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Biomarkers & Applications

Research Article

Chakravadhanula M, et al. Biomark Applic: BMAP-124.
DOI: 10.29011/2576-9588. 100024

Study of Stem Cell Marker Proteins in Atypical Teratoid Rhabdoid Tumor

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Citation: Chakravadhanula M, Ozols VV, Bhardwaj RD, Kala M (2018) Study of Stem Cell Marker Proteins in Atypical Teratoid Rhabdoid Tumor. Biomark Applic: BMAP-124. DOI: 10.29011/2576-9588. 100024

Received Date: 04 April, 2018; **Accepted Date:** 13 April, 2018; **Published Date:** 19 April, 2018

Abstract

Number of studies have shown that tumor biology and resistance to treatment are closely connected to the existence of Cancer Stem Cells (CSCs). Sox2, a stem cell marker belongs to a family of transcription factors that are known to regulate gene expression in normal developmental processes. Sox2 is also pivotal for the maintenance of undifferentiated stem cells. Along with Sox2, other stem cell proteins such as CD133, Olig, and Oct4 are known to have clinical relevance as cancer markers, since they have prominent roles to play in the regulation of pluripotency. Proteins such as cMyc and β-catenin are known to be involved in the regulation of pluripotency. Atypical Teratoid/Rhabdoid Tumors (AT/RTs) are classified as aggressive CNS embryonal tumors with a dismal prognosis. A characteristic feature of AT/RTs is an aberration of chromosome 22, which results in a loss of the gene SMARCB1. In this study we utilized a flow cytometry to examine the expression of CSC markers in AT/RT cells. Analysis of the cell surface or intracellular expression of CSC markers indicated that Sox2, Oct3/4, cMyc, and β-catenin showed increased expression in AT/RT compared to the control neural stem cells. In order to measure the mRNA profile, qRT-PCR analyses were performed on these cell lines. The mRNA profile however did not always correlate with the CSC marker protein expression profile, but indicates that CSC markers in AT/RT can serve as potential biomarker.

Keywords: AT/RT; Beta-Catenin; Cmyc; FACS; Flow Cytometry; Oct; Olig; Stem Cell Factors; Sox2

Introduction

In recent years, studies have shown that tumor biology and resistance to treatment are closely connected to the existence of Cancer Stem Cells (CSCs) [1,2]. CSC research currently suggests these cells have unique self-renewal capabilities thereby sustaining tumor growth, in contrast to the other tumor cells. Moreover, in several studies CSCs have been shown to have tumorigenic potential in addition to enhanced resistance mechanisms [3-7]. Sox2, a stem cell marker is a Sry-containing protein belonging to a family of transcription factors, which are known to regulate gene expression involved in normal developmental processes. Therefore, Sox2 is pivotal for early development and maintenance of undifferentiated embryonic stem cells [8]. SOX2 down regulation after embryogenesis is correlated with loss of pluripotency and self-renewal [9].

Recent studies suggest that SOX2 over expression has been associated with cancer development. In a study by Gangemi et al [10], knockout of SOX2 in a Glioma model caused loss of tumorigenicity. Along with Sox2, other stem cell proteins have been shown to have clinical relevance as cancer markers [11].

CD133 or Prominin is one such stem cell cancer marker, which is a 5-pass transmembrane glycoprotein located in the membrane of human hematopoietic cells and in neural progenitor cells [12,13]. CD133 has been shown to be associated with poor outcomes in brain tumor types such as astrocytoma [14,15], and glioblastoma [16]. Studies have shown a correlation between expression of CD133 and grade level of the brain tumor type [17].

Oct4 is another stem cell marker protein that is expressed in pluripotent embryonic stem cells, where it is a regulator of self-renewal and differentiation [18-20]. It is expressed in several cancer types [18-21]. In an earlier study, knock-out of Oct4 in lung cancer

enhanced sensitivity towards chemotherapy and radiotherapy and also increased apoptotic activity [19]. Elevated levels of Oct4 were found in both high-grade and low-grade astrocytomas in 41 patients examined in a study [20].

Olig 1, Olig 2, and Olig 3 are basic helix-loop-helix transcriptional regulators. The expression of OLIG 1 and 2 is usually associated with early specification of the oligodendrocyte lineage [22,23], thus used as stem cell markers. OLIG 1, 2 are also essential for the maintenance of oligodendrocytes in the adult CNS. Studies have demonstrated high levels of OLIG1 AND OLIG2 mRNA transcript expression in oligodendrogliomas [24,25]. Other studies demonstrated that high levels of Olig protein expression were present in most diffuse gliomas [26,27], such as oligodendrogliomas, astrocytomas and mixed gliomas [28,29].

Myc belongs to a family of helix-loop-helix/leucine zipper transcription factors including cMyc and N-Myc, and has been shown to play a role in embryonic stem cell maintenance in human small cell lung carcinomas [30]. Elevated Myc levels have been shown to interfere with early embryonic development [31-35]. cMyc has been known to be a frequently de-regulated oncogene in human cancers. Recently, studies have shown that as a transcription factor, cMyc alters the expression of many target genes including tumor suppressors and oncogenes. This leads to an acceleration of the intrinsic mutation rate and causes genomic instability commonly seen in tumor genotypes [36]. High cMyc levels were detected in Medullomyoblastoma, a subtype of primitive neuroectodermal brain tumors [37, 38].

