

# **Gene Expression During Neural Tube Closure.**

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### **Abstract**

Neurulation is the process of neural tube formation in embryos. The neural tube will develop into the brain and spinal column. Pax3 is a transcription factor that is important for neural tube closure. Two good copies of the Pax3 gene are present in normal individuals. Splotch mice are heterozygous mutant for the gene; they have one good copy of the gene and one bad copy of the gene. As a result of this they express a white splotch on their bellies. The white splotch is present because the neural crest cells that give rise to melanin cells have failed to migrate down to the belly section. Mice that are homozygous or have two bad copies of the Pax3 gene die at embryological day 14. Neural crest cell migration is necessary for proper closure of the neural tube. Studies have shown that Pax3 may affect cell adhesion properties. In particular, in mice with two bad copies of the gene and in humans with one bad copy of the gene and one good copy these defects lead to Spina bifida and other disorders of the neural tube. It is not known how Pax3 works. Mayanil et. al. (2001) identified several potential downstream target genes for Pax3. Downstream target genes are genes that Pax3 could bind to and activate. The purpose of the studies presented here was to further investigate if these genes are truly down stream target genes for Pax3. Genes selected had the highest ranking for properties that would allow them to interact with the Pax3 transcription factor, these genes included TGF $\beta$ 2, NeuroD, and Pre pro $\alpha$ 2 (1) collagen gene. In order to visualize if the genes were present and active during neural tube closure normal and splotch mouse embryos were prepared and taken through a staining process for NeuroD, TGF $\beta$ 2, and Pax3. The staining was done at a point in development when Pax3 should be interacting with these genes. The results showed that Pax3 does co-localize with Pre-pro $\alpha$ 2(1) collagen gene and TGF $\beta$ 2. These findings indicate that Pax3 probably acts through these two genes for some of its affects on neural tube closure, which again develops into the brain and spinal column. Future studies will focus on the role of the Pax3 gene in neural tube closure.

### **Neurulation**

In between conception and birth there are many important processes that give rise to a normal, functioning human being. One such process is neurulation. Neurulation takes place just after gastrulation, a process that results in a developing embryo that is only three layers thick and no larger than a grain of rice. These three layers are the internal endoderm, the intermediate mesoderm, and the external ectoderm layers. It is interactions between the mesodermal layer and the ectodermal layer that begin another important process, organogenesis. It is during organogenesis, the formation of tissues and organs, that neurulation begins. Through neurulation the ever changing and growing embryo will develop a neural tube that will eventually become the brain and spinal column.<sup>15</sup>

The neural tube forms in a coordinated and simultaneous sequence of events where every step is critical. The external ectoderm divides into three new cell types. Some cells receive chemical signals to elongate, by doing so they create the neural plate. This neural plate will become the inside of the neural tube. Other cells receive chemical signals to flatten, and these cells will become the epidermis. The epidermis is a layer of cells that will separate from the newly formed neural tube and form skin. The third type of cells formed from the external ectoderm, neural crest cells, migrate out from in between the epidermis and the neural tube, these cells will form much of the peripheral nervous system.<sup>15</sup>

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The neural tube starts to form at separate times and at five different locations along the dorsal or back-side of the embryo. These five sites extend from where the tail bone will be located to where the brain will be located. Failure of the neural tube to form properly at any point will lead to a disease state or death of the embryo.

Anencephaly (without brain) results when the portion of the neural tube that will become the brain fails to form properly. The neural folds in this location do not come together and the neural tube does not close completely.<sup>10</sup> This leaves the developing brain exposed to the mother's amniotic fluid which deteriorates the brain and leads to the death of the embryo.<sup>7</sup>

Another developmental abnormality that results from improper closure of the neural tube is Spina Bifida. Spina Bifida results when the posterior portion of the neural tube, where the spinal cord will form, does not close completely.

