Development of the Vestibular Apparatus in the Zebrafish Embryo: Analysis of the cloudy Mutant

Acknowledgements:

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Abstract:
The semicircular canals, utricle and endolymphatic duct form the vestibular system of the zebrafish, which has a major role in balance, acceleration and gravity-sensing. It is known that normal ear development relies on the expression of both competence and inducing factors and so a loss of function of either factor results in the loss or reduction of tissue. What is unknown is all the genes that are needed for normal semicircular canal development. We aim to identify the cause of the cloudy mutant, starting with a strong candidate gene, bmper. The cloudy mutant ear has defects in formation of the anterior and posterior semicircular canals. Findings may further understanding of the development of the ear and of balance disorders in both fish and humans. Using various methods we find that the cloudy mutant phenotype is first visible between 35 - 70 hours post fertilisation. I have found that the bmper gene is expressed strongly in the ear of wild type embryos in a pattern that fits with the mutant phenotype. Expression appears to be weaker in the cloudy mutant embryos. These results strengthen the interpretation that mutations in bmper are causative for the semicircular canal defects in the cloudy mutant.

Introduction:
In the zebrafish, the ear becomes morphologically distinguishable at 13.5 to 14 hours post fertilization (hpf). The first sign of canal formation is the appearance of finger-like protrusions of epithelium at about 42 hpf, which push inward towards the centre of the otic vesicle. Expression of dachA marks the anterior and posterior semicircular canal projection at 48 hours post fertilization.

The genetic defect underlying the cloudy mutant is currently unknown. The cloudy mutant ear has defects in development of the anterior and posterior semicircular canals, easily visible at day 4 of development. One of the aims of the project was to identify when the mutant phenotype of cloudy is first visible. I set out to examine and photograph the cloudy ear at different stages to see when the defect first arises.

A second aim was to identify the genetic lesion underlying the cloudy mutation. As part of a collaboration, the Megason lab have identified the gene bmper using RNA sequencing as the best candidate gene for the cloudy phenotype. RNA Sequencing identifies both the region of the genome linked to a mutation and candidate lesions that may be causal for the phenotype of interest. Whole genome sequencing identifies regions of homozygosity within mutants at genetic markers (infrequent microsatellite markers); this is laborious and low resolution.

Next-generation sequencing (NGS) identifies the most abundant class of marker, the single nucleotide polymorphism (SNP), in order to map mutations. The data from NGS identifies candidate mutations within the region of linkage that may be causal for the phenotype. [2][3]

Signalling by secreted BMPs regulates numerous processes of embryogenesis and organogenesis, including development of the zebrafish ear. The action of BMP in organ development therefore makes bmper a good candidate gene to study. bmper has been shown to be a BMP agonist and antagonist: bmper protein undergoes proteolytic cleavage and this cleavage converts bmper from an anti- to a pro-BMP factor. Zebrafish bmper is required in a positive feedback loop to promote BMP signalling during embryonic dorsoventral patterning. bmper’s pro-BMP action is partly due to bmper’s ability to compete with chordin for binding to BMPs, whilst bmper’s anti-BMP action is thought to be due to the association of uncleaved bmper with the extracellular matrix. [4] As a first step to confirm whether mutations in bmper are causative for the cloudy phenotype, I used in situ hybridisation to examined bmper expression in the ear of wild type embryos, and to
compare expression in wild-types against expression in mutant embryos, so that we can identify any differences.

Methods:

1) Dissecting, compound and confocal microscopy:
A dissecting microscope was used to sort and mount embryos; the compound microscope was used to image the live embryos, embryos stained by in situ hybridisation and antibody stained embryos. The confocal microscope was used to image the GFP line cloudy engrailed embryos.

2) In situ hybridisation:
We hybridized embryos with a bmper probe to show where the gene bmper is expressed and how, or if, this is affected in the cloudy mutant.

3) PSmad antibody staining:
We used an antibody that attaches to phosphorylated Smads, which are found in the BMP signalling pathways. This will give an indication as to where there is a lot of BMP signalling.

4) Primer design and PCR:
To amplify the bmper gene

Results:

The images in Figure 1 have been taken with a confocal microscope and edited in ImageJ. They show clearly the structure difference between the ears of cloudy sibling (normal phenotype) and cloudy mutant zebrafish. In the mutant the anterior and posterior canals fail to form the kidney-like shapes seen at stage 3 and instead an unclear ‘cloudy’ structure forms.

![Figure 1](image_url)

Figure 2 shows an in-situ hybridization of wild type embryos at different stages of development using a bmper probe. From 20 somites to 24 hours post fertilisation it can be deduced that bmper is strongly expressed dorsally early on. Wild type embryos older than 30 hours indicate there is more expression ventrally later on in development. 60x Magnification at the 55 hour stage tells us there is strong expression in the sensory patches, tail and branchial arches.
Figure 2

Figure 3 shows an in-situ hybridisation of cloudy mutant embryos at different stages of development using a bmper probe. Comparing the 55 hour and 56 hour stage of the wild type and cloudy embryos it is evident that both express bmper strongly in the dorsal ear and less-so in the ventral ear. It seems that bmper expression is weaker in cloudy mutants. Another in-situ hybridisation needs to be done to confirm this.

Figure 3

The fluorescence in Figure 4 represents the areas of phosphorylated Smads and therefore BMP signalling. This preliminary experiment suggests that positive staining is seen throughout the embryo and there are no clear differences visible between the dorsal and ventral parts of the ear. This experiment would need to be repeated, using a ‘no primary antibody’ control, to check that the staining is real. Also higher power imaging, using a confocal microscope, would help to see more detail.
Discussion:

Live imaging using the compound microscope shows the phenotype is visible earlier than previously described, between day 1 and 3. I have not been able to examine earlier time points, mainly due to time constraints; clear pictures were obtained towards the end of the project as I improved my mounting and imaging technique but by then I had run out of time.

*In situ hybridisation* in wild type embryos, using a *bmper* probe showed that *bmper* is strongly expressed in the dorsal ear, sensory patches and branchial arches. At the 56 hour stage of the cloudy mutant there was expression of *bmper* like in the wild type but perhaps a little weaker; this may be due to the use of a diluted probe.

Conclusion:

During my 6 weeks working in the Whitfield lab I have improved on basic skills such as pipetting and making solutions as well as performing more complex tasks that we as undergraduate students only read about during term time, such as *in situ hybridisation* and PCR. This has given me a better contextualization of the course material. The project has also given me more confidence in my scientific ability and an invaluable insight into the life of a researcher.

My results were entirely consistent with the interpretation that mutations in *bmper* are causative for the *cloudy* mutant phenotype, but further work will be required to validate this fully. Results show that bmper is expressed in the dorsal part of the ear from early stages
and that is where the defect occurs. It may be speculated that the defect does not manifest at the earlier stages because maternal contribution in the egg might be sufficient.

For me at least, the experience has been totally worthwhile. I have made mistakes along the way and feel I need to improve on my organisation most of all; keeping thorough notes in the lab book and thinking independently, ensuring as many controls as possible are performed to ensure validity of results.

References:


