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Characterization of an individual neural crest-like cell lineage in the invertebrate chordate Ciona intestinalis

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2008

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Characterization of an Individual Neural Crest-like Cell Lineage in the Invertebrate Chordate *Ciona intestinalis*

A Dissertation submitted in partial satisfaction of the requirement for the degree

Doctor of Philosophy

in

Biology

by

Angela C. Cone

Committee in charge:

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Professor James Posakony

San Diego State University

Professor Robert W. Zeller, Chair
Professor Greg Harris
Professor Elizabeth Waters

2008
The dissertation of Angela C. Cone is approved, and it is acceptable in quality and form for publication on microfilm:

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Chair

University of California, San Diego
San Diego State University
2008
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<tr>
<td>A\P</td>
<td>anterior-posterior</td>
</tr>
<tr>
<td>artmiR</td>
<td>artificial micro RNA</td>
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<tr>
<td>BMP</td>
<td>bone morphogenic protein</td>
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<tr>
<td>Ci</td>
<td>Ciona intestinalis</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double-stranded RNA</td>
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<tr>
<td>EMT</td>
<td>epithelial to mesenchymal transition</td>
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<tr>
<td>ESTs</td>
<td>expressed sequence tags</td>
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<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
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<tr>
<td>GRN</td>
<td>gene regulatory network</td>
</tr>
<tr>
<td>Hr</td>
<td>Halocynthia roretzi</td>
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<tr>
<td>HRP</td>
<td>horse radish peroxidase</td>
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<tr>
<td>Kb</td>
<td>kilobase</td>
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<tr>
<td>MB</td>
<td>megabase</td>
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<tr>
<td>miRNA</td>
<td>micro RNA</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>NCC</td>
<td>neural crest cell</td>
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<tr>
<td>nt</td>
<td>nucleotide</td>
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<tr>
<td>PCL</td>
<td>pigment cell lineage</td>
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<tr>
<td>PCP</td>
<td>pigment cell precursor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
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<tr>
<td>RISC</td>
<td>RNA induced silencing complex</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RPE</td>
<td>retinal pigmented epithelium</td>
</tr>
<tr>
<td>siRNA</td>
<td>short interfering RNA</td>
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I would like to acknowledge Dr. Robert W. Zeller as the chair of my dissertation committee. His endless patience, guidance, and encouragement have been invaluable.

Professor Mark Q. Martindale performed crucial microinjection experiments that made some of the work in chapter 2 possible. Special thanks are due to him and the Embryology Course at the Marine Biological Laboratory in Woods Hole, MA where some of this research was performed. The dissertation author was the primary investigator and author of this chapter.

Chapter 4, in full, is a reprint of the material, as it appears in the Canadian Journal of Zoology 83: 75–89, 2005, Using Ascidian Embryos to Study the Evolution of Developmental Gene Regulatory Networks by Cone, Angela C. and Zeller, Robert W. The dissertation author together with the chair of the committee were both authors of this paper.

Chapter 5, in full, is a reprint of the material, as it appears in Developmental Dynamics 235:1921–1932, 2006, Predictable Mosaic Transgene Expression in Ascidian Embryos Produced with a Simple Electroporation Device by Zeller, Robert W.; Virata, Michael J. and Cone, Angela C. The dissertation author was a co-author of this paper.
EDUCATION

Doctorate of Philosophy in Biology

*December 2008*

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*December 2000*

*Humboldt State University*

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*Cone, Zeller, and Martindale. 2008.* A neural crest-like cell lineage in the invertebrate chordate *Ciona intestinalis*. (manuscript in progress).


COURSES ATTENDED

Embryology 2004 ~ Marine Biological Laboratory, Woods Hole, MA.

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*Spring 2002 – Spring 2008*

- Microscopy: Confocal, Epifluorescent, Nomarski, Time-lapse, optical sectioning and 3D reconstruction
- Embryology: Production and culture of transgenic ascidian embryos, Microsurgical manipulations, Microinjections
- Design and construction of tissue specific GFP reporters and protein misexpression vectors
• Whole mount *in situ* hybridization, Genomic DNA and RNA extraction
• Quantitative PCR
• Immunofluorescent labelling
• Bioinformatic analyses including basic genome mining, phylogenetic footprinting, and transcription factor binding site prediction

**Rational Protein Design ~ Dr. John Love's Lab SDSU**  
*Spring 2002*

• Writing basic python scripts
• Designed a script to use with the PYMOL protein visualization software to help analyze the top 100 output results from modeling of *de novo* protein docking

**Phage Display ~ Dr. Kathleen McGuire's Lab SDSU**  
*Winter 2002*

• Phage Display: library design and construction, isolation of chicken ScFv genes
• Protein purification
• SDS-PAGE and Western analysis

**Marine Bacteriophage ~ Dr. Forest Rohwer's Lab SDSU**  
*Fall 2001*

• Phage isolation from environmental samples using a CsCl gradient
• Bioinformatic identification of signature genes in known phage genomes
• Design and optimization of degenerate PCR primers

**Microbial Ecology of Extreme Environments ~ Dr. Mark Wilson's Lab HSU**  
*Fall 2000*

• Field work, environmental sample collection using GPS
• Extraction of DNA from soil and sediment samples
• Amplification of 16s and 18s rDNA sequences using universal primer sets
• Basic cloning
• DNA sequencing
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**PRESENTATIONS AND AWARDS**

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A neural crest-like cell lineage in the invertebrate chordate *Ciona intestinalis*.  
(Poster) American Society for Cell Biology 46\textsuperscript{th} Annual Meeting. December 2006

Artmiracles: Developing tools in an emerging model organism.  
(Oral presentation) SDSU Graduate Student Seminar Series. October 2006

Melanocyte development of the invertebrate chordate *Ciona intestinalis*.  
(Poster) Society for Developmental Biology 64\textsuperscript{th} Annual Meeting. August 2005

Characterization of an individual neural plate cell lineage in a basal chordate provides insight to the evolutionary origin of neural crest-like cells and promotes a model system for the study of gene regulation in developing chordate melanocytes and possibly the progression of melanoma.  

A neural crest-like cell lineage in the invertebrate chordate *Ciona intestinalis*.  
(Poster) 3\textsuperscript{rd} Annual International Tunicate Conference. July 2005

An integrative approach to studying gene regulatory networks.  
(Poster) SDSU Cell and Molecular Biology Graduate Student Symposium. May 2004

It’s just a phage I’m going through: Generating a naïve phage display library from chicken short-chain variable fragments.  
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The phage genomic tree.  
(Poster) Ocean Sciences Conference. February 2002

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**Bio 202 – Evolution, Biodiversity and Organismal Biology**
Miracosta Fall 2007  
\textit{Instructor.} A comprehensive lower-division biological "foundation" for science majors that surveys the "organismal/meta-organismal half" of biological disciplines. Lecture and laboratory components cover topics including the taxonomy and physiology of protists, fungi, and plants; the taxonomy, developmental biology, and physiology of animals; single-species population dynamics and interspecies interactions in communities. The laboratory emphasizes comparative anatomy/physiology of plants, invertebrates, and vertebrates, phylogeny
reconstruction, life history evolution, and surveys of ecological habitats through field studies.

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**Bio 576 - Developmental Biology.**  
*SDSU Fall 2006*  
*Guest lecturer.* Evo/Devo: the evolution of developmental strategies among metazoans. Recent changes in chordate phylogeny and the evolutionary origin of neural crest cells were discussed.

**Bio 350 - Microbiology**  
*SDSU Fall 2001, Spring 2002, & Spring 2004*  
*Laboratory instructor.* Classical microbiological lessons and experiments in this course have aseptic technique and problem solving as the underlying goals for our students to attain. Responsibilities included lesson plan preparation, quiz writing, grading of quizzes and lab reports, and office hours.

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**OTHER TEACHING EXPERIENCE**

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*Summer 2005 & 2006*  
*Teaching assistant.* Instructed and led sea squirt lab sections in an unusually intensive summer course for graduate and post-graduate scholars from around the world. Assisted students round-the-clock in advanced scientific inquiry that included
confocal and epifluorescent microscopy, immunofluorescent staining, microinjection, classical embryological manipulations and tool making.

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*December 2000-August 2001*

Substitute teaching in San Diego's bilingual and special education elementary school classrooms. Maintained classroom order and a pleasant environment for students to learn and interact in the absence of their regular teacher.

**San Diego Zoo - Education Department**


*Educator Guide.* During summer 2000, I created and implemented a six-week science-based curriculum for 6th and 7th graders. This included the study of plants and animals from 5 different biomes to increase student awareness of current environmental issues and food webs. Other responsibilities included conducting a variety of programs for adults and children throughout the zoo, driving double-decker buses while narrating, as well as up close presentations of exotic and endangered animals.
During embryogenesis, all chordate embryos undergo neurulation to form a dorsal, hollow nerve cord. Neural crest cells (NCC), considered a vertebrate innovation, arise during neurulation and later differentiate into a multitude of tissues that account for much of the structural complexity that distinguishes craniates from invertebrate chordates [1, 2]. NCCs are induced and specified at the border of the neural and non-neural ectoderm by a complex network of inductive signals and
transcriptional regulators then migrate throughout the embryo prior to differentiating [3, 4]. Invertebrate chordates, such as ascidians and amphioxus, possess cell types such as melanocytes, sensory neurons and even migratory cells [5] that resemble neural crest derivatives. However, it is not known whether these invertebrate cells share developmental specification mechanisms with vertebrate NCCs. Here we show that neural crest-like cells descending from the pigment cell lineage (PCL) of the ascidian *Ciona intestinalis* (*Ci*) originate at the neural plate border, express NCC transcriptional regulatory genes, migrate at the time of neural tube closure and differentiate into NCC derivatives including melanocytes and sensory neurons.

Importantly, this careful analysis of the PCL has been carried out with single-cell resolution within a single ascidian species. Our results suggest that the specification and development of neural crest-like cells at the neural plate border is a symplesiomorphy of olfactores and provides insight into how this gene regulatory network was co-opted for NCC specification during vertebrate evolution.


I. Introduction

Evolutionary origin of a new cell type

All chordates undergo neurulation during embryogenesis to form a hollow nerve cord. This morphogenic event is directed by changes in the neural ectoderm and neighboring epidermis that result in the neural plate forming a dorsal tube along the anteroposterior axis that is covered with epidermal ectoderm. During neurulation in vertebrate embryos a cell type arises, the neural crest, that accounts for most of the structural complexity that distinguishes craniates from invertebrates (Holland et al., 1996).

Neural crest cells (NCCs) have long been considered to be a vertebrate synapomorphy not found in any other metazoans. They are referred to as an evolutionary novelty that arose at the base of the vertebrate radiation. In recent years they have been described in the most basal vertebrate model, lamprey (McCauley and Bronner-Fraser, 2003; Meulemans et al., 2003; McCauley and Bronner-Fraser, 2004; Nikitina and Bronner-Fraser, 2008), as well as in developing hagfish (Ota et al., 2007), which is a craniate but technically not a vertebrate. This logically leads to the question of when and how did this new cell type come about during the evolution of chordates. This dissertation research searches for signs of the process of NCC evolution in an extant protochordate from the subphylum most closely related to the craniates. In order to approach this problem we must first review recent changes in deuterostome phylogeny and also address the question of, what is a "new" cell type?
A review of the current understanding of the relative evolutionary relationships between the deuterostomes is shown in Figure 1.1. Deuterostomes can be categorized into the six phylogenetic groupings shown here: echinodermata, hemichordata, xenoturbellida, cephalochordata, urochordata, and craniata. The relative positions of four of those have recently changed. Based on morphological characterization previously hemichordates were considered part of the chordate branch and cephalochordates were placed as the closest living relatives of craniates (Peterson, 2004). Sequence data from more deuterostome genomes, like sea urchin, amphioxus, Oikopleura and Xenoturbella, have allowed for more extensive sequence comparisons in recent years. This resulted in the formation of a new clade called olfactores that includes craniata and urochordata (Delsuc et al., 2006). This moved cephalochordates down to the most basal branch among chordates and confirmed that hemichordates belong with the echinoderms in a clade called ambulacraria. The neodeuterostome, Xenoturbella, has now been placed in a group of its own as the sister group of ambulacraria (Bourlat et al., 2006).

From an evolutionary point of view, there are several notable novelties and peculiarities among various groups of deuterostomes. Early in echinoderm evolution the water vascular system evolved. It is a system of canals that branch and form tube feet that function in locomotion, feeding, and gas exchange and it is unique to echinoderms. It consists of a ring-shaped canal in the center of the animal with connections to five radial canals. The radial canals branch further into the tube feet which each consist of a bulb-like structure and a suckered podium. Muscle cell
contractions in the walls of the tube feet can cause the podium to extend or bend by forcing water into them or out of them. By this method a sea star or urchin, for example, can slowly walk across a substrate with coordinated movements of the tube feet. The sea cucumber even has modified tube feet that are used to shovel food into the mouth. Additionally, the highly branched nature of the water vascular system gives it high surface area for performing gas exchange. The madreporite is a small opening near the central ring that allows water to flow into and out of the water vascular system from the surrounding seawater (Campbell and Reece, 2005). The water vascular system is an example of a novel multifunctional system that was generated during the early evolution of echinoderms. It has been maintained in all six of the extant classes of this phylum and modified differently for various functions within each of these lineages.

Additionally, with the exception of sea cucumbers, adult echinoderms are peculiar in that they have all adopted a pattern of radial symmetry although they are bilaterally symmetric at embryonic and larval stages (Campbell and Reece, 2005). The method by which this radical evolutionary change in body patterning occurred is not fully understood. This highly derived body plan leaves its bilaterian roots hardly recognizable. Even the adult sea cucumbers, the only non-radially symmetric echinoderms, retain 5 rows of tube feet implying that their elongated body plan could be a further derivation upon a pentaradial past, and not a basal bilaterian layout. Comparisons of morphology and gene expression patterns have not determined the orientation of the ancestral anterior-posterior (A/P) axis in echinoderms. One
hypothesis is that each arm of a starfish, for example, could represent a duplicated A/P axis, while another hypothesis proposes that the oral-aboral axis of the starfish represents a single A/P axis of the animal (Carroll et al., 2001).

Although echinoderms have evolved to be morphologically unrecognizable as bilaterians and have developed novelties not found in any other phylum, they have done so using genes that are all shared with other phyla. These novelties are the outcome of co-opting developmental regulatory genes for use in different cell types by acquiring new regulatory linkages. The echinoderms are a good example of how new regulatory circuits generated by co-option can lead to dramatic changes in the body plan, and an evolutionarily novel circulatory/locomotion system. Additionally, both of these examples demonstrate that new regulatory circuits, like those that were generated at the base of the echinoderm radiation, have served as a foundation for further diversification (Carroll et al., 2001).

These same mechanisms have led to evolutionary novelties among chordates like the notochord, the morphogenic process of neurulation, NCCs, and bones to name a few (Figure 1.2). In some ways each of these developmental novelties laid the foundation for the next. The notochord serves as an embryonic organizer for the central nervous system (CNS) in addition to providing structural support for the spinal cord after differentiation. Co-option of the T-box gene Brachyury to specify the notochord was an important step in laying the foundation for the dorsal CNS of chordates (Carroll et al., 2001). The morphogenic process of neurulation of the dorsal ectoderm to form the dorsal neural tube is a process that is fundamental to CNS
formation in all chordates and is unique to this phylum. In what seems to be a related invention, craniates have a population of embryonic cells that are specified during neurulation known as NCCs that migrate away from the dorsal neural tube and have numerous cell fates in extant vertebrates. Higher up the branches of the chordate tree, came the evolutionary novelty of mineralized bone. Interestingly, the very first mineralized bone-like structures found in vertebrates are teeth (Donoghue and Sansom, 2002), which in part are generated from NCC lineages. By examining the novelties in vertebrate evolution that are marked with a star in figure 1.2, it can be inferred that the innovation of NCCs is another example of a co-opted gene regulatory network being deployed in a new embryonic cell lineage that set up a foundation for further diversification. The developmental gene regulatory network for NCCs has further co-opted numerous cell fates for diverse functions during the course of vertebrate evolution. These include melanocytes, sensory neurons, sympathetic neurons, enteric neurons, glia, smooth muscle, neuroendocrine cells, cartilage, bone, dentine, and mechanoreceptors of the lateral line system neuromasts. Intriguing questions remain about the basal characteristics of NCCs and their original functions in the soft, non-bony ancestors of craniata.

In figure 1.2, the placement of the branch points is well accepted in the literature and textbooks (Campbell and Reece, 2005) with the exception of the branch points shown for urochordates and cartilaginous fishes. Urochordates would normally be shown diverging from the ancestors of tetrapods before the evolution of NCCs, but I have chosen to place them as shown because of the evolutionary implications of the
research discussed in this dissertation. There is not sufficient fossil evidence to
determine if the split of the chondrichthyan lineage from tetrapod ancestors occurred
before or after the evolution of endochondral bone. Fossil evidence shows that the
common gnathostome ancestor had a highly mineralized skeleton. However extant
members of this clade, like sharks and rays, have a highly derived almost entirely
cartilaginous skeleton (Donoghue and Sansom, 2002).

It seems that the origin of bone during vertebrate evolution may in fact be
mosaic in nature. It has been shown in chick development that the skull is made up of
bone from three different embryonic origins; the cranial neural crest (ectoderm), the
paraxial cephalic mesoderm, and the somitic mesoderm (Couly et al., 1993). It is clear
that mineralization of the vertebrate skeleton began in the head, but it remains unclear
which embryonic cells were the first to contribute to mineralized bone. Bone tissue is
generally thought of as a mesodermally derived tissue with some contribution from
neural crest “ectomesoderm” lineages. The ability of ectoderm lineages to undergo
mesenchymal transition and give rise to mesodermal tissue types is not novel to
vertebrates or NCCs. In fact, ectomesoderm is found even in Cnidarians and
Ctenophores and is common to all metazoans (Martindale and Henry, 1999; Seipel and
Schmid, 2005).

It is plausible that the teeth and jaw of vertebrates were made possible by
NCCs first and then those developmental gene regulatory circuits were co-opted by
mesoderm lineages (Shimeld and Holland, 2000), or vice versa. Alternatively, it is
possible that bone tissue evolved convergently in these different lineages. It is
interesting to think about the sequence of the evolutionary processes that involved the
development of NCCs in craniate embryos long before the invention of bones and then
allowed for the acquisition of the bone cell fate for use by the NCC lineages. This line
of thinking complicates the issue about what an evolutionarily “new” cell type really
is. NCCs take on fates like melanocytes, bone, cartilage, neuroendocrine cells,
neurons and glia of the peripheral nervous system (PNS), all of which can also
develop from non-neural crest lineages in vertebrates and/or are also found in
invertebrates without NCCs. It could be argued that NCCs evolved more like a
recycle bin where existing gene regulatory networks can be reused in a neural plate
border cell lineage. This gets to the point of a need for defining a cell type by more
than its differentiated cell fate (which may not be novel), but by its embryonic origin,
and the gene functions that regulate its specification and determination.

In the case of NCCs, their definition must include at least four different aspects
in order to understand them well enough to begin addressing their evolutionary
origins. First, of course, they are a multipotent set of embryonic cells (Bronner-Fraser
and Fraser, 1988b; Fraser and Bronner-Fraser, 1991). Their differentiated cell types
are many, but they alone do not constitute the evolutionary novelty. Secondly, they
have a very specific embryonic origin at the edges of the border between the neural
and non-neural ectoderm (Scherson et al., 1993; Birgbauer et al., 1995; Bronner-Fraser
and Fraser, 1997). Thirdly, these cells also have the ability to migrate near the time of
the end of neurulation (Bronner-Fraser and Fraser, 1988a; Serbedzija et al., 1989). It
can be noted that this migratory property does not constitute any evolutionary novelty
on its own either. This could be described as another instance of recycling or co-opting a preexisting molecular regulatory pathway for migration machinery that has been reused in neural plate border cell lineages for NCC development (Kee et al., 2007). The fourth aspect of the NCC definition is the gene regulatory cascade that specifies their position on the neural plate border and initially defines them as NCCs (Meulemans and Bronner-Fraser, 2004; Meulemans and Bronner-Fraser, 2005). Only when examined on all four levels are NCCs considered a “new” cell type that could have been an important key to the success of craniata.

Although this seems like a comprehensive definition for NCCs, unfortunately there are other cell lineages that compose parts of the elaborate vertebrate brain that almost match these same four criteria but are not considered NCCs. There is a lot of room for interpretation in the field of vertebrate neuroanatomy, and the embryonic origin of all the parts of the complex brain is not fully understood. The diencephalon region of the developing nervous system gives rise to pigmented melanocytes that function in photoreception in vertebrate eyes. In fact there are three sources of melanocytes in vertebrates: the skin (neural plate border, NCCs), the retina (medial anterior diencephalon), and the pineal organ (neural plate border). In addition to producing melanocytes, cells from the anterior medial diencephalon that contribute to retinal pigmented epithelium (RPE) express some of the same genes as developing NCCs and have migratory properties. However, they have a different embryonic origin than NCCs (Etchevers et al., 1999). The pineal organ of vertebrates seems to share embryonic origin with NCCs and is thought to develop bilaterally from cells of
the neural plate border at the most anterior domain that gives rise to NCCs (Etchevers et al., 1999). The pineal is referred to as the “third eye” or “medial eye” in non-mammalian vertebrates because it functions in photoreception. In mammals this organ has taken on a neuroendocrine function and controls melatonin production. In culture, pineal cells can differentiate into neural crest and placode cell types like lens, muscle and neurons (Araki, 2001). There is no evidence that cells constituting the pineal organ migrate at any time during development. But gene expression matches that of developing NCCs with the exception of the transcription factor Snail which is expressed in NCC lineages and is required for their migration. The question of whether the pineal organ is in fact constituted by NCCs that do not migrate away from the neural tube but instead remain at the site of their origin in the dorsal neural tube remains to be addressed (Araki, 2001).

Morphologically the ocellus of ascidians like *Ciona intestinalis* (*Ci*) has been compared to both the pineal and the lateral eyes of vertebrates (Dilly, 1964; Eakin, 1973). The pigment cells of vertebrate eyes that are involved with light sensing, RPE, are *not* neural crest derived cells and do *not* originate from the neural plate border. The developmental origin of the vertebrate eyes is not the same as that of the cephalic neural crest and the pineal at neural plate stages (Adelmann, 1936b; Adelmann, 1936a). The putative location of the pineal rudiments at the edges of the neural plate and in the neural fold corresponds to the embryonic origin of the PCL in *Ci*. Recent analysis of visual cycle genes in *Ci* supports the hypothesis that the ocellus organ is homologous to the vertebrate medial eye (the pineal organ) and not to the lateral eyes
(Takimoto et al., 2006; Kusakabe and Tsuda, 2007). Although the term pineal organ is only used in reference to the vertebrate brain, homology with photoreceptive organs of invertebrates like ascidians has been implied (Dilly, 1964; Eakin, 1973; Torrence, 1986). This dissertation work supports the hypothesis that the ocellus of Ci is pineal-like and comes from a lineage of cells with all the basic properties of developing NCCs. Furthermore, I propose that the PCL of Ci represents a “basal” NCC population that lends insight to the tools present in the evolutionary toolbox of the Olfactores ancestor and implies that NCCs and the pineal organ may share a common evolutionary origin.

Invertebrate chordates neurulate and have cell fates that are similar to vertebrate neural crest derivatives, namely melanocytes and neurons of the PNS, despite the fact that they lack NCCs by definition. Questions about the evolutionary origin of the neural crest have previously been pursued by examining neurulation and the development of these neural crest-like cell types in cephalochordate and urochordate model systems (Meulemans and Bronner-Fraser, 2002; Meulemans et al., 2003; Jeffery et al., 2004). These studies were not able to address all four aspects of the definition of NCCs and provide a convincing argument for the presence of, or lack of, neural crest-like cells in their model system.

Until recently cephalochordates, like Amphioxus, were considered to be the invertebrates most closely related to vertebrates, while urochordates, like the ascidian Ci, were the most basal chordates sharing a common chordate ancestor with vertebrates. Sequence data from more deuterostome genomes, like starfish,
amphioxus, *Oikopleura* and *Xenoturbella*, have allowed for more extensive sequence comparisons in recent years. Comparison of nuclear genes and release of the completed Amphioxus genome confirmed that they are the most basal chordates (Bourlat et al., 2006; Delsuc et al., 2006). Although cephalochordates were phylogenetically placed as the sister group to vertebrates when this project began, it has always been clear to me that urochordate embryos of *Ci* are better suited for comparisons with vertebrate embryos especially with regards to neurulation and neural cell types. Neurulation in amphioxus embryos is distinctive from other chordate embryos. In the cephalochordate embryo, non-neural ectoderm detaches from the edges of the neural plate and slides over it to meet at the midline before the neural plate rolls into a tube. These morphogenetic movements and the gene expression of cells at the neural plate border, in Amphioxus, can only be seen by careful cross-sectioning of *in situ* hybridized embryos at multiple neurula stages and at multiple levels along the A-P axis. This difference in the order of neurulation events along with the need for embryo sectioning to view the neural plate border cells are the most significant problems in pursuing the origin of neural crest in Amphioxus. Additionally, seasonal availability of embryos for only a few weeks in the summer has impeded the development of tools for functional analysis.

The pursuit of identifying neural crest-like cells in urochordates was discouraged because of gene expression analysis that implied that most of the genes involved in NCC specification were not expressed at the neural plate border in amphioxus (Meulemans and Bronner-Fraser, 2004; Meulemans and Bronner-Fraser,
2005). This seemed to negate the purpose of pursuing the same question in the, then basal, urochordates because it would be less likely that NCC arose at the base of Chordata and were subsequently lost in the cephlochordate lineage, than the hypothesis that they simply arose at the base of the vertebrate radiation. However, after carefully reviewing the same literature cited in the above-mentioned publication, I disagree with their analysis of the gene expression patterns, and their failure to clearly state where they define the neural plate border in this embryo. It is my opinion that the majority of neural crest specifiers are in fact expressed at the neural plate border in amphioxus embryos in addition to the neural plate border specifiers and that morphological differences in the process of neurulation have clouded this issue. It is still very important for the embryonic origin and gene regulation of developing melanocytes and the lamellar organ in amphioxus to be determined.

The search for the evolutionary origin of a new cell type arising in the vertebrate radiation, like the neural crest, should involve analysis of which parts of the molecular toolbox were present in the common ancestor of olfactores and even those tools present in the common chordate ancestor. This is important because a novel cell type and the mechanisms by which it develops cannot just spontaneously arise in a clade, but must involve the elaboration upon an existing regulatory system by cooption of factors and formation of new circuits within an already existing regulatory network. Translating what is known about NCCs in vertebrate embryos onto a closely related invertebrate embryo has to be done with careful considerations for the differences in developmental strategies and morphologies between the two species being compared.
The morphogenic processes of gastrulation and neurulation are extremely compacted temporally in *Ci* development compared to that of vertebrates. We expected the gene regulatory network controlling specification of the neural crest-like cells in *Ci* to be deployed more quickly and perhaps begin earlier in embryonic development.

The dorsal CNS of *Ci* is only present in the larval stage and consists of only about 330 cells. This non-feeding larva is formed from the limited resources that are already present in the unfertilized egg. Although extant tunicates have been evolving for just as long as vertebrates since their divergence, it has been to very different ends. The filter-feeding lifestyle of protochordates has maintained a simple CNS and sensory organs that for many species, like *Ci*, are used only temporarily in life to distribute offspring before metamorphosis into their sessile adulthood. Although the single neural crest-like cell lineage in *Ci* is not ancestral, it is likely to represent the most basal example of what was accomplished using the gene regulatory network of the neural plate border that was present in the common ancestor of Olfactores. The limited scale of the *Ci* embryo with a small number of cells, a small genome lacking the gene duplications that occurred in the vertebrate radiation, and a simple temporary head that never hunts or feeds, reveals the neurosensory function and multipotent nature of basal NCCs in chordate evolution all within an individual embryonic cell lineage.

**The ascidian model**
The ascidian embryo is an ideal invertebrate model for analyzing gene regulation in melanocyte development. Ascidians are marine organisms and, as chordates, share many of the same embryological processes and gene regulatory networks with vertebrates that are not present in either *Drosophila* or *C. elegans* (Dehal et al., 2002). Three species are actively studied, *Halocynthia roretzi*, *Ci intestinalis* and *C. savignyi*, but this research is restricted to *C. intestinalis*.

The ascidian tadpole consists of about 2500 cells, has a well-defined cell lineage and hatches just 18 hours after fertilization. *Ci* embryos are available year round in San Diego Bay and can be cultivated in the lab. Unlike most chordate embryos, ascidians utilize a cell-lineage based developmental program and most of the lineages have been well described at least through neurulation (Conklin, 1905; Nishida, 1987; Nicol and Meinertzhagen, 1988b; Nicol and Meinertzhagen, 1988a; Taniguchi and Nishida, 2004). Its small size and relative transparency allow the entire period of embryonic development to be documented with high-resolution microscopy with single cell resolution of cells near the surface of the embryo. Extensive efforts by laboratories in the United States and Japan have produced a wealth of ascidian biological sequence data including over 750,000 ESTs (only humans and mice have more ESTs), two complete genome sequences from the closely related species, *Ci* and *C. savignyi* (Dehal et al., 2002; Vinson et al., 2005), and a systematic effort at characterizing the expression of all ascidian genes by *in situ* hybridization (Satou et al., 2002). Analysis of the 155 MB *C. intestinalis* genome indicates there are few
examples of large-scale gene duplications and thus gene regulation is expected to be much less complicated than in vertebrates.

The real advantages of the ascidian embryo system are in the area of gene regulatory analysis (Cone and Zeller, 2005). A simple electroporation procedure, allows the creation of hundreds of transgenic ascidian embryos (Corbo et al., 1997; DiGregorio and Levine, 2002; Zeller, 2004; Zeller et al., 2006b). Because of the rapid development of the embryo, gene expression experiments are completed within a 24-hour period. The available genomic information for two Ciona species has allowed for the successful prediction of regulatory regions controlling key genes in neural ectoderm and melanocyte development. Regulatory sequences from numerous gene promoter regions have been used to create tissue specific expression vectors used in this research, such as Tyrosinase (pigment cells), Pax 3/7 (neural plate border), MsxB (neural plate border), and Elongation Factor 1α (ubiquitous). The first and most valuable promoter that I analyzed and cloned for this research project was that of the Tyrosinase gene which is expressed in a total of four cells during larval brain development: the two pigment cells, a/a10.97 and briefly in their sister cells a/a10.98. Phylogenetic foot printing analysis was performed using the VISTA alignment tool (Mayor et al., 2000) to identify conserved regions of non-coding DNA upstream of the Tyrosinase coding region. Three putative regulatory elements (figure 1.5, dark grey peaks) were identified in the 2.1 kb region upstream of the Tyrosinase coding region (figure 1.5, exons in light gray). This 2.1 kb region was cloned from genomic DNA isolated from Ci sperm. Oligonucleotide primers were designed to amplify the
promoter and include a Sal I restriction enzyme recognition site at the 5’ end of the 
promoter and an Asp 718 restriction enzyme recognition site at the 3’ end (Appendix). 
The promoter included a recognizable TATA box, an ATG translational start site and 
the first ten amino acid codons from the endogenous Tyrosinase gene.

To generate a mis-expression vector with this gene's expression pattern the 
promoter was cloned into a pSP72-1.27 backbone vector (Promega). The sequence of 
the protein to be mis-expressed was fused in-frame with the first few codons of the 
gene thus preserving the context of the endogenous promoter's transcriptional and 
translational start signals. This has been an effective way of reproducing the tissue 
specific expression pattern of a gene using its computationally predicted cis-regulatory 
DNA in Ci and is modeled on methods that have been previously described (Corbo et 
al., 1997; Zeller et al., 2006b). I have used this gene fusion method for cloning 
expression vectors to create a variety of transgenes mis-expressing fluorescent 
proteins, transcription factors, and toxin proteins in the Zeller Lab. (Figure 1.6) 
Although finding, cloning, and testing these cis-regulatory DNAs can be time 
consuming, once in hand, they can easily be sub-cloned into a variety of cassettes to 
mis-express any of four different color variants of fluorescent proteins (Zeller et al., 
2006b). Additionally, nine different transcription factors that are implicated in 
melanocyte development have been mis-expressed in Ci embryos during the course of 
this project.

Another important part of gene regulatory analysis in any model system is the 
use of molecular techniques to knock-down or eliminate the expression of a gene
product in vivo. Many invertebrate and vertebrate model systems use techniques like morpholino oligos and RNA interference (RNAi) to perform these analyses. These two methods for disrupting gene expression both operate at the post-transcriptional level but in very different ways. Morpholinos are antisense oligonucleotides designed to complement and bind to a target transcript near the translational start site. These oligos disrupt gene expression by physically preventing translational machinery from gaining access to the transcript. Morpholinos have different internucleotide linkages than normal DNA or RNA with morpholine rings replacing the ribose or deoxyribose rings in the sugar phosphate backbone making them extremely long lasting and resistant to nuclease activity. In the ascidian model, morpholinos are the only molecular technique that has been used repeatedly to disrupt gene expression (Imai et al., 2006).

RNAi is an endogenous pathway for post-transcriptional gene silencing originally discovered and characterized in the C. elegans model system (Fire et al., 1998; Montgomery and Fire, 1998; Montgomery et al., 1998; Timmons and Fire, 1998). This pathway is required for regulation of genes that are important during development and may also be a protective mechanism against infection by dsRNA viruses. There are microRNA (miRNA) genes within an organism’s genome that are transcribed into non-coding single-stranded RNA transcripts that fold back on themselves to form hairpin structures held together by hydrogen bonds. By enzymatic processing these precursor miRNA transcripts are trimmed into short double-stranded RNA molecules by enzymes called Drosha and Dicer. A large protein complex called
the RNA induced silencing complex (RISC) utilizes one of the RNA strands as a template to target mRNAs for silencing.

One publication reported the ability to significantly knock-down gene expression in a colonial ascidian called *Botryllus* using short interfering RNA (siRNA) introduced by a soaking technique (Nyholm et al., 2006). Very recently another publication reports the use of long dsRNA introduced by electroporation to silence the expression of a gene in *Ci* embryos (Coric et al., 2008). The development of an electroporation-based technique for generating the continuous knock-down of a specific gene target through the RNAi pathway has been a goal of my doctoral research that will be discussed thoroughly in chapter three of this dissertation.

*Ci* is an ideal model system for the study of melanocyte development and the pursuit of the evolutionary origin of neural crest-like cells among chordates. The well-characterized lineages and gene-expression patterns of this particular species have formed a solid foundation from which to generate discreet testable hypotheses. An important issue in the progression of understanding NCC development and evolution has been that they are studied in a variety of very different model systems. The vertebrate model systems used to study the expression and function of NCC genes include Xenopus, Chicken, Zebrafish, and Mouse. The results of studies from all of these models have been combined to create a bigger picture of the gene regulation in neural crest development, despite significant discrepancies in some gene expression patterns between these vertebrate models. Similarly, gene expression data from a variety of invertebrate chordate species has been combined in some publications.
(Meulemans and Bronner-Fraser, 2004) confusing the true sequence of genetic events that is occurring in any one of them. It has been an important goal of this research project to provide a comprehensive analysis of the expression of neural crest gene homologues in a single organism. Ci has provided the ideal platform for a descriptive study of what has happened to the tools provided in the evolutionary toolbox handed down from the ancestor of Olfactores and how they have been utilized in combination on this branch of the chordate tree to specify neural crest-like cell types and sensory structures.

Central nervous system formation

Lineage analyses have thoroughly described the early development of the cells of the nervous system in Ci from the gastrula stage through neurulation to the time of dorsal neural tube closure (Nicol and Meinertzhagen, 1988b; Nicol and Meinertzhagen, 1988a). The initial neural plate is composed of two rows of 5 cells and through a series of canonical cell divisions that occur over the next six hours produces a neural plate of 75 cells arranged in 12 rows (Nicol and Meinertzhagen, 1988a). The clearly defined lineages of the ectoderm in Ci embryos and ease of visualization allow for careful distinction of gene expression along the neural plate border, which is made up of six cells on each side of the embryo at the gastrula stage. Three of these cells are on the neural plate side of the border and three are on the non-neural side of the border (Nishida, 1987; Nicol and Meinertzhagen, 1988a).
In Ci, neurulation proceeds similarly to vertebrate embryos. The neural plate invaginates, rolls into a tube and its two borders come together and meet at the dorsal midline zipping up from posterior to anterior. The well-defined canonical cell lineage allows distinction of ectodermal cells that will give rise to neural fates from those that are primarily epidermal in fate - an advantage compared to vertebrate embryos. Ascidians are the only chordate embryos where the neural plate border has been clearly defined and it is literally a line between two columns of cells on the left and right side of the embryo that can be observed through the microscope.

The entire larval CNS is composed of less than 400 cells. The fate and mitotic events of the neural tube cells has been difficult to study after neural tube closure when these cells are no longer visible by light microscopy. The possibility of migratory events by the neural plate border cells, like the descendants of a/a8.25 (Figure 1.4), has never been eliminated, but instead supported by consistencies in lineage analyses of at least two publications. In the ascidian Halocynthia, HRP injections of a8.25 at the gastrula stage results in the formation of a stained cell with neuronal morphology from this lineage at the larval stage that lies separate from and posterior to the sister cells, implying that it may have migrated to that location during development (Nishida, 1987; Nishida and Satoh, 1989; Taniguchi and Nishida, 2004). Another study of the mitotic history of all neural tube cells in Ci using serial fixation and fluorescent staining of carefully staged embryos showed that certain members of the a/a8.25 lineage could not be kept track of by this method. The authors implied that a possible migratory event caused them to lose track of these particular cells after
neural tube closure (Cole and Meinertzhagen, 2004). This literature provided the foundation for my hypothesis that some descendents of the a/a\textsubscript{8.25} lineage migrate shortly after neural tube closure similar to NCCs. Additionally, there have been several morphological analyses of the otolith pigment cell that briefly mention how it arrives to its final position attached by a small stalk to the ventral floor of the sensory vesicle. These publications refer to the otolith’s “migration” from its original position at the dorsal neural tube but cannot specify if this movement is within the sheet of its neighboring cells or on its own as they were written long before modern tools for cell labeling were available (Kowalevsky, 1866; Willey, 1893; Whittaker, 1973b).

The Zeller laboratory has cloned and characterized the promoters of useful genes that are expressed in cells of the neural plate border. Transgenes driven by these promoters are used to label cells of interest with fluorescent proteins and also for mis-expression experiments. Pax3 is considered one of the earliest markers of developing NCCs in vertebrate embryos. The Pax3/7 promoter of Ci is transiently expressed in all six of the neural plate border cells, including the melanocyte precursors, a/a\textsubscript{8.25}, at the gastrula stage. Its expression is maintained in the single row of cells on the non-neural side of the border throughout neurulation (Figure 1.3). Tyrosinase is a required enzyme for melanin synthesis in the melanocytes. The promoter of this melanocyte lineage specific gene is active in four cells at the dorsal midline at the time of neural tube closure, the a/a\textsubscript{10.98} and a/a\textsubscript{10.97} pairs. Expression is maintained in the two cells that will give rise to the otolith and ocellus, a/a\textsubscript{10.97}. These two cells lie posterior to all of their sister descendents lined up on the dorsal
midline of the neural tube at the time of its closure and become post-mitotic, never
dividing again (Figure 1.4) (Cole and Meinertzhagen, 2004).

In vertebrate embryos, the neural plate border can be composed of hundreds or
thousands of cells with no clear line to distinguish neural ectoderm from non-neural
ectoderm until after neural tube closure. This in combination with a non-canonical
cell lineage makes it difficult to say whether NCCs arise from the neural plate border,
the non-neural ectoderm border or from cells on both sides of this border. In early
neurula stage vertebrate embryos the neural plate border is usually defined in terms of
the expression pattern of neural crest specific genes like Pax 3 or Slug. Pax3/7
expression in Ci embryos has been demonstrated to be quite dynamic, with expression
on both sides of the neural plate border in early neural plate formation then restricting
its expression to only the column of cells on the non-neural ectoderm border (figure
1.3) during middle and late neurula stages. These nuances of changes in gene
expression patterns are difficult to define in vertebrate embryos where the cells have
no names and their individual fates are not predictable. It is not known whether all
cells at the neural plate border express the same repertoire of genes or if
subpopulations of neural crest progenitors can be identified that differentially express
some of these transcription factors. This question remains because of the lack of
specific antibodies and the level of resolution of in situ hybridization in vertebrate
embryos (Huang and Saint-Jeannet, 2004). An advantage to understanding the true
embryonic origin of NCCs would be to have a model embryo with fewer cells,
allowing more clearly defined earlier lineages correlated with later fates, where gene
expression can be analyzed with cellular resolution. The invertebrate chordate model system Ci has these advantages.

**Pigment cells**

Two melanin-containing cells are present in the brain of the Ci larva. Other pigmented cells are present in the adult body after metamorphosis. These cells are found near the siphons within the tunic and are orange in color. The embryonic origin of the adult pigment cells remains unknown, but they have been recently shown to contain melanin beginning at juvenile stages (Jeffery, 2007).

An extensive amount of work has been published characterizing the lineage, morphology and functions of the two embryonic melanocytes called the otolith and the ocellus. Like vertebrates, the ascidian embryo melanocytes are pigmented by a biosynthetic pathway homologous to vertebrate melanocytes. These melanocytes have arrested their cell cycles near the end of neurulation (Cole and Meinertzhagen, 2004), and they do not increase in number after the larva metamorphoses into the juvenile. These melanocytes are present in the juvenile for several days to a few weeks and then disappear by an unknown mechanism.

Lineage tracing experiments performed in the ascidian *Halocynthia roretzi* utilizing microinjection of HRP tracer enzyme have shown that the melanocytes and neurons of the PNS are derived from cells at the neural plate border, just like these neural crest-derived cell types in vertebrate embryos. The two melanocytes of the tadpole larva are descendants of the a8.25 pair that are on the neural plate side of the
border, while sensory neurons of the PNS are derived from adjacent cells on the non-neural side of the border (Nishida, 1987). This cell lineage shows that neural crest-like cell types come from both sides of the border between the neural and non-neural ectoderm. In addition to showing that the melanocyte lineage is neural crest-like with regards to its position in the embryo, the HRP lineage analysis suggested that this lineage may give rise to a neuron and a migratory cell (Taniguchi and Nishida, 2004).

My research has demonstrated that the melanocytes of Ci, which are derived from a pair of cells at the border of the neural and non-neural ectoderm, possess many of the same characteristics of vertebrate NCCs (chapter 2). Research from other laboratories has shown that FGF and BMP signaling is required for pigment cell development in ascidian embryos (Darras and Nishida, 2001; Hudson and Lemaire, 2001; Bertrand et al., 2003; Miya and Nishida, 2003). We have cloned and analyzed the expression of most of the key transcription factor genes implicated in neural crest development in the Ci embryo. Many of these have been previously described but not in correctly staged embryos to examine the neural plate border with single cell resolution. In translating the gene expression profiles during neurulation of vertebrate embryos to an invertebrate chordate model for comparison, it is important to consider, especially for Ci, that there are fewer cells and fewer cell cycles in which this gene regulatory network may be operating. In Ci, neurulation begins before gastrulation is completed and ends in about 3 hours with differentiation and melanization of the otolith just over two hours after that. Some of these gene expression patterns last less than a cell cycle and could be easy to miss especially if citing expression data where
the neural plate was not being emphasized and carefully examined. I have re-examined these in gastrula and neurula stage embryos as well as cloned and examined expression patterns of some new genes in *Ci*. After examinations of all but one of the transcription factors listed as neural plate border specifiers and neural crest specifiers in a review paper of vertebrate neural crest (Meulemans and Bronner-Fraser, 2004), I have found that all but two of them are expressed in this individual cell lineage either at the neural plate border or at the midline after neural tube closure, consistent with the hypothesis that these genes may function in a gene regulatory network.

The expression patterns of these genes in ascidian embryos are consistent with their role in melanocyte development and NCC development in vertebrates. Evidence from our cell lineage tracing experiments suggests that derivatives of the melanocyte cell lineage also produce two types of neurons and demonstrates that cell migration is occurring. Importantly, this data demonstrates that the cells of the ascidian melanocyte lineage have the same four basal characteristics as vertebrate NCCs.

**Methods**

**Transgenic and cleavage-arrest embryo production.**

A 3 Kb region of the *Ci* Pax3/7 gene regulatory region was amplified by PCR and subcloned into an expression vector containing an optimized Green Fluorescent Protein (Zeller et al., 2006b). A 2.1 Kb region of the *Ci* Tyrosinase gene regulatory region was amplified by PCR and subcloned into a vector containing a membrane-bound form of optimized Yellow Fluorescent Protein (Zeller et al., 2006b). PCR
primer sequences can be found in the Appendix. Fertilized and dechorionated eggs were electroporated with 35 µg of the construct using a custom electroporation device (Zeller et al., 2006a) in a total volume of 800 µl. At neurula stages, some embryos were cleavage arrested with 2 µg/mL cytochalasin B in seawater (Whittaker, 1973a) and allowed to develop until melanin formation at 12-15 hpf at 18°C.

**Figures**

**Figure 1.** Recent changes in deuterostome phylogeny. Urochordates, like *Ciona intestinalis*, are now accepted as the sister group to craniates, while the cephalochordate model, *Amphioxus*, has been demoted to the position of basal chordate. The newcomer to the group, *Xenoturbella*, has found its place among deuterostomes as the sister of ambulacraria.
Figure 1.2. Hypothetical sequence of some of the steps during the evolution of developmental novelties in chordates from the ambulacraria/xenoturbellida split to tetrapods. Novel tissues, structures, body systems, or cell types that were acquired during the evolution of chordates are listed on the left in the hypothetical order that they were acquired from top (chordate ancestor) to bottom (tetrapods). Branch points are shown splitting off to the right for extant and fossilized groups within olfactores at various points in this evolutionary scheme. Stars indicate novel cell types, tissues, or systems that are constituted fully or in part by differentiated NCCs.
notochord

neurulation

neural crest cells

-cartilagenous skull

& cartilagenous vertebrae

-inner ear with two semicircular canals for balance

-divergence of urochordate ancestor

-divergence of hagfish ancestor

-differentiation of lamprey ancestor

-divergence of conodont ancestor

-mineralized teeth

-cartilage containing type II collagen

-starved endoskeleton (dentine, enamel, perichondral bone)

-jaws & paired fins (lateral appendages)

-divergence of cartilagenous fish (chondrichthyes) ancestor

-divergence of ray-finned fish (actinopterygi) ancestor

-ossified endoskeleton (endochondral bone with calcium phosphate matrix)

-lungs

-divergence of ray-finned fish (actinopterygi) ancestor

-limbs with feet
Figure 1.3. Cleavage-arrested Pax3/7 transgenic embryo.
The embryo was cleavage-arrested with continuous treatment of 2 µg/ml cytochalasin B starting at 7.5 hpf and imaged at 14 hpf. The transgene contains a 3 kb region of the Pax3/7 cis-regulatory domain fused to Green Fluorescent Protein which accurately reflects endogenous Pax3/7 expression in the early embryo. Transgene expression (green) marks the cells on the non-neural side of the border. The neural tube has not closed, allowing both a/a9.49 cells to differentiate into the default otolith pigment fate at the neural plate border. Anterior is up.
Figure 1.4. Mitotic cell divisions in the bilaterally symmetrical right or left half of the PCL. Embryonic stages listed across top. The a10.97 cell is post-mitotic and differentiates into one of the pigment cells. Dashed lines indicate cell divisions which have not been previously characterized, but our results indicate 9-12 total descendants from each half PCL. Although a10.100 was not reported to be post-mitotic in previous studies, our research implies that it does not divide again, only migrates (Figure 2.8). The a8.26 cell, the sister cell of the PCP, gives rise to anterior dorsal neural tube (Cole and Meinertzhagen, 2004).
Figure 1.5. Computational analysis can predict the location of cis-regulatory DNA within *ascidian* genomes.

VISTA analysis (Mayor et al., 2000) comparing 9 kilobases of the Tyrosinase genes from *Ciona intestinalis* and *Ciona savignyi*. Exons are shaded light gray and shown with light gray boxes at the top of the graph. Highly conserved non-coding regions (putative regulatory modules) are identified with dark gray shading. The number of nucleotides in each of the sequences being compared is shown across the bottom axis and the percent similarity between the two genome sequences is shown on the vertical axis.
Figure 1.6. The Tyrosinase promoter can be used to drive expression of various proteins in the pigment cells of *Ciona intestinalis* embryos.

a. A close-up photo of the otolith pigment cell expressing the Tyrosinase::GPI-YFP construct. Here the pigment cell promoter is driving a membrane bound version (Passamaneck et al., 2006; Rhee et al., 2006) of the optimized yellow fluorescent protein (Zeller et al., 2006b). In this mosaic larva only the otolith pigment cell is transgenic; the unlabelled ocellus can be seen in the background. The membrane anchored fluorescent protein allows us to see the substantial foot of the otolith cell that is embedded in the sensory vesicle wall in addition to the narrow stalk by which the main cell body containing the melanin and the nucleus is attached.

b. An albino tadpole generated with the Tyrosinase::DTA construct. Here the pigment cell promoter is driving the diphtheria toxin A gene. This toxin protein results in translational inhibition and essentially kills any cell that expresses it. This larva is transgenic in both pigment cells resulting in the albino phenotype. The pigment cell reporter does not work in combination with this construct because its expression is also inhibited by the toxin, so a nervous system GFP reporter was included to verify transgenics and identify dorsal orientation (not shown).
Appendix

Tyrosinase Promoter Forward:
CGGTCGACGAATTTTCTTTCACAGATTTCATG
\text{SalI}

Tyrosinase Promoter Reverse:
CGCTGGTACCTGTTAAATACCATCTTTTGAAG
\text{Asp718}

\text{Pax3/7 Promoter Forward:}
GCGCTCGAGTGATATCGCCAAATCTCAGGGCGATAAT
\text{XhoI}

\text{Pax3/7 Promoter Reverse:}
CGGCGGCCGTACGGAATTAGACCCTGTGATGCTCATCAT
\text{Asp718}

MsxB Promoter Forward:
GCGCTCGAGACTACACACTACGCATTGATGTCGC
\text{XhoI}

MsxB Promoter Reverse:
CGGCGGCCATTCCGCAATACAGAGATTGAGAATACG
\text{Asp718}

Elongation Factor 1\alpha Promoter Forward:
GCGCTCGAGATTTTTGTAAACCACCCCTCCTCCC
\text{XhoI}

Elongation Factor 1\alpha Promoter Reverse:
CGCTGGTACCTAATAGTGCTTTGTCTTTTCAT
\text{Asp718}

\text{GFPci Forward:}
CGGCGGTAACCCATGGTCCAAAGGTGAAGAAC
\text{Asp178}

\text{GFPci Reverse:}
CGCGAATTCTATTATATGAGATGCTCTTCTCTCAT
\text{EcoRI}

Membrane-bound YFPci Forward:
GCGCCATGGTACAGATGCTGGCAACTGTCGC
\text{NcoI}
Membrane-bound YFPci Reverse:
CGCGAATTCCTTACAGAGAAATGAAATGCAGCCAGGCC
  EcoRI

Membrane-bound YFPci oligos to knock-out NcoI site:
ACCACGCTGCAAGGATTCGCCACATATGGTGTCAGAAGGTTG Sense
CACCTTTGGACACCAAGCATGTCGGCGAAGCTCCTGACGCTGGT Antisense

Tyrosinase Promoter Xba Forward for membrane-bound YFPci:
CGCTCTAGAACCATTTTTTCATGCGCTGACAAATGGCGG
  XbaI

Tyrosinase Promoter Nco Reverse for membrane-bound YFPci:
CGCGGTACCATGGTTGGAAGTGCTAGACAAATGTGTGAAC
  NcoI

Diptheria toxin A Forward:
CGCGGTACCAATGGGCCTGTATGTGTTGTTTA
  Asp718

Diptheria toxin A Reverse:
CGCGAATTCCTTATCGCTGACAGATTTCC
  EcoRI
References


II. A Basal Neural Crest Cell Lineage in *Ciona intestinalis*

**Introduction**

NCCs are defined as a population of 1) multipotent embryonic cells that 2) arise at the border of the neural and non-neural ectoderm, 3) employ a characteristic set of genes organized in a multitiered regulatory network and 4) migrate at the end of neurulation prior to differentiation (Meulemans and Bronner-Fraser, 2004; Meulemans and Bronner-Fraser, 2005). Because vertebrate embryos have large numbers of cells, it is technically difficult to profile the transcriptome of individual NCCs (Huang and Saint-Jeannet, 2004) and it remains unclear if individual NCCs express the complete suite of regulatory genes. There are also differences in the patterns of NCC gene expression in various vertebrate embryos, highlighting the importance of describing these processes in a single species and then making comparisons among different vertebrate embryos. Recently molecular (Bourlat et al., 2006; Delsuc et al., 2006) and morphological (Ruppert, 2005) analyses place urochordates as the sister group of vertebrates. Ascidians, like *Ciona intestinalis* (*Ci*), are thus ideally positioned phylogenetically to investigate the evolutionary origin of NCCs. I have therefore characterized the development of descendants of an individual neural plate border cell lineage in the *Ci* embryo with regard to the definition of NCCs.

**Beginning at the border**

The two melanocytes of the ascidian embryo, called the otolith and the ocellus, are descended from a neural plate border cell lineage and are present in the larval brain
where they perform sensory functions (Nishida, 1987; Nicol and Meinertzhagen, 1988b; Nicol and Meinertzhagen, 1988a; Tsuda et al., 2003) (Figure 2.1a). The embryonic neural ectoderm develops from 22 precursor cells present in three rows at the 110-cell stage (Figure 2.2). Subsequent rounds of cell division along the anterior-posterior axis generate 42-cells of the ninth generation making up the neural plate (Figure 2.3). Previous lineage studies by Nishida in another urochordate, Halocynthia roretzi (Hr), have shown that although a8.25 and a8.26 are sister cells, only the a8.25 pair will give rise to pigment cells (Nishida, 1987; Nishida and Satoh, 1989; Taniguchi and Nishida, 2004). By microinjection of DiI lineage tracer into either one of the a8.25 cells at the 110-cell gastrula stage, we confirmed these findings to be true in Ci as well (Figure 2.1). Hence this pair (a/a 8.25) will be referred to as the pigment cell precursors (PCPs) while all the descendants of this pair will be referred to collectively as the pigment cell lineage (PCL) (Figure 2.1).

The PCL develops on the edge of the neural plate adjacent to, and contacting, cells of the non-neural ectoderm (Figure 2.1b-e, and Figure 2-4). Either the otolith or the ocellus can originate from the right (a8.25) or the left (a8.25) PCP. At the time of neural tube closure, the neural and non-neural ectoderm border is organized with four pairs of the PCL on the neural plate side of the border (Nicol and Meinertzhagen, 1988a) and the cells that will give rise to the dorsal midline epidermis on the non-neural ectoderm side (Nishida, 1987). In figure 2.5, the four pairs of the PCL can be identified in the first frame of the time-lapse movie that starts around the time of neural tube closure. The four “white” labelled cells (top, right panel) are the labelled
members of the PCL from one half of the embryo, while the dark spaces between these cells represent the unlabelled PCL cells of the same names from the other side of the embryo. Here they have intercalated between one another to *briefly* form a straight line on the dorsal neural tube.

Consistent, predictable cleavage patterns and identification of single cells with high resolution allow the reliable tracking of individual neural plate cells during *Ci* development because cells do not wander unpredictably across the border. Although the positions of some cells change relative to one another, as shown in figures 2.2-2.4 (*), notice that the *same* cell lineages are included within the black border outlining the neural plate in each diagram. These flattened, two-dimensional representations of neural plate cell lineages are my interpretation and adaptation of lineage studies from the Meinertzhagen Laboratory (Cole and Meinertzhagen, 2004). Some differences from previously published neural plate diagrams are that I have included cell lineages on both sides of the neural plate border, properly placed b-line cells posterior of the PCL, and eliminated muscle cell lineages in the final diagram. Although these changes help to provide a clearer view of how neurulation is proceeding, they cannot be interpreted as entirely accurate. In fact, by the developmental stage represented in figure 2.3, cell divisions are asynchronous (as shown in Figure 2.1e) and in reality all the ninth generation cells are not present in the embryo at the same time. Additionally, morphogenesis is dynamic as this apparently flat sheet of neat columns and rows is actually getting stretched and curled into a very narrow cylinder with a large anterior bulge.
**Multipotent cell fates**

In addition to melanocytes, our lineage-tracing experiments reveal that in *Ci* the PCPs give rise to multiple cell types. Studies have established that just after neural tube closure in *Ci*, there are a total of ten PCP descendants (Cole and Meinertzhagen, 2004), however the fates of only the two pigment cells are known. By examining DiI labeled and Hoechst stained embryos, we estimate that there are 9-12 descendants from each of the PCPs in the trunk of the larva at 20hpf. The otolith and ocellus pigment cells are post-mitotic after neural tube closure (Cole and Meinertzhagen, 2004), implying that at least two more cell divisions take place among their sister cells located more anteriorly. In addition to the melanocytes, our lineage tracing experiments indicate that several other neural crest-like derivatives are derived from the PCPs as has been reported in the ascidian *Hr* (Taniguchi and Nishida, 2004). Some of the a/a8.25 derivatives contribute to wall of the brain vesicle (Figure 2.1f-j). Although we are not claiming homology with the *Ci* brain vesicle wall, the telencephalic meninges that covers and cushions the vertebrate brain has been shown to be a NCC derived structure in the chicken (Le Douarin et al., 1993).

We also observed other PCP derivatives including two different pairs of neurons. In larvae of several ascidian species, a pair of "spring-like" sensory neurons contacting the otolith cell has been described (Torrence, 1986; Sakurai et al., 2004) that are likely descendents of the PCL in *Hr* (Taniguchi and Nishida, 2004). Our confocal images of larvae with DiI labelled otolith cell lineages show individual projections (Figure 2.1i-j, arrowheads) protruding from the sensory vesicle wall like
those previously described SEM imaging (Sakurai et al., 2004) suggesting that this pair of neurons comes from the PCL in Ci. The coiled spring-like projection can be seen contacting the otolith pigment cell (Figure 2.1j, arrowhead), which is thought to facilitate transmission of sensory information to the CNS (Sakurai et al., 2004). We have not determined whether these cells are always associated with the otolith fate or if they can also arise from the part of the lineage that gives rise to the ocellus pigment cell. In addition to these sensory neurons, a second pair of neurons extends posteriorly. One neuron consistently arises from each of the right and left PCPs (Figure 2.1f-h), and lays apart from its sister cells which all reside in close proximity of one another within the brain vesicle. Because the melanocytes are post-mitotic at the end of neural tube closure (Cole and Meinertzhagen, 2004), I hypothesized that these neurons must descend from the anteriorly positioned sister cells of the lineage and tested this hypothesis with time-lapse analysis discussed below.

**Migratory cells**

Our analysis of the PCL indicates that at least two types of cells undergo migration: the pair of posterior neurons and the otolith cell. Our cell lineage experiments combined with time-lapse imaging confirm that the two neurons have migrated posteriorly after neural tube closure in Ci (Figure 2.5) as previously suggested in the ascidian Hr (Taniguchi and Nishida, 2004). The melanocytes and their sister cells are aligned dorsally at neural tube closure, but the otolith soon translocates to the ventral floor of the brain vesicle (Cole and Meinertzhagen, 2004).
Several other cell types in ascidians have been shown to migrate during development including heart precursors (Davidson et al., 2005) and body pigment cells of a colonial ascidian that were labelled and followed after neural tube closure (Jeffery et al., 2004). Because of the small size and temporary lifestyle of the Ci larva, we hypothesized that cell movements among PCL descendents would not be extensive, perhaps only one or two to several cell diameters in distance. This would be consistent with the lineage tracing data from other ascidian species where the a8.25 lineage has been labeled (Nishida, 1987; Taniguchi and Nishida, 2004). Further analyses employing time-lapse microscopy have allowed us to identify and further characterize the posterior migratory neurons of the PCL. Unlike work done by Jeffery to describe migratory pigment cells in the ascidian Ectienascidia turbinata and other ascidians (Jeffery et al., 2004; Jeffery, 2007), which 1.) is not done with time-lapse analysis, 2.) has no correlation with cells originating at the neural plate border, and 3.) makes no mention of epithelial to mesenchymal transition (EMT) as a means of migration, our observations are the first to describe cell movements in a neural crest-like cell lineage that arises at the border of the neural/non-neural ectoderm in a protochordate.

It could be assumed that the posterior neurons arise from one of the anterior sister cells of the PCL because it has been demonstrated previously that the pigment cells (a/a10.97), the most posteriorly located members of the lineage at neural tube closure, are post mitotic (Cole and Meinertzhagen, 2004). Our time-lapse analysis of the DiI labeled a8.25 lineage reveals that it is, in fact, the most anterior pair (a/a10.100) that begins to dive ventrally and migrate immediately following neural
tube closure (Figure 2.5). These cells send out a projection that then pulls the cell body forward to meet it several times during their journey along the ventral edge of the sensory vesicle (Figure 2.5). We demonstrate here for the first time that these posterior migratory neurons are descendants of the PCP, a/a8.25. They likely correspond to the recently described “ventroposterior sensory interneuron” of the Ci nervous system (Imai and Meinertzhagen, 2007).

Evidence of potentially migratory pigment cells in the larvae of a large colonial ascidian, has been previously described (Jeffery et al., 2004; Jeffery, 2007). It is unclear how those cells relate to the PCL of Ci because there is no way to distinguish which “anterior neural tube cells” were injected in that study. Additionally, the cells were likely labeled well after neural tube closure and there is no evidence that the labeled migrating cells originated from the neural plate border. No evidence was presented that those labeled cells were multipotent, or even bipotent, or that they undergo any type of EMT. They were characterized only by examination of expression of one gene and one epitope that are not neural crest specific. In contrast, the two posterior migrating neurons described herein are descendants of a multipotent precursor cell that arises on the neural plate border and expresses a majority of the genes implicated in NCC development in vertebrates as discussed below. Additionally, they each display the morphological characteristics of a cell that has undergone EMT with fillopodia-like extensions during migration.
**Gene expression**

By carefully documenting temporal and spatial gene expression patterns with single cell resolution, we present the first comprehensive analysis of NCC gene expression in a single ascidian species focusing on a neural crest-like lineage. In *Ci* the PCPs and their descendants express homologues of eight neural crest transcriptional regulatory genes and a gene encoding a melanocyte enzyme. Although previous reports have surveyed expression patterns of NCC genes in several different ascidian species, in many cases descriptions of gene expression during significant developmental time points have not been carefully examined or reported. Problems have arisen first by generation of gene expression data that is not of cellular resolution in an organism like *Ci* that is known to have single-cell resolution and well-known early cell lineages; and second by the flurry of review articles that claim to have gained insight into the evolution of neural crest by examining the existing literature regarding expression, or more alarmingly the lack of expression, of neural crest gene homologs among protochordates (Meulemans and Bronner-Fraser, 2004; Meulemans and Bronner-Fraser, 2005). Additionally, authors of review articles are summarizing the gene expression patterns from multiple species and genera not only within the same paragraph but even within single sentences. Using DAPI counterstaining to positively identify PCL cells, we find that patterns of gene expression within PCL derivatives is very dynamic, often changing from one cell cycle to the next. Examination of NCC gene expression profiles in multiple vertebrate species indicates that there are differences in the presumed neural crest gene regulatory network (GRN)
(Meulemans and Bronner-Fraser, 2005) clearly indicating the importance of developing a GRN model within a single species.

In vertebrates, patterning at the neural plate border results from the interaction of several different signalling cascades such as BMP, FGF, Wnt and Notch (Meulemans and Bronner-Fraser, 2004; Meulemans and Bronner-Fraser, 2005). Ci orthologues of these signalling molecules are expressed in temporal and spatial patterns consistent with them functioning in an analogous manner in Ci (Bertrand et al., 2003; Imai et al., 2004) and we did not re-examine their expression here. In vertebrates, the neural plate border specifier genes include Msx, Dll, Zic and Pax3 and 7. The Ci AP-2, Dll-b and ZicL homologues are expressed early in the PCL, but by neurulation are no longer expressed (Figure 2.6a and b, ZicL data - (Anno et al., 2006)). The Ci MsxB and Pax3/7 genes are expressed from late gastrulation through early neurulation in the PCL (Figure 2.6d and e). Msx-b expression is maintained in the PCL through the neural plate stage, but Pax3/7 expression in the PCL is extinguished just as neurulation commences. Ci orthologues of NCC specifier genes Snail(slug), Ap-2, FoxD and Id are expressed in the PCL (Figure 2.6b, c, f and g), however we were unable to detect SoxE and Meis expression in the PCL. Id and Snail are expressed in the late gastrula through early neurula stages in the PCL and thereafter PCL expression is extinguished, although these genes are expressed in neighbouring, non-PCL cells. FoxD is expressed late in the development of the PCL just after neural tube closure (Figure 2.6g), but only in the ocellus, prior to pigmentation, and not in the otolith.
In vertebrates, FoxD3, has two distinct functions in chick and in zebrafish neural crest development (Kos et al., 2001; Lister et al., 2006); an early role in segregating NCCs from the neural epithelium and a later role repressing melanogenesis thus allowing other neural crest fates to appear before melanoblast specification. Expression of \( Ci \) FoxD in the ocellus precursor is consistent with the late function in vertebrates and suggests that early FoxD3 expression in vertebrate NCCs may represent a novel co-option of function. In \( Ci \), expression of both Meis (Moret et al., 2005) and SoxE (Figure 2.6j) is evident in a small number of cells located adjacent to the melanocytes that are not of the PCL. The functions of these genes in NCC specification have likely been co-opted during vertebrate evolution.

In vertebrates, the neural crest effector gene MITF-M is expressed exclusively in NCC-derived melanocytes. Expression of \( Ci \) MITF is first expressed in four members of the PCL just prior to neural tube closure, but its expression is only maintained in the two PCL descendants fated to become the melanocytes (Figure 2.6h). The \( Ci \) Tyrosinase gene, which encodes a melanin synthesis enzyme, is probably a direct target of MITF regulation and follows MITF expression in the melanocytes (Figure 2.6i). A complete summary of the temporal expression patterns of the NCC genes in the PCL is presented in figure 2.7. These results demonstrate that in \( Ci \), individual descendants of the PCL express eight NCC transcriptional regulatory genes and indicate that the possible absence of Meis and SoxE expression in these cells is either a loss of expression in \( Ci \) or represents a co-option of function in the NCC regulatory network of vertebrates.
Evolutionary implications

Current hypotheses suggest that NCCs in vertebrates did not arise de novo, but evolved by elaboration of ancestral neural tube cells that had only a subset of the molecular and migratory properties of NCCs (Meulemans and Bronner-Fraser, 2004; Meulemans and Bronner-Fraser, 2005). Previous analyses of gene expression data compiled from both amphioxus and ascidian literature concluded that the function of neural crest specifier genes such as Id, FoxD, AP-2 and others was co-opted by neural plate border cells in vertebrates resulting in the NCC GRN (Meulemans and Bronner-Fraser, 2005). Our careful analysis of PCL gene expression patterns and cell lineage suggests that even fewer changes in the neural crest GRN account for the differences and elaborations in vertebrate NCCs.

Recent computational analyses support our evidence that the novel thing about vertebrate NCCs is not primarily the result of NCC specifier genes in the multi-tiered GRN. By comparing gene emergence rates within different tissues among phylogenetic categories of deuterostomes, they confirm that the rate of emergence of new neural crest genes is increased in the vertebrate and mammalian subcategories over that of the deuterostome and chordate categories, consistent with the idea that NCCs evolved dramatically in vertebrates. However, 91% of the 615 neural crest genes, including all the key TFs that have been deemed “neural plate border specifiers” and “neural crest specifiers,” can be traced back to the base of all metazoans. Surprisingly, of the new neural crest genes that appear first in vertebrates, half of them are extracellular ligand genes (Martinez-Morales et al., 2007). This
emphasizes the important role for diversification of receptor ligands in vertebrate NCC evolution that was likely allowed for by the duplications of the genome that occurred early in the vertebrate radiation. The emergence of these novel ligand genes is proposed to be a mode of evolution for the novel tissue types that have been acquired by vertebrate NCC lineages like bone. This computational insight together with our research paints a clearer picture of where the differences are between the basal NCCs of urochordates and vertebrate NCCs. This lays a foundation to propose a model for explaining the acquisition of two fundamental properties of NCCs in vertebrate evolution: the pluripotency of NCC precursors, and the novel cell types that are not present in invertebrates.

We have demonstrated that descendants of the *Ci* PCL arise at the border of the neural and non-neural ectoderm and differentiate into at least three neural crest-like cell types. These cells express genes from all four organizational levels of the NCC GRN, indicating that significant portions of the GRN were present in the last common chordate ancestor. Lastly, our cell lineage analysis indicates that some of the PCL descendants migrate, albeit very short distances within the small *Ci* embryo. Our results support the hypothesis that the last common ancestor of Olfactores had an extensive GRN capable of generating neural crest-like cells in an embryonic cell lineage that is extensively similar to extant NCCs.

An alternative argument for the evolutionary comparison of the PCL of *Ci* is to the origin of vertebrate eyes and ears. The otolith has been morphologically and functionally compared to placode derivatives like the vertebrate ear and the lateral line
system of fish, also called the acousto-lateralis system. Both of these structures are functionally used in the detection of pressure waves using neuromast mechanoreceptors. Although they are normally thought of as placode-derivatives, it was shown that NCCs contribute to the neuromasts mechanoreceptors of the acousto-lateralis system in the development of fish and frogs (Collazo et al., 1994).

Morphologically the ocellus and its ciliary photoreceptors have been compared to both the pineal and the lateral eyes of vertebrates (Dilly, 1964; Eakin, 1973; Whittaker, 1973). The pigment cells of the eyes that are involved with light sensing, retinal pigmented epithelium (RPE), are *not* neural crest derived cells and do *not* originate from the neural plate border. The developmental origin of the vertebrate eyes is not the same as that of the cephalic neural crest at neural plate stages (Figure 2.8a); instead the eyes are derived from medial neural plate cells (Le Douarin et al., 1993). The lateral eyes of vertebrates do not share a developmental origin with the PCL of *Ci*.

Recent analysis of visual cycle genes in *Ci* supports the hypothesis that the ocellus organ is homologous to the vertebrate medial eye (the pineal organ) and not to the lateral eyes (Figure 2.8). RPE65 is expressed in cells of RPE in vertebrate lateral eyes, a pigmented cell type that is *not* neural crest derived. *Ci*RPE65 is not expressed in larvae, but only in the neural complex of adults (Takimoto et al., 2006). In addition to lacking expression of this RPE specific gene, the ocellus shares function at larval stages with the pineal organ of *Xenopus* tadpoles by allowing for shadow responses
before the lateral eyes are developmentally capable of responding to light (Kusakabe and Tsuda, 2007).

The cells of the pineal organ of vertebrates are very neural crest-like. Cultured pineal cells exhibit multipotentiality; they are primarily neurogenic but can be stimulated to form lens cells and even muscle cells (Araki, 2001). The pineal organ is not a term used to describe non-vertebrates and its embryonic origin in vertebrates has not been directly characterized. There have been suggestions that pineal rudiments originate bilaterally in the neural folds and meet at the midline during neural tube formation (Le Douarin and Kalcheim, 1999). Their final location is consistent with them originating at the anterior edges of the caudal diencephalon, in contrast to the vertebrate lateral eyes which arise from the medial cells of the anterior diencephalon (Figure 2.8a). This putative location of the pineal rudiments at the edges of the neural plate and in the neural fold corresponds to the embryonic origin of the PCL in Ci (Figure 2.8b). It is important to note that the suggested origin of the pineal organ corresponds to “the most anterior boundary of neural crest formation” (Le Douarin and Kalcheim, 1999). An important question remaining is whether the pineal organ is fully or partially constituted by NCCs that remain at their place of embryonic origin instead of migrating away (Araki, 2001). The pineal organ of mammals has evolved to have endocrine function and is part of the “diffuse neuroendocrine system” that consists of mostly NCC derivatives (Ralph, 1983). This leads to the question of whether the neural crest and the pineal are evolutionarily linked by their embryonic origin.
Two requirements that must have occurred during this evolutionary scenario were for pineal cells to 1) gain the ability to migrate away from the neural tube and 2) co-opt the use of key TFs in their developmental GRN. Both of these are intertwined with the need to cope developmentally with the much larger size of vertebrate embryos which includes both increased scale and cell numbers. Not, also called Floating Head, is a TF expressed in the pineal and also in one of the cells of the PCL. In zebrafish mesoderm, Floating Head represses Snail TF expression. Snail TF function is required for NCC migration. Sox10 is expressed in early development of NCCs and in the pineal, but not in the PCL of Ci. There is evidence that FoxD is expressed in the developing pineal gland of zebrafish (Gilmour et al., 2002; Concha et al., 2003; Gamse et al., 2005), and we have demonstrated for the first time that it is expressed specifically in the pineal-like ocellus of Ci. I hypothesize that transcriptional regulators, like SoxE and FoxD, were recruited and deployed early in NCC development to delay differentiation by maintaining a “stem-cell” like state. This accommodates the slower temporal progression needed to cope developmentally with the increased scale, cell numbers, and travel distance required of vertebrate NCCs (Lacalli, 2004).

Perhaps the common ancestor of Olfactores had a pineal-like organ that functioned in light sensing and originated from cells of the neural folds that developed with a multi-tiered GRN much like the ocellus of Ci and the pineal complex of early Xenopus tadpoles. With this in the ancestral toolbox, urochordates have evolved neural crest-like migratory neurons and the otolith for sensing gravity as additions to
the ontogeny of this embryonic lineage. The vertebrate radiation allowed for dramatic expansion of the numbers of cells and cell types to develop along this pathway by co-opting the use of key TFs early in the regulatory network and expanding the region of origin of these cells along the anteroposterior axis. The pineal organ was maintained but its functions entirely converted to neuroendocrine use later in mammals (again very neural crest-like), while the neural crest evolved from it to migrate to diverse locations and a variety of fates. A key question remaining is whether there are migratory cells that migrate away from, or migrate during development of, the pineal complex in vertebrates.

Ascidian NCCs lack extensive migration, because of the small size of their embryos, and lack extensive pluripotency, with fewer cell types, but have all the basic properties of vertebrate NCCs. Vertebrates inherited basal NCCs then redeployed FoxD expression early and added SoxE to establish NCC pluripotency. The larger size of vertebrate embryos required an anterior-posterior expansion of this basal cell population and necessitated extensive cell migration. Large scale gene duplications during vertebrate evolution allowed for the acquisition of novel cell types through associated novel ligands.

**Methods**

**Cell lineage analysis.**

The left or right a8.25 cells of 110-cell gastrula stage _Ci_ embryos were pressure injected with DiIC\textsubscript{18} (Invitrogen, Carlsbad, CA) dissolved in soybean oil
using glass microelectrodes (Martindale and Henry, 1995). Some of the properly injected embryos were sorted according to which of the precursor pair, left or right, had been labelled before neurulation was complete. Injected embryos were allowed to grow to 20-24 hpf then fixed in 3.7% formaldehyde in seawater with 50mM EDTA for 15 minutes. The fixed embryos were washed and stored in phosphate buffered Tris (pH 8.0) containing 0.05% Tween 80, and 50 mM EDTA at 4°C. The larvae were imaged on a Zeiss Pascal Confocal microscope and on a Zeiss Axiosplan II epifluorescent microscope. Reconstructions of optical sections were generated using Zeiss LSM and Axiovision software.

**Gene expression analysis.**

*C i* neural crest gene homologues were PCR amplified from staged cDNA using oligonucleotide primer pairs designed to amplify the entire coding regions and subcloned into pBluescriptII (Stratagene, La Jolla, CA). Primer sequences can be found in the Appendix. Digoxigenin labelled RNA probes were generated from linearized templates. All embryos were reared at 18°C and a standard in situ hybridization protocol (Satou et al., 2001) was employed with the following modifications: 1) hybridization reactions (pH4.5) were conducted at 56-60°C for 48-72 hours, probe concentration was 0.5-5 ng/µl; 2) anti-Digoxigenin antibody (Roche) was pre-absorbed against fixed embryos for at least one hour before use; 3) Blocking reagent (Roche) was dissolved in maleic acid buffer.
Figures

**Figure 2.1. The PCL of Ci.**

**a.** The otolith and ocellus pigment cells can be seen in the brain of a late larva (anterior is left). **b-e.** Development of these pigment cells from their precursors during gastrulation is demonstrated in fixed DAPI stained embryos (anterior is up). The anterior neural plate is comprised of six columns of cells. The PCP and its descendents after the tissue restricted stage are indicated with red arrowheads while its sister cell, a8.26, is indicated with white arrowheads. These all develop in bilateral pairs in the outermost columns on the edge of the neural plate with four columns of nuclei between them (a dashed white line indicates the midline of each embryo). **d. and e.** show the same embryos used in Figure 2.6. **c. & d.** and illustrate asynchronous cleavages of the neural plate border cells between the eighth and ninth generation. **f-j.** Confocal imaging of the labelled pigment cell and adjacent sister cells. Optical sections have been projected and rotated via reconstruction software. Whole mount of Ci larvae, 22-24hpf @ 18C, with DiI labelled descendants of a8.25 in red. **f-h.** A neuronal axon extends posteriorly in each labelled embryo. **i. and j.** Both of the examples shown are of embryos microinjected with DiI in the right PCP, a8.25, which gave rise to an Otolith fate. Putative sensory neurons (arrows) projecting from the sensory vesicle wall (arrowheads) can be seen contacting the otolith cell body.
Figure 2.2. A fate map of cells at the border of the neural and non-neural ectoderm of a 110-cell embryo (~5 hpf).

Only the right half cells of the bilaterally symmetrical neural plate have been labeled with the cell names (top) and their corresponding fates (bottom). Grey cells occupy the non-neural ectoderm side of the border and give rise to epidermis (Ep). The dorsal midline epidermis arises from the b8.20 and b8.18 pairs from which also arise the dorsal portion of the peripheral nervous system called epidermal sensory neurons (ESNs). Uncolored cells within the black border have neural fates: Br, Brain; SC, spinal cord (=neural tube); PC, pigment cell (dotted border); Palp, anterior adhesive papillae. Cells marked with an asterisk are dragged posteriorly relative to neighboring neural plate cells due to morphogenic movements of gastrulation and neurulation (Figure 2.3 and 2.4).
Figure 2.3. Neural plate cells of the ninth generation.

Only cells on the right half of the bilaterally symmetrical neural plate have been labeled with the cell names (top) and their corresponding fates (bottom). Grey cells occupy the non-neural ectoderm side of the border and give rise to epidermis (Epi). Uncolored cells within the black border have neural fates: Br, Brain; SC, spinal cord (=neural tube); PC, pigment cell (dotted border); Palp, anterior adhesive papillae. Cells marked with an asterisk are dragged posteriorly relative to neighboring neural plate cells due to morphogenic movements of gastrulation and neurulation (Figure 2.2 and 2.4). One of the descendants of A8.16, A9.31, has muscle cell fate and will not be included in figure 2.4.
Figure 2.4. Neural plate cells of the tenth generation.

Only the right half cells of the bilaterally symmetrical neural plate have been labeled with the cell names (top) and their corresponding fates (bottom). Grey cells occupy the non-neural ectoderm side of the border and give rise to epidermis (Epi). Uncolored cells within the black border have neural fates: Br, Brain; SC, spinal cord (=neural tube); PC, pigment cell (dotted border); Palp, anterior adhesive papillae. Cells marked with an asterisk are dragged posteriorly relative to neighboring neural plate cells due to morphogenic movements of gastrulation and neurulation (Figure 2.2 and 2.3).
Figure 2.5. Migration of the most anterior member of the PCL a10.100.

Seven selected frames are shown from a time lapse movie containing 130 total frames. One of the PCPs (a8.25) was labeled with DiI at the 110-cell gastrula stage. The tailbud embryo was imaged every three minutes from ~9 hpf through ~15.5 hpf in a small petri dish of sea water. Merged images of DIC and fluorescence are shown on the left, with corresponding black and white images of DiI labeled cells on the right. a. – c. Anterior is at bottom right corner. d. – g. Anterior is down. In the first frame (top, right panel) the four labeled cells of the PCL (white arrowheads) are lined up on the dorsal neural tube with their unlabelled pair mates of the same name in the dark spaces between them. They can be identified from posterior (upper, left) to anterior (lower, right) as a10.97 (the pigment cell), a10.98, a10.99, and a10.100 (the migratory neuron).
**Figure 2.6. Neural crest transcription factor homolog expression in the Ci PCL.**

Whole mount in situ hybridizations of the following genes: a. Dll-B, 5 hpf; b. AP-2, 5.5 hpf; c. Id, 5.75 hpf; d. Pax3/7, 6 hpf; e. Mxs-b, 6 hpf; f. Snail, 6 hpf; g. FoxD, 11.5 hpf; h. MITF, 11.5 hpf; i. Tyrosinase, 11.5 hpf and j. Meis, 11.5 hpf. DAPI counterstaining in a.-f. Green arrowheads indicate gene expression in the PCL and black arrowheads indicate melanin formation in the developing otolith pigment cell. a-f dorsal view, anterior is up; g.-j. dorsal is up, anterior is left. In a.-e., the blue/purple in situ staining was false colored red and superimposed with the blue DAPI image to conclusively identify PCLs; in f. the DIC image was superimposed with the corresponding DAPI image. Melanin accumulation in the developing otolith pigment cell is an endogenous marker of the post-mitotic a/a10.97 cells (green arrowheads in h. and i.). The PCP assuming the otolith fate is always anterior to the ocellus and melanin is visible four hours prior to ocellus melanization. FoxD is only expressed in the ocellus (g), while MITF and Tyrosinase are expressed in both pigment cells (h. and i.). SoxE expression (data not shown) in the brain of the early larva is indistinguishable from Meis (j.). Scale bars: a.-f. = 25µm and g.-j. = 20µm.
Figure 2.7. Temporal expression of *Ciona* NCC gene orthologues in the PCL

*Neural plate border specifier gene; †NCC specifier gene; ‡NCC effector gene; £NCC melanocyte differentiation gene. First column: hours post fertilization at 18°C. Black boxes indicate the gene was expressed, empty circles indicate gene expression was examined but was not present in the PCL. Note: from 5-6 hpf, a/a8.25 are the only PCLs, later they will divide into a/a9.49 (PCL) and a/a9.50. The a/a9.49 cells will divide once more to produce a/a10.97 (otolith and ocellus) and a/a10.98. The timing of Zic L* expression within the PCL matches that of Dil-B and AP-2 (Anno et al., 2006).
Figure 2.8. Homologous photoreceptive organs among chordates.

a. The embryonic origin of the eyes (blue) is anterior and medial compared to that of the pineal complex or epiphysis (green stars) in vertebrate embryos. The epiphysis demarcates the anterior-most domain of cephalic neural crest formation (red).

b. The lateral and posterior position of the PCPs in *Ciona* (broken border) corresponds to that of the epiphysis in vertebrates and not the lateral eyes. The region of the neural plate that is homologous to vertebrate lateral eyes instead gives rise to the oral siphon of the adult ascidian (blue). Vertebrate NP based on chick from *The Neural Crest*, p.84 (Le Douarin and Kalcheim, 1999). *Ciona* neural plate is the same as figure 2.3.
Appendix

Neural plate border specifier gene homologs:

MsxB Forward:
CGCGGTACCAATGACAGTAAACGAATCCGATCG
    Asp718

MsxB Reverse:
GCGAAGCTTCTAACGACTCTCAGTTGGGTAGTA
    HindIII

DllB Forward:
CGCGGTACCAATGTCAGCGTCGGTTGAATCTCC
    Asp718

DllB Reverse:
GCGAAGCTTACCAAAAGTTTTTTTATACGA
    HindIII

ZicL Forward:
CGCGGTACCTATGTATTCTAATGTAATGGTT
    Asp718

ZicL Reverse:
CGCGGATTCAGCAACGAAGTGAGCTGACAA
    BamHI

Pax3/7 Forward:
CGCGGTACCAATGATGCATCCACGGTCTAATTTCG
    Asp718

Pax3/7 Reverse:
CGCGGATTCATATGCTCATGCTGAACGCTCGGTCCTCC
    BamHI

Pax3/7 oligos to knock-out internal BamHI site:
CGAACGGAAGTGAGCGAACAGTGAGTCAG Sense
CTGACTCAGCATGCTCGACCTCCGACTTCGTTCG Antisense
Neural crest specifier gene homologs:

AP2 Forward:
CGCGGTACCAATGAGTGATATTCTGTC
\underline{Asp718}

AP2 Reverse:
CGCGGATCCCTTTGGTGCCTTTTCGAAATATT
\underline{BamHI}

Id Forward:
CGCGGTACCAATGGTTAAAGTTGTTGCAAAATC
\underline{Asp718}

Id Reverse:
CGCGAATTCAAACTGCTTGCACCTTGTCTG
\underline{EcoRI}

Snail Forward:
CGCGGTACCAATGACCTCCGTCGAGCCCATG
\underline{Asp718}

Snail Reverse:
CGCGGATCCCTTTAGGATGCTGTCTTGGCTTG
\underline{BamHI}

FoxD Forward:
CGCGGTACCAATGATGACAGTCAGTGTTGT
\underline{Asp718}

FoxD Reverse:
CGCGAATTCTTTAAGTCTGCCAAAACAAGGCCA
\underline{EcoRI}

SoxE Forward:
CGCGGTACCATGACAAACGATATGACGCTTTT
\underline{Asp718}

SoxE Reverse:
CGCGAATTCTTAATGCGCCGGGCGAACAACCTCAGC
\underline{EcoRI}

Meis1 Forward:
CGCGGTACCAATGGCGACGTCACATGTTTTGA
\underline{Asp718}
Meis1 Reverse:
CGCGAATTCTAGCAAGTATGGCCGAGCATATG  
EcoRI

Neural Crest effector gene homologs:

MITF Forward:
CGCGGTACCAATGGGTAGTCGCGCTAACATGAAAGATG  
Asp718

MITF Reverse:
CGCGGATCCCTGGTTAGCATCGGACAGAAATATATC  
BamHI

Tyrosinase Forward:
GCGCGGTACCACAGTGTGTTCAGTGACTCTGT  
Asp718

Tyrosinase Reverse:
GCCGCTCTAGACGCATGGATGTCATCAGCAATA  
XbaI
References


III. Manipulating Gene Expression in *Ciona intestinalis*

**Introduction**

RNA interference (RNAi) is a natural process by which small double-stranded RNA (dsRNA), of approximately 21–23 nucleotides, directs sequence-specific silencing of homologous genes. This process is evolutionarily conserved and has been found in a wide range of eukaryotic organisms (Hammond et al., 2001; McManus and Sharp, 2002). The actual mechanisms used to silence the gene expression are diverse and have been found to include, degradation of target RNA, repression of translation, and chromatin remodeling. This variety of phenomena that comprise the RNAi pathway have been implicated in important biological processes such as the control of gene expression during development (Grishok et al., 2001; Carrington and Ambros, 2003; Chen et al., 2004), the establishment and maintenance of heterochromatin (Hall et al., 2002; Volpe et al., 2002; Fukagawa et al., 2004; Lippman and Martienssen, 2004), suppressing transposon activity (Tabara et al., 1999; Pal-Bhadra et al., 2004; Pelisson et al., 2007), and immunity against viruses (Keene et al., 2004; Li and Ding, 2005).

One feature that is shared by all of the mechanistic pathways that are associated with RNAi is processing of the dsRNA by Dicer, an RNAse III type endonuclease that generates 21 to 23 nucleotide duplexes with 5’ phosphates and 3’ dinucleotide overhangs (Bernstein et al., 2001). The resulting short dsRNA duplexes are sometimes called short interfering RNA (siRNA) and are incorporated into a
protein complex called the RNA induced silencing complex (RISC) that then recognizes target RNA within the cell based on homology to the complex-bound short RNA (Figure 3.1). Regardless of the mechanism used by the RNAi pathway (target degradation, translational inhibition, or chromatin remodeling) the result is sequence-specific gene silencing.

microRNAs (miRNAs) are a class of small dsRNA that can induce silencing by targeting mRNA. Genes encoding miRNAs are typically located in non-protein-coding regions of plant and animal genomes. Gene silencing that is mediated by these endogenous miRNAs is essential for plant and animal development (Pasquinelli et al., 2000; He and Hannon, 2004). miRNAs are generally 21–25 nucleotide, non-coding dsRNAs that are derived from larger precursors that form imperfect stem-loop structures. Dicer further processes the imperfect RNA hairpins. Then the mature miRNAs are incorporated into a RISC complex and, depending on their degree of complementarity to the target mRNA, they elicit either translational repression or mRNA cleavage (Figure 3.1).

Long dsRNA is a sign of viral infection and vertebrate cells have evolved the capacity to mount a strong innate immune response against viruses by activating the interferon system. One of the molecules responsible for mediating this recognition and response to the presence of long dsRNA was recently identified as Toll-like receptor 3 (Matsumoto et al., 2004). The consequences of activation of the interferon system include initiation of apoptosis and total inhibition of protein synthesis, therefore long dsRNA cannot be introduced into mammalian cells for experimental
purposes as it can be in the invertebrate models Drosophila and C. elegans. Although this antiviral response is thought to be exclusive to vertebrates, the recognition of dsRNA by the RNAi pathway is widely distributed among invertebrates, like Drosophila and C. elegans, and is likely an important component of their invertebrate antiviral protection.

The question of whether an invertebrate organism more closely related to vertebrates, like the chordate Ciona intestinalis (Ci), would mount an antiviral response like that of vertebrate cells remained unexplored until recently. This year authors report being able to knock-down the expression of an Acid-sensing ion channel (ASIC) gene to 40% of the level in control larvae by introducing long dsRNA into fertilized eggs by electroporation (Coric et al., 2008). Interestingly, quantitative PCR was used to analyze the levels of RNA in samples extracted from individual larva, which helped to clarify the results as the experimental embryos fell into two distinct categories: affected and unaffected. The control embryos were electroporated with dsRNA generated with GFP sequence. Disappointingly, the authors did not report whether they are able to use that same dsRNA to knock-down the expression of GFP in transgenic larvae. The results of this experiment are intriguing as one of the first to demonstrate RNAi in this model organism, but leave me curious about the levels of other mRNA that would be expected to remain constant in the experimental embryos. There were no controls included to demonstrate the specificity of the gene silencing.
This chapter will describe our pursuit of a technique to knock-down gene expression in Ci embryos by taking advantage of the endogenous RNAi pathway. These experiments have been modeled on techniques that are continuously being developed in mammalian cell culture systems. Because of the close evolutionary relationship between tunicates and vertebrates, we began a path of experimentation that avoided the use of long dsRNA and any antiviral response that it may cause. Additionally, we have chosen to pursue a technique that is fundamentally based on plasmid DNA in order to accommodate our transfection technique and the rapidly developing Ci embryo.

It is important to consider why gene knock-down and knock-out strategies are the most valuable tools that need to be developed for future ascidian research. Ascidians are a valuable model system for studying gene regulatory networks and gene function because they have all the same genes and tissue types as vertebrates but have fewer cells and less gene duplication than vertebrates making their gene regulatory networks possible to decipher (Cone and Zeller, 2005). In order to examine gene function in molecular embryology we want to manipulate expression of the gene of interest, and then look at the outcome of that manipulation in terms of the embryonic phenotype and expression of putative downstream genes. The most physiologically relevant way to perform this manipulation is to decrease the amount of gene expression instead of using over-expression.

In Ci, electroporation of expression constructs into fertilized eggs results in over-expression because many more copies of the gene and the corresponding
regulatory DNA are present than would normally be found in an individual cell and may be maintained in a sort of extra chromosomal array (Zeller et al., 2006). This can result in absorption of endogenous DNA binding factors by the numerous exogenous copies of a promoter that were introduced and result in decreased ability for those factors to perform other normal functions in that embryo. Additionally, the expression of the gene products from the exogenous DNA plasmids will be at a level far greater than would ever normally be experienced during development. The stoichiometry of the molecular interactions is dramatically changed to levels that would never be seen in the normal developmental scheme. For this reason the phenotypes generated in over-expression experiments are not necessarily reflective of the normal function of that gene, but may primarily demonstrate an artifact of how a gene could function when expressed at a level far beyond what is found in the physiological context. Instead, eliminating gene expression is a more biologically relevant way to perform the manipulation because the levels of gene expression do not leave the range of levels that are found in normal cells and tissues.

I am interested in doing functional analysis of the TFs involved with the NCC GRN. Over-expression experiments are quite simple to perform in Ci but can be difficult to understand (figures 3.2-3.4). In these over-expression experiments, the ubiquitous promoter of the Elongation Factor gene (EF) is used to express Ci transcription factor homologs of four neural plate border specifiers (Pax3/7, ZicL, DllB, and MsxB) and one neural crest specifier (AP2). Each TF is expressed as a fusion protein with CFP and its pattern of expression can be identified by cyan
fluorescence in the right column of figures 3.2-3.4. The punctate appearance of the CFP in these transgenic larvae indicates the nuclear localization of the TF-CFP fusion protein that would be expected for the exogenous TFs to be functional. Each TF was mis-expressed throughout the embryo either alone or in combination with Pax3/7. In order to determine the effects of these manipulations on pigment cell development a Tyrosinase reporter construct was included in each electroporation. Activation of the Tyrosinase promoter drives transcription of a histone-YFP fusion protein that can be identified by yellow fluorescence in the right column of figures 3.2-3.4. The Tyrosinase:H2-YFP transgene is normally expressed in the two pigment cells within the brain of the Ci larva (Figure 3.4). Sometimes the sister cells of the two pigment cells are also YFP positive because Tyrosinase expression begins before the final mitotic event, so at most there are four cells in the Ci brain that are labeled with this reporter construct. Ubiquitous overexpression of MsxB, ZicL, and AP2 either alone or in combination with Pax3/7 overexpression causes ectopic activation of the Tyrosinase reporter construct in six to thirty cells per embryo but does not result in the formation of extra pigment cells (Figures 3.2-3.3). Co-transfection of all five TFs results in delayed pigmentation relative to the other experimental and control embryos, but did not result in extra pigment cells (Figure 3.2a-b). Gene specific silencing of these genes of interest with RNAi would be an ideal way to compliment over-expression studies like those described above. However, reports of methods to knock-down or knock-out gene expression in Ci are limited.
In summer of 2002 my first project in the Zeller Laboratory was to design and implement a technique for using RNAi to knock down gene expression in Ci. At that time, RNAi was being used extensively in mammalian cell culture systems, but its use in whole organisms was limited primarily to C. elegans, Xenopus, zebrafish, and Drosophila. It is reasonable to assume that ascidians have all the machinery for RNAi to function endogenously because the genome sequence shows the presence of genes for Dicer and other Argonaut members as well as Exportin 5. Additionally, a large number of miRNA genes have been identified in the Ci genome (Keshavan, 2006; Norden-Krichmar et al., 2007).

A gene’s function from a developmental point of view can best be determined by manipulating its expression in a whole developing organism where conditional specification and communication between tissues and cells can still occur. The roles that a gene may play in morphogenesis and even cell fate determination cannot be demonstrated and studied completely in cell culture. A tool for down regulating expression of a gene during Ci development will ideally be inexpensive and fast to build by cloning. These two characteristics can make it scalable, so that it can be done on a high-throughput level for use in genomic screens in the future.

From the very beginning certain goals were developed with respect to the development of RNAi as a tool to use in ascidians. Most importantly we desired a system that would be plasmid based to take advantage of the electroporation technique that is used to transfect fertilized eggs with constructs that will be expressed during embryogenesis. This would allow for the gene-silencing RNA molecules to be
continuously generated in vivo by zygotic transcription during development. This is an important concept for use in this organism. There are methods for generating siRNA molecules in vitro and then putting them into the experimental system to be recognized by Dicer and used by the endogenous RNAi machinery to silence gene expression. This did not seem like the best approach for ascidian RNAi because of the rapid cell divisions that would essentially cause a ~1/2500 dilution of those cytoplasmic factors during the first 18 hours of development. This is one of several characteristics of the ascidian model organism that have made RNAi a difficult new technique to develop. An additional reason for pursuing a plasmid-based RNAi system is the possible ability to generate transgenic lines of knock-down ascidians in the future by allowing the transgenic larvae to develop into adult ascidians and pass on the transgenesis to the next generation (Matsuoka et al., 2005).

There is a precedent for silencing gene expression in ascidians. The first use of RNAi in ascidians came from work in the DeTomaso Lab. They reported to have silenced a gene using RNAi in adult colonial ascidians called *Botryllus* by soaking the adult colonies in siRNAs that were generated in vitro (Nyholm et al., 2006). They generated long double-stranded RNA by in vitro transcription from the cDNA clone, then used human recombinant Dicer enzyme to digest the long double-stranded RNA into siRNA bits. This was very encouraging news demonstrating that ascidians have the required mechanisms downstream of Dicer for carrying out gene silencing by RNAi, but the techniques used did not conform to our long-term goals for using RNAi as a tool in *Ci*.
Currently the use of microinjected morpholino oligos to inhibit translation is the most commonly used way to silence transcripts in Ci (Imai et al., 2006). A large proportion of morpholino oligos tested in Ci have been shown to have no effect (Yamada et al., 2003). This could result from alternative splicing of the first coding exon of transcripts, which is more common in this organism. Morpholino oligos are made to complement and bind to the 5-prime end of a target transcript near the translational start site. Alternatively spliced gene transcripts would not be bound by the MO allowing the transcripts to be expressed. Therefore, it can be required to design and test many different MO sequences to perform a single experiment (Yamada et al., 2003), dramatically raising the expense of this technique so that most laboratories, because of the cost, cannot scale it up.

Much like the siRNA approach used by the DeTomaso Laboratory, this method does not overcome the caveat of the rapid cytoplasmic dilution of these nucleic acids caused by cell divisions during early development of Ci embryos even though morpholinos are extremely long lived molecules. One advantage of using MO injection to decrease gene expression is that they immediately start silencing transcripts that may already be present in the early embryo. Maternally deposited mRNA will not be inhibited by a plasmid based RNAi system because it relies upon zygotic transcription that begins around the 32-cell stage in Ci embryos. However, microinjection of these embryos is difficult and this method will only produce a handful of experimental embryos to examine because of the relatively small number of microinjections that can be performed before the fertilized eggs begin to undergo
cleavage. This is a disadvantage compared to the large numbers of experimental embryos, hundreds that can be generated by electroporation of fertilized eggs.

Another way that gene expression has been decreased in Ci embryos has been to generate dominant negative TFs. A way this has been done is by reengineering the TF so that it has a transcriptional repression domain instead of a transcriptional activation domain while still retaining its same DNA binding domain. The repressor domain from Engrailed (Mita and Fujiwara, 2007) and a Hairy domain called WRPW (Corbo et al., 1998; Beh et al., 2007) are examples that have been used in building these TF-repressor fusions for use in Ci. This re-engineered version of the transcription factor is then over-expressed from a plasmid in transgenic embryos. These exogenous TF-repressor fusion proteins can still bind and occupy their normal sites within the regulatory DNA of the genome and potentially out-compete the endogenous transcription factor inhibiting its function completely. Both Engrailed repressor fusions and WRPW domain containing fusion proteins have been successfully used to create dominant negative transcription factors in ascidians (Wada et al., 2002; Beh et al., 2007; Mita and Fujiwara, 2007).

Over-expression of the dominant negative TF knocks out the function of the endogenous transcription factor even though it is still expressed. Although this is not really a method for gene silencing it is a way of getting at the function of a transcription factor and its place in a gene regulatory network. It does rely on over-expression, and may still be susceptible to artifacts resulting from changes in binding kinetics. Transcription factor binding site recognition is quite dynamic and could
definitely be changed by the substantially increased concentration of the over-expressed exogenous version of the TF. This could allow them to bind to sites that would not normally be bound by this factor, excluding other DNA binding proteins from doing their jobs and causing an embryonic phenotype unrelated to the TF’s true function. The transcription factor-repressor fusion proteins will only be able to act in a dominant negative fashion if the original TF is, in fact, a transcriptional activator. If it has a repressive function then over-expression of the engineered protein could only exacerbate its function instead of knocking it out.

Over-expression of both the endogenous form and the dominant negative TF form of FoxD are approaches I have used to functionally characterize its role in the PCL of Ci (Figure 3.5). FoxD is expressed in only one cell of the PCL, the ocellus pigment cell (Figure 2.6g) which allows for a very discreet hypothesis: FoxD functions to delay melanogenesis in this developing melanocyte. To examine the function of FoxD it was overexpressed in both pigment cells using the Tyrosinase promoter. Additionally, this construct was re-designed to express a FoxD-WRPW dominant negative fusion protein in both pigment cells. Transgenic embryos generated with each of these experimental constructs displayed the same phenotype of delayed pigmentation (Figure 11). Examination of the experimental embryos at the time of otolith pigment formation, 12-13 hpf, revealed that embryos with transgenic otolith cells had no sign of pigment granule accumulation while non-transgenic otolith cells in the same dish had visible melanin (Figure 11a-b). These results are consistent with my hypothesis that mis-expression of FoxD in the otolith cell would delay or
prevent melanization of this pigment cell which normally creates visible pigment granules four hours earlier than the ocellus. Additionally, I hypothesized that inhibiting the function of FoxD in the ocellus cell with the dominant negative form would result in precocious pigmentation of the ocellus which normally does not form visible pigment granules until 16 hpf. However, the consistency of the results in both experiments may indicate that FoxD inhibits melanization through a repressive mechanism which cannot be abrogated by the WRPW hairy domain fusion.

Vertebrate neural crest cell development utilizes FoxD expression for dual functions: early expression during neural crest cell specification, and to inhibit melanogenesis in early migrating melanoblasts (Kos et al., 2001). Our data suggests that only this second function of FoxD is utilized in a single cell (the ocellus precursor) of the PCL in Ci. This experimental question could be further addressed in the future by using RNAi to inhibit FoxD function in the PCL.

**Engineering gene knock-down techniques for use in Ci**

Considerable thought was given to deciding which gene should be targeted for silencing. It needs to be easy to determine if the gene's expression decreased in response to the RNAi inducing signal. Additionally, the target gene needs to be present in a single copy within the Ci genome with no functionally redundant duplicate genes. Alkaline phosphatase is an enzyme normally expressed in the endoderm cells of the trunk and tail of the Ci larva. The presence of this enzyme can be visualized with a simple histochemical stain allowing for quick comparison of
experimental and control embryos. An alternative target is the Brachyury TF gene that is required for notochord development. Brachyury is one of the most well characterized TF genes in *Ci* with many known target genes (Corbo et al., 1997; Takahashi et al., 1999) and the distinctive knock-down phenotype of a stumpy tail previously demonstrated using MO (Yamada et al., 2003).

Another excellent target gene candidate is that of the Tyrosinase enzyme that is required for melanin synthesis in the two pigment cells that develop in the larval brain. No staining would be required to see a decrease in this gene's expression, which should cause albinism, faint pigmentation, or delayed pigmentation in a developing larva. These results would be visible under the dissection scope with no treatment at all. We hypothesize that the Tyrosinase gene is most surely regulated by the MITF TF as in the melanogenesis pathway of other organisms. Therefore, knocking down MITF expression would cause a subsequent knock-down in Tyrosinase gene transcription and result in the same phenotype as silencing Tyrosinase.

An experimentally interesting RNAi target for my research is the FoxD TF that is expressed in the ocellus cell. I hypothesize that it functions to delay melanogenisis in this cell based on overexpression studies described above, and that knocking-down FoxD expression will result in precocious pigmentation of the ocellus.

**shRNA**

In addition to identifying an appropriate target gene for testing the RNAi techniques, a suitable promoter also needed to be identified and cloned. The plan was
to generate short hairpin RNAs (shRNAs) transcribed by RNA polymerase III, which transcribes relatively short RNA transcripts that are usually not translated and its transcription terminates upon reaching a stretch of at least four consecutive T nucleotides. Three different RNA polymerase III recruiting promoters were cloned from the Ci genome to drive transcription of short double-stranded RNAs; U6, H1, and a tRNA promoter. Many hairpin producing constructs have been generated with a variety of loop sizes and stem lengths based on developing literature from vector-based RNAi that is done in mammalian cells (Paddison et al., 2002).

The most well characterized RNA polymerase III recruiting promoter is that of the U6 RNA gene which encodes a small nuclear RNA. This gene was identified in the Ci genome and 1KB of its upstream regulatory DNA was cloned. The promoter regions of two other genes transcribed by RNA polymerase III, H1 and a tRNA gene, were also identified and cloned. The H1 promoter normally controls expression of an RNA component of the splicosome. The RNA nucleotides that make up tRNA are also transcribed by RNA polymerase III. The promoter DNA is followed by a 21- to 29-nucleotide sequence matching the target gene and the inverted repeat of that sequence separated by a short linker. (Figure 10a) The short RNA should fold back on itself after transcription to form a hairpin structure with a 21 or 29 nucleotide stem and a loop at one end. Numerous constructs were generated varying in stem length, loop size, promoter, and target location. (Figure 10)

The short hairpin RNA structure is intended to be recognized by the endogenous components of the RNAi pathway. They should be cleaved by Dicer to
enter the pathway and become templates for targeting matching RNA transcripts for silencing. Initially we targeted Alkaline Phosphatase transcripts with these hairpin RNAs. Histochemical staining of the experimental larvae and the transgenic controls, to examine alkaline phosphatase enzyme activity, revealed no distinguishable difference between them. It also became evident that there was another copy of an Alkaline Phosphatase gene in the Ci genome. We could not be sure that the one we were targeting with shRNA sequence was responsible for the enzymatic activity. To avoid this complication we refocused efforts on silencing expression of the Tyrosinase gene that is present in a single copy in the Ci genome.

In order to further narrow down the number of constructs to be generated and tested the three promoters, U6, H1, and tRNA, were tested for their ability to activate gene expression. The coding sequence of green fluorescent protein (GFP) DNA was cloned near the transcriptional start site of the promoters. None of the three promoters normally drive transcription of a protein coding sequence to be exported from the nucleus and translated, but this was the easiest way to find out if they were functional in numerous tissues of the embryo. All three of the promoters were able to express GFP in numerous tissues of the developing embryos. The U6 promoter is the best choice for this project because it is well characterized enough in other organisms, and well conserved enough in Ci, to know the position of the first nucleotide to be transcribed. It is more likely to generate the predicted RNA transcript without the addition or deletion of any 5’ nucleotides that could alter the ability of the hairpin to induce RNAi.
The U6 promoter was used to build constructs for 19 and 29 nucleotide hairpins with 6 and 9 nucleotide linkers between the inverted repeat sequences targeting Tyrosinase (Figure 3.6). The majority of the resulting transgenic embryos in these experiments had normal pigment cells. A small percentage showed a decreased amount of melanin in the pigment cells (Figure 3.7). Because transgenes are incorporated in a mosaic fashion, a tyrosinase reporter construct (Figure 1.6) was built and included in the electroporations to help identify whether one or both pigment cells were transgenic. Many of the mosaic embryos generated by electroporation have either the left or the right half of the embryo containing the exogenous DNA. Because one pigment cell comes from the right side and one from the left during embryogenesis, in half-labeled mosaic embryos only one pigment cell receives the plasmid DNA. In the Tyrosinase knock-down experiments, even when only one pigment cell had a melanin phenotype, often times both pigment cells would be transgenic like the example shown in figure 3.7.

Only a small percentage of the transgenic embryos exhibited a pigment cell phenotype like the larva in figure 3.7. We hypothesized based on the initial RNA hairpin expression results that the hairpins were triggering the RNAi responses only in a small number of the cells that experienced them due to poor recognition and recruitment into the endogenous RNAi pathway. I have also tried including a U6 leader sequence in the hairpin RNA transcript that is supposed to aid entry into the RNAi pathway (Paddison et al., 2004). This leader sequence is thought to aid in the recognition and initial cleavage of the RNA hairpin to initiate the RNAi response. A
method for including the leader sequence to be transcribed in front of the hairpin RNA was incorporated into our approach to try and optimize the RNAi technique (Figure 3.8). Incorporation of the U6 leader sequence did not improve the proportion of transgenic pigment cells that exhibited the Tyrosinase knock-down phenotype. Our hypothesis remained that the shRNA molecules were not being properly recognized and processed by the endogenous RNAi machinery.

siRNA

The next approach in troubleshooting the RNAi technique was to bypass Dicer cleavage entirely by transcribing short interfering RNAs (siRNAs) directly. The two short strands of complementary RNA, 21 to 23 nucleotides long, were generated separately by the U6 gene promoter. This approach requires the two constructs to be co-electroporated together (Figure 3.9). Although transcribed from separate plasmids the two strands of short RNA should find one another and anneal after transcription to mimic Dicer products and be incorporated into the RISC complex. Additionally, these RNAi constructs have been directed at two different target genes and at multiple target locations within each transcript based on mRNA structure prediction analyses and empirical algorithms for siRNA design (Zuker, 2003; Amarzguioui and Prydz, 2004; Reynolds et al., 2004).

Although initial attempts to target Brachyury transcripts for silencing with siRNAs were encouraging, only a small proportion of the transgenic larvae had tail
phenotypes indicating that the RNAi pathway was still not being triggered consistently by the RNA molecules that were introduced (Figure 3.10).

**Antisense**

Another approach has been to directly transfect antisense S-oligos into the embryos. These should bind to the targeted mRNA and induce cleavage in an RNAi independent manner. Although the use of antisense MOs to specifically decrease expression of a gene had already been eliminated because of the cost and experimental disadvantages, less costly versions of nucleic acids were still tried in combination with electroporation. S-oligos have phosphorothioate linkages which are a variation of normal DNA backbone structure that replaces one of the oxygens attached to the phosphate bridge with a sulfur atom. The presence of the sulfur between the nucleotides decreases endonuclease and exonuclease activity preventing degradation of the oligonucleotide. Our next approach was to design S-oligos targeting Tyrosinase transcripts. We ordered S-oligos targeting three different locations within the Tyrosinase transcript and transfected them into fertilized Ci eggs in a very small volume electroporation of 100 µl in order to maintain high concentration. Each of the S-oligos was transfected individually with a Tyrosinase:GFP reporter and then all 3 were combined and co-electroporated. All of the oligonucleotides were used up in the triple target trial which generated just one completely albino tadpole (Figure 3.11). All of the other experimental embryos were phenotypically normal.
Artificial miRNA

miRNAs are naturally occurring genes found throughout metazoans that play a role in switching genes on and off during development (Pasquinelli et al., 2003). They can regulate their targets directly by mRNA cleavage or by repressing their translation, depending on the degree of complementarity between the miRNA and the target. *Ci* miR195 is a good candidate to provide the structural context for our artificial miRNA genes (artmiRs) because it is found in a 1.5kb intron and is transcribed from the same strand of DNA as the gene it is within (Keshavan, 2006).

In order to build the construct EF::artmiR195-RFP (Figure 3.12), the entire fourth intron of the predicted gene ci0100143475 (that contains *Ci* miR195) was cloned including the sequence for six to ten amino acids from the flanking exons on each side. The 5’ exon sequence was cloned in frame with the beginning of the EF gene promoter and the 3’ exon sequence was cloned in frame with RFP. Keeping the entire intron intact, including the splice locations at each end, will allow for the intron to be excised *in vivo* from the RNA that is generated from this transgene. After the intron is spliced it leaves behind a sequence encoding a few random amino acids from the EF and ci0100143475 genes followed in frame by RFP. The RFP expression in transgenic embryos allows for localization of the artmiR expression in mosaic embryos.

The intron was cloned in two pieces with XhoI in the middle of the two pieces. Primers were used to incorporate EcoRV and BglII restriction endonuclease sites flanking each side of the location of the miRNA gene. These restriction sites are used
to insert artificial sequence that will form a transcript that folds into the same shape as the original miR195 gene (Figure 3.13) and can target an mRNA of our choosing.

**Future directions**

I think that the extensive amount of polymorphism present in the *Ci* genome has prevented us from using vector-based RNAi to knockdown transcripts with reproducible results. One way to avoid the possibility that polymorphisms are preventing the siRNAs and shRNAs from matching exactly and inducing target cleavage by RNAi would be to target the entire length of the transcript. This could most easily be accomplished by generating full-length dsRNA in vitro and "Dicing" it into siRNA sized products with Dicer enzyme. *Ci* has a single 6Kb Dicer gene that could readily be used for protein expression in bacterial cells, then purified and used to dice dsRNA in vitro. Transfecting this population of siRNA molecules will ensure that there are some active siRNAs with an exact match to an available region on the targeted mRNA. A method similar to this has been successfully used to knockdown genes in a colonial ascidian species (Nyholm et al., 2006).

More recently, the genes involved in mediating the antiviral response in vertebrate cells were identified as an interferon mediated pathway. The genes for interferons and their receptors are now reported to be absent from invertebrate genomes including that of *Ci* by various authors (Robalino et al., 2005; Coric et al., 2008). One report of using long dsRNA to elicit gene specific silencing by RNAi has been published so far (Coric et al., 2008). In my opinion their experimental method in
combination with their single-embryo quantitative PCR analysis technique are the best reported approach demonstrating the use of the RNAi pathway for functional analysis in *Ci*.

Electroporation of long dsRNA is the only RNAi method we currently have with published, believable evidence in *Ci*. It is a good method to pursue for further use in *Ci* because it is technically simple to generate the needed dsRNA material. This method utilizes electroporation and lends itself to being scaled up for larger numbers of gene knock-down experiments. It is a technically much more difficult to make the constructs that are needed for the plasmid-based RNAi techniques that are described in this chapter. It is also likely that some of the plasmid-based approaches, like artmiR expression, may not result in target cleavage but translational inhibition and therefore the effects will not be directly analyzable with quantitative PCR. Although there is no evidence that the artmiR constructs were able to elicit post transcriptional gene silencing of any of the targeted genes, one major advantage of the artmiR plasmid design is that it could have allowed for tissue-specific gene silencing with *Ci* promoters. For example, this is the only design that would allow me to knock-down expression of FoxD in the PCL without affecting its expression in other parts of the embryo that are required for normal development.
Figure 3.1. A model for post-transcriptional gene silencing by miRNAs and siRNAs. Primary miRNA transcripts are first processed into precursor miRNAs of about 70 nucleotides in length by the enzyme Drosha within the nucleus. Exportin 5 is responsible for transporting these precursor miRNA hairpins to the cytoplasm where they will undergo further processing by the enzyme Dicer to form mature miRNAs that are short RNA duplexes containing mismatched base pairs. Dicer also processes long dsRNA into small RNA duplexes without mismatches called siRNAs. Only one strand of the siRNA or miRNA duplex will be utilized by the RNA-induced silencing complex (RISC) to identify mRNA targets based on sequence complementarity. The mechanism of silencing (translational repression or target mRNA cleavage) caused by miRNAs can depend on whether the small RNA and its target have mismatches or perfect complementarity.
Figure 3.2. Overexpression of neural crest transcription factor homologs can disrupt pigment cell development and affect expression of a Tyrosinase reporter construct.

Each experimental electroporation in a.-h. included 20 µg of the Tyrosinase:Histone2A-YFP reporter construct and 20 µg of the following transcription factor misexpression constructs: a. and b. EF:Pax3/7, EF:AP2-CFP, EF:DllB-CFP, EF:ZicL-CFP, EF:Mx-B-CFP. c. and d. EF:AP2-CFP and EF:Pax3/7. e. and f. EF:AP2-CFP g. and h. EF:DllB-CFP
Figure 3.3. Overexpression of neural crest transcription factor homologs can disrupt pigment cell development and affect expression of a Tyrosinase reporter construct.

Each experimental electroporation in a.-h. included 20 µg of the Tyrosinase:Histone2A-YFP reporter construct and 20 µg of the following transcription factor misexpression constructs: a. and b. EF:MsxB-CFP and EF:Pax3/7  c. and d. EF:MsxB-CFP  e. and f. EF:ZicL-CFP and EF:Pax3/7  g. and h. EF:ZicL-CFP
Figure 3.4. The Tyrosinase reporter is expressed in two pigment cells within the brain at larval stages.

a. and b. Example control larvae electroporated with 20 µg of the Tyrosinase:Histone2A-YFP reporter at the same developmental age as the experimental embryos in figures 3.2-3.3. Transgenic larvae will have two to four YFP-positive cells, the pigment cells and their two sister cells, because Tyrosinase expression begins before the final cell division.
Figure 3.5. Misexpression of FoxD in both pigment cells inhibits melanogenesis.
a. and b. Overexpression of FoxD (b.) or a Dominant negative form (a.) in the pigment cells results in the same phenotype showing delayed and diminished melanization of the otolith and ocellus. Yellow fluorescence has been false colored red. Melanin accumulation can be seen in the developing otolith on non-transgenic (b.,white arrow) and control embryos as early as 12hpf.  
c. Overexpression of FoxD or a dominant-negative form in the pigment cells results in the same phenotype showing delayed and diminished melanization of the otolith and ocellus. In this larva, the Tyrosinase promoter regulates expression of FoxD-WRPR dominant-negative fusion protein and the Tyrosinase:Histone2A-YFP reporter construct labels the nucleus of the pigment cells. Three focal planes of the same transgenic larva are shown. A small amount of melanin can be detected in the transgenic pigment cells of older larvae like this one.
Figure 3.6. Design of RNA hairpin expression constructs.

a. The 1kb U6 promoter was cloned from *Ciona intestinalis* genomic DNA. A sequence of DNA either 19 or 29 nucleotides in length that matches the target gene was cloned at the transcriptional start site of the U6 promoter followed by a short linker and the inverted repeat of the target gene sequence ending with a string of T nucleotides. b. and c. The short RNA transcript is expected to fold back on itself because of the inverted repeat sequence with the linker nucleotides in the middle forming a loop at one end. These examples show the expected structure of a hairpins designed to target transcripts from the Alkaline Phosphatase gene. b. This hairpin has a 19 nucleotide stem that matches a region of the target gene and a 6 nucleotide linker. c. Another design plan includes 29 nucleotides of the target sequence for the stem of the hairpin. This example also shows a longer linker sequence of 9 nucleotides.
Figure 3.7. A transgenic larva expressing shRNA targeting Tyrosinase transcripts for degradation by RNAi exhibits diminished melanin formation in the Otolith pigment cell. This 18 hour larva was electroporated with a hairpin construct driven by the U6 promoter transcribing a hairpin with a 29 nucleotide stem and a 9 nucleotide loop, see figure 3.6c. The pigment cell reporter construct expressing GFP driven by the Tryosinase promoter demonstrated that both of the pigment cells in this embryo are transgenic, however only the otolith pigment cell appears to be affected. Dorsolateral view of trunk. Anterior is at bottom right corner.

```
TC
T  G
C  G
G  C
C
T  A
C  G
G  C
T  A  TTCA
```

```
5’G CATATACCTAGTACACGACGCTGAATGACTGCAAGAG
   CATGTCGCTGCGACTTTACTGACGTTCTTC
   G
3’
```

Figure 3.8. Predicted structure of RNA hairpins when expressed with the U6 leader sequence at the 5’ end.
Figure 3.9. A two-plasmid system for generating siRNA in vivo.

a. A sequence of DNA either 23 or 21 nucleotides in length that matches the target gene was cloned at the transcriptional start site of the U6 promoter followed with a string of T nucleotides. Separately, the reverse complimentary sequence for that same target was cloned at the transcriptional start site of the U6 promoter followed with a string of T nucleotides.

b. Predicted structure of the siRNAs generated in vivo by co-electroporation of the sense and antisense construct pairs shown in a.

Target: Brachyury nt 879-901

```
GAUUAUCCUCAAUUCAACGGACCCAUU
UUCUAUUAGGAUUAGGCUGGUG
```

Target: Tyrosinase nt 1081-1105

```
GAUAACGCAAACACUUAAUCAGCUU
UUCUAUUUGCGUUGGAAUAAGTCG
```
Figure 3.10. Very few transgenic larvae expressing siRNA molecules targeting Brachyury transcripts for degradation by RNAi exhibit poor tail formation and elongation. a. and b. Example larvae demonstrating a tail phenotype similar to that of embryos injected with MO against Brachyury. c. Control larva with normal tail development. a. – c. Brachyury:GFP expression labels the notochord cells.
Figure 3.11. The only truly albino larva ever generated with antisense oligonucleotides. This larva was co-electroporated with the Tyrosinase::GFP reporter construct and three different S-oligos containing antisense sequences targeted to three different locations on Tyrosinase transcripts. It had very normal morphology and swimming behavior, but had no sign of melanin pigment accumulation at any time up to 18 hpf. Both pigment cells could be located by GFP expression, only one of them is shown in this focal plane.

Figure 3.12. Re-engineering the Ci miR195 miRNA gene to generate artificial miRNAs (artmiRs) in vivo.
Figure 3.13. Predicted structure of *Ci* miR195 and an artificial miRNA targeting FoxD transcripts.

a. The predicted folding pattern for the primary transcript of the *Ci* miR195 gene.  

b. The folded structure of an artmiR designed to target FoxD mRNA at nt position 358. The 2nd half of the stem structure contains the antisense portion of the target expected to be incorporated into a RISC complex after two rounds of enzymatic cleavage. Black arrowheads show the predicted site of Drosha cleavage.

artmiR target sequence complementary to FoxD358

BglII restriction site

EcoRV restriction site
Appendix

Elongation Factor 1α Promoter Forward:
GCGCTCGAGATTTTGTAAACACTCAAATCCC
\textit{XhoI}

Elongation Factor 1α Promoter Reverse:
CGCTGGTGACCCCTCAATATGAGTCTTGCTTTCAT
\textit{Asp178}

Tyrosinase Promoter Forward:
CGCGTGCAGCTTAATTTTTCTTCACAGATTCATG
\textit{SalI}

Tyrosinase Promoter Reverse:
CGCTGGTGACCTGTTAAATACATCTTTGGAAG
\textit{Asp178}

MsxB Forward:
CGCGGTACCAATGACAGTAAACGAATCCGATCG
\textit{Asp718}

MsxB Reverse (no stop codon):
CGCGGATCCACGACTCTCAGTGGGTAGTA
\textit{BamHI}

DllB Forward:
CGCGGTACCAATGTCAGAAGCGTCGTTGAATCTCC
\textit{Asp718}

DllB Reverse (no stop codon):
CGGGGATCCCAAAAAGTTTGTATACGA
\textit{BamHI}

ZicL Forward:
CGCGGTACCTATGTATTCTAATGTAATGGTT
\textit{Asp718}

ZicL Reverse (no stop codon):
CGCGGATCCAGCAACGAATGAGCTGTACA
\textit{BamHI}

Pax3/7 Forward:
CGCGGTACCAATGATGCATCCACGGTCTAATTTCG
\textit{Asp718}
Pax3/7 Reverse:
CGCGGATCCATATGCATGCTGAACGCTCGGTCCTCC
  BamHI

Pax3/7 oligos to knock-out internal BamH1 site:
CGAACGAAAGTCGGAGGGTCCGACAGTGAAGTCAG Sense
CTGACTCAGTGGCAGACGACTTGTTCCG Antisense

AP2 Forward:
CGCGGTACCAATGAGTGATATTCCGAAATTCTGTC
  Asp718

AP2 Reverse (no stop codon):
CGCGGATCCCTTTTGTGTTTTTTGTGGAAATATT
  BamHI

FoxD Forward:
CGCGGTACCAATGAGTGAGCTTCGAAATTCTGTC
  Asp718

FoxD Reverse (no stop codon):
GCGCAGATCTAGTTGCTGCCAAAACAAGGCC
  BglII

WRPW Forward; the hairy domain from Ci0100139157
CGCGGATCCATGTTTTCTCAGCATTGCTTCACC
  BamHI

WRPW Reverse; the hairy domain from Ci0100139157
CGCGAATTCTTTACCATGGTCTCCCATACTGGATCAGATCT
  EcoRI

RNA polymerase III promoters:

U6 Promoter Forward:
GCGCCTCGAGGTTGTATGTTGCTGACCAGCTG
  XhoI

U6 Promoter Reverse:
GCGCGGATCCAGCTGTTCATCTATACCATCGG
  BamHI BsrGI
H1 Promoter Forward:
GCGCTCGAGTCTCTCTGGCTAACAAGGTGCT
XhoI

H1 Promoter Reverse:
CGCGGATCCGTGTAACACACCACGAGTCAATGTATGACGG
BamHI BsrGI

tRNA Promoter Forward:
GCGCTCGGACATATGTTAGTTTTATATCAATCTAGTATGC
XhoI

tRNA Promoter Reverse:
GCGAAGCTTGTACAAGGTTGATGCTTGTCTGCTACC
Hin3 BsrGI

U6 Leader Sequence Forward:
GCGTGTAAGGTTGATGGTTGCTGCTACC
BsrGI

U6 Leader Sequence Reverse 1:
GCCGAACCGCAGCAGCTCATCTATACC

U6 Leader Sequence Reverse 2:
TAGTATATGCTGAGCGAGC

shRNA targets:

Alkaline Phosphatase 29nt stem, 9nt loop:
A Forward:
GTACAGCGACGCTGAAATGACTGCAGAAG
A Reverse:
TCTCTTGAAGGCTGCTGCTAAGGCTGCTACTTTTTT

Tyrosinase 21nt stem, 6nt loop:
A Forward:
GTACAGTTAGGTCAGACAGC
A Reverse:
TCTCTTGAACTTGTTCTGACTGACCTA
B Forward:
GATCTAAAAAGTACAGTTAGGTCACAGAGCC
B Reverse:
TTCAAGAGGGCTCTGTGACCTAATGTACTTTTTTA

Tyrosinase 29nt stem, 6nt loop:
A Forward:
GTACAGTTAGGTCACAGAGCC
A Reverse:
TCTCTTGAAAAGTCATCGGCTCTGTGACCTAACT
B Forward:
GATCTAAAAAGTACAGTTAGGTCACAGAGCC
B Reverse:
TTCAAGAGGGCTCTGTGACCTAATGTACTTTTTTA

Antisense S-oligos to target regions of Tyrosinase mRNA:

Tyros 977:
GGTCCCTCGATGCCCCTGT
Tyros 759:
GTCACGCAGCTCTTGGTTCCC
Tyros 239:
GCTCGCGCCAGAATGCTCT

siRNA targets:

Tyrosinase 1081-1105:
Sense Forward:
GATAACGCAACACTTATCAGTTTTTTA
Sense Reverse:
GATCTAAAAAGCTGATAAAGTGTGTCGTTATC
Antisense Forward:
GCTGATAAATGTTGCGTTATCTTTTTA
Antisense Reverse:
GATCTAAAAAGAACAACCTATCAGC

Brachyury 879-901:
Sense Forward:
GATTATCCTCAATTCCAGACCACCTTTTTA
Sense Reverse:
GATCTAAAAAGTGCGTGAAATTGAAGATAATC
Antisense Forward:
GTGGTGCGTGAATTGAGGATAATCTTTTTA
Antisense Reverse:
GATCTAAAAAGATTATCCTCAATTCACGACCAC

Artificial miRNA expression vector:

Primers to clone the 4th intron of the predicted gene ci0100143475 (that contains Ci miR195) in two pieces:

miR195 A Forward:
CGCGGTACCAACACTGCTACAGGACAGTGGAATCG
  Asp718

miR195 A Reverse:
CGCCTCGAGATCTGTAGATTTAAATATGCTGTATTTATAG
  XhoIBglII

miR195 B Forward:
GCGCTCGAGGATATGCTCTTACACCCCTTTCTATAATATGC
  XhoI  EcoRV

miR195 B Reverse:
CGCGGTACCAACACTGCTACAGGACAGTGGAATCG
  Asp718

ArtmiR targets:

Antisense target sequence on 1\textsuperscript{st} half of stem structure:
Tyros1353 Sense:
CGCAGATCTCGCGGGTTCTAACTGTACACCTTTCTGTGACATATTTGCATC
  BglII

Tyros1353 Antisense:
GCGCTATAGTGTCATTGGACATAGAGAAGAGATACAGTTATATTTGACATC
  EcoRV(rev)

Antisense target sequence on 2\textsuperscript{nd} half of stem structure:
Tyros1353 Sense:
CGCAGATCTCGCGGGTTATCACAGAAGAGATACAGTTATATTTGACATC
  BglII

Tyros1353 Antisense:
GCGCTATAGTGTCATTGGACATAGAGAAGAGATACAGTTATATTTGACATC
  EcoRV(rev)
FoxD358 Sense:
GCGAGATCTCGCGGGTCTGTCAACAAAAAGAAATCACCAATATTTGC
   BglII

FoxD358 Antisense:
GCTCTATAGTGTTCCATTACAGTGTTTCAGTAGTGTTTCGTTTATAAAA
   EcoRV(rev)

MITF337 Sense:
GCGAGATCTCGCGGGTCTCGCCGTATAGAACAAACACATATTTCGTT
   BglII

MITF337 Antisense:
CGTCTATAGTGTTCCATAACGGCAATAGAGGTTGCTTTGCGTTTATACAA
   EcoRV(rev)
References


Using ascidian embryos to study the evolution of developmental gene regulatory networks

Angela C. Cone and Robert W. Zeller

Abstract: Ascidians are ideally positioned taxonomically at the base of the chordate tree to provide a point of comparison for developmental regulatory mechanisms that operate among protostomes, non-chordate deuterostomes, invertebrate chordates, and vertebrates. In this review, we propose a model for the gene regulatory network that gives rise to the ascidian notochord. The purpose of this model is not to clarify all of the interactions between molecules of this network, but to provide a working schematic of the regulatory architecture that leads to the specification of endoderm and the patterning of mesoderm in ascidian embryos. We describe a series of approaches, both computational and biological, that are currently being used, or are in development, for the study of ascidian embryo gene regulatory networks. It is our belief that the tools now available to ascidian biologists, in combination with a streamlined mode of development and small genome size, will allow for more rapid dissection of developmental gene regulatory networks than in more complex organisms such as vertebrates. It is our hope that the analysis of gene regulatory networks in ascidians can provide a basic template which will allow developmental biologists to superimpose the modifications and novelties that have arisen during deuterostome evolution.

Résumé : Les ascidiers occupent une position taxonomique idéale à la base de l’arbre évolutif des chordés pour fournir un point de comparaison des mécanismes régulateurs du développement qui sont en opération chez les protostomes, les deuterostomes non chordés, les chordés invertebrés et les vertébrés. Nous proposons dans notre rétrospective un modèle du réseau génique de régulation qui mène à la formation du notocorde des ascidiers. Le but du modèle n’est pas de faire la lumière sur l’ensemble des interactions entre les molécules du réseau, mais plutôt de fournir un schéma de travail de l’architecture régulatrice qui mène à la spécification de l’endoderme et à la structuration du mésooderme chez les embryos d’ascidiers. Nous décrivons une série de méthodologies, tant informatiques que biologiques, d’usage courant ou en développement, pour l’étude des réseaux génétiques de régulation chez les embryos d’ascidiers. Nous croyons que, compte tenu du mode simplifié de développement et de la taille réduite du génome chez les ascidiers, les outils actuellement disponibles aux chercheurs intéressés à la biologie des ascidiers permettront une dissection accélérée des réseaux génétiques de régulation du développement par comparaison à ce qui se passe chez les organismes plus complexes, tels que les vertébrés. Nous espérons que l’analyse des réseaux génétiques de régulation chez les ascidiers fournira une maquette de base sur laquelle les biologistes du développement pourront superimposer les modifications et les nouveautés qui sont apparues durant l’évolution des deuterostomes.

[Traduit par la Rédaction]

Introduction

Ascidians are marine invertebrate chordates and are the largest class within the subphylum Urochordata. Commonly called sea squirts, these bilateral animals have been studied by developmental biologists since their chordate affinity was first described in the late-19th century (Kowalevsky 1866; Kowalevsky 1871). Adult ascidians are sessile filter feeders that reproduce via motile tadpole larvae which possess key features of the chordate body plan such as notochord flanked by muscles and a dorsal, hollow neural tube (Satoh 1994). There has been a renewed interest in recent years to exploit the ascidian embryo as a means to understand the molecular mechanisms underlying the developmental programs of the
ancient chordate, as well as modern chordates such as hu-
man (for reviews see Di Gregorio and Levine 2002; Di
2002; Satou and Satoh 1999; Satoh 2001, 2003; Satoh et al.
2003).

From an embryological perspective, the ascidian is an id-
eal experimental system in which to decipher cell fate
specification mechanisms at the molecular level. The
ascidian tadpole larva consists of approximately 2500
cells that form a limited set of tissues including epidermis, central
and peripheral nervous systems, notochord, muscle, endo-
derm, and mesoderm (Satoh 1994). This simple configura-
tion of the tadpole larva represents the basic chordate body
plan. Development in ascidians is extremely rapid — about a
dozen cell divisions produces a fully developed Ciona
intestinalis (L., 1758) larva that hatches 18 h post fertiliza-
tion when reared at 18 °C. Extensive research over the last
century has defined the nearly complete cell lineage of
the ascidian embryo up to the gastrula stage and most of
the major cell lineages are well characterized (Conklin 1905;
Oort 1953, 1957, 1962; Nishida and Satoh 1983; Nishida
and Satoh 1985; Nishida 1987). The early blastomeres of the
embryo are uniquely shaped and positioned, and are thus
amenable to experimental manipulation. In situ hybridization
may be used to visualize the differential patterns of gene ex-
pression with high cellular resolution. Two commonly
studied ascidians, C. intestinalis and Ciona savignyi
Herdman, 1882, have relatively short life cycles of about
3 months that have facilitated the implementation of cultur-
ing techniques and mutagenesis screens (Moody et al. 1999;
Nakatani et al. 1999; Sordino et al. 2000, 2001; Deschet et al.
2003).

From a molecular perspective, ascidians provide an ideal
platform with which to examine developmental gene regu-
lation. A key feature is the ability to generate hundreds, or
even thousands, of transgenic embryos using a simple
electroporation technique (Corbo et al. 1997b; Di Gregorio
and Levine 2002; Zeller 2004). Although these embryos of-
ten express transgenes mosaically, they have proved in-
valuable for examining the expression of tissue-specific
regulatory modules that function during embryogenesis.
Recently, a simple to build electroporator was reported to allow
the precise creation of transgenic embryos with predictable
levels of mosaic transgene expression, thus controlling the
extent of mosaic expression. The functions of development-
ally important genes may be assayed by misexpressing
genes under the control of tissue-specific regulatory modules
via electroporation or by microinjection of RNA (e.g.,
Corbo et al. 1998; Takahashi et al. 1999; Imai et al. 2000; Di
Gregorio and Levine 2002). Recently, the use of morpholino
antisense oligonucleotides has been demonstrated to effec-
tively suppress gene expression during ascidian develop-
ment (Satou et al. 2001a, 2001b; Imai et al. 2002a, 2002c;
Wada and Saiga 2002; Miyawaki and Nishida 2003) and has even been used in a large-scale screen to analyze genes of unknown
function that are conserved between ascidians and verte-
brates (Yamada et al. 2003). It is possible to generate stable,
transgenic lines of ascidians (Deschet et al. 2003), and re-
cently, a transposon system has been demonstrated to func-
tion in ascidians (Sasakura et al. 2003) that will compliment
traditional chemically induced mutant screening strategies.

From a genomics perspective, ascidians provide an exten-
sive collection of sequence resources that include two draft
genome sequences, a collection of nearly 500,000 expressed
sequence tag (EST) sequences deposited in GenBank, and an
extensive set of in situ hybridization-based expression data
of over 5000 genes. The draft genome of C. intestinalis
was reported in 2002 (Dehal et al. 2002) and the draft sequence
of C. savignyi was recently released by the Whitehead
Center for Genome Research (http://www.broad.mit.edu/annotation/
Ciona/). The C. intestinalis genome is predicted to express
nearly 16,000 genes and there are few examples of gene
duplication events, suggesting that gene regulatory analysis
in ascidians should relatively simple compared with verte-
brates. An extensive set of EST sequence data and in situ
hybridization data is available on the Web (http://ghost.zool.
kyoto-u.ac.jp/index1.html) and has been published in a series
of papers on both embryonic ESTs (Nishikata et al. 2001;
Satou et al. 2001c; Fujikawa et al. 2002; Kusakabe et al.
2002) and adult/juvenile tissue ESTs (Takamura et al. 2001;
Inaba et al. 2002; Ogawara et al. 2002). Comparative
genomics-based methods for gene regulatory analysis, such
as phylogenetic footprinting techniques (e.g., VISTA analy-
sis (Mayor et al. 2000; Loots et al. 2002)), promise to ac-
celerate the rate at which gene regulatory research may be
pursued in ascidians. At the present time, ascidians are cur-
rently the only deuterostome group in which the genomes
from related species within the same genus have been se-
quenced.

A proposed model of the ascidian notochord gene regulatory network

We propose a working model of the gene regulatory net-
work (GRN) that specifies notochord cell fate in ascidian
embryos. This model, shown in Fig. 1, is based on the ex-
perimental results from a number of laboratories and will be
briefly described here. A detailed accounting of the key ex-
periments used to construct this model will be discussed
later in this review. During early embryogenesis, the differ-
ential nuclear localization of β-catenin in the endoderm lin-
edge is believed to activate the transcription of several key
genes including a fibroblast growth factor (FGF) ligand
(FGF9/16/20) and the transcription factor FoxD (Imai et al.
2002a, 2002b). This FGF ligand, and possibly other FGF
ligands as well, produced from the endodermal cells signals
the surrounding mesoderm and patterns this mesoderm along
the anteroposterior axis (Kim et al. 2000; Kim and Nishida
2001; Nishida 2002). An Ets class transcription factor has
recently been shown to mediate this signal (Miyawaki and
Nishida 2003). A maternally inherited transcription factor
called Macho-1 that localizes in the posterior mesoderm
serves as a “molecular switch” to determine if mesoderm re-
ceiving the FGF signal will adopt an anterior (notochord) or
posterior (mesenchyme and muscle) fate (Nishida and
Sawada 2001; Kobayashi et al. 2003). In addition to FGF
signaling, recent experiments have suggested that bone
morphogenetic protein (BMP) signaling also plays an im-
portant role in notochord induction (Darras and Nishida 2001).
One of the genes downstream of this FGF-signaling event is
the gene encoding the conserved transcription factor Brachy-
ury (Yano and Satoh 1994; Nakatani et al. 1996; Corbo et al.
1997b). The synergistic activity of Brachury together

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with the transcription factor FoxA2 specifies a notochord cell and presumably activates a series of genes that are required in the differentiation of the notochord cells (Yasuo and Satoh 1998; Di Gregorio and Levine 1999; Hotta et al. 1999, 2000; Takahashi et al. 1999; Shimauchi et al. 2001a). It is unclear how the FoxA2 gene is regulated, but it is known to be expressed in the endoderm, ventral cells of the neural tube, and the notochord (Corbo et al. 1997a; Shimauchi et al. 1997). In addition to these key regulatory genes, several other regulatory genes have also been implicated in the formation of the ascidian notochord including a Zic-type transcription factor (Imai et al. 2002c; Yagi et al. 2004), a winged-helix transcription factor called FoxD, and Notch signaling (Imai et al. 2002b). Additional details of this model will be provided in the following discussion of the key experiments supporting this GRN. Readers should continue to consult Fig. 1, as details of the experiments implicating the various regulatory genes are described.

The cell lineage of the notochord and inductive events

The notochord of the ascidian tadpole larva is composed of exactly 40 cells in a single file “stack-of-coins” arrangement as shown in Fig. 2. The cell lineage of the ascidian embryo, first described by Conklin (Conklin 1905) with subsequent refinements by Ortolani (Ortolani 1953, 1957, 1962), was verified with modern cell lineage analysis techniques by Nishida and Satoh (Nishida and Satoh 1983, 1985; Nishida 1987). The notochord cells are derived from two cell lineages that arise in the early embryo. The anterior 32 cells are descendants of the A4.1 blastomere pair (anterior-vegetal blastomeres) of the 8-cell-stage embryo and are referred to as the primary lineage. The secondary lineage, consisting of the 8 posterior notochord cells, is derived from the B4.1 descendants (posterior-vegetal) of the 8-cell-stage embryo. The notochord cells are specified during early embryogenesis and later will undergo convergent extension movements to arrange their final axial orientation along the anteroposterior axis (Miyamoto and Crowther 1985; Clooney 1990; Momma and Odell 2002a, 2002b). These movements are completed at about 12 h post fertilization in the C. intestinalis embryo. From this point forward the notochord cells differentiate by forming an extracellular component called the notochordal sheath and produce numerous vacuoles that eventually will form the lumen of the notochord (Clooney
There are three pairs of blastomeres at the 16-cell stage that are fated to give rise to both endoderm and endoderm (A5.1, A5.2, and B5.1). At this time, transcripts of the transcription factors FoxA2 in Halocynthia roretzi (von Drasche, 1884) (Shinomiya et al. 1997) and FoxD in C. savignyi (Imai et al. 2002b) are detected in these blastomeres, but the early embryonic expression pattern of Ciona FoxA2 gene has not been reported. In the ascidian Molgula oculata Forbes, 1848, expression of the FoxA gene begins around the 44- to 64-cell stage and inhibiting the function of this gene with antisense oligonucleotide treatment disrupts migration of both endoderm and notochord cells (Olsen and Jeffery 1997). At this time, maternally supplied β-catenin protein is probably beginning to differentially localize to the nuclei of endodermal cells, a process that appears to be complete by the 32-cell stage (Imai et al. 2000). By the 32-cell stage, the endoderm (A6.1, A6.3, and B6.1) and notochord (A6.2, A6.4, and B6.2) fates have separated in the A- and B-lineages (Fig. 2). The three pairs of notochord precursors are situated at the vegetal-marginal zone immediately flanking the three pairs of endoderm precursors. The A-lineage notochord founder cells (A7.3 and A7.7) are born at the 44-cell stage (Darras and Nishida 2001), although in most reports these cells are reported as appearing at the 64-cell stage. These two pairs of the primary lineage founder cells become fate restricted at this time and divide three more times to form the 32 primary notochord cells. The secondary notochord precursors (B8.6) do not become restricted to notochord fate until the 110-cell stage. These B8.6 blastomeres will divide two more times to form the last 8 cells (Nishida 1987).

Early experiments with partial ascidian embryos suggested that the notochord formed autonomously, consistent with the notion that ascidian cell specification was mosaic in nature. In embryos of C. intestinalis and H. roretzi, B4.1 and A4.1 partial embryos produced notochord cells (Crowther and Whittaker 1986; Nishikaia and Satoh 1990) Early experiments with H. roretzi showed that isolated blastomeres from 110-cell embryos could autonomously differentiate into notochord cells. We now know that both the primary lineage and the secondary lineage of the notochord have already been specified by the 110-cell stage, allowing for autonomous differentiation from this point forward. With the
nootchord cell lineage well characterized, Nishida’s laboratory examined notochord cell specification on a much finer scale. When grown in a state of continuous dissociation until the 1.11-cell stage, embryonic cells displaced into notochord (Nishida 1992). This not only revealed that inductive interactions between cells must be necessary for ascidian notochord formation, as in the development of the vertebrate notochord, but that the induction occurs early. Complementing this study, Nishida’s laboratory conducted a similar experiment in which embryos were dissociated at different cleavages from egg to the 64-cell stage, and then cultured as partial embryos in normal seawater from the 110-cell stage onward (Nakatani and Nishida 1994). The results from this series of experiments indicated that embryos which dissociated around the 32-cell stage failed to produce notochord cells, but notochord cells were produced in embryos that dissociated after this time or when the dissociation was stopped prior to the 32-cell stage.

The preceding sets of experiments established the 32-cell stage as a critical time in ascidian notochord induction but did not identify which cells in the early embryo produced notochord. Therefore, Nishida’s laboratory proceeded to carry out a series of elegant experiments using partial ascidian embryos (Nakatani and Nishida 1994). Partial embryos consisting of presumptive A-lineage notochord cells generated at the 8-, 16-, 64-, or 110-cell stage produced notochord cells, whereas partial embryonic cells failed to produce notochord cells. Notochordal cells could not be produced from B-lineage partial embryos produced prior to the 64-cell stage, suggesting that an induction takes place around this time in development. Similar types of partial embryo experiments examining notochord specification, other than creating A- or B-lineage quarter embryos, have not yet been reported in embryos of C. intestinalis.

To examine which molecules might confer the notochord-inducing signal in ascidians, factors implicated in vertebrate embryo mesoderm induction were tested for their ability to induce notochord fate in isolated presumptive notochord blastomeres from H. roretzi. When applied exogenously to isolated presumptive notochord blastomeres, low levels of recombinant human bFGF, but not activin, successfully induced notochord features in these cells (Nakatani et al. 1996). Additional experiments demonstrated that this FGF treatment was also sufficient for activating the expression of the notochord-specific transcription factor Brachury less than one cell division later at the 64-cell stage (Nakatani et al. 1996). Six FGF genes have been identified in the recently released genome of C. intestinalis (Satou et al. 2002), an FGF receptor has been characterized from H. roretzi (Kamei et al. 2000; Shimauchi et al. 2001a), and experiments inhibiting FGF signaling in embryos of H. roretzi (Kim and Nishida 2001; Minokawa et al. 2001) have all implicated endogenous FGF signaling in the induction of notochord cells. The function of one of the FGF cell stage of FGF signaling, FGF-H, has been more thoroughly examined in C. savignyi embryos (Imai et al. 2002a). When an antisense morpholino oligonucleotide was used to perform a gene “knock-down” of the FGF9/16/20 gene, the initial stages of notochord induction were blocked, but notochord gene expression was observed later in development, suggesting that perhaps additional FGF ligands are present. FGF9/16/20 gene is thought to be a downstream target of the maternal B-catenin that localizes to the nuclei of endoderm cells (Imai et al. 2002a). We should note that these FGF signals are involved in the general patterning of the mesoderm along the antero-posterior axis of the ascidian embryo (reviewed by Nishida 2002) and the notochord represents the anterior mesoderm in the ascidian embryo.

Unlike the A-lineage notochord cells, the B-lineage notochord precursor cells did not respond to induction by exogenously applied bFGF (Nakatani et al. 1996). In the search for additional signaling molecules, the Nishida laboratory discovered that BMP is likely the second signaling molecule (Darras and Nishida 2001). In their model, beginning at the 24-cell stage, an FGF signal from the anterior and posterior endoderm cells initiates the first phase of notochord induction. At the 48-cell stage, BMP signaling only from the anterior endoderm cells, and not the posterior endoderm cells, completes the induction of the notochord precursors. This observation may also explain why only the combination of an anterior endoderm precursor, but not a posterior endoderm precursor, with a B-lineage notochord precursor will result in specification of notochord (Kim et al. 2000). These experiments also demonstrated that inhibition of BMP signaling, by overexpression of its antagonist chordin, resulted in the reduced formation of both primary and secondary notochord cells, indicating the importance of FGF and BMP signals for the induction of both notochord lineages.

Transcriptional activation of Brachury, a notochord-specific transcription factor

In ascidians, the transcription factor Brachury is the earliest expressed notochord-specific gene. The Brachury gene has been identified and at least partially characterized in a number of different ascidian species including C. intestinalis (Corbo et al. 1997b), C. savignyi (Imai et al. 2000), H. roretzi (Yasuo and Satoh 1993), several Molgula species (Takada et al. 2002), and several larvacean (pelagic urochordates) species (Bassham and Postlethwait 2000; Nishino et al. 2001). In all cases examined, Brachury is expressed in the notochord lineage. Within the last few years, our understanding of Brachury gene regulation has dramatically improved, as results from many experiments have linked several signal transduction pathways and transcription factors to Brachury. There are at least three different pathways that positively regulate Brachury expression: (1) FGF signaling, (2) BMP signaling, and (3) Notch signaling. There is also an interaction with the Snail repressor protein that mediates transcriptional repression (Fig. 1). In this section, we
will summarize the relevant experiments that led us to the GRN model we presented in Fig. 1.

We have previously mentioned that in early ascidian embryos between the 24- and 44-cell stages, FGF and BMP signaling is required to induce the formation of both primary and secondary lineage notochord. When bFGF is exogenously applied to isolated notochord precursor cells, these cells are induced to form notochord and they activate the expression of the Brachyury gene (Nakatani et al. 1998). One possible FGFR ligand is encoded by the FGF9/16/20 gene isolated from C. savignyi. The Ciona FGF9/16/20 gene is a downstream target of β-catenin and when the function of FGF9/16/20 is knocked-down by injecting early embryos with a specific morpholino oligonucleotide, early Brachyury expression is inhibited. However, Brachyury expression in the notochord will begin later in development (Imai et al. 2002a). Consistent with this data, when endogenous FGF signaling was chemically inhibited in H. roretzi embryos or by the overexpression of a dominant-negative ras, which is a component of the FGF signaling pathway, Brachyury was not expressed and the notochord was not formed (Nakatani and Nishida 1997; Kim et al. 2000). Finally, a recent study has demonstrated that an Ets class transcription factor in H. roretzi mediates FGF signaling in ascidian embryos. Interfering with the function of this transcription factor prevented the expression of Brachyury and the formation of the notochord (Miya and Nishida 2003). Similarly, when endogenous BMP signaling was inhibited with overexpression of ascidian chordin or Xenopus noggin, both BMP antagonists, Brachyury expression and notochord formation were abrogated (Darras and Nishida 2001). These results clearly demonstrate that Brachyury expression is dependent on both FGF and BMP signaling pathways, as indicated in Fig. 1, although it is unclear if transcription factors that mediate these signaling events, namely Ets (for FGF) and SMADs (for BMP), directly bind to the Brachyury cis-regulatory domain.

A third signaling pathway has been implicated in the activation of the Brachyury gene. There are several lines of evidence that support this hypothesis. An analysis of the Brachyury cis-regulatory domain demonstrated that several putative binding sites for the Suppressor of Hairloss (Su(H)) transcription factor, a mediator of Notch signaling (reviewed by Artavanis-Tsakonas et al. 1995), was required for transcriptional activation of the gene (Corbo et al. 1997b). Subsequent experiments demonstrated that a C. intestinalis Su(H) ortholog is bound to these sites to transcriptionally regulate the Brachyury gene (Corbo et al. 1998). So what regulates Notch signaling in notochord induction? One clue comes from the analysis of the C. savignyi FoxD gene, which encodes a winged helix class transcription factor. Like FGF9/16/20 gene, this gene is a downstream target of β-catenin and is transiently expressed in the early endoderm lineage (Imai et al. 2002b). Inhibition of the function of FoxD with specific morpholinos prevented the expression of Brachyury and the formation of the notochord — both primary and secondary lineages. This phenotype could be partially rescued by the co-injection of an activated form of the C. savignyi Notch receptor, but this would only occur in the B-lineage secondary notochord lineage (Imai et al. 2002b). These results suggest that FoxD may be regulating notochord specification through at least two mechanisms: (1) Notch signaling in the secondary lineages and (2) some other signaling pathway in the primary and (or) secondary lineages. Since FoxD knock-downs prevent notochord formation in both lineages, some common signaling pathway is likely to mediate this interaction. By itself, activated Notch rescues only the B-lineage notochord fate; therefore, Notch and this unknown signaling pathway may be functionally redundant in this lineage. Perhaps this second signal could be BMP signaling (indicated with a dotted line in Fig. 1), although in vertebrates, FoxD3 is a transcriptional repressor that inhibits BMP expression (Sasaki et al. 2001).

In addition to inducing the fate of the anterior mesoderm (notochord), FGF signaling also patterns the posterior mesoderm (mesenchyme and muscle) in ascidian embryos (Nishida 2002). If FGF signaling activates Brachyury expression, then how is Brachyury transcriptionally repressed in the posterior mesoderm? The Nishida laboratory has recently reported that a maternal mRNA encoding the transcription factor Macho-1 in H. roretzi is selectively sequestered to the posterior embryo as development proceeds (Nishida and Sawada 2001). Recent experiments have demonstrated that Macho-1 function, which is normally only present in the posterior mesoderm, acts as a switch to modulate the actions of FGF signaling in the mesoderm (Kobayashi et al. 2002). Notochord cells do not normally inherit Macho-1 mRNA or protein, so they express Brachyury in response to FGF signaling. In the posterior mesoderm, Macho-1 activates the expression of the ascidian Snail gene that encodes a transcriptional repressor (Kobayashi et al. 2003). Previous experiments in C. intestinalis have demonstrated that Snail mediates transcriptional repression of the Brachyury gene in the posterior mesoderm (Fujiiwara et al. 1998). In these experiments, Snail was shown to bind to specific target sequences located in the Brachyury cis-regulatory domain.

We have now accounted for three activating interactions (FGF, BMP, and Notch signaling) and the single repressive interaction (Snail) that regulate Brachyury expression (Fig. 1). There is a fourth positively acting interaction that has been recently defined. In both H. roretzi and the two Ciona species, genes encoding a Zic-type zinc finger transcription factor have been shown to regulate a variety of developmental processes, including notochord specification (Imai et al. 2002c; Wada and Saiga 2002; Yagi et al. 2004). When the function of either gene is inhibited by morpholino injection, notochord development is abrogated and Brachyury is not expressed. In both Ciona species, a series of functional experiments have demonstrated that ZicL is downstream of both β-catenin and FoxD function (Imai et al. 2002c; Yagi et al. 2004), although it is unclear if β-catenin activates ZicL directly or indirectly via FoxD. ZicL is first expressed at the 32-cell stage in the A-lineage notochord precursor cells (A6.2 and A6.4, Fig. 2). It is unclear if ZicL expression in these cells is mediated by a FoxD-regulated signaling pathway such as Notch. It is possible that the expression of FoxD at the 16-cell stage in A5.1 and A5.2, the cells that will give rise to A6.2 and A6.4, is sufficient to directly activate ZicL in A6.2 and A6.4 when the 32-cell stage is reached. We have now accounted for all of the early interactions, depicted in Fig. 1, that lead to Brachyury expression.
Identification of downstream Brachyury target genes

Experiments in Xenopus had previously demonstrated that the function of Brachyury alone was not sufficient to promote notochord fate in animal cap assays. Only when Brachyury and the FoxA orthologue Pintallavis were injected together in animal caps did those transform to notochord cells (O’Reilly et al. 1995). Ascidians also have a FoxA orthologue that has been characterized from both H. roretzi and C. intestinalis. This gene begins expression at the 16-cell stage (Shimauchi et al. 1997) and is expressed in tissues that include the endoderm, notochord, and ventral neural tube (Corbo et al. 1997a; Shimauchi et al. 1997). It is unclear how the ascidian FoxA2 gene is regulated, but an analysis of the C. intestinalis FoxA2 cis-regulatory domain has implicated the Snail repressor and a possible T-box protein like Ci-VegTR (which is expressed in early vegetal blastomeres) as possible transcriptional regulators (Di Gregorio et al. 2003). To test whether the synergistic functions of Brachyury and FoxA2 were required to specify ascidian notochord cells, a transgene was constructed that expressed the Brachyury cDNA under the control of the FoxA2 promoter. When this construct was electroporated into fertilized eggs, the resulting embryos displayed a phenotype consistent with the ectopic production of notochord cells (Takahashi et al. 1999). Essentially, all cells that expressed both Brachyury and FoxA2 were at least partially converted to a notochord fate. This experiment has also been repeated using mRNA injections of both transcription factors and the same results were obtained (Yoshio and Satoh 1998; Shimauchi et al. 2001a).

Because great quantities of transgenic embryos may be obtained via electroporation (Corbo et al. 1997b; Di Gregorio and Levine 2002; Zeller 2004; Zeller et al. 2004), large numbers of transgenic embryos ectopically expressing Brachyury were generated and used in a subtractive hybridization screen to identify potential downstream targets of Brachyury (Takahashi et al. 1999). Nearly 40 genes were identified that were predominately expressed in the notochord and subsequent experiments characterized the temporal and spatial expression patterns of these genes (Hotta et al. 1999, 2000). As might be expected, these genes are expressed after Brachyury begins to be expressed in the notochord founder cells of the early embryo. Some of the target genes initiate expression about a cleavage after Brachyury, while others initiate expression much later in development. These varied temporal expression patterns suggest that some genes may be direct targets of Brachyury function, while others may be indirect targets. As expected, many of these genes encode the types of molecules that would be expected to be produced in the differentiated notochord.

Validating the ascidian notochord GRN

The summary of experimental data in the previous section supports the GRN model that we have presented in this review. The goal in designing this model is to determine the overall regulatory architecture of the network, without necessarily knowing all of the minute details. In the case of the ascidian notochord GRN, we now have a reasonable view of the overall regulatory architecture. However, further experimentation is required to validate this model and expand it to encompass additional regulatory factors and downstream targets as these molecules are identified. The GRN provides a framework for designing experiments that can determine the functional relationships between the molecules portrayed in the network, as well as identifying additional molecules that play important roles in the development of the notochord in chordates.

In the last few years, advances in gene regulatory experimentation in ascidians have reached a point where GRNs models may now be rigorously tested. To test GRNs, the functions of key regulatory molecules must be modulated both positively and negatively, and the results of these manipulations monitored by observing the resulting embryonic phenotypes as well as by identifying changes in the expression of downstream genes. With the completion of both C. intestinalis and C. savignyi genomes, microarrays have begun to be used to assay global gene expression during ascidian embryogenesis (Azumi et al. 2003; Ishibashi et al. 2003). Once readily available, microarrays may be used to examine the expression of target genes in embryos in which the GRNs have been experimentally manipulated. It will be necessary to manipulate GRNs at different times and in different cell types during embryonic development, necessitating the use of appropriate methods for misexpressing genes. In addition to misexpressing genes with wild-type functions, it will be necessary to express genes that have been mutated to have positively or negatively acting functions. For example, the DNA-binding domain of a transcription factor may be fused to either an activation or a repression domain to alter the function of the protein. Similar types of modifications and (or) truncations may be made to receptors to alter their functions as well. These types of approaches, first developed in other systems and used extensively in embryos such as the sea urchin embryo (Davidson et al. 2002a, 2002b; Oliveri et al. 2002; Rast et al. 2002), have now begun to be employed in ascidians. The next key step in the analysis of ascidian GRNs will be to combine these genetic manipulations with microarray analysis to examine changes in the global patterns of gene expression during embryonic development.

Two strategies are currently used in ascidians to mis- or over-express genes: mRNA injections and transgene-based expression. Microinjection in ascidians is relatively simple to perform, although the throughput is quite small compared with producing transgenic embryos with electroporation. One of the advantages of mRNA injection is that there is very little lag time for producing protein off of the mRNA template; protein is likely produced prior to first cleavage and may be produced in all blastomeres that inherit the injected mRNA. This is an important consideration when trying to manipulate GRN function in early development. Although transgenes have been used to ectopically express genes in ascidian embryos, those genes are expressed with the temporal, spatial, and quantitative regulation provided by the cis-regulatory DNA used to create the transgene. The earliest reported zygotic transcription occurs at the 16-cell stage in ascidians (Shimauchi et al. 1997; Imai et al. 2002a), and transgene-based expression will not be suitable for early GRN manipulations unless cis-regulatory DNAs are identified that confer earlier zygotic expression. A drawback of mRNA injections is that the injected mRNAs are di-
ulated during embryogenesis as blastomeres divide, mRNAs are degraded during this time, and there is no spatial control. For manipulating later aspects of GRN function, transgene-based methods are ideal. Large numbers of transgenic embryos may be produced via electroporation (Di Gregorio and Levine 2002; Zeller 2004) and a variety of cis-regulatory DNAs have been identified that will confine temporally and spatially restricted patterns of expression on targeted genes (e.g., Harafuji et al. 2002).

To complement ecotopic expression experiments, targeted gene knock-down using morpholinos antisense oligonucleotides have proven to function well in ascidians (Satou et al. 2001a). Morpholinos, designed against specific mRNAs, are microinjected into fertilized eggs where they effectively prevent the translation of the targeted mRNA. Although designed to be resistant to degradation, morpholinos tend to lose effectiveness as development proceeds. Because ascidian embryogenesis is rapid, this potential problem is minimized. As with mRNA injections, injections of morpholinos provide no means to control the spatial or temporal distribution of the knock-down effect. Morpholinos should prove effective at preventing the translation of maternal as well as zygotically transcribed genes. To date, morpholinos have been used to knock-down the functions of the following genes relevant to the development of the ascidian notochord: Brachury (Yamada et al. 2003), FoxD (Sasa et al. 2001), H/ZipC (Wada and Saita 2002), FGFR/FGF20 (Imai et al. 2002a), Macho-1 (Nishida and Sawada 2001; Kobayashi et al. 2003), HrEts (Miya and Nishida 2003), and C. elegans (Imai et al. 2002c). Although RNA interference (RNAi) techniques have been successfully employed to knock-down gene function in other invertebrate embryonic systems such as Drosophila melanogaster Meigen, 1830 and Caenorhabditis elegans (Maupas, 1900) Dougery, 1955, (e.g., Bargmann 2001; Schmiid et al. 2002), there have been no published reports of this technique being successfully used in ascidian embryos.

Manipulations of Brachury expression during ascidian embryogenesis have been accomplished by both ecotopic expression, using both mRNA injections (Yanou and Sato 1998; Shimauchi et al. 2001a) and transgene expression (Takahashi et al. 1999), as well as by gene knock-down approaches using morpholinos (Yamada et al. 2003). Results from these experiments are consistent with Brachury acting as a transcriptional activator on the downstream notochord target genes; however, these experiments do not address if these are direct or indirect targets. The Brachury target genes initiate expression at several different times during development, suggesting that there are both direct and indirect targets (Hottu et al. 1999, 2000). Some initiate expression one cleavage after the Brachury gene begins to be expressed, indicating that these may be direct transcriptional targets, while others initiate expression much later in embryogenesis, which is consistent with the idea that they may represent indirect targets. Experiments on a single Brachury target gene, tropomyosin-like, have identified putative Brachury-binding sites in the cis-regulatory domain and have demonstrated that these sites are required for proper gene expression (Di Gregorio and Levine 1999). Similar types of experiments should be conducted on the remaining sets of notochord genes, although this will require a significant labor investment.

Given that the genomes of the related Ciona species are now available, comparative genomics and bioinformatics approaches promise to expedite the analysis of the Brachury target genes and should prove invaluable for the analysis of genes in other ascidian GRNs. Reports from a number of different laboratories, using a variety of bilaterian embryo models, have demonstrated that comparative genomics methods are useful for identifying potential cis-regulatory modules from genomic sequences (e.g., Blanchette et al. 2002; Blanchette and Tompa 2002; Halfon et al. 2002). Although several different methods have been reported, they all essentially identify what are called "phylogenetic footprints"—sequence regions that have been constrained during evolution. The coding regions of a pair of homologous genes would be expected to be present within a phylogenetic footprint analysis, since the exons encode similar proteins like the Brachury gene from C. intestinalis and C. savignyi. An interesting observation from these types of analyses is that there are often extensive regions of non-coding sequence that are also present within phylogenetic footprints. It is these non-coding regions that are often found to identify important cis-regulatory DNAs, when tested using appropriate experimental procedures.

Our laboratory has used phylogenetic footprinting techniques with the two Ciona genome sequences to identify putative cis-regulatory domains for a variety of ascidian genes. We have found that these computational methods are useful for identifying where to design primers to amplify by polymerase chain reaction the 5’-cis-regulatory domains directly from genomic sequence. When assembled into a transgene to drive green fluorescent protein (GFP) expression, these cis-regulatory regions quite often express GFP with the expected temporal and spatial patterns. We have successfully designed a number of transgenes in this manner, including genes that are expressed in the central nervous system, epidermis, muscle, and endoderm of the ascidian embryo.

A phylogenetic footprint analysis of a 5-kb region of the Brachury gene, using the VISTA web-based service (Mayor et al. 2000; Loots et al. 2002), is displayed in Fig. 3A. Peaks indicate the positions of conserved sequences, dark gray peaks denote exons, and light gray peaks denote non-coding regions. The "full length" cis-regulatory region from the Brachury gene, as originally reported (Corbo et al. 1997a), extends about 3.5 kb upstream of the start of transcription (left-most dark gray peak in Fig. 3). From the first draft of the C. intestinalis genome, we have determined that about 1.2 kb of this region actually extends into an additional gene near the Brachury gene and has been removed from the analysis shown in Fig. 3A. The set of light gray peaks immediately adjacent to the first exon detected by the VISTA analysis roughly corresponds to the 434-bp minimal Brachury enhancer, and the inclusion of the second set of light gray peaks corresponds to the 790-bp enhancer, both of which have been extensively tested experimentally (Corbo et al. 1997b). Phylogenetic footprinting thus promises to expedite the identification of cis-regulatory DNAs and should prove useful in the analysis of developmental gene regulation.

Phylogenetic footprinting techniques will probably be most useful for identifying cis-regulatory DNAs on the level of a regulatory module. To computationally identify actual transcriptions factor binding sites, additional bioinformatics

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Fig. 3. Computational analyses can predict molecular interactions within ascidian gene regulatory networks. (A) VISTA analysis (Mayor et al. 2000) comparing the Brachury genes from Ciona intestinalis and Ciona savignyi. Exons are shaded dark gray and highly conserved non-coding regions (putative regulatory modules) are shaded light gray. Transcription factor binding sites within these light gray peaks were predicted with MatInspector (Quandt et al. 1995), and are indicated as SMAD, Ets, Su(H), or Brach.

(B) Brachury::lacZ transgene expression in normal C. intestinalis embryos. Fertilized egg embryos were electroporated with a GBrac::lacZ reporter construct and stained for LacZ expression, which is only present in the notochord. (C) The Brachury::lacZ transgene is misexpressed in embryos expressing ectopic Brachury. Fertilized eggs were co-electroporated with GFOXa2::Brachury and the GBrac::lacZ reporter construct. Ectopic expression of Brachury activates ectopic expression of the Cbrac::lacZ transgene consistent with the hypothesis that Brachury may positively autoregulate its expression.

Approaches are becoming available, but there are a number of hurdles that must be overcome before these methods will become more generally useful. Transcription factors tend to bind to short degenerate sequences, so simple sequence searches are not always useful for identifying biologically meaningful regulatory sites. Additionally, only a small number of the transcription factors encoded in animal genomes have characterized binding sites, most sites are of unknown composition. Other computational approaches, beyond the scope of this review, are currently being developed to identify over-represented motifs that may prove useful for identifying the binding sites of uncharacterized transcription factors (e.g., GuhaThakurta et al. 2002a, 2002b; Storno and Tan 2002; Wang and Storno 2003).

Despite these drawbacks, potentially useful gene regulatory predictions may be obtained by combining phylogenetic footprint analysis with the search for characterized transcription factor binding sites (e.g., Wang and Storno 2003). Since the DNA-binding domains of homologous transcription factor proteins are conserved, these proteins are liable to bind to similar DNA target sequences. This has been demonstrated extensively with the Brachury proteins of many different animals in both ascidians (Satoh et al. 2000) and vertebrates (Marcellini et al. 2003). Since the Brachury-binding site has been well characterized (Kasper and Herrmann 1993), we performed a search for putative Brachury-binding sites, using the Transfac database (Quandt et al. 1995; Wingender et al. 1996), within the phylogenetically footprinted regions of the Brachury gene. As shown in Fig. 3A, our analysis detected the presence of putative Brachury-binding sites within the 434 minimal enhancer element, suggesting that the Brachury gene may regulate its own expression. In an initial analysis of this hypothesis, we electroporated C. intestinalis embryos with either the Brachury::lacZ reporter gene alone or together with the FOXa2::Brachury transgene that drives expression of Brachury in embryonic cells that normally express Foxa2. As shown in Fig. 3B, the Brachury::lacZ reporter gene is correctly expressed only in the notochord cells, while ectopic expression of this reporter gene is evident in embryos ectopically expressing Brachury (Fig. 3C), suggesting that Brachury may regulate its own expression.

Using a similar computational approach, we have searched the Brachury cis-regulatory domain for the presence of binding sites for Suppressor of Hairless (Su(H)), Ets, and SMAD proteins that are downstream effectors of the Notch, FGF, and BMP signal transduction pathways. The Su(H)-binding sites identified in this computational analysis are the same Su(H) sites that have been experimentally demonstrated to regulate Brachury expression in ascidian embryos (Corbo et al. 1997b). As described earlier, both FGF and BMP signaling regulate notochord specification in early ascidian embryos (Nakatani et al. 1996; Darras and Nishida 2001). Recently, the Ets protein has been shown to mediate the FGF-inducing signal in ascidians (Miya and Nishida 2003). The computational analysis has also identified puta-
tive binding sites for factors that mediate the three signal transduction pathways involved in notochord specification, although it is not known if these sites functionally regulate Brachyury gene expression (R.W. Zeller, unpublished observations). Thus, phylogenetic footprinting, coupled with searching for characterized transcription factor binding sites, should prove to be a powerful tool for deciphering gene regulation during development.

**Comparing notochord gene expression between ascidians and other deuterostome embryos**

The analysis of developmental GRNs in ascidians will provide an important data set that may be used to understand how gene regulation has changed during evolution. As an example, we will discuss the evolution of the notochord within the deuterostomes. The animals within the deuterostomes are either chordates (like ascidians, amphioxus, and vertebrates) or non-chordates (like the sister-groups of echinoderms and hemichordates). Neither echinoderms nor the sea urchin, nor hemichordates have a notochord, but both sea urchin and hemichordate embryos express Brachyury in endodermal tissues (Tagawa et al. 1998; Peterson et al. 1999a, 1999b). In these organisms, Brachyury functions control cell movements during gastrulation and endoderm development, which is believed to be the ancestral role of Brachyury function (Rast et al. 2002). In chordates, Brachyury regulation of notochord genes is believed to be a co-opted function. Therefore, in chordates, Brachyury would likely regulate multiple sets of targets: one group of genes that regulate cell movement during gastrulation and another of genes that make the notochord (Rast et al. 2002). A screen to identify sea urchin embryo Brachyury target genes has also been performed, but there is little overlap between these genes and the Brachyury targets identified in the Ciona screen (Rast et al. 2002). In the sea urchin screen, many of the Brachyury targets encode genes that are involved in cell movement or in the differentiation of the endoderm, while the Ciona screen primarily identified genes involved with notochord differentiation. These results support the hypothesis that Brachyury function was co-opted in chordates to make the notochord and suggest that the Ciona screen may have missed Brachyury targets that regulate cell movements. Future experiments could address this issue by characterizing putative Brachyury target genes that regulate cell movements.

Within the chordates, in addition to the ascidian embryo, there exists extensive research detailing the development of the notochord in amphioxus (e.g., Holland et al. 1995: Shimeld 1997), as well as in representative vertebrates such as Xenopus, zebrafish, chickens, and mice. The expression patterns and functions of signal transduction pathway genes, such as BMP, FGF, and Wnt (e.g., Isaacs 1991; Casey et al. 1998; Dickmeis et al. 2001; Yasuo and LeMaire 2001; Faure et al. 2002), as well as several transcription factors, including Brachyury (Wilkinson et al. 1990; Smith et al. 1991; Kispet et al. 1994; Schulte-Merker et al. 1994), FoxA2 (Ruiz i Altaba and Jessell 1992; Ang et al. 1993; Kaestner et al. 1993; Strahle et al. 1993; Ang and Rossant 1994), and Xnot or floating head (von Dassow et al. 1993; Stein and Kessel 1995; Talbot et al. 1995; Gott et al. 1996; Stein et al. 1996), that are involved in notochord development are well described in many vertebrate species. Significant research has also focused on the regulation of key notochord genes such as Brachyury (e.g., Schulte-Merker et al. 1994; Griffin et al. 1995; O'Reilly et al. 1995; Schulte-Merker and Smith 1995; Clements et al. 1996; Casey et al. 1998) and mutant screens in zebrafish have identified numerous alleles that effect notochord development (Odenthal et al. 1996; Steemple et al. 1996), although the molecular identity of many of these genes remains unknown. In vertebrates, the notochord arises from the chordamesoderm of the Organizer, a specialized group of cells that defines the dorsal-most region of the embryo (recently reviewed by De Robertis et al. 2000). The Organizer forms in response to several different signaling pathways, including β-catenin, Wnt, and TGFβ family members. Brachyury is expressed in the prospective mesoderm of the early vertebrate embryo and is involved in the development of the notochord as well as in regulating cell movements during gastrulation (reviewed by Herrmann and Kispert 1994; Smith 1997, 1999; Papaloisou and Silver 1998; Technau 2001).

Ascidians are not known to possess a vertebrate Organizer, so early specification events regulating the expression of Brachyury may not be completely conserved between ascidians and vertebrates. Although there are a number of Organizer genes that are present in the ascidian genome, few have been characterized. One of the genes that has been characterized is the chordin gene from H. roretzi. It is expressed in the primary lineage notochord cells at the 64-cell stage and overexpression of this gene, or the Xenopus noggin gene, early in embryogenesis disrupts notochord formation in ascidians (Darras and Nishida 2001). Despite the fact that ascidians do not have an Organizer, many of the signaling pathways that are required for Organizer function in vertebrates, as mentioned above, may also be required for Brachyury expression in ascidians. Interestingly, Brachyury indirectly autoregulates its own expression in Xenopus embryos by activating the expression of embryonic FGF (Casey et al. 1998). Recall that our computational analysis and preliminary experiment suggests that Ciona Brachyury also regulates its own expression (Fig. 3). The computational analysis suggests that this interaction is direct, but we cannot rule out indirect regulation via FGF signaling, as in Xenopus. Future experiments should address these questions.

Brachyury function in ascidians and vertebrates is likely to be conserved, although at the present time there is limited molecular evidence to support this hypothesis. The large-scale mutant zebrafish embryo screens identified a number of loci that are involved in the specification, formation, and maintenance of the notochord (Odenthal et al. 1996; Stemple et al. 1996). Unfortunately, the identities of the genes corresponding to most of these mutants are unknown. Some vertebrate Brachyury targets, such as eFGF and the Bix transcription factors, identified from screens in Xenopus embryos (Tada et al. 1997; Casey et al. 1998; Tada and Smith 2001) have not been analyzed in ascidians. Clearly, the screens in zebrafish, Xenopus, and ascidians have failed to identify all of the loci regulating notochord development. We know the identities of most of the characterized Brachyury target genes from ascidians. Perhaps the identities of some of the
remaining zebrafish mutants correspond to some of the Ciona Brachury targets, although we are unsure if this analysis has been reported.

Some genes implicated in the development of the vertebrate notochord have not been examined in ascidian embryos. It is likely that some of these genes will not have ascidian orthologues and will represent vertebrate novelties. For example, the zebrafish one-eyed pinhead (oep) gene is required for pre-chordal plate and endoderm formation and is also expressed in the notochord (Zhang et al. 1998; Feldman and Stemple 2001; Griffin and Kimelman 2003), but there is no oep orthologue present in the current release of the C. intestinalis genome (R.W. Zeller, unpublished observations). In other cases, ascidians will have orthologues to vertebrate genes required for notochord development. For example, the Xenopus Xnot gene (floating head in zebrafish) encodes a homeodomain transcription factor that is required for early notochord specification (von Dassow et al. 1993; Talbot et al. 1995; Gont et al. 1996). This gene has been isolated and characterized in sea urchins (Peterson et al. 1999a) and is present in the ascidian genome, but it has not been characterized (R.W. Zeller, unpublished results). As more notochord genes are identified in vertebrates, their ascidian orthologues should be characterized as well, to develop a more complete model of the notochord GRN operating in the ascidian embryo.

The Brachury gene is central to notochord formation in chordates, although it is also expressed in non-chordate deuterostome and protostome embryos. Because ascidians are situated at the base of the chordate tree, they are ideally suited for comparisons among ‘higher’ chordates (vertebrates), as well as non-chordate deuterostomes. To understand how gene regulatory mechanisms have evolved over time, we must be able to identify the large sets of genes that function together in a developmental process and then construct and test a GRN model. The large-scale efforts to understand gene regulation in the sea urchin embryo (Davidson et al. 2002a, 2002b; Oliveri et al. 2002; Rast et al. 2002) will provide us with a detailed example of developmental mechanisms in a non-chordate deuterostome. Acsidians are now poised to exploit the recent advances in large-scale gene regulatory analysis to provide us with a detailed understanding of developmental gene regulation in a basal chordate. Compared to vertebrates, gene regulatory analysis in ascidians is relatively straightforward. By assembling the basic framework of important developmental GRNs in ascidians, we may be able to provide researchers with a ‘blueprint’ with which to model vertebrate GRNs. Comparisons of the developmental mechanisms between ascidians and sea urchins may shed light on the evolution of chordates from a deuterostome ancestor. Similarly, comparisons between ascidian and vertebrates will provide clues to the evolution of vertebrates from a common chordate ancestor.

Summary

Acsidians are ideally suited for gene regulatory analysis. Development is rapid, producing a 2500 cell tadpole larva composed of few tissue types. The availability of sequenced genomes from two closely related species will accelerate research efforts as computational genomics methods are im-

proved. The ability to easily create transgenic embryos, as well as the ability to modulate the expression of genes, are all key requirements for analyzing gene regulation on a global scale. Acsidians are now poised to exploit this embryo for defining and testing developmental GRNs that may provide “blueprints” for examining gene regulation in vertebrate embryos. Because ascidians are basal chordates, comparisons between developmental gene regulatory mechanisms in ascidians, vertebrates, and non-chordate deuterostomes will provide us with the data required to investigate not only how chordates evolved, but how vertebrates evolved from a basal chordate ancestor. This new era of gene regulatory experimentation in ascidian embryos promises to unify the efforts of the disciplines of evolution, development, genomics, and genetics towards understanding and deciphering the gene regulatory code.

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Predictable Mosaic Transgene Expression in Ascidian Embryos Produced With a Simple Electroporation Device

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Two customized electroporators were specifically designed for creating transgenic ascidian embryos. These electroporators were simple to build, inexpensive, and produced transgenic embryos with efficiencies that equaled or rivaled commercially available machines. A key design feature of these machines resulted in the generation of consistent electroporation pulses providing repeatability between experiments. These devices were used to optimize experimental parameters allowing for the creation of transient transgenic embryos with predictable patterns of mosaic transgene expression. We used these new electroporators to examine the expression of two different fluorescent protein reporter genes with regard to embryonic cell lineage. In general, transgene expression followed the embryonic cell lineage and coelectroporated transgenes were always expressed in the same embryonic cells. Our analysis also indicated that, during development, transgenes could be lost from embryonic cells, suggesting that transgenes may be present in extrachromosomal arrays, as has been observed in other organisms. Our new electroporator designs will allow ascidian researchers to inexpensively produce transgenic ascidians and should prove useful for adapting the electroporation technique to other marine embryo systems. Developmental Dynamics 235: 1921–1932, 2006. © 2006 Wiley-Liss, Inc.

Key words: ascidian embryo; Ciona intestinalis; electroporation; electroporator; green fluorescent protein; DsRed; transgenic embryo; cell lineage

Accepted 12 December 2005

INTRODUCTION

In recent years, there has been a renewed interest in studying the evolution of developmental mechanisms in the ascidian embryo. Ascidians, a group of strictly marine animals, were first recognized as chordates in the mid-nineteenth century (Kowalevsky, 1871). They are considered to be basal chordates, located at the evolutionary boundary between invertebrates and vertebrates (Sato, 2003). The rapidly developing, 2,500 cell ascidian embryo consists of approximately six major tissues and has a dorsal hollow neural tube flanked by muscles and a notochord, both characteristics of a chordate body plan (Sato, 1994). The blastomeres of the early embryo are uniquely shaped and positioned, allowing for easy visual identification, and are amenable to experimental manipulation. The cell lineage of the embryo is well documented (Nishida and Sato, 1983, 1985; Nishida, 1987), allowing the gene expression patterns in the early embryo, detected by whole-mount in situ hybridization, to be correlated with cell fate specification decisions. Ascidians have a short life cycle and small genomes, only 5% the size of a typical vertebrate genome (Dehal et al., 2002), and genetic screens are under way in two different species (Moody et al., 1999; Sordino et al., 2001). The recent sequencing of the complete genomes of two closely related ascidians, Ciona intestinalis and C. navigata, and an extensive collection of expressed sequence tag sequences, has provided valuable data that will enhance the
experimental analysis of gene regulation during embryonic development (Dehal et al., 2002; Satoh, 2003; Satoh et al., 2000). An electroporation technique, developed in the late 1990s, permits the generation of thousands of transgenic ascidian embryos in a short period of time (Corbo et al., 1997b; Di Gregorio and Levine, 2002; Zeller, 2004). Because of this extensive collection of “tools,” ascidians are perhaps one of the best suited chordates for gene regulatory analysis (Satoh et al., 2003).

An analysis of the molecular mechanisms governing the development of the ascidian embryo will provide insight into the evolution of chordates and of vertebrates. A key experimental breakthrough that enables the dissection of chordate gene regulatory networks in ascidians was the development of an electroporation technique for generating transgenic embryos (Corbo et al., 1997b). This procedure is surprisingly simple and takes approximately 30 min. After fertilization, the protective chorion is removed from the 1-celled zygote by protease treatment, the eggs are washed and then mixed with plasmid DNA, and the egg/DNA mixture is then electroporated. The resulting embryos are transgenic and often express the transgene in a mosaic manner reminiscent of transgene expression in sea urchin embryos (Hough-Evans et al., 1988). Because development is completed in less than 1 day, the rate-limiting step in the generation of transgenic ascidian embryos is the actual construction of the transgenes. Although electroporation may be used to produce stable transgenic ascidians (Matsuoka et al., 2005), the vast majority of transgenic embryos produced in the laboratory are transient in nature and to date, this has not been an issue. Electroporation has been used to create a variety of transgenic ascidians, from embryos expressing green fluorescent protein (GFP) and lacZ reporter genes (Corbo et al., 1997b; Zeller et al., in press), to “mutant” embryos producing phenotypes induced by transgene expression (Corbo et al., 1998; Takahashi et al., 1999; Di Gregorio et al., 2002; Keys et al., 2002).

We have constructed an electroporation device that is specifically designed for use with marine embryos, such as the ascidian. Our electroporator has several features that, to our knowledge, are not found in commercially available units. The simple electronic design provides reproducible, exponentially decaying pulses, ensuring consistency from experiment to experiment. The machine is inexpensive to build and may be assembled by anyone with a basic knowledge of electronics. Although the ascidian electroporation technique was developed some time ago, there has been no reported optimization of the parameters. We, therefore, have optimized the electroporation parameters and demonstrate that we can generate transgenic embryos using our machine with high efficiencies. Because our machine produces pulses with low variability, compared to commercially available machines, we find that we consistently can generate transgenic embryos with predictable levels of mosaic transgene expression. For example, we have identified a setting in which most embryos express lineage-specific transgenes in 50% of the expected cells (i.e., 50% mosaic expression) and other settings in which most embryos express transgenes with very little or no mosaicism. The ability to predict the extent of mosaic expression in transgenic embryos will allow researchers to produce embryos that are most appropriately suited to their particular experimental needs. We used our electroporators to carefully examine the expression of two transgenes with regard to embryonic cell lineage and show that these transgenes are expressed in a lineage-specific manner. Our observations are consistent with the notion that electroporated transgenes form one or more aggregates that become “inherited” by early blastomeres during the first few cleavages as has been described for the sea urchin embryo (Livan et al., 1991). It is not known currently if these aggregates become integrated into the genome, as they do in sea urchins; however, our observations indicate that these aggregates may be differentially “lost” in cell lineages during later cleavages. We expect that our electroporators would be useful for adapting electroporation methods to produce transgenic embryos in other marine animal systems.

RESULTS

Electroporator Design

Two different electroporators were used in these studies (schematics diagrammed in Fig. 1). These devices are inexpensive, approximately 10% the cost of a commercial unit, and simple to build for someone with basic electronic skills. The first electroporator design provides fully adjustable resistance and capacitance settings and was used to optimize the specific parameters for creating transgenic ascidian embryos (Fig. 1A). The electroporator requires a 50 VDC power source that may be built into the unit or supplied from a standard laboratory electrophoresis power supply. The controls to operate the device are simple: two buttons provide charging and discharging capabilities, six parallel capacitor banks ranging in value from 125 μF to 2,000 μF may be selected using a series of toggle switches and a series of timing resistors may be added to the circuit using a rotary switch. The total capacitance of the device is 4,000 μF, and smaller values are user selectable in multiples of 125 μF. The device outputs an exponentially decaying pulse typical of a capacitor discharging across a resistor. A series of user-selectable timing resistors, ranging in value from 5 Ω to 100 Ω and in parallel to the electroporation cuvette, may be added to the circuit providing control over the time constant of the discharge pulse. To use the device, the desired capacitance is set with a series of toggle switches and the timing resistor is set with a rotating dial. The cuvette containing DNA and dechorionated, fertilized eggs is placed in a cuvette holder, and the charge button is depressed and held for a few seconds until the capacitors charge to 50 VDC as measured by a built-in volt meter. The charge button is quickly released, and the discharge button is then immediately depressed. The electroporated eggs are carefully removed from the cuvette and spread in a 60-mm dish as described in the Experimental Procedures section.

The second electroporator is a non-adjustable device that delivers reproducible pulses for a single setting (Fig.
1B). Unlike the adjustable device, charging and discharging operations are controlled from a single button that controls a single-pole, double-throw (SPDT) relay. When the button is depressed, capacitors are charged to 50 VDC from an externally supplied power source. Upon release of this button, the stored energy is discharged across the timing resistor and cuvette. The relay-based charge-discharge system is superior to the two-button arrangement described above, and could easily be adapted for the machine diagrammed in Figure 1A. One of the benefits of the nonadjustable device is that it is small and very inexpensive, making it suitable for use in remote laboratory locations or in a classroom environment. Additionally, because it is fixed at a single setting, experiments will always be performed under the same electroporation conditions.

One of the critical parameters for generating transgenic ascidian embryos is the length of the electroporation pulse, measured by the time constant of the circuit. The time constant (τ) is equivalent to the total resistance multiplied by the total capacitance (τ = RC) and indicates the amount of time it takes for the capacitance to discharge to approximately 37% of the initial voltage (Andreason and Evans, 1988; Shiokawa and Dower, 1988; Zeller, 2004). Commercial electroporation devices, such as the Bio-Rad Gene Pulser II, used to develop the original asidian electroporation technique (Corbo et al., 1997b; Zeller, 2004), often produce quite variable time constants and have been reported to range from 15 to 25 msec (Erives et al., 1998). One of the benefits of the designs reported here is the low variability in time constants achieved through the use of a timing resistor placed in parallel with the electroporation cuvette. The measured resistance of the electroporation cuvette filled with electroporation medium is typically much greater than the timing resistor; therefore, the total circuit resistance is approximately equal to the value of the selected timing resistor. Because this total resistance does not significantly vary from experiment to experiment, the timing constant produced is extremely reproducible. An additional benefit of this design is that, because of this large difference in resistance, the majority of the discharge current will flow through the timing resistor (reducing potential heating of the sample), whereas the voltage across both the cuvette and timing resistor will be equal. To our knowledge, there are no commercial electroporator designs that incorporate equivalent low-value resistors together with low-voltage pulse discharges.

Time constants from three independent experiments, using a variety of electroporator settings, were measured with an oscilloscope and are plotted in Figure 2. In each of the 16 tested settings, the time constants varied by a small percentage. This finding is in contrast to tests with the Bio-Rad machine, where we measured a time constant of 16.6 ± 2.8 msec (range 14.3–20.3 msec, 960 μF setting). We found that by adding a resistor in parallel to the cuvette, the variability of the time constants of the Bio-Rad device could be significantly reduced. For example, by adding a 30 Ω resistor in parallel to the cuvette, the time constant was 13.6 msec ± 0.3 msec (range, 13.2 to 13.9 msec; 960 μF setting). Researchers who currently use commercial electroporators may improve their ability to generate transgenic embryos by adding a timing resistor in parallel to the sample cuvette. Using our new machine and selecting appropriate electroporation parameters, we can generate trans-
genic ascidian embryos with efficiencies approaching 90% or greater, as discussed below. Electroporation has been reported to create transgenic embryos from the following species: C. intestinalis (Corbo et al., 1997b), C. savignyi (Deschet et al., 2003), Botryllia villosa and Phallusia mammilata (Di Gregorio and Levine, 2002). In this study, we only report our results with Ciona intestinalis embryos; however, our machines will create transgenic embryos from C. savignyi, Aiscidiella aspera, and Styela plicata (R.W. Zeller, unpublished observations).

Parameters Affecting Transgenic Embryo Production

Although the development of an electroporation technique to generate transgenic ascidian embryos was reported some time ago (Corbo et al., 1997b), a detailed examination of the parameters affecting the outcome has not been presented. We, therefore, used both electroporators and well-characterized, lineage-specific GFP-expressing transgenes to examine the parameters affecting the generation of transgenic ascidian embryos. The electroporation procedure induces varying degrees of embryonic lethality as well as alterations in normal embryonic development and morphology. Therefore, we set about to minimize both of these side effects. If transgenes expressing altered forms of genes are used to generate phenotypes in ascidian embryos, then it is imperative to generate these embryos under electroporation conditions that minimize electroporation-induced effects. It is also critical to identify parameters that maximize transgene expression and provide consistent levels of mosaic transgene expression in these same embryos. In the following experiments, we assayed embryological development using a variety of criteria. First, morphologically normal embryonic development, relative to dechorionated but not electroporated control embryos, was scored. Second, embryos were scored if they expressed the transgene in any cell regardless of the level of mosaic expression. Lastly, the level of mosaic transgene expression was scored. In these experiments, there was usually a range of transgene expression patterns produced from the electroporation. To illustrate this point, we electroporated 40 μg of the Brachyury::GFP transgene into fertilized eggs (3,000 μF and 10 Ω setting) and plotted some of the resulting mosaic expression patterns, from over 150 embryos examined, in Figure 3. The Brachyury::GFP transgene is expressed exclusively in the 40 notochord cells of the tadpole larva, and in this example, over 90% of the resulting morphologically normal embryos expressed the transgene in the patterns depicted in Figure 3A. We also show an embryo expressing the transgene in all notochord cells, labeled 32-8, for comparison, although none of the embryos from this particular experiment expressed the transgene in all notochord cells as indicated in Figure 3B. Most of these embryos expressed the transgene in 25% to 50% of the expected cells, i.e., 25% to 50% mosaic expression (Fig. 3B).

In the first set of experiments to optimize parameters, we examined the effects of different electroporator settings on the generation of transgenic embryos (Fig. 4A). The amount of DNA used was held constant at 60 μg in a total volume of 800 μl, and the capacitance and timing resistors were varied as indicated in Figure 4A. Several conclusions are apparent. First, as the amount of capacitance is increased, the percentage of normal embryonic development, relative to dechorionated control embryos, generally decreases (Fig. 4A, open bars). At low capacitances (i.e., 1,000 μF), approximately 80% of the resulting embryos are morphologically normal, relative to control dechorionated embryos, whereas at larger capacitance settings, only 30–50% of the embryos develop normally. At a setting of 1,000 μF, normal embryonic development is consistently 80% or greater over the four different timing resistor settings tested. Second, electroporations using larger capacitances resulted in a higher percentage of morphologically normal embryos expressing the transgene of interest at the expense of the percentage of normally developing embryos. For example, using the 1,000 μF setting, the percentage of embryos expressing the Brachyury::GFP transgene increased from approximately 10% to 80% when the timing resistor was changed from 5 Ω to 30 Ω. At 3,000 μF and 4,000 μF, essentially 100% of the embryos expressed the transgene, but only approximately 50% of the initial fertilized eggs developed into morphologically normal embryos. Lastly, as the amount of capacitance was increased, the level of mosaic transgene expression decreased.
Fig. 3. Typical observed patterns of mosaic transgene expression. For this experiment, the adjustable electroporator was used (50 VDC input, 3,000 μF \times 10 \Omega, 40-μg supercoiled Brachyury::GFP plasmid) and over 150 embryos were scored. A: Diagrams of mosaic patterns of green fluorescent protein (GFP) expression in the Ciona intestinals notochord. The ascidian notochord is composed of exactly 40 cells: 32 anterior cells (1° lineage) and 8 posterior cells (2° lineage). An 8-0 pattern indicates that there are eight 1° and no 2° cells expressing GFP, i.e., an embryo with ~20% mosaic expression. The 32-8 pattern, indicating all cells are expressing GFP is shown for comparison. B: The percentages of expression patterns observed for this experiment. Approximately 60% of these embryos have an 8-0 or 8-4 mosaic pattern of GFP expression.

Fig. 4. Optimization of electroporation parameters. A: Electroporator settings. For each experiment, 60 μg of DNA was electroporated into fertilized eggs at the indicated electroporator settings and the resulting embryos scored. B: Voltage. For each experiment, 60 μg of DNA was electroporated at the indicated voltages and the resulting embryos scored. C: DNA concentration. For each experiment, a variable amount of DNA was electroporated as indicated. The fixed value electroporator, 50 VDC input, was used for the data presented in B and C. The supercoiled Brachyury::GFP transgene plasmid was used in these experiments. For each graph, open bars indicate the percentage of normal embryonic development relative to dechorionated controls, the filled bars indicate the percentage of those normal embryos that are transgenic, and the solid lines indicate the percentage of expected cells expressing the transgene. In other words, a measure of mosaic transgene expression. A variety of transgenic embryos may be generated using the adjustable electroporator. In general, electroporating with increased voltage and DNA concentration generates more transgenic embryos with reduced levels of mosaic transgene expression. For example, when using the fixed device with 50 VDC input and 100 μg DNA, there is 80% normal embryonic development, nearly 100% of these embryos are transgenic, and the transgene is expressed in approximately 80% of the expected cells.

For example, the 1,000 μF \times 30 \Omega electroporator setting generated approximately 80% morphologically normal embryos in which 80% of these embryos express the Brachyury::GFP transgene. These embryos expressed the transgene mosaically, with approximately 60% of the expected cells expressing GFP. Settings of 3,000 μF \times 10 \Omega generated embryos that express the transgene in nearly all expected cells (10% mosaic expression), although the percentage of morphologically normal embryos is only 50%.

The next set of experiments measured the effect of voltage on the generation of transgenic embryos. For cells to become permeable by means of electroporation, the voltage potential developing across the cell membrane must exceed a threshold level during the applied pulse (Andreason and Evans, 1988; Shigekawa and Dower, 1988). To examine the effects of voltage on the generation of transgenic embryos, we set the electroporator to
1,000 µF and used a 30 Ω timing resistor. DNA concentration was adjusted to 60 µg per 800-µl reaction volume, and a cuvette with a 0.4 cm electrode gap was used. As can be seen in Figure 4B, increasing the voltage up to 50 VDC (125 V/cm across the cuvette electrodes) resulted in a higher percentage of morphologically normal embryos expressing the transgene. Electroporation of zygotes using voltages below 30 volts (75 V/cm) failed to generate transgenic embryos. This finding suggests that voltages less than 75 V/cm are below the threshold membrane breakdown voltage of ascidian eggs. We did not examine voltages greater than 125 V/cm.

The last parameter examined was the effect of adding increasing amounts of DNA during electroporation. The nonadjustable electroporator, fixed at 1,000 µF and 30 Ω, was used for these experiments. In a series of electroporations, the Brachyury-GFP transgene was used in increasing amounts to generate transgenic embryos. As shown in Figure 4C, as the concentration of DNA increased, the percentage of normally developing embryos decreased slightly, but the percentage of embryos expressing GFP increased to nearly 100%. At the highest concentration of DNA used (100 µg), 80% of the input zygotes developed into morphologically normal embryos, nearly all of which expressed GFP, and the level of mosaic expression was reduced to approximately 20%.

Although difficult to quantify, the time required to fertilize and dechorionate the eggs, as well as temperature fluctuations during this time, also affected the success of the electroporation procedure. We suspect that this finding correlates with the two phases of opalescent segregation that occur after ascidian eggs are fertilized (Satoh, 1994). In our experience, embryos produced from electroporations approximately 15–20 min postfertilization tend to develop more normally and express transgenes at higher percentages than embryos from electroporations that are performed much later (>25 min after fertilization) when the second phase of opalescent streaming is occurring. To minimize temperature fluctuations that may affect the electroporation parameters and embryonic development, all of our manipulations are performed at 18°C, either on cold plates or in water-cooled incubators. Manipulations performed at temperatures greater than 18°C will still take the same amount of time, however; embryonic development will be accelerated and electroporations will be performed when opalescent streaming is occurring. Because our embryos are grown at a constant temperature, we are able to compare timed embryonic development from experiment to experiment.

**Transgenes Are Expressed in a Lineage-Specific Manner**

In both ascidian and sea urchin embryos, transgenes are usually expressed mosaically and only a portion of the cells capable of expressing a given transgene actually express a given transgene. In sea urchin embryos, microinjected DNA is thought to accumulate in multiple extrachromosomal assemblages that are differentially incorporated into the genomes of early blastomeres during the first few cleavages (Hough-Evans et al., 1988). The level of mosaic expression in sea urchin embryos can be reduced or eliminated by microinjecting DNA constructs several times before first cleavage (Livant et al., 1991). This process presumably forms larger numbers of extrachromosomal aggregates, thus increasing the probability of a chromosomal integration event before first cleavage. To gain insight into the fate of electroporated DNA, we examined the expression of notochord-specific and muscle-specific transgenes with regard to the embryonic cell lineage.

The cell lineage of ascidian notochord and larval tail muscles is well documented (Nishida and Satoh, 1983, 1985; Nishida, 1987) and is depicted in Figure 5A. First cleavage in ascidian embryos occurs along the left–right plane of bilateral symmetry, so the lineage for only half of the embryo is shown. For simplicity, only the notochord and muscle cell lineages are detailed. Both the notochord and muscle tissues are composed of two different cell lineages, the primary (1st) and secondary (2nd) lineages. Cells in the anterior of the embryo are derived from the 1st lineages, whereas cells in the posterior of the embryo are derived from the 2nd lineages (Fig. 5B). In the notochord, the 32 primary notochord cells are derived from two pairs of founder cells that are born at the 64-cell stage (A7.3 and A7.7, Fig. 5A). These cells undergo three additional cell divisions to form the complete complement of 32 anterior notochord cells. The secondary lineage of the notochord is derived from a single pair of cells that are born at the 110-cell gastrula stage. These cells undergo two subsequent cell divisions to form the eight secondary notochord cells.

Ascidian embryos were electroporated with the Brachyury-GFP transgene using a variety of electroporator settings, and the resulting patterns of GFP expression were analyzed with regard to the notochord cell lineage. Some of these expression patterns were already presented in Figure 3, and many other patterns were also observed (data not shown). The mosaic expression of nearly all of these patterns is consistent with the electroporated DNA being assembled into aggregates that will become inherited by early blastomeres during the first few cleavages. For example, the 8-0 pattern indicates that 8 primary and no secondary notochord cells expressed the GFP transgene (Fig. 3A). The simplest explanation for this pattern is that a transgene aggregate became stably incorporated at the 16-cell stage in one of the A5.1 cells, or their A6.2 or A7.3 descendants (Fig. 5A). Alternatively, the aggregate could have become stably incorporated in either A7.7 cell of the 64-cell stage embryo (Fig. 5A). In either case, our observations are consistent with the formation of extrachromosomal arrays as has been demonstrated in C. elegans (Stinchcomb et al., 1985; Mello et al., 1991). The pattern 16-4 (Fig. 3A) could result from the stable expression of a single transgene aggregate in either cell of the two-cell stage as each half of the embryo contributes 16 primary and 4 secondary notochord cells (Fig. 5A). More complex expression patterns suggest that more than one transgene aggregate became stably expressed. For example, the pattern 4-4 suggests that transgene aggregates become stably expressed at the 110-cell stage in one of the four primary notochord cells (A8.5, A8.6, A8.13, or A8.14) and in one of the secondary notochord cells.
(B8.6). The pattern 8-4 suggests stable incorporation into a primary notochord lineage cell at the 64-cell stage (A7.3 or A7.7) and into one secondary notochord lineage cell at the 110-cell stage (B8.6). In over 100 embryos examined, the mosaic pattern of transgene expression could be explained by the differential incorporation of one or more DNA aggregates into blastomeres up to the 110-cell gastrula stage.

In two cases, we observed embryos with an odd number of notochord cells expressing the GFP transgene. The simplest explanation for this pattern is that the transgene aggregate was stably incorporated during an early cleavage but was subsequently lost in a small number of notochord cells later in development. A less likely explanation requires that multiple aggregates become stabilized in many different cells at different times during development. Although we rarely saw embryos in which an odd number of notochord cells expressed GFP, these observations did suggest that selective loss of transgene aggregates could be responsible for generating some of the mosaic expression patterns we observed. In the experiments described above, we were unable to distinguish an expression pattern that resulted from selective loss of DNA aggregates versus a pattern that resulted from the inheritance of DNA aggregates by multiple blastomeres. We reasoned that we would be able to distinguish between these two possibilities by examining the expression patterns of two different transgenes coelectroporated into the same fertilized egg.

**Coelectroporated Transgenes Are Always Expressed in the Same Cells**

We analyzed the expression pattern of two different Brachyury transgenes that were coelectroporated into fertilized zygotes. Each transgene contained the exact same 3.5-kb Brachyury cis-regulatory domain as in our previous experiments; however, two different reporter molecules, GFP and DsRed, were expressed. In this experiment, equal amounts of each transgene were coelectroporated into fertilized, dechorionated eggs and the resulting morphologically normal embryos scored for GFP and DsRed expression (Fig. 6A–F). We observed the same types of mosaic transgene expression patterns as reported in Figure 3A in which the Brachyury::GFP transgene alone was electroporated into eggs (data not shown). In over 95% of these transgenic embryos (98/103), GFP and DsRed expression was observed in exactly the same cells, no matter what mosaic expression pattern was observed. If DNA aggregates are forming during early cleavages, both transgenes are likely to become part of the aggregates, and selective loss of an aggregate would be expected to alter the expression of the two transgenes equally.

We decided to use transgene coelectroporation to re-examine some of the more complicated expression patterns we observed in the Brachyury::GFP experiments. We reasoned that, by analyzing the expression of two different transgenes expressed in independent cell-lineages (muscle and notochord), we would be able to more easily detect if selective loss of transgene aggregates from early blastomeres occurred. Like the notochord cells, the muscle cells are divided into a primary and secondary lineage (Fig. 5A,B). The 14 cells of the primary lineage are derived from B7.5 (2 cells), B7.4 (8 cells), and B7.8 (4 cells). The four cells of the secondary lineage are derived from A8.16 (2 cells) and B8.17 (2 cells). Because the first embryonic
Fig. 6.
cleavage occurs along the plane of bilaterally symmetric, muscle cells from the right side of the embryo remain on the right side of the larva, and vice versa (Nishida and Satoh, 1983, 1985; Nishida, 1987). The examination of muscle transgene expression, therefore, can be used to determine whether transgene aggregates are inherited by blastomeres on the right or left side of the embryo. Unlike the muscle lineage, both the primary and secondary notochord lineages are formed from a mixture of right and left embryo derivatives. The primary notochord and secondary muscle cell lineages are both derived from the A4.1 cell of the eight-cell stage (Fig. 5A). Transgene aggregates inherited in this cell, therefore, should be expressed in both lineages in the resulting embryos. Similarly, the secondary notochord and primary muscle lineages are both derived from the B4.1 cell of the eight-cell stage (Fig. 5A). If the transgene aggregates become incorporated in this cell, then both cell lineages should express the transgenes.

Equal amounts of the Brachury::RFP and HrM::GFP transgenes (20–50 μg each in a series of experiments) were coelectroporated into fertilized eggs, and the resulting embryos were scored for fluorescent protein expression (Fig. 6C–U). The first example embryo from this experiment (Fig. 6G–I) expressed the muscle::GFP transgene in all of the primitive muscle cells along one side of the embryo (Fig. 6G) and the notochord::RFP transgenes in 50% of the secondary notochord cells (Fig. 6H). Similarly, all of the primary notochord cells expressed red fluorescent protein (RFP), and all of the right and left A-lineage derived secondary muscles expressed GFP. The inheritance of two DNA aggregates during early cleavage, one into one cell of the two-cell embryo and the other into the A4.1 cell from the opposite side of the embryo, can explain this mosaic expression pattern. The embryo shown in Figure 6J–L expressed both transgenes in 50% of each cell type in both primary and secondary lineages. Because all of the muscle cells on one side of the embryo expressed HrM::GFP, a DNA aggregate was probably inherited by one cell at the two-cell stage. In Figure 6M–O, the embryo is expressing the transgenes in 50% of all notochord cells and 50% of all muscle cells, however, the primary and secondary GFP expressing muscle cells are present on opposite sides of this embryo. This expression pattern may be explained if one B4.1 cell and the opposite A4.1 cell inherited the transgenes at the eight-cell stage. The embryo shown in Figure 6P–R displays an even more complicated expression pattern and is an example where selective loss of transgenes has likely occurred. In both primary lineages, 100% of the cells expressed the transgenes, whereas in both secondary lineages, 50% of 2nd notochord cells and 50% of the A-lineage derived 2nd muscle cells expressed the transgenes. The simplest interpretation of this pattern is that transgene aggregates were selectively lost in one A7.8 cell (2nd muscle), one B8.6 cell (2nd notochord), and the B8.17 cells (2nd muscle). The alternative explanation is that half of the two-cell embryo inherited the transgenes and that additional DNA aggregates were inherited by the following cells, on the opposite side of the embryo, during early development: A5.1 (or derivatives) plus A7.7, B7.4, and B5.2. The occurrence of these events seems much less probable than the selective loss of DNA by several blastomeres of the early embryo. Lastly, the embryo shown in Figure 6S–U shows transgene expression in the right and left halves of the 1st muscle lineage, although the B7.4 derivatives are missing. In addition, the entire A4.1-derived 2nd muscle cells expressed GFP. Approximately 75% of the 1st notochord cells and all of the 2nd notochord cells expressed GFP. In this embryo, transgene aggregates were probably lost in both B7.4 cells and in one of the A7.3 or A7.7 cells of the 64-cell embryo. Thus, we find that most transgene expression patterns result from the incorporation of DNA aggregates into one or more early blastomeres. Complicated patterns of transgene expression are likely due to the selective loss of these aggregates from small numbers of cells within particular cell lineages. These findings are consistent with observations in C. elegans in which the size of extrachromosomal arrays affects their stability (Mello et al., 1991).

DISCUSSION

A key development in the use of ascidians to study gene regulation was the implementation of an efficient procedure for generating transgenic embryos (recently reviewed by Di Gregorio and Levine, 2002; Satoh, 2003; Satoh et al., 2005; Zeller, 2004). Although transgenic embryos, for several ascidian species, can be produced by traditional micromanipulation methods, this process is tedious and inefficient. In addition, microinjection methods are simply not practical for generating large numbers of transgenic Ciona intestinalis—the most commonly used ascidian species in developmental studies. Initially, transgenic ascidian embryos expressing GFP or lacZ-based reporter genes were generated...
to identify and examine tissue-specific transcriptional enhancers for several genes, including Brachyury (Corbo et al., 1997b), FoxA2 (Corbo et al., 1997a), Snail (Erives et al., 1998), and a tropomyosin-like gene (Di Gregorio and Levine, 1999). In addition to creating transgenic embryos expressing reporter genes, the electroporation method has also been used to mis-express regulatory molecules in embryos producing “mutant” phenotypes that have become invaluable for dissecting apart gene regulatory networks. The first use of transgenes to create mutant embryos was designed to express the Brachyury transcription factor under the control of the FoