Mesenchymal-epithelial transition and stemness in sarcoma

Alexander J. Hish

Under the supervision of Dr. William Eward,
Cancer Institute and Department of Medicine, Duke University

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Durham, North Carolina

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ABSTRACT

Clinical evidence reveals that upregulation of epithelial markers is prognostic for better survival in patients with sarcoma, while mesenchymal markers are prognostic for worse survival. We previously observed that forced induction of mesenchymal-epithelial transition (MET) not only decreased the cancer-like aggression of rhabdomyosarcoma (RD) cells *in vitro*, but also produced downregulation of key stem cell marker Zeb1. From this result, we hypothesized that MET reduces aggression in sarcoma by inhibiting stemness. If this is the case, then inducing differentiation without a concurrent MET should produce the same reduction in aggression we observe following forced MET induction. Here we attempted to address this possibility by inducing differentiation and testing for aggression and MET induction, and by isolating stem-like cells and testing for aggression, MET induction, and differentiation. Our use of all-trans retinoic acid (ATRA) to induce differentiation in RD cells caused a decrease in Zeb1 expression, but did not reliably increase expression of muscle differentiation genes. Unexpectedly, ATRA treatment also caused an increase in anchorage-independent growth. RD stem-like cells isolated via Hoechst dye exclusion exhibited increased expression of Zeb1, but did not show increased expression of muscle differentiation markers. Isolated stem-like cells were also not better at forming clones. Overall, our results do not provide evidence that a reduction in stemness is related to the reduction in aggression observed following MET. Contrary to expectation, we cannot rule out the possibility that an increase in stemness may instead be associated with reduced aggression in sarcoma.

INTRODUCTION

Stem cells possess two defining characteristics: self-renewal and potency (Morrison & Kimble, 2006; Tajbakhsh et al., 2009). Self-renewal refers to the ability to proliferate without undergoing differentiation. Potency refers to the ability to produce many cell types. Recent hypotheses have placed stem cells at the center of cancer research, proposing a hierarchical organization of tumors in which only particular cells are able to recapitulate the tumor when transplanted. These so-called cancer stem cells (CSCs) are able to reinitiate tumorigenesis *de novo*, unlike the differentiated cells of tumors. CSCs are thought to exist as distinct populations in tumors, causing relapse and metastasis by giving rise to new tumors through the stem cell process of self-renewal.

Historically, the existence of CSCs has been hotly debated, partly for definitional reasons. True stem cells are able to recapitulate an organism's anatomy in its entirety, whereas CSCs can only recapitulate tumors. However, the debate most prominently centers around whether this distinct population can actually be isolated, and whether CSCs possess the characteristics they are hypothesized to have. Some of the earliest evidence in favor of the existence of CSCs came from Lapidot et al. (1994), who isolated human acute myeloid leukemia cells that were able to proliferate rapidly and initiate leukemia upon transplantation into severe combined immune-deficient mice. This result is hard to ignore, as *in vivo* repopulation is considered the gold standard for stem cell activity (van Os et al., 2004). Subsequent studies have validated this proposed hierarchical organization in leukemias and other cancers, such as gliomas (Bonnet & Dick, 1997; Ignatova et al., 2002). Furthermore, Kong et al. (2015) demonstrated that stem cell-like characteristics of prostate cancers were shown to be associated with progression to an aggressive castration-resistant phenotype. Therefore, whether we refer to them as "cancer

stem cells" or not, it is clear that some cancer cells can give rise to new tumors, while others cannot. The role of these tumor-propagating cells in cancer progression, recurrence, and metastasis marks them as an important focus of study—both to better understand cancer biology, and as a potential clinical target. Throughout this paper, I will continue to refer to these tumor-propagating cells as "cancer stem cells" or CSCs, with the understanding that this name is used purely for convenience without any broader biological implications.

The current paper focuses on the relationship of CSCs to sarcoma. Sarcomas are rare cancers derived from mesenchymal tissue, making up less than 1% of adult cancers, but at least 12% of pediatric cancers (Fletcher et al., 2013; Siegel et al., 2015). Relative to epithelial-derived carcinomas, sarcomas tend to be more aggressive with worse survival outcomes. Interestingly, clinical evidence reveals that upregulation of E-cadherin, an epithelial marker, is prognostic for better survival in patients with sarcoma, while some mesenchymal markers (such as Zeb1, Twist, and Vimentin) are prognostic for worse survival (Tian et al., 2013; Wang et al., 2015; Yin et al., 2012). These results suggest that induction of mesenchymal-epithelial transition (MET) in sarcoma may reduce the aggressive characteristics of sarcomas, driving them to a more indolent epithelial-like state. MET is a normal process of human biology in which the more motile, invasive, and typically spindle-shaped mesenchymal cells transition to the more stationary, planar epithelial cells; the reverse process is called epithelial-mesenchymal transition (EMT). Importantly, both types of phenotypic transitions (EMT and MET) are important during normal development and wound healing (Hay, 1995; Polyak & Weinberg, 2009; Shook & Keller, 2003; Yan et al., 2010). It should be noted that the epithelial-mesenchymal dichotomy does not represent two distinct states of cell programming, but rather a spectrum of intermediary states, as

evidenced by the existence of hybrid epithelial/mesenchymal states in clinical specimens (Armstrong et al., 2011).

