture in an in vitro tumor assay. Cell aggregates are shown in comparison with iPSC-derived embryoid bodies. Note the round, smooth morphology of the embryoid bodies formed by iPSCs as compared with the more ragged cell aggregates formed by the ABCG2+ and ABCG2- retinoblastoma cells.

The aggregates varied in size, but were similar between Rb cell populations.

ABCG2+ and ABCG2- Rb cells in three dimentional clusters express beta-tubulin but neither alpha-fetoprotein nor smooth muscle actin

ABCG2+ and ABCG2- growths were prepared for histological analysis and stained for embryonic markers alpha-fetoprotein, smooth muscle actin and beta-tubulin. Results are shown for WERI-Rb27 cells, with similar results for Y79 cells (not shown) (Figure 2). Both ABCG2+ and ABCG2- Rb cells show intense immunoreactivity to beta tubulin (ectoderm), but no immunoreactivity to alpha-fetoprotein and smooth muscle actin. Induced pluripotent stem cell embryoid bodies are shown for comparison as positive controls for each marker, as well as negative controls.

ABCG2+ cells retain ABCG2 immunoreactivity, as well as stem cell markers

ABCG2+ and ABCG2- cell aggregates from the *in vitro* tumor assay were examined for stem cell associated markers by immunofluorescence. The aggregate material

