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# Role of SOX18 in Promoting Tumorigenesis in Pediatric Cancer Cell Lines

Cherubina Rubannelsonkumar
rcherry2000@gmail.com

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by

Cherubina S. Rubannelsonkumar

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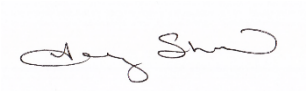

Dr. Terry Shackleford

Associate Professor of Biological Sciences

Dr. Richard Cardenas

Director, Honors Program

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Abstract

Sarcomas constitute a high percentage (~13%) of cancer-related deaths among pediatric patients between 0-19 years of age, with Rhabdomyosarcoma (RMS) being the most common pediatric soft tissue sarcoma and Ewing Sarcoma being the second most common malignant bone tumor in children. Yet, survival for those who develop such metastatic sarcomas remains below 20-30%. Interestingly, SOX family proteins are known to be up regulated in various cancer types and play a role in cancer progression (tumorigenesis, metastasis, etc.). More specifically, targeted knockdown of SOX18 has been shown to suppress various tumorigenic properties in cancer cell lines including osteosarcoma cells, hepatocellular carcinoma cells and breast cancer cells. Additionally, prior studies showed inhibition of IGFR leads to compensatory pathway activation via other RTK receptors resulting in continued cell survival. Upregulation of SOX18 was observed in such cases. Thus, it is important to clarify the role of SOX18 in RMS and ES. Ewing Sarcoma (ES8) cell line was infected with shSOX18 to produce knockdown and selected via puromycin resistance. Cell migration, cell proliferation and colony formation assays were used to characterize effects of knockdown. Results showed that the shRNA was effective in SOX18 knockdown and lead to reduced cell proliferation in ES8 cells. Further evaluation of knockdown effects on angiogenesis will be analyzed using chick chorioallantoic membrane (CAM) assays. We anticipate characterization of the role of SOX18 in RMS and ES may inform future development of novel targeted treatments and increased understanding of role of SOX18 in activation of RTK compensatory pathways during IGFR inhibition in pediatric sarcomas.

Introduction

It is well known that cancer is one of the leading causes of death worldwide, accounting for around 9.6 million deaths in 2018, according to the WHO Global Cancer Observatory data (Bray et al, 2018). In the United States it is the second leading cause of death (Siegel et al, 2021). In 2021 alone, it is estimated that approximately 1,898,160 patients will be diagnosed with cancer in the United States, which translates into an equivalent of 5,200 new cases per day (Siegel et al, 2021). Although cancer is rare in children and adolescents, it is the leading cause of death by disease past infancy in this age population, with an estimated 15,590 cases to be diagnosed in children and adolescents between the ages of 0-19 in 2021. These statistics provide substantial evidence for the importance of cancer research in general but especially the targeted research to improve the current knowledge and understanding of pediatric cancers.

This paper explores the characterization of SOX18 in the bone and soft-tissue cancers Ewing Sarcoma (ES) and Rhabdomyosarcoma (RMS). Interestingly, the overall five-year survival for patients with pediatric cancers improved from 63% to 83% from 1970s to 2000s, however, in cancers including bone and soft-tissue cancers like ES and RMS, progress has been limited for the past 40 years (Siegel et al, 2020).

Ewing sarcoma is derived from the primordial bone-marrow-derived mesenchymal stem cell (PDQ Pediatric, 2021) and is characterized by small round blue cell tumors. Genetically, ES is characterized by a translocation mutation of the EWS gene found on chromosome 22 with an E26 transformation-specific transcription factory family of genes. Most commonly the fusion gene EWS FLI1 is found in ES tumors in this translocation (Casey et al, 2019). Notably, ES is the second most common malignant bone tumor in children (Gurria et al, 2018).

Rhabdomyosarcoma is the most common form of pediatric soft tissue sarcoma. Specifically, it accounts for around 5% of all pediatric cancers. Similar to ES, RMS is a malignant tumor that originates from the mesenchymal stem cells and is also characterized by small round blue cell tumors. Furthermore, RMS is broken down into two subdivisions: alveolar (ARMS) and embryonal (ERMS) (Gurria et al, 2018). Importantly, although ERMS and ARMS differ in morphology and genetic make-up. In terms of morphology, ERMS cells are composed of round or spindle shaped cells while ARMS cells are characterized by a more alveolar structure (Wang et al, 2012). Although ES and RMS are distinctive cancer types, it is important to note that the basic properties of these tumors are still uncontrolled proliferation, elevated survival, unlimited replicability, increased angiogenesis behavior, and/or invasion and metastasis potential (Hanahan et al, 2011). Thus, it is important to identify targetable genes and proteins that have an effect on these tumorigenic properties.

One such targetable gene is SOX18 of the SOX family. The SOX family of transcription factors have become a topic of research as they were shown to be involved in tumorigenesis and cancer, as mediators of DNA binding critically controlling cell fate and cancer cell differentiation (Grimm et al, 2020). Interestingly, SOX genes contain the highly conserved high-mobility group (HMG) box from SRY gene, an important gene for male sex determination. SOX stands for SRY-related HMG box. SOX transcription factors bind to the minor groove of DNA and allow for development of various tissues and primary sex determination. Due to it important function, SOX family proteins are maintained under tight regulation, via expression level regulation, post-translational modifications (i.e., phosphorylation, acetylation, methylation, glycosylation and sumoylation), and recruitment of partner proteins (Grimm et al, 2020).. Additionally, microRNAs assist in SOX protein modulation. SOX proteins are classified into

various letter groups, with SOX18 classified as a SoxF protein. SoxF proteins contain a short amino acid motif in the transactivation domain that uniquely helps mediate and coordinate gene transcription. This is consistent with findings implicating SOX18 as having a major role in angiogenesis in wound healing and tissue repair (Grimm et al, 2020). These functions could also contribute to tumor growth if overactive.