We have found evidence that an MET-induced reduction in aggression may be related to stemness. Here, "stemness" refers to the degree to which a cell is like a CSC, measured by the characteristic abilities of stem cells to self-renew and remain undifferentiated. We observed that forced induction of MET not only decreased the cancer-like aggression of rhabdomyosarcoma cells *in vitro*, but also produced downregulation of Zeb1, a driver of EMT and stemness (Li et al., 2012; Preca et al., 2015; Wu et al., 2013). Based on these data, we hypothesize that MET reduces aggression in sarcoma by inhibiting stemness. To test this hypothesis, we have developed the following conceptual framework of stemness and epithelial/ mesenchymal phenotypic pathways in sarcoma (Fig. 1): In the context of sarcoma, differentiation of a mesenchymal stem-like cell can occur in one of two ways: it can proceed towards reduced stemness on the mesenchymal side of the mesenchymal-epithelial spectrum, or it can proceed towards reduced stemness on the epithelial side of the spectrum. If MET reduces aggression in sarcoma by inhibiting stemness, then inducing differentiation towards the mesenchymal side of the spectrum (i.e. reducing stemness only, without a concurrent MET) should produce the same reduction in aggression we observe following forced MET induction.

To address these possibilities, we attempted to induce differentiation in sarcoma cultures to reduce their stemness and observe the effects on aggression. We also attempted to isolate CSC populations from sarcoma cultures to compare their aggression to non-CSC sarcoma cells. Ultimately, our results do not provide strong evidence that we were able to induce differentiation, nor isolate a stem-cell like population in rhabdomyosarcoma or osteosarcoma cell

lines. Contrary to expectation, we cannot rule out the possibility that an increase in stemness may instead be associated with reduced aggression in sarcoma.

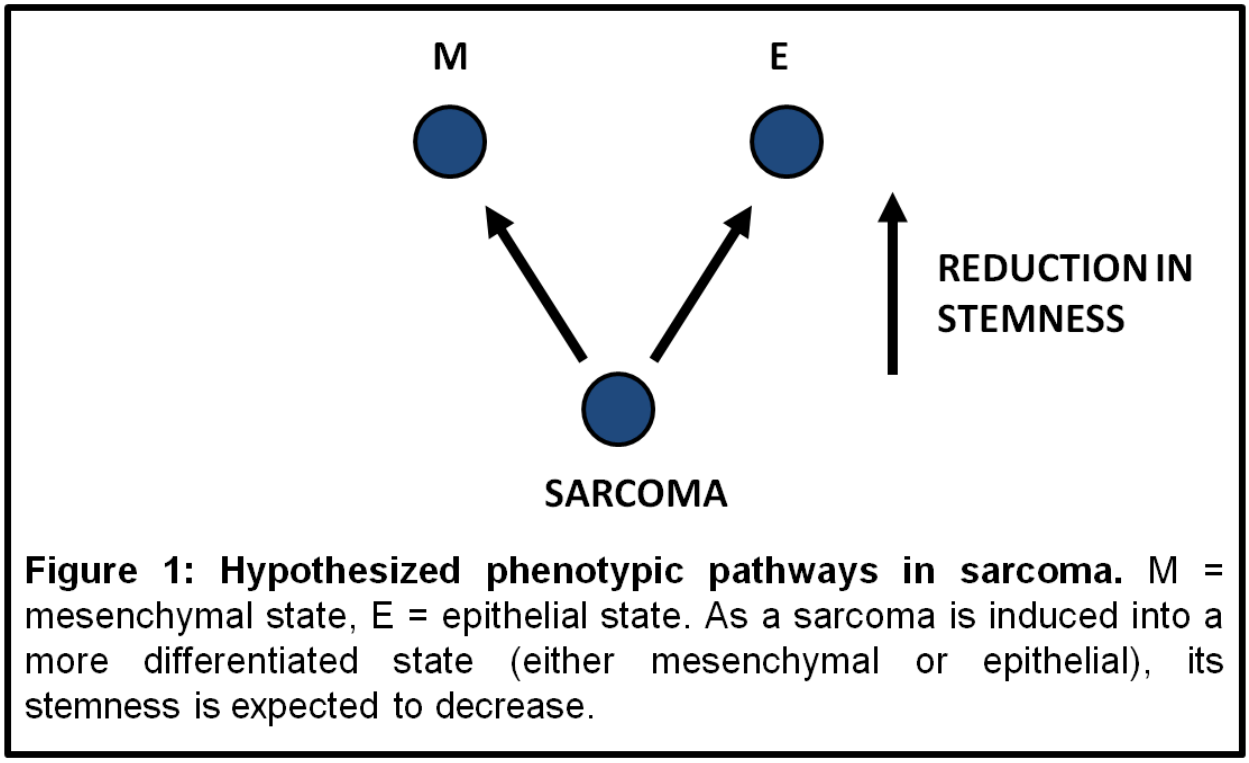

Figure 1: Hypothesized phenotypic pathways in sarcoma. M = mesenchymal state, E = epithelial state. As a sarcoma is induced into a more differentiated state (either mesenchymal or epithelial), its stemness is expected to decrease.

MATERIALS AND METHODS

Cell lines

Our model system includes human-derived cell lines of rhabdomyosarcomas (RD) and osteosarcomas (143B) grown in DMEM.

ATRA treatment

The methodology used to perform all-trans retinoic acid (ATRA) treatment was previously published in Barlow et al. (2006). ATRA stocks (5 mM) were dissolved in DMSO. A total of 500,000 cells per well were plated, then media was changed to include either DMSO in

DMEM or 5uM to 40uM of ATRA in DMSO in DMEM. Media was refreshed every two days for four to 14 days.

qPCR

RT-PCR and qPCR were performed using standard technique, and the following primers were used: GAPDH, E-cadherin, Zeb1, MyoD1, Myogenin, Myf-6 (Table 1).