Wnt/beta-catenin signaling is a crucial factor in the development of many cancers [39-42]. Studies have shown that aberrant Wnt/beta-catenin signaling plays an important role in atypical teratoid/rhabdoid tumors [39], and glioblastoma [43,44]. In meningiomas, studies have indicated that CTNNB1 is upregulated and localized to the nucleus [45].

Atypical Teratoid/Rhabdoid Tumors (AT/RTs) are classified as aggressive CNS embryonal tumors with a dismal prognosis. A characteristic feature of AT/RTs is an aberration of chromosome 22, which results in a loss of the gene SMARCB1. An earlier study showed expression of CD133 in AT/RT by FACS analysis [2]. Studies done in AT/RT thus far, have not shown the protein expression of CSC markers such as, Sox2, Olig, and Oct by flow cytometry analyses. Expression of oncogenic markers such as cMyc and β-catenin by flow cytometry have not been shown in AT/RT. Therefore, we hypothesized that the above mentioned CSC markers and oncogenic markers will indicate higher protein expression levels in AT/RT compared to a control human neural stem cell line (H9). In the present study we examined the protein expression of these CSC and oncogenic markers in AT/RT cell lines and H9 cells by flow cytometry. The protein expression of these markers was also compared to mRNA expression of the markers by qRT-PCR analyses. The markers included in this study were, Sox2, CD133, Oct3/4, Olig2/3, cMyc, and β-catenin.

Materials and Methods

ATRT Cell Line Establishment, Characterization and Maintenance

The AT/RT cells CHLA-04 was obtained from a 20-month male, and CHLA-05 from a 2-year male. Informed consent was obtained. Tissues were prepared as described [21] and initially cultured as Neurospheres in modified Neurobasal medium consisting of 1:1 DMEM:F12, HEPES (15mM), Sodium Pyruvate (110mg/L), Sodium Bicarbonate (1.2g/L), 1xB27 (Invitrogen, cat#A1895601), EGF (20ng/mL, Invitrogen, cat# 10605HNAE5), bFGF (20ng/mL, Invitrogen, cat#PMG0034). During first two weeks of growth gentamycin 25μg/ml was used. Passaging was at ratio of 1:2-3 with 25% conditioned medium [22]. Loss of SMARCB1 was confirmed by immunohistochemistry, western blotting, qRT-PCR and G-band karyotyping. CHLA-266 and BT-12 were obtained from Children's Oncology Group (COG) and were maintained in culture similar to the AT/RT cell lines mentioned earlier in this section.

Flow Cytometry

The AT/RT cell lines used for this study include, CHLA-04, CHLA-05, BT-12, and CHLA-266. A commercially available human neural stem line, H9 was used as a control cell line. Staining for cell surface markers like CD133 (Miltenyi Biosciences, cat#130-080-801) was performed by washing the appropriate cell line in PBS. About $1x10^6$ cells were stained with different amounts of CD133 and its isotype control. Staining for intracellular markers was done after fixing and permeabilizing the cells with Fix:Perm solution (Ebioscience, cat# 00-5523-00) for 30min at room temperature followed by the Perm buffer (Ebioscience, cat# 00-5523-00). Appropriate amounts of antibodies and isotype controls were added to these fixed cells for 30min at room temperature. The antibodies used included: Sox2 (Ebiosciences, cat# 50-9811-82), cMyc (R&D systems, cat# IC36964), Olig (R&D systems, cat# IC2230P), β-Catenin (Ebiosciences, cat# 12-2567-41), and Oct3/4 (Ebiosciences, cat# 53-S841-82). The cells were washed with PBS and analyzed using the BD Aria II (BD Bioscience) cytometer. Data analysis was done using FCS Express Research Edition software.

qRT-PCR

The AT/RT cell lines used for this study include, CHLA-04, CHLA-05, BT-12, and CHLA-266, along with the control H9 cells. RNA was isolated (Qiagen, cat# 80204) and cDNA synthesized using Superscript II® cDNA kit (Life Technologies, cat# 18064-014). For quantitative PCR, SYBr green I master mix (Roche, cat# 04 707 516001) was used. One set of forward and reverse primers for the genes studied were used (detailed primer information in

Supplementary Methods). Quantitative PCR was run on a real-time PCR system (Roche Light Cycler™ 480 system). Each sample was run in triplicate and Roche software was used to analyze data. The expression of each gene was compared to expression in H9 cells and normalized against GAPDH. Expression was calculated as fold changes using the standard ΔCp value calculation.

Results

The ATRT cell lines, CHLA-04, CHLA-05, CHLA-266, and BT-12, were used to study the protein and mRNA expression of each of the CSC and oncogenic markers. The protein and mRNA expression of each marker in ATRT cell lines was compared to expression in the H9 human neural stem cell line. In all Figures, Figure I shows either cell surface or intracellular expression of various markers and II) shows the corresponding mRNA expression for these markers, in each cell line studied. For each cell line shown line graphs show, isotype control (black) with the plot marked as isotype control showing the corresponding cell population, and protein expression (red) with the plot marked as antibody showing the corresponding cell population.

