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Overexpression of SOX18 increased cell proliferation, migration and colony formation in pediatric sarcomas

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Overexpression of SOX18 Increased Cell Proliferation, Migration and Colony Formation in Pediatric Sarcomas

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Abstract

Cancer has become one of the leading causes for premature deaths and continues to increase in rank across the world. An estimated 19.3 million new cases of cancer were reported in 2020 and 10 million cancer related deaths occurred worldwide. Ewing sarcoma and Rhabdomyosarcoma are two pediatric cancers that have not made significant improvements to patient survival for a metastatic diagnosis and the current chemotherapy regiment can cause several undesirable side effects immediately after treatment or later in life. SOX18 has been observed to play some role in various tumorigenic properties like proliferation, invasion, and resisting cell death signals in cancers such as breast cancer, hepatocellular cancer, and osteosarcoma. It also has been observed increasing expression of some receptor tyrosine kinases leading to increased survival in the presence of cancer therapeutic inhibitors. In order to test its effects on tumorigenic properties, Western blot, cell viability, colony formation, and migration assays were performed using Ewings sarcoma (ES8) and Rhabdomyosarcoma (RD) cells transfected with pCDNA and pLenti plasmids with negative control and overexpression of SOX18. Results displayed a trend of increased migration and colony formation capacity for pLenti SOX18 transfected cells and significantly increased cell viability in pLenti SOX18 transfected cells. Western blot displayed greater fold difference for pLenti transfected cell. Further studies of overexpression effects on angiogenesis will be analyzed using chick chorioallantoic membrane (CAM) assay. We anticipate further identification of the role SOX18 plays in tumorigenic properties in RMS and ES may bring to light another possible target for novel targeted treatments.

Keywords: Ewings sarcoma, Rhabdomyosarcoma, SOX18, Sox18, overexpression, Western blot, cell viability, migration

Introduction

Cancer is a leading cause of premature death in many post-industrialized countries and continues to increase across the world and is characterized by uncontrolled cellular proliferation (Naveen et al, 2018). Cancer's increased rank is partially reflective of the decrease in mortality rates of other diseases relative to cancer, such as coronary heart disease and strokes. As the average age and size of populations continue to increase, the burden of cancer increases. In 2020, an estimated 19.3 million new cases and 10 million cancer related deaths were recorded worldwide (Sung et al, 2021). Currently, the most commonly diagnosed cancer is female breast cancer, followed by lung and colorectal cancer (Sung et al, 2021). The NIH has provided an impressive amount of funding dedicated to cancer research which has led to advancements in cancer treatments, but not as much goes towards pediatric cancers compared to other common cancers. This has led to only a small shift in mortality rates for most cancers. While many cancers can be cured or improved through drug treatments, the survival rate of advanced metastatic forms have not significantly improved (Naveen et al, 2018). Additionally, pediatric cancer is the leading cause of death by disease for children and adolescence ages 1-19 with it being 9% of all deaths per year of that age range (Cunningham et al, 2018). As a result, there is a need for novel and effective therapies.

Cancer development is a multistep process that relies on mutations and selection of cells that contain certain traits like increased capacities for survival, proliferation, invasion, and metastasis. There are many causes of cancer, and substances that facilitate the development of cancer are called carcinogens. These substances have been identified in experimental animal studied and analysis of cancer in human populations. Carcinogenic substances act by damaging DNA which induces mutations or by stimulating cell proliferation (Cooper, 2000). One such

mutation can occur to the gene SOX18, a gene that encodes for the Sox18 transcription factor. This paper explores the effects of Sox18 upregulation in the pediatric cancers Rhabdomyosarcoma (RMS) and Ewing Sarcoma (ES).

Ewing Sarcoma is described by the appearance of small round blue cells and originates from a primordial bone marrow-derived mesenchymal stem cell. The incidence has remained unchanged for the past 30 years and is between nine and ten cases per one million people in patients aged 10-19 years old (PDQ Pediatrics, 2002). There are three main categories of ES: round cell sarcomas with EWSR1 gene fusion with non-erythroblast transformation specific (ETS) family members, round cell sarcomas (CIC-rearranged sarcomas), and malignant mesenchymal tumors classified and treated as ES or as undifferentiated round cell sarcomas (BCOR-rearranged sarcomas) (Sbaraglia et al, 2020). When genetically sequence, ES can be characterized by a translocation involving Ewing Sarcomas gene (EWS) on chromosome 22 with a E26 transformation-specific transcription factor family gene (Casey et al, 2019). In the majority of all ES tumors, the fusion gene EWS-FLI1 is found, and it plays a role in the proliferation of ES. Currently, it is the second most common malignant bone tumor in both children and young adults (Casey et al, 2019). The current standard of care involves initial cytoreduction using chemotherapy (vincristine, actinomycin D, and cyclophosphamide) to destroy the micro metastatic disease and facilitate effective local control, definitive radiation or surgical procedures to kill all known disease following up with consolidation therapy for eradication of any hidden residual disease. However, relapse is a common occurrence, and the prognosis is very poor when compared to osteosarcoma (Casey et al, 2019). Additionally, late term effects of the chemotherapy, surgery, or radiation can impair limb function, cause second malignancies, or cause other diseases such as chronic cardiomyopathy (Jain et al, 2010).