### **Spina Bifida**

The symptoms of Spina Bifida vary with each patient, the type of Spina Bifida, and with the location of the abnormality along the neural tube. The less severe form of Spina Bifida is Spina Bifida Occulta. In this form a small dimple or a tuft of hair will appear at the location where the neural tube did not close completely.<sup>10</sup>

More severe forms of Spina Bifida are marked by either a tumor or cyst at the site where the neural tube failed to close. In one of the more severe forms of Spina Bifida a small fatty tumor (lipoma) forms beneath the skin at the location where the neural tube failed to close which in most of these cases is at the base of the spine or the lumbosacral spine.<sup>10,14</sup> Another possible

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deformity occurs when a cyst protrudes at the site where the neural tube failed to close completely. This cyst is filled with cerebral spinal fluid and may contain the meninges which is the lining of the spinal column, this type of cyst is termed a meningocele.<sup>10,14</sup> Yet another type of deformity and a more severe one is a cyst that protrudes from the opening where the neural tube failed to close. This cyst is filled with cerebral spinal fluid, meninges, and a portion of the spinal cord and spinal nerve roots. This type of cyst is known as a myelomeningocele.<sup>10,14</sup>

The symptoms of Spina Bifida vary among patients. Factors involved in the severity and increase in complications of Spina Bifida include not only the type of deformity but also the location of the deformity along the spinal column. The spinal cord serves as a messenger between the brain and rest of the body. Our bodies rely on this free flow of information to grow, move, talk, and to feel our surroundings. Because of this, children afflicted with the types of Spina Bifida in which the meninges, cerebral spinal fluid and parts of the nerves and spinal cord are involved will generally have more severe symptoms.

Communication with the brain is necessary for the body to carry out its proper functions because of this a key factor in terms of patient complications is the location of the deformity along a child's spine. All of the severe forms of Spina Bifida will include as symptoms bladder and bowel dysfunctions and complications of the lower extremities because the nerves to these areas of the body are located at the base of the spine. Typically, for the same reason the lower limbs of these children tend to show atrophy, they are smaller and thinner. This results from the lack of communication to the area during pregnancy.<sup>14</sup> As the cyst occurs higher up on the spinal column the symptoms and complications increase. This is because as the cyst gets higher on the

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spinal column more nerves are involved and become none functional. With greater loss of nerve function paralysis occurs. This paralysis can range from slight paralysis and loss of sensation in the extremities to total paralysis.<sup>14</sup> Most children with the severe forms of Spina Bifida will also have hydrocephalus which means water in the head. This “water” is really cerebral spinal fluid that is no longer circulating as it would under normal conditions. Although folic acid may help to prevent some forms of Spina Bifida, a woman must have taken it for at least one month prior to becoming pregnant and continue taking it for at least a few months following conception. Folic acid can stave off 70 percent of Spina Bifida cases.

There are many treatments for the multitude of symptoms and complications of the disease and patients usually see a team of specialists for this. Treatments range from orthopedic devices to help with walking to surgical implantation of a shunt in the brain to help circulate the cerebral spinal fluid in a more normal fashion.<sup>14</sup> Although there are treatments to help with the symptoms there is no 100 percent solution to the problem. Both environmental and genetic factors may play a role in Spina Bifida so it is important to identify the genes involved in neural tube closure so that preventative treatments can be found.

### **Possible Downstream targets of Pax3**

Many genes appear to be critical for neural tube closure. Genes are specific sections of one’s deoxyribonucleic acid (DNA). Every gene codes for a specific protein. In order for a gene to produce protein it must first be activated. Activation of a gene requires the help of many different proteins. One very important type of protein is a transcription factor. A gene cannot be activated without the help of a transcription factor. This factor must first bind to the gene at a

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specific section known as the promoter region. A transcription factor must be specific for the DNA sequence of the gene's promoter region for it to be able to bind to it. Once the transcription factor has bound, other needed proteins are assembled so that an intermediate product, messenger ribonucleic acid (mRNA) is first made with the use of the assembled proteins as they travel down the length of the gene. The intermediate product is separate from the gene and it is then translated or made into protein with the help of yet other proteins.