Gene	Forward Primer	Reverse Primer
GAPDH	5'- AGC CAC ATC GCT CAG ACA C -3'	5'- GCC CAA TAC GAC CAA ATC C -3'
E-cadherin	5'- TGG AGG AAT TCT TGC TTT GC -3'	5'- CGC TCT CCT CCG AAG AAA C -3'
Zeb1	5'- GCA TAC AGA ACC CAA CTT GAA CGT C -3'	5'- CGA TTA CAC CCA GAC TGC GTC -3'
MyoD1	5'- CAC TAC AGC GGC GAC TCC -3'	5'- TAG GCG CCT TCG TAG CAG -3'
Myogenin	5'- GCT CAG CTC CCT CAA CCA -3'	5'- GCT GTG AGA GCT GCA TTC G -3'
Myf-6	5'- CTT CAG CTA CAG ACC CAA ACA -3'	5'- CCC TGG AAT GAT CGG AAA CA -3'

Table 1: qPCR primers.

Soft agar

Soft agar plates were prepared by heating 1.0% agarose in a microwave until well dissolved, and mixing 1:1 with 2x DMEM. A total of 1.5 mL of this mixture was added to each well of a 6-well plate with swirling to ensure formation of an even gel layer without bubbles, and allowed to harden for 30 minutes at room temperature. Next, 0.6% agarose was heated in a microwave until well dissolved, and mixed 1:1 with 2x DMEM seeded to plate 50,000 cells per well. A total of 1.5 mL of this mixture was added to each well of the 6-well plate on top of the previous layer, again with swirling, and allowed to harden for 30 minutes at room temperature. DMEM (2 mL) with either DMSO or 0 to 40 μM ATRA in DMSO were added to each well. Media plates were then allowed to incubate at 37°C for three weeks, with media and ATRA treatments replaced once per week. After three weeks, wells were incubated with 300uL 1 mg/mL Nitrotetrazoleum Blue overnight, then imaged by taking photographs with a light microscope.

Hoechst 33342 staining for stem cell purification

The methodology used to perform Hoechst 33342 staining was previously published in Goodell et al. (1996). Cells were suspended in DMEM at 10^6 cells per ml. Hoechst 33342 dye was added to a final concentration of 5 μg/μl. Verapamil was added to half of the samples to provide a gate control, to a final concentration of 50 μM. Cells were placed in a 37°C water bath for 80-90 minutes, and mixed every 15 minutes. Cells were then centrifuged at 250 xg and 4°C, then resuspended in cold HBSS. Propidium iodide (PI) was added for dead cell discrimination to a final concentration of 2 μg/ml. Cells were placed on ice and transported to FACS.

Flow cytometry and side population purification

The methodology used to perform flow cytometry and side population purification was previously published in Goodell et al. (1996). In particular, the Hoechst 33342 dye was excited at 350 nm and its fluorescence measured to distinguish side population cells from non-side population cells. Inclusion of PI allowed for dead cell discrimination and exclusion without affecting Hoechst analysis. Cells were sorted on a FACS machine between cells with low Hoechst expression (side population cells) and cells with moderate-to-high Hoechst expression (non-side population cells). 10,000 cells of each type were collected.

Clonogenic growth assay

Following side population purification, sorted cells were plated at 100 cells per well. After 1-3 weeks of growth, wells were stained with 200uL crystal violet (4% PFA, 0.5% crystal violet) for 30 minutes, rinsed with tap water to remove excess dye, then imaged. Side population and non-side population wells were evaluated based on the number of colonies formed from single cells.

RESULTS

ATRA treatment

Naïve rhabdomyosarcoma (RD) cells were treated with all-trans retinoic acid (ATRA) to observe if differentiation occurred with treatment, as reported in Barlow et al. (2006). Levels of mRNA expression in these cells were examined via qPCR, for the following genes: E-cadherin (to examine epithelial character); Zeb1 (to examine mesenchymal character); and MyoD1, Myogenin, and Myf-6 (three muscle differentiation factors). GAPDH was used as an endogenous control. After 6 days of treatment, Zeb1 expression decreased, Myogenin expression increased, MyoD1 and Myf-6 expression neither increased nor decreased consistently, and levels of Cdh1 expression were too low to be determined (Fig. 2a). After 10 days of treatment, Zeb1 and MyoD1 expression decreased, and levels of E-cadherin, Myogenin, and Myf-6 expression were too low to be determined (Fig. 2b). After 14 days of treatment, RNA extraction failed to yield sufficient levels of mRNA to accurately measure mRNA expression via qPCR (data not shown). Figure 2c shows a second trial: at 6 days of treatment, Zeb1 and MyoD1 expression decreased, and levels of other mRNA were too low to be determined.