There have been multiple studies identifying mutations of the SOX-containing genes as they play an important role in many developmental, physiological and pathological processes and functions in the cell. Interestingly there is a trend of SOX mutations leading to mainly developmental disorders often due to de novo alterations negatively affecting one of the functional alleles and have come to be known as “SOXopathies”. Interestingly, since the first SOX18 mutation discovery in 2003 there was no further development in the discovery of new mutations until 2015. Most SOX genes are separated from coding regions nearby by flanking regions which include multiple enhancers that are an important method of gene regulation for the SOX genes, thus it is understandable that mutations in these regions have been shown to lead to multiple diseases (Angelozzi et al, 2019).

The first SOX18 loss-of-function mutation was discovered in 2003, as mentioned previously, and led to Hypotrichosis-Lymphedema-Telangiectasia syndrome (HLTS). These variants were heterozygous and nonsense mutations that led to early truncation of the SOX18 gene with its transactivation domain. Other mutations that were identified included homozygous missense mutations in consanguineous families, but with this mutation heterozygotes remained unaffected. Interestingly, as the heterozygotes with this mutation remain unaffected, this mutation remains the first and only recessive SOXopathy. In the other SOX gene groups, however, there have been multiple chromosomal rearrangements discovered including in SOXA,

SOXB1, SOXC, SOXD, and SOXE. SOXopathies related to SOXA genes have been characterized with translocations and deletions, SOXB1 has translocations, deletions, duplications and inversions, SOXC has translocations and deletions, SOXD has translocations and deletions, and SOXE has translocations, deletions, duplications and inversions (Angelozzi et al, 2019).

A study looking into further understanding these mutations in SOX18 leading to detrimental effects such as the development of HLTS, focused on a dominant -negative SOX18 mutation. What they realized was that in these dominant-negative cells there was effective transcriptional repression that resulted. As it normally functions as a transcription factor, the truncated protein was only able to bind DNA but not activate gene transcription. Additionally, this mutation led to reduction in the ability of SOX18 to bind chromatin in comparison with wild-type cells, along with affecting other transcriptional requirements. Moreover, by some unknown mechanism this study showed that the mutation was somehow interfering with the ability of other SOXF members, SOX7 and 17, from rescuing the phenotype (McCann et al, 2021).

Another study further developed the current understanding of hereditary lymphedema, which is a developmental disorder leading to chronic swelling of extremities due to malfunctioning lymphatic vessels. Microsatellite analysis was to narrow down the responsible gene, excluding VEGFR3, involved in vascular endothelial growth, and FOXC2, a related transcription factor. It was later identified through study of a similar murine phenotype that this lymphatic dysfunction was actually due to homozygous missense mutations in the SOX18 gene, while heterozygous nonsense mutation led to death. Interestingly, these mutations were de novo mutation in the germline and were not inherited from the parents. Overall, this study was able to

implicate mutations in SOX18 as essential in development of functional lymphatic vessels (Irrthum et al, 2003).

Additional research into SOX18 and its implications in vascular diseases like Hypotrichosis-Lymphedema-Telangiectasia and Renal Syndrome (HLTRS) due to various mutations in the SOX18 gene (Overman et al, 2019) helps to identify its important role in vascular formation. HLTS, which is the syndrome lacking the additional sign of renal dysfunction, was first described in 2001 and since this first case there have only been 10 individuals with the SOX18 mutation from 8 different families (Coulie et al, 2021). HLTS is the major syndrome that is known to be associated with SOX18 mutations, however SOX18 also seems to be playing a role in allowing survival of cancer cells, as there is overexpression of this gene in various cancer types (Wang et al, 2015).

Our current understanding of SOX18 and its role within the cell lends itself to the therapeutic potential in cancer treatment, as it has been shown to be vital in vessel formation and transcription. Thus, although a complete deletion would be detrimental targeted knockdown of SOX18 could be beneficial.

A few studies have already begun exploring the mechanistic role of SOX18 in these cancers. One study indicated that knockdown of SOX18 in hepatocellular carcinoma cells led to inhibition of proliferation, migration and invasion which are all tumorigenic properties that allow for metastasis of a tumor. (Wang et al, 2015). In osteosarcoma knockdown of SOX18 also allowed for suppression of proliferation and metastatic properties (Wu et al, 2016). In breast cancer, knockdown via siRNA led to inhibition of growth and invasion of the cancerous cells (Zhang et al, 2016).

All of these findings make logical sense as SOX18 is well known to be a transcription factor promoting functions such as vascular and lymphatic development (Kamachi et al, 2013). HLTRS on the other hand is known to be caused by a premature truncated SOX18 gene at the C-terminus which results in a defective (by dominant negative) protein (Overman et al, 2019).

Not many studies have begun fully dissecting treatment options targeting the SOX18 transcription factor, however there are few that have identified the therapeutic potential of this target. One such study identified R-propranolol as a small molecule inhibitor of the SOX18 transcription factor as a possible treatment for HLTRS and even hemangioma. More specifically, the R(+)-enantiomer of propranolol, which is already FDA -approved, can be repurposed to alleviate some of the detrimental effects caused by vascular diseases and even cancers caused by SOX18 (Overman et al, 2019). Another article studied the association of SOX18 with the Hedgehog (HH) signaling pathway which is vital in the cell differentiation process as well as embryonic development. It is well known that HH signaling and SOX genes are in functional partnership to facilitate embryonic development, and this article elucidated the crosstalk that occurs between the two. This article identified that expression of the SOX18 gene is regulated by GLI1 and GLI2 transcription factors which is part of the hedgehog signaling cascade, specifically in cervical carcinoma cell line (Petrovic et al, 2015).

Given the importance of understanding SOX18 in various diseases including cancers, it is important to characterize the transcription factor as a possible target for therapeutics. In this paper it was hypothesized that knockdown of SOX18 will lead to decreased tumorigenic effects such as colony formation, proliferation and migration in .

Methods

**Cell Culture**

ES8 and RH41 cells were utilized for these experiments. All cell lines were cultured in RPMI-1640 medium (1X) (Cytiva, cat. No SH30027.01), supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich) and 1% Penicillin and Streptomycin solution (50X) (Corning). Cells were washed with HBSS (1X) (Corning). Cell lines were grown at 37 °C with 5% CO2.

**Formation of Competent Cells**

Lysogeny broth (LB) media was prepared to allow E. coli growth. Single colony of JM109 cells (Promega) were inoculated into the broth. The cells were grown overnight at 37 °C at 250 rpm in the shaking incubator, alongside a control flask. The next day, a 1:50 dilution of cells were reinoculated into a new flask and grown for 2-3 hours in the shaking incubator with the same parameters in incubator. After incubation for 1.5 hours, optical density (OD) was checked and then every 20 minutes after until OD was within the range of 0.5-0.7. The culture was then aliquoted into 50-ml prechilled sterile tubes with 50 ml of culture and tubes were left on ice for 10 minutes. Tubes were then centrifuged for 7 min at 1600 x g at 4 °C and supernatant was discarded. Pellet was resuspended in 10 ml chilled CaCl2 solution. Tubes were then centrifuged for another 5 min at 1100 x g and 4 °C. Supernatant was discarded and pellet was resuspended in 10 ml chilled CaCl2 solution. Suspension was then kept on ice for 30 minutes. A final centrifugation at 1100 x g and 4 °C was done for 5 minutes. Supernatant was discarded and cell pellet was resuspended in 2 ml chilled CaCl2 and glycerol solution. For long-term storage, cells were stored in 100 uL aliquots in the -80 freezer.

**Plasmids and Lentiviral Infection**

pCMV-SOX18 from Dharmacon was used (MHS6278-202759994, Clone Id: 6183010). Lentivirus was produced by transfecting HEK293T cells with shRNA expression plasmids shSOX18-1, shSOX18-2 and shSOX18-3 (Mission shRNA, Sigma-Aldrich), psPAX2 packaging plasmid, pMD2.G envelope plasmid and the lentiviral backbone plasmid pLKO1. Virus was collected 72 hours grown in 20% FBS-containing DMEM medium after transfection and filtered through 0.45 um membrane. Cells were plated at $5x10^5$ cells/well in a 6-well dish and were transduced with virus by spinfection by centrifugation at 800 x g for 1 hour in the absence of polybrene. Cells for stable cell lines were selected using puromycin (1ug/mL). Target sequences can be found table 1.

**Table 1.** shRNA target sequences utilized in the SOX18 knockdown in ES8 cell line.

| Construct | Source | Identifiers | Target Sequence (5' to 3') |
|---|---|---|---|
| shControl | Sigma-Aldrich | SHC202 | CAACAAGATGAAGAGCACCAA |
| shSOX18_1 | Sigma-Aldrich | TRCN0000017448 | CCGGCCTCACCGAGTTCGACCAGTACT CGAGTACTGGTCGAACTCGGTGAGGTT TTT |
| shSOX18_2 | Sigma-Aldrich | TRCN0000017451 | CCGGGCCGCTCGCTGGCCTGTACTACT CGAGTAGTACAGGCCAGCGAGCGGCT TTTT |
| shSOX18_3 | Sigma-Aldrich | TRCN0000017452 | CCGGCAAAGCGTGGAAGGAGCTGAAC TCGAGTTCAGCTCCTTCCACGCTTTGTT TTT |

**Reverse Transcription and Real-Time PCR**

RT-qPCR was done to analyze expression of SOX18 (Forward primer: CGCGTGTATGTTTGGTTC; Reverse primer: ATGTAACCCTGGCAACTG) in comparison with housekeeping gene, EEF1G (Forward primer: CTGTCTTGGGCGAAGTG; Reverse

primer: TTCTCCTCCTTCCGCTC). RNA was extracted from shSOX18 control, shSOX18-1, shSOX18-2 and shSOX18-3 cells using RNA extraction buffer provided by Quick-RNA MiniPrep Kit (Zymo Research, cat. no R1055). Cells were lysed in RNA lysis buffer. RNA (600 ng) was used for cDNA synthesis. 1:20 dilution was done to cDNA samples and gene-specific primers were utilized for amplification of specified genes. RT-qPCR was performed using the AzuraView GreenFast qPCR Blue Mix HR (Azura Genomics) and amplification was performed on the CFX96 Real-Time System (Bio-Rad, C1000 Touch Thermal Cycler). The thermal cycling conditions were 2 minutes at 50 °C followed by denaturation for 10 minutes at 95 °C, and then 40 cycles of annealing at 95 °C for 15 seconds/cycle and finally and extension step at 60 °C for 1 minute. Samples were analyzed in triplicate and relative gene expression was determined by delta-delta-CT method. The delta-CT values were compared with the control.

**Western Blot Analysis**

shSOX18 control, shSOX18-1, shSOX18-2 and shSOX18-3 cells were collected from a 10 cm plate. Old media was removed from cells. Next, cells were first washed with cold PBS. After wash, PBS was aspirated. Then, 1 ml of PBS was added to the plate. Cells were scraped off of plate using a pipette tip. Cells were then placed into a 1.5 ml Eppendorf tube. Lysis buffer with PMSF was added to the collected cells and mixed well. Cells were stored in the -80C freezer until ready to perform western blot analysis. For western blot analysis, protein concentrations in samples were measured using nanodrop (Thermo Scientific, NanoDrop 2000 Spectrophotometer). Then, nano pure water, beta-mercaptoethanol (Sigma-Aldrich, EC no 200-464-6), 2X Laemmli sample buffer (Bio-Rad, cat. no 1610737), and equal amounts of protein (70 ug) were mixed to prepare samples for loading into Mini-PROTEAN TGX Precast Gels (Bio-