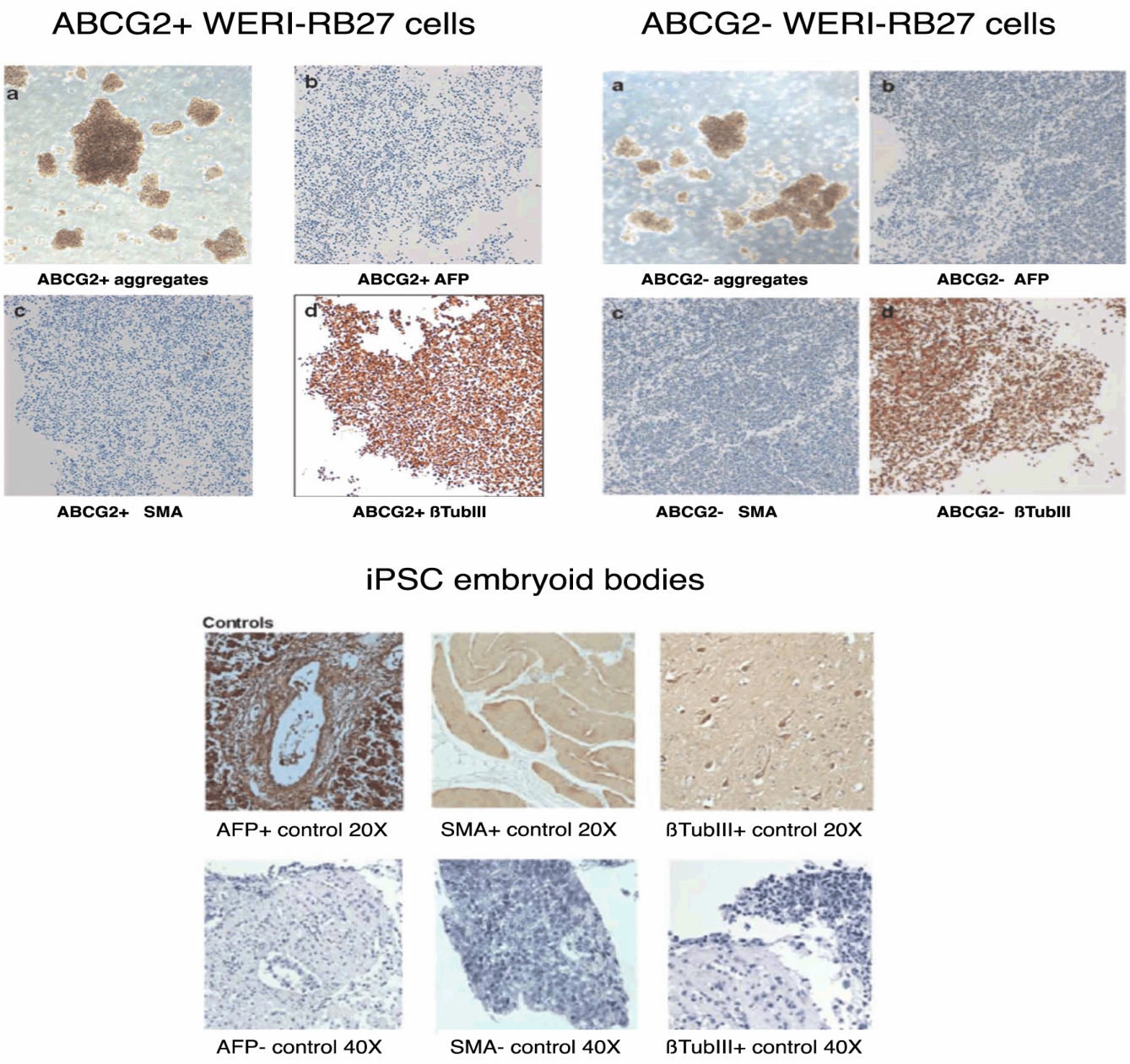

Figure 2: ABCG2+ and ABCG2- retinoblastoma cells express beta-tubulin, but neither alpha fetoprotein nor smooth muscle actin. ABCG2+ and ABCG2- enriched WERI-Rb27 cells were grown in three-dimensional culture and analyzed for embryonic markers beta-tubulin, alpha-fetoprotein (AFP) and smooth muscle actin (SMA). Both ABCG2+ and ABCG2- cells were immunoreactive for only beta-tubulin and not AFP or SMA. Positive and negative control staining is shown for embryoid bodies in the lower panel.

prepared for immuno staining was too small for statistically significant quantitation, but provided convincing photo micrographic evidence of staining intensity. In Figure 3, ABCG2+ enriched WERI-Rb27 cells retained some ABCG2 immunoreactivity from the original separation over several passages. ABCG2+ enriched cell populations also displayed greater immunoreactivity to CD164, a stem cell-associated marker as compared with ABCG2- cells. ABCG2+ cells also had greater immunofluorescence for ALDH1A1 but comparable levels of immunoreactivity for nestin in comparision to ABCG2- cells. Embryoid bodies exhibited immunoreactivity to CD164, ALDH1A1 and Nestin.

ABCG2- cells exhibit greater immunoreactivity for mature retinal markers MAP2 and S-Antigen, but not PAX6, as compared with ABCG2+ cells

We continued our investigation of three-dimensional aggregate cultures of ABCG2+ and ABCG2- enriched cells with more mature retinal markers. We tested the hypothesis that ABCG2- cells would display more immunoreactivity for mature markers than the more primitive ABCG2+ cells. Figure 4 illustrates that ABCG2- aggregates show greater immunoreactivity for MAP-2 and S-Antigen than ABCG2+ aggregates. The degree of PAX6 immunoreactivity was similar in both populations. In contrast, embryoid bodies displayed little to no immuno reactivity for MAP2, S-Ag and PAX6.

DISCUSSION

In this study, we examined potential differences between ABCG2+ and ABCG2- enriched populations in a three-dimensional culture system. We examined our ABCG2+ and ABCG2- enriched Rb cells in order to determine their degree of developmental potency. We used a novel in vitro approach by adapting what would normally have been embryoid body assay [22, 23] used for pluripotent iPSCs and fashioned it into an in vitro tumor assay. We chose this method to examine Rb cells in three-dimension but without confounding host factors of animal xenografts or human tumors. One potential hypothesis was that ABCG2+ stem-like cells would behave as iPSCs and form embryoid bodies that would express all three