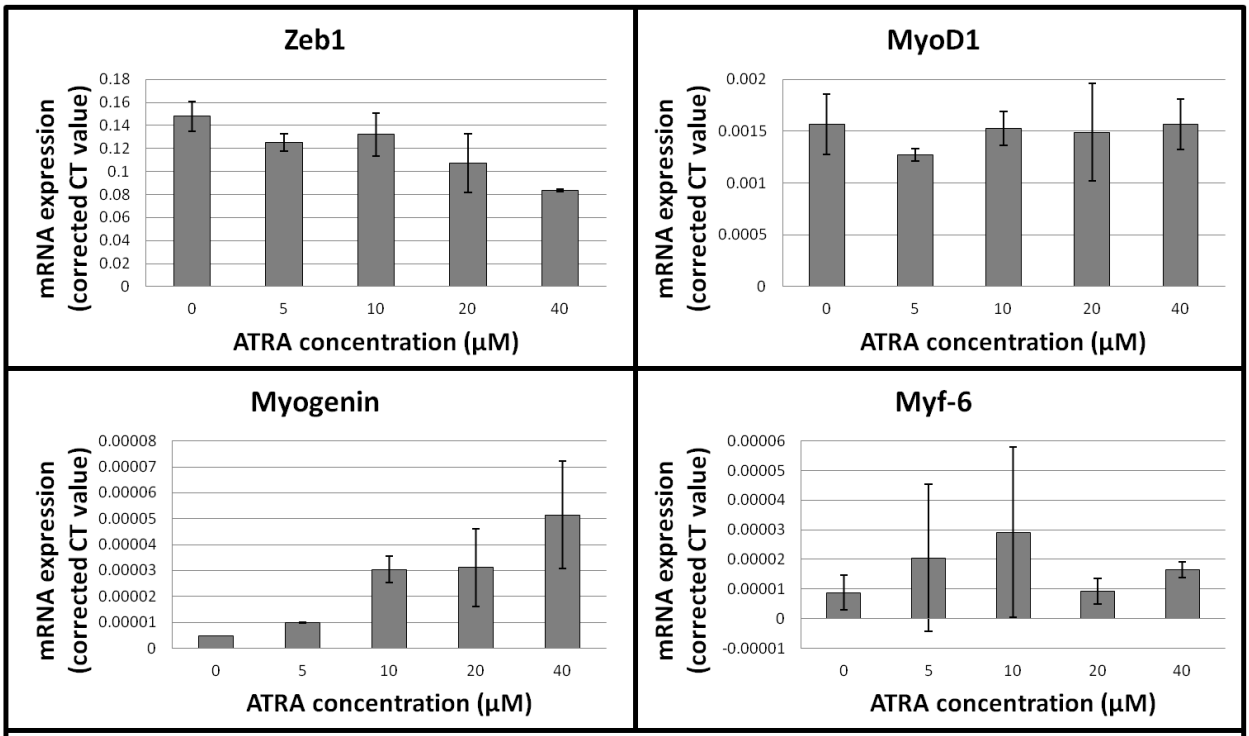

Figure 2a: ATRA treatment results, 6 days. qPCR analysis of mRNA expression. Levels of E-cadherin expression were too low to be determined. Error bars represent standard deviation of three replicates.

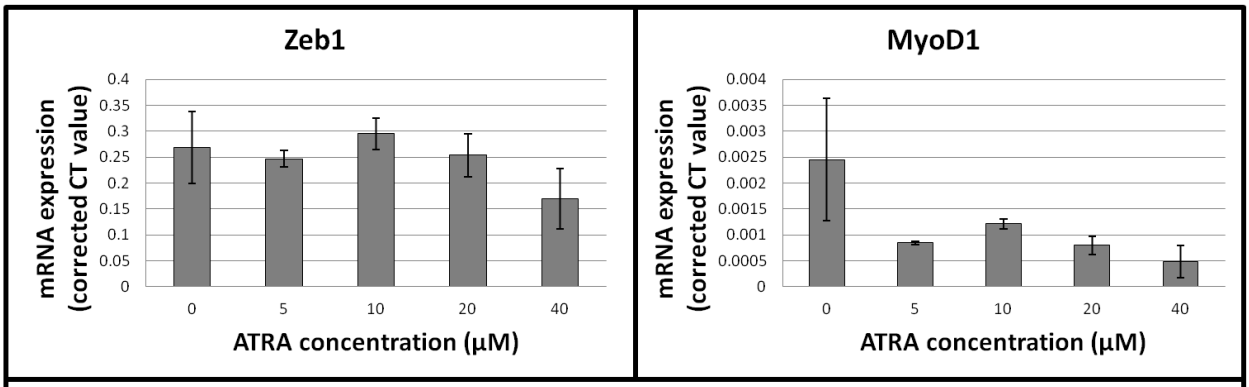

Figure 2b: ATRA treatment results, 10 days. qPCR analysis of mRNA expression. Levels of E-cadherin, Myogenin, and Myf-6 expression were too low to be determined. Error bars represent standard deviation of three replicates.

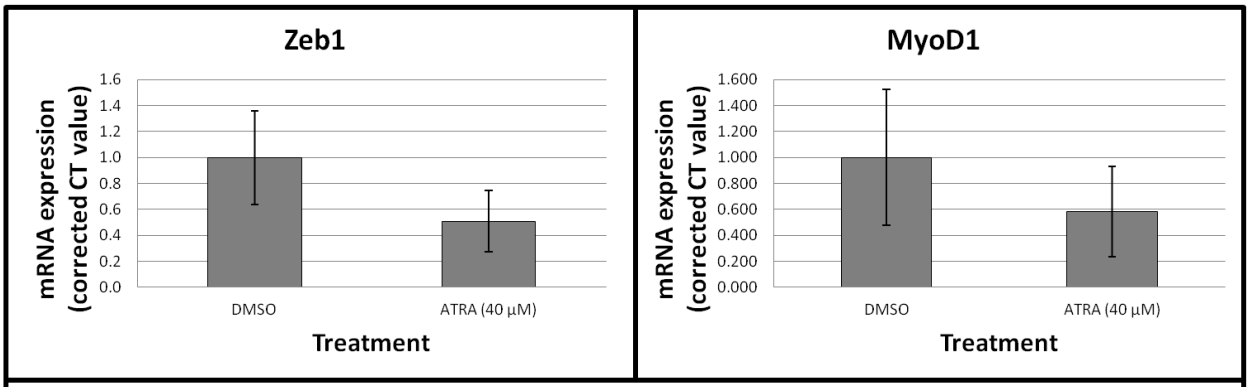

Figure 2c: ATRA treatment results, 6 days. qPCR analysis of mRNA expression. Levels of E-cadherin, Myogenin, and Myf-6 expression were too low to be determined. Error bars represent standard deviation of three replicates.

The data from qPCR does not support the conclusion that differentiation of RD cells occurred with ATRA treatment. Barlow et al. (2006) showed that levels of MyoD, Myogenin, and Myf-6 protein expression, measured by Western blot, increased after only 4 days of treatment with ATRA and its derivative 9-cis retinoic acid (CRA). Even after 10 days of ATRA treatment, mRNA expression of MyoD, Myogenin, and Myf-6 did not reliably increase in our RD cells, suggesting that differentiation did not occur. Zeb1 expression did decrease at both 6 days and 10 days, perhaps suggesting a reduction in stemness.

Soft agar assays were performed on ATRA-treated RD cells to examine anchorage-independent growth, a cancer phenotype associated with increased aggression. In two of three trials, we observed a statistically significant increase in anchorage independent growth following ATRA treatment (Fig. 3). This result was not consistent with our expectations: if ATRA-treated cells were truly differentiated, we would expect them to have decreased anchorage independent growth due to their less cancer-like phenotype.

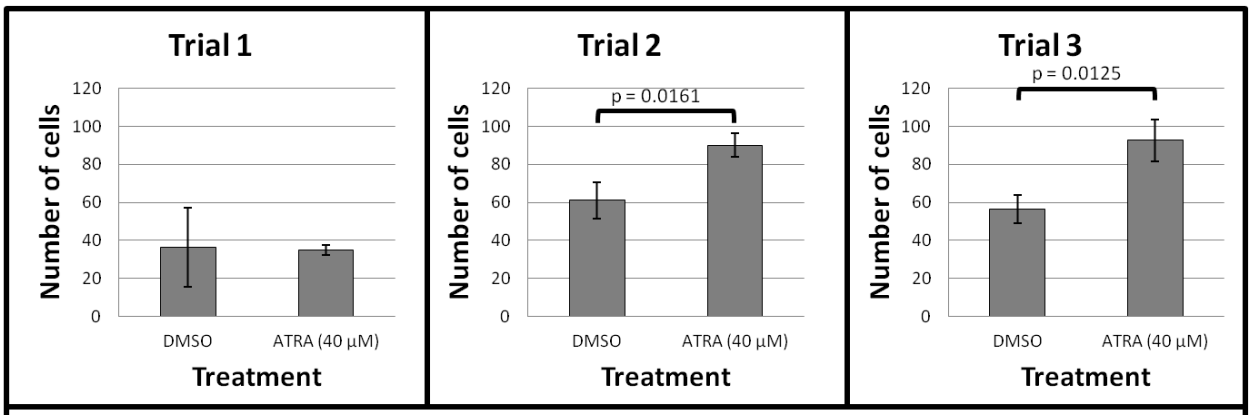

Figure 3: Soft agar results following ATRA treatment. In two trials (Trial 2 and Trial 3), a statistically significant increase in anchorage independent growth was observed. Error bars represent standard deviation of three replicates.

Side population analysis

Hoechst 33342 dye exclusion is a useful technique for isolating cancer stem cells, based on the ability of stem cells to exclude Hoechst dye by expression of the ATP binding cassette protein transporter ABCG2 (Haraguchi et al., 2006; Ho et al., 2007; Scharenberg et al., 2002). The cells with the highest ability to exclude Hoechst are dubbed "side population" (SP) cells. These side populations have been shown to be enriched with stem-like cells (Yang et al., 2011). Verapamil is an inhibitor of drug efflux pump proteins such as ABCG2, and is used to provide a gate within which low Hoechst expression is indicative of the action of these pumps (Bellamy 1996).

RD and osteosarcoma (143B) cells were stained with Hoechst, Verapamil, and PI, then sorted on a FACS machine. A sizable side population was observed in both RD and 143B samples based on their low Hoechst expression (Fig. 4). Verapamil was effective as a gate control: without Verapamil, an average of 1.73% (s = 0.42) RD cells and 3.07% (s = 0.32) 143B cells were identified as side population cells based on gating, while only 0.1 % (s = 1.7×10^{-17}) RD cells and 1.07% (s = 0.21) 143B cells were within the same gate after addition of Verapamil.

Cells were collected from both the side population (SP) and the non-side population (non-SP) for qPCR analysis and clonogenic growth analysis.

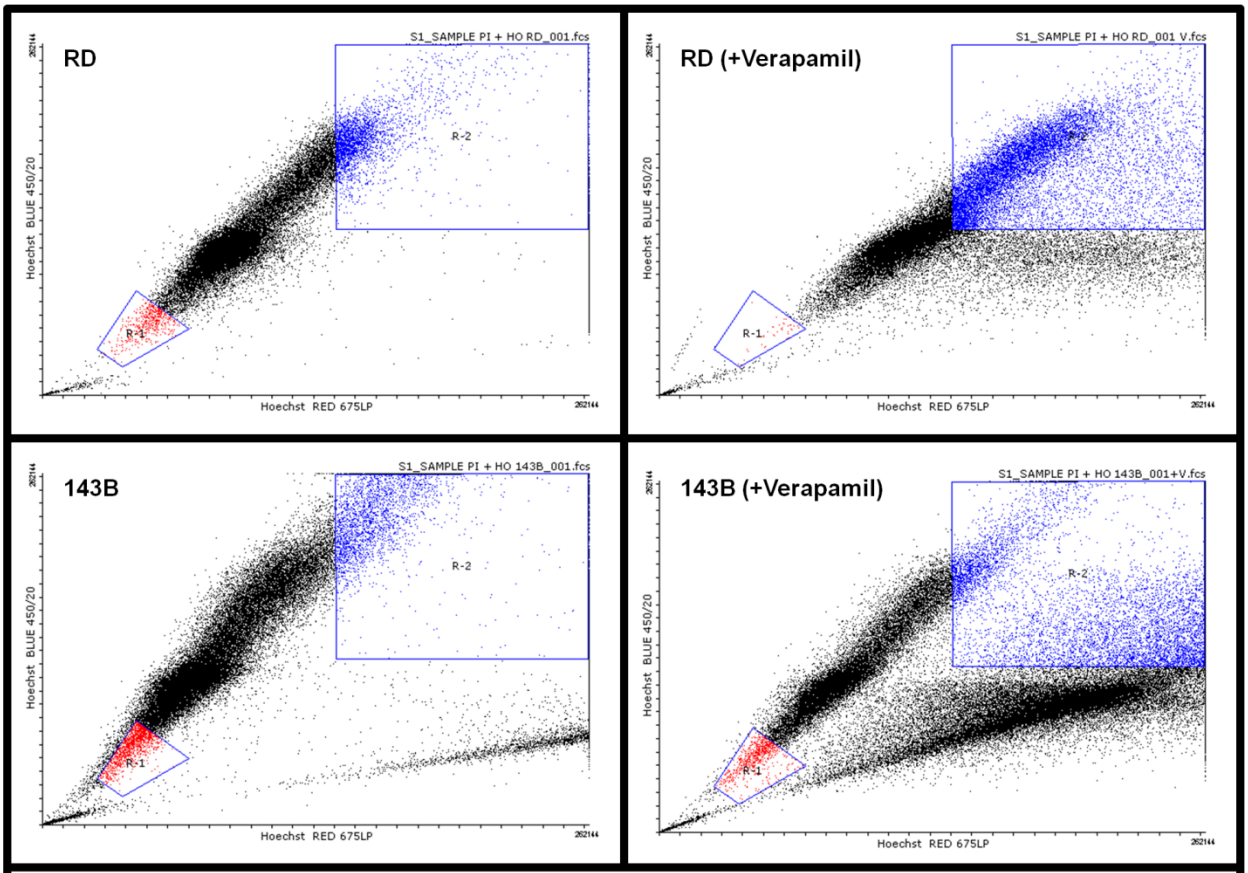

Figure 4: Side population purification, FACS analysis and collection. x-axis = Hoechst RED 675LP expression, y-axis = Hoechst BLUE 450/20 expression. Each point represents a single sorted cell. Region R-1 (red points) represents the side population based on its low Hoechst expression. Region R-2 (blue points) represents non-side population cells.

Over three trials, RD cells were evaluated via qPCR for their expression of E-cadherin, Zeb1, MyoD1, Myogenin, and Myf-6. 143B cells were evaluated for expression of E-cadherin and Zeb1. In the first trial, relative to non-SP cells, SP cells from both cell lines had a decreased expression of Zeb1, and RD cells had a decreased expression of MyoD1 and an increased expression of Myogenin (Fig. 5). E-cadherin was undetectable in both cell lines, and Myf-6 was undetectable in RD cells.

The first trial only involved single replicates, so we performed additional FACS trials in triplicate to see if any markers were consistently changed. In the second and third trials, only Zeb1 was detectable in all populations (Fig. 5). In RD cells, the second and third trials showed increased expression of Zeb1 in SP cells, though this difference was only statistically significant in the third trial. In 143B cells, the second and third trials showed conflicting results for SP cells, with Zeb1 significantly decreased in the second trial and significantly increased in the third trial. Overall, RD cells were more consistently seen to have increased expression of Zeb1 in SP cells, suggesting increased stemness in the RD SP population as expected. However, inconsistent results in the 143B population with Zeb1 expression make it difficult to conclude whether our method always successfully isolates stem-like cells. Additionally, the mostly-undetectable muscle differentiation markers give little insight as to whether SP cells are less differentiated relative to non-SP cells.