Pax3 is an important protein involved in neural tube closure. This protein is a transcription factor that is found in developing embryos at the time of neurulation.<sup>8,11</sup> Pax3 is expressed in developing mouse and chick embryos at specific times and locations that are important for neural tube closure.<sup>8,11</sup> These locations include the neural plate, the neural folds, and the neural crest cells which will migrate out from the area of neural tube closure.<sup>11</sup> Due to its location and documented expression pattern it is most likely that Pax3 can bind to and regulate the activation of gene's that are necessary for proper neural tube closure. If Pax3 is a transcription factor for other genes involved in neural tube closure then it must be able to bind to their promoter regions.

### **Study of Pax3's Regulatory Affects**

Mayanil et al. (2001) wanted to investigate if Pax3 could be binding to and regulating other important genes involved in neural tube closure. In order to do this the lab first had to isolate genes that Pax3 might bind to.

One way to determine if Pax3 was a transcription factor for other genes was to transfect or insert extra copies of the Pax3 gene into a Daoy medulla blastoma cell. This is a cancer cell

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line. Cancer cell lines are often used in research because they continuously divide and therefore do not die out. Daoy medulla blastoma cells do not normally express large amounts of the Pax3 protein. The extra copies of the gene were inserted with the use of a plasmid, a circular section of DNA into which the selected gene has been spliced.

The transfectant cells or cells that have accepted the plasmid with the extra copies of the gene are selected for by growing them on petri dishes with a specific antibiotic. Along with the extra copy of Pax3, the plasmid also contains a gene that will make the cells resistant to the specific antibiotic. In this way only the cancer cells that have the plasmid with the antibiotic resistant gene can survive in the petri dish. After the cells that had accepted the extra copies of the Pax3 gene were selected they along with untransfected cells of the same type were screened through a complex process known as Affymetrix.<sup>12</sup>

Affymetrix screens for differences in gene activation. Cells with transfected Pax3 express more Pax3 protein than non transfected cells. This protein in turn will up-regulate or down-regulate gene expression. When cells transfected with the Pax3 transcription factor gene are compared to the cells that were not transfected the difference in their levels of downstream target gene regulation is revealed.

### **Results of Affymetrix**

Out of the estimated 30- 50,000 genes in the body, one thousand possible downstream target genes of Pax3 were screened through Affymetrix. The results of the Affymetrix showed that 270 genes out of the thousand screened had mRNA protein expression levels that had been

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altered in comparison with the nontransfectant cancer cell line. These two-hundred and seventy genes are all possible downstream target genes of Pax3.

As a transcription factor Pax3 must be able to bind to the promoter region of its possible downstream target genes. Pax3 has three ways of binding to the DNA of the promoter region of its downstream target genes. Pax3 has two DNA binding domains; it has a paired binding domain and a homeo (single) binding domain. These two DNA binding domains are separated by a length of the protein that does not participate in binding to downstream target DNA.<sup>1,12</sup> The Pax3 transcription factor is a protein that can bind to a downstream target gene's promoter region using its paired domain, its homeodomain, or by using both its paired and its homeodomain at the same time. Each specific binding domain has one to three specific DNA sequences of the target gene's promoter region that it recognizes and binds to.

In order to categorize the genes selected through Affymetrix by order of importance, a computer program was used that would assign a score to a possible downstream target for each time that one of the Pax3's binding sequences appeared in the genes promoter region. This method generated a new list of the previously selected genes in order of the highest number of times the binding pattern appeared to the lowest number of times that the binding pattern appeared.

Mayanil et al. (2001) went on to investigate some of the genes identified with Affymetrix using other techniques to find if they truly are Pax3 downstream target genes. Their group selected genes based on high Pax3 binding scores and based on the findings of other studies that

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implicated the genes as being involved in neural tube closure. The selected genes included Pax3, NeuroD, TGF $\beta$ 2, and pre-pro  $\alpha$ 2 (1) collagen.

