Characterizing the meiotic failure and transcriptional aberrations caused by mutation of A-Myb in mice

Honors Thesis

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by

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Hypothesis:

The *A-Myb*^{Repro9} male mutant is sterile because of a mutation on transcription factor A-Myb. This mutation changes the transcriptional function of A-myb, which causes differential expression of genes that are fundamental to the processes of meiosis and spermatogenesis.

Significance of Project:

This project aims to understand the role of the transcription factor A-myb and its targets in spermatogenesis. If the project is successful, we will know how a mutation in the *MybL1* gene leads to male sterility in the mouse. This information will give us a more detailed picture of the processes of meiosis and spermatogenesis, not just in the mouse but in all organisms with homologous genes and mechanisms.

Ultimately, knowledge of the inner workings of meiosis and spermatogenesis will allow for advances in human and veterinary medicine. A knowledge of which genes and proteins play a major role in spermatogenesis may allow doctors to reverse sterility in humans, and may help couples who cannot conceive children at the moment to be able to in the future. For example, if a mutation in *MybL1* is causing male mice to be sterile because of an affected downstream gene, then researchers will be able to devise a simple genetic test for male sterility that will look for mutations in either of these two genes. Males with mutations in these genes may one day be prescribed a drug that will "cure" them by giving them the exact protein that they are unable to produce.

Another potential application of this knowledge is new forms of birth control. If this research finds that a gene product specific to spermatogenesis is the cause of male

sterility in mice, then this product could be blocked or degraded using a drug. In this way, males could be temporarily sterilized with a male version of the birth control pill.

Finally, because the Myb class of proteins is expressed in tissues other than the testis, our research may facilitate research in other areas. Our understanding of the way *MybL1* affects spermatogenesis has the potential to unlock the functions of the Myb class of proteins in breast tissue, among others.

Theory:

Spermatogenesis:

Spermatogenesis functions to produce haploid gametes, allowing sexual reproduction to occur. These haploid gametes, sperm, are produced in the testes. When the sperm fuse with the haploid gametes produced in the ovaries (the ovum), they produce a single-celled organism called a zygote, which eventually grows into a multi-cellular organism of the same species as the parents.

Spermatogenesis forms sperm cells by an elaborate differentiation process that includes meiosis, which is a special type of nuclear division that segregates one copy of each homologous chromosome from the diploid progenitor into each new "gamete." Meiosis, unlike mitosis, reduces the number of sets of chromosomes by half, so that when gametic recombination (fertilization) occurs, the ploidy of the parents will be reestablished (Alberts, 2002).

At early stages in meiosis, homologs become coaligned along their lengths; the formation of meiosis-specific double-strand breaks (DSBs), which initiates genetic recombination, drives this pairing. At intermediate stages, the coaligned homolog axes

become intimately connected via a prominent zipper-like structure, the synaptonemal complex (SC); at about this same time, some or all DSBs are converted to double Holliday junctions, and recombination begins. At the next stage of meiotic prophase, the pachytene phase, SCs occur along the entire lengths of all homolog axes. The recombination process is completed at about the end of the pachytene phase; mature crossover and noncrossover recombination products both occur at about this time. At pachytene, chromatin loops are tightly condensed. Exit from pachytene is signaled by the disassembly of SCs and the reorganization of the chromosome structure in preparation for the first nuclear division. In organisms with large chromosomes, dramatic compaction can occur at this stage. During the next stage, diplotene, the SC degrades and homologous chromosomes begin to separate. The transition from pachytene to metaphase I is an important point for regulation of meiotic development in many organisms (Xu, 1995).

This process requires that homologous chromosomes pair, synapse, and recombine. In many organisms including mammals, mutations that disrupt pairing, synapsis, and/or homologous recombination confer a meiotic arrest phenotype resulting in sterility. Experiments with *Saccharomyces cerevisiae* (yeast) have shown that many recombination checkpoints exist during meiosis (Reinholdt and Schimenti, 2005). Meiotic cells monitor the progression of chromosome metabolism and arrest at pachytene in response to certain critical defects (Xu, 1995).

However, of all the genes required for the induction and initial processing of meiotic DSBs in yeast, only three are present in mammals (Keeney 2001). Therefore, it is likely that the majority of mammalian genes required for initiation of meiotic

recombination remain to be identified (Reinholdt and Schimenti, 2005). Similarly, very little is known about transcription during spermatogenesis, so research in this area is very important.

Mutagenesis:

Mutations are the basic tools of genetic analysis: altering genes one at a time by inducing mutations allows us to see what functions each gene has. This can be done in mice by simply breeding and waiting for mutations to occur, but this process is extremely slow. The effectiveness of the hunt can be improved dramatically by using mutagens that will induce mutations and allow for faster identification of mutations that cause the desired phenotype. A major problem with chemically-induced and irradiation-induced mutations, however, is that they are generated essentially at random. In order to identify a mutant phenotype of interest, a laborious screen for mutants needs to be conducted by close examination of the phenotypes following mutagenesis (Alberts, 2002).

We are using forward genetic screens to find new autosomal recessive mutations affecting meiotic recombination in mammals (Reinholdt and Schimenti, 2005). A genetic screen is a procedure that is used to identify individuals with a phenotype of interest. In a forward genetic screen, mice that exhibit a mutant phenotype (sterility, in our case) are selected in order to find genes that are causing this phenotype.

One such recessive mouse mutant, the *Repro9* mutant, encounters problems during the meiosis stage of spermatogenesis, when homologous chromosomes normally align and recombine before the spermatocytes divide and eventually differentiate into sperm. The *Repro9* mutant has a mutated allele at the locus that produces the

transcription factor A-Myb. This project aims to elucidate the functions of this key transcription factor in spermatogenesis.

Myb Class of Genes:

A-Myb is one of a class of Myb genes, all of which are very similar structurally. The Myb proteins are not limited to expression within the testis or in cancerous cells. B-Myb is expressed in all dividing cells, and c-Myb and A-Myb are often co-expressed. In addition to homologous structure, all three proteins (A-, B-, and C-Myb) bind the same DNA sequence *in vitro* and all activate the same reporter gene constructs in transient transfection assays, suggesting that they should have similar activities on endogenous genes (Rushton, 2003). However, several studies have found that this is not the case. Over expression of each version of Myb in the MFC7 cell type, for example, has a unique activity and leads to the activation of a different set of endogenous genes. This implies that the Myb proteins differ not by their relative strengths as transcriptional activators but in some qualitative feature, such as relative access to promoters or in ability to interact with cofactors (Ness, 2003). The expression patterns of Myb in testis have not been studied in depth.

We know that an alternate version of the transcription factor A-Myb that results from a point mutation is expressed in the *Repro9* mutant. In studying which genes' transcription is directly and indirectly affected, we can learn about the functions of these genes and of A-Myb. This will allow us to increase our knowledge of transcription and the role of genes and transcription factors in spermatogenesis, and potentially meiosis in general.

Histones:

Histones are the proteins that act as spools around which DNA winds in the nucleus of the cell. They enable the large genomes of eukaryotes to fit inside a cell: the compacted molecule is 50,000 times shorter than an unpacked molecule. Combinations of histone modifications are thought to constitute a code, the so-called "histone code," which determines which DNA is wound or unwound so that transcription can occur. Histone modifications act in diverse biological processes such as gene regulation, DNA repair and chromosome condensation (mitosis) (Alberts, 2002).

Characterization of the meiotic defects in the homozygous *Repro9* mutant males indicated aberrant staining of DNA repair foci, indicating possible defects in chromatin organization. Many core histone replacements and modifications occur during mouse spermatogenesis (Lewis, 2003). We thus used immunohistochemical staining of testicular cross-sections to look for potential differences in the histone code that may have produced the effects seen in the mutant. We examined a subset of known histone modifications using innumohistochemical staining of wild-type and mutant testis. This allowed us to identify yet more differences between our *Repro9* mutant and the wild-type testes.

Earlier Work:

In order to identify genes that play a role in spermatogenesis, we used a forward genetic screen, a process in which affected phenotypes are used to identify new genes. Mice were mutated using the chemical N-ethyl-N-nitrosourea (ENU). Mutant pedigrees

were screened for infertility, and the *Repro9* mutation was discovered. *Repro9* was found to cause male-specific infertility.

Male sterility indicates a problem with spermatogenesis, and so the testes of the mutant male mice were studied in further detail to find the effects of the newly discovered mutation. First, an overall comparison in body size, weight, testes size, and other factors was performed to learn about the major effects of this mutation. Next, cross-sections of the testes were prepared. We used microscopy to look at these cross-sections. Spermatocytes were immuno-labeled using antibodies directed against axial and central elements of the synaptonemal complex so that problems with synapsis and other meiotic defects could be identified.

Recombination mapping was used to define the critical region harboring the gene of interest. This method involved mating mice heterozygous for the mutation and screening recombinant male progeny for sterility. If a mouse was a product of recombination in one region of its chromosome but still had the sterile phenotype, then that region was ruled out as a potential location for the mutation. Recombination events were used to narrow the location of the mutation down to a small region, a centromere-proximal 7Mb region on chromosome 1. Genes within this critical region were considered as potential candidates in causing the observed phenotype.

The *MybL1* gene was found and sequenced in order to find the mutation. Once the knockout mouse was available to us[1], complementation analysis was used to tell whether the *Repro9* mutation was indeed in the *MybL1* gene. This analysis depends on

[1] The mouse was not available earlier in our lab because another lab, which had been studying the A-Myb expression in breast tissue, had already developed a knockout mouse (a mouse with that gene inactivated). Because it is extremely tedious to create a knockout from scratch, our lab requested that an A-Myb knockout mouse be sent to us. See the section entitled "Mighty Mouse" below for a discussion of the sharing of mice between labs.

the phenomenon of genetic complementation, that is, the restoration of the wild-type phenotype by mating of two different mutants. If two recessive mutations, A and B, are in the same gene, then a diploid organism heterozygous for both mutations will exhibit the mutant phenotype. However, if mutation A and B are in separate genes, then heterozygotes carrying a single copy of each mutant allele will exhibit the wild-type (normal) phenotype. In this case, the mutations are said to complement each other. (Lodish, 2000).

Once the affected gene product had been identified as a transcription factor, A-Myb, we became interested in the targets of its transcription.

Methods:

Microarrays:

In order to see which genes are affected by the mutation, microarrays were performed. Microarrays measure the expression of mRNA (the intermediate between DNA in the nucleus and the translated protein that is the eventual product). The mRNA expression is a good measure of how much a certain gene is transcribed, with the caveat that certain mRNA is degraded more quickly than other mRNA. Still, relative expression of the same mRNA gives a good measure of how much a certain gene is transcribed.

Microarray analysis was performed on RNA obtained from ground-up testes tissue of both wild-type and mutant mice, both in 17-day post conception (dpc) and 14-dpc mice. The genes that were differentially expressed by two-fold or more in the wild type versus the mutant were noted.

The results of the microarray were in spreadsheet form with no way to sort results by gene function or location, though this information is included in the data set. These results were transformed into a list that could be sorted by gene function and location. This information was used to group the genes that were of interest to us.

This list of genes was reduced in two ways. The genes we considered had a Log2 value above 1 or below -1, meaning there was a two-fold difference in expression between wild-type and mutant. In addition, because the Schimenti lab has microarray expression data on various other meiotic mutants that exhibit male sterility, the A-Myb data were compared against these other mutants. Those genes that were similarly differentially expressed in other mutants were removed from the list as well since they were not specific to the mutation and are likely affected due to the similarity in arrest point of these mutants rather than A-Myb regulation and not necessarily a direct by-product of our mutation. We will re-visit these genes once we have studied the others.

Verification:

The final list of potentially regulated genes needed to be verified. Primers, which are strands of DNA that serve as a starting point for DNA replication, were created for each of the genes using AlleleID 3 software. This was done so that each gene could be amplified in a Polymerase Chain Reaction (PCR) in order for genetic expression to be measured. The list of primers is as follows:

Gene	Sense Primer	Antisense Primer
RIKEN cDNA 1700029I01 gene	TTAGCGATGTGATACTTGAAGCC	TCCCTGATTGAGACTAACGGTAG
RIKEN cDNA 4930428B01 gene	GGGTACTCATGTTCAACTGTG	ATGCCTTGCTTTCATCCC
RIKEN cDNA 4930515G13 gene	AGGGATAAGAGGTTCACGAG	TTGCTGGCGAGATGGTAG
PHD finger protein 7	TTGTGTCAACGACTAAAG	GGTAACAGTCAGAAGAGG