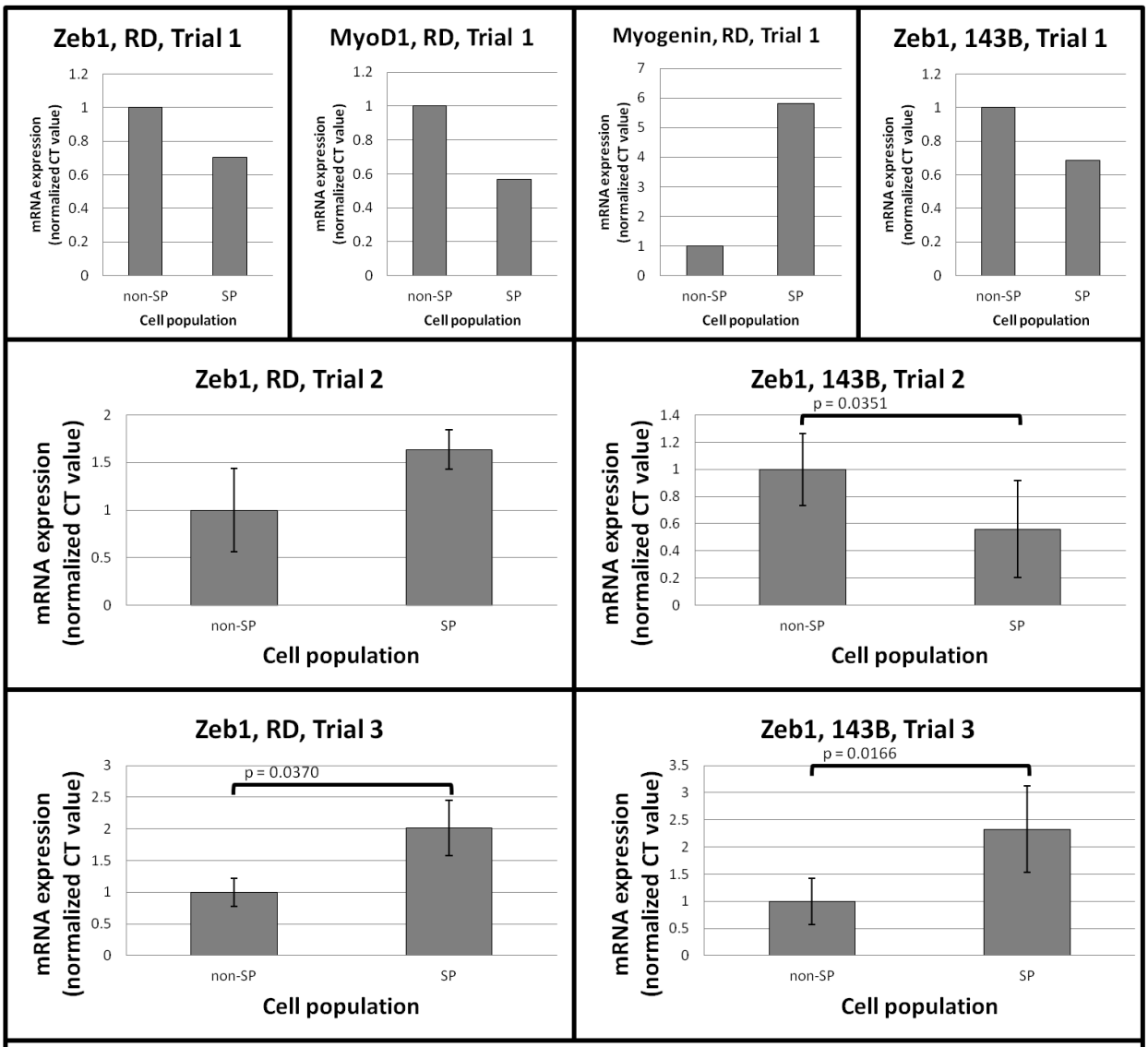

Figure 5: Side population purification, qPCR analysis of mRNA expression. Trials 1-3. SP = side population, non-SP = non-side population. Genes not shown for a trial were undetectable in that trial. mRNA expression CT values were normalized to the non-SP population. Trial 1 involved only one replicate; error bars in Trials 2 and 3 represent standard deviation of three replicates.

In the RD line, single cells formed clones more often in the non-SP populations (avg = 1.33, s = 1.15) than in the SP populations (avg = 1, s = 1) (p = 0.7250) (Fig. 6a). In the 143B line, single cells formed clones more often in the non-SP populations (avg = 14, s = 6.56) than in the SP populations (avg = 4.67, s = 3.51) (p = 0.1163) (Fig. 6b). Both results are contrary to expectation, though neither result is statistically significant.

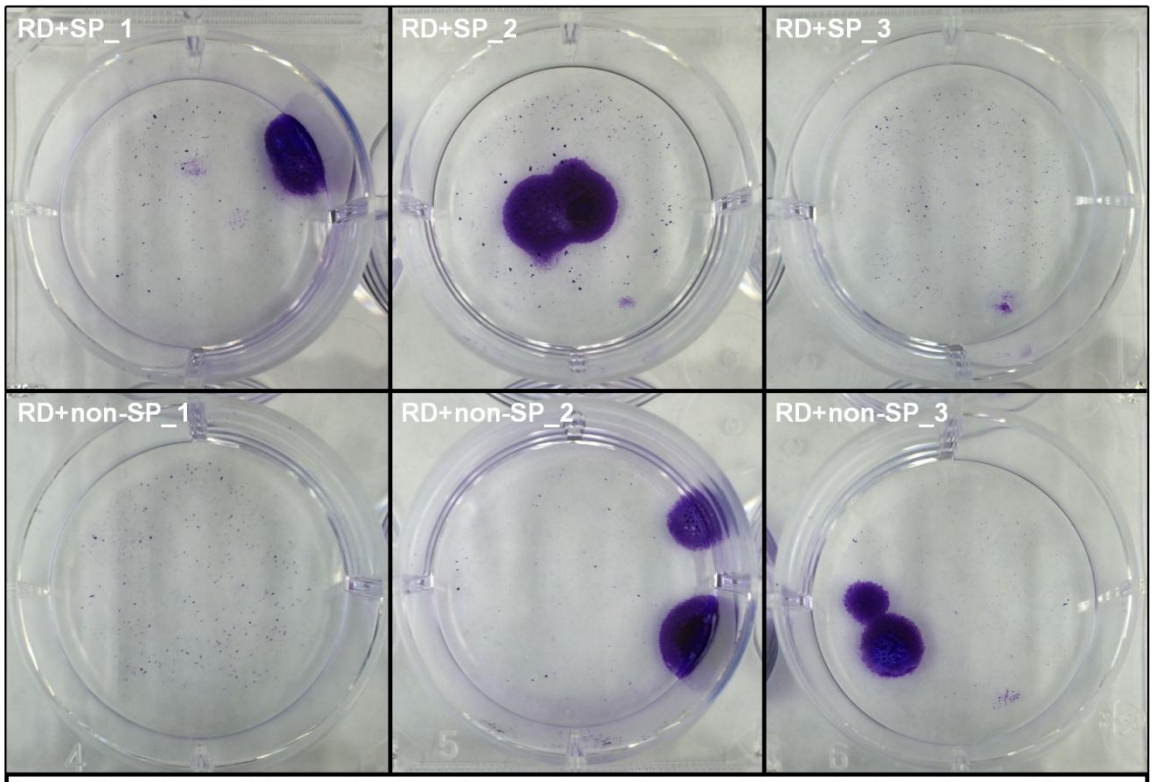

Figure 6a: Side population purification, clonogenic growth assay. RD results. SP = side population, non-SP = non-side population.

