Expression of Wnt genes in regenerating colonies of *Botrylloides violaceous*

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Abstract

The expression of Wnt genes will be studied in marine invertebrate *Botrylloides violaceous* to provide information about the regeneration pathway of this colonial ascidian. The close evolutionary relationship between *Botrylloides* and vertebrates (Delsuc, 2006) makes it a good candidate for the study of regeneration with the hopes that any advances made will further the understanding of disease, healing, and regeneration in humans. Colonial ascidians are the only chordates that are able to undergo whole body regeneration after complete ablation or all zooids and buds. The colony completely regenerates from the peripheral vascular system, indicating that pluripotent stem cells exist and differentiate in the blood (Brown, 2009). The Wnt family of proteins is known to be involved in regeneration mechanisms, and has been well studied in mammals. However, the Wnts have not been well examined in other phyla, such as the colonial ascidians. We hypothesize that, upon ablation of all members of the colony, Wnt secretion from the pluripotent blood cells is up regulated and initiates the regeneration of the colony from the vasculature.

Project Summary

The proposed project will study the expression of Wnt proteins in regenerating colonies of *Botrylloides violaceous*, a tunicate found in Morro Bay, CA, USA. This organism is of interest due to its close evolutionary relationship to vertebrates. Despite this compelling relationship, tunicate regeneration has not been well studied. This project attempts to elucidate the expression patterns of genes known to be involved in development, regeneration, and cell proliferation. To study these patterns, the researchers will examine known Wnt gene sequences from related organisms to find the most homologous regions and will use inverse PCR to obtain all or part of the gene sequence. The expression of this gene following ablation of all zooids from a colony will then be discovered using real-time PCR (qPCR). Further, qPCR will be used to determine which cells are expressing the Wnt gene in question to help determine which of the lymphocytes has stem-cell potential.

Intellectual Merit

The proposed study will aid in the understanding of the process of regeneration in vertebrates' closest relative, the tunicates. Homology between the vertebral spinal cord and the ascidian neural tube is suspected (Sasakura, 2000). Thus, determining the expression patterns of the Wnt genes in *Botrylloides violaceous* during regeneration may contribute to knowledge about how development, healing, and disease functions in humans.

Broader Impacts of the Proposed Research

1. *Undergraduate participation in research*. Undergraduate students will do the majority (if not all) of the work required for this project. Giving students the opportunity to participate in research better prepares them for professional careers outside of the classroom.
2. *Participation of underrepresented groups*. This project involves participation of women in science. Women involved in the project include the principal investigator, the project advisor, and the secondary investigator.
3. *Development of standard procedures*. Inverse PCR has not been used frequently on the Cal Poly campus, although it is a powerful tool for molecular research. This project will aid in the development of a reliable technique for inverse PCR that will be useful for other students and projects in the future.
4. *Potential for advancement in the medical field*. Understanding the regeneration of *Botrylloides violaceous* has the potential to help elucidate the method of vertebrate healing, disease, and development. The Wnt signaling pathway has been implicated in cancers of the lungs, mammary tissue, and pancreas. Determining the implications of this pathway will lead to a better understanding of oncogenesis and potential therapies for these, and potentially many other, cancers.

Project Description

1. Introduction

1.1 Undergraduate senior project research.

This project investigates the expression of a Wnt gene during the process of whole colony regeneration from the vasculature in *Botrylloides violaceous*. The project will use the methods of inverse polymerase chain reaction (IPCR) and real-time polymerase chain reaction (qPCR) to determine the expression patterns of Wnt. The impetus for this research is the lack of information concerning vertebrates' closest relative capable of total regeneration.

