Analysis of multiple genes expressed in the murine eye and lymphatic system during embryonic development.

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Abstract:

My senior research project was completed at St. Jude Children's Research Hospital in Memphis, TN. I did my work in the Genetics department in the lab of Dr. Guillermo Oliver during the summers of 2001 and 2002. Dr. Oliver's lab uses mice as a model to study developmental genetics and embryology. I had a different project each summer, and both of these projects are related in many aspects. The overall goal of both of my projects was to try to understand the function of genes that are expressed in the developing mouse embryo. The first summer I focused mostly on the gene Six 2, and the second summer I focused on gaining a better understanding three genes expressed in the developing lymphatic system. The first summer I learned a tremendous amount about cloning techniques, and I make a the knockout construct for the gene *Six* 2. The second summer I made the conditional knockout construct for the gene *Prox* 1, since the complete knockout is lethal before birth. I undertook another project the second summer, which was very educational, but unfortunately unsuccessful. In this project, I attempted to do a yeast two-hybrid screening of a mouse E10.5 cDNA library to look for genes whose protein products physically interacted with the products of *VEGF-C* and *Flt-4.* Although many of the results of these experiments are not complete, I think they will be very interesting.

***Six* 2**

The *Six* family of homeobox genes acts as transcription factors activating other genes, and initiating some developmental processes (1). The *Six* family of genes are evolutionarily conserved and can be found in organisms ranging from *Drosophila* to humans (1). The *Six* family was identified with the discovery of its first member, *Drosphila sine oculus*, a gene very important in compound eye formation (1).

Most members of the *Six* family contain a very similar domain called the "*Six* Domain," which contains 110-115 amino acids. Since these genes are also homeobox genes, they also contain a conserved homeodomain which is around sixty amino acids in length. This family's homeodomain is quite unique when compared to that of other homeobox genes. The domain does not include an arginine at position five or a glutamine at position twelve in the first helix. These residues are usually responsible for the binding of the transcription factor to the TAAT binding sequence. So, as would be expected, the *Six* family of genes does not bind to DNA containing the TAAT sequence.

Six 2 is one of the members of this family, and this is the gene on which part of my project was focused. *Six* 2 is located on mouse chromosome 17, and its expression is not seen before day E8.5 in development (2). So far only a single transcript of *Six 2* has been found, which is approximately 2.3 KB (2). *Six* 2 is expressed largely in the developing connective tissues of the body such as tendons (2). However *Six* 2 is also expressed in the sensory placodes of early embryos around E9.5, which will later give rise to the eyes and the ear (1). Other areas of *Six* 2 expression include the head mesenchyme E8.5, brachial arches E9.5, primitive kidneys E10.5, and genitals E11.5 (2). To try and better understand the function of the gene, I began a loss of function

experiment or a knockout. When I arrived, Dr. Oleg Lagutin had already cloned the genomic fragment of *Six* 2 into a plasmid he named CE5I. He had also designed the construct, and the majority of my project was creating this knockout construct.

First, I started with the plasmid CE5I (*Six* 2) which was digested with *EcoR1* to obtain the 2.5 K.B. and 1.6 K.B. *Six* 2 / *EcoR1* fragments. These fragments were isolated using gel electrophoresis and gel extraction of the DNA. The 2.5 K.B. fragment was used as the 3' arm of the knockout construct. The 1.6 K.B. fragment was used to detect the proper recombination of the construct with the genomic DNA. Both of these fragments were cloned separately into the vector pbluescript II KS. In preparation for this cloning, pbluescript II KS was digested with *EcoR1* to linearize the vector. Next, the 5' phosphates were removed to prevent self-ligation of the vector. The two *EcoR1* fragments were ligated (sticky/nondirectional) into separate vectors resulting in plasmid CI, which contains the 2.5 K.B. Fragment, and CJ, which contains the 1.6 K.B. fragment.