Rhabdomyosarcoma is a malignant tumor that, similar to ES, originates from mesenchymal stem cells. It is the most common soft-tissue sarcoma for children, with approximately 65% if cases diagnosed in children younger than six years old and the remaining cases in the 10-18-year-old age group (Dagher et al, 1999). The annual incidence of RMS s four-point-five cases per one million children. RMS has two major subtypes, Alveolar Rhabdomyosarcoma (ARMS) and Embryonal Rhabdomyosarcoma (ERMS). ERMS usually manifests in early childhood and typically effect the head, neck, and genitourinary regions. ARMS usually manifests in late childhood and adolescent years and typically effect the trunk and extremities. ERMS has a higher incidence rate of 65-75% while ARMS has a 25-32% incidence rate (Gurria et al, 2018). The two subtypes also have different histopathology. ERMS is made up of round or spindle-shaped cells that resemble embryonic muscles while ARMS have alveolar architecture with aggregates of small round undifferentiated cells (Wang, 2012). Some ARMS frequently involve the lymph nodes, which has a poor prognosis of a 45% survival rate after five years (Gurria et al, 2018). Similar to ES, RMS uses chemotherapy that is comprised of vincristine, actinomycin-D, and cyclophosphamide (VAC) (Chemotherapy, 2018). When they have an intermediate-risk group, they may use VAC/VI, which means they alternate vincristine with irinotecan. For high-risk groups, VAC regiment us used but other drugs such as docorubicin, ifosfamide, and etoposide may be used to make the treatments more intense. Radiation and surgery are also useful tools of treatment depending on the stage the sarcoma was detected. Some immediate side effects to the chemotherapy drugs include hair loss, nausea, and vomiting, diarrhea, and increase chance of infections. There are also possible long-term effects, such as nerve damage, decreased fertility, damage to bladder, and a second type of cancer could develop (Chemotherapy, 2018). While both ES and RMS are different cancers, both cancers still

display the hallmarks of cancer, which is why it's important to find targetable genes or proteins that are involved in tumorigenic properties.

One group of biomolecules involved in tumorigenic properties is the Sox family of transcription factors, which are well-established regulators of what happens to a cell (cell fate) during development. Research for this gene family started with the discovery of the *Sry* gene, also known as the mammalian testis-determining factor. The distinction between the Sry gene and Sox proteins lies within a region called the high-mobility group (HMG). If a protein contains an HMG domain that is at least 50% similar to the HMG domain of Sry, then it is considered a Sox protein (Sarker et al, 2013). An important feature of the Sox transcription factor is its ability to bind to the minor groove of DNA. The SOX family has no specific biological function, but among their many roles include proliferation, multipotentiality, and differentiation (Chew et al, 2009). They have also been noted to be involved with the wound healing process which can make tumors more resilient.

Within the mouse and human genome, twenty homologous sequence pairs of the SOX gene were discovered and an additional eight in *Drosophila melanogaster* (Schepers et al, 2002). These were then sub-group into ten distinct families and denoted by letters A-J. Sox families B-H have been found to be upregulated in various cancers, such as breast, prostate, and lung cancer. Sox18 is a member of the SoxF family and is characterized by a distinct C-terminal transactivation domain and a short amino acid motif inside the transactivation domain that mediates β-catenin interaction and the regulation and coordination of gene transcription (Grimm et al, 2020). This analysis is further verified by the finding that Sox18 is implicated in having a major role in angiogenesis throughout the wound and tissue repair process which was later confirmed when an increased gene expression in the SoxF family was recorded in the vascular

endothelial cells of invasive ductal carcinomas (Grimm et al, 2020). As a result of these findings, Sox18 makes a very promising target for cancer therapy.

Studies have already begun to determine the role Sox18 plays in various cancers, including breast cancer. Since breast cancer is the most common malignancy in women around the world, a targeted therapy would prove quite useful. Sox19's importance in tumorigenesis and tumor progression could have a mechanism that leads to proliferation and invasion of the cancer cells (Zhang et al, 2016). When investigated further, it was determined that Sox18 is highly expressed in various human breast cancer cell lines. By performing a knockdown of the gene, the study shows decreased cell proliferation. Completely silencing the gene induced cellular apoptosis, decreasing its ability to invade other tissues, and reduced the expression of many receptors that are often overexpressed in cancer cells (Zhang et al, 2016). Similar results were found in other common cancers, such as hepatocellular carcinoma (Sun et al, 2019) and osteosarcoma (Wu et al, 2015). In hepatocellular carcinoma (HCC), downregulation also activated the autophagy signaling pathways in the HCC cells (Sun et al, 2019). Sox18 overexpression has also been found in 88% of participants in the study conducted by Wu et al.

