Patterns of Shh and Fgf8 expression in mouse embryonic development

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Introduction

When a new gene has been discovered, the first step is to figure out its possible finction. Certain kinds of birth defects are associated with mutations in genes, certain birth defects are results of mutations far away from genes in the non-coding sequences. In this project, we have studied the effects of mutations in the non-coding sequences around Sonic Hedgehog gene (Shh). Mice are chosen model animal because mouse and human genome are very similar in the Shh non-coding regions.

Expressed genes in a cell is trancribed into small strands of mRNA. When a gene is off, no RNA is being transcribed and thus no protein produced. Gene could be turned off when the protein is no longer needed in that particular part of the body. During embryonic development genes are constantly being turned on and off. At different times and different places along the body, a perfect tuned orchestra of gene expressions turns a zygote to fully developed embryo.

Sonic Hedgehog gene (Shh) is located on fifth chromosome and makes a Sonic Hedgehog protein which is essencial for embryonic development of the central nervous system, limbs, eyes, genitals and cell specification.

Our task is to understand the functions of the non-coding sequences in the Shh region. To do this we observed the effects of the deletions in this non-coding region on the development of mice embryos. In order to find out what kind of deletions did our embryos have, we performed PCR and the agarous gel electrophoresis. We have studied the expression of Shh gene in mouse embryos by using in situ hybridization to stain the parts of the embryo where is expressed. Using the same in situ hybridization method we also tried to detect the expression of Fgf8 gene and its relation to Shh expression.

Material and methods

1. *Dissections*

Mouse (*Mus musculus*) embryos were dissected in phosphate-buffered saline (PBS), then fixed in 4% paraformaldehyde (PFA) in PBS at 4°C and washed two times in PBS with Tween 20 (PBST) for 5 minutes.

2. *Hybridization*

This DNA probes are made by incorporating special nucleotides that have a region called DIG, which can be recognized by antibody. After the antibody is bound, treatment with NBT and BCIP, which are staining chemicals, that can indicate the region of antibodies are bound with deep blue colors. Hybridization of DIG-labeled probes to Shh and Fgf8 mRNAs was done in 12-well plates.

Embryos were bleached with 6% H_2O_2 in PBST for one hour. They were then treated with 100 ng/ml proteinase K in PBS for 7 minutes. Digestion was stopped by two glycine washes on ice for 5 minutes and three times in PBST on ice for 5 minutes each. Fixation of embryos was done with 4% PFA for 20 minutes. Washing of embryos was done in following steps: four times for 5 minutes in PBST on ice each, one time for 5 minutes in PBST at room temperature and one time for 10 minutes in W1 buffer (5x SSC buffer, pH 4.5; 50% formamide; 1% SDS; 0.1% Tween 20; 100ml dH_2O) at 65°C. DIG- labeled probes were denatured at 90°C for 10 minutes and on ice at least 2 minutes before overnight hybridization with probes in H2 buffer (W1; torula yeast RNA, 250mg; heparin, 2.5mg) overnight.

Next day, embryos were washed in four different washing buffers: three times in W1 buffer for 30 minutes as 65°C, three times in W2 buffer (2x SSC buffer; 50% formamide; 0.1% Tween 20; 100ml dH_2O) for 30 minutes at 65°C, once in W3 buffer (2x SSC; 0.1% Tween; 100ml dH_2O) for 15 minutes at 65°C and three times in TBST (1x Tris-buffered saline (TBS); 1% Tween) at room temperature. Blocking was done in blocking solution (1x TBST; Bovine Serum Albumin (BSA), 100mg/ml) at room temperature for 1-2 hours. Embryos were incubated overnight in 1:3000 dilution of anti-DIG antibody in blocking solution.

Our next step was to wash embryos once for 5 minutes and four times over a perid of 48 to 72 hours in TBST in order to wash excess of antibodys that could not link with the DNA probes and made Shh expression area undefined. Staining was done with NBT and BCIP in NTMT at room temperature in the dark and when embryos had clearly defined Shh expression area, the embryos were washed twice for 5 minutes in PBS.

