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DNA MUTATION ANALYSIS OF THE VH4 INTRON LOCUS IN SMURF2 KNOCKOUT MICE

A Major Qualifying Project Report

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in

Biology and Biotechnology

by

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Abstract

The Smurf2 gene in mice encodes a protein that mediates ubiquination and degradation of a germinal center transcription factor responsible for B-cell proliferation. Knockouts of the Smurf2 gene have been shown in our lab to increase the formation of Diffuse Large B-Cell Lymphomas. The purpose of this project was to clone and sequence the VH4 intron locus in the DNA from the germinal centers of spleens from Smurf2-KO mice to determine whether they have an increased DNA mutation rate prior to tumor formation. The cloning of the DNA was unsuccessful, indicating a need to conduct further experiments.

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Acknowledgements

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Background

Diffuse Large B-Cell Lymphoma

Diffuse Large B-Cell Lymphoma is the most common type of Non-Hodgkin Lymphoma, comprising about thirty percent of all cases of the disease (Morton et al., 2006). This type of lymphoma affects the B-cells of a patient's blood, and is an aggressive, fast growing tumor. There are multiple forms of this cancer, most of which are determined by where the tumors are located in the body, and which other cells are involved. Although treatment has greatly improved, around fifty percent of patients do not respond to current chemotherapy treatments, or they relapse after treatment (Shipp et al., 2002). The cause of this lymphoma is still not known (Ramkumar et al., 2013). Knowing the cause would help determine therapeutic targets to form more effective treatments.

The Zhang Laboratory

Recently, the Zhang laboratory at the University of Massachusetts Medical School has found that the Smurf2 gene plays a role in the regulation of B-cell proliferation, and in the development of diffuse large B-cell lymphoma. Smurf2 is an E3 ubiquitin ligase protein which mediates the degradation of proteins associated with tumor formation (Ramkumar et al., 2012). Using Smurf2-knockout mice, the Zhang laboratory demonstrated that Smurf2 deficiency decreased the expression of p16, a known tumor suppressor. In addition, the Zhang laboratory found that when Smurf2 was suppressed, by either complete knockout or heterozygous knockout, mouse embryonic fibroblasts

showed an impaired senescence response and became immortalized (Ramkumar et al., 2012) (**Figure 1**).

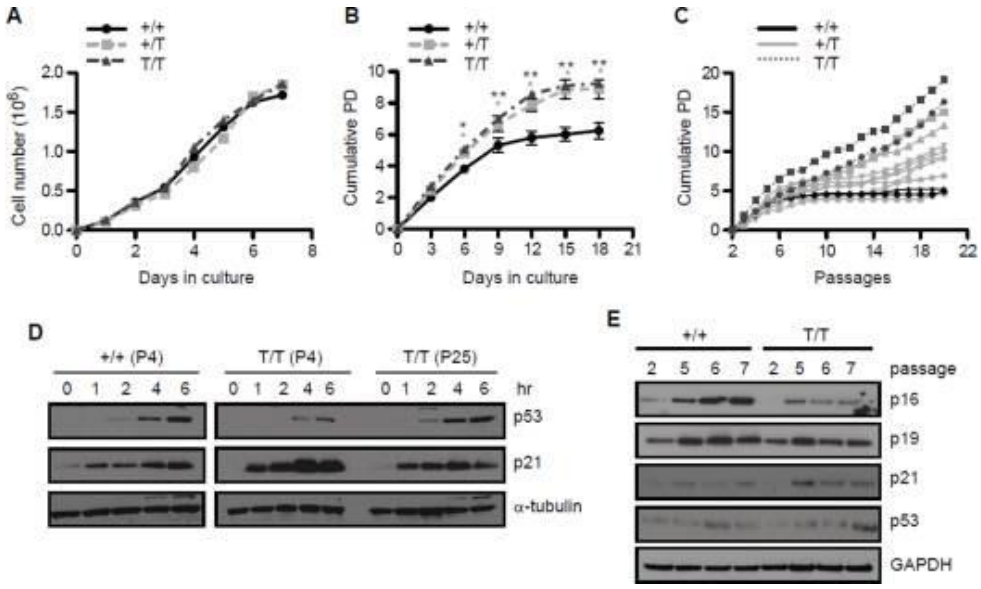

Figure-1: Impaired Senescence in Smurf2-Deficient MEF's. Panels B and C show that mouse embryo fibroblast cells lacking Smurf2 (T/T or +/T) show increased population doublings compared to WT cells. Panels D and F show that cells lacking Smurf2 have an increased expression of p21 and a decreased expression of p16 tumor suppressor (Ramkumar et al., 2012)

Using the same knockout mice, the Zhang lab also showed that Smurf2-deficient mice show splenomegaly and develop tumors spontaneously at a far greater rate than WT mice (**Figure-2**). While the WT mice showed no tumor formation over 20 weeks, the heterozygous Smurf2 knockout mice showed a 23.8% tumor development, and the homozygous Smurf2 knockout mice showed a 30.6% rate of tumor development. Of the tumors that formed, 72.7% were lymphomas (Ramkumar et al., 2012).

