THE EXPRESSION OF ALKALINE PHOSPHATASE IN COLONIAL ASCIDIANS

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ABSTRACT

The goal of this project was to better understand anatomical and physiological characteristics of the colonial ascidian, *Botrylloides violaceus*, a marine tunicate species. One reason for studying *Botrylloides violaceus* is that it is an invertebrate that is especially closely related to vertebrates. *Botrylloides violaceus* has the ability to reproduce sexually and asexually (through a process called "budding"), and to regenerate an entire body from stem cells in the circulatory system. The regeneration process is not well understood and does not occur in the better studied species of solitary ascidians. To better understand the process of regeneration, this study focused on the expression of the enzyme alkaline phosphatase. This

enzyme has been used as a marker for stem cells, which differentiate into other cells, a vital part of regeneration. However, the enzyme is expressed in other tissues and its specific role in development is still unknown. To better understand the anatomy of a *Botrylloides violaceus* colony, tissue sections stained with hematoxylin and eosin were analyzed. To perform a preliminary examination of the role of alkaline phosphatase in development, colony tissue sections were stained for the expression of the enzyme. Expression was seen in adult endodermal tissue and in scattered cells within the circulatory system.

BACKGROUND

The colonial ascidian *Botrylloides violaceus* is a filter-feeding marine tunicate species belonging to the phylum Chordata. Tunicates are very closely related to vertebrates, and although it is debated, some scientists argue that they are in fact vertebrates' closest relatives (Delsuc et al. 2006). Therefore, understanding more about regeneration in tunicates may shed light on regeneration in vertebrates. Colonial ascidians form colonies of identical zooids, or adults, from a single settled larva (Burighel & Cloney, 1997). The colony shares a circulatory system, but each zooid has its own organs and reproductive capabilities.

Botrylloides violaceus has the ability to reproduce both sexually and asexually (through a process called "budding"), and it also has the ability to regenerate an entire body from stem cells in the circulatory system (Manni & Burighel, 2006; Rinkevich et al., 1995). Sexual reproduction occurs seasonally. Zooids develop testes and ovaries in order to form sperm and eggs. Once fertilization occurs, an embryo is produced which will develop into larvae in a brood chamber within the colony. These larvae are released and are then free to start a new colony. During asexual reproduction, a bud forms from the body wall and becomes an identical copy of the parent (Oka & Watanabe, 1957). If all body parts are removed, leaving only the circulatory system, the organism can regenerate itself entirely from some subset of circulatory blood cells. These cells must be stem cells since they have the ability to give rise to all differentiated cell types (Rinkevich et al., 1995). There are many questions about the similarities and differences between asexual

reproduction and regeneration, how regeneration is regulated, and which cells are the stem cells.

Alkaline phosphatase is a membrane-bound enzyme widely expressed across different species and developmental stages, and it has many different biochemical functions. It plays a major role in the hydrolysis of phosphates and also regulates the transport of several different compounds, such as calcium, fats, and proteins. Alkaline phosphatase is expressed in proliferating cells and in cells with a high rate of metabolism (Iida et al., 2007). Many questions have arisen about this enzyme's role in the process of proliferation, cell substrate adhesion, and also whether this enzyme is active in inducing differentiation of cells (Hui et al., 1996).

Alkaline phosphatase is commonly found in the intestine of numerous organisms and has been found in early developmental stages of the endoderm, a tissue that gives rise to the gut. This pattern of expression has been seen in solitary ascidians, for example (Whittaker, 1990). Alkaline phosphatase has also been found on the membranes of germ cells and stem cells (Akhmadieva et al., 2007; Iida et al., 2007). This is of great importance because there are still questions surrounding the morphological characteristics of stem cells. The expression of alkaline phosphatase by stem cells has mostly been studied in mammals, but two recent studies of species in the botryllid family have revealed the expression of alkaline phosphatase in stem cells of colonial ascidians (Akhmadieva et al., 2007; Rinkevich et al., 2007). Using light and electron microscopy, these studies revealed prospective stem cells that appeared to be hemoblasts; they were characterized as having a high nucleus to cytoplasm ratio, dense hematoxylin staining, and a small, round cell shape. In one of these studies, an ascidian showed differentiation of hemoblasts into germ cells (Akhmadieva et al., 2007). These findings give rise to more questions concerning the specific subpopulation of hemoblasts that are stem cells.