The three genes selected along with the Pax3 gene for further studies serve different purposes during neurulation. The NeuroD gene codes for a transcription factor protein that seems to activate other genes that are involved in neuralation.<sup>10</sup> The transforming growth factor beta 2 (TGF $\beta$ 2) gene codes for a type of protein that can regulate cell growth, division and death.<sup>4</sup> This gene is found on human chromosome 19, and on mouse chromosome 7.<sup>2,4</sup> The Pre-pro  $\alpha$ 2 (1) collagen gene codes for collagen. Collagen is important in the development of the early embryo.<sup>3</sup>

These genes were examined in a cell system, it was found that the genes seem to be downstream target (DST) genes of Pax3. The question now was would the same genes show positive results in an animal system.

### **Whole Mount *In Situ* Hybridization**

To help in analysis of a genes function it is necessary to examine the genes expression in relation to time and space within the organism or tissue being studied.<sup>16</sup> Whole mount *in situ* hybridization (WISH) is used for this purpose. WISH gives three-dimensional visualization of the mRNA being expressed. WISH is the labeling of a specific genes mRNA by hybridizing it with a labeled anti-sense probe. To understand how WISH works it is necessary to know a little more about DNA and mRNA structures.

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DNA is made up of four nucleic base molecules. They are adenine, cytosine, guanine, and tyrosine. DNA is double stranded and the two strands bond with each other in a specific manner. The two strands always come together with the bases on one strand binding with their corresponding bases on the other strand. Adenine always binds with tyrosine and guanine always binds with cytosine.

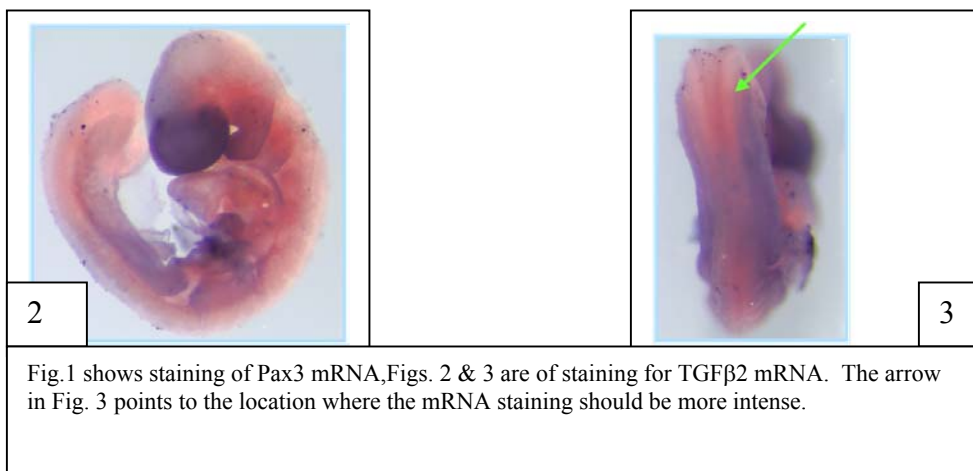
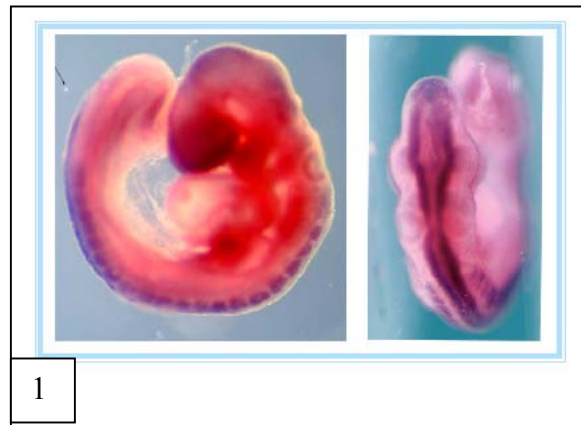
Messenger RNA is a very short and somewhat altered copy of DNA; this is because it codes for one specific gene. Messenger RNA is single stranded and tyrosine is replaced with uracil, another base molecule. The mRNA is considered a sense strand and the anti-sense probe is a strand that will pair up with the sense mRNA strand in much the same way as two DNA strands bind with each other. The only exception is that adenine will bond with uracil. Once the probe and the targeted gene's mRNA have bonded, a color reaction takes place that will help to visually localize the mRNA. Sometimes though this resulting dye or color is difficult to see in a large, whole embryo so that the embryo needs to be sectioned and placed on slides for the color labeled probe to be seen. The sections are then looked at under a microscope to visualize the gene's mRNA.<sup>16</sup>

The whole mount *in situ* hybridization was performed on mouse embryos at a critical age of 9.5 days old when the neural tube is still being formed. The mRNAs that were targeted were those of the genes that scored highly for Pax3's binding motif and that also were significant per previous studies. These genes were Pax3, TGF $\beta$ 2, and Neuro D.

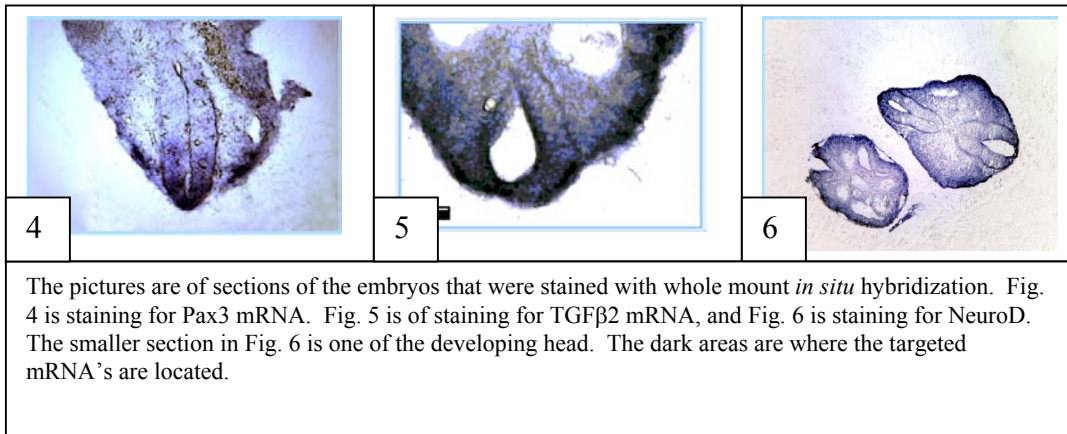
The results of the whole mount *in situ* hybridization in the embryo showed low intensity with the dye (Figures 1-3). Therefore, to get a better view, the embryos were sectioned. Within

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the sections it was easy to see the location of the staining for the targeted genes mRNA. All the genes showed staining within the same areas and at the same time of embryological development (Figures 4-6). This is important because it shows that because they are all being activated within the same vicinity as Pax3, Pax3 could be activating them to produce mRNA.



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### Fluorescent Immunohistochemistry

The purpose of fluorescent immunohistochemistry is to label a specific gene's protein. This process is accomplished with the use of protein specific antibodies. Antibodies are “Y” shaped proteins that are part of an organism's immune system's defense against foreign substances.<sup>6</sup> The top “V” shaped portion of the antibody is specific for binding to one foreign substance, in this case it is specific for the targeted protein. This is the role of the primary antibody. Once the primary antibody has bonded to the targeted protein, a secondary antibody that has been labeled with a fluorescent dye is applied. This secondary antibody targets the primary antibody. The fluorescent dye is observed in the location of the targeted protein.

This fluorescent immunohistochemistry was performed on two different types of mouse embryos. The first type was the wild type, this mouse has two normal copies of the Pax3 gene

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and this is denoted by a (+/+) that represents the two identical, functioning copies for that gene. Two identical copies of a gene are termed homozygous.