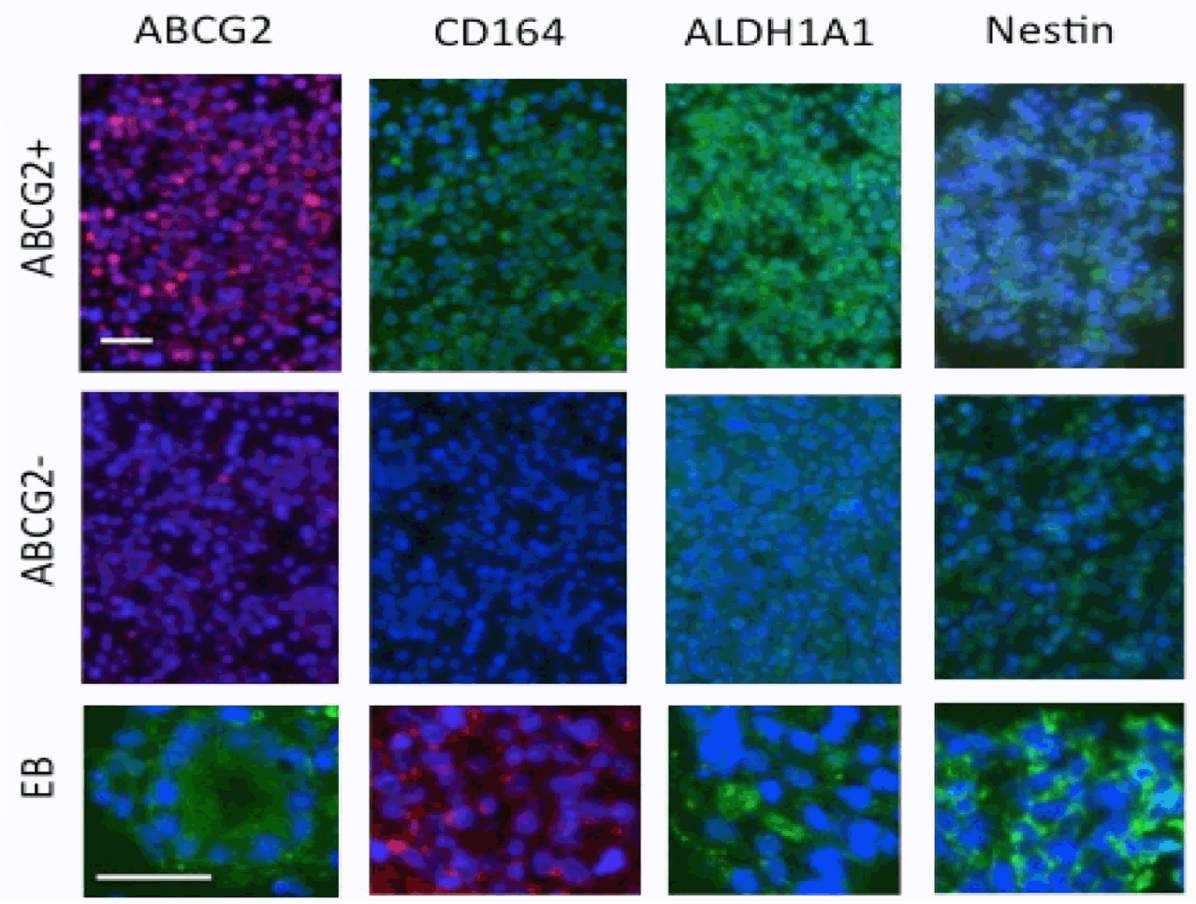

Figure 3: ABCG2, CD164 and ALDH1A1 immunoreactivity is higher in ABCG2+ enriched cells. ABCG2+ and ABCG2- enriched WERI-Rb27 cell aggregates were grown in 3-dimension and immunostained for the stem cell-associated markers ABCG2, CD164 and ALDH1A1. ABCG2+ cells retained some ABCG2 immunoreactivity and exhibited more intense CD164 and ALDH1A1 staining as compared with the ABCG2- aggregates. Embryoid bodies displayed immunoreactivity for CD164, ALDH1A1 and Nestin. (scale bars = 20 microns).

markers of pluripotency, i.e., alpha-fetoprotein, smooth muscle actin and beta-III tubulin. This was not the case in our study. Instead, we found that Rb aggregates did not physically resemble embryoid bodies. Both ABCG2+ and ABCG2- enriched cells were immunoreactive for tubulin III (ectoderm), but immunonegative for smooth muscle actin (mesoderm) and alpha-fetoprotein (endoderm). It is somewhat surprising that ABCG2+ Rb cells do not express mesodermal or endodermal markers, but are indistinguishable from the ABCG2-cells in this regard. These results suggest that although ABCG2+ cells exhibit many cancer stem cell properties, including human embryonic stem cell markers such as Nanog and Oct-3/4 [1–3], they differ from pluripotent embryonic stem cells and appear to be more restricted to an ectodermal lineage. This places ABCG2+ Rb cells in contrast to developmental stem cells that are capable of differentiating into all developmental lineages. This is an important finding that demonstrates a limitation in the stem cell phenotype of ABCG2+ Rb cells that has not been reported previously.

Despite these similarities in the expression of the three markers commonly used for the embryoid body assay, we did see differences between ABCG2+ and ABCG2- enriched aggregates in terms of other stem cell markers, particularly CD164 and ALDH1A1. CD164 is important because it is a cell adhesion molecule found on the surface of stem cells [21] and is reported to play a role in the growth, mobility, and metastasis in colon cancer cells [24]. ALDH1A1 is another marker common to both developmental stem cells [25] and cancer stem cells [26, 27] that seems to be highly immunoreactive in ABCG2+ enriched aggregates. Other markers, such as nestin and PAX6 did not exhibit major differences between ABCG2+ and ABCG2- enriched cells. For the more mature markers, we saw more MAP-2 and S-Antigen immunoreactivity in ABCG2- cells.

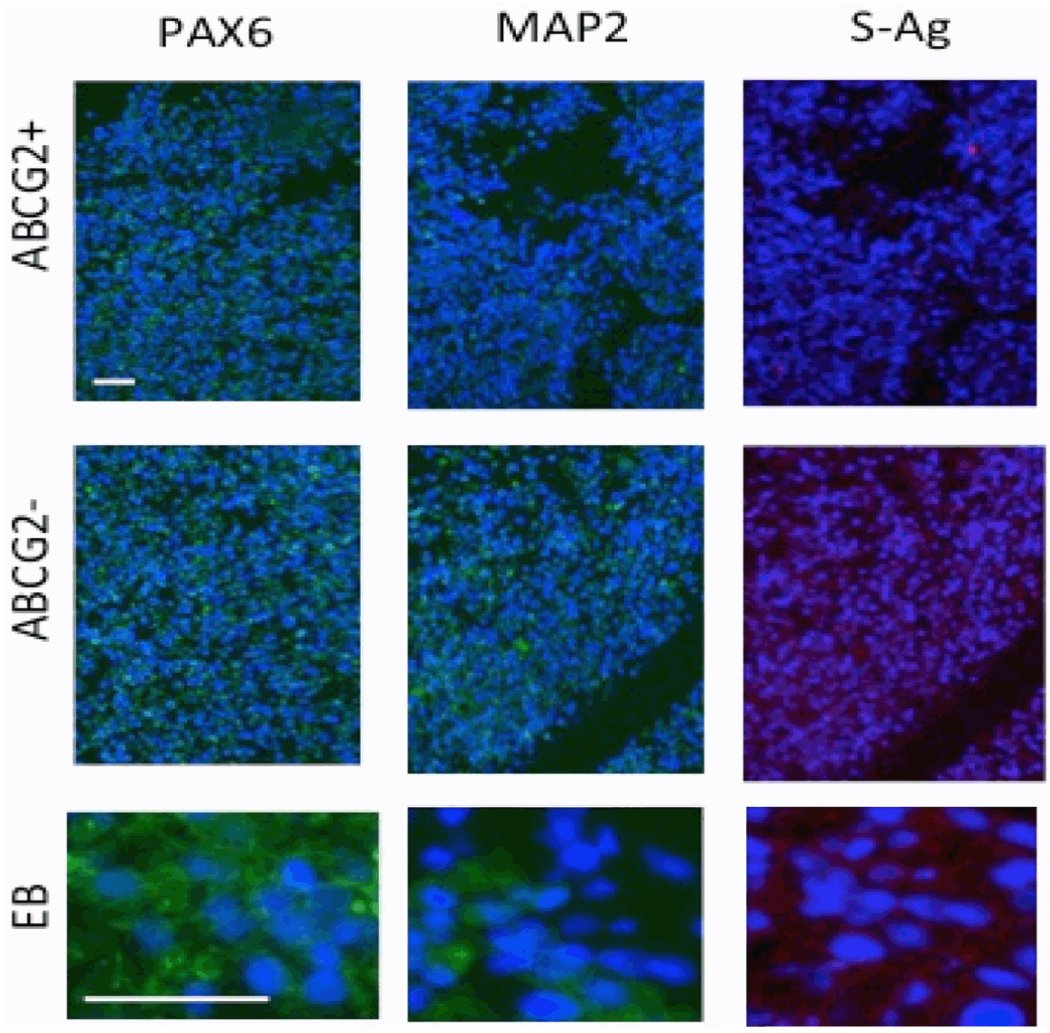

Figure 4: MAP-2 and S-Antigen, but not PAX6, are more immunoreactive in ABCG2- cells than ABCG2+ cells. ABCG2+ and ABCG2- enriched WERI-Rb27 cell aggregates were grown in 3-dimension and immunostained for the more mature retinal markers MAP-2, PAX6 and S-Antigen (Arrestin). MAP-2 and S-Antigen but not PAX6, were more highly immunoreactive in ABCG2+ aggregates compared with ABCG2- aggregates. Embryoid bodies displayed little or no immunoreactivity for these markers. (scale bars = 20 microns).

CONCLUSION

In summary, although both ABCG2+ and ABCG2- cells appear similarly restricted to an ectodermal lineage, they do show some differences in immunoreactivity to specific stem cell-associated and mature markers. A better understanding of these differences may lead to important strategies to target ABCG2+ and other chemoresistant cells in retinoblastoma and other malignancies.

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Author Contributions
Linda Cassidy – Substantial contributions to conception and design, Acquisition of data, Analysis and interpretation of data, Drafting the article, Revising it critically for important intellectual content, Final approval of the version to be published
Robert Diaz – Design and acquisition of embryoid body and cell aggregate data. Analysis and interpretation of data, Drafting the article, Revising it critically for important intellectual content, Final approval of the version to be published
Ruby Yanru Chen-Tsai – Analysis and interpretation of data, Drafting the article, Revising it critically for important intellectual content, Final approval of the version to be published
Gail M Seigel – Analysis and interpretation of data, Drafting the article, Revising it critically for important intellectual content, Final approval of the version to be published

Guarantor
The corresponding author is the guarantor of submission.

Conflict of Interest
Authors declare no conflict of interest.

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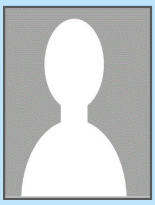

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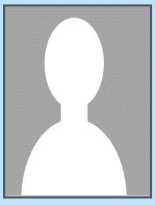

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