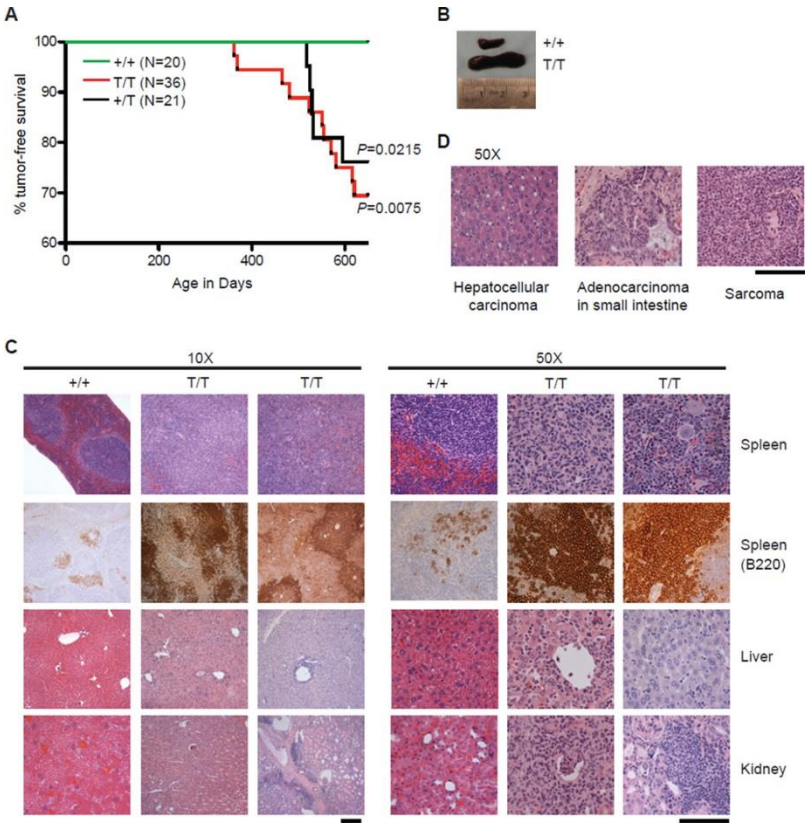

Figure-2: Increased Spontaneous Tumor Formation in Smurf2-Deficient Mice. Panel A shows the tumor free survival rates of WT (+/+), heterozygous Smurf2-KO (+/T) and homozygous Smurf2-KO (T/T) mice. Only the WT mice remain tumor-free. Panel B shows the spleen size of T/T and age-matched WT mice, the T/T mice show splenomegaly. Panel C shows H&E staining and B220 staining of lymphomas in spleen, liver, and kidneys of T/T and WT +/+ mice (Ramkumar et al., 2012).

The Smurf2 knockout was also found to inhibit spleen senescence (**Figure-3**). In premalignant 12 week old mice, the senescence response was inhibited in Smurf2 deficient mice compared to the same age wild type mice. It was also found that p16, an important tumor suppressor, was decreased in Smurf2-deficient mice, while protein Id1 was increased. Thus, Smurf2 plays a role in senescence through the Smurf2-Id1-p16

tumor suppressor pathway to induce senescence, which could explain why Smurf2 deficiency promotes spontaneous tumor formation (Ramkumar et al., 2012).

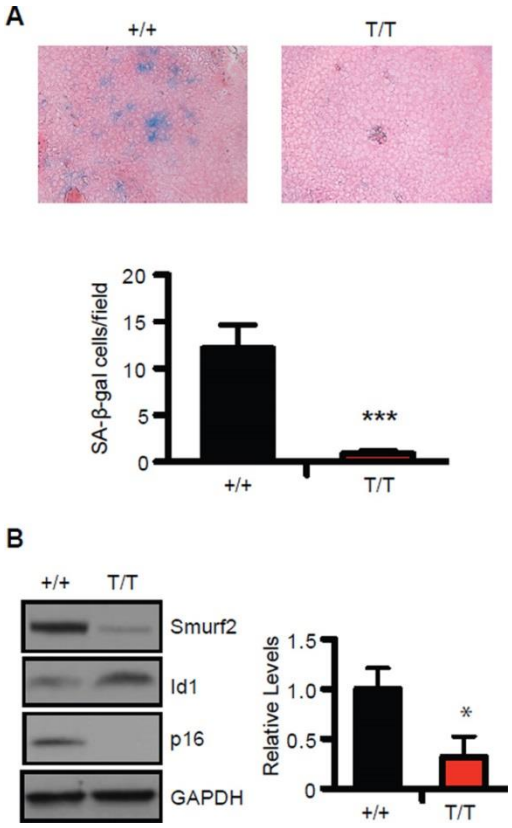

Figure-3: Impaired Senescence Response in Spleens of Aged Smurf2-Deficient Mice. Panel A shows SA- β-gal activity of WT (+/+) and homozygous Smurf2-KO (T/T) mice, indicating a decrease in senescence in T/T mice. Panel B shows Western and RT-PCR analyses of p16 levels in WT (+/+) and Smurf2-KO (T/T) mice, showing a decrease in p16 levels in KO mice (Ramkumar et al., 2012).

In their next paper (Ramkumar et al., 2013), the Zhang laboratory further examined the role of Smurf2 in the development of diffuse large B-cell lymphoma. In this study, they demonstrated that Smurf2 is also involved in the YY1-c-Myc pathway. YY1 is a germinal center transcription factor that trans-activates the transcription of c-Myc (Ramkumar et al., 2013). c-Myc is an oncogene that is critical for human development. It is also known that c-Myc plays a crucial role in the cell cycle,

prolonging the doubling time of rat fibroblast cells when removed (Dang, 1999). c-Myc is known to play a large role in the development of cancer, with an estimated 70,000 cancer deaths per year associated with c-Myc mutations or changes in c-Myc expression (Dang, 1999).

In their experiment, the Zhang laboratory showed that Smurf2 regulated the stability of YY1, showing that Smurf2 is responsible for mediating the ubiquination and degradation of YY1 (**Figure-4**). They demonstrate that Smurf2-deficient

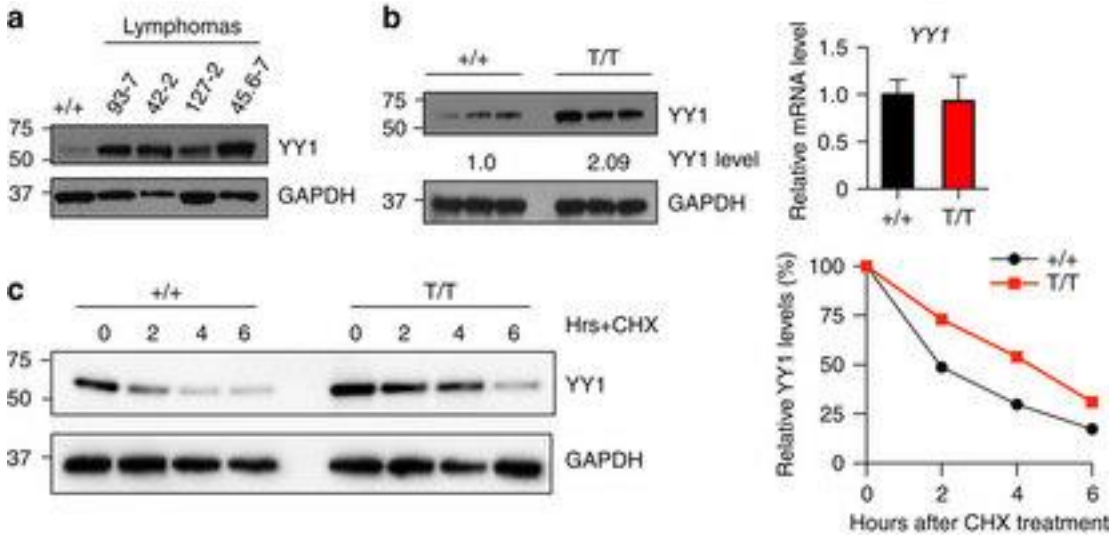

Figure-4: Smurf2 Regulates the Stability of Protein YY1. Panel A shows the expression of YY1 transcription factor in WT (+/+) spleens compared to age-matched lymphomas using Western analysis. YY1 is increased in the lymphomas. Panel B shows the expression of YY1 in spleens of 2-month old WT mice and Smurf2-KO (T/T) mice. YY1 increases in the KO mice (Ramkumar et al., 2013).

To further show that Smurf2 plays a crucial role in the YY1-c-Myc pathway, the Zhang lab examined the c-Myc levels in Smurf2-deficient mice compared to wild type mice. Their results (**Figure-5**) showed that Smurf2-deficient mice with increased levels

of YY1 also show increased levels of c-Myc. Thus, there is a direct correlation between Smurf2 expression and the expression of oncogene c-Myc, which is mediated by the Smurf2-YY1-c-Myc pathway (Ramkumar et al., 2013).

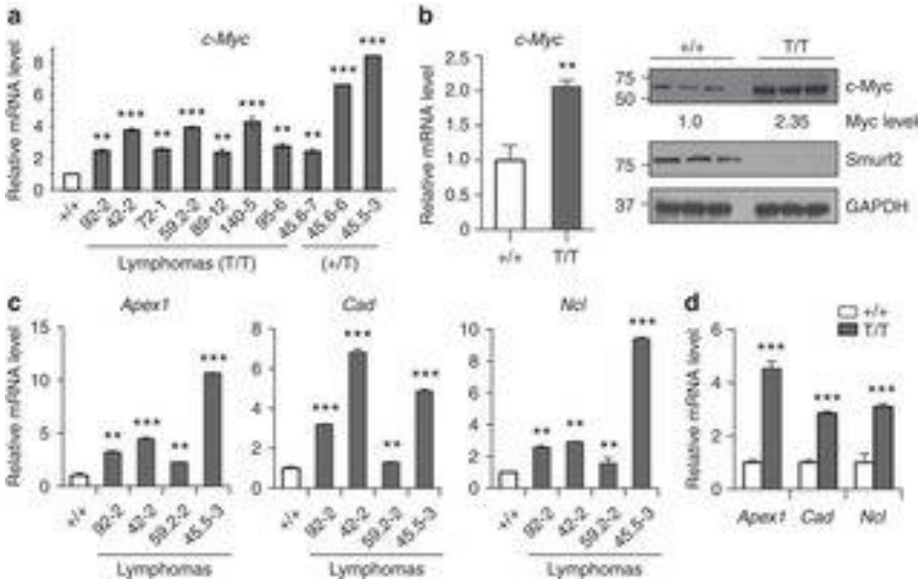

Figure-5: Elevated c-Myc Expression in Smurf2-Deficient Mice. Panel A shows that lymphomas in Smurf2-KO (T/T) and (+/T) mice demonstrate an increased expression of c-Myc compared to aged WT (+/+) mice. Panel B shows that c-Myc expression is increased in the spleens of 2-month old T/T mice compared to WT (+/+) mice (Ramkumar et al., 2013).