The other type of mouse used was a splotch mouse, which only has one functional copy of the Pax3 gene. The other copy of the gene is dysfunctional. This is denoted by a (+/-), and is termed heterozygous. The bad copy of the gene does not produce protein. Splotch mouse, although it does only have one good copy of Pax3, does have a properly formed neural tube; however, the mouse tends to be smaller and has a white splotch on its belly from where the neural crest cells have failed to migrate. Neural crest cells will give rise to skin cells that produce melanin. Due to failure of the neural crest cells to migrate to the belly area of the mouse it remains without pigmentation and is therefore white.<sup>9</sup>

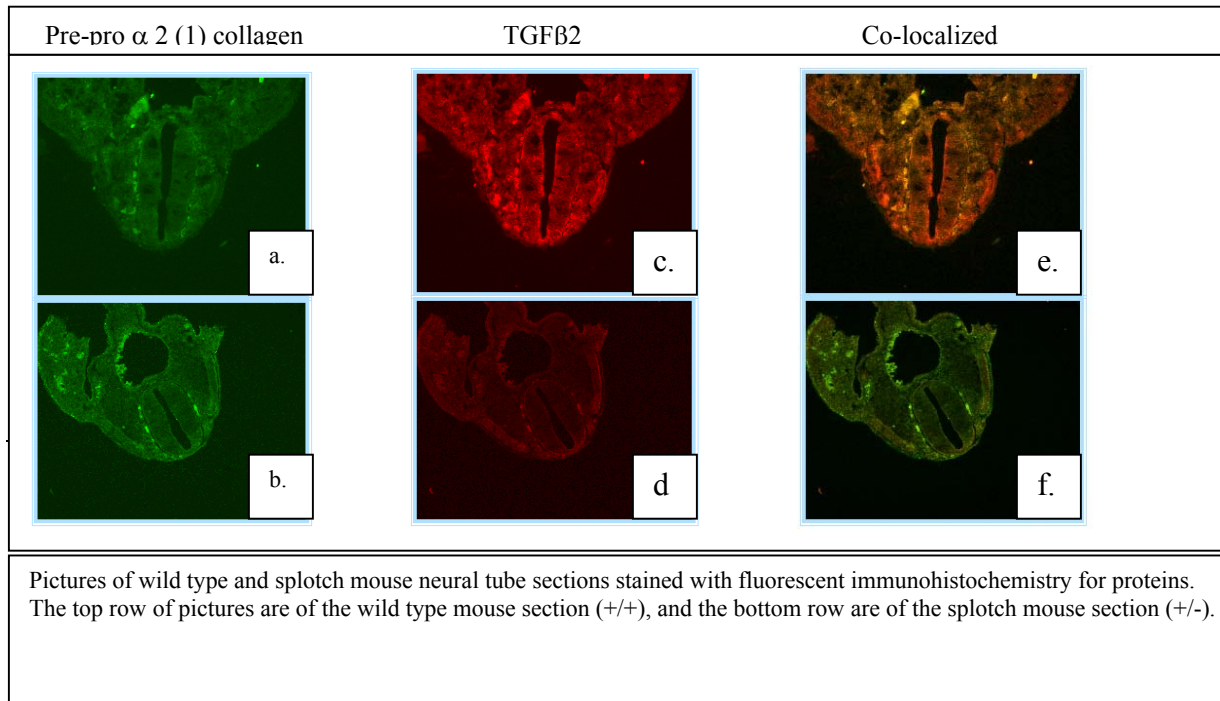
### **Targeted Proteins**

The results of the Affymetrix suggested that Pax3 upregulates the protein expression of the TGF $\beta$ 2 gene and that it down regulates the protein expression of the Pre-pro $\alpha$ 2 (1) collagen gene. Due to the factors mentioned earlier for selection of genes to test and based on this suggestion, these two genes were selected for fluorescent immunohistochemistry. All of the sections both wild type and splotch were stained for each of the two gene's proteins to compare the levels and to see if the suggestion from Affymetrix was correct.

The results of the of the fluorescent immunohistochemistry showed that in the wild type there was less Pre-pro  $\alpha$  2(1) collagen as compared to the splotch sections in which the staining was more intense (Figures 7a and 7b). The sections showed the reverse in regards to the TGF $\beta$ 2

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protein. In the wild type the staining for TGF $\beta$ 2 protein was more intense when compared to the staining for the same protein in splotch (Figures 7c and 7d).



