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Cytosolic Carboxypeptidase 5 and Cilia Development in Zebrafish

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ABSTRACT

My research project is focused around a specific gene—cytosolic carboxypeptidase 5 (CCP5). Previous research suggests that the CCP5 gene is essential for the modification of α-tubulin which is necessary for normal growth of cilia in the olfactory pit of zebrafish. Using a CCP5-MO to knockdown the gene I was able to compare the resulting phenotype to that of a control specimen in order to determine any visible differences with respect to cilia morphology. Through analysis by scanning electron microscopy I was able to see that cilia morphology did appear to be effected by the knockdown of CCP5. However, similar results were also displayed by one of our control groups unaffected by CCP5-MO, therefore no conclusive results could be drawn from this experiment.
INTRODUCTION

Cytosolic carboxypeptidase 5 is a gene found in zebrafish that codes for proteins found in its olfactory pit (Berezniuk et al., 2013). Previous research suggests that the CCP5 gene is essential for the modification of α-tubulin which is necessary for normal growth of cilia in the olfactory pit of zebrafish (Rodriguez et al., 2013). The CCP5 enzyme has been shown to be capable of cleaving α- and γ-linked glutamates from the C-termini of both α- and β-tubulin (Lyons et al., 2013). Cilia are essential structural figures found in many organisms. They serve important functions in both motile and sensory capacities. In zebrafish, cilia are located in the olfactory pit, among other places. Since we know that CCP5 is expressed in the olfactory pit we wanted to analyze how the knockdown of CCP5 would affect cilia growth specifically in this region (Lyons et al., 2013).

In order to analyze the effects of this gene we chose to inhibit, or knockdown, CCP5 with the use of a morpholino (MO). Morpholinos are oligonucleotides that knockdown the expression of a specific gene by interfering with the mRNA splicing of the gene (Brent et. al, 2009). By using a CCP5-MO to knockdown the gene, and scanning electron microscopy to analyze the results, we were able to compare the resulting zebrafish phenotypes to those of control specimens in order to determine any visible differences in the cilia of the olfactory pits. By injecting zebrafish embryos immediately following fertilization (while they are still susceptible to the effects of the MO) with a CCP5-MO we were able to analyze the effect of the MO on the CCP5 gene in regards to cilia growth and morphology. Specimens were analyzed with a scanning electron microscope (SEM) in order to characterize any changes in cilia morphology.
METHODOLOGY

_Zebrafish Breeding and Care_—Zebrafish (Danio rerio) were cared for and bred in a controlled environment. They were kept in 10 gallon fish tanks filled with freshwater. Zebrafish were fed twice a day while breeding with fish flakes and once a day otherwise. To breed, zebrafish of mixed genders were placed in small breeding tanks in groups of three. To regulate the circadian rhythms of the zebrafish they were kept in a room absent of natural lighting and their sunrise and sunset were emulated with artificial lighting that ran on a timer. Zebrafish usually breed shortly after sunrise; the lights came on at 9AM to provide time for all necessary preparations prior to fertilization.

_Nanoinjector Set-up_—A pressurized nanoinjector was used to inject the zebrafish with CCP5-MO. To set up the nanoinjector a needle puller was used to pull very small glass needles necessary for the injection. After pulling the needles the tips were broken with a pair of fine-tipped tweezers. The tips were limited to approximately 10 microns in diameter to allow for injections of very small amounts. Next the needles were loaded with phenol red and test injections were done into a drop of oil to ensure that an appropriate amount of CCP5-MO was injected into each zebrafish embryo. A micrometer was used to determine the diameter of the bubble of phenol red injected into the oil. Using the volume and the concentration of the CCP5-MO, the exact amount of CCP5-MO used in each injection was determined. Approximately 2.3nL of CCP5-MO at a concentration of 1.5ng/nL was injected into each embryo for a total of 3.5ng of CCP5-MO in each developing zebrafish.

_Injection and Storage_—As soon as possible after the zebrafish eggs are fertilized they were collected from the breeding tanks using a plastic pipette and transferred in a petri dish to the lab. The embryos were moved to a new petri dish fill with agarose gel molded specifically to
funnel the embryos into a linear arrangement for efficient injection. A pressurized nanoinjector was used to inject each embryo with 3.5ng of CCP5-MO. Approximately 25 embryos were injected. Following injections they were stored in a petri dish filled with E3 buffer solution. Twenty five control embryos were injected with phenol red and kept under the same conditions. Twenty five more un-injected control embryos were preserved. Both petri dishes were stored in an incubator set at 30° C. Three days following fertilization five embryos were removed from the control groups and five from the injected group. The embryos were euthanized with a 4% paraformaldehyde solution and stored in 1.5mL test tubes filled with E3 buffer solution in the refrigerator. This process was repeated at 4dpf in order to track cilia structure and development at different time points.

*Scanning Electron Microscope (SEM) Analysis*— Specimens from each time group prepared for analysis using the SEM. To prepare each sample they were first removed from their E3 solution. Next seven different ethanol washes, all of different concentrations, were applied to the specimens for half an hour each in order to dehydrate the specimens. The concentrations of ethanol washes in increasing order were 30%, 50%, 70%, 85%, 95%, and 100%. One wash of 100% amyl acetate was applied for half an hour. The samples sat in a second wash of amyl acetate overnight. A critical point dryer was then used to remove all liquid from each sample while preserving the sample’s structural integrity. Each sample was given a light coat of gold with a sputter coater to allow them interact with the electrons used in the SEM. Finally, each specimen was be analyzed with a SEM at the appropriate magnification in order to observe the development of cilia at different time periods in the injected embryos and control embryos.
RESULTS

SEM analysis of olfactory pits at least 50 hours post-fertilization, when the pits have completely formed, allowed us to observe and catalogue any changes in cilia morphology (Hansen, et al., 1993). SEM imaging of the head region of a control zebrafish at 4 days post-fertilization (dpf) showed olfactory pits developing normally (Figure 1.1), in an oval shape adjacent to the eye (Hansen et al., 1993).

Figure 1.1 Uninjected control zebrafish (3 days post-fertilization) analyzed with scanning electron microscopy at a 544x magnification. White arrow indicate the olfactory pits, asterisks indicate the location of the eyes, and the black arrow points out the mouth.

In order to analyze the olfactory pit specifically we increased the magnification to 3030x. We were able to observe a clear example of what we expected as a standard for olfactory cilia. Along the outer edge of the olfactory pit we can see longer, flowing, cilia that extend outside of the pit (Figure 1.2). These cilia are classified as kinocilia. Further inside the pit we can see...
smaller, stub-like cilia on the surface of olfactory epithelium. These cilia are classified as ciliated and microvillous endings of receptor cells (Hansen et. al, 1993).

Figure 1.2 Olfactory pit of uninjected control zebrafish (3 days post-fertilization) analyzed with scanning electron microscopy at a 3030x magnification. White arrow point to kinocilia extending from the outer edge of the olfactory pit.

We observed a close-up of the olfactory pit of a CCP5-MO injected zebrafish. In this image it is immediately apparent that there is an alteration, or a significant depletion, of kinocilia at the surrounding edge of the pit. In the place of long, flowing kinocilia we see a few scattered clumps of what may be kinocilia at the edge of the olfactory pit (Figure 2). These results are consistent with our hypothesis that the CCP5-MO would alter the morphological structure of cilia. As for the cilia lining the inside of the pit no drastic or significant differences were noted when compared to the cilia of the wild type zebrafish at 3 days post-fertilization (Figure 1.2).
Phenol red was injected to serve as a control to account for the variable of the actual injection process—phenol red is not known or expected to have any effect on tubulin or cilia. Despite this, in a phenol-red injected zebrafish we saw an olfactory pit with altered kinocilia (Figure 3), similar to that seen in the CCP5-MO injected samples (Figure 2). The kinocilia that we would expect to see flowing from the outer edge of the pit are absent. Also, the olfactory pit is comparatively smaller than the pits seen in control and CCP5-MO injected specimens. It should also be noted that there appears to be a flattening of cells surrounding the olfactory pit in the phenol red-injected specimen (Figure 2).

*Figure 2* CCP5-MO injected zebrafish (3 days post-fertilization) analyzed with scanning electron microscopy at 544x magnification (Panel A). Panel B illustrates an enlarged portion of the white box from panel A and depicts the olfactory pit of the specimen at a 2990x magnification.
Figure 3 Phenol red injected control zebrafish (3 days post-fertilization) analyzed with scanning electron microscopy at a 696x magnification in Panel A. Panel B illustrates an enlarged portion of the white box from panel A and depicts the olfactory pit of the specimen at a 3020x magnification.
In analysis of a separate batch of zebrafish, all at 4 days post-fertilization we found results that were similar to those described above from the batch of zebrafish analysed at 3 days post-fertilization. The control specimens from each batch mimicked each other in regard to cilia morphology and distribution. Figure 4 shows the olfactory pit of a wild type control zebrafish at 4 day post-fertilization. The presence of long, flowing kinocilia at the outer edge of the pit, along with shorter more stub-like cilia lining the inside of the pit, provided for us the same expected observations as we saw in our first control zebrafish (Figure 1.1).