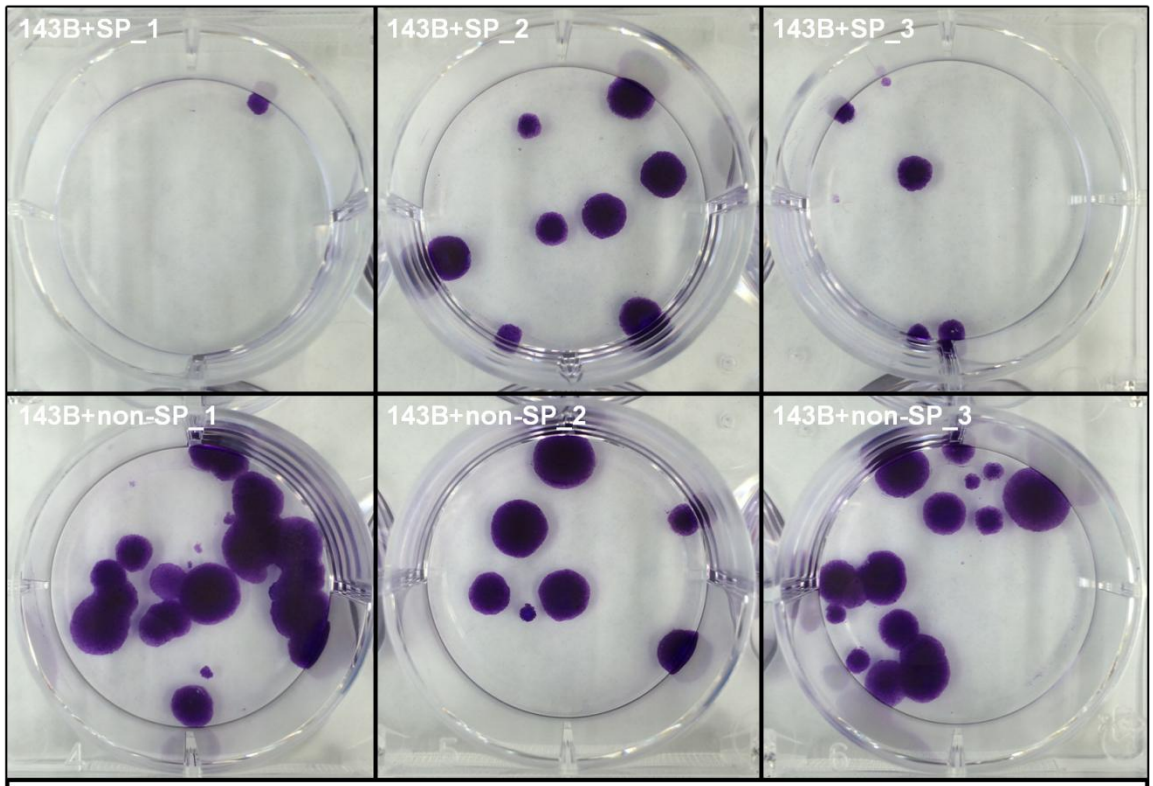

Figure 6b: Side population purification, clonogenic growth assay. 143B results. SP = side population, non-SP = non-side population.

DISCUSSION

Our results do not provide strong evidence that we were able to induce differentiation, nor that we were able to isolate a stem-like cell population. Therefore, we were unable to determine whether a reduction in stemness is related to the reduction in aggression observed following MET. Indeed, assuming that ATRA treatment successfully induced differentiation, and assuming that stem-like side population cells were successfully isolated, several of our results suggest that stemness may instead be associated with a reduction in aggression. Soft agar reveals that ATRA treatment actually increases anchorage independent growth of RDs, a marker of cancer-like aggression. Similarly, more clones were formed from single cells of non-SP populations than from SP populations. Increased clonogenic growth is associated with increased metastasis and tumorigenesis; therefore, this characteristic should instead be observed in SP populations if they are truly enriched in stem-like cells (Buick & Pollak, 1984).

Based on levels of mRNA expression, differentiation of RDs did not seem to occur with ATRA treatment. RD cells express, at baseline, some skeletal muscle proteins due to their origins as striated muscle tissue. However, the normal myogenic differentiation program has been halted in RD cells because many terminal morphological and functional changes are absent, and they continue to proliferate unlike terminally differentiated muscle cells (Saab et al., 2011). Therefore, retinoic acid has been studied for its role in inducing differentiation of myoblasts, as a means to similarly induce differentiation and loss of proliferative capabilities in RD tumors (Hansen et al., 2000; Sidell et al., 1983; Zhu et al., 2009). Treatment of RD cells with retinoic acid in vitro has previously been shown to slow cell proliferation and increase the expression of genes associated with muscle differentiation, including Myogenin and Troponin T (Al-Tahan et

al., 2012; Barlow et al., 2006). In our experiments with RD cells, Myogenin did show a modest increase in expression following ATRA treatment, and Zeb1 expression decreased. These changes are both consistent with a reduction in stemness—however, variable MyoD and Myf-6 expression results make it difficult to conclude that differentiation occurred. Adding the analysis of protein expression, and including other terminal myocyte differentiation markers such as Troponin T, to our differentiation assays may help to determine more definitively whether differentiation is occurring with ATRA treatment.

SP cells isolated via Hoechst dye exclusion and cell sorting did not seem to be more stem-like than non-SP cells. In our experiments, both RD and 143B sarcoma lines were shown to have a sizable side population. Relative to non-SP cells, SP cells from the RD line exhibited a higher expression of Zeb1—a finding consistent with increased stemness and consistent with our results following ATRA treatment of RDs. However, unlike our ATRA experiments, we were unable to consistently detect expression of any of the muscle differentiation markers, making it difficult to conclusively determine whether SP cells were indeed more stem-like. Additionally, in different trials, 143B cells showed both significant increases and decreases in Zeb1 expression in SP cells. These conflicting patterns of expression mean that we again cannot make a definitive conclusion that SP cells are more stem-like than non-SP cells. Results from functional assays such as soft agar and clonogenic growth should therefore be assessed critically.

Unfortunately, due to this uncertainty in the identity of both our ATRA-treated cells and isolated side population cells, we cannot at present determine conclusively whether inhibiting stemness reduces aggression in sarcoma. We will have to refine our methods or attempt other methods to more successfully isolate populations of cancer stem cells and more differentiated cancer cells, in order to gather evidence for or against the hypothesis. Once we have more

confidently understood the relationship of MET and stemness to cancer aggression *in vitro*, future studies will involve using patient-derived xenografts to confirm our results *in vivo*.

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