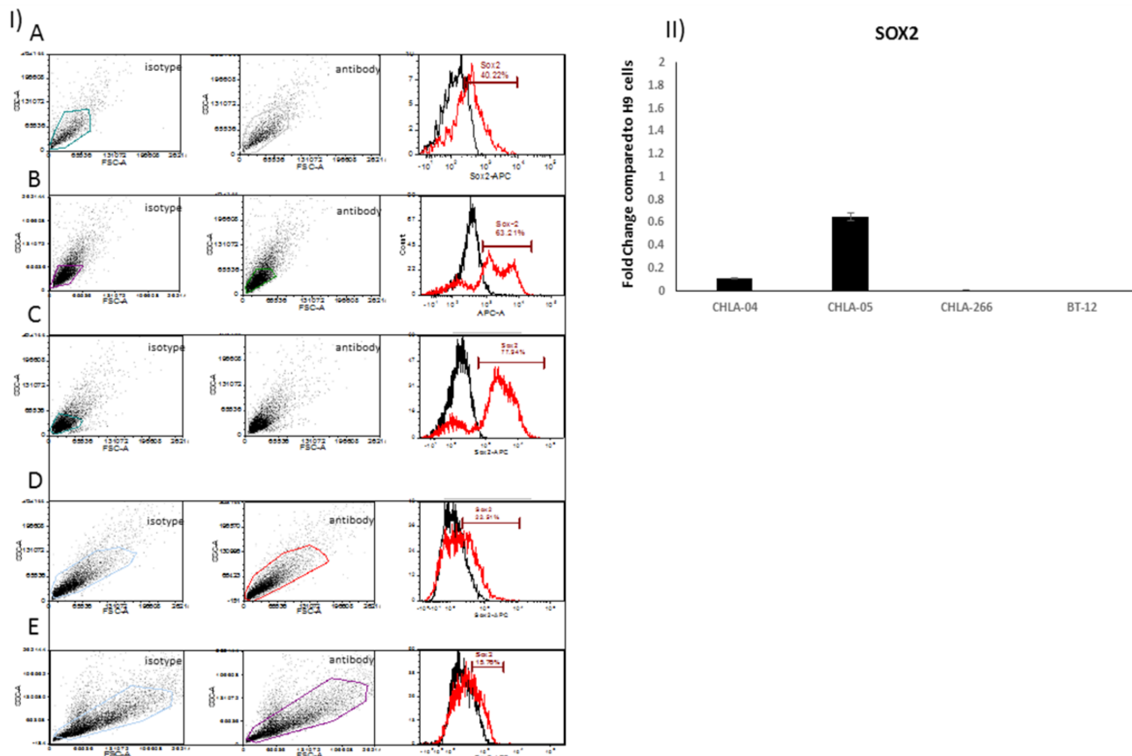

Figure 1: Expression of Sox2 protein by flow cytometry (I), and SOX2 mRNA by qRT-PCR (II). (IA) For H9 control neural stem cells, the histogram shows isotype control (black) with the dot plot marked as isotype control showing the corresponding cell population. Sox2 protein expression is shown in the histogram (red) with the dot plot marked as antibody showing the corresponding cell population. Similarly, Sox2 expression is shown for CHLA-04 (IB), CHLA-05 (IC), CHLA-266 (ID), and BT-12 (IE). (II) Real-time qRT-PCR analyses using cell lines to investigate differential expression of the SOX2 gene (shown on the X-axis). Differential expression of these genes was calculated as a fold change compared with H9 cells (Y-axis). The dashed lines above a bar indicate a fold change >2.

The stem cell factor, Sox2 was first studied as a marker for ATRT (Figure 1). Compared to isotype controls, CHLA-05 (Figure 1-IC) shows the highest Sox2 protein expression at 77.94%, followed by H9 (Figure1-IA) at 40.22%, and CHLA-04 (Figure 1-IB) at 63.21%. CHLA-266 (Figure 1-ID) shows an expression of 22.51%, while BT-12 (Figure 1-Ie) shows an expression of 15.76%. However, compared to SOX2 mRNA expression in the H9 sample, CHLA-04 and CHLA-05 do not show a significantly higher fold change in expression (Figure 1-Ii).

Figure 2 shows results of the Olig2/3 stem cell marker. Compared to isotype controls, H9 shows the highest Olig 2/3 expression at 46.10% (Figure 2-IA), followed by CHLA-04 at 16.32% (Figure 2-IB). CHLA-05 (Figure 2-IC) shows an expression of 0%, CHLA-266 (Figure 2-ID) shows an expression of 6.75%, and BT-12 shows an expression of 0.31% (Figure 2-IE). Compared to OLIG mRNA

expression in H9 cells (Figure 2-II), the mRNA fold expression of OLIG is significantly higher only in CHLA-266 (above 2-fold). This does not correlate with protein expression. Oct3/4 was the next stem cell factor studied in the AT/RT cell lines. Compared to isotype controls, Oct3/4 expression is highest in CHLA-05 at 71.41% (Figure 3-IC), followed by H9 at 46.79% (Figure 3-IA) and CHLA-04 at 34.70% (Figure 3-IB). CHLA-266 shows an Oct3/4 expression of 19.64% (Figure 3-ID), while BT-12 shows an Oct3/4 expression of 13.66% (Figure 3-IE). Interestingly, the mRNA fold expression of OCT3/4 is significantly higher only in CHLA-266 (above 2-fold) compared to the expression in control H9 cells (Figure 3-II). These results do not correlate with the protein results.