Rad, cat. no 456-1085) for electrophoresis. Samples with buffer were then heated on heat block for 5 minutes at 90-100 °C. While sample was heated, electrophoresis chamber was set up, then 1X running buffer was used to fill inner chamber and outer chamber until bottom of gels were covered. 1X running buffer was prepared by diluting 10X Tris/Glycine/SDS Buffer (Bio-Rad, cat. no 1610772) with nanopure water. Samples and Novex Sharp Pre-Stained Protein Standard ladder (Invitrogen, cat. no LC5800) were loaded into wells. Wells were made sure to be covered with running buffer at all times during electrophoresis. After electrophoresis transfer of bands onto a nitrocellulose membrane was performed. Semi-dry transfer was conducted using the Trans-Blot Turbo Transfer System (Bio-Rad) and Trans-Blot Turbo 5X Transfer Buffer (Bio-Rad, cat. no 10026938) diluted to 1X. After transfer was completed, 5% nonfat milk was used to block non-specific proteins prior to antibody incubation. 1X TBST was used to wash membranes, and membranes were then incubated in primary mouse monoclonal IgG (SantaCruz Biotechnology, cat. no sc-166025) overnight in 4 °C. Membranes were then washed with 1X TBST. Then membranes were incubated in secondary anti-mouse IgG, HRP-linked antibody (Cell Signaling, cat. no 7076) for 1 hour. A final wash with 1X TBST was done and finally ECL, SuperSignal West Femto Stable Peroxide Buffer (Thermo Scientific, prod. no 1856190) and SuperSignal West Femto Luminol/Enhancer Solution (Thermo Scientific, prod. no 1856189), was used to fluoresce bands during imaging with UVP ChemStudio system (AnalytikJena). After SOX18 band imaging was completed, membranes were stripped using NewBlot Nitro Stripping Buffer (Li-Cor, Part no. 928-40030) for 5 minutes after diluting 5X buffer to 1X with water.

**Cell proliferation Assay**

shSOX18 control and shSOX18-1 cells were collected and counted using a hemocytometer. shSOX18 control (shC) and shSOX18-1 cells were collected and counted using a hemocytometer. Cells were collected by removing old media from cells. Then 5 ml of HBSS was used to wash the cells and remove any remaining media. After aspiration, 2 ml of trypsin was used to detach cells from flask. After 10-minute incubation cells were placed into a 15 ml conical tube for counting. On day 0, cells were plated into 96-well plate at a concentration of 5x10^3 cells per well in 100ul of RPMI-1640 media, supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin and Streptomycin solution (50X) (Corning). Media was changed every 2 days. On day 5, Alamar blue (Bio-Rad) was added to cells (10 ul Alamar blue/100ul media). Incubate cells in Alamar Blue for 2-3 hours. Plate was read and fluorescence was measured as an indicator of cell concentration using SpectraMax iD3 system (Molecular Devices).

**Colony Formation Assay**

shSOX18 control, shSOX18-1, shSOX18-2 and shSOX18-3 cells were collected and counted using a hemocytometer. shSOX18 control (shC) and shSOX18-1 cells were collected and counted using a hemocytometer. Cells were collected by removing old media from cells. Then 5 ml of HBSS was used to wash the cells and remove any remaining media. After aspiration, 2 ml of trypsin was used to detach cells from flask. After 10-minute incubation cells were placed into a 15 ml conical tube for counting. On day 0, cells were plated into 12-well plate at a concentration of 1000 cells per well in 2 ml of RPMI-1640 media, supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin and Streptomycin solution (50X) (Corning). Cell were

fixed and stained on day 7. Media was changed every 2 days. ImageJ 1.53 software was used to quantify results.

**Migration Assay**

shSOX18 control (shC) and shSOX18-1 cells were collected and counted using a hemocytometer. Cells were collected by removing old media from cells. Then 5 ml of HBSS was used to wash the cells and remove any remaining media. After aspiration, 2 ml of trypsin was used to detach cells from flask. After 10-minute incubation cells were placed into a 15 ml conical tube for counting. On day 0, each cell line (shC and shSOX18-1) was plated into 3 wells each for triplicate measurements in a 24-well plate. These plates included inserts. 40,000 cells were plated into the inner chamber of the well with serum-free media. FBS-containing media was added to the outer chamber to attract cells inducing migration. Plate was placed in incubator at 37 °C for 36 hours. After 36-hour incubation, cells in the inner and outer chambers were fixed with 4% paraformaldehyde (PFM). PFM was then removed, and cells were stained with 0.5% Crystal Violet (VWR, cat. no 0528), by incubation at room temperature for 20 minutes. Stain was then washed off with nanopure water and fresh Q-tip was used to remove any unmigrated cells from inside the inner chamber and pictures of the migrated cells were taken and counted using ImageJ 1.53 software.

**Statistical Analysis**

Experiments were performed in triplicate and results were analyzed with mean and standard deviation. One-way ANOVA test was used for all statistical analyses involving more than two groups. This test was done in conjunction with the Tukey posttest to identify significance

between any two of the groups involved in the experiment. Unpaired t-test was conducted in conjunction with the Welch's test for any experiments or data that involved only two groups. Welch's test was used to confirm if the variances from the two groups were from the same populations. GraphPad Prism 9.3.0 software (GraphPad Software Inc.) was utilized to perform all statistical analyses. $P<0.05$ was considered to be statistically significant.