With cancer cases on the rise, it is becoming increasingly important to discover new novel therapies to combat cancer. With data supporting the importance of Sox18 in many tumorigenic properties in various types of cancer, we can view Sox18 as a possible target for future drug therapies. In this paper it was hypothesized that overexpression of the SOX18 gene will lead to increased tumorigenic properties, such as colony formation, migration, and cell proliferation.

Materials and Methods

Cell Culture

ES8 and RD cells were used for these experiments. Each cell line was cultured in RPMI-1640 medium (Hyclone, cat no. SH30027.01) and supplemented with 10% fetal bovine serum (FBS) (Corning, ref 35-01-CV) and 1% Penicillin/Streptomycin solution (50X) (Corning). The cells were washed with Hank's Balanced Salt Solution (HBSS) (1X) (Corning) and Trypsin (Corning, ref 25-050-CI) was used to remove cells from the plate. The cells were incubated at 37 ℃ with 5% CO_2.

Incubation of Transformed Bacteria

Luria Broth (LB) was added to flasks and one colony of the transformed bacteria was added to each flask. A control and overexpressed SOX18 variant of both pCMV (MHS6278-202759994, Clone Id: 6183010) and pLenti (OriGene Technologies, cat no. PS100093) were used. Ampicillin (Research Products International, cas no. 69-52-3) was added to the flasks containing pCMV for a final concentration of 100 µg/mL and Chloramphenicol (Research Products International, cas no. 56-75-7) was added to the flasks containing pLenti for a final concentration of 34 µg/mL. The flasks were placed in a mixing incubator overnight at 37 ℃ at 250 rpm.

Midi-Prep

After one day of incubation for the transformed bacteria, ZymoPURE Midiprep Kit (cat no. D4213-A) was used to prepare the DNA. From the flask 25 mL was transferred into a 50 mL tube and 8 mL of ZP Express Lysis Buffer was added to the 25 mL aliquot and mixed by inverting the tube six times. The tube was rested at room temperature for three minutes. Once the

solution looks clear, viscous, and blue, 10 ML of ZP Express Neutralization Buffer was added and mixed by inverting 8-12 times. After the sample turned yellow and precipitate formed, the lysate was added to the ZymoPURE Syringe Filter-X. Once the precipitate floats to the top after 8 minutes, the plunger was added and pressed the solution through the syringe until about 35-36 mL of cleared lysate was in a clean 50 mL tube. The cleared lysate was saved and 10 mL ZP Express Binding Buffer was added and inverted 8 times. Zymo-Spin V-PS Column Assembly was connected to the vacuum manifold. All of the clear lysate was added to the Column and the vacuum was turned on until all the liquid passed through. The 50 mL reservoir will be discarded from the top of the Column and 5 mL of ZymoPURE Wash 1 will be added to the Column. The liquid was vacuumed through the Column and 5 mL of ZymoPURE Wash 2 was then added to the Column. The liquid was vacuumed through the Column and Wash 2 was repeated one more time. The 15 mL Reservoir-X was removed and discarded, and the Column was placed in a Collection Tube. The Column was centrifuged at 16,000 x g or greater for one minute. The column was transferred into a clean 1.5 mL microcentrifuge tube and add 200 µL of ZymoPURE Elution Buffer directly to the column matrix. After two minutes, centrifuged again at 16,000 x g for one minute. The eluted plasmid DNA will be stored at a temperature of 20 ℃ or less.

Transfection

Two 1.5 mL tubes were labeled separately. The final concentration of cells was $1x10^6$ cells/mL for ES8 and $2x10^6$ cells/mL for RD. In one tube, 500 µL Opti MEM (Gibco, ref 31985-062) and 21.7 µL Lipofectamine 3000 (Invitrogen, P/N 56531) were added and mixed. In the second tube, 500 µL Opti MEM, 28 µL P3000 (Invitrogen, P/N 100022057), and 5-10 µg of the collected DNA were added and mixed. Both tubes were then combined and allowed to incubate

for 10-15 minutes. The solution was added to 10 cm dishes with 10 mL RPMI supplemented with 10% FBS and inoculated for 2-4 days at 37℃.

Cell Proliferation Assay

Transfected cells were collected and counted using a hemocytometer. The cells were collected by removing the old media, washing with 5 mL HBSS, and 2 mL of trypsin was used to detach cells from plate. After a ten-minute incubation, trypsin was neutralized with RPMI and placed into a conical tube to be counted. On day 0, the cells were plated in triplicate into a 96-well plate in triplicate with a final concentration of $5x10^3$ cells/well in 100 µL of RPMI with 10% FBS and 1% Penicillin and Streptomycin solution. After 96 hours, 10 µL Alamar Blue (Bio-Rad) was added to the cells and incubated for 2 hours. The plate was read, and fluorescence was measured using SpectraMax iD3(Molecular Devices) at 550 nm excitation and 590 nm emission. It was placed back in the incubator and allowed to incubate for another 2 hours and read again. The data was then analyzed for statistical significance.