1.2 About Botrylloides violaceous *and its relationship with vertebrates.*

Botrylloides violaceous is a marine invertebrate found in Morro Bay, CA. It belongs to the subphylum Tunicata, under the phylum Chordata. Tunicates are sessile organisms that attach to hard surfaces. The colonial ascidians, class Ascidiacea, are a group of organisms that act together as a colony. Each individual organism, or zooid, takes in food through its own oral siphon and expels unused product through a shared anal siphon. *Botrylloides* violaceous, among other colonial ascidians, are able to reproduce sexually or asexually. Asexual reproduction, called palleal budding in these organisms (Oka and Watanabe, 1957 & 1959), results in a new but genetically identical member of the colony that starts with a growth on the side of a zooid. After development of simple organs, such as a heart, cardiac tissue, and simple neural cord, the new organism then buds off from the original zooid. The colonies also have a shared circulatory system, through which the stem cells of interest in this project pass (Brown, 2009).

The colonial ascidians are the only chordates that are able to undergo whole body regeneration (WBR) through a process called vascular budding. After complete ablation (removal) of all zooids, pluripotent hemocytes in the circulatory system interact with the vasculature epithelium and are able to differentiate and proliferate (Rinkevich, 1995). This process creates an entirely new, complete organism, a phenomenon relatively rare in the animal kingdom. While evidence is abound for the existence of these pluripotent stem cells in the vasculature (Brown, 2009), the nature of these cells remains unknown.

Historically, cephalochordates were regarded as the closest extant relative to vertebrates. However, a recent phylogenetic analysis of nearly 150 genes in 14 dueterosome species showed tunicates to have the closest evolutionary relationship to vertebrates (Delsuc, 2006). This information helped elucidate the suggestion by Stone, et al. (2004) that the evolutionarily novel vertebrate neural crest cells may trace back to origins in the chordate lineage. Further, tunicates have shown to have migratory neural crest cells (Jeffrey 2004) while cephalochordates have not proven to have this attribute. This evidence suggests that tunicates, such as *Botrylloides*, make a good model system for studying regeneration. The knowledge uncovered may be useful in later applications in vertebrates.

1.3 Current research in this lab.

The current research in this lab focuses on quantitation and classification of blood cells in *Botrylloides*, methods for maintaining colonies in vitro, evaluating telomerase activity and its potential effect on whole colony regeneration, and studying patterns of regeneration following ablation of the colonies. The classification of blood cells is in hopes of determining the types of blood cells in *Botrylloides violaceous*. Blood is drawn from healthy colonies, fixed onto microscope slides, stained, and then viewed under a microscope. Nearly ten cell types that differ in morphology and function have been identified in this lab and others (Brown, 2009). The abundance of these types of blood cells is being investigated in both the veins and the ampulle. The ampulle are the ends of the vasculature, which are visible at the surface of the colonies and are thought to be where stem cells gather. Knowledge about the types of blood cells that exist in *B. violaceous* and the quantities of these various cells will help elucidate which cells may contain stem cell-like behavior.

The affect of telomerase is also being studied in this lab. Telomerase is the protein responsible for maintaining the integrity of chromosomes by adding six-nucleotide repeats to the ends of chromosomes to protect against degradation. It has also been implicated in cell immortality and is commonly found in stem cells. The expression of telomerase has been shown to increase in hepatic regeneration in pigs (Wege, 2007). The specific mechanism that involves telomerase in initiating regeneration remains unknown. The current projects in this lab aim to determine if telomerase expression is higher in developing buds than in adult tissues, and if expression is higher in the ampulle due to the increased stem cell presence.

Other members of the lab have focused on methods for growing *Botrylloides* colonies in the lab. Larvae released from the colonies are captured and allowed to attach themselves to slides. After their growth patterns are documented, the colonies are used in experiments regarding regeneration. In these experiments, all zooids are removed from the colony, leaving only the vasculature. Successful regrowth has occurred.

Work has previously been done on the Wnt genes; however, an inability to successfully sequence the gene had hindered progression in this area. The previous researchers used traditional PCR with two gene-specific primers to attempt to isolate the Wnt3 and Wnt5 genes. However, this method was not successful and the project was put on hold. In the mean time, the lab has focused on cloning the β-catenin gene from Botrylloides, another gene involved in Wnt signaling. This project proposes an alternate method for cloning all of part of the Wnt gene, as well as a method for determining expression of this gene.

