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Targeted Germ Cell Knockdown of Xlr3 in Mus musculus

Caity Miller
University of Connecticut - Storrs, caity.miller@uconn.edu

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Caity Miller
Department of Molecular and Cell Biology,
University of Connecticut
Honors Program
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Honors Thesis Advisor: Dr. Michael J. O'Neill
Graduate Advisor: Natali Naveh

Abstract:

The X chromosome is rather distinct from autosomes due to the unique regulatory and functional characteristics it exhibits. *Xlr3* is just one gene in a superfamily of highly related, and homologous genes found on the X chromosome in mice. *Xlr3* is part of a complex, imprinted locus, of which the function is not well understood, although its protein product can be found in testis and oocyte. However, it is theorized that the region may be implicated in the progression of meiosis, due to localization of XLR3. This study sought to characterize the function of *Xlr3* through the use of a short hairpin knockdown model, findings for which can hopefully be used to better understand broader implications of X chromosomal imprinting, regulatory patterns, and gene duplication in fertility, development, and even cognition. In our findings, no phenotype was observed in heterozygote knockdown testis tissue when examined using Hematoxylin & Eosin staining. Additionally, there was no difference in the average weight or gross morphology between the heterozygote and wild-type samples. However, a reduction in the expression of *Xlr3* was achieved in the heterozygotes and the use of a short hairpin did not generate any observable immune response within our samples. Further work with homozygote knockdown samples will be examined in the future to further elucidate the function of *Xlr3*.

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Background:

Although development in any organism is inarguably complex, when one examines the variance of paternal and maternal influences on development in mammalian species, the extent of this complexity only grows. Regulation and function of genes on the X-chromosome are particularly interesting areas of research, both of which are currently examined in the laboratory of Dr. Michael O'Neill at the University of Connecticut.

Gene regulation and function on the X chromosome is characteristically distinct from autosomal genes. Many of the primary distinctions involve complex mechanisms of dosage compensation, most of which have evolved to account for the differences between the X and Y chromosome. These compensatory mechanisms enable control over, and balance between, expression levels of X-linked genes in both males and females. The occurrence of Barr bodies, products of the random inactivation of a single X chromosome in female somatic cells, is one such mechanism. Random X inactivation prevents females, with two X chromosomes, from having twice the X-linked gene product as males, with only one X chromosome. Upregulation of X-linked genes has also evolved for dosage compensation purposes. Unlike autosomal genes, which are present in two copies, both males and females exhibit only one active copy of the X chromosome. As a result, some evolved compensatory mechanisms increase transcription of X-linked genes and affect RNA stability to facilitate these higher levels of transcription (Deng et al., 2014). As suggested by the work of Prestel et al. (2010), autosomal 'buffering' or downregulation may also contribute to the highly intricate and complex dosage compensation systems diploid organisms have evolved in order to maintain an impressive balance of gene expression across sexes.

In addition to these distinct evolutionary regulatory mechanisms, many of the genes found on the X chromosome have also adopted unique, rather specialized functions. In females, many X chromosome genes are highly expressed in ovaries and support reproductive functions. Increased expression of these select genes is crucial for the development of proper ovarian function, supported by the finding of ovarian dysgenesis in Turner Syndrome individuals (Deng et al., 2014). Although the role of the X chromosome in female development may seem rather intuitive, there are a surprising amount of genes on the X chromosome that play a role in male development as well. Expression of these male-specific genes is mainly observed in testis during pre and post meiotic stages, although the regulation and potential upregulation of these genes is still not well understood (Deng et al., 2014). Interestingly, X-linked genes are highly specific to the brain and reproductive tissue, rather than other somatic tissue. These findings support the role of X-linked genes in cognition and neurological function, and how sexual selection is key in passing on these genes due to the relationship that has been drawn between testis and brain gene expression (Deng et al., 2014).

Also consistent with these findings is the idea of sexual antagonism and its contribution to the distinctions seen on the X chromosome. This theory seeks to explain the finding of paralogs and multi-copy genes on the X chromosome, another of its many unique features. According to the theory, certain genes may benefit one sex while being highly disfavored in the other (Rice, 1992). The existence of a nearly identical gene pool between males and females does not facilitate selection and instead gives rise to a genetic "tug of war" between the sexes, each of which desires to pass on genes that promote its own selection (Connallon and Clark, 2011). To resolve this conflict, gene duplication and differentiation between male and female genes has arisen. Gene duplicates are especially common among sex-biased genes. Paralogs

have, in many cases, adopted different functions for one or both sexes, further seeking to resolve this conflict and promote differentiation between males and females (Wyman et al., 2011). Male-biased genes are found to have more paralogs than female-biased or un-biased genes, supported by studies that demonstrate an increased susceptibility of genes with male-related functions to sex-specific selection. Therefore, the observance of paralogs and an increased rates of gene duplication in male-biased genes may arise from an increased need to resolve the previously described sexual "tug of war," consistent with the theory of sexual antagonism (Wyman et al., 2011).

The abnormal nature of the X chromosome presents itself more poignantly through the role it plays, or may play, in a variety of disorders. One such disorder includes Turner's syndrome, a condition in which an individual exhibits a genotype of XO, inheriting a single X chromosome from either the mother (Xm) or father (Xp). Of the many phenotypes these individuals exhibit, one that is of particular interest is the affect of the disorder on both executive and social-cognitive tasks. Although all Turner syndrome individuals exhibit deficits of this nature, research by Skuse et al. (1997) showed that differences in performance on cognitive tests was dependent on the parental origin of the patient's X chromosome. Xm Turner Syndrome individuals were much less successful on cognitive tasks than Xp patients. The phenotypic variability between Xm and Xp individuals indicated that an associated imprinted locus was involved, for which it was theorized to be maternally silenced and paternally expressed. It was believed that this difference in gene expression between the maternally and paternally derived X chromosome could account for the variation in social and learning difficulties presented in the patient population (Skuse et al. 1997). Autism Spectrum Disorder (ASD), Prader-Willi and Angelman syndromes, even cancer, are just a few examples of additional disorders influenced by

parent-of origin effects, or imprinting, although the exact mechanisms for many of these are still under investigation (Raefski and O'Neill, 2005; Hall, 1990).

Research in the O'Neill laboratory at the University of Connecticut investigates regulation and function of the X chromosome, particularly epigenetic regulatory mechanisms, imprinting, and implications of these areas in development and cognition. The first X-linked imprinted gene cluster, the *X-linked lymphocyte receptor locus, Xlr*, was not identified until 2005 in the O'Neill laboratory by Dr. Adam Raefski (Raefski and O'Neill, 2005). The imprinted locus of paralogs, *Xlr3b/4b/4c*, was identified using a Turner syndrome mouse model, with which experiments identified a small, yet significant difference in transcription levels of Xp and Xm in *Xlr3*. This imprinted locus is paternally silenced and maternally expressed, opposing the pattern seen in Turner syndrome patients, and compromises a small part of a larger group of highly similar, multi-copy genes (Raefski and O'Neill, 2005).

In autosomes, imprinting is characterized by a parent-of-origin effect on the expression of certain genes. One of the most highly understood cases involves the closely linked and reciprocally imprinted *Igf2* and *H19* genes. *Igf2* is expressed only on the paternal chromosome, and *H19* only on the maternal chromosome (Kaffer et al., 2001). Differences in the expression of these genes at the *Igf2/H19* locus are explained by differences in methylation patterns, regulated by an imprinting control region (ICR) found upstream of the *H19* promoter (Yang et al., 2003). Zinc finger protein CTCF produces an insulating, or silencing, effect when bound to the unmethylated maternal allele, preventing enhancers from accessing *Igf2* promoter regions and silencing the gene while allowing *H19* enhancers to access its promoter region. The paternal allele, however, is methylated and CTCF proteins cannot bind, therefore *Igf2* will be expressed at this allele, resulting in the differential expression of these two genes at the *Igf2*/*H19* locus (Yang

et al., 2003). Understanding of the imprinted *Igf2*/*H19* locus has facilitated the understanding of other identified imprinted genes, of which a majority are autosomally linked (Henckel and Arnaud, 2010).

Xlr is of particular interest due to the involvement of *Xlr* superfamily proteins, whose members include XLR3, XLR6, SLY and SLX, in meiosis, specifically at Synaptomenal Complex Protein 3 (SYCP3). SYCP3, along with SYCP2, is involved in the meiotic chromosome axis and sister chromatid cohesion during meiosis (Kouznetsova et al., 2011). A 2005 study performed by Kouznetsova et al. showed that without SYCP3, spermatocyte development halts at meiotic prophase I, resulting in male infertility, and while some effects may be seen in the cohesion complex, there are little functional effects of the loss of SYCP3 in oocytes (Kouznetsova et al., 2005). Previous studies have performed knockdowns of *Sly* and *Slx* to examine the effects of the loss of these genes and their related proteins on development. Cocquet et al. characterized a murine knockdown of *Sly* using in vivo RNA interference. Results demonstrated abnormal sperm head abnormalities, issues with spermatogenesis, gender bias in offspring, infertility, as well as interference with the regulation of X-Y genes and histone modification proteins when *Sly* was deficient (Cocquet et al., 2009). Due to the relationship between SLY and XLR, their involvement in SYCP3, their multi-copy status and their role as *Xlr* superfamily proteins, we determined that similar mechanisms could be utilized to analyze the functions of *Xlr3.*

In our research, we seek to better characterize the function of *Xlr3* and its role in development. As a multi-copy gene, like *Sly*, *Xlr3* is not a suitable candidate for conventional knockdown techniques such as targeted mutagenesis and CRISPR technology. These techniques cannot successfully knock down *Xlr3* fully or specifically due to its paralog status and its

location on the X chromosome. In vivo RNA interference, as also utilized by Cocquet et al. (2012) in their *Sly* knockdown, is a much more suitable candidate for knocking down a gene of this character. RNA interference (RNAi) was thus used to knockdown *Xlr3* using an *in vivo,* with a targeted short hairpin mechanism (shRNA). Using the mechanism designed previously in the O'Neill laboratory by Dr. Robert Foley, we examined transgenic, *Xlr3* knockdown mice for the purpose of further characterizing *Xlr3* and its implications in meiosis, gender bias in offspring, spermatogenesis, and other aspects of development.

A potential undesired outcome of using RNA interference is off-target effects that can trigger an interferon response within the organism (Bridge et al., 2003). Recognition of RNA interference using a short hairpin mimics the mechanisms through which viral double stranded RNA (dsRNA) is recognized, subsequently inducing an immune response through the activation of the NF-kβ pathway and ultimately interferon release (Pulit-Penaloza et al., 2012). Therefore, throughout our experiment, we monitored any potentially generated immune response within our samples to account for off-target effects of the short hairpin knockdown that may have implications within our results.

The *Xlr3b/4b/4c* locus demonstrates many of the key distinctions that are seen in X-linked genes. *Xlr3* is expressed in reproductive tissue, as well as brain, and is regulated under complex mechanisms, as demonstrated by the characterization of this locus as an imprinted cluster. The expression in both brain and testis/oocytes (although the actual XLR3 protein is only seen in testis/oocytes and not the brain) provide evidence for sexual selection, as previously described. This information, in conjunction with the cluster's paralog status, the relationship between *Xlr3* and other highly similar, multi-copy genes and the involvement of the XLR3 protein in SYCP3 during meiosis provides compelling evidence for *Xlr3* to be examined through

the theoretical lens of sexual antagonism. Therefore, this study serves to better characterize *Xlr3* as a candidate in the theory of sexual antagonism. Specifically, the study will examine this through the function *Xlr3* may have in meiosis to understand where and how it is important in development. Variation in phenotypes and other qualities may be observed when *Xlr3* is made deficient and shed light on its function. Though little is currently known about the *Xlr* gene cluster and its function, identification of the imprinted *Xlr3b/4b/4c* locus demonstrated evidence of imprinting beyond autosomes, holding large implications on research into the mechanisms that underlie a multitude of disorders including Autism, Turner's Syndrome, and others particularly related to cognitive function (Skuse et al., 2000). Work on *Xlr3* and other X-linked genes may help illuminate the development, progression and inheritance of these aforementioned disorders, among others.

Materials and Methods

Primer Design

Gene targets were selected and examined using the Ensembl genome browser. Gene sequences, in FASTA format obtained from the genome browser, were entered into Primer3 to look at specificity and select primers for each target. In addition to *Xlr3* exons, *Stat1, Oas2,* and *Oas1b* were selected in order to quantify any immune response elicitation due to the shRNA knockdown mechanism. The annealing temperature (Tm) for all primer sets was 58°C and all primer sequences are presented in Table 1.

Target	Forward Primer Sequence (5' to 3')	Reverse Primer Sequence (5' to 3')
Xlr3 Exon 3	GTCCTTGATGCTGGTAGGGA	CCTCCATGGCACTGAAAGATG
Xlr3 Exon 6	ACTTCGGATGCATACAAACTCA	AGTACCTCCAGTTTCTCCAAGT
Stat1	CGCTGCTTGGCTCTCTTATC	TTCCGTTCCCACGTAGACTT
Oas2	GCCCAACAAGCTCTTCCTAAA	CTCAAACGTCACCTCCCACT
Oas1b	CAGACTCCAGGCTTCTGTCC	GGGTTGGCTTACAGGTTCAG

Table 1: Primer sequences for all gene targets.

RNA Extraction

Following dissection, liver, lung spleen, testis, brain, and ovary tissues were homogenized using a polytron and RNA extraction was performed using a Macherey-Nagel NucleoSpin RNA kit. A maximum of 30 mg of each tissue sample was prepped for homogenization using a Kinematica Polytron.

cDNA Synthesis

From the extracted RNA, cDNA was obtained for further analysis using a Quanta qScript cDNA supermix kit. A final concentration of 500 ng/mL was targeted for each sample and remaining RNA was stored at -80°C for future cDNA synthesis and experimental purposes.

PCR

PCR was used to assess the progress of the experiment at multiple points including genotyping and characterization of mice, and assessing cDNA quality. PCR products were analyzed using agarose gel electrophoresis with ethidium bromide for visualization. To analyze the quality of our immune response primer targets, PCR protocol using Promega GoTaq was followed whereas for genotyping experiments, a Takara Clontech PrimeSTAR protocol was used.

qRT PCR

Using the synthesized cDNA from each sample, relative expression of the genes of interest in both heterozygotes and wild-type controls were examined using a BioRad iTAQ Universal SYBR Green SuperMix Real-Time qPCR. A beta-Actin control was used as a baseline in order to determine relative expression levels for each gene of interest in each sample and data was analyzed using double delta CT analysis.

Tissue Fixation

Following dissection, tissues were fixed using a 4% paraformaldehyde solution and stored at 4°C and transferred to a 70% ethanol solution for dehydration. Tissues were submitted for paraffin processing at the Connecticut Veterinary Medical Diagnostic Laboratory in the Department of Pathology and Veterinary Science at the University of Connecticut.

Microtome

To section tissue samples for slide preparation, a Thermoscientific Microme Model HM325 microtome was used. Paraffin embedded tissue samples were sliced into ribbons at a thickness of 6 μm after excess paraffin was removed. Ribbons were transferred to a 42°C water bath for flattening. Flattened sections from the water bath were adhered to charged microscope slides. Slides were stored at 37°C overnight for drying before proceeding to subsequent staining and analysis.

Hematoxylin and Eosin (H & E) Staining

Regressive H & E staining was performed using Ricca Chemical Company Hematoxylin and Eosin Y alcoholic stock solution, according to the Ricca Chemical Company protocol for regressive staining on testis samples from heterozygote and wild-type tissue. Harris hematoxylin was utilized and a working solution of eosin Y was prepared during each staining procedure. The

following changes were made: instead of Xylenes, Histoclear was used, and hematoxylin was applied directly to the slide as was the eosin Y working solution. Following staining and mounting with Thermoscientific Cytoseal 60, slides were observed using a Olympus CKX 41 light microscope to examine any differences in the phenotype between heterozygote and wild-type samples. Histology pictures were taken using Infinity Capture camera and software at 10X magnification for all images.

Results

Xlr3 Knockdown

In order to decrease expression of *Xlr3*, rather than conventional knockout mechanisms, RNA interference using a short hairpin was performed. Although *Xlr3* will still be transcribed from the host genome, the short hairpin is designed to target the mRNA for degradation in the cytoplasm, therefore inhibiting translation and protein production. This is achieved through a pathway involving the RNA-induced silencing complex (RISC). First, following transcription, the short hairpin transcript is processed by a protein, Dicer, allowing binding to the mRNA taret and RISC complex. RISC guides the short hairpin to the complementary mRNA sequence in the cytoplasm where it binds. Argonaute (Ago) proteins, part of the RISC complex, cleave the target mRNA and it is degraded (Moore et al., 2010). The short hairpin for this study was designed to target all active copies of the XLR3 protein, and previous tests in cell culture demonstrated successful reduction of *Xlr3* using this short hairpin design (Foley, 2015). Transgenic mice were generated in collaboration with Jackson Laboratories, and breeding schemes included the use of Flippase and Cre to activate the short hairpin. Eight total mice were used for, four of which were

wild-type (WT) controls, and four of which were the sibling paired heterozygote knockdown (KD +/-) samples.

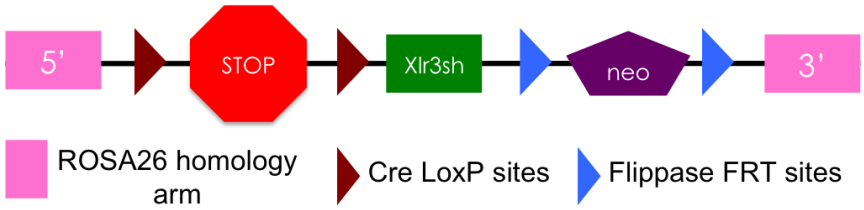

Figure 1: Schematic of short hairpin construct used to generate knockdown mouse model. Figure provided by Natali Naveh.

This study examines murine samples heterozygous for the short hairpin construct. As such, an exampled reduction of *Xlr3* by ~50% was expected. This reduction in the expression of *Xlr3* was observed in testis, and across other tissue types, as seen in Figures 2 and 3, respectively. Therefore, it was demonstrated that a ~50% knockdown of the gene of interest was achieved in these heterozygote samples, an observation consistent across all tissue types.

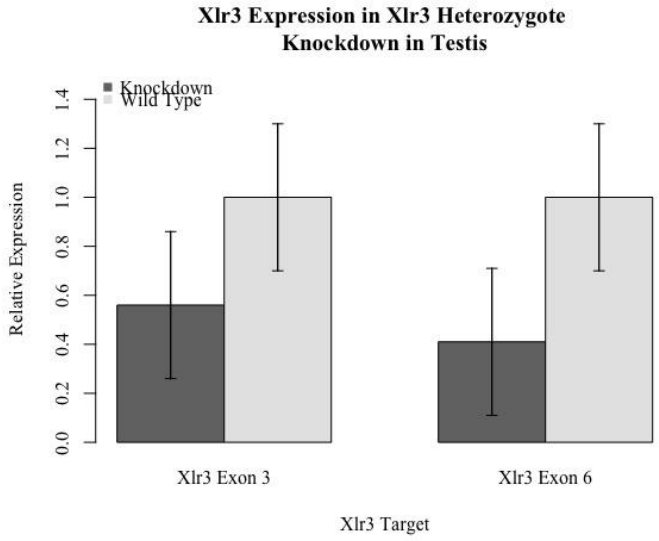

Figure 2: Demonstration of reduction of *Xlr3* expression in heterozygote knockdown testis. Data was standardized against a beta-Actin control and wild-type expression has been standardized to 1 using double delta CT analysis. A reduction in expression by ~50% can be observed in the heterozygotes.

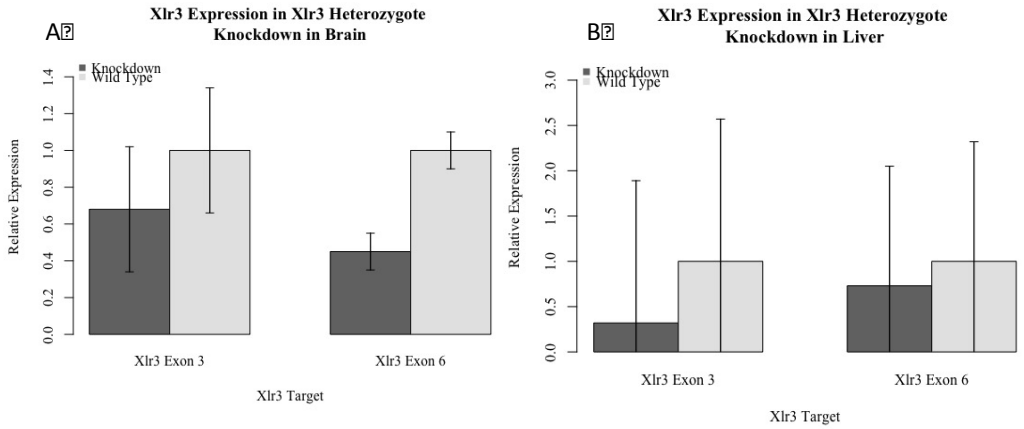

Figure 3: Relative expression of *Xlr3* in knockdown and wild-type (A) brain and (B) liver samples.

Immune Response

When using a shRNA knockdown mechanism, there is a potential that an immune response may be elicited (Bridge et al., 2003). Activation of this response may then activate additional genes, potentially confounding experimental results with off-target gene or protein interactions. To monitor the immune response throughout the duration of the study, and to quantify any significant differences between control and heterozygote knockdown, three target genes were chosen: *Stat1*, *Oas2* and *Oas1b.* Data regarding the relative expression of these genes against a beta-Actin control in five tissue types – liver, lung, spleen, brain, and testis – was observed and collected.

Murine oligoadenylate synthetases (OASs) are enzymes that are induced by interferon release, making them strong candidates to monitor any interferon response throughout this experiment (Pulit-Penaloza et al., 2012). *Oas2* is one well-characterized interferon response marker, used commonly for assessment in other shRNA knockdown studies. Cocquet and al. (2012) selected *Oas2* to monitor an immune response in their *Sly* knockdown experiment and on

these bases it was selected as one immune response marker in this study. *Oas1b* was selected as an additional marker from this family of OAS enzyme-encoding genes to provide further insight into any interferon response. An additional gene,*signal transducer and activator of transcription 1*, or *Stat1*,was selected for immune monitoring for this experiment as it is highly responsive to viral infections and is upregulated in response to multiple interferon types (Gil et al., 2006).

Interferons are involved in the innate immune response. Therefore, in addition to the tissues – testis and brain – in which *Xlr3* expression is observed, organs highly active in the innate immune response were selected for additional analysis. The liver is active in the innate immune system, acting as one of the primary defense mechanisms between the host and its external environment. The organ's rapid activation of the immune response, coupled with the presence of ligands with the ability to detect dsRNA make it a viable candidate monitor a potential response to shRNA (Jenne and Kubes, 2014). The lungs are another primary defense organ, also active in interferon pathways that could be activated by any adverse effect of the short hairpin (Martin and Frevert, 2005). Furthermore, the spleen is well-known for its function in both the innate and adaptive immune

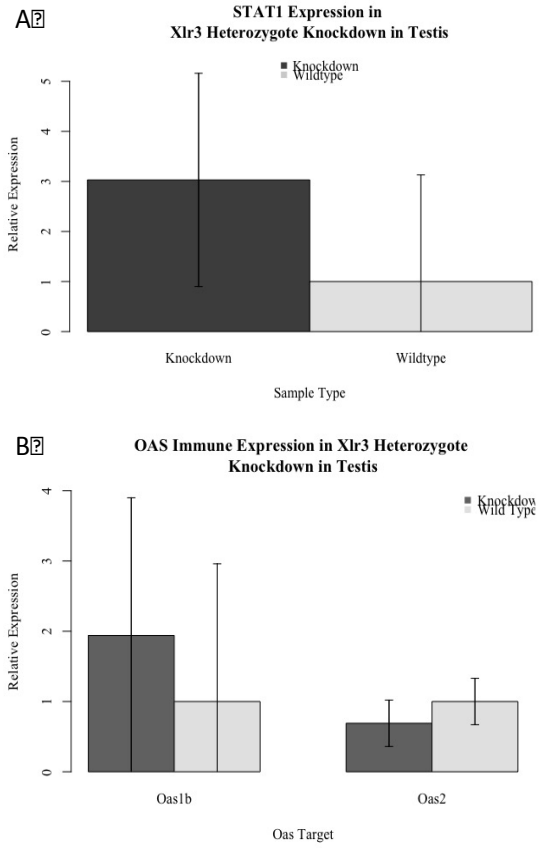

Figure 4: Immune response for all target genes in testis samples. Panel A shows the expression of Stat1 in knockdown and wildtype samples, and panel B shows the expression of both Oas1b and Oas2 across these two groups.

system, and its involvement with the regulation of the immune system in regards to viral infection make it another suitable candidate for characterization of the immune response in this study (Bronte and Pittet, 2013).

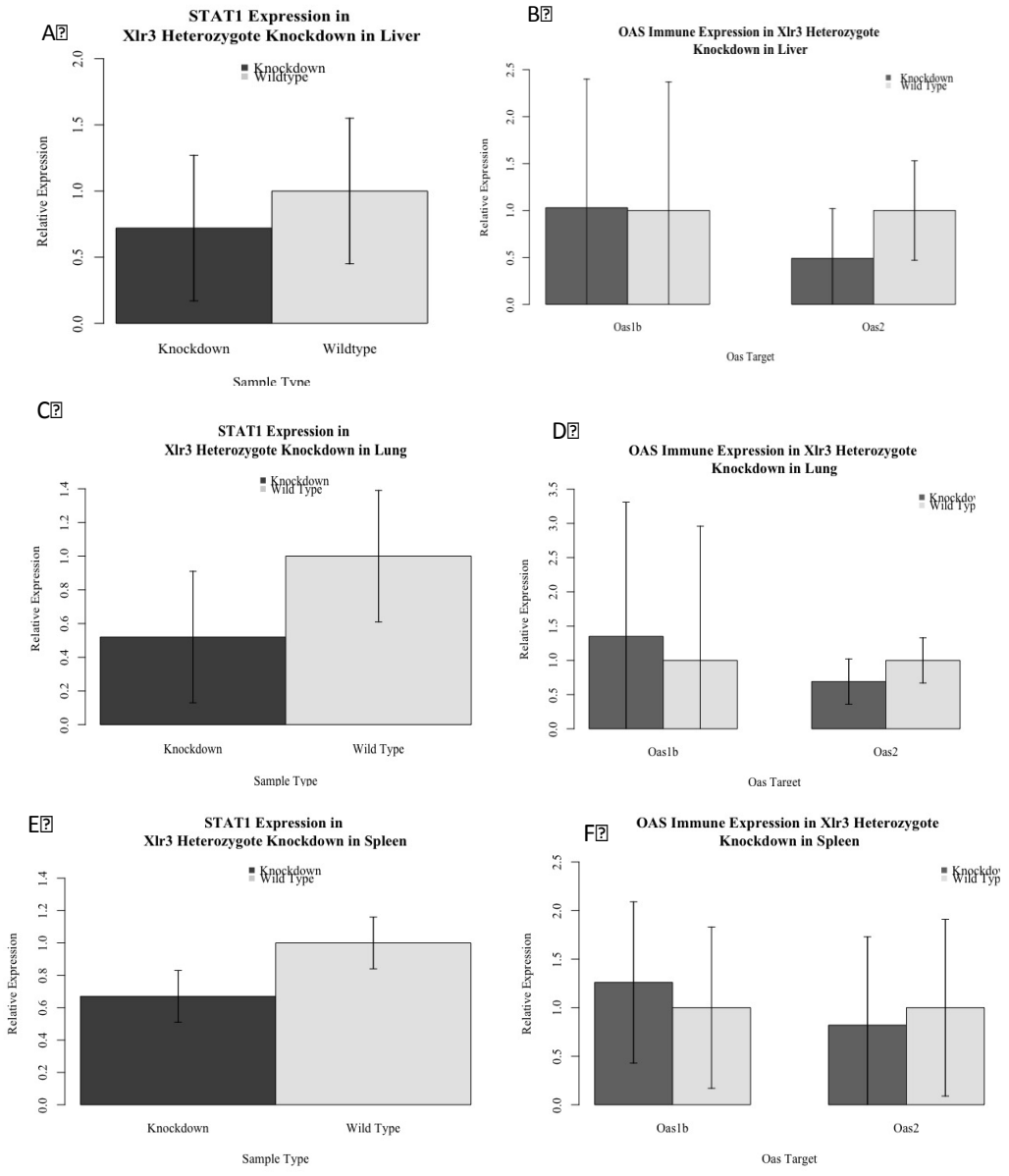

Figure 5: Relative expression of immune targets in immune surveillance tissue: (A) liver *Stat1* and (B) *Oas1b* and *Oas2*, (C) lung *Stat1* and (D) *Oas1b* and *Oas2*, and finally (E) spleen *Stat1* and (F) *Oas1b* and *Oas2*.

As depicted in Figure 6, one can observe a high degree of variability within samples. In testis, the tissue of interest for this experiment, no difference can be observed between the wild-type and heterozygote samples. Both are highly variable, and due to the observed overlap of error bars and lack of significant difference between the groups, it can be concluded that no immune response was elicited by the short hairpin knockdown mechanism. Although the data is not depicted in this paper, this variability continues to be seen across all other tissue types for all gene targets. No trend across any one target tissue or gene can be extracted. These observations are consistent with a low sample size, highlighting the individual differences across samples.

Testis Cell Population Morphology

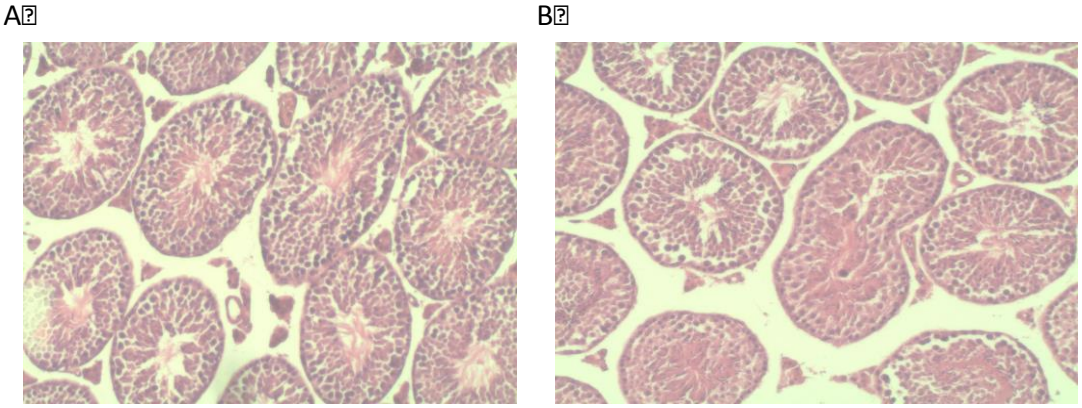

Figure 6: (A) Regressive H & E Staining of WT testis compared to (B) heterozygote knockdown sample at a magnification of 10X under a light microscope. All observations are consistent with a normal phenotype.

Figure 4 depicts the seminiferous tubules of wild-type (panel A) and heterozygote (panel B) testis samples. In each, normal phenotypic characteristics are observed. Due to the presence of XLR3 at SYCP3 at the XY body, theoretically a knockdown could interfere with spermatogenesis, as previously described. Within each seminiferous tubule, spermatogenesis occurs from the outside in. Spermatogonia are found in the outermost shell, primary

spermatocytes following slightly closer to the center, then secondary spermatocytes and spermatids. Approaching the center, the distribution of differentiation is relatively heterogeneous, however spermatozoa are found furthest from the outer circle of the seminiferous tubule, the tails of which collect and point towards the center. The sperm heads are oriented away from the lumen. If spermatogenesis were being halted in the heterozygote samples, production of spermatozoa would be decreased, and therefore one would observe relatively obvious phenotypic differences between wild-type and heterozygote knockdown samples. The lumen would be theoretically more cavernous due to the deficiency in production of spermatozoa, and these further differentiated cells would not be observed. However, differentiated cells are present and this, or another, phenotype is not observed. Thus, it can be concluded that there is no affect on spermatogenesis and the phenotype observed in testis in the heterozygote knockdown population appears not to be affected.

Additionally, the weights of all dissected testis samples were collected and compared to account for any potential differences in the size, weight, and therefore potentially the

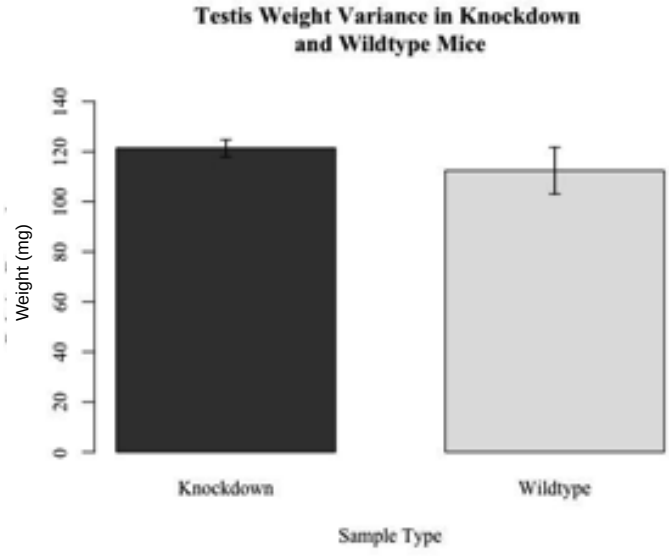

Figure 7: Average weight (in mg) for heterozygote knockdown and wild-type testis samples.

development of the testis in heterozygote samples. However, no significant differences were observed and it can be concluded that there is no affect in this way.