Migration Assay

Transfected cells were collected and counted using a hemocytometer. The cells were collected by removing the old media, washing with 5 mL HBSS, and 2 mL of trypsin was used to detach cells from plate. After a ten-minute incubation, trypsin was neutralized with RPMI and placed into a conical tube to be counted. The cells were placed in SF RPMI for this experiment. On day 0, 750 µL RPMI with 10% FBS was pipetted into each well of the 24 well plate in duplicate. Fluoroblok Transwell Inserts (Corning) were added to each well and 500 µL of the cells in SF media were added to the Transwell Inserts at a final concentration of $5x10^4$ cells/insert. After 48 hours, the plate was read, and fluorescence was measured using SpectraMax

iD3 at 487 nm excitation and 517 nm emission. The data was then analyzed for statistical significance.

Colony Formation Assay

Transfected cells were collected and counted using a hemocytometer. The cells were collected by removing the old media, washing with 5 mL HBSS, and 2 mL of trypsin was used to detach cells from plate. After a ten-minute incubation, trypsin was neutralized with RPMI and placed into a conical tube to be counted. On day 0, the cells were plated into 6-well plates in triplicate with a final cell concentration of 500 cells/well in 2 mL of RPMI with 10% FBS. On day 7, the media was removed, and each well was washed with 1.0 mL PBS. Then 1 mL of 4% paraformaldehyde (PFA) was added and let sit for 15-30 minutes. PFA was disposed of, and 0.5 mL 0.05% (weight/volume) crystal violet was added to each well and let sit for 60 minutes. The crystal violet was removed, the wells were washed with distilled water and the dishes were allowed to air dry. Colonies were quantified using ImageJ software version 1.53 and analyzed or statistical significance.

Western Blot Assay

The remaining transformed cells underwent protein lysis. The cells were pelleted, and media was replaced with 1.0 mL 1X PBS. The cells were resuspended and placed into a microcentrifuge tube and spun at 10,000 rpm for two minutes at 4℃. The PBS was removed and Lysis buffer with PMSF was added and stored at -80 ℃ until assay was ready to be performed. For western blot, protein concentrations were determined using nanodrop (Thermo Scientific, NanoDrop 2000 Spectrophotometer). To create the protein samples, nano-pure water, beta-mercaptoethanol (Sigma-Aldrisch, EC no 200-464-6), 2X Laemmli sample buffer (Bio-Rad, cat.

no 1610737), and equal amounts of protein (50 ug) were mixed for loading into Mini-PROTEAN TGX Precast Gels to run electrophoresis. Mixed samples were heated on a heat block for five minutes at 95 ℃. During this time, the electrophoresis chamber was set up and 1X running buffer was used to fill both the inner chamber completely and until the bottom of the outer chamber was covered. The 1X running buffer was prepared by diluting 10X Tris/Glycine/SDS Buffer (Bio-Rad, cat. No 1610772) with nano pure water. The mixed sample and Novex Sharp Pre-Stained Protein Standard ladder (Invitrogen, P/N 57318) were loaded into the wells and ran at 90-120 volts for about 60 minutes or until protein reached the bottom of the gel. Once complete, transfer of bands onto a nitrocellulose membrane was performed. Semi-dry transfer was conducted with Trans-Blot Turbo Transfer System (Bio-Rad) and Trans-Blot Turbo 5X Transfer Buffer (Bio-Rad, cat. No 10026938) diluted to 1X. Once transfer was complete, 5% nonfat milk dissolved in 1X TBST was used to block any non-specific protein prior to antibody incubation. The membranes were washed with 1X TBST and then incubated in 1:500 dilution primary anti-SOX18 antibody (Santa Cruz Biotechnology, Sc-166025) overnight at 4 ℃. Membranes were then washed with 1X TBST and incubated with secondary anti-mouse monoclonal mouse antibody, diluted 1:5000 (Santa Cruz Biotechnology, D-8, Sc-166025) for 1-2 hours at room temperature. The membrane was washed with 1X TBST and ECL was added to the membrane one minute prior to chemiluminescent imaging using UVP ChemStudio (AnalytikJena). ECL was a 1:1 mix of SuperSignal West Femto Stable Peroxide Buffer (Thermo Scientific, prod no 1856190) and SuperSignal West Femto Luminol/Enhancer Solution (Thermo Scientific, prod no 1856189). After imaging for Sox18 bands, membranes were stripped with NewBlot Nitro Stripping Buffer (Li-Cor, Part no. 982-40030) diluted from 5X to 1X with nano pure water for five minutes. Bands were quantified using ImageJ software version 1.53.

Statistical Analysis

Experiments were performed in triplicate, except for the migration assay which was performed in duplicate, and the data was analyzed using mean and standard deviation. For statistical analyses involving more than two groups, a one-way ANOVA test was performed. One-way ANOVA test was done concurrently with the Tukey posttest to determine significance between any two of the groups in the experiment. Unpaired t-test and Welch's test were done concurrently for experiments/data that contained only two groups. All statistical analyses were performed on GraphPad Prism (9.3.1) software (GraphPad Software Inc.). $P < 0.05$ was considered statistically significant.