The objective of this project was to better understand the anatomy of the colonial ascidian, *Botrylloides violaceus*, as well as to examine its cellular expression of alkaline phosphatase. Neither endoderm expression nor stem cell expression of

alkaline phosphatase has been studied in *Botrylloides violaceus*, and the enzyme's role in the organism is not understood. In this project, the first steps were taken toward examining this question. The basic tissue stain hematoxylin and eosin was performed on thin colony slices of *Botrylloides violaceus* in order to highlight important anatomical structures. Other colony tissue slices were stained for the presence of alkaline phosphatase.

METHODS

Botrylloides violaceus tissue samples, embedded in polyester wax, were used to prepare slides with serial sections of the organism. Using a microtome, 7-micron tissue slices were cut and mounted onto gelatin-subbed slides. The slides were allowed to air-dry for 48 hours and were then placed in the refrigerator until staining (Presnell & Schreibman, 1997).

To perform a basic hematoxylin and eosin tissue stain, subbed slides with mounted tissue sections were treated with pure ethanol to remove the wax and then gradually rehydrated through an ethanol series of decreasing strengths. After staining with hematoxylin for four minutes, the slides were rinsed with deionized water and placed in Scott's Solution (0.02 M $NaHCO_3$ and 0.16 M $MgSO_4$), followed by an eosin counterstain for three minutes. The stained slides were gradually dehydrated in another ethanol series of increasing strengths, followed by toluene for two minutes and mounted in Permount (Presnell & Schreibman, 1997). Hematoxylin stains the nucleus of cells blue and eosin stains the cytoplasm of cells pink.

To test for the expression of alkaline phosphatase, several slides with mounted tissue sections of *Botrylloides violaceus* colonies were rehydrated through an ethanol series of decreasing strengths, and placed in phosphate buffer saline (PBS). The slides were then placed in Buffer 3 pH 8 (100 mM Tris-Cl pH 8.0, 100 mM NaCl, and 50 mM $MgCl_2$), followed by Buffer 3 pH 9.5 (100 mM Tris-Cl pH 9.5, 100 mM NaCl, and 50 mM $MgCl_2$). The tissue sections on the slides were covered with Buffer 3 pH 9.5, NBT, and BCIP, and then developed in a dark, moist chamber. BCIP is a colorless compound that contains a phosphate group.

If alkaline phosphatase is present, it will cleave the phosphate group from BCIP. NBT is a yellow compound that will then bind with the dephosphorylated BCIP to produce a blue precipitate. The presence of blue precipitate indicates alkaline phosphatase expression. The slides were then rinsed with PBS and dehydrated through an ethanol series of increasing strengths. They were counterstained with eosin for approximately two minutes and mounted in Permount (adapted from Whittaker, 1990).

RESULTS

The hematoxylin and eosin stain highlighted the tissues, as seen in Figures 1 and 2, although very little eosin counterstain was observed. This stain highlighted major anatomical structures within a *Botrylloides violaceus* colony. In Figure 1 the colony is seen in cross-section revealing three adjacent adult zooids, or individual bodies, across the bottom of the figure (a). Cross-sections of two buds, flanking the center zooid, are visible (b). Cross-sections through blood vessels are also seen (c). Within each individual zooid, the pharynx or branchial basket is seen (a). In order to feed, water filters into the pharynx through the oral siphon of a zooid and food is caught by cilia on the endostyle (indicated by white arrows), where the food is taken in. A close-up view of an endostyle and cilia of an individual zooid can be seen in Figure 2.

