Comparison of Cilia Densities in Sea Urchin Gastrula via Immunofluorescent Staining

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Independent Research Project
BIO 254 - Developmental Biology
May 3, 2012

Introduction

Sea urchins are echinoderms that serve as extremely useful model organisms in the study of ciliary function, as they are ciliated as early as the blastula stage and during gastrulation when the archenteron is present (Wilt and Hake 2004). Cilia are organelles consisting of an axoneme covered by a ciliary membrane that protrude from the cell body. The axoneme is made of bundles of microtubules—polar cytoskeletal filament polymers of α-tubulin dimers (Hammond et.al. 2008)—in a 9+2 or 9+0 arrangement (Satir and Tvorup Christensen 2007).

Cilia have many different functions which depend on their location and post-translational modifications to their tubulin (Hammond et.al. 2008). Some functions include ciliary beating, cell division, and cell signaling (Eggenschwiler and Anderson 2007). One such modification is known as acetylation, whereby an acetyl group is added onto the lysine 40 of an α-tubulin. A recent study showed that α-tubulin is acetylated upon its incorporation into the axoneme, and thus microtubules of all cilia and flagellum are acetylated. (MacRae 2004). This acetylation adds stability and structure to the microtubule (Hammond et.al. 2008). It has also been found that Kinesin-1 has a higher binding affinity for acetylated microtubules (Hammond et.al. 2008).

All animals have some type of cilia, and in humans they occur in almost all polarized cell-types (Fliegauf et.al. 2007). These cilia play very important roles in many mammalian organs, such as the eyes and intestines. Recent research is revealing that many human diseases are caused by a malfunction of a particular subset of cilia (Fliegauf et.al. 2007). These diseases are known as ciliopathies, some of which include polycystic kidney disease and hydrocephalus (Satir Tvorup Christensen 2007). It is the functional diversity of cilia that causes their malfunctions to be so devastating (Fliegauf et.al. 2007). For this reason, it

is important that we learn as much about the nature of cilia as possible, for any new information could potentially help identify or treat ciliopathies.

Most cilia are small, and as such detection often requires a microscope. The use of immunofluorescent staining, adding directly conjugated fluorescent antibodies to a sample, can aid in the detection of the cilia. Immunofluorescence works by adding antibodies against the molecule that the researcher wishes to locate. This causes the epitope, or variable region, of the antibody to bind to that molecule, its antigen (Raven et.al. 2002). If a fluorescent compound, known as fluorochrome, is directly conjugated to the antibody, wherever its antigen occurs will appear fluorescent under a given light frequency. The fluorescent quality of these molecules is exhibited when a photon excites the molecule, and due to the fact that these molecules are ring structures, and therefore have resonance, this energy will be distributed around the molecule before being re-emitted as light. To increase the amount of fluorescence, the fluorochrome can be directly conjugated to a secondary antibody can be used, which has the primary antibody as its antigen. (Moore 2012)

In this study, we immunofluorescently labeled sea urchin (*Lytechinus pictus)* gastrulas to detect the presence of DNA, α-tubulin, and acetylated tubulin. There will be more cilia present on the epidermal ectoderm than in the archenteron of sea urchin gastrula as evidenced by the ratio of α-tubulin to acetylated tubulin in each of these structures.

Materials and Methods

The lab procedure according to Morris et.al. 2012 was followed for this experiment with the following modifications and clarifications. For step 14, rather than transfer dry coverslips to pedestals, coverslips were allowed to incubate in a 6-well plate in block buffer for 71 hrs before the liquid was gently pipetted off, and forceps were used to transfer them onto the pedestals. In step 15 a polylysine solution was used, not protamine sulfate and let sit for 1hr. In section D, the concentrations of all antibody solutions (steps 23, 25, 25) were doubled to ensure sufficient staining. Steps 26 and 27 were combined so that anti-acetylated tubulin antibody was applied to all coverslips. After the application of the first primary antibody the coverslips were incubated for 6hrs 50mins. After the third wash described in step 35, 1mL block buffer was added and the dish was returned to the

refrigerator. Embryos were allowed to incubate in secondary antibody for 1.5hrs. The embryos were incubated in FITC-conjugated DM1A primary antibody for 1hr. For step 60, neon nail polish was used to seal the chipchamber in lieu of VALAP. Slides were then labeled with names, embryo stage, whether it was experimental or negative control, and what batch of Alexafluor456 GAM was applied.

A Nikon 80-I microscope was used to view the slides under white, UV, blue, and green light (See Moore 2012 for further background). Each time the type of light, the intensity of light, the exposure, or the focus was changed a picture was taken, limiting total exposure as much as possible to avoid dimming the embryo stains. Background procedure regarding quantification of immunofluorescent images was followed according to Moore 2012 and Morris 2012. The pictures of the stains were viewed analyzed using the programs Adobe Photoshop and Image J. An overlay of corresponding images of the immunofluorescence of alpha-tubulin and acetylated tubulin was overlaid, and the amount of pixels per brightness value (on a scale of 0-255) was calculated for each of the different colors in the archenteron and epidermal ectoderm, separately. This was done by selecting the entire region of the archenteron and using the color histogram tool. The same was done for the epidermal ectoderm. These numbers were then copied into excel where the data could be worked with. From these numbers the mean values were determined and used to calculate the ratio of tubulin to acetylated tubulin in both regions of interest, and this data was then converted into graph form.

The control for this experiment consisted of the coverslip with embryos that were stained only with the anti-acetylated tubulin antibody. Thus, if any fluorescence appeared on these embryos when viewed with the Nikon 80-I, it could only be attributed to the autofluorescence of the embryos or the anti-acetylated tubulin antibodies, not to the presence of a fluorescent antibody.
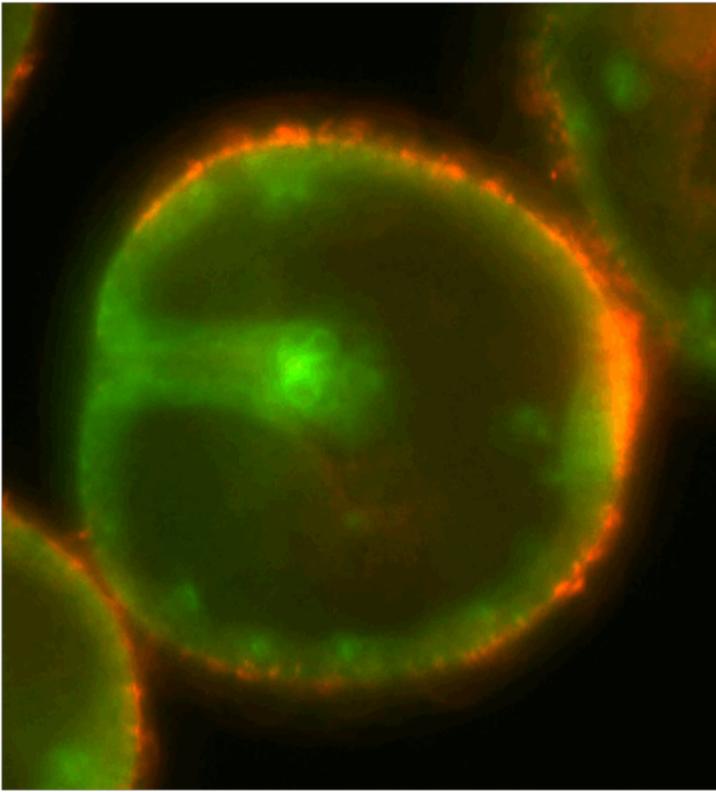
Results

Figure 1. This is a 24-bit image overlay of two sea urchin gastrula. The image of these embryos exposed to blue light, showing the presence of α-tubulin in green, and the corresponding image of the same embryos exposed to green light, showing the presence of acetylated tubulin, were overlaid to produce this image. The bright green that protrudes into the embryo is the archenteron, and the outer edge of the embryo that appears red and green is the epidermal ectoderm.
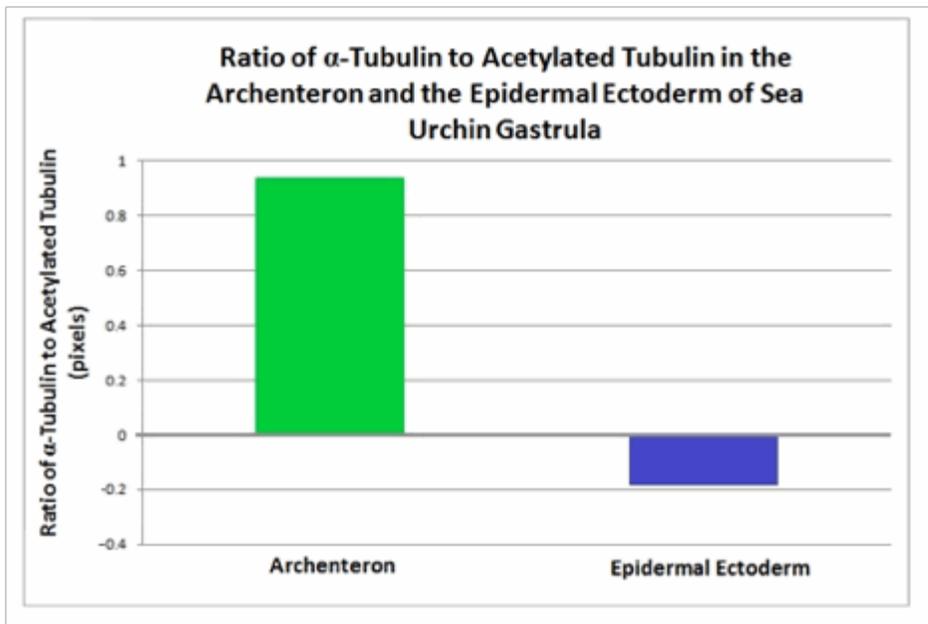

Figure 2. This bar graph demonstrates the ratio of tubulin to acetylated tubulin in the archenteron as green and the same ratio in the epidermal ectoderm as blue.

The value for the epidermal ectoderm as seen in Figure 2 is given as a negative value due to the fact that there was a greater amount of acetylated tubulin than α-tubulin located in this structure. These results show that there is a greater amount of α-tubulin than acetylated tubulin in the archenteron. When calculated, the ratio of α-tubulin to acetylated tubulin in the archenteron is 1.9/1 and in the epidermal ectoderm it is 0.8/1. This is a total difference of 1.1 between the two ratios of α-tubulin.

The red that is apparent in Figure 1. shows where the acetylated tubulin, the cilia, is located. The green color in Figure 1. shows where the α-tubulin is located.

Discussion

The results of this study support the hypothesis that there is a greater amount of cilia present on the epidermal ectoderm than on the archenteron as evidenced by the ratio of tubulin to acetylated tubulin (Figure 2).

According to Amemiya (1979), cilia exist in the gut of sea urchin pluteus, the antecessor of the archenteron. This gives support to the fact that the dim red pixels seen in Figure 1 are indeed cilia. Based on these data, the question arises as to why there are less cilia in the archenteron. One of Cilia's primary functions in a sea urchin gastrula is motility (Katow et.al. 2010), thus, it is plausible that there is less cilia in the archenteron because this function is not necessary on the interior of an organism. Based off of current research on Kartagener's Syndrome, which is caused by primary ciliary dyskinesia, one of the major functions of cilia in the archenteron is to determine the left-right asymmetry of internal organs (Lillington 2001). One future study would be to test whether or not cilia in the archenteron have any function in the motility of the gastrula, or if they are solely for signaling purposes. This could be done by removing all of the cilia from the epidermal ectoderm of a blastula and observing whether or not the embryo still has the ability to swim.

There are many possible future studies that can be done on this topic. One would be to go more in depth with the analysis by looking at the specific number of cilia present in the archenteron and the epidermal ectoderm, rather than using relative counts based on immunofluorescent image brightness. Another study would be to track the formation of cilia in these two regions from the earliest blastula stage through gastrulation. This could

point to differences between the cilia in the two regions and help determine more of the biology behind the vagrancies in the distribution of cilia in all regions of sea urchin embryos. This knowledge may help us to better understand when and where ciliopathies arise, which will direct researchers where to study for the cellular mechanisms behind these diseases and hence develop potential treatments.

References

Amemiya, S., Akasaka, K., Terayama, H. (1979). Reversal of polarity in ciliated cells of the isolated sea urchin pluteus gut. *Journal of Experimental Zoology,* 210, 1:177-182. DOI: 10.1002/jez.1402100120.

Eggenschwiler, J. T., & Kathryn, A. V. (2007). Cilia and developmental signaling. *Annu Rev Cell Dev Biol, 23*: 345-373.

Fliegauf, M., Bensing, T., Omran, H. (2007). When cilia go bad: cilia defects and ciliopathies. *Nature Reviews Molecular Cell Biology,* 8, 880-893. DOI:10.1038/nrm2278.

Hammond, J. W., Cai, D., & Verhey, K. J. (2008). Tubulin modifications and their cellular functions. *Current Opinion in Cell Biology*, 20:71-76.

Katow, H., suyemitsu, T., Ooka, S., Yaguchi, J., Jin-nai, T., Kuwahara, I., Katow, T., Yaguchi, S., Abe, H. (2010). Development of a dopaminergic system in sea urchin embryos and larvae. *Journal of Experimental Biology,* 213, 2808-2819. doi: 10.1242/jeb.042150.

Lillington, G.A. (2001). Dyskinetic Cilia and Kartagener's Syndrome: Bronchiectasis with a Twist. *Clinical Reviews in Allergy and Immunology,* 21, 1:65-69. DOI:10.1385/CRIAI:21:1:65

MacRae. (1997). Tubulin Post-Translational Modifications: Enzymes and their mechanisms of action. *European Journal of Biochemistry,* 244, 2:265-278.

Moore, S. (2012) Lab Notebook BIO 254 Developmental Biology, Wheaton College. Pages 4/5/2012 (1) (2) SAM, See 4/10/2012 (2) SAM, 4/19/2012 (1) SAM, 4/26/2012 (1) SAM.

Morris, R. (2012). Handout BIO 254 Developmental Biology. *How to create three-color overlays of your fluorescent pictures using Adobe Photoshop*. Wheaton College. Available at http://icuc.wheatonma.edu/bio254/2012/rmorris/How_to_create_three_color_overlays_in_photoshop.htm

Morris, R., Henson, J., and Shuster, B. Immunofluor staining of SU embryos – MeOH fixation. (for classroom use, adherent embryo tecnique) 2008, updated 2012. Available at http://icuc.wheatonma.edu/ bio254/2012/rmorris/Immunofluor_Protocol_2012.htm

Raven, et.al. (2002) Biology 8th ed.

Satir, P., Tvorup Christensen, S. (2007). Overview of Structure and Function of Mammalian Cilia. *Annu Rev Physiol,* 69:377-400.

Wilt, F. H., & Hake, S. C. (2004). *Principles of Developmental Biology*. New York, London: W.W. Norton & Company.