Results

Overexpressed SOX18 Increased Viability

To determine the effect overexpressed SOX18 has on ES8 and RD cells viability, the Alamar Blue assay was performed. As shown in Figure 1A, ES8 transfected with the pLentiSOX18 plasmid displayed significantly greater cell viability ($P < 0.05$) of 95% compared to the control. A similar outcome was observed in the RD cell line transfected with the pLentiSOX18 plasmid with a significant increase of 85% (Figure 1B). These results point to SOX18's overexpression promote cell survival in ES8 and RD cells.

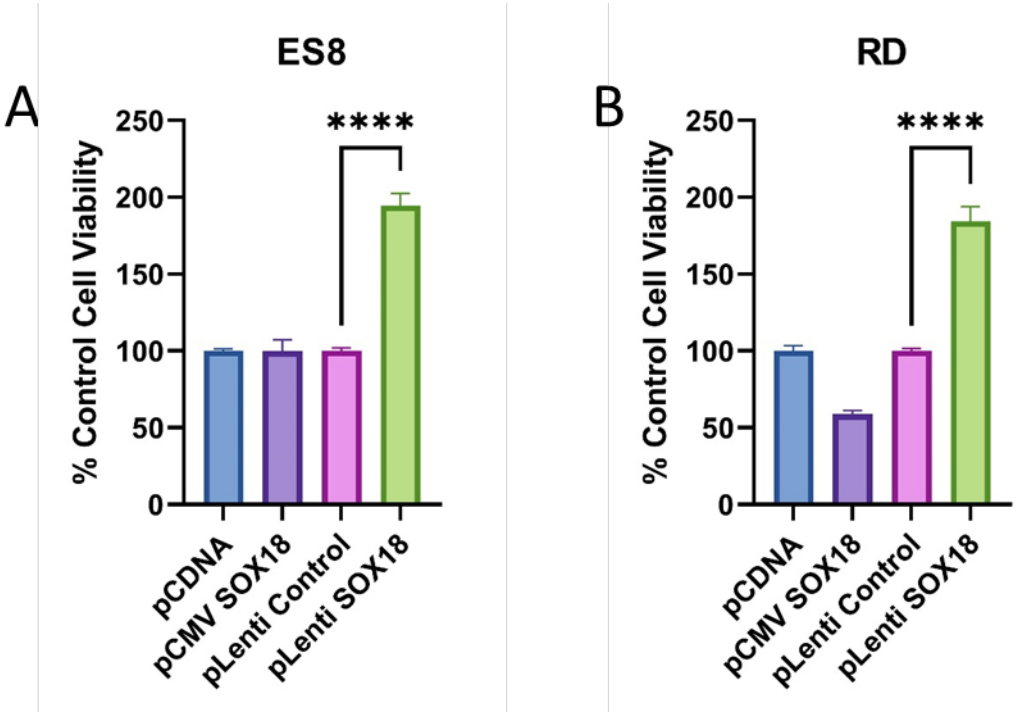

Figure 1 Overexpression of SOX18 resulted in increased cell viability. Cells were transfected with overexpression plasmids and replated 48 hours following transfection in triplicate. Cell viability was assessed by Alamar Blue assay. Fluorescence was detected after 72-hour incubation using SpectraMax iD3 at wavelengths of 550 nm excitation 590 nm emission. **(A)** ES8 cell survival with a significant increase between pLetnti control and pLenti SOX18. **(B)** RD cell survival with a significant increase between pLenti control and pLenti SOX18. The data was analyzed by GraphPad Prism. Values are expressed as means ± standard deviation. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Overexpressed SOX18 increased migration

To investigate how overexpression of SOX18 affects migration capabilities of ES8 and RD cell lines, a migration assay was performed. The observed trend displayed increased migration for cells infected with the overexpression plasmid when compared to the controls, about (Figure 2A and B). The increase was 23% for ES8 pCMV SOX18, 50% ES8 pLentiSOX18, 14% RD pCMV SOX18, and 29% RD pCMV SOX18. These results suggest that overexpressed SOX18 promotes cell migration in ES8 and RD cells.