Having established that the Smurf2-YY1 pathway helps regulate the expression of the oncogene c-Myc, the Zhang laboratory then examined human diffuse large B-cell lymphoma tumors for Smurf2 expression (**Figure-6**). They found that Smurf2 expression directly correlates with patient survival probability. In particular, they found that decreased levels of Smurf2 expression in human tumors correlated to a poorer survival prognosis (Ramkumar et al., 2013).

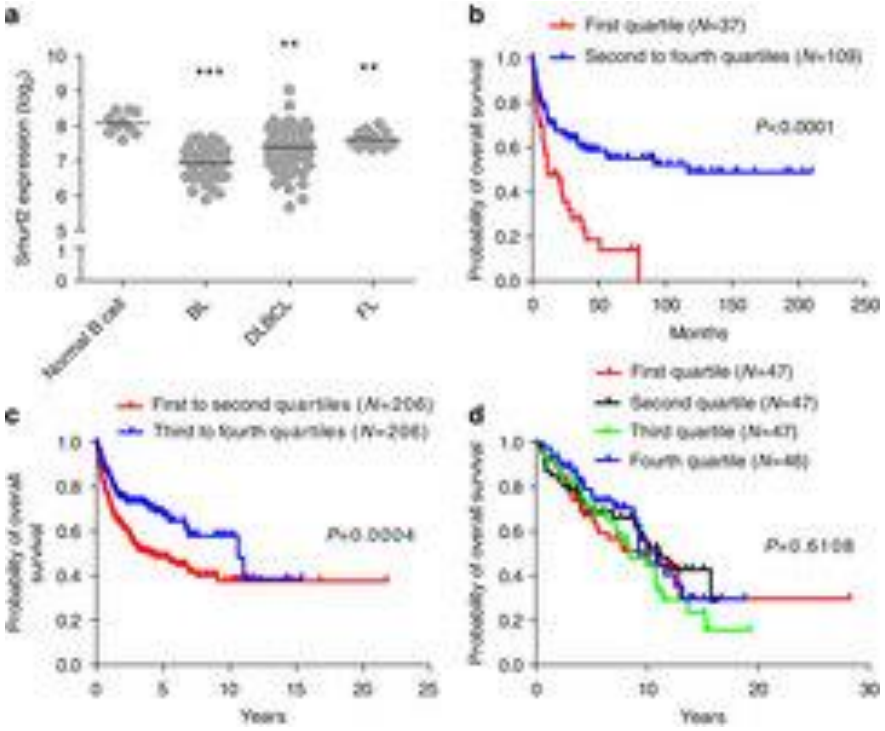

Figure-6: Smurf2 Expression Correlates With Overall Survival of Human DLBCL Patients. Panel A shows that Smurf2 expression in human lymphomas is decreased when compared to normal B cells. Panels B-D show that in human diffuse large B-cell lymphoma tumors, when Smurf2 expression is low, the patient survival prognosis is poor (Ramkumar et al., 2013).

Altogether, the Zhang laboratory's findings demonstrate that the loss of Smurf2 plays a crucial role in the development of diffuse large b-cell lymphomas, playing two roles in tumor suppression: 1) increasing senescence through the Smurf2-Id1-p16 pathway, and 2) suppressing oncogene c-Myc through the degradation of YY1 using the Smurf2-YY1-c-Myc pathway, which can result in the spontaneous development of diffuse large B-cell lymphomas.

The VH4 Family

The variable heavy-4 (VH4 or V_H4) gene family encodes a portion of the variable heavy chain present in the immunoglobulin gene. Variable heavy, and variable light (V_L) chains are formed through recombination of the V (D) and J gene segments, as well as random pairing of the heavy and light chains (Bende et al., 2002). Additional variability is added through class switch recombination and through somatic hyper-mutation. All of these gene rearrangements and mutations occur so that during the course of a normal humoral response, specific antibodies can become selected for their affinity to a pathogen (Schrader et al., 2013). In the V regions of the Ig genes, however, somatic hyper-mutation is the most likely cause of diversity. The VH4 intron is the upstream leader primer of the VH region (**Figure-7**). The VH region is also known to be associated with multiple forms of lymphoma. In particular, the IgVh region has been shown to be involved in two V4-34-expressing lymphomas, a follicular lymphoma, and a V4-34 expressing diffuse large B-cell lymphoma (Bende et al., 2002). Because mutations in the VH4 intron locus have previously been associated with diffuse large B-cell lymphomas, we chose to analyze whether mutations in this locus might be induced by Smurf-2 knockout in mice.

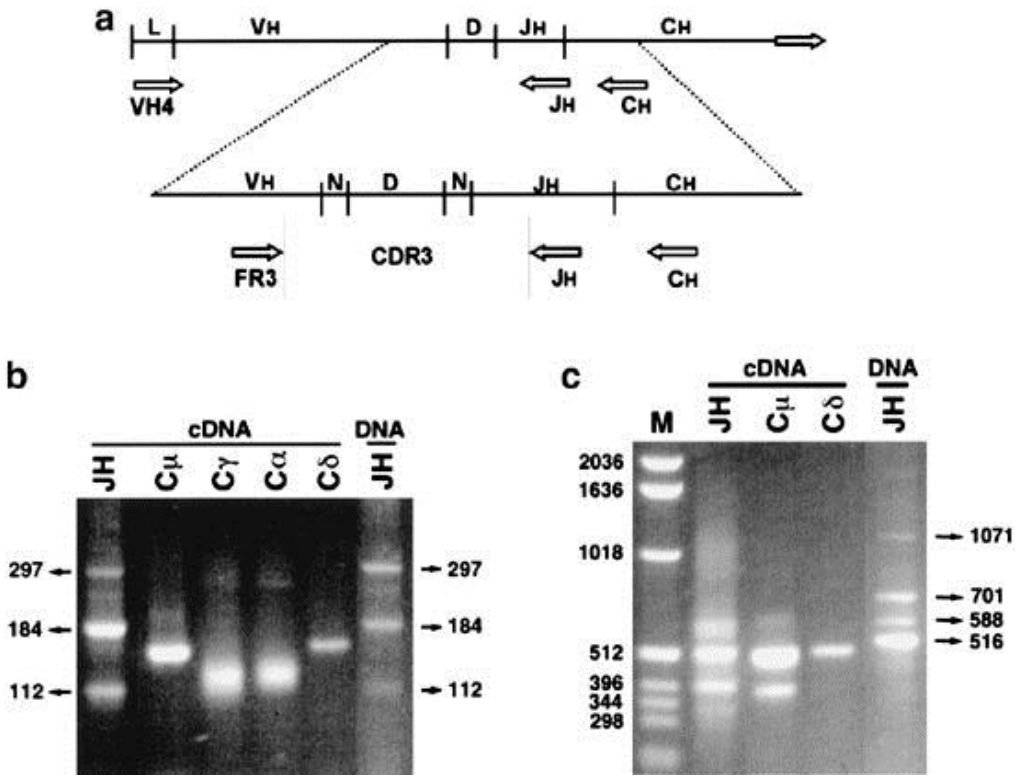

Figure-7: The IgH Locus. Panel A shows the IgH locus, and the VH region of this locus. The VH4 intron (white arrow, upper left side) can be seen as the lead primer on the upstream end of the VH region. (Bende et al., 2002)

Project Purpose

The purpose of this project was to clone and sequence the VH4 Intron portion of spleen germinal center cells in Smurf-2 knockout mice to determine whether mice lacking Smurf2 show increased mutation rates at this locus prior to tumor formation. Mutations in the VH4 locus (Bende et al., 2002) and decreased levels of Smurf2 (Ramkumar et al., 2013) have both previously been associated with the formation of diffuse large B-cell lymphomas. The VH4 intron is the lead upstream primer to the variable heavy region of the IgH locus. In this position, mutations to the VH4 locus could disrupt transcription of the variable heavy region of the IgH locus, or could disrupt the effects of somatic hyper-mutation on this region.

The germinal center B-cells within the spleen will be isolated by FACS sorting for cellular markers B220+, CD95+, and GL7+ which are markers for germinal center B-cells (Schader et al., 2013). Genomic DNA will be isolated from these cells in WT and in Smurf2-KO mice before and after tumor formation, and then PCR will be used to amplify a 600 bp amplicon of the VH4 intron locus. The amplicons will be d3' tailed, cloned into a TOPO plasmid, and sequenced.

Methods

Fluorescent Activated Cell Sorting

Wild type and Smurf2 knock mice were provided by the Zhang lab. The spleens from six week old un-immunized WT and Smurf2-knockout mice were minced using the rough edges of two Fisherbrand slides. The crushed spleens were then mixed with staining reagent, and centrifuged for 6 minutes at 1300 RPM. This was aspirated to 100 uL, and vortexed. To lyse the red blood cells, 9.0 mL of cold Tcd water was added to the pellet, and mixed well. 1.0 mL of 10X PBS was then added, and mixed well. SM was then added to the mixture, filling to 13 mL. Using a nylon mesh filter, tissue and dead cell debris were removed from the mixture. Around 1 mL of newborn calf serum was then applied to make a layer on the bottom of the tube. This was then spun for 6 minutes at 1300 RPM, and the supernatant was aspirated off. The cells were then re-suspended in 10 mL of SM buffer, and subjected to FACS analysis at the University of Massachusetts Medical School (UMMS), selecting for cellular markers B220+, CD95+, and GL7+ which are markers for Germinal Center B-Cells (Schader et al., 2013).

Isolation of Genomic DNA

To isolate genomic DNA, the cell pellet was re-suspended in 100 uL STE buffer (0.1M NaCl, 20 mM Tris pH 8.0, 1mM EDTA), 5 uL proteinase K, and 2.5 uL 20% SDS. This was placed at 55°C for 2 hours. This was then cooled, spun down quickly, and a mixture of 15 uL 3M sodium acetate at pH 5.2 and 450 uL of 95% EtOH was added. This was left at -20°C overnight, and then spun at 14,000 RPM, at 4°C for 30 minutes. The

EtOH was poured off, and 500 uL 70% EtOH was added. This was then spun down at 14,000 RPM at 4°C for 10 minutes, and allowed to air dry for around 5 minutes. The pellet was then re-suspended in 20 uL TE-4 (10 mM Tris pH 7.4, 0.1 mM EDTA).

Nested PCR of FACS Product

Following the isolation of genomic DNA, a nested PCR was performed to amplify a 600 base pair amplicon in the VH4 intron DNA. The first amplification used the forward primer 5' AGCCTGACAT-ACTGAGGAG and the reverse primer 5'GTGTTCCTTT-GAAAGCTGGAC. Phusion (New England Biolabs Inc.) was used as the polymerase instead of Taq, due to its higher fidelity, and Phusion buffer, dNTP's, and the genomic DNA. Water was used as a control. The reaction conditions were 95°C for 30 seconds, 57°C for 30 seconds, 72°C for 1 minute, and this was repeated for 30 cycles. The second PCR amplification used the same reagents, but the forward primer was 5' CCGGAATTCCTGACATCTGAGGACTCTGC, and the reverse primer was 5' GATGCCTTTCTCCCTTGACTC. The reaction conditions were also the same as the first amplification, but run for 35 cycles instead of 30 (Schrader et al., 2013).

Gel Electrophoresis of PCR Amplicons and Band Extraction

The PCR products of the second PCR were electrophoresed in a 1% agarose gel. The PCR reactions were loaded into the gel using a blue loading dye, and were run at 70 volts for about one hour. Following electrophoresis, the bands at the 600 base pair range for each lane were removed using a scalpel. They were placed into previously weighed tubes, and were then weighed again to determine the weight of the extracted band. After

this, gel extraction was performed in order to remove the DNA from the agarose using the Qiagen QIAquick Gel Extraction Kit. The gel was first dissolved using buffer QG in a 50°C water bath. After the gel was completely dissolved, isopropanol was added to the sample and mixed. This mixture was then added to the QIAquick spin column, where the DNA absorbs to the silica gel of the column, and contaminants pass through the column. The column wash was spun down, and QG buffer was added once more, and spun down again. Buffer PE was then added, and the column was spun down twice. Elution Buffer EB was then added, and the column was spun down to elute the purified amplicon DNA from the QIAquick spin column.

dA Tail Extension

After the gel extraction of amplicon DNA, a 3' dA extension was performed to add a short adenosine tail to the DNA to facilitate cloning. To perform this, each gel extraction product was mixed with 1 µl of Taq, and 1 µl of dNTPs. The reaction conditions used for this extension were 72°C for 10 minutes, and then cooled to 4°C for 2 minutes. Following the extension, the DNA with a 3' dA tail was purified again using the Qiagen QIAquick gel extension kit. The same protocol was used as before, skipping the initial dissolving of the gel in 50°C water.

TOPO Cloning

Following purification of the dA-tailed amplicon, the DNA was cloned into a TOPO plasmid to facilitate sequencing using the Invitrogen TOPO TA cloning kit. 4.0 µl

of the amplicon DNA was added to 1 µl of salt solution, and 1 microliter of TOPO vector, to a final volume of 6 µl. This was incubated for 5 minutes at room temperature. Immediately following the incubation, the mixture was placed on ice. The solution was then transfected into Mach1-T1 competent *E. coli* cells using the Transform One Shot Invitrogen protocol. 2 µl of the solution was added to the *E. coli* cells provided. This mixture was incubated on ice for 5 minutes, heat shocked at 42°C in a hot water bath for 30 seconds, and then placed back on ice. 250 µl of room temperature S.O.C. medium was then added, and the mixture was shaken gently for one hour at room temperature. 100 µl of the mixture was then added to pre-warmed LB ampicillin selective plates, and these were left to incubate overnight at 37°C.

Miniprep DNA Isolation

After the transfection and plating, three random colonies were chosen from each plate based on their color, size, and shape (circular, with a solid outline). These colonies were then placed into separate LB media in test tubes and pelleted. The cell pellet was then added to Buffer P1 and resuspended. Buffer P2 was then added, and the solution was mixed by inversion. After no more than 5 minutes, Buffer N3 was added, and the solution was again mixed gently by inversion. This was then centrifuged for 10 minutes at 13,000 RPM, and the supernatant was applied directly to the QIAquick Spin column. This was then centrifuged for 60 seconds, and then washed with buffer PB, and centrifuged again for 60 seconds. The column was washed again using buffer PE and centrifuged. The flow-through was discarded, and the column was centrifuged once more for 60 seconds. Finally, the DNA was eluted by applying water directly to the center of

the column, allowing the column to sit for one minute, and then centrifuged for one minute.

DNA Sequencing

The purified plasmids were sent to Macrogen USA to be sequenced. Upon receiving the sequence results, they were examined using MacVector software to identify potential mutations.

RESULTS

In this project, we attempted to isolate the germinal center cells containing B-cells from the spleens of WT and Smurf2-knockout mice, amplify the VH4 intron region by PCR, and clone and sequence the amplicons to determine whether DNA mutations at this locus precedes cancer formation. The first thing we did was to isolate the germinal center cells from 6-week old spleens from WT and Smurf2-KO mice using FACS (**Figures 7 and 8**). The markers used to select for the germinal center cells were B220+, CD95+, and GL7+, which when all three are present represent a strong selection for germinal center cells (Schrader et al., 2013). The data show that we were able to obtain a usable amount of germinal center cells from the spleens of unimmunized mice. The data also show that immunizing the mice with NP-chicken gamma-globulin or isolating Peyer's Patch cells is not needed to obtain a usable amount of material.

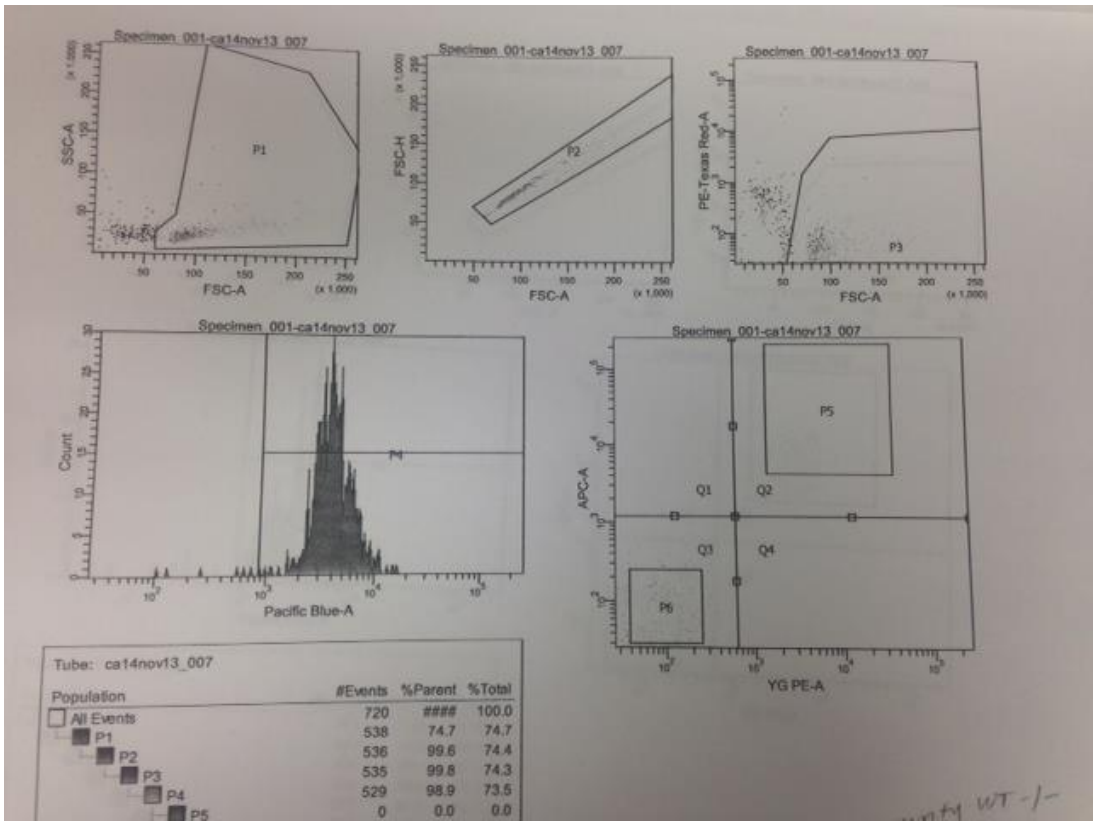

Figure-7: FACS Analysis of Spleen Cells in Wild Type Mice.
Shown are the sorting data for B220+, CD95+, and GL7+ markers indicating germinal center cells.

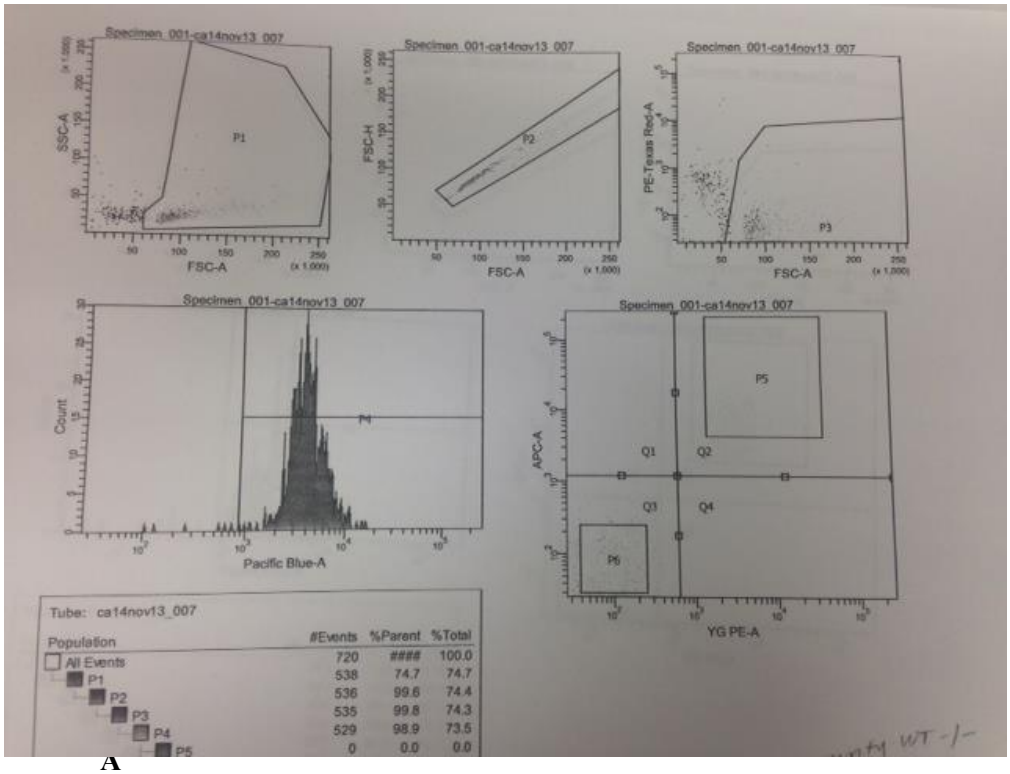

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Figure 8: FACS analysis of Spleen Cells in Smurf2-Knockout Mice. Shown are the data sorting for B220+, CD95+, and GL7+ markers indicating germinal center cells.

Genomic DNA was isolated from the spleen germinal center cells, and was then used in a nested PCR to amplify the VH4 intron locus. Based on the primers used, we expected amplicons of approximately 600 bp (**Figure-9**). Amplicons of the correct size were obtained for WT and Smurf2-KO samples (blue arrow in the figure), although the amplicons were not discrete and appeared as smears.

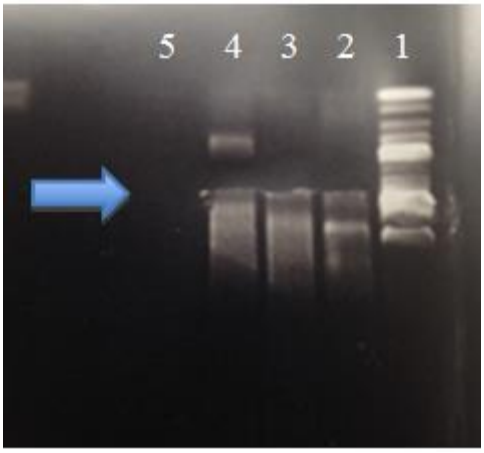

Figure-9: Nested PCR of the VH4 Intron Locus of DNA Isolated from Germinal Center Cells. Lane 1 (on the right) is a DNA ladder; Lanes 2 and 3, Smurf 2-knockout; Lane 4, WT; Lane-5, negative control (dH_2O). The 600 bp range where the amplicons were excised from is denoted by a blue arrow. Amplicons were excised from lanes 2, 3, and 4.

Following the PCR, a band gel extraction was performed to extract the DNA from the gel, and 3' dA tails were added to the amplicons using Taq polymerase to facilitate cloning in the TOPO dT-tailed vector. Unfortunately, it was not determined in advance whether a sufficient amount of amplicon DNA was obtained for cloning, so this could have caused a problem with the cloning from the outset. In addition, the serious smearing of the amplicons could also have caused cloning problems. The 3' dA-tailed DNA was purified again, and then ligated into a T-tailed TOPO plasmid vector. The ligated samples were transformed into competent *E. coli* cells provided with the cloning kit. Three "positive" colonies from each selection plate were used to purify plasmid DNA, which was then sent for sequencing analysis at Macrogen USA.

The sequence information was analyzed using MacVector software. Unfortunately, the transformation of the bacteria did not occur properly. Although

"positive" ampicillin-resistant colonies were found on the plates, the sequence data indicated that no VH4 intron was inserted. Because of this we were unable to determine whether there was an increased mutation rate in the VH4 intron region of the germinal center DNA when Smurf2 is knocked out in mice, so that remains a future experiment.

DISCUSSION

The purpose of this project was to determine whether the mutation rate in the VH4 intron region increases when Smurf2 is knocked out in mice. Unfortunately, we were unable to achieve this goal because we were unable to successfully clone the VH4 locus. Because of this, we were not able to successfully make any conclusions about the main purpose of the project.

However, the FACS analysis appeared to work well. We determined that the spleens of non-immunized six week old mice (WT and Smurf2-KO) allow the isolation of a sufficient amount of germinal center cells to isolate genomic DNA. Thus, immunization with NP-chicken gamma-globulin was not needed to boost the B-cell yield, which could have been problematic when performing future projects. If immunized mice were used, their germinal center cells reproduce at a very high rate, increasing the likelihood of natural DNA mutations which could obscure the mutations induced by the Smurf2 KO.

PCR was the main problem encountered in this project. In many cases, a 600 bp band appeared in the negative control samples which were the same size DNA as the expected VH4 intron amplicon. After extensive testing of the reagents, we found the source of this contamination to be in the micropipettes. However, even after eliminating the pipette contamination other contaminations remained, likely from the dH_2O samples used in the PCR reactions. In addition, the amplicons when they did appear were smears and not clear discrete strong bands, so this too could have caused cloning problems. In

the future, the isolated genomic DNA should be tested for quality by determining whether primers for other genes work well. If so, the problem lies with the VH4 primers.

The other problem encountered in this project came from not being able to transfect a sufficient amount of plasmid containing the VH4 intron into the *E. coli* to obtain a high number of "positive" colonies on the plates. This could have been caused by very low yields of amplicon DNA resulting from the contamination mentioned previously, or from the low transformation efficiency (TFE) of the competent cells used. In the future, a different strain of bacteria will be tested for transfection, or a new batch of commercial highly competent cells will be tested.

Due to its scientific importance, this project should be continued. As mentioned in the Background section, our laboratory has shown that Smurf2 knockout in mice causes the spontaneous development of B-cell tumors closely resembling diffuse large B-cell lymphoma in humans; and suppressed Smurf2 expression in human diffuse large B-cell Lymphoma tumors correlates with a poor patient prognosis (Ramkumar et al., 2013). This clearly shows that the Smurf2 pathway plays a major role in the progression of these lymphomas, so it is necessary to better understand what the suppression of Smurf2 expression does to B-cells, and how it may affect DNA mutations. In future projects, it would be necessary to ensure that a sufficient amount of high-quality genomic DNA is obtained from the spleen cells (PCR for genes other than VH4 could be used as positive controls), to test other VH4 primers until a strong discrete amplicon is obtained, to make sure the gel purification protocols provide a high yield of amplicon DNA, and to switch to other commercially available strains of high TFE competent cells for the

transformation experiments. Finally, using a different method for selection other than ampicillin resistance may more accurately select true positives.

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