1.4 The Wnt pathway

The Wnt signaling pathway is highly conserved across all phyla of the animal kingdom and is implicated in control of stem cells during embryogenesis and regeneration. It has been implicated in development and disease in a variety of mammals, including humans (Fuerer, 2008). The Wnt proteins are characterized by conserved amino acid residues rather then function. Typical features of these proteins include a distribution of 22 cysteine residues along with several highly charged amino acids and numerous glycosylation sites (Nusse, 2008). The canonical Wnt pathway involves the binding of Wnt to Frizzled (Fz), which involves Wnt "pinching" Fz with two extending fingers (Bienz, 2012). After binding to Fz, Disheveled (Dsh) is activated, which interacts with the Axin complex. This complex, when not disbanded by Dsh, normally acts to degrade beta-catenin through the ubiquitin pathway. However, the presence of Dsh interrupts the phosphorylation of B-catenin and causes its release from the Axin complex. The stabilized B-catenin then builds up in the cell and is able to enter the nucleus. Upon entering the nucleus, B-catenin acts as a transcription factor for the Wnt target genes (Nusse, 2008).

The Wnt family of genes is large and incomplete. Over 80 different Wnt genes have been discovered, and more are still being added (Schubert, 2000). An organism is most likely to have more than one Wnt gene, each of which may have a unique function. Relatives of

colonial ascidians have been found to have Wnt genes. For example, five Wnt genes, Wnt1, -7, -4, and -11, have been discovered in cephalocordate amphioxus (Schubert, 2000). Wnt3 was shown to be important the driving factor in Hydra head formation and regeneration (Chera, 2009 & Lengfield, 2009). Wnt5b expression has been shown to inhibit regeneration in zebrafish (Stoick-Cooper, 2007). *Botrylloides*' sister genus, *Botryllus*, has been found to have multiple Wnt genes, including Wnt5 and Wnt8. Wnt5 was also implicated in the regulation of nuclear β-catenin in embryos of the ascidian *Halocynthia roretzi*. Due to their direct implication in regeneration in similar species, Wnt3 and Wnt5 will be the targets of this project.

1.5 Research Objectives

Knowledge concerning the expression patterns of Wnt in *Botrylloides violaceous* could potentially be useful in the understanding of oncogenesis, healing, and development in humans. The primary objectives of this research are:

1. Use known Wnt sequences to determine a potential target area of a *Botrylloides* Wnt gene, which will be used to clone the gene.
2. Create a cDNA library from *Botrylloides* cells, and then use inverse polymerase chain reaction (PCR) to clone the gene of interest.
3. Use real-time reverse transcription PCR (qPCR) to determine the change in expression of the Wnt genes during regeneration and to establish the cells in which these genes are expressed.

2. Study Area

The research will take place at California Polytechnic State University, San Luis Obispo, in the lab of Dr. Elena Keeling. Cal poly offers a wonderful study area with access to many of the tools needed to successfully complete this research. *Botrylloides violaceous* is found and collected in nearby Morro Bay, CA, where it grows on docks, rocks, and the like. The permits for the collection of these organisms has already been obtained by Dr. Elena Keeling for use in her lab.

3. Methods

3.1 Wnt sequence analysis

To determine where a protein is expressed in any system, the sequence of that protein or its encoding mRNA or DNA must be known. This information allows for the cloning and probing that will be used later in the project. The mRNA sequences of known Wnt genes will be analyzed for sequence homology. mRNA will be used because of its accessibility. Using mRNA in cloning is very useful because the product is often much shorter than the genomic DNA. Introns in the genomic DNA can be very large and distracting from even a small product. Using genomic DNA in inverse PCR, the method that will be used to clone all or part of the Wnt gene, often makes it very difficult to get a useful sequence. Because introns are so large, the product of inverse PCR may contain only part of the target sequence and a small portion of the unknown sequence, but a large part of the intron. Knowledge of the intron sequences is not useful in the discovery of where a gene is expressed. Genes that are expressed are transcribed into mRNA, which lacks the introns. This mRNA is what we will be searching for in the final step of the project, real-time PCR (qPCR). Determining the sequence of just the mRNA for the Wnt gene eliminates the problem caused by introns in the genomic DNA.