The purpose of creating CI was to conveniently modify the 3' arm by removing a *Hind* III site, without altering the final construct. The removal of the *Hind* III site within the 3' arm was used as part of the strategy to detect correct construct genomic recombination. To remove this site, CI was partially digested with *Hind* III. Gel electrophoresis and extraction of the DNA was preformed to isolate the fragments only cut once in the desired place. The ends were then blunted with *Klenow*, and the plasmid was self-ligated. This self-ligation will result in the elimination of the *Hind* III site in the 3' arm of the construct, and they new plasmid was called CK.

The vector for the final construct, pKO Scrambler NTKV-1901, contains two scrambler sites for the introduction of the 5' and 3' arms of the construct. It also contains

a neomycin resistance gene as positive selection and thymidine kinase as negative selection. This vector was digested with *EcoR1* and dephosphorylated at the scrambler B site. CK was digested with *EcoR1* to remove the 2.5 K.B. 3' arm of the construct. Both the vector and insert were ran out on an agarose gel and purified by gel extraction to be ligated together (sticky/nondirectional). The resulting plasmid is called CL.

CE5I was used again to obtain the 5' arm of the construct. CE5I was digested with *Nco*1, which will linearize the plasmid, and *Klenow* was used to blunt the ends of the fragment. It was then digested with *Hind* III. The 5' arm of the construct can be isolated as a 6.2 K.B. fragment upon gel electrophoresis and extraction of the DNA. CL, which is pKO scrambler NTKV-1901 already containing the 3' arm, was first digested with Xho1, and the ends were blunted with Klenow. It was then digested with *Hind* III. The ends of the insert and vector were blunted due to the incompatibility of the *Nco*1 and *Xho*1 ends. A ligation was set and the resulting plasmid was the final construct and named CM (blunt/sticky/directional). This construct was sequenced and all junctions were correct, so I concluded that CM was the desired final *Six* 2 knockout construct. Appendix page 1 shows CM map, and page 2 details how the construct will recombine with the genomic DNA.

The proper recombination of the construct with the genomic DNA will be detected using CJ, which contains the 1.6 K.B. *EcoR1* fragment that will be used as a probe in a Southern blot. After electroporation of the embryonic stem cells, the genomic DNA will be digested with *Hind* III, and the radioactive probe will be used to identify the fragment that includes the 1.6 K.B. probe area. If recombination has occurred in the proper place, then one of the *Hind* III sites will be absent and the probe will adhere to a

larger fragment. If the construct has not recombined in the proper place, then the probe will adhere to a 3.0 K.B. fragment.

Another member of the lab, Michelle Self, took over the *Six* 2 knockout project while I returned to school. She did the electroporation of the embryonic stem cells and selected cells that had undergone the proper recombination with the knockout construct. The original plan was for me to return to St. Jude the next summer and analyze the *Six* 2 mutants, but unfortunately the knockout mice were not born in time for me to analyze them.

Results & Discussion:

On October 2, 2002 I received notification that the first *Six* 2 knockout litter had been born and the mice died at birth. The mice appeared to be normal from the exterior of the body, but upon dissection, it was discovered that the mice had no kidneys. Preliminary analysis has shown that the mesonephros and metanephros are present E9.5 through E14.5. The metanephros, which would persist to function as the kidney degenerates sometime between E14.5 and birth.

Another phenotype of the knockout has recently been discovered. Although this is preliminary data, it seems as if all the cells in the retina look morphologically abnormal. It has been hypothesized that there is a defect in amacrine cell differentiation. Experiments are currently being performed to better understand this.

There are still a tremendous number of experiments to be done. It would be very interesting to see how the connective tissues of the knockout mice are affected, since *Six* 2 is highly expressed in this tissue (2). I am also anxiously awaiting the results of experiments which detail how the expression of other genes are affected by the absence

of *Six* 2. These experiments would help to elucidate developmental pathways. Since this gene is a transcription factor, it is expected to affect to have some very interesting results. I also think it would be beneficial to make a conditional knockout, since the mutation is lethal. This is the same type of experiment that I did in another part of my project, and it will allow us to see the tissue specific effects of the gene while avoiding lethality. Although my aspect of the project is finished, I plan to keep in touch with the lab and stay updated on any new results that are obtained.