Results

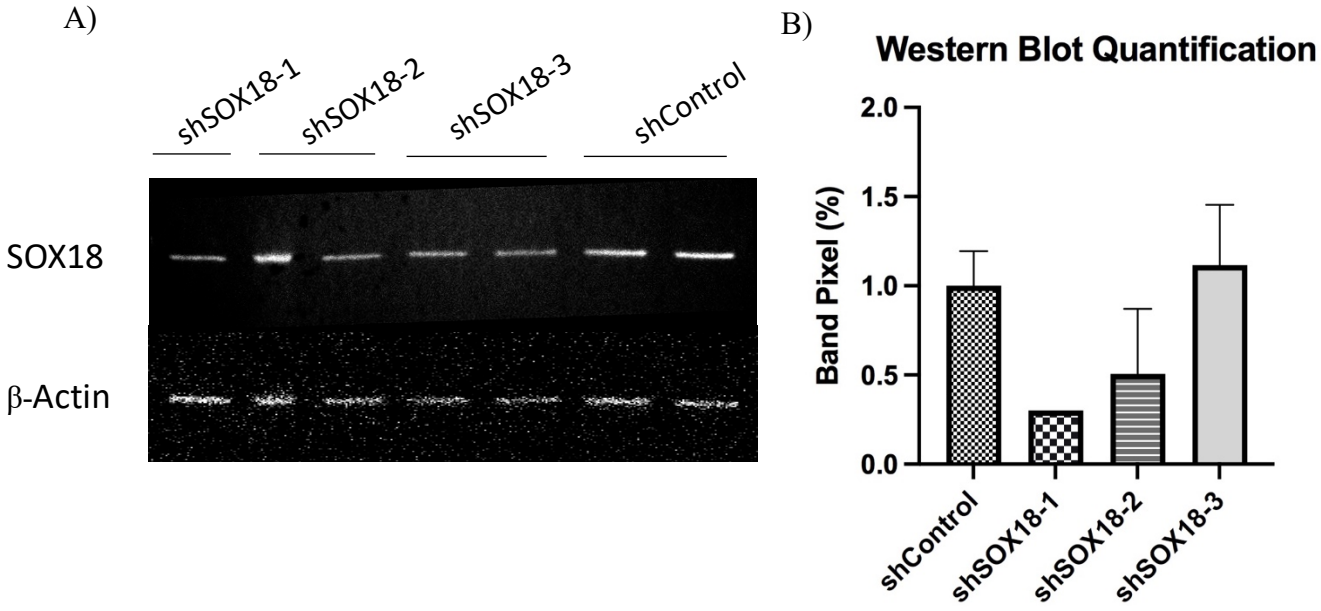


**Figure 1.** A) Qualitative figure of western blot bands indicating expression of SOX18 in the knockdown cell lines (shSOX18-1, -2 and -3) and the shControl. B) Quanitative figure of western blot bands indicating expression of SOX18 in the knockdown cell lines (shSOX18-1, -2 and -3) and the shControl. Values are expressed as means ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p<0.0001$.

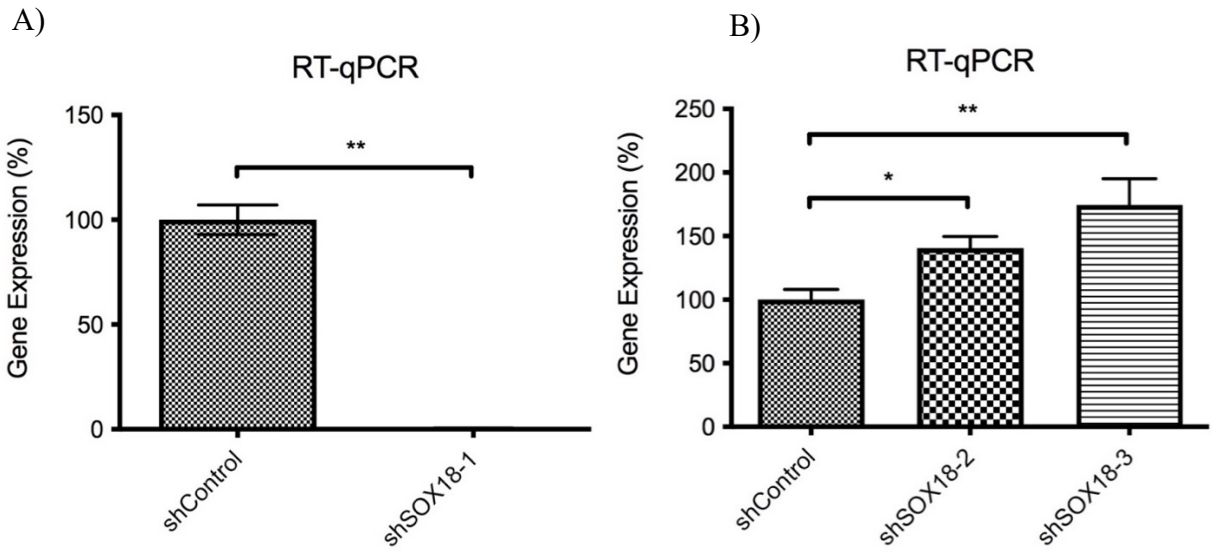


**Figure 2.** A) RT-qPCR indicating significantly decreased gene expression confirming knockdown of SOX18 by shRNA-1 in the ES8 cell line. B) RT-qPCR indicating significantly increased gene expression after transfection of shSOX18-2 and shSOX18-3 in ES8 cell line. Values are expressed as means ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p<0.0001$.

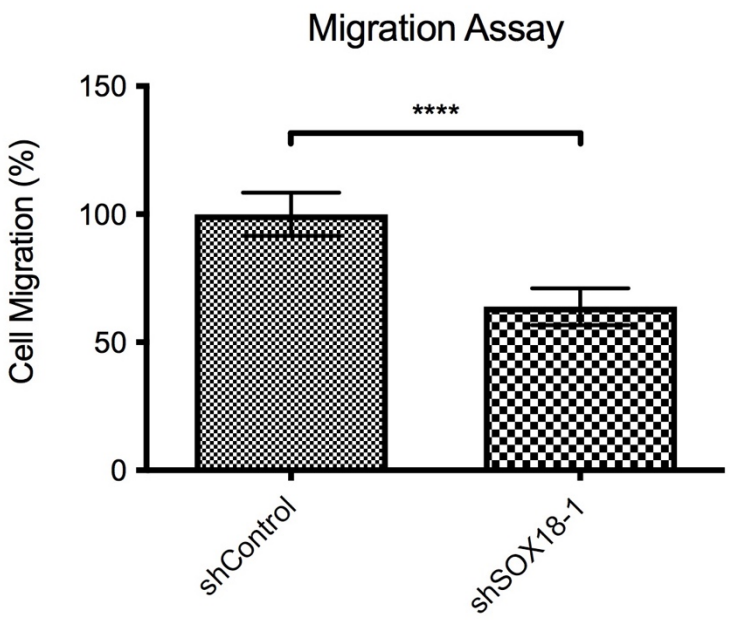


**Figure 3.** Migration Assay indicating significantly decreased cell migration after knockdown of SOX18 by shSOX18-1 in ES8 cell line. Values are expressed as means ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p<0.0001$.