RIKEN cDNA 4933401I19 gene	GACAATCTCGTGGCAATC	TAGGCTTATTCTTGACTTGAG
RIKEN cDNA 1700047I16 gene	TCGTGTCAACATTCCAAG	TGAACCACTCCTAAAGGG
RIKEN cDNA 4930524B15 gene	GGAGCAAAGTACAGCAAGTC	ACCCAGGAAGGTGTCAGG
RIKEN cDNA 4930503B16 gene	TTGCCTGTGTCGTTGCTG	GAATGCTTGGAGTCTTGAGATAG
RIKEN cDNA 4930542C12 gene	TCACAGGTCAACCATCCATCAAAC	CCAGGCAGGAGAATTAAGGCTATC
centromere protein P	GCCTGCTAGGTGTACTTGG	AAAGAGAAATGCTCATAGTCAAC
cyclin N-terminal domain containing 1	GAGAAAGGGAAGGAAGAATC	AGTCACTACAGTTCACCAG
fibronectin type 3 and ankyrin repeat domains	GCCATTGCTCTGTGATTGAC	GCGTCTTTCCATCTTTGTCC
RIKEN cDNA 1700001L05 gene	CATCAGTTGCTTGGAGTCACATAG	TCTGGCTTCCTGGTCCTGTAG
metallothionein-like 5, testis-specific (tesmin)	CGTGTTATCGTCGTTACTTC	GGGATGATGGGAACTTGC
ropporin 1-like	GTGAGGACAAAATAGAGTGG	ATAGGCAAAGGTTTCAAAGG
solute carrier family 2	CCCTGAGAGTCCAAGATTC	CTCCTGTGACATCCGAAC
sorbitol dehydrogenase	TGCGGCTCAGATGTTCACTAC	CAGATGTTTCACCAACTCTCCTAC
TAF15 RNA polymerase II	TCAAGGCAGTCAAGGCTATGG	TGTCCCTGGTTATTGTATGATTGC
RIKEN cDNA 1700108N07 gene	GAATCTTTAAGGGCGAATCTG	CTCCATTGTCCTCTTCTTCC
C2 and coiled-coil domain containing 2	CCAGGACGCACTCAGGAC	TCATTTAACCGCACATATTCATCG
RIKEN cDNA 1700110I01 gene	CCATAGGAGCCATCAAGCAAG	CTAAGCCTGGTGGTGTAAGC
Beta Actin	CGTGCGTGACATCAAAGAG	TGCCACAGGATTCCATACC
bromodomain, testis-specific	TTAAGCGGCAAGAGATTC	GTGGTAGGAGTTGTTGTG
Cancer/testis antigen 3	CCTCTCAAGACTTCTCCTACTCAG	ATCACCTAACAAACTGCCTTCTG
	AGTGAATCCAGATACACCAGAA	GCTGTTCCACTGTATAGACATCAA
RIKEN cDNA 4930528F23 gene	GCCAATATCCTAC	CTATTGTGTTTAAG
RIKEN cDNA 4932415G12 gene	ATTGCCAAGTAACTGCTTCTCAC	ACCCAAAGTCAACCTCTGTCC
RIKEN cDNA 4930418G15 gene	AGTCACTAACCTGAATTACACCAC	TTCTTCTAATCCTTCCAAGCCTTC
H2A histone family, member X	CCCTCGCCTGGACCTTTG	CTAGAGAAACTGGGTTCACATTAC
hematopoietic cell transcript 1	ACTACTGGTGGCTTGGACAATGC	CCGAATGTTCACACGAAAGGAGAC
RIKEN cDNA 1700027M21 gene	GCGATGATAGCATAGTTCTTTCC	GGCATATCTCCACTTCCTTCC
RIKEN cDNA 1700016M24 gene	GGTGGTCAATAAGATCAAGAAC	GAGGTGGAAGAAGAAAGCC
/ubiquitin-conjugating enzyme E2T	GAGGTCACGGAGAGTCTG	GCATGATCTCAAGTGAAAGG
RIKEN cDNA 4930521A18 gene	TGGAAGCAAAGAGCAAGGAAGG	TCGGGATTGAGGAGACTGGATAG

RIKEN cDNA 24 0004F06 gene	GGATTCCTTGACAGACTCG	GCAGTGGCTTCTCA
	GCCTTCCGTGTTCCTACC	GCCTGCTTCACCACCTTC
RIKE cDNA 2610020H08 gene	TG TGG GCAAGAGAAGG	AAAG GGCAGAAATTAGAGG
RIKEN cDNA 4933409K07 gene	CAGACCAGATTGAATGACCTC	GGCTTCTCATCTTCTTCTCG
dehydrogenase	GATTCCTGCTTCTGCTCTCTC	ACACGGGCTCCTCTTTGG
stearoyl Coenzyme		
	AGGCGAGCAACTGACT	GTGGTGGTGG
AN1 ubiquitin like, homolog		
(Xenopus laevis)	CTGCTGAAGGCTAAGATGGAGAAC	GGCTGCTGCTGTAGAACTGC

Table Primers used each potentia

A YBR Green Quantitati vmerase Chain Reacti qPCR used fy the differe al pressed enes. Y g Gree used instead f TaqM probes, because the latter costly results slightl prec Because we were ooking for el large differences pressi in so many genes, so precision term of expressi difference coul be if ced in der to

Homebrew Preparation.

T cut costs further eventuall developed homebrew master mix PCR, mix mad from sc tc rather than urchasing the App ed tems YBR Green Master Mix. A homebrew Y R green mix was touted to work as the commercial abl m at half the st. The do des to the mebrew ix that take ghtly onger to up the reacti each time and that several the components need be re-di uted and re-made every two weeks, else TC will de in ty thus ed ed urnal art to fi th best

homebrew SYBR green mix. One of the articles referred me to usenet and Yahoo! Groups, both of which are online resources where researchers can swap recipes and ask each other questions (Bustin, 2002). used these resources to determine which components were necessary and to get a sense of what concentrations were permissible.