Discussion and Conclusion

Results from the *Xlr3* expression data, as shown in Figures 1 and 2, demonstrate that about a 50% reduction in the expression of *Xlr3* was achieved, as expected, in the heterozygote knockdown samples. High variability among the expression of immune-related genes, combined with the lack of a consistent trend across gene and tissue targets do not demonstrate that an immune response was generated by the shRNA mechanism. Therefore, off-target effects are not interfering with experimental results in the heterozygote knockdown samples. However, despite the achievement of the reduction in its expression, no notable phenotype was observed in the testis in heterozygotes.

One reason a phenotype may not have been observed is because enough reduction in the expression may not have yet occurred. In order to fully understand this, further work must be done on homozygote samples in the future to observe additional levels of gene expression reduction, and any resulting phenotypes. A 50% reduction in *Xlr3* may not be sufficient to elicit a phenotype, or prove detrimental in whatever its function may be in SYCP3 at the XY body, or other unknown interactions. Other compensatory mechanisms may also be involved. Earlier, it was discussed how that *Xlr3* is part of this greater superfamily of highly related proteins and genes. It is also present as paralogs, in a highly duplicated region on the X chromosome. This status as a multi-copy gene with highly related regions within the genome may imply that other genes and/or proteins can compensate for a deficiency in *Xlr3* function. These other related genes may serve similar functions, due to the highly related nature of the *Xlr* superfamily.

Consistent with the theory of sexual antagonism, many genes that are present in multiple copies are highly favorable in reproduction and development, and therefore it can be theorized that compensatory mechanisms may have evolved on the X chromosome in order to account for any deficiencies in the expression of *Xlr3*. Overall, it demonstrates the sheer complexity of this locus in terms not only of its function, but its regulation and relationship with other genes on the X chromosome. In order to completely account for the function of *Xlr3* in testis, further work will be done to examine the affect of the knockdown in homozygote mice, through which further deficiency of *Xlr3* can be observed.

Perhaps the function of *Xlr3* is not of most significance in testis. Although the protein is expressed in this tissue, expression of *Xlr3* can also be found in neonatal brain and fibroblasts, where *Xlr3* also demonstrates imprinting (Raefski and O'Neill, 2005). Although the protein is not expressed in these two regions, there may be additional gene interactions or some other implication of its function. Further research will be necessary to determine the extent of these implications if they are indeed present

XLR3 can also be found in oocytes, localized to nucleolus organizer regions (NORs) (Foley, 2015). Previous work in the lab by Dr. Robert Foley demonstrated this localization, as shown in Figure 7. In this figure, XLR3 is shown in green, SYCP3 in red, and in the bottom panel Fibrillarin, a marker for NORs is stained in red adjacent to XLR3. An overlap in the expression of SYCP3 and XLR3 can be observed, however the relationship between Fibrillarin and XLR3 is even more consistent and provided evidence for localization of XLR3 at NORs (Foley, 2015). Further examination into the function of XLR3 in oocytes must be conducted in order to better understand the role it is playing in females, a function that at this point remains relatively uncharacterized.

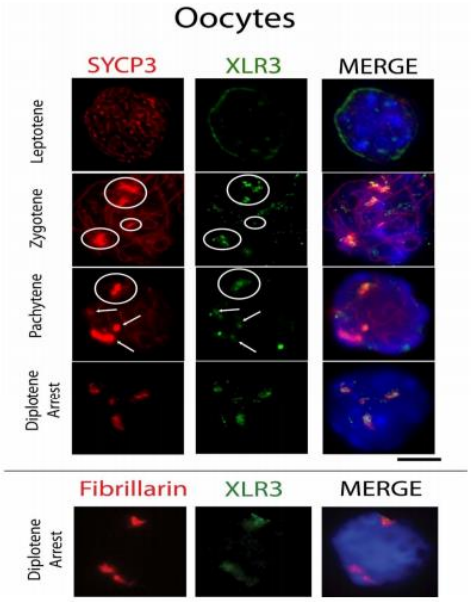

Figure 8: Immunohistochemical analysis of XLR3 in oocytes during meiosis. XLR3 appears in green in both panels, found highly expressed around the same regions as SYCP3, present in red. In the bottom panel, a NORs marker, Fibrillarin, is presented in red XLR3 is again in green. Co-localization of Fibrillarin and XLR3 provides evidence for its localization to NORs. Image courtesy of Dr. Robert Foley.

After examining a heterozygote, short hairpin mouse model, the function of *Xlr3* remains unclear. This highly complex, imprinted locus of paralogs, may still be implicated in meiosis in both males and females, however additional work must be conducted to demonstrate any affect of the knock down model in homozygote males, and additionally females due to the presence of XLR3 in oocytes. Expression of *Xlr3* in murine brain provides the foundation for additional future studies in this tissue. Research in all of these described domains may lead to better understanding of this gene's function as well as the implications of other, multi-copy, imprinted genes on the X chromosome. The theory of sexual antagonism provides the initial context for this understanding due to its explanation of the development of multi-copy genes and paralogs, as seen with *Xlr3* and related genes, and future work will hopefully elucidate *Xlr3* function.

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