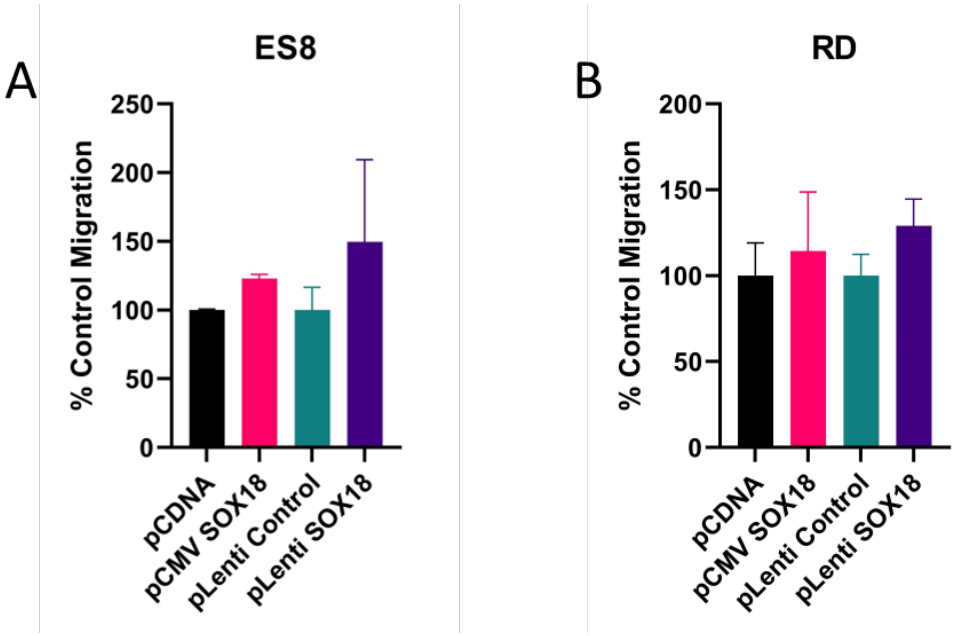

Figure 2 Overexpression of SOX18 resulted in increased cell migration. Cells were transfected with overexpression plasmids and replated 48 hours following transfection in duplicate. Migration ability was assessed by migration assay using Fluoroblok Transwell Inserts. Fluorescence was detected after 48 hr incubation using SpectraMax iD3 at wavelengths 487 nm excitation and 517 nm emission. **(A)** ES8 cell migration shows a trend with more overexpressed cells migrating. **(B)** RD cell migration with a slight increase in overexpression migration. The

data was analyzed by GraphPad Prism. Values are expressed as means ± standard deviation. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Overexpressed SOX18 produces more protein

To determine if the plasmids were successful in transfecting and producing more protein, a Western blot was performed. The Western blot displayed bright bands for RD cells that were transfected with pLentiSOX18 and less bright bands for ES8 cells transfected with pLentiSOX18 (Figure 3A). The other bands were not easily visible which shows that the pLentiSOX18 plasmids upregulates SOX18 more than the other plasmids. The quantification of the bands shows a fold difference 2.8x higher than the control for ES8 pLentiSOX18 and 5.8x higher than the control for RD pLentiSOX18 (Figure 3B and C).