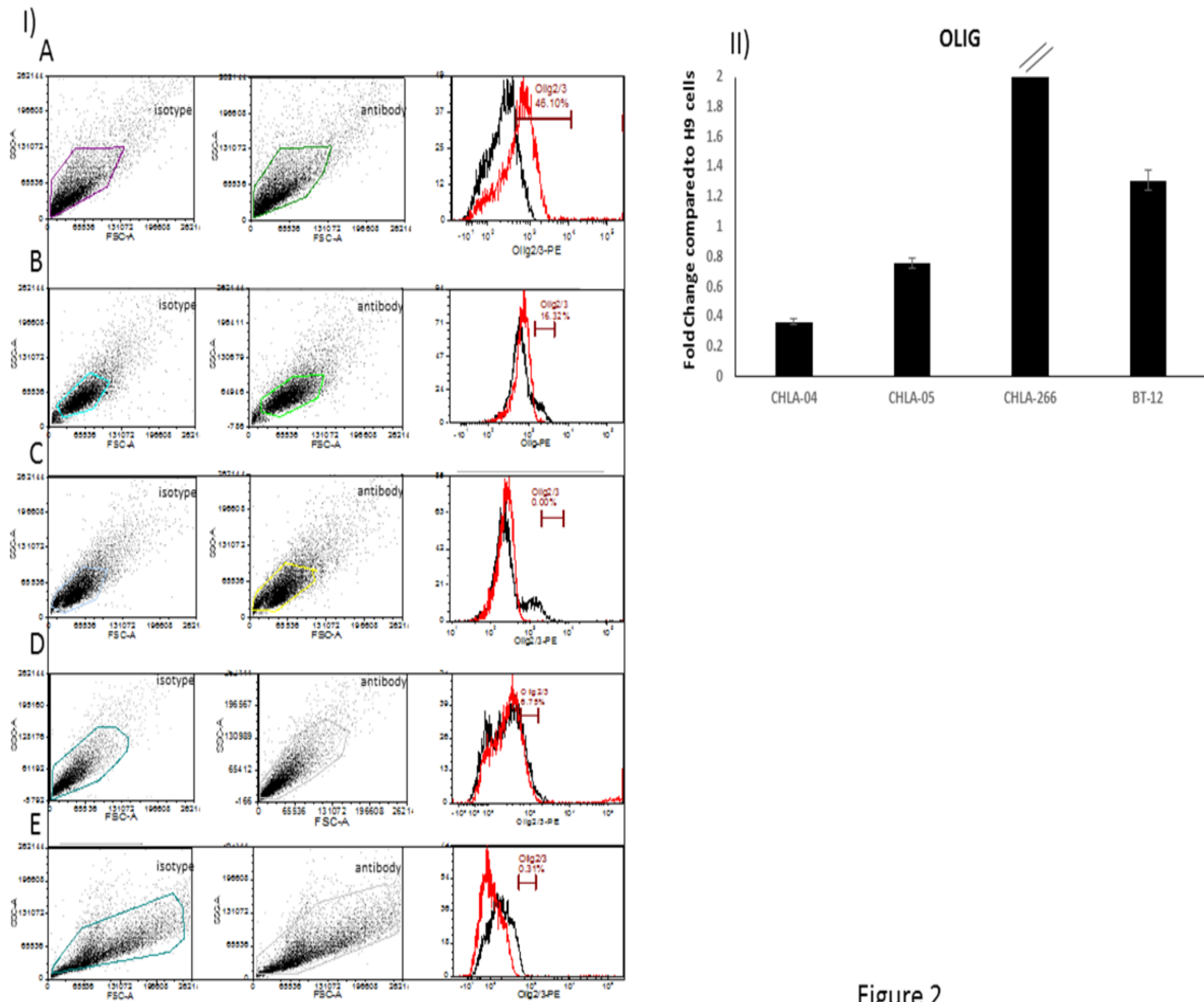

Figure 2: Expression of Olig2/3 protein by flow cytometry (I), and OLIG mRNA by qRT-PCR (II). (IA) For H9 control neural stem cells, the histogram shows isotype control (black) with the dot plot marked as isotype control showing the corresponding cell population. Olig2/3 protein expression is shown in the histogram (red) with the dot plot marked as antibody showing the corresponding cell population. Similarly, Olig2/3 expression is shown for CHLA-04 (IB), CHLA-05 (IC), CHLA-266 (ID), and BT-12 (IE). (II) Real-time qRT-PCR analyses using cell lines to investigate differential expression of the OLIG gene (shown on the X-axis). Differential expression of these genes was calculated as a fold change compared with H9 cells (Y-axis). The dashed lines above a bar indicate a fold change >2.

The stem cell marker, CD133 was studied in the ATRT cell lines. Compared to isotype controls, CD133 expression is highest in H9 cells at 70.11% (Figure 4-IA), followed by CHLA-04 at 59.67% (Figure 4-IB) and CHLA-05 at 29.03% (Figure 4-IC). CHLA-266 shows CD133 expression of 14.37% (Figure 3-ID), while BT-12 shows CD133 expression of 22.38% (Figure 3-IE). The mRNA fold expression of CD133 in AT/RT cell lines is not significantly higher than expression in H9 cells (Figure 4-II).

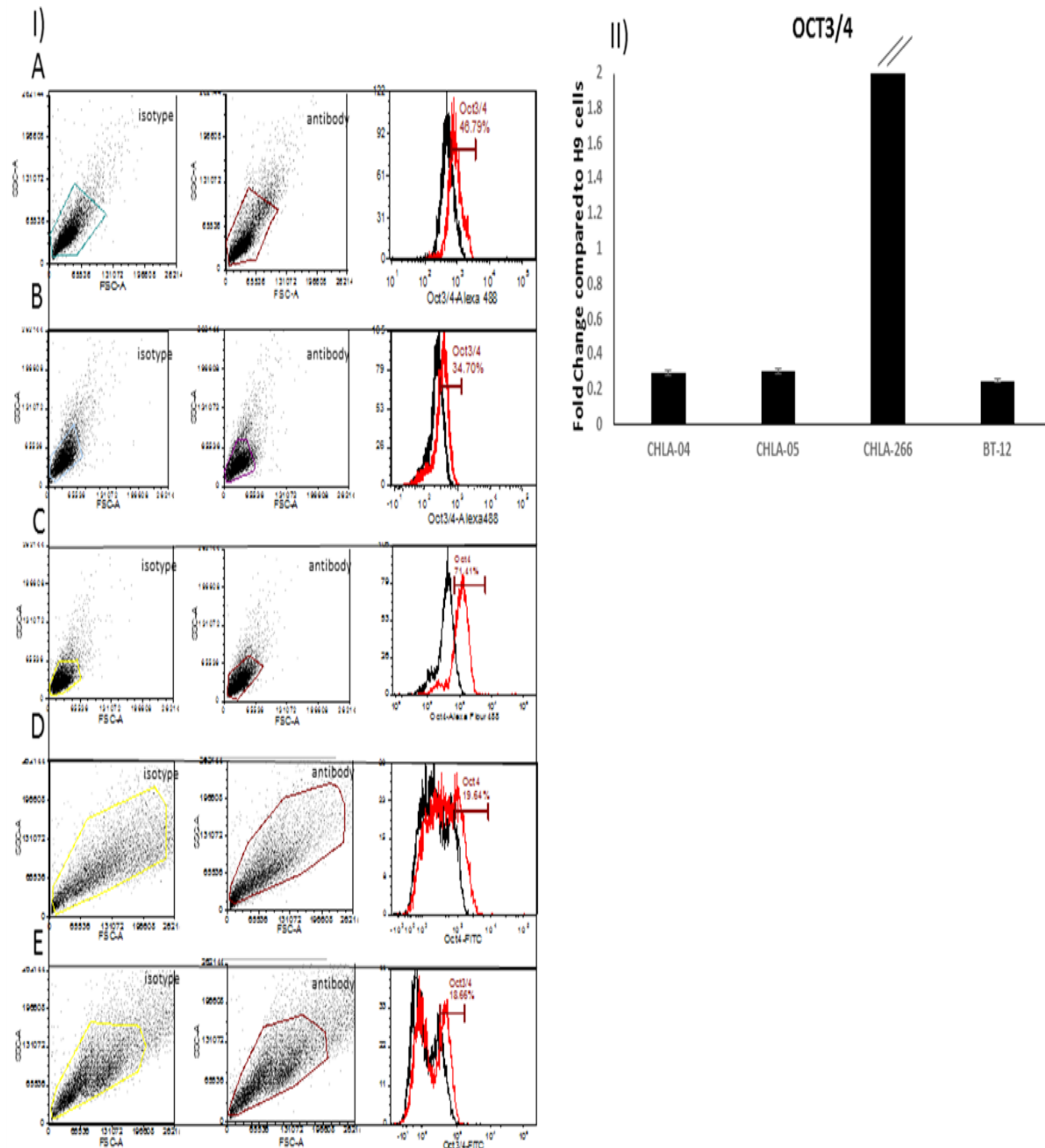

Figure 3: Expression of Oct3/4 protein by flow cytometry (I), and OCT mRNA by qRT-PCR (II). (IA) For H9 control neural stem cells, the histogram shows isotype control (black) with the dot plot marked as isotype control showing the corresponding cell population. Oct3/4 protein expression is shown in the histogram (red) with the dot plot marked as antibody showing the corresponding cell population. Similarly, Oct3/4 expression is shown for CHLA-04 (IB), CHLA-05 (IC), CHLA-266 (ID), and BT-12 (IE). (II) Real-time qRT-PCR analyses using cell lines to investigate differential expression of the OCT gene (shown on the X-axis). Differential expression of these genes was calculated as a fold change compared with H9 cells (Y-axis). The dashed lines above a bar indicate a fold change >2.

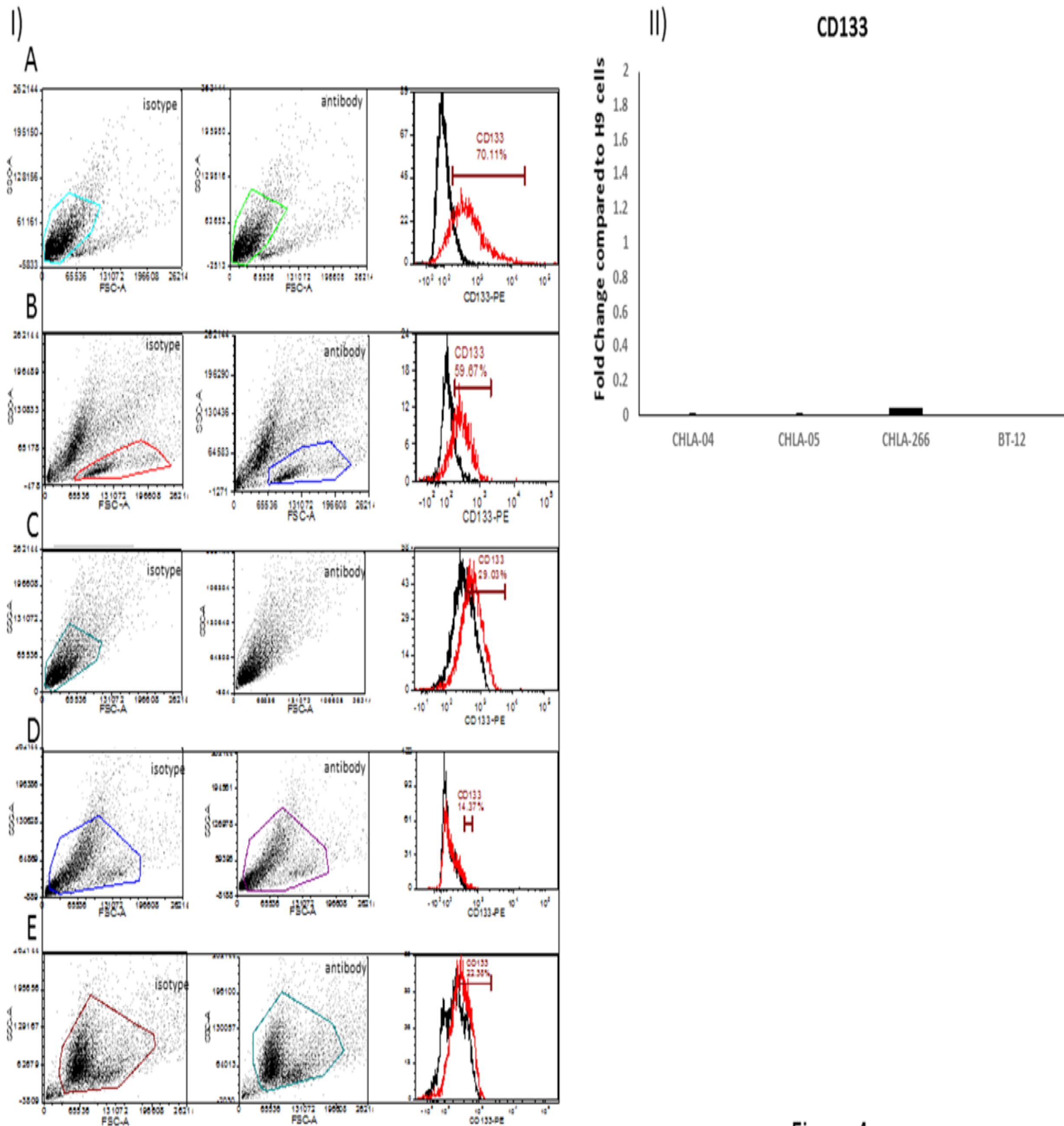

Figure 4: Expression of CD133 protein by flow cytometry (I), and CD133 mRNA by qRT-PCR (II). (IA) For H9 control neural stem cells, the histogram shows isotype control (black) with the dot plot marked as isotype control showing the corresponding cell population. CD133 protein expression is shown in the histogram (red) with the dot plot marked as antibody showing the corresponding cell population. Similarly, CD133 expression is shown for CHLA-04 (IB), CHLA-05 (IC), CHLA-266 (ID), and BT-12 (ID). (II) Real-time qRT-PCR analyses using cell lines to investigate differential expression of the CD133 gene (shown on the X-axis). Differential expression of these genes was calculated as a fold change compared with H9 cells (Y-axis). The dashed lines above a bar indicate a fold change >2.

Expression of oncogenic markers such as, cMyc and β-catenin were also studied in AT/RT. Compared to isotype controls, cMyc expression is highest in CHLA-04 cells at 62.27% (Figure 5-IB), followed by CHLA-05 at 54.22% (Figure 5-IC) and H9 at 45.57% (Figure 4-IA). CHLA-266 shows cMyc expression of 17.03% (Figure 3-ID), while BT-12 shows cMyc expression of 35.22% (Figure 3-IE). The mRNA fold expression of cMYC is significantly higher (above 2-fold) in BT-12, CHLA-266 and CHLA-04 compared to the expression in H9 cells (Figure 5-II). Compared to isotype controls, β-catenin expression is highest in CHLA-05 cells at 72.41% (Figure 6-IC), followed by CHLA-04 at 70.15% (Figure 6-IB) and H9 at 25.27% (Figure 5-IA). CHLA-266 shows β-catenin expression of 11.31% (Figure 3-ID), while BT-12 shows β-catenin expression of 0.14% (Figure 3-IE). The mRNA fold expression of CTNNB1 in AT/RT cells is not significantly higher that expression in H9 cells (Figure 6-II).

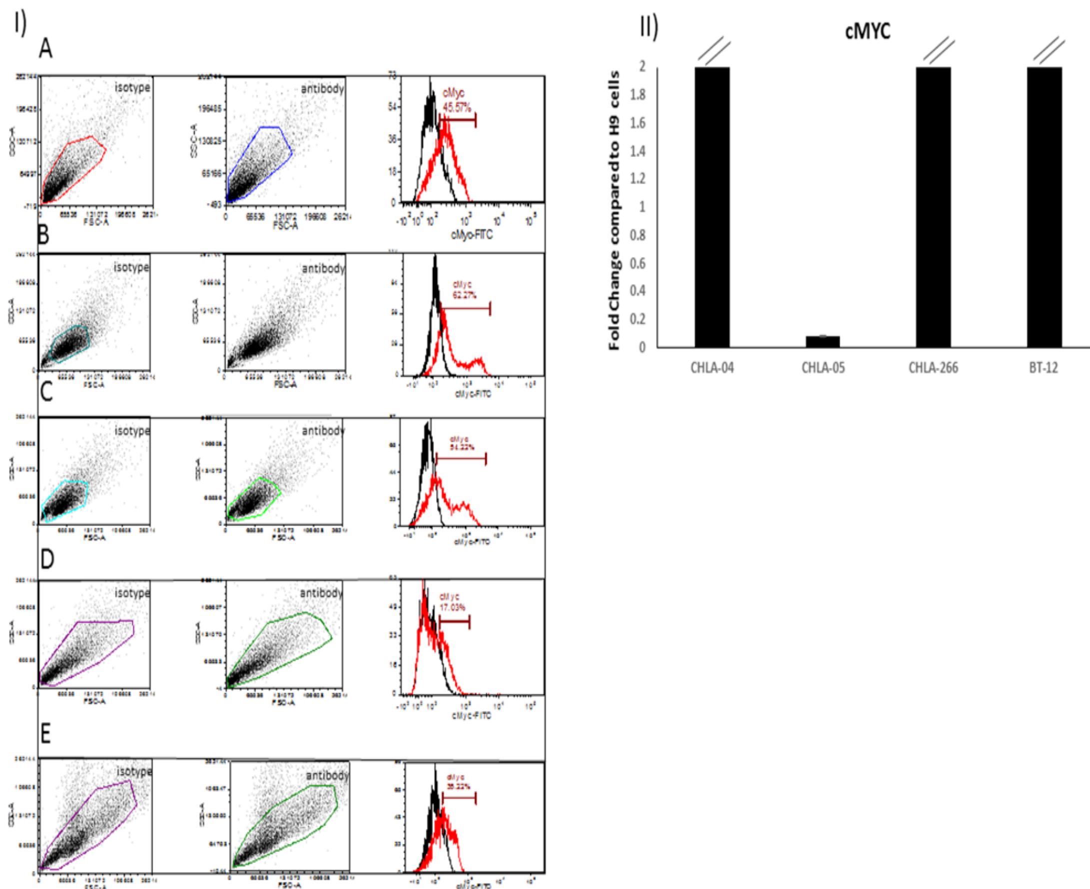

Figure 5: Expression of cMyc protein by flow cytometry (I), and cMYC mRNA by qRT-PCR (II). (IA) For H9 control neural stem cells, the histogram shows isotype control (black) with the dot plot marked as isotype control showing the corresponding cell population. cMyc protein expression is shown in the histogram (red) with the dot plot marked as antibody showing the corresponding cell population. Similarly, cMyc expression is shown for CHLA-04 (IB), CHLA-05 (IC), CHLA-266 (ID), and BT-12 (ID). (II) Real-time qRT-PCR analyses using cell lines to investigate differential expression of the cMYC gene (shown on the X-axis). Differential expression of these genes was calculated as a fold change compared with H9 cells (Y-axis). The dashed lines above a bar indicate a fold change >2.

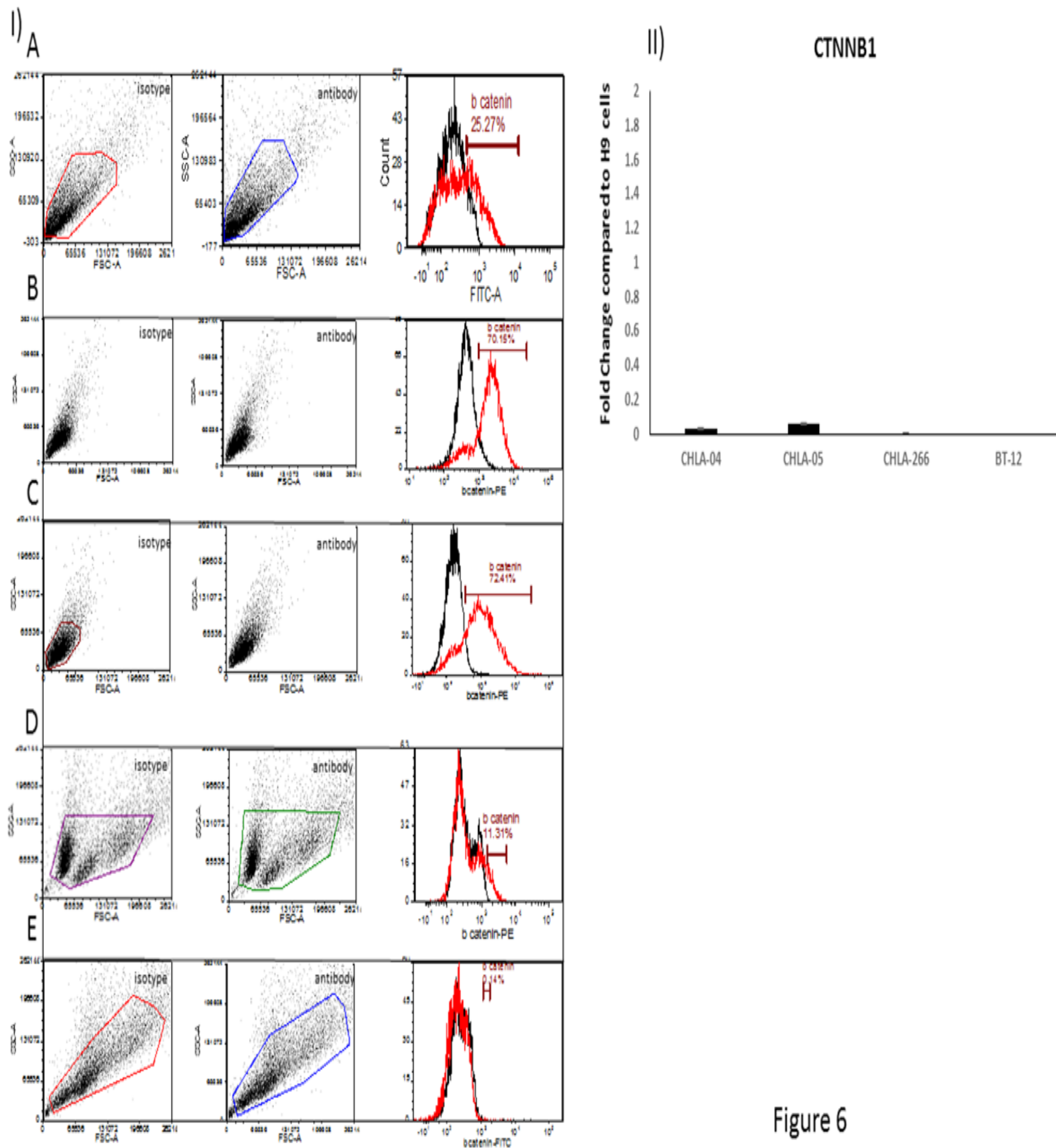

Figure 6: Expression of β-catenin protein by flow cytometry (I), and CTNNB1 mRNA by qRT-PCR (II). (IA) For H9 control neural stem cells, the histogram shows isotype control (black) with the dot plot marked as isotype control showing the corresponding cell population. β-catenin protein expression is shown in the histogram (red) with the dot plot marked as antibody showing the corresponding cell population. Similarly, β-catenin expression is shown for CHLA-04 (IB), CHLA-05 (IC), CHLA-266 (ID), and BT-12 (ID). (II) Real-time qRT-PCR analyses using cell lines to investigate differential expression of the CTNNB1 gene (shown on the X-axis). Differential expression of these genes was calculated as a fold change compared with H9 cells (Y-axis). The dashed lines above a bar indicate a fold change >2.

Discussion

AT/RTs are CNS tumors where information about the tumor originating cells is unclear. In this study, expression of some of the commonly studied neural stem cell markers was examined in AT/RT. Oct3/4, Sox2, and Nanog have been shown to function as core transcription factors in maintaining pluripotency [46,47]. Oct3/4 and Sox2 were identified as being essential for the generation of induced pluripotent cells in a study by Takahashi and Yamanaka [48]. In the same study, c-Myc and Klf4 were identified as essential factors to induce pluripotency. Another study indicated that c-Myc protein may induce global histone acetylation [49], thus allowing Oct3/4 and Sox2 to bind to their specific target loci.

The AT/RT cells, CHLA-04 and CHLA-05 show the highest protein expression of Sox2 more than the percent expression seen in neural stem cell controls. Also, the protein expression of Sox2 in all AT/RT cells correlates with the protein expression of Oct3/4 where CHLA-04 and CHLA-05 show higher Oct3/4 expression than the neural stem cells followed by CHLA-266 and BT-12. Similarly, the protein expression profile of cMyc in AT/RT cells correlates with the expression of Sox2 and Oct3/4 where CHLA-04 and CHLA-05 show higher Oct3/4 expression than the neural stem cells followed by CHLA-266 and BT-12. These protein expression results indicate that a majority of the AT/RT cell population may be pluripotent cells. Protein expression of Olig2/3 is high only in CHLA-04 compared to the neural stem cells. Olig is a lineage marker for oligodendrocytes [22,23], and the results with AT/RT cells seem to indicate that these cells may not have an abundance of oligodendrocyte lineage cells. Interestingly, the mRNA profile of CD133, OCT3/4, OLIG, CTNNB1, and cMYC in ATRT cells does not correlate with the corresponding protein profile. This discrepancy between the mRNA and protein for these CSC markers needs to be analyzed further.

Activation of Wnt/β-catenin signaling has been linked to maintenance of the pluripotent state in human and mouse ESCs [50]. Studies by Kelly et al. [51] have shown that β-catenin can form a complex with and modulate the activity of Oct-4, thus having an influence on regulating the differentiation of mouse ESCs. In another study [52] Wnt/β-catenin signaling was shown to be repressed by Oct4 in the context of self-renewing human ESCs and de-repressed when human ESCs differentiate. In CHLA-04, CHLA-05, and CHLA-266, elevated β-catenin protein levels correlate with elevated Oct3/4 protein levels. This is further indication that AT/RT cells may be in a self-renewing undifferentiated state.

Conclusion

Stem cells protein expressed on cell surface and intracellularly can serve as potential biomarker in ATRT

Acknowledgements

We wish to acknowledge the organization, Students' Supporting Brain Tumor Research (SSBTR) as a funding source. We wish to acknowledge the flow cytometry core facility at the University of Arizona, College of Medicine. We thank Dr. Anat Erdreich-Epstein at the Children's Hospital in Los Angeles, for the AT/RT cell lines CHLA-04 and CHLA-05. We thank Dr. David Azorsa from the RMIM institute at Phoenix Children's Hospital for the AT/RT cell lines, CHLA-266 and BT-12. The authors declare no conflict of interest.

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