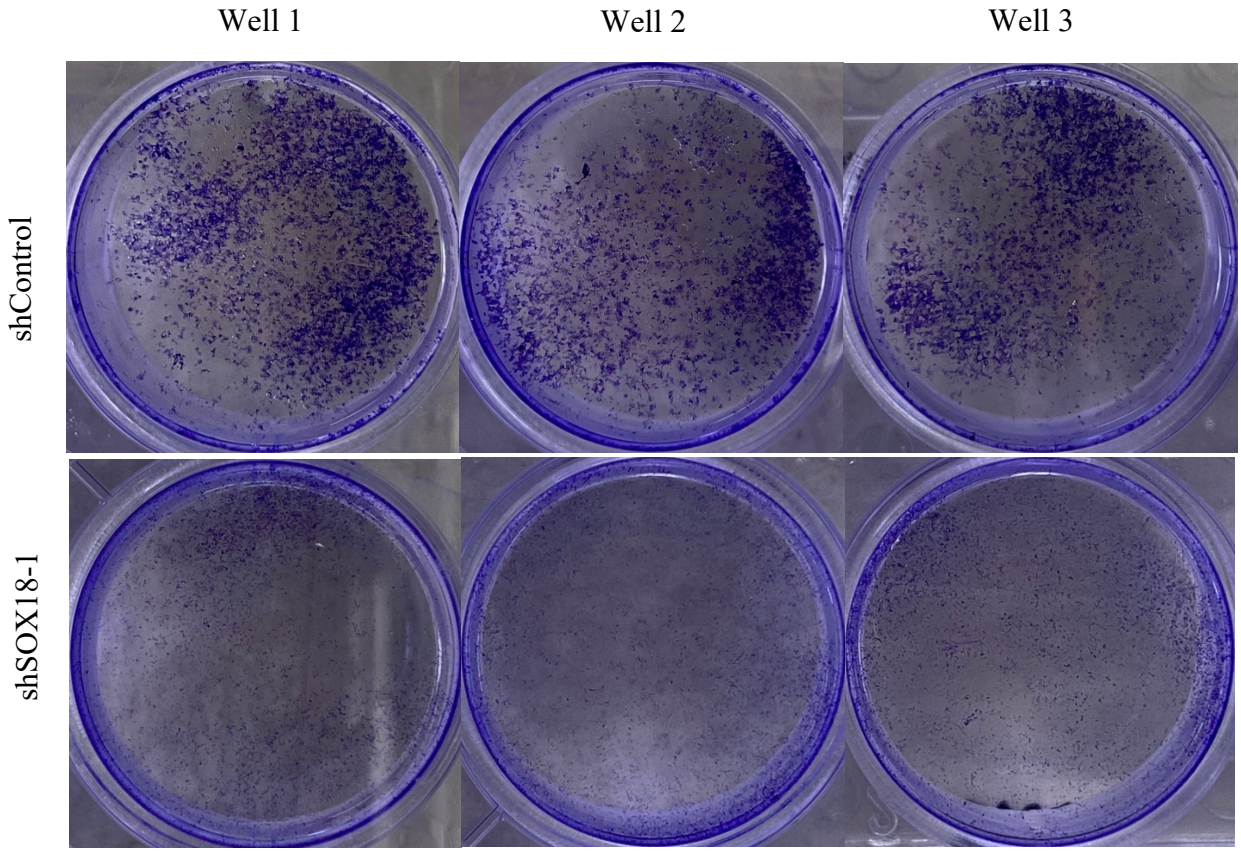


**Figure 4.** Decreased cell survival and colony formation in colony formation assay of ES8 cells with SOX18 knockdown by shSOX18-1 in comparison with shControl. Values are expressed as means ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p<0.0001$.

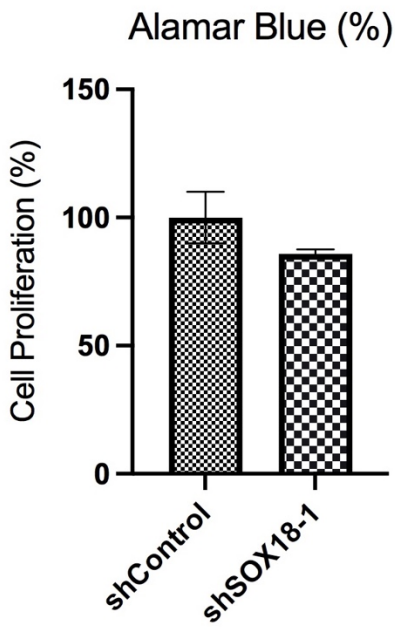


**Figure 5.** Cell Proliferation assay indicating observable decreased proliferation after knockdown of SOX18 by shSOX18-1 in ES8 cell line in comparison with shControl. Values are expressed as means ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p<0.0001$.

Discussion

In order to characterize SOX18, it was necessary to create a stable cell line that had the gene for SOX18 knocked down allowing for decreased expression of the transcription factor itself. Thus, the effects of decreased expression could be studied to understand the normal function and characteristics of SOX18 in cells. In this project, SOX18 was knocked down by spinfection in the ES8 cells and SOX18 in these cells was characterized. In order to confirm whether the knockdown worked RT-qPCR and western blotting was done. Western blotting was done initially to get an idea of which shRNA, shSOX18-1, -2 or-3, worked best. These observations were later confirmed using quantification of the western blot bands as well as RT-qPCR as seen in Figures 1 and 2.