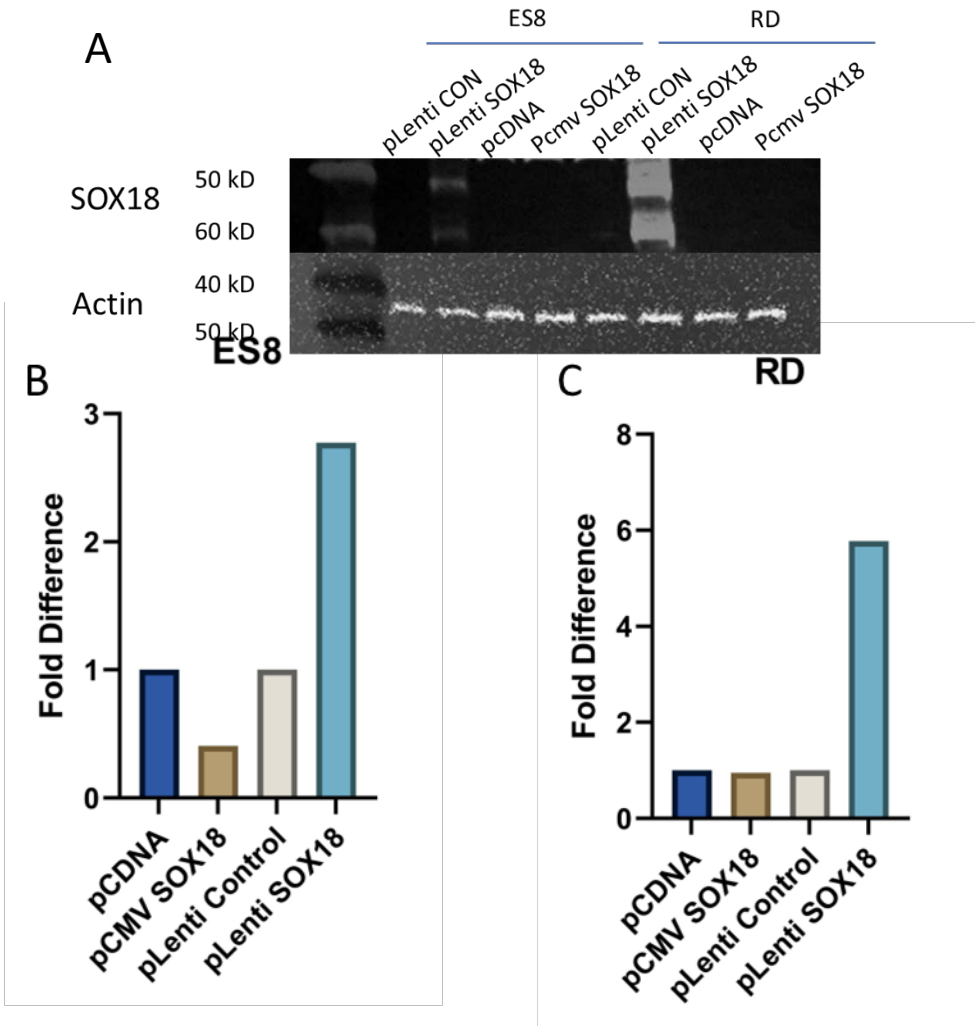

Figure 3 Overexpressed transfected cells were lysed and protein was collected. Protein comparison was performed by Western blot. Data was quantified using ImageJ software and analyzed with GraphPad Prism. **(A)** Qualitative figure of Western blot bands indicating overexpression of SOX18 in ES8 and RD cell lines (pCDNA, pCMV SOX18, pLenti Control, pLenti SOX18). **(B)** Quantitative figure of Western blot bands indicating increased expression of SOX18 in pLenti SOX18 for the ES8 cell line. **(C)** Quantitative figure of Western blot bands indicating increased expression of SOX18 in pLenti for the RD cell line.

Overexpressed SOX18 increased colony formation

To evaluate how overexpression of SOX18 functions in a cell's ability to multiply on its own, a colony formation assay was performed. Figure 4A-D show the colonies observed on the dishes. After analysis, a trend displaying increased colony formation in ES8 cells transfected with pCMV SOX18 and pLentiSOX18 plasmids by 70% (pCMV SOX18) and 130% (pLentiSOX18) when compared to the control (Figure 4E). The RD cells only displayed this trend when transfected with pLentiSOX18 and showed an increase of 270% when compared to the control (Figure 4F). These results suggest that overexpression of SOX18 enhances colony formation in ES8 and RD cells.