3. *PCR and Agarose Gel Electrophoresis*

Polymarase Chain Reaction (PCR) was done to confirm the genotypes of th embryos. It was done with 4 different primer pairs that correspond with the non-coding regions that had

deleted. All of the PCR reactions, are run on agarose gel electrophoresis in order to determine genotype of embryos.

Results and discussion

1. *Identifying Deletions in the Sonic Hedgehog non-coding region*

Figure 1 shows the Shh region where the deletions have been made. Regions adjacent to the Shh gene (4a, 5b-5a, -33) have been deleted to see if they are involved in the regulation of the Shh. The image shown in the Figure 1 was made on the UCSC browser by inserting the primers where the deletions have been made.

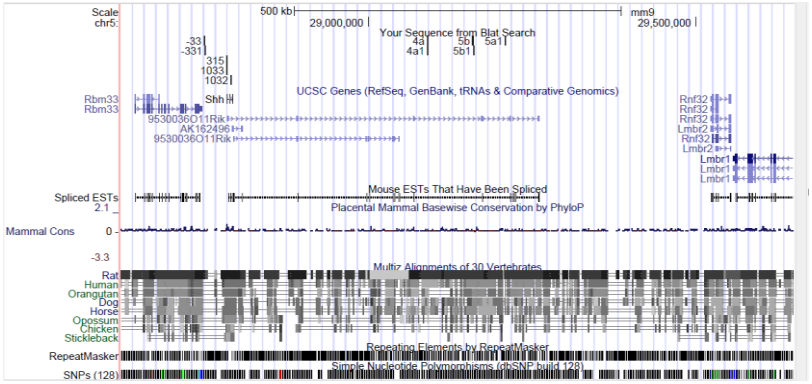

Figure 1 The organization of the genes and evolutionary conservation in the non-coding Shh region

2. *PCR Results*

We are going to explan the results of the PCR and ggarose gel electrophoresis experiments conducted in EMBL. These results will show us the genotype and the phenotype of each of the mice embryos.

Table 1 shows us 6 embryos 11,5 days after fertilization who come from a mother that has a deletion in the -33b region of the Shh genetical region and a father that has a deletion of the complete Shh gene. Embryos from these parents can either have a homozygous genotype with both alleles without any deletion (Wild Type), or a heterozygous genotype which can either have one deletion in one allele or two deletions on each of the two alleles. Compoud heterozygous are suposed to not be properly developed because in one alllele they don't have the Shh gene and in the other allele the don't have one of the genes that is suposed to be necessary for the expression of Shh. In the table we can see that there are two wild type embryos (3,4), two embryos that have a deletion in -.33b region (1, 5) and two embryos that have a deletion of Shh (2, 6).
Table 2 shows us 7 embryos 10,5 days after fertilization who come from a mother that has a deletion in 5b-5a genes and a father that has a deletion of Shh. From these 7 embryos we found three compound heterozygous, which means that they have a deletion on both alleles (1, 2, 6), four of them have a deletion in 5b-5a region (3, 4, 5, 7).

Table 3 shows us 6 embryos 12,5 days after fertilization who come from a mother that has a deletion in 4a region, and a father that has a deletion of Shh. Only one of the embryos is a compound heterozygous (3), four of the embryos have a deletion in 4a region (1, 2, 5, 6).and the last one has a deletion of Shh (4).

We used the *Figure 2* to construct the tables 1, 2, and 3. The figure shows the elecrophoresis results made from the embryonic membrane DNA. To read it we need to compare the DNAs with the control sample, if they are the same, that means that they have a deletion in this region.

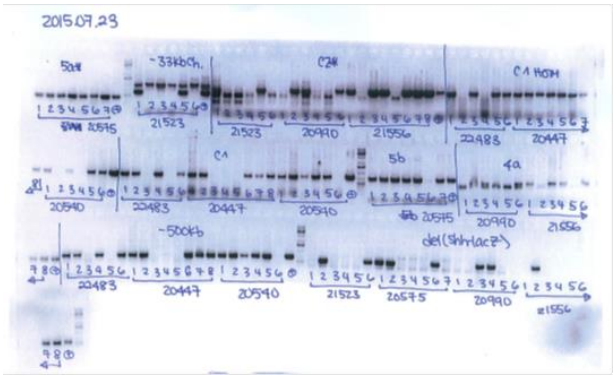

Figure 2 The results of the electrophoresis that were prepared prior to S3++.