Looking at the western blot analysis it can be observed that the shSOX18-1 band is lighter than that of the shControl bands. This can also be seen in the second shSOX18-2 band and both shSOX18-3 bands (Figure 1A). To further analyze these bands, they were quantified using ImageJ. After quantification, there was an observable decrease in SOX18 expression in ES8 cells transfected with shSOX18-1 and -2, but not for shSOX18-3. However, the decrease was not statistically significant (Figure 1B). According to the RT-qPCR conducted there was a significant decrease in the expression of SOX18 in the cells transfected with the shSOX18-1 in comparison with the shControl. There was almost complete absence of expression (Figure 2A). Through t-test and Welch's posttest it was identified that the difference between expression had p-value<0.01. This also confirmed the observable qualitative and quantitative results from the western blotting analysis. Interestingly, shSOX18-2 and -3 didn't show any decrease in the SOX18 expression (Figure 2B). In fact, it seemed that there was actually an increase in the expression of SOX18 which was statistically significant (Figure 2B).

Based on these baseline measurements, the ES8 cells treated with shSOX18-1 were selected for further experimentation and stable cell line was maintained, while other cells were frozen and stored. In order to characterize the effects of SOX18 in cancer cells, tumorigenic properties such as proliferation, migration and colony formation were studied. These assays were performed on the ES8 SOX18 knockdown cell line selected and studied in comparison with the shControl cells.

The first of these properties that was studied was cell migration via a cell migration assay. This assay is different from invasion assay which includes Matrigel permeance, whereas in the migration assay cells must simply cross a membrane from serum-free media to FBS-containing media. In cancer development and progression, migration/invasion and metastasis

allow a primary tumor to spread throughout the body via circulatory or lymphatic systems. Thus, distant tissues are able to be colonized having further detrimental effects (Pijuan et al, 2019). For this reason, understanding whether SOX18 plays an important role in Ewing sarcoma cancer cell's ability to migrate is essential to identifying it as a target for cancer therapy.

There was a decrease in cell migration of cells with SOX18 knockdown in comparison with the control cells. In fact, this decrease was statistically significant as indicated by the t-test and Welch's posttest with a p-value<0.0001 (Figure 3). Only 63.9% of cells with the knockdown migrated in comparison with control set at 100%. This indicates that SOX18 could play a major role in the cancer cell's ability to migrate in vitro. Further studies to analyze if these results are observed in invasion could form a more complete picture of SOX18's role in cell migration and invasion.

The next tumorigenic property that was studied was colony formation. This property was studied by plating very few cells on 6-well dish and later analyzing the ES8 cell's ability to form colonies with little to no cell-to-cell communication. Generally, clonogenic or colony formation assays are performed as an indicator of cell survival. Cancer cells have an impressive ability to maintain survival and thus by studying colony formation SOX18's role in cell survival can be studied (Franken et al, 2006).

There was obvious decreased cell survival in the knockdown cells in comparison with the control cells. As seen in Figure 4, the top three wells indicate triplicates of the shControl cells, where there is increased cell survival as indication by the staining. The bottom three wells are the triplicates of the shSOX18-1 knockdown cells where there is decreased staining and thus decreased cell survival. Interestingly, neither the control nor the knockdown cells formed colonies after cell plating and 36-hour incubation (Figure 4).

Cell Proliferation was the final tumorigenic property of ES8 cells studied. Cell proliferation, like colony formation, is a commonly studied property that is an indicator of cell survival. Cell proliferation is central to the cancer cell survival as they often have dysregulated, or unregulated cell growth and division caused by malfunctioning of a variety of genes. One of these genes is possibly SOX18. As seen in Figure 5, there was an observable decrease (85.8% of the control cell proliferation count) in the ES8 cell's ability to proliferate, however the decrease was not statistically significant, as indicated by the t-test and Welch's posttest. Thus, it can be concluded that SOX18 may not play a vital role in cell proliferation, however it does seem to have an effect on it causing the observable decrease.

## Conclusion

These results taken together show promise that SOX18 may be a player in various tumorigenic properties in Ewing Sarcoma cells. Specifically, it was shown that there is a statistically significant decrease in migration in the SOX18 knockdown cells in comparison with control cells, and an observable decrease in colony formation and cell proliferation. Future studies may focus on identifying whether these results and characterization of SOX18 is conserved in other pediatric sarcomas such as Rhabdomyosarcoma.

Research has only begun to explore the therapeutic potential of SOX18, thus there is much more to be learned. Since all cancers have a basic similarity in terms tumor mechanism it can be assumed that SOX18 is relevant for certain tumorigenic properties seen in multiple cancer types, however further and more detailed studies could allow for specific results. Additionally, further research could be conducted on identifying more FDA-approved drugs that could also target SOX18 and/or other molecules in its cascade pathways for further treatment.

Resources


1. Angelozzi, M., & Lefebvre, V. 2019. SOXopathies: Growing Family of Developmental Disorders Due to SOX Mutations. *Trends in genetics: TIG*, *35*(9), 658–671. https://doi.org/10.1016/j.tig.2019.06.003
2. Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: a cancer journal for clinicians*, *68*(6), 394–424. https://doi.org/10.3322/caac.21492
3. Brown, A., Shao, S., Murray, J., Hegde, R. S., & Ramakrishnan, V. 2015. Structural basis for stop codon recognition in eukaryotes. *Nature*, *524*(7566), 493–496. https://doi.org/10.1038/nature14896
4. Casey, D. L., Lin, T. Y., & Cheung, N. V. 2019. Exploiting Signaling Pathways and Immune Targets Beyond the Standard of Care for Ewing Sarcoma. Frontiers in oncology, 9, 537. https://doi.org/10.3389/fonc.2019.00537
5. Coulie, R., Niyazov, D. M., Gambello, M. J., Fastré, E., Brouillard, P., & Vikkula, M. 2021. Hypotrichosis-lymphedema-telangiectasia syndrome: Report of ileal atresia associated with a SOX18 de novo pathogenic variant and review of the phenotypic spectrum. American journal of medical genetics. Part A, *185*(7), 2153–2159. https://doi.org/10.1002/ajmg.a.62205
6. Franken, N., Rodermond, H., Stap, J. et al. 2006. Clonogenic assay of cells in vitro. Nat Protoc **1,** 2315–2319. https://doi.org/10.1038/nprot.2006.339