The pictures of the sections stained for Pre-pro  $\alpha$  2(1) collagen and TGF $\beta$ 2 in wild type were overlapped to find the areas where both proteins were being expressed simultaneously (Figure 7e). The pictures of the slides of the splotch sections were also overlapped (Figure 7f).

These results were expected based on the Affymetrix results. Affymetrix suggested that Pax3 down regulates Pre-pro  $\alpha$  2(1) collagen and that it up regulates TGF $\beta$ 2. The Splotch section has less Pax3 since it only has one functional copy of the gene. The Splotch section showed more staining for Pre-pro  $\alpha$  2(1) collagen than the wild type section. This is because there is less Pax3 to down regulate it. The reverse is seen for TGF $\beta$ 2 for the same reason. Pax3

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is shown to up regulate TGF $\beta$ 2. The Splotch has less Pax3 and therefore it is less able to up regulate TGF $\beta$ 2. This results in less TGF $\beta$ 2 in Splotch. The wild type has two functional copies of Pax3 and therefore it shows less Pre-pro $\alpha$  2(1) collagen due to it's down regulation and more TGF $\beta$ 2 due to it's up regulation.

### **Conclusion**

Neurulation is the formation of the neural tube which is important because it will become the brain and spinal cord. If the neural tube does not form properly then it will lead to disease or death of the embryo. Pax3 is an important gene in neurulation because of it's location during neurulation and because it is a transcription factor. It is necessary to find out what downstream target genes Pax3 activates so that it's role in neurulation and ultimately an understanding of the complete process of neurulation can be gained.

Mayanil et. al. (2001) investigated possible downstream target genes of Pax3 for the number of times that these genes contained the Pax3 binding motif. From the 1,000 genes screened for the binding motif three genes were selected for closer inspection based on the binding score and the available literature. These genes were NeuroD, TGF $\beta$ 2, and Pre-pro $\alpha$  2 (1) collagen. Work in a cell system suggested that these genes are downstream target genes of Pax3. These genes were further investigated in a living system to see if the results would show the same thing as seen in the cell system.

Whole mount *in situ* hybridization in mouse embryos was used to see if NeuroD, TGF $\beta$ 2 were actively producing mRNA at the same time and within the same location as Pax3. The

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results of this showed that both NeuroD and TGF $\beta$ 2 were actively producing mRNA within the same time and location as Pax3 expression.

Fluorescent immunohistochemistry was used to see if TGF $\beta$ 2, and Pre-pro  $\alpha$  2(1) collagen were producing protein in the same areas and times when it has been shown that the Pax3 gene is active. The results of the fluorescent immunohistochemistry correlated with the results of the Affymetrix which indicated that Pax3 upregulates the expression of TGF $\beta$ 2 and that it down regulates the expression of Pre-pro  $\alpha$  2(1) collagen.

Proper neural tube closure is very important because the neural tube will become the brain and spinal cord. Failure of the neural tube to develop properly will result in a disease state and can result in death. One of the disease states resulting from improper neural tube formation is Spina Bifida.

Spina Bifida Aperta is a more severe form of Spina Bifida and this is because the Pax3 gene is affected and so too are all of its downstream target genes. The body has to maintain a regulated amount of proteins in order to work properly. If Pax3 is mutated so that it cannot

produce protein then all of its downstream target gene's proteins go unregulated and the natural balance is disturbed.

Spina Bifida Occulta is a less severe form of Spina Bifida, this is because Pax3 is not mutated but rather only one other gene is mutated so that only one protein goes unregulated and this is not as critical in this situation. The results of this study and those of on going ones may

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lead to possible preventative treatments that will work in 100 percent of all cases and not just in 70 percent of cases.

Although people afflicted with Spina Bifida live as normal a life as possible, they must undergo surgical procedures and treatment for life. Ongoing studies of Pax3 and it's downstream target genes and their roles in neural tube closure are very important to finding alternative preventative and therapeutic treatments.

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