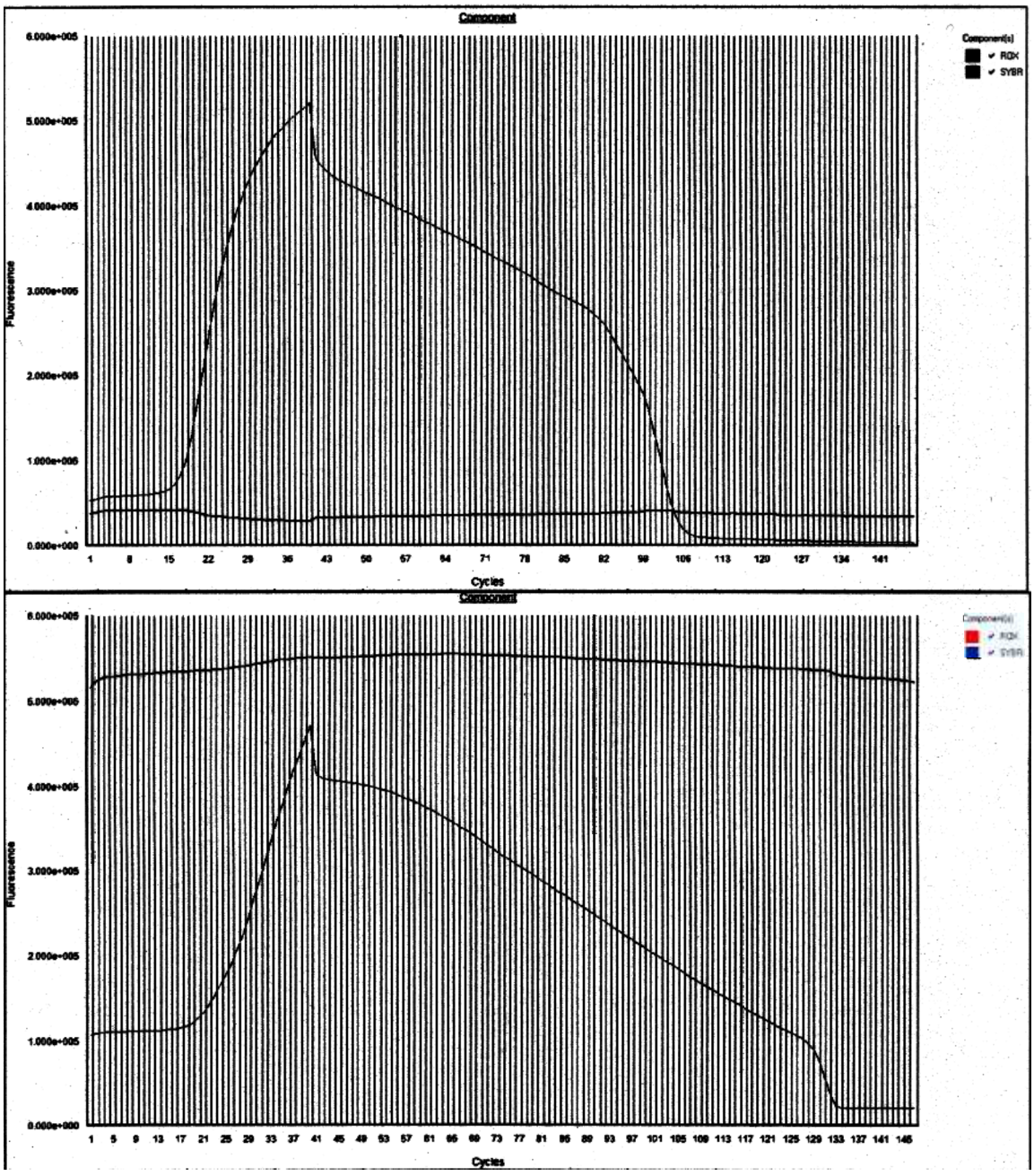
I used a journal article to learn about how each component in my mix would affect the sensitivity of my qPCR reaction. This paper scientifically tested how different dilutions of SYBR green and different substances in which the SYBR could be diluted affected the outcome of a reaction (Karsai, 2002). However, I needed a measurement of background fluorescence, and the article did not discuss this at all. Thus, I used the online resources to find people who had used 6-ROX, which is the background fluorescence component that is included in the commercial SYBR Green mix.

In my next step, I compared the efficiency of my homebrew mix to that of the commercial Applied Biosystems mix. This ensured that my mixture worked similarly to the commercially available mix I was trying to emulate. I set up equivalent reactions using both mixes on one 96-well qPCR plate, so that both mixes would be tested on the same primer sets and under the same exact conditions.

The online resources I used do not go into as much detail as journal articles with regard to which components to use and in what amount. Thus, when I finally ran the commercial mix against my homebrew, the fluorescence was much lower in the homebrew mix (on the order of 10-fold). I found the same results both in repeated testing with the same primers and with other primer sets, thus confirming that it was not user error or a primer-dependent problem. I ran the same three primer sets and the same

cDNA with both my homebrew and the commercial mix to test the efficiency of my new reagents.

When I looked back into the resulting graphs and charts in the software, I was shocked to find that the true fluorescence of SYBR Green in each mix was almost the same (see Figure 1). The problem was that the fluorescence of my baseline reading, the 6-ROX, was entirely too high. Since the software was comparing the SYBR green level against the fluorescence of 6-ROX, the result was a very low fluorescence. Thus, I needed to decrease the level of 6-ROX in the mix.



Figu Top T ia YBR G ee ivtu ompon plo

B ttom: Th omebrew mi itl imi YBR el RO lev ls.

leci d deci th ROX my fo Thi as based

expe th changin BR gree concent ati my had

previously tried to increase the fluorescence of my mix by boosting the SYBR green 10-fold, with almost no results. This happened for two reasons. First, SYBR green inhibits PCR reactions when it is present at high levels (Karsai, 2002). Second, the level of fluorescence of SYBR green was increasing, but I could not see this because the baseline of the 6-ROX was just too high. The raw fluorescence data showed me that the 10-fold increase in SYBR boosted its raw fluorescence about 2/3 as much as I needed to decrease the ROX. Thus, I presumed that a 15-fold dilution of the 6-ROX would be necessary to get similar readings to a commercial mix.

The final mixture was as follows:

2X homemade SYBR green premix: Makes 5mL, thus 400 reactions at 1X
1000 ul 10X PCR Gold buffer (ours has 15mM MgCl2)
2035 ul H2O
1000 ul 10mM MgCl2 (because we have the 15mM in the buffer)
80 ul 25mM dNTPs
25 ul 1:100 diln SYBR Green I nucleic acid gel stain (in DMSO)
1 ul 1ug/ul 6-ROX (in DMSO)
800 ul DMSO
50 ul 5U/ul AmpliTaq Gold
(5ul Tween 20)

Identifying A-Myb Binding Sites:

It is important to see which of the genes being studied have *in silico* evidence of A-Myb binding sites in their promoters, since only these genes can be directly regulated by this transcription factor. Transcription factors bind to particular sequences which are usually unique to one or a few factors. The sequence of the binding site for A-Myb is known to be AAC(G/T)G(G/C)C, with letters in parenthesis representing "wobble" sites that can be either of the two bases. Online software called MATInspector

(http://www.genomatix.de/online_help/help_matinspector/matinspector_help.html) was used to identify potential Myb binding sites in the promoters (1kb upstream region) of each prospective gene. The gene sequences were found at the Mouse Genome Informatics (MGI) website site.

Because the sequence is a short degenerate consensus site, we expect the site to be found relatively frequently. We should expect to find a non-degenerate 7-mer approximately every 1 per 16 kb in random sequence. The frequency increases as we add 'wobble' sites to approximately every 1 per 4 kb. In addition, the sequence is not specific for A-Myb because B-Myb and C-Myb bind the same promoter sequences.

Immunohistochemistry for Staining Histones:

Testes from 21 day juvenile wild type and mutant males were fixed in Bouin's Solution,and paraffin-embedded . Four micron sections were placed on polylysine-coated microscope slides. Slides were deparaffinized by washing in Xylene and subsequent Ethanol dehydration. Antigen retrieval was carried out by boiling slides in a solution composed of Citiric Acid and Sodium Citrate. After 3 washes, 3 minutes each in 1X Phosphate-buffered saline (PBS), slides were placed in primary antibody overnight at 4 degrees Celsius (1:100 dilution). Rabbit polyclonal antibodies used in staining included: Anti-dimethyl-Histone H3 (Lys9) (Upstate #07-441), Anti-acetly-Histone H3 (Lys14) (Upstate #07-353), and Anti-acetyl-H4 (Lys 12) (Upstate #07-595), all from Upstate Cell Signaling Solutions. Slides were once again rinsed thrice in PBS and then placed in secondary antibody (Goat anti-rabbit Alexaflour488; Molecular probes; 1:2000) for 1-2

hours at 37 degrees Celsius. Slides were rinsed in 1X PBS 3 times, counterstained with DAPI (1:10,000), and mounted for microscope analysis.