Mother Genotype	Del(X)		
Father Genotype	Del(Shh)		
Stage of Embryos	E11.5		
	Del(X)	Del(Shh)	Genotype
Embryo #1	+	-	Del(X)
Embryo #2	-	+	Del(Shh)
Embryo #3	-	-	wildtype
Embryo #4	-	-	wildtype
Embryo #5	+	-	Del(X)
Embryo #6	-	+	Del(Shh)

Table 1 The genotypes and phenotypes of the six embryos we studied. The parents of this six embryos are also shown here.

Mother Genotype	Del(Y)		
Father Genotype	Del(Shh)		
Stage of Embryos	E10.5		
	Del(Y)	Del(Shh)	Genotype
Embryo #1	+	+	Del(Y)/Del(Shh) Compound Het
Embryo #2	+	+	Del(Y)/Del(Shh) Compound Het
Embryo #3	+	-	Del(Y)
Embryo #4	+	-	Del(Y)
Embryo #5	+	-	Del(Y)
Embryo #6	+	+	Del(Y)/Del(Shh) Compound Het
Embryo #7	+	-	Del(Y)

Table 2 The genotypes and phenotypes of the seven embryos we studied. The parents of this seven embryos are also shown here

Mother Genotype	Del(Z)		
Father Genotype	Del(Shh)		
Stage of Embryos	E10.5		
	Del(Z)	Del(Shh)	Genotype
Embryo #1	-	-	Wildtype
Embryo #2	+	-	Del(Z)
Embryo #3	+	+	Del(Z)/Del(Shh) Compound Het
Embryo #4	-	+	Del(Shh)
Embryo #5	-	-	Wildtype
Embryo #6	+	-	Del(Z)

Table 3 The genotypes and phenotypes of the six embryos we studied. The parents of this six embryos are also shown here

3. *Sonic Hedgehog*

After comparing the staining patterns of three groups of embryos at the same stage of development (E10.5) that come from different parents found that most of them looked the same and had a proper Shh expression (*Figure 3*).

Nonetheless, one embryo did not have any of the Shh expression. This could be due to two reasons. The first reason is that the procedure was not done correctly or it could be that the embryo had a mutation in the region that is responsible for the development of the limbs and the brain (*Figure 4*).

For embryos with compound heterozygous embryos we did not see any embryo with obvious phenotypes or difference in staining pattern. The reason could be that the deleted region is not important for development of embryos.

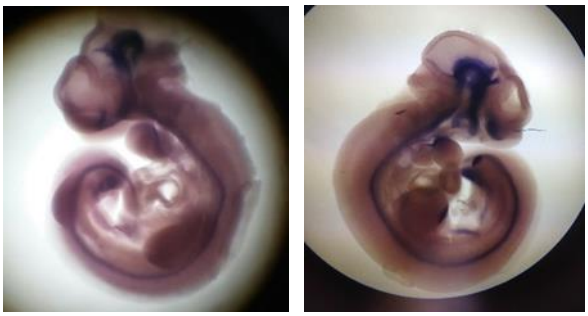

Figure 3 A comparison between two embryos who come from different families and have the same stage (E10.5).

Figure 4 The embryo without the expression of the Shh gene.

4. *Fgf8*

Table 4 shows the comparison of the expression of the Fgf8 gene on three groups of embryos that come from different families, and how it changes ad the embryos grow older (E10.5 (*Figure 5*), E11.5, and E12.5 (*Figure 6*).

We found an embryo that could match the compound heterozygous from the *Table3* because it does not have the expression of Fgf8 and is not properly developed (*Figure 7*).