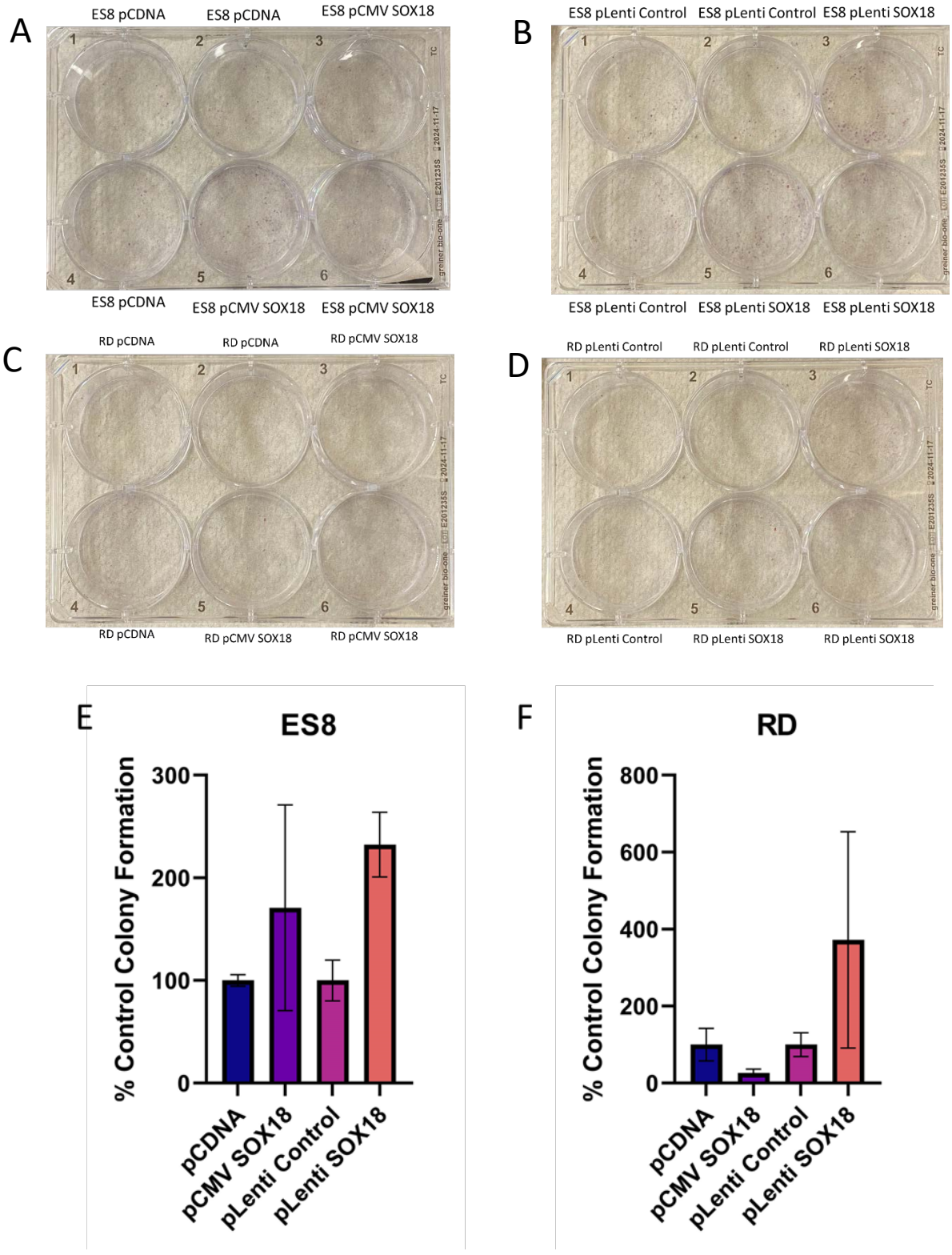

Figure 4 Overexpression of SOX18 resulted in increased colony formation. Cells were transfected with overexpression plasmids and replated 48 hours following transfection in

triplicate. Colony formation ability was assessed by the colony formation assay. Following 7-day incubation, cells were fixed, stained, quantified with ImageJ software and analyzed with GraphPad Prism. **(A-D)** Qualitative figures of colony formation of the ES8 and RD cell lines. **(E)** ES8 cells display a trend of increased capabilities for overexpressed cells. **(F)** RD cells display a trend of increased capabilities for pLenti SOX18 only. Values are expressed as means ± standard deviation. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Discussion

Transfection was used to characterize the overexpression of SOX18 which allowed for increased expression of the Sox18 transcription factor. Both the pLenti and pCDNA plasmid were tested in this experiment to determine which one worked best. The effect of increased expression could then be studied to discern the impact SOX18 has on normal function and behavior. Western blotting was performed to determine which plasmid worked best and the observations were later quantified using the Western blot bands seen in Figure 3.

The Western blot analysis shows the pLenti SOX18 band is brighter than all the other bands for the RD cell line (Figure 3A). When quantified with ImageJ, there was an observable increase in protein produced by cells transfected with the pLenti SOX18 plasmid for both ES8 and RD cell lines (Figure 3B and C). Statistical significance was not able to be obtained as the samples were not performed in replicates, therefore no statistical analysis can be performed. However, the fold difference observed is 2.77x in ES8 cells and 5.77x larger in RD cells (Figure 3B and C). While the difference in protein production was large between the two plasmids, both plasmids were used for the other tests performed to determine if the initial Western blot data was

accurate. To characterize the effects of SOX18's effect on tumorigenic properties, the following studies were conducted: migration, cell viability, and colony formation.

The migration assay had the cells cross a membrane from serum-free media to FBS-containing media. An important landmark in cancer development and progression is its ability to migrate and metastasize to other tissues, allowing for the primary tumor to spread to various regions of the body through the circulatory or lymphatic systems. The current data was not statistically significant, but the trend seen in Figure 2 was opposite of that seen in a knockdown study performed by Wang et al, 2015. Further studies into refining the migration experiment and studying invasion could provide a clearer picture into how SOX18 effects migration and invasion in cancerous cells.

Cell viability was performed to test the effect SOX18 had on the survival of the cells. There was a significant difference in the viability of both ES8 and RD cells transfected with pLenti SOX18 with a p-value < 0.0001 (Figure 1A and B). This finding was consistent with another study who found significant increase in cell viability of hepatocellular cells containing overexpressed SOX18 gene (Sun et al, 2019). There was a dip in cell viability of 41% with RD cells transfected with pCMV SOX18 when compared to the control, which is an anomaly. Further testing needs to be conducted to determine if this is due to experimental error or a result of the plasmid.

The final assay that was performed was the colony formation assay which assesses the cell's ability to form a colony with little to no cell-to-cell communication. This assay is also another indicator of cell survivability as it is important for cancer cells to proliferate on their own after they metastasize. The stained colonies show that pLenti SOX18 for both ES8 and RD cells have the most formed colonies in their respective cell line (Figure 4A-D). Analysis of the colony

count confirmed the trend that the pLenti SOX18 transfected cells had an increased ability to form colonies for both ES8 and RD cell lines (Figure 4E fand F). The trend shown in Figure 4 was consistent with data reported by Miao et al, who observed a similar trend with overexpressed SOX18 cells. Similar to cell viability, there was an anomaly with pCMV SOX18 transfected RD cells where they saw a decrease compared to the control of 73%. Further studies need to be done to refine and gather more data to determine if these findings are significant and if that anomaly was due to experimental error or a result of the plasmid.

Conclusion

These results indicate SOX18 may be a big player in various tumorigenic properties in both ES8 and RD cells. It was specifically shown to statistically increase cell viability in comparison to the control cells and shows a trend of increasing the ability for the cells to migrate and form colonies. Future studies may focus on testing invasion or further validating the results by testing for significant data in migration and colony formation assays. They may also determine if SOX18 plays a role in other pediatric cancers or cancers that afflict similar tissue to Ewings sarcoma and Rhabdomyosarcoma.

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