Chromatin Immunoprecipitation:

The genes that are verified will eventually be tested using chromatin immunoprecipitation (ChIP). ChIP cross-links the transcription factor to the DNA, not allowing it to disassociate. This will allow us to determine which genes are being directly transcribed by transcription factor A-Myb, and which are not. The genes that are not directly transcribed by A-Myb are probably regulated downstream by directly affected genes.

This process will allow us to formulate a model of how A-Myb and the affected genes are involved in spermatoegensis.

Results:

A-myb knockout mice exhibit impaired spermatogenesis in male mice and breast duct epithelium differentiation problems in females (Toscani, 1997). Our mutation in this gene, *Repro9*, produces mutants that do not exhibit the latter effect.

It is evident that *Repro9* mutant testes are reduced in size when compared with those of the normal male mouse. When sections were studied under the microscope, the seminiferous tubules (ST) of adult wild-type testes have a much wider range of cells, from spermatogonia to mature sperm, whereas mutant testes exhibit very few cells past the pachytene stage of meiosis. This led us to believe that the problem in these mutants occurred between the diplotene and pachytene stages of meiosis. There are several important checkpoints during the pachytene stage of meiosis, so this was not entirely unexpected with a meiotic arrest mutant. Inside the nucleus of the wild type,

chromosomes were fully paired. Mutant spermatocytes exhibited regions of incomplete synapsis and nonsynapsed chromosomes. The *Repro9* mutation caused incomplete synapsis to occur during meiosis.

Recombination mapping was used to narrow the critical region (the region that contained the induced mutation) to proximal Chromosome 1 Most recently, complementation analysis performed between *Repro9* and the targeted *MybL1* allele was successful in showing that our mutation is, indeed, in the gene for A-Myb.

The affected *MybL1* gene encodes the 751 aa A-Myb transcription factor. Sequencing showed that the *Repro9* mutation causes an A213<E change in the amino acid sequence. This change did not occur in the conserved DNA binding motif of the transcription factor, yet it has profound effects on affected mice. As stated earlier, Myb proteins work with cofactors, meaning that our mutation may be affecting A-Myb's affinity for binding site or cofactors and thus leading to the observed phenotype. This transcription activates other genes, some of which probably play an important role in spermatogenesis and its checkpoints.

The microarray identified 208 differentially regulated genes in the 14d *Repro9* mutant and 824 differentially regulated genes in the 7d *Repro9* mutant. After narrowing down the list of genes as discussed above, 38 genes remained. These genes are likely to be directly or indirectly regulated by the A-Myb transcription factor. Each of the genes was downregulated either in early-(14dpp) or mid-pachytene (17dpp) testes, or both, when compared with wild-type. A wide variety in the number of binding sites was found in the selected genes. The following table summarizes these results:

Symbol	Description	ΔFold 14d	ΔFold 17d	Myb Sites
1700001L05Rik	RIKEN cDNA 1700001L05 gene	2.046748	2.63056	0
1700016M24Rik	RIKEN cDNA 1700016M24 gene	47.39512	7.863234	5
1700027M21Rik	RIKEN cDNA 1700027M21 gene		26.29962	0
1700029I01Rik	RIKEN cDNA 1700029I01 gene	5.92439	2.563349	1
1700047I16Rik	RIKEN cDNA 1700047I16 gene		2.898963	N/A
1700110I01Rik	RIKEN cDNA 1700110I01 gene		14.59936	2
2410004F06Rik	RIKEN cDNA 2410004F06 gene	2.046748	2.54894	1
2610020H08Rik	RIKEN cDNA 2610020H08 gene	2.639016	2.268107	1
4930418G15Rik	RIKEN cDNA 4930418G15 gene		5.440716	1
4930428B01Rik	RIKEN cDNA 4930428B01 gene	2.046748	4.126864	1
4930503B16Rik	RIKEN cDNA 4930503B16 gene	2.244924	4.766179	0
4930515G13Rik	RIKEN cDNA 4930515G13 gene		2.260276	1
4930521A18Rik	RIKEN cDNA 4930521A18 gene		5.125084	0
4930524B15Rik	RIKEN cDNA 4930524B15 gene	1.823445	2.052448	0
4930528F23Rik	RIKEN cDNA 4930528F23 gene	2.619842	2.376432	0
4930542C12Rik	RIKEN cDNA 4930542C12 gene		5.580487	0
4932415G12Rik	RIKEN cDNA 4932415G12 gene	2.19366	3.473115	0
4933409K07Rik	RIKEN cDNA 4933409K07 gene	2.962195		1
Anubl1	AN1, ubiquitin-like, homolog (Xenopus laevis)	2.578741	6.55276	2
Brdt	bromodomain, testis-specific	1.866086	2.900613	0
Cage1	Cancer/testis antigen 3		6.179938	2
Cenpp	centromere protein P	2.462289	1.693443	1
Cntd1	cyclin N-terminal domain containing 1	5.527662	6.416576	3
Fank1	fibronectin type 3 and ankyrin repeat domains 1	5.278032	5.379372	0
H2afx	H2A histone family, member X	2.046748	2.826852	0
Hemt1	hematopoietic cell transcript 1	4.093495	5.055852	2
Mti5	metallothionein-like 5, testis-specific (tesmin)	5.656854	1.451823	2
Phf7	PHD finger protein 7	3.402669	13.60141	0
Pomt1	N/A	2.40605		2
Rdh11	retinol dehydrogenase 11	2.244924	5.563943	1
Ropn1l	ropporin 1-like	6.240458	4.916782	1
Scd1	stearoyl-Coenzyme A desaturase 1	4		2
Slc2a3	solute carrier family 2 (facilitated glucose transporter)	3.402669	33.06639	1
Sord	sorbitol dehydrogenase	2.094588	6.864824	1
Taf15	TAF15 RNA polymerase II	3.324952		2
Tex24	testis expressed gene 24		4.127569	0
Tkti2	transketolase-like 2	3.482202		4
Ube2t	ubiquitin-conjugating enzyme E2T (putative)	2.14547	1.092407	1
Wwc2	WW, C2 and coiled-coil domain containing 2		2.610097	1

Both 14d and 17d mutant

14d mutant only

17d mutant only

Table 2. Genes that are potentially affected by the mutation in A-Myb

Analysis of the 1kb upstream region of the chosen genes showed that genes with Myb sites had a much greater frequency for the consensus sequence about 200-300bp upstream of + , as should be expected for a binding site for a transcription factor.

Our real-time PCR results suggested that 7 of the primer sets that were used are not optimal. Thus, these primers were re-designed using the same AlleleID software. All 7 of the new primer sets have been verified.

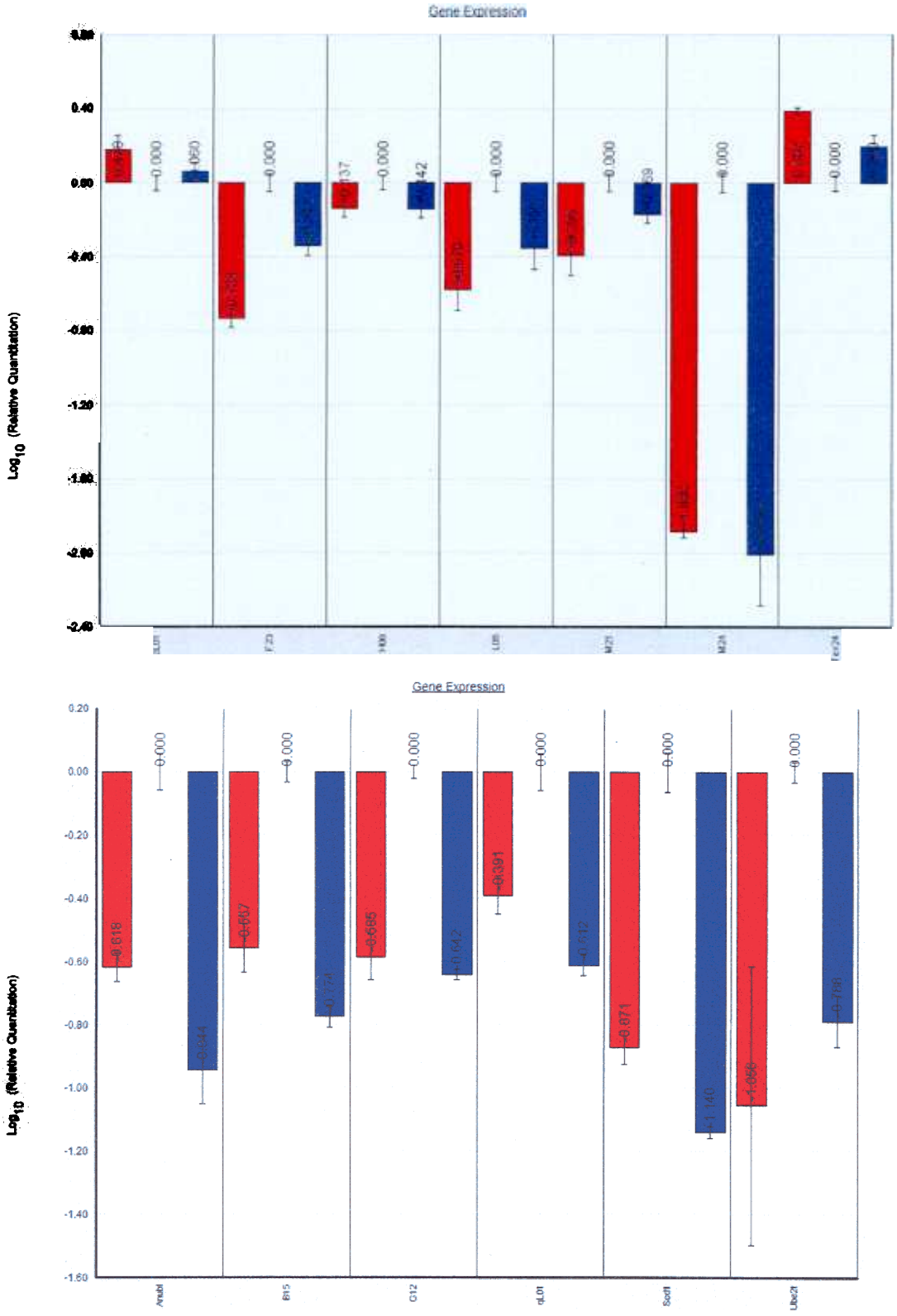


Figure 2: qPCR Results – The middle bar is the wild-type mouse, while the red and blue bars to either side are homozygous mutant mice.

The real-time PCR data thus far has verified the microarray data relatively well (See Figure 2). This means that the genes that were thought to be affected by our mutation are actually being affected. Verification of the expression of the other genes is ongoing.

Histone Staining:

In staining Acetylated Lysine 12 of Histone 4 (21-day Sections), we found staining of this marker in the spermatogonia of the wild-type, as expected, but not in the mutant testes. In staining for Acetylated Histone 3 Lys14 (21-day Sections), wild-type stained in spermatocytes but did not stain in the mutant. The Dimethyl Lysine 9 of Histone 3 antibody (21-day Sections) stained spermatogonia of both mutant and wild type in the same way (See Figure 3). This data must be further studied but it suggests that the *Repro9* mutation seems to be causing differential modifications in histones.

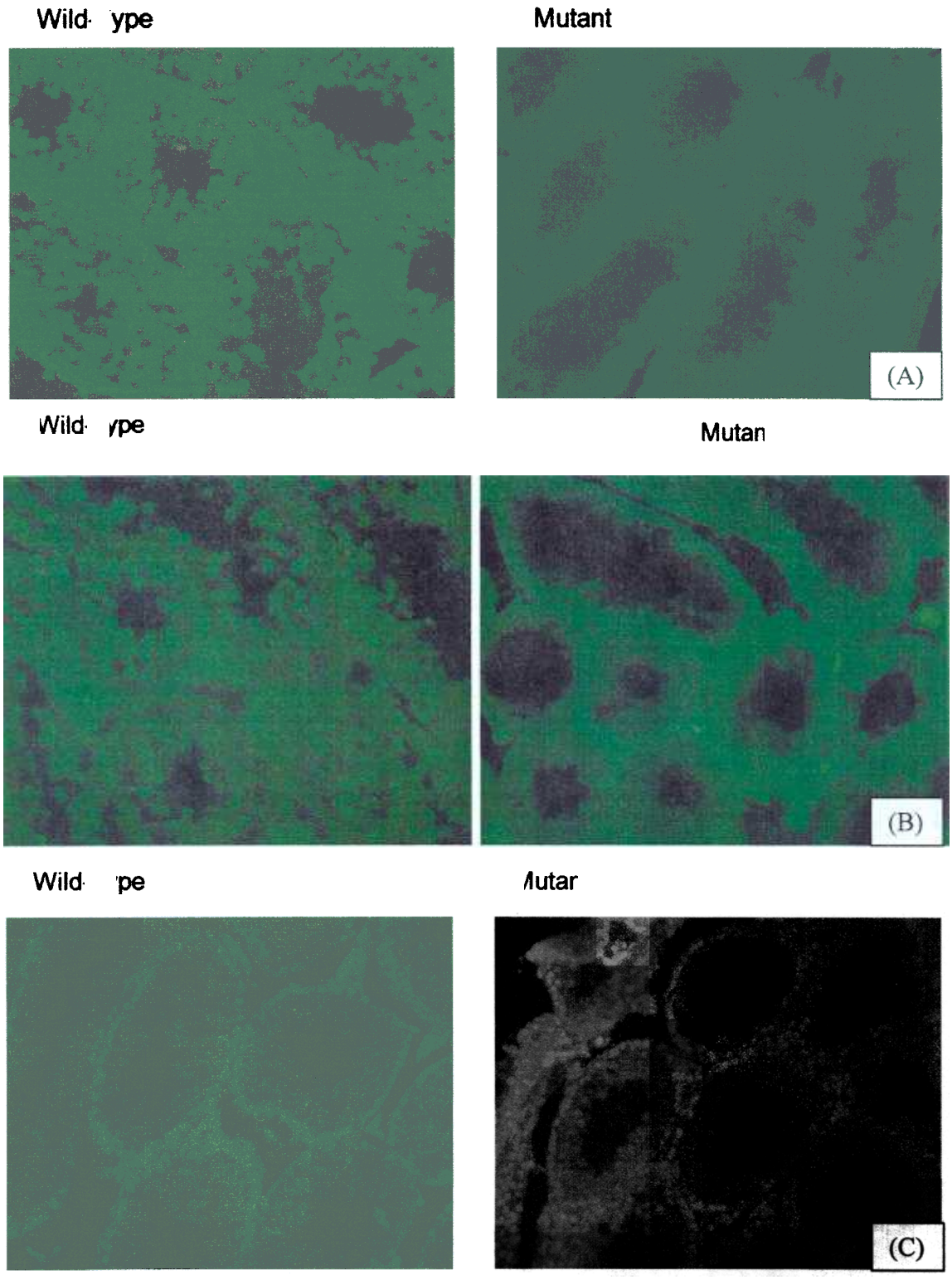


Figure 3: Histone Immunostaining. Top to bottom: (a) Staining of H4 Lys12 (21-day Sections), (b) Staining of Acetylated H3 Lys14 (21-day Sections) (c) Staining of H3 Dimethyl Lys 9 (21-day Sections). Images were captured with 40X objective lens.

Summary of Experimental Findings:

The *Repro9* mutation results in male mice that are sterile and that have decreased testis size. The sterility stems from problems in meiosis that trigger a meiotic checkpoint. Thus, the seminiferous tubules of affected mice contained no cells past the pachytene stage of meiosis. Complementation data shows that the *Repro9* mutation affects the *MybL1* gene and creates a modified A-Myb protein that is the cause of this phenotype.

As a transcription factor, A-Myb affects many genes within the testis, directly affecting those genes that contain a Myb promoter and indirectly affecting downstream genes. Microarray data showed 208 differentially regulated genes in 14d and 824 genes in our 17d mutant, while real-time PCR verified that selected genes are indeed differentially expressed in the *Repro9* mutant.

Mighty Mouse[2]:

The laboratory mouse has been the source of many recent breakthroughs in cancer research, the discovery of stem cells, and insight into how cells differentiate 'self' from 'non-self,' something that is crucial to solving the problem of rejection in organ transplantation. "Mouse work has even begun to revolutionize basic Mendelian assumptions, especially the assumption that a gene's expression is independent of the parental origins of the chromosome" (Rader, 2004).

Why the Mouse?

Human testing can be prohibitively expensive, unethical, or downright impossible. A model organism that will be used to study human disease and physiology must be as

[2] Thanks to Professor Judith Reppy for suggesting this title.

similar as possible to the human while alleviating the problems that plague human testing. The model organism must thus be much less expensive and much easier to work with genetically than humans. *In vitro* cell culture systems are very powerful, but they are too simple. With *in vitro* cultures, we cannot study physiological systems in their entirety, and certainly not their interactions, which are many (Paigen, 2003).

There is a reason that mice are so commonly used: they are simple to breed and make great model organisms.

> Mice are small, relatively tame animals, which makes them easy to handle, house, and feed. They breed readily and often (several times per year), and three weeks after the females have mated, good-sized litters of pups are born, which allows for a quick yield of search results, whether in terms of providing a large sample or observing generational patterns. Finally, mice are mammals with a 99 percent genetic homology to humans, and they happen to get many of the same diseases as us (cancer, heart disease, etc.), which (by extrapolation) makes it possible to track and experiment on many human health conditions in situ. (Rader, 2004)

Because the mouse has served as a model organism for so long, there is a very sophisticated genetic system with hundreds of inbred strains, even more mutants, a dense genetic map, sophisticated mapping resources, and a well-established husbandry system (Paigen, 2003). The rewards from this are faster search results and greater consensus over their meaning. Genetically standardized mice and rats now represent at least 70 percent of all animals used in research.

According to Kenneth Paigen, the director of Jackson Laboratory in Bar Harbor, Maine from 1989 to 2003, "more than 95 percent all mouse models in the world come from Jackson Laboratory." The 2001 JAX annual report concluded that "Researchers around the world agree that JAX mice are the 'gold standard' of genetic purity in mouse models" (Rader, 2004).

It is common to hear of scientific breakthroughs arising from experiments in mice. However, this was not always the case. Only in the twentieth century did the laboratory mouse become an "iconic [symbol] of the value of standardization." (Rader, 2004)

The Mouse Becomes A Model Organism:

Before there was mouse research, there were breeders that bred the strangest mice they could find. The furriest, most hairless, darkest, or any other variation of weird, was treasured in these circles (Rader, 2004). These breeders were not breeding their mice for any purpose other than for pleasure and profit, but their animals would eventually become the basis for murine model organisms.

Interestingly enough, Gregor Mendel, the father of genetics, originally wanted to study the inheritance of coat color in mice. The Bishop of Australia at the time did not allow Mendel to carry out his tests using mice because the animals copulated. Consequently, Mendel turned to peas to discover his laws (Paigen, 2003).

One man, a geneticist by the name of Clarence Cook Little, almost single-handedly made the mouse into what it is today. At Harvard's Bussey Institution, Little was put in charge of tending a small colony of mouse mutants which was being used to study Mendel's laws at the time. These mice were not ordinary mice, but rather mice obtained from fancier organizations in Boston and beyond (Rader, 2004).

The public at this time was very resistant to animal testing, with many debates on the ethics of testing on animals, specifically cats and dogs, which were of sentimental value. Mouse fanciers provided scientists with both mice as a resource and a context in which

mouse breeding was an accepted activity (Rader, 2004). It was partly for this reason that murine research did not have as many hurdles as research on higher-order mammals.

'True' Strains:

In 1909, Ernest Tyzzer published a crucial paper on the inheritance of mice of resistance to growth of transplanted tumors (Paigen, 2003). In a climate of evolution, genetics, and the validation of Mendel's laws in nearly every kind of plant and animal, Little was sure that Tyzzer's results could be explained by Mendelian mechanisms, and thus worked to develop the mice he tended as genetically stable research organisms (Rader, 2004). Little was the first to construct an inbred mouse strain so that reproducible genetic crosses would be possible. (Paigen, 2003).

By the late 1910's, Little had articulated an ambitious vision for a scientific identity of inbred mice as a means of true cooperation between medical and genetic research. As he continued to breed 'true' strains of mice, he also defended his views on the mouse as a model organism, both in research journals meant for the scientific community and in popular magazines (Rader, 2004). At this stage, Little had laid the foundation for the mouse as a research organism by inbreeding to create distinct genetic lines. He also cemented the mouse as a model organism in the research community with his continuing articles and research.

Standardized Protocols:

The research community had begun to look to the mouse as a model organism. The next step was to standardize breeding and research protocols so that results could be more

easily and definitively replicated. Little moved on to the Station for Experimental Evolution (SEE) in Cold Spring Harbor, NY. There, he continued to breed his mice and founded a group called the Mouse Club of America (MCA) that formalized the exchange of material and practical information about mouse breeding. Mouse researchers came to SEE each summer to exchange breeding tips, information about mutant stocks, and breeding experiments. MCA members also collectively communicated these things through periodicals throughout the year (Rader, 2004).

Many of the inbred mouse strains most commonly used today were bred within 20 years of Little's initial inbreeding efforts as a response to the need for genetically uniform stocks in the study of the cancer problem. In no other eukaryote is such a variety of genetically uniform stocks available for genetic work. In fact, the genotypic and phenotypic diversity across the strains often exceeds that of the human population (Paigen, 2003). This diversity allows for research into a broad range of topics using a single organism.

Mouse as a Model for Human Disease:

Little presented mice as models for cancer research during a time when more and more were becoming concerned with this disease. In addition, shifts in the patronage of American scientific work, away from small grants to individual researchers and towards funding for cooperative discipline-building, helped Little to garner more support for his ideas of standardization (Rader, 2004). Roscoe B, Jackson, a wealthy engineer, took an interest in Little's ideas and pushed Little to pursue finances to further his goals. In 1929, with the financial backing of five wealthy Detroiters including Mr. Jackson, Little

founded the Jackson Laboratory (JAX) in Bar Harbor, Maine, whose mice are now considered the gold standard within the scientific community.

At the newly created Jackson Laboratory, Little continued to advance his programmatic goals for mouse work so that he could increase "the recognition and appreciation" of mouse work among medical men. Little strove to create an interdisciplinary exchange among physicians, medical researchers, geneticists, public health advocates, and social reformers (Rader, 2004). His reputation and visibility as a cancer researcher continued to grow, and this afforded him many opportunities both to finance JAX and to collaborate and spread his scientific ideas. Once again, Little spent much of his time attempting to convince both the public and the scientific community of the mouse's ability to model human disease.

Selling Mice:

The Jackson Laboratory opened its doors immediately after the stock market crashed, and money was tight. Little was eventually forced to mass produce and sell Jackson Lab's mice to outside researchers in order to keep the institution alive (Rader, 2004). Lab design and mouse breeding techniques were refined in order to create the best product and to make as many mice as possible, as cheaply as possible; many of these techniques are still being used today. This decision paved the way for the true standardization and industrialization of the laboratory mouse. As a major center for mouse breeding, JAX became a source for standardized research organisms for all mouse researchers.

Mouse breeding was one of the only sources of income for the Jackson Lab, and Little worked hard to sell as many mice as he could to keep the laboratory afloat during the depression. He looked for new markets for his mice and sought to expand the scope of the laboratory mouse (Rader, 2004). As a result of Little's efforts, both researchers and the public began to see the mouse as a model for all diseases, and not just as a model for tumor research. Mice today are used to study all sorts of diseases, from cancer to mental problems like autism, from stroke to heart disease.

George Snell helped push the standardization of mouse genetics by creating reading materials. The publication of *Biology of the Laboratory Mouse* in 1938 was an important milestone in mouse genetics. This book consolidated all established facts on the practice of mouse work that crossed disciplinary boundaries and codified a body of universal scientific knowledge that was once limited to JAX workers. The volume also emphasized the JAX mouse as a gold standard (Rader, 2004). Snell also took over as editor of *The Mouse Newsletter*, which became a centralized listing of all available mouse mutants for genetic study.

Diseases Other Than Cancer:

The next evolution in mouse genetics was away from simple short-term mutational effects and towards long-term studies. This occurred after the Second World War, when the public became concerned about radioactive fallout s a result of the stories and images that came out of Japan. Researchers began to use mice to study radiation's effects, both in terms of dominant and recessive mutations, and the mouse became even more suitable for modeling human disease with this new use (Rader, 2004).

The development of the transgenic mouse allowed for the expression of human and other genes in the mouse. In a dramatic demonstration that captured the imagination of both the lay public and scientists, Richard Palmiter and Ralph Brinster et. Al. fused the rat growth hormone to a mouse promoter and inserted it as a transgene, obtaining very high levels of gene expression and giant mice that made the cover of Nature (Paigen, 2003). This was yet another example of public interest in mouse science.

The steady miniaturization of physiological measuring techniques and introduction of imaging technologies (MRI, CT, PET, DEXA, etc.) are making it easier to use mice and their genetic systems for phenotype exploration (Paigen 2003). Human diseases that were too difficult to observe in the past, such as asthma, inflammatory bowel disease, Type II diabetes, and osteoporosis, can now be modeled in the mouse.

Our Lab's Role:

Our lab's work reinforces the mouse as a model organism for studying human disease. C-Myb, the potential proto-oncogene, was originally studied because the entire Myb family has very close relatives in humans (Gonda, 1982). The gene we study also has a human homolog and is thus applicable to humans. Any information we find about the gene has a high probability of being relevant to humans, and can be used to develop therapies for people and other animals which have homologs of *MybL1*.

In fact, the *MybL1* knockout mouse that is currently being used in our lab comes from a different lab that was using this same mouse to study defects in breast duct epithelium. While female Myb knockout mice have breast duct epithelium problems, affected males are infertile. Our *Repro9* allele has male infertility only, suggesting "separate functions."

Because the mouse is such a standardized model organism, our lab was able to request the knockout mouse and use it in our research. It is tedious and expensive to create a knockout organism, and thus the ubiquity of the mouse as a model organism helped to speed up our research. The availability of the *MybL1* knockout mouse made both projects, as well as any future projects that may involve this gene, possible

This gene is not unique in its homology to humans. The high amount of overlap between mouse and human genes, as discussed above, means that most human genes have mouse homologs and can potentially be studied in mice. The more information we gain from the laboratory mouse, the more socially acceptable and prominent mouse research will become.

In publishing a paper about the mouse, we are reinforcing its use in scientific research. The published material will reach scientists in related fields, and will show them that mouse research is still very relevant to human disease (male sterility, in this case). Electronic access of published papers has made them easier to distribute. Thus, anyone who is interested in sterility or meiotic defects will be able to find our paper and learn about ways in which mice can be used to study human disease.

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