Figure 4 Uninjected zebrafish (4 days post-fertilization) analyzed with scanning electron microscopy at 434x magnification (Panel A). Panel B illustrates an enlarged portion of the white box from panel A and depicts the olfactory pit of the specimen at a 3070x magnification.

CCP5-MO injected zebrafish at 4 days post-fertilization (Figure 5), similar to CCP5-MO injected zebrafish at 3 days post-fertilization displayed a depletion of kinocilia flowing from the edge of the olfactory pit (Figure 5). We also noticed a flattening of cells surrounding the pit.
Figure 5 CCP5-MO injected zebrafish (4 days post-fertilization) analyzed with scanning electron microscopy at 358x magnification (Panel A). Panel B illustrates an enlarged portion of the white box from panel A and depicts the olfactory pit of the specimen at a 3070x magnification.

DISCUSSION

Through our experimentation with CCP5-MO, in regards to cilia growth and morphology in the olfactory pit of zebrafish, we were not able to arrive at any conclusive results. Because of CCP5’s effects on tubulin we expected to see structural variation in cilia; though there was certainly variation between the control and injected specimens we cannot conclude that this was due to the work of the CCP5-MO. Many of our specimens that were injected only with a phenol red solution (as controls) also showed obscure cilia morphology.

Previous research confirms the role of CCP5 in zebrafish olfactory placode cilia morphology. Confocal microscopy was used to display the effects of CCP5 knockdown in this region (Lyons et al., 2013). CCP5 knockdown was shown to cause a decrease in the amount of tubulin present in the olfactory organ of zebrafish, suggesting a change in amount or size of cilia (Lyons et al., 2013). Analysis with scanning electron microscopy has allowed us to determine more about the morphology of the cilia in the olfactory pit. Our results are consistent with this
research—in both cases CCP5 knockdown led to alteration in the amount of cilia in the olfactory pit.

The olfactory pit appears in developing zebrafish between 34 and 36 hour post-fertilization in the zebrafish. As the pit enlarges the amount of cilia present increases (Hansen et al., 1993). Our control and injected specimens were all analyzed at the same time (either 3dpf or 4dpf), so comparatively the abundance of cilia should not have been affected by the maturity of the organism. One characteristic of CCP5-MO injected specimens was a smaller than usual olfactory pit (Lyons et al., 2013). This diminishing effect of CCP5-MO on the olfactory pit was consistent with our data as well. This suggests that CCP5-MO may have a stunting effect on zebrafish embryonic development (Lyons et al., 2013).

The presence of both kinocilia and receptor cilia are expected in the olfactory pit of zebrafish (Hansen et al., 1998). Kinocilia function in the olfactory placodes in a motile fashion and are used to propel water and/or mucus over the lamellae of the olfactory pit (Hansen et al., 1998). Kinocilia also have dynein arms, indicative of their motile function (Zeiske et al., 1992). In comparison receptor cell cilia in the olfactory pit are equipped with odorant reception and function to detect stimuli and transfer the information to the olfactory bulb (Buck et al., 1991; Nagi et al., 1993). In our analysis of the cilia located in the olfactory pit we found that the most notable effects of CCP5-MO were visible on kinocilia which protrude from the outer rim of the olfactory pit. Since CCP5-MO effects \( \alpha \)-tubulin we would expect to have consequential effects on both kinocilia and receptor cilia (Rodriguez et al., 2013). Kinocilia are characteristically long (7-8 um) (Hansen et al., 1998). It is possible that the effects of the CCP5-MO were more visible on kinocilia because its effect was exaggerated due to kinocilia’s comparatively long structure.
Although some of our results were consistent with our hypothesis we cannot make any significant conclusion from our experimentation. One of the main questions from this experiment is why the control specimens with phenol red displayed a similar phenotype to those that were injected with a CCP5-MO. In an effort to further this research I would suggest analysis of a significantly larger sample size would prove useful in determining if the results we saw, especially those of the phenol-injected specimens, were continuous throughout many different samples. Also, since phenol-red gave unexpected results, I would suggest running several samples injected with other non-effecting substances in an effort to determine whether or not the actual injection process plays a role in the alteration of olfactory cilia.
BIBLIOGRAPHY