***Prox* 1**

The next gene studied was *Prox1*, a vertebrate homeobox gene related to the *Drosophila* gene *prospero* (3). *Prox1* is expressed in the CNS, liver, heart, lens, retina, pancreas, skeletal muscle, and lymphatic endothelial cells of mice (3). The polarized expression of this gene by a subset of cells the cardinal vein gives these cells a bias towards becoming lymphatic tissue (4). Upon the expression of other genes, one of which will be discussed later, these cells bud off and become the first lymphatic endothelial cells (4). A knockout of *Prox1* was developed, and the mice died before birth (3). Analysis of the knockout embryos showed that cells did begin to bud from the cardinal vein, but did not continue to develop into lymphatic vessels (3). It was concluded that *Prox1* expression is required to maintain budding and develop lymphatics (3).

Analysis of *Prox1* heterozygotes has been done, and it has yielded some interesting results, all of which indicate a disruption in lymphatic development (3). The mice have lymph edema, a build up of chyle in the intestines, and abnormalities in the

positions of lymphatic vessels (see appendix page 3) (3). It is desirable to make a conditional knockout of *Prox1* to see how inactivating the gene at particular times and places in development will affect the tissue while avoiding lethality.

Dr. Natasha Harvey had already designed the construct and cloned part of the genomic sequence of *Prox* 1 in plasmids P23 and P5. My job was to execute the plans and create the knock-out construct. The first step was to cut the 3' arm of the construct out of the P23 plasmid. This arm contains exons 2 and 3 of the *Prox* 1 gene. P23 was digested with *Cla* I (sticky) and *EcoR*V (blunt) to generate the 8.8 KB insert fragment. Then next step was to digest the vector, pNeotkLoxP, with *Sal* I and then blunt the end with *Klenow*. The vector was digested with *Cla* I. The first ligation was set with the 3' arm and pNeotkLoxP (directional/sticky/blunt). To prepare for the ligation of the 5' arm into the construct P5 was digested with *Not* I and blunted with *Klenow*. This generates the 2.2 KB insert fragment. To prepare the vector for this ligation, pNeotkLoxP + 3' arm was digested with *EcoR*V (blunt) and *Hind* III, which was blunted with *Klenow*. The second ligation is a blunt, nondirectional ligation.

The next steps for the conditional knockout will be to electroporate the embryonic stem cells and select cells which have under gone the proper recombination. Dr. Harvey introduced LoxP sites into the intron sequence of *Prox* 1 as can be seen in figure 1 on the next page. When the construct recombines with the genomic DNA it will appear as in the first line of the figure. When a mouse who is homozygous for the Prox 1 conditional knockout is crossed with a line of CreLoxP mice, their offspring will be the ones to express the desired phenotype. Depending on the line of Cre mice the used, Cre recombinase will be expressed in a certain tissue, at a certain time and cleavage of the

construct will occur. The ultimate result will be the bottom line, in which Prox 1 is not functional due to the removal of exons 2 and 3.

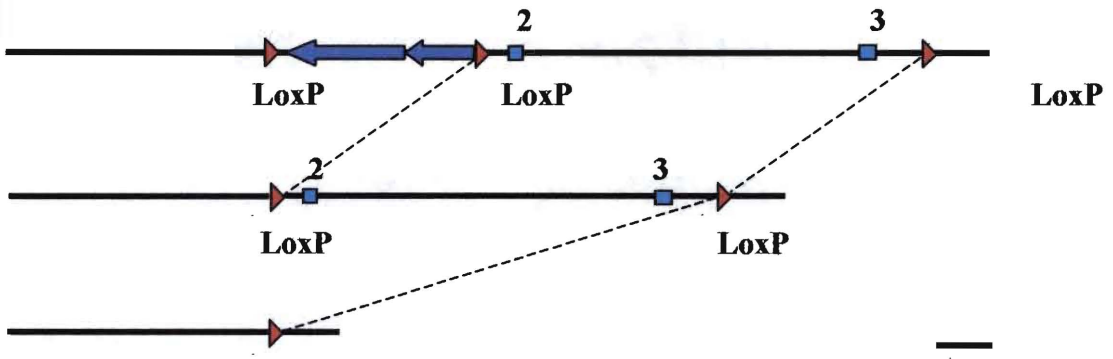

Figure 1. This describes the way this construct will work in a developing embryo expressing CreRecombinase. The end result will be the cleavage and removal of exons 2 and 3. This will leave *Prox* 1 nonfunctional.

Results & Discussion:

After three ligation attempts, one arm of the *Prox1* conditional knockout was successfully ligated in the proper orientation. When I left St. Jude, colonies were still being screened to see if any of the ligations for the second arm were successful. I was notified in September that a colony hybridization revealed that the construct was complete. Sequencing has been ordered to check the junctions in the construct. Dr. Natasha Harvey has taken over the project, and I expect to have the first *Prox* 1 conditional knockout mouse around December 2003.

***VEGF-C* and *Flt*-4 Yeast-two hybrid library screening**

The other genes being studied in the yeast-two hybrid library screening are *Flt-4* and *VEGF-C*. *Flt-4* (also known as *VEGFR-3*) is first expressed in developing blood vessels and later in lymphatics (4). *Flt-4* is a vascular endothelial growth factor receptor. It is one of the first genes expressed by cells that bud off from the cardinal vein to develop into lymphatics (4). Later in adulthood, *Flt-4* is expression is restricted to

lymphatic tissue (4). A missense mutation in *Flt-4* results in a genetic disorder called Milroy disease (4). This disease results in lymph edema and is due to a missense mutation that disrupts the tyrosine kinase activity of *Flt-4* (see figure 2 below) (4). *VEGF-C* is a vascular endothelial growth factor, and it is the ligand of *Flt-4* (4). Over expression of this gene induces lymphangiogenesis, which could be important in tumor growth and metastasis (4). It would be interesting to see what other proteins or co-factors these molecules bind, since they are both present in lymphatic vessels. This will be accomplished by a yeast-two-hybrid library screening.

Fig. 4. 9 year-old girl with chylous reflux and left leg lymphedema. Injection of radiotracer only into the right foot refluxes into the left leg all the way to the foot.

Figure 2. A 9 year old child with Milroy Disease. Note the lymph edema that results from a mutation in *Flt*-4.

Flt-4 and *VEGF-C* DNA was obtained for the library screening constructs by RT-PCR from cDNA. The PCR primers were designed to incorporate an additional *Not* I and *Sal* I site on the ends of the fragment to facilitate future cloning. The PCR products were ligated into pgem-T-easy because the PCRs were very sensitive, and only small amounts of DNA could be made. The next step was to clone the genes into the vector that would be used for the library screening, PC97B.

The library screening will help to identify any genes in the mouse E10.5 cDNA library that physically interact with the bait gene (*VEGF-C* or *Flt*-4). This is done by

cloning genes in frame with domains of a transcription factor. The only way that the transcription factor can activate its target gene is if the bait protein and the library protein bind to one another. The concept is that if the bait and library proteins interact the two domains of the transcription factor will be in the correct locations to activate the reporter genes. A figure on appendix page 4 illustrates this concept. The reporter genes were *lac Z*, *HIS 3*, and *URA 3*. The *lac Z* provided the opportunity to use IPTG and X-GAL to test for positive (blue) colonies. The *HIS 3* and *URA 3* genes allowed for selection of positive colonies as ones that can survive on plates lacking histidine and uracil.

Results & Discussion

Seven ligation attempts were made to ligate *Flt-4* into PC97B, and none were successful. *VEGF-C* was cloned parallel to *Flt-4*, and the second ligation attempt was successful for *VEGF-C*. During this time both genes were sequenced in pgem-T-easy, and both were found to have missence and nonsense mutations as a result of PCR. Unfortunately due to the sensitivity of the reactions, none of the high fidelity polymerases worked. The PCRs were very hard to optimize, and successful reactions were not reproducible. The mutation in *Flt*-4 produced a stop codon in the middle of the sequence, so this clearly means that we must go back and obtain *Flt*-4 sequence that is not mutated either by PCR or other means. At the end of my time at St. Jude, no clonings of *Flt-4* had been done without mutations. In order for the library screening to be successful, a fully functional protein had to be produced, so this project has not made any significant progress. There were two mutations in *VEGF-C*. One involved changing a tyrosine to methionine and the other changed a proline to leucine. These mutations are clearly not extreme changes, and they are not in functional or conserved domains. However, since