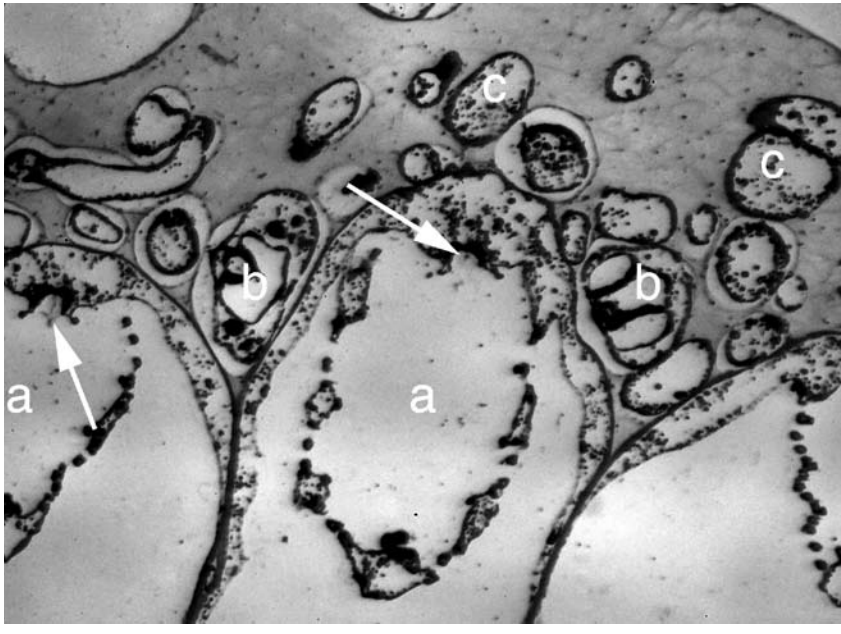

Figure 1. Hematoxylin and eosin stain of a Botrylloides violaceus colony section. (a) adult zooid branchial basket, (b) buds showing earlier stages of development, (c) cross sections through blood vessels, Arrows: endostyles in adult zooid.

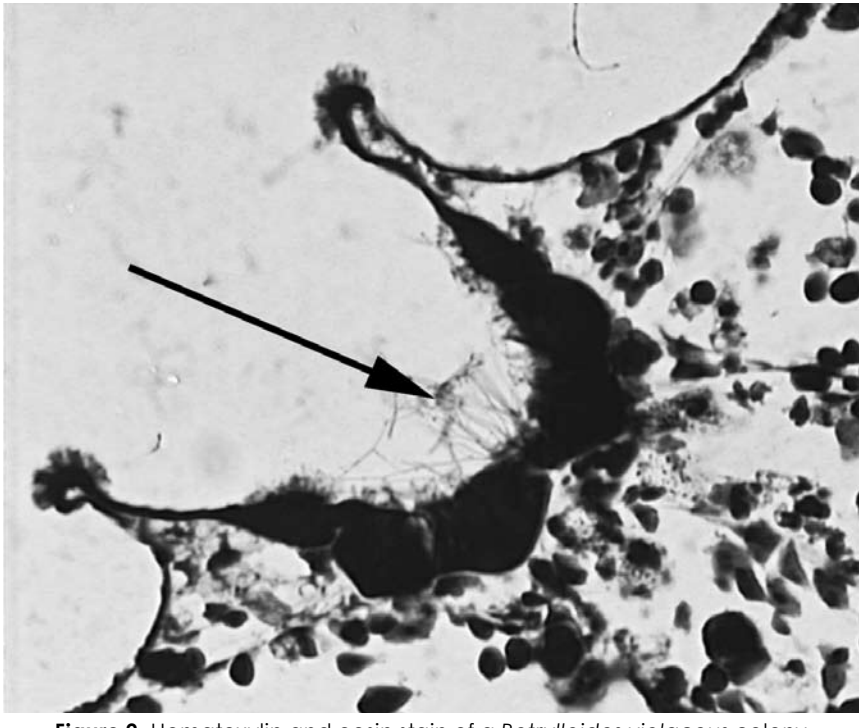

Figure 2. Hematoxylin and eosin stain of a *Botrylloides violaceus* colony section—close-up view of endostyle within pharynx of adult zooid. Cilia are indicated by arrow.

Alkaline phosphatase expression was indicated by the brown and blue pigment seen within the cells. Figure 3a shows extensive expression on a large stomach from an adult zooid. Figure 3b shows the same adult stomach showing expression, and above this stomach a smaller, less developed stomach showing no expression is seen. This smaller stomach appears to be that of a large bud. Figure 3c shows a brood chamber surrounding an embryo extensively expressing alkaline phosphatase activity. Figure 3d shows expression in scattered cells in the circulatory system, some of which may be stem cells. It is important to note that expression is not seen in all circulating cells.

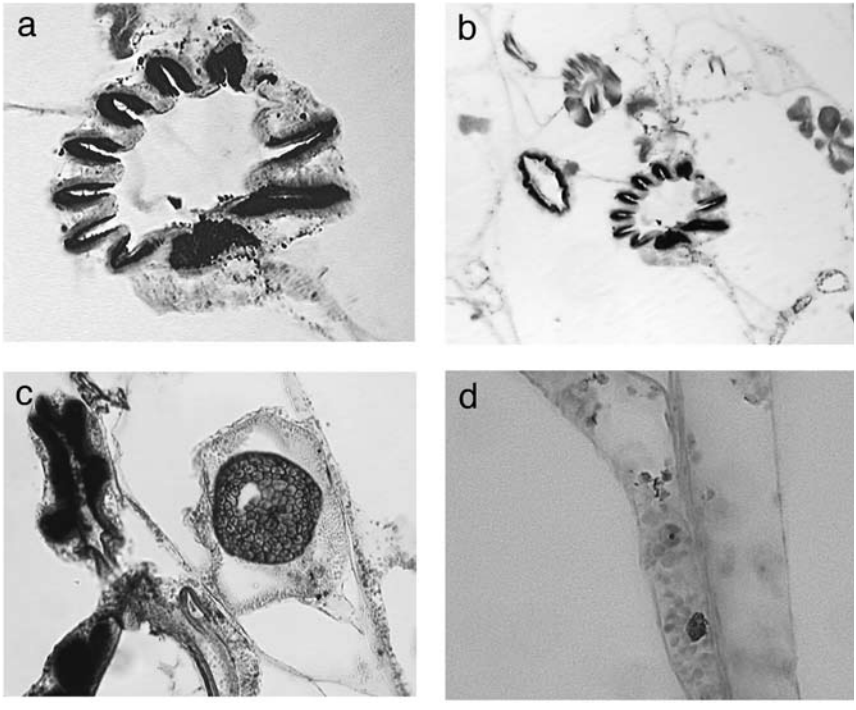

Figure 3. Alkaline phosphatase expression in *Botrylloides violaceus* tissue sections. (a) Stomach of a mature zooid showing positive staining for alkaline phosphatase. (b) Colony section showing the stomach of a mature zooid, showing positive staining for alkaline phosphatase, and the smaller stomach of a large bud, showing a lack of staining for alkaline phosphatase. (c) Colony section showing a brood chamber with an embryo stained positively for alkaline phosphatase, (d) Blood vessel showing scattered blood cells positively staining for alkaline phosphatase.

DISCUSSION

The purpose of this project was to section tissues of *Botrylloides violaceus*, perform a basic tissue stain on the sections to better understand the colonial anatomy, and stain for the activity of alkaline phosphatase. The hematoxylin and eosin staining procedure allowed the anatomy of the colonial ascidians to be viewed and better understood. However, the eosin counterstain did not stain the tissues well. There was no pink color to offset the dark purple of the hematoxylin stain. Increasing the time of incubation in eosin up to five minutes produced the same results. It is possible that the *Botrylloides violaceus* tissue has slightly different properties than

other tissues successfully stained with hematoxylin and eosin. More research must be done to fully understand the reason for minimal eosin staining.

An effective staining procedure for the alkaline phosphatase was successfully developed. With this procedure, tissue sections of colonies were stained, and structures within the colonies were compared for the expression of alkaline phosphatase. There were different patterns of expression observed in the various tissues, especially in the developing endoderm. It appears that smaller stomachs of the buds did not show expression of the enzyme, whereas the larger, more mature stomachs did. This could potentially mean that there are different amounts of expression at varying developmental stages of the gut in colonial ascidians. This is interesting because it suggests a potential difference in alkaline phosphatase expression between solitary ascidians and colonial ascidians. In solitary ascidians, alkaline phosphatase expression has been seen in the very early embryo in cells fated to give rise to the gut, before any visible differentiation occurs (Whittaker, 1990). It is also possible that the pattern of expression differs between embryonic development of the endoderm and its development during asexual budding. To investigate these possible differences further, more stomach sections at different stages of development need to be examined in colonies, and it should be determined at what stage of development or size threshold the expression is first seen. Examination of more sections will allow confirmation that there is a genuine size difference rather than simply artifact of sectioning angle. In addition, expression of alkaline phosphatase in the stomachs of embryos and larvae should be examined.

Scattered blood cells within the tissue also showed varying amounts of alkaline phosphatase expression. In the future, a closer examination of the expression within the circulatory system will be made to identify the specific cell types that express alkaline phosphatase. Based on previous studies (Akhmadieva et al., 2007), it is predicted that alkaline phosphatase is expressed in hemoblasts in *Botrylloides violaceus*. It is not known whether all or only a subset of hemoblasts show expression, nor is it known whether other blood cells show expression. If only stem cells express alkaline phosphatase within the circulatory system, this

stain may serve as a marker to differentiate the stem cells from other circulating blood cells as it has in studies of other ascidians (Akhmadieva et al., 2007). A method of extracting blood from a live colony of *Botrylloides violaceus* will be developed in order isolate these cells away from the surrounding tissue to make cell type identification easier and to clarify enzyme expression patterns. Finally, to confirm stem cell identity we will examine expression of other proteins indicative of stem cells and compare to alkaline phosphatase expression.

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WORKS CITED

Akhmadieva, A.V., Shukalyuk, A.I., Aleksandrova, Y.N., & Isaeva, V.V. (2007). Stem cells in asexual reproduction of the colonial ascidian *Botryllus tubaratus* (Tunica: Ascidiacea). *Russian Journal of Marine Biology*, 33(3), 181-186.

Burighel, P. & Cloney, R.A. 1997. Urochordata: Ascidiacea. *Microscopic Anatomy of Invertebrates, Volume 15: Hemichordata, Chaetognatha, and the Invertebrate Chordates*. 221-347.

Delsuc, F., Brinkmann H, Chourrout D, Philippe H. (2006). Tunicates and not cephalochordates are the closest living relatives of vertebrates. *Nature*, 439, 965-968.

Hui, M., Sukhu, B., & Tenebaum, H.C. (1996). Expression of tissue non-specific alkaline phosphatase stimulates differentiated behaviour in specific transformed cell populations. *The Anatomical Record*, 244: 423-236.

Iida, M., Ihara, S., & Matsuzaki, T. (2007). Hair-cycle dependent changes of alkaline phosphatase activity in the mesenchyme and epithelium in mouse vibrissal follicles. *Development, Growth & Differentiation*, 49: 185-195.

Manni, L. & Burighel, P. (2006). Common and divergent pathways in alternative developmental processes of ascidians. BioEssays, 28, 902-912.

Oka, H. & Watanabe, H. (1957). Vascular budding, a new type of budding in botryllus. *Biological bulletin*, 112, 225-240.

Presnell, J.K., & Schreibman, M.P. (1997). Animal tissue techniques (5th ed.), pp. 101-113. Baltimore: John's Hopkins University Press.

Rinkevich, B., Shlemberg, Z., & Fishelson, L. (1995). Whole-body protochordate regeneration from totipotent blood cells. *Proceedings of the national academy of sciences*, 92, 7695-7699.

Rinkevich, Y., Paz, G., Rinkevich, B., & Reshef, R. (2007). Systemic bud induction and retinoic

acid signaling underlie whole body regeneration in the urochordate Botrylloides leachi. *Public Library of Science*, 5(4), 900-913.

Whittaker, J.R. (1990). Determination of alkaline phosphatase expression in endodermal cell lineages of ascidian embryo. *Biological bulletin*, 178, 222-230.