Expression of the *FGF8* gene	
E10.5/E11.5*	**E12.5***
Tip of the tail	All the tail
Top of the hand	Back part of the head
On the back back part of the head	No expression on the hand
On the mouth and nose	No expression on the nose and little on the mouth

Table 4 *E10.5, E11.5, E12.5 are the days after the fertilization. In this table are shown the difference in Fgf8 expression between embryos that are different days old

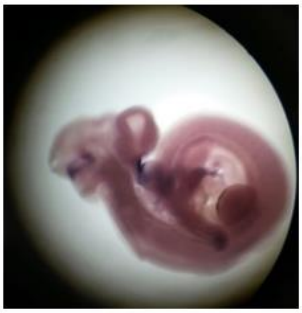

Figure 5 The expression of Fgf8 in the 10.5 and 11.5 embryos

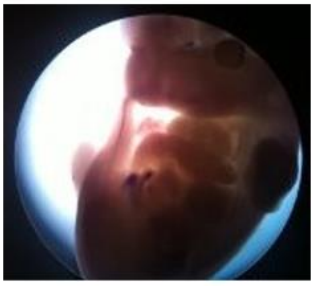

Figure 6 The expression of Fgf8 in the 12,5 embryos

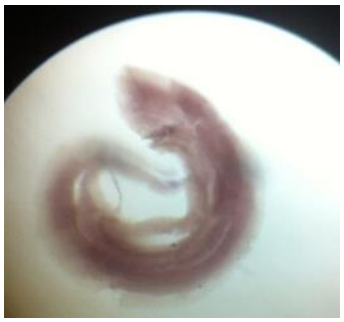

Figure 7 Embryo that could much as a compound heterozygous

5. PCR and elecrophoresis results

Since we did not have a lot of time, most of the embryos were genotyped in EMBL. We only ran the PCR and electrophoresis experiments on four different embryos that have not been genotyped yet. The results that we got were that one of the embryos was a wild type, one had the deletion in the genetical area between 5b-5a, and two of them had a deletion of the Shh. The results of the Agarose Gel Electrophoresis are shown in the *Figure 8*. Since the results are not very visible, we drew them and the drawing is shown in the *Figure 9*.

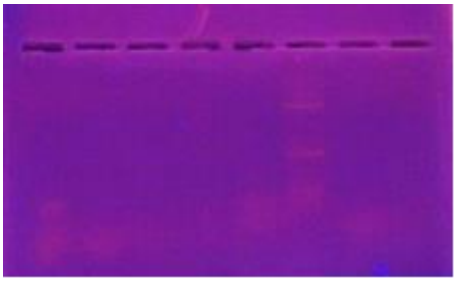

Figure 8 Elecrophoresis results

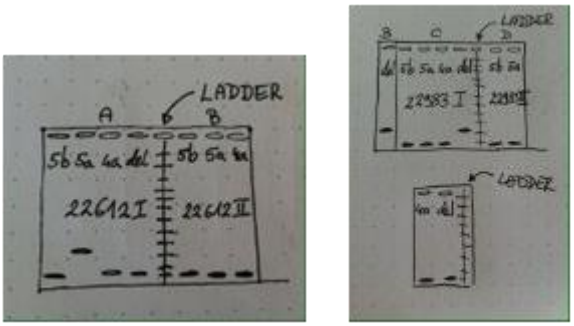

Figure 9 The drawings of the electrophoresis results

Conclusions

As embryos develope (E10.5-E12.5), FGF8 expression pattern in the embryos is changing. However, from this project we could not conclude if Shh expression chaanges in development because all embryos used in this part of experiment were E10.5. Moreover we have found that only one normal copy of Shh is required for proper embryo development. However, we could conclude that FGF8 is independent from Shh because when in embryos with two mutated allels, FGF8 was still expressed. To understand the effects of deletion of non-coding region near Shh, we need embryos in order to obtain all possible genotypes and their effects on phenotypes. We have also discovered that dehydratation and rehydratation in the original in situ protocol are not necessary because even though we omitted these steps of protocol, we embryos were stained.

References

https://genome.ucsc.edu/mm9

http://www.emouseatlas.org/emap/home.html