7. Griffiths, A. J. F., Gelbart, W. M., Miller, J. H., et al. 1999. Modern Genetic Analysis. New York: W. H. Freeman. Chromosomal Rearrangements https://www.ncbi.nlm.nih.gov/books/NBK21367/
8. Grimm, D., Bauer, J., Wise, P., Krüger, M., Simonsen, U., Wehland, M., Infanger, M., & Corydon, T. J. (2020). The role of SOX family members in solid tumours and metastasis. *Seminars in cancer biology*, *67*(Pt 1), 122–153. https://doi.org/10.1016/j.semcancer.2019.03.004
9. Gurria, J. P., & Dasgupta, R. 2018. Rhabdomyosarcoma and Extraosseous Ewing Sarcoma. Children (Basel, Switzerland*)*, 5(12), 165. https://doi.org/10.3390/children5120165
10. Hanahan, D., & Weinberg, R. A. 2011. Hallmarks of cancer: the next generation. Cell, 144(5), 646–674. https://doi.org/10.1016/j.cell.2011.02.013
11. Irrthum, A., Devriendt, K., Chitayat, D., Matthijs, G., Glade, C., Steijlen, P. M., Fryns, J. P., Van Steensel, M. A., & Vikkula, M. 2003. Mutations in the transcription factor gene SOX18 underlie recessive and dominant forms of hypotrichosis-lymphedema-telangiectasia. American journal of human genetics, *72*(6), 1470–1478. https://doi.org/10.1086/375614
12. Kamachi, Y., & Kondoh, H. 2013. Sox proteins: regulators of cell fate specification and differentiation. Development (Cambridge, England), 140(20), 4129–4144. https://doi.org/10.1242/dev.091793
13. McCann, A. J., Lou, J., Moustaqil, M., Graus, M. S., Blum, A., Fontaine, F., Liu, H., Luu, W., Rudolffi-Soto, P., Koopman, P., Sierecki, E., Gambin, Y., Meunier, F. A., Liu, Z., Hinde, E., Francois, M. 2021. A dominant-negative SOX18 mutant disrupts multiple

regulatory layers essential to transcription factor activity. Nucleic Acids Research; gkab820, https://doi.org/10.1093/nar/gkab820

14. Overman, J., Fontaine, F., Wylie-Sears, J., Moustaqil, M., Huang, L., Meurer, M., Chiang, I. K., Lesieur, E., Patel, J., Zuegg, J., Pasquier, E., Sierecki, E., Gambin, Y., Hamdan, M., Khosrotehrani, K., Andelfinger, G., Bischoff, J., & Francois, M. 2019. R-propranolol is a small molecule inhibitor of the SOX18 transcription factor in a rare vascular syndrome and hemangioma. eLife, *8*. https://doi.org/10.7554/eLife.43026
15. PDQ Pediatric Treatment Editorial Board. Ewing Sarcoma and Undifferentiated Small Round Cell Sarcomas of Bone and Soft Tissue Treatment (PDQ®): Health Professional Version. 2021. In: PDQ Cancer Information Summaries [Internet]. Bethesda (MD): National Cancer Institute (US); 2002-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK66045/
16. Petrovic, I., Milivojevic, M., Popovic, J., Schwirtlich, M., Rankovic, B., & Stevanovic, M. 2015. SOX18 Is a Novel Target Gene of Hedgehog Signaling in Cervical Carcinoma Cell Lines. PloS one, 10(11). https://doi.org/10.1371/journal.pone.0143591
17. Pijuan, J., Barceló, C., Moreno, D. F., Maiques, O., Sisó, P., Marti, R. M., Macià, A., & Panosa, A. 2019. In vitro Cell Migration, Invasion, and Adhesion Assays: From Cell Imaging to Data Analysis. Frontiers in cell and developmental biology, *7*, 107. https://doi.org/10.3389/fcell.2019.00107
18. Siegel, R. L., Miller, K. D., Fuchs, H. E., & Jemal, A. 2021. Cancer Statistics, 2021. *CA: a cancer journal for clinicians*, *71*(1), 7–33. https://doi.org/10.3322/caac.21654
19. Siegel, D. A., Richardson, L. C., Henley, S. J., Wilson, R. J., Dowling, N. F., Weir, H. K., Tai, E. W., & Buchanan Lunsford, N. 2020. Pediatric cancer mortality and survival in

the United States, 2001-2016. Cancer, 126(19), 4379–4389. https://doi.org/10.1002/cncr.33080

20. Torella, A., Zanobio, M., Zeuli, R., Del Vecchio Blanco, F., Savarese, M., Giugliano, T., Garofalo, A., Piluso, G., Politano, L., & Nigro, V. 2020. The position of nonsense mutations can predict the phenotype severity: A survey on the DMD gene. PloS one, *15*(8), e0237803. https://doi.org/10.1371/journal.pone.0237803
21. Wang C. 2012. Childhood rhabdomyosarcoma: recent advances and prospective views. Journal of dental research, 91(4), 341–350. https://doi.org/10.1177/0022034511421490
22. Wang, G., Wei, Z., Jia, H., Zhao, W., Yang, G., & Zhao, H. 2015. Knockdown of SOX18 inhibits the proliferation, migration and invasion of hepatocellular carcinoma cells. Oncology reports, 34(3), 1121–1128. https://doi.org/10.3892/or.2015.4112
23. Wu, Z., Liu, J., Wang, J., & Zhang, F. 2016. SOX18 knockdown suppresses the proliferation and metastasis and induces the apoptosis of osteosarcoma cells. *Molecular medicine reports*, *13*(1), 497–504. https://doi.org/10.3892/mmr.2015.4541
24. Zhang, J., Ma, Y., Wang, S., Chen, F., & Gu, Y. 2016. Suppression of SOX18 by siRNA inhibits cell growth and invasion of breast cancer cells. Oncology reports, *35*(6), 3721–3727. https://doi.org/10.3892/or.2016.4746
25. Zhou, X., Iversen, E. S., Jr, & Parmigiani, G. 2005. Classification of Missense Mutations of Disease Genes. Journal of the American Statistical Association, 100(469), 51–60. https://doi.org/10.1198/016214504000001817