wild-type protein conformation is essential in this experiment the results obtained using the mutated constructs would have to be repeated to confirm that there is an interaction between the wild-type *VEGF-C* and the library protein. When the sequencing errors were discovered, the *VEGF-C* library screening was already underway. Nineteen clones had passed the His selection and other screenings were underway. New *VEGF-C* PCR cloning attempts were also started. Currently another member of the lab, Jaime Morgan, is attempting to clone both of these genes, and she has had no success.

Conclusion:

The final result of my senior research project, as of Nov. 14, 2002 is to have developed the knock out construct for *Six* 2 and the conditional knock out construct for *Prox* 1. Overall, I feel that my project was a success most importantly because I learned so much information in those two summers. I also feel that in doing my project I utilized and combined the information from almost all of my classes, and it was truly an educational conclusion to my undergraduate education.

References:

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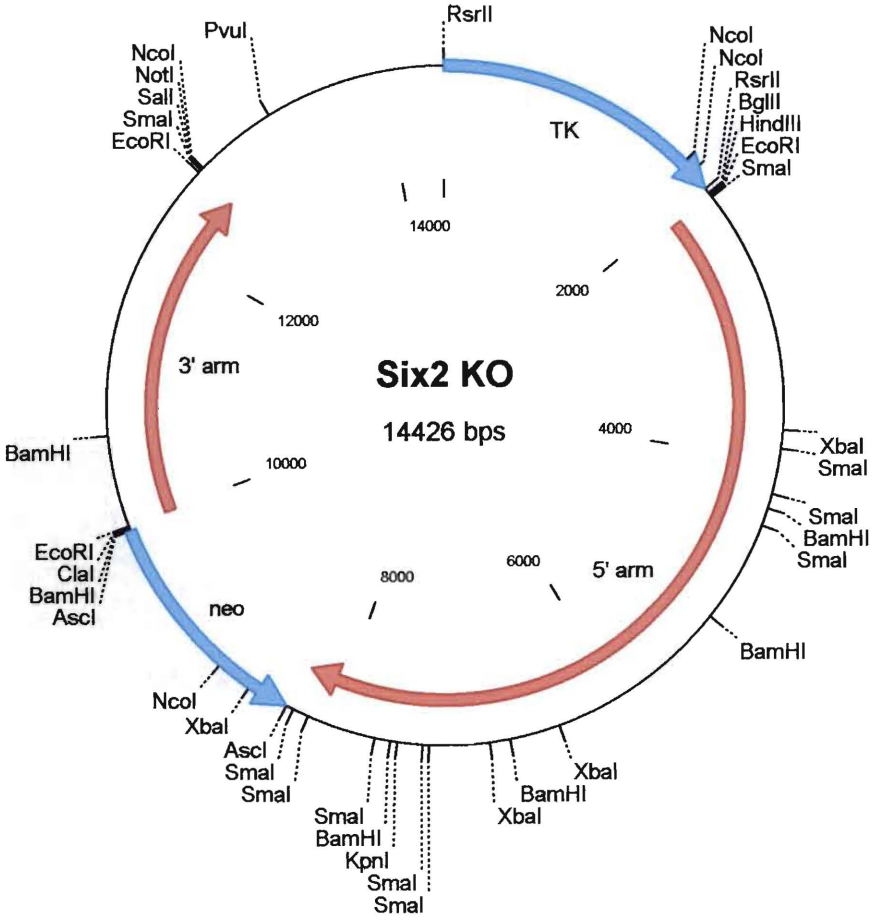
2. Oliver, G. Wehr, R., Jenkins, N.A., Copeland, N. G., Cheyette, B.N.R., Hartenstein, B., Zipursky, L.S., Gruss, P.(1995). Homeobox genes and connective tissue pattering. *Development* 121:693-705.
3. Wigle, J. and Guillermo Oliver (1999). *Prox1* function is required for the development of the murine lymphatic system. *Cell* 98, 769-778.
4. Oliver, G. and Michael Detmar (2002). The rediscovery of the lymphatic system: old

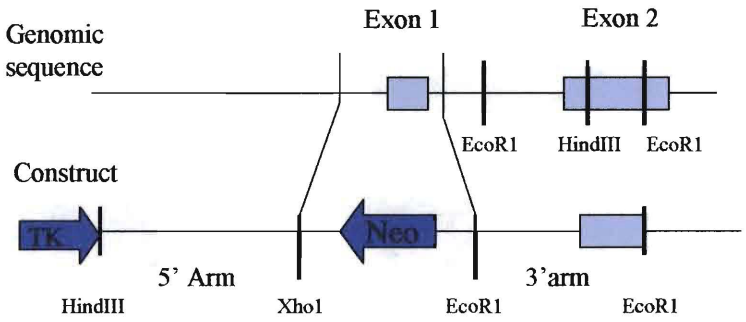
and new insights into the development and biological function of the lymphatic vasculature. *Genes & Development* 16, 773-783.

Appendix pg 1



Appendix pg 2

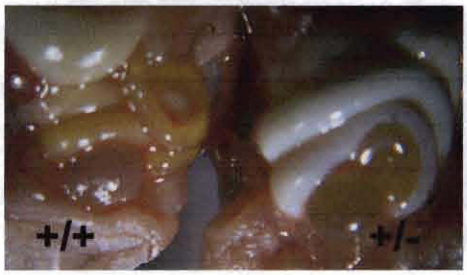
Six 2 Knock Out Construct



Appendix pg 3

Prox 1 +/- Phenotype

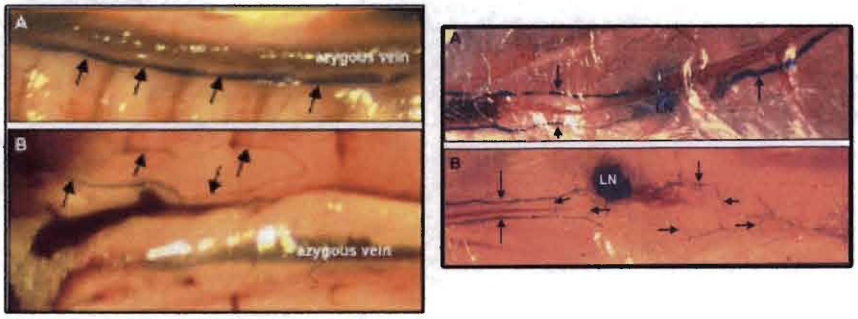
edema



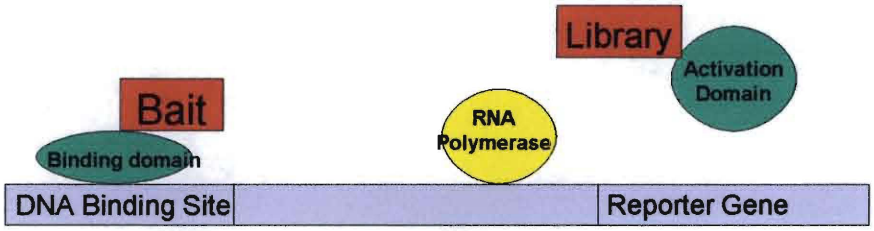
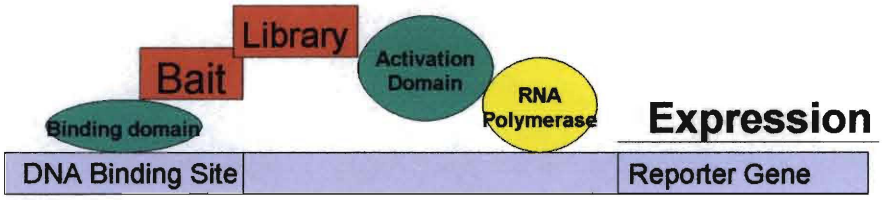
chyle

+/+

+/-



lymphatic vasculature abnormalities

Appendix pg 4

Yeast Two-hybrid Screening