Thus, mRNA sequences of known Wnt genes will be analyzed for their homology using the NCBI BLAST tool. The part of the gene that is most homologous across many different Wnt genes from various sources will be used in inverse PCR. The inverse PCR technique is unique in that it does not require two known sequences, so the single most homologous coding region will be used.

3.2 Inverse PCR

Inverse PCR (IPCR) is a powerful tool that allows amplification of genetic data from very limited sequence information, as we are dealing with here. IPCR is unique in that, though only one segment of sequence information is required, it still entails the use of *two* gene-specific primers. First, a cDNA library will be made. mRNA will be purified from currently regenerating *Botrylloides* cells using column purification that binds the Poly-A tail of the mRNA. An oglio-dT primer is used with reverse transcriptase to make a complimentary

strand to the mRNA. The mRNA is degraded, leaving a single-stranded cDNA (sscDNA) strand. Terminal transferase adds a poly-C tail to the end of the sscDNA, which is bound by an oglio-dG primer to synthesize a double strand. The new double-stranded molecules constitute the cDNA library.

Restriction enzymes are added to the cDNA library and the library allowed to digest. The restriction digestion cuts the DNA at predictable sites that usually result in homologous ends. The restriction digest would theoretically cut two areas of the cDNA, whose ends would therefore be homologous. The homology of the end sites would create an affinity between the ends. The addition of ligase will result in the circularization of the cDNA strands where the homologous ends come together. This results in a small circular segment of DNA with one known sequence region. A primer will be added that anneals to the middle to end of this known region, along with replication machinery and materials. The reaction mixture will then undergo a few cycles of standard PCR, which results in the amplification of the plasmid, beginning with the known sequence. The resulting PCR product is a linear piece of DNA, with the known sequences on the ends of the strand and the flanking unknown portions in the middle. This product will be sequenced and analyzed using programs such as Sequencher. The upstream and downstream portions will be differentiated using the homologous ends created by the restriction enzyme digestion.

3.3 Real-time reverse transcriptase PCR

The innovation of real-time reverse transcription PCR (qPCR) has made possible the monitoring of PRC products as they are formed. This is an important advancement of the traditional method of PCR, which allows quantification only after a set number of cycles. qPCR enables the measurement of a nuclear transcript in a particular tissue type. The sequence information obtained through iPCR will be used to probe for expressed Wnt seqnences in *B. violaceous* cells. This will help determine the levels of expression of the Wnt3 and Wnt5 genes in regenerating colonies of the colonial ascidian. First, qPCR will be used to determine whether or not Wnt expression increases during regeneration of the colony following ablation of all zooids. If Wnt expression increases, two suppositions are made; 1) Wnt is not constitutively expressed and 2) the Wnt pathway may be involved in the vascular budding seen in *Botrylloides violaceous*. If Wnt expression does not increase, this would

indicate either that 1) the cloned Wnt gene is constitutively expressed in the colony, but its product is hindered from activating the Wnt pathway, or 2) that specific Wnt gene is not involved in vascular budding, or 3) the Wnt pathway in general is not involved in vascular budding in *Botrylloides* (unlikely).

4. Budget Information

Materials:

Product	**Company**	**Price**
dNTP Mix	BioRad	$115
oligo(dT) Primer Mix	Sigma Aldrich	$300
Reverse Transcriptase	Sigma Aldrich	$252
Terminal Transferase (TdT)	Thermo Scientific	$261
dCTP	Thermo Scientific	$57
oligo(dG) primer	Sigma Aldrich	$150
Restriction Enzymes	Various	$100-$200
Ligase	Sigma Aldrich	$70
Buffer components	Various	$50-$150
Custom primers	Various	$200-$400
Sequencing	Various	$500-$1000
Other reagents	Various	$500-$1000

The scientists will be paid for their work. The primary researcher will receive an annual salary of $50,000 USD per year. Two secondary researchers will receive an annual salary of $35,000 USD per year. This project is expected to take a maximum of two years to complete. Costs for machinery required to perform the procedures is not included. California Polytechnic State University, San Luis Obispo, will provide all necessary machinery. The researches will work within all reasonable limits of the machinery provided to minimize costs.

Other unexpected expenses may add up to a total of $10,000 per year. Each year will require $140,000 of funding to ensure proper resources for the completion of the project. The total sum requested is $280,000.

References Cited

Bienz, M., & He, X. (2012). A Lipid Linchpin for Wnt-Fz Docking. *Science Signalling*, *337*(6090), 44.

Brown, F. D., Keeling, E. L., Le, A. D., & Swalla, B. J. (2009). Whole body regeneration in a colonial ascidian, Botrylloides violaceus. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, *312*(8), 885-900.

Chera, S., Ghila, L., Dobretz, K., Wenger, Y., Bauer, C., Buzgariu, W., ... & Galliot, B. (2009). Apoptotic Cells Provide an Unexpected Source of Wnt3 Signaling to Drive *Hydra* Head Regeneration. *Developmental cell*, 17(2), 279-289.

Delsuc, F., Brinkmann, H., Chourrout, D., & Philippe, H. (2006). Tunicates and not cephalochordates are the closest living relatives of vertebrates. *Nature*,*439*(7079), 965-968.

Fuerer, C., Nusse, R., & Ten Berge, D. (2008). Wnt signalling in development and disease. *EMBO reports*, 9(2), 134-138.

Jeffery, W. R., Strickler, A. G., & Yamamoto, Y. (2004). Migratory neural crest-like cells form body pigmentation in a urochordate embryo. *Nature*, 431(7009), 696-699.

Lengfeld, T., Watanabe, H., Simakov, O., Lindgens, D., Gee, L., Law, L., ... & Holstein, T. W. (2009). Multiple Wnts are involved in *Hydra* organizer formation and regeneration. *Developmental biology*, 330(1), 186-199.

Liu, P., Wakamiya, M., Shea, M. J., Albrecht, U., Behringer, R. R., & Bradley, A. (1999). Requirement for Wnt3 in vertebrate axis formation. *Nature genetics*, 22(4), 361-365.

Nusse, R. (2008). Wnt signaling and stem cell control. *Cell research*, *18*(5), 523-527.

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

Oka, H., & Watanabe, H. (1959). Vascular budding in Botrylloides. *The Biological Bulletin*, 117(2), 340-346.

Rinkevich, B., Shlemberg, Z., & Fishelson, L. (1995). Whole-body protochordate regeneration from totipotent blood cells. *Proceedings of the National Academy of Sciences*, 92(17), 7695-7699.

Sasakura, Y., & Makabe, K. W. (2000). Ascidian Wnt-7 gene is expressed exclusively in the tail neural tube of tailbud embryos. *Development Genes and Evolution*, 210(12), 641-643.

Schubert, M., Holland, L. Z., Holland, N. D., & Jacobs, D. K. (2000). A phylogenetic tree of the Wnt genes based on all available full-length sequences, including five from the cephalochordate amphioxus. *Molecular Biology and Evolution*, 17(12), 1896-1903.

Stoick-Cooper, C. L., Weidinger, G., Riehle, K. J., Hubbert, C., Major, M. B., Fausto, N., & Moon, R. T. (2007). Distinct Wnt signaling pathways have opposing roles in appendage regeneration. *Development*, 134(3), 479-489.

Stone, J. R., & Hall, B. K. (2004). Latent homologues for the neural crest as an evolutionary novelty. *Evolution & development*, 6(2), 123-129.

Wege, H., & Brummendorf, T. H. (2007). Telomerase activation in liver regeneration and hepatocarcinogenesis: Dr. Jekyll or Mr. Hyde?. *Current Stem Cell Research & Therapy*, 2(1), 31-38.

Biographical Sketch

Raquel Kahler is a fourth year undergraduate student at California Polytechnic State University, San Luis Obispo. She is a double major; her primary major is Molecular and Cellular Biology, and her secondary major is Microbiology. She currently works at Applied Biotechnology Institute as a research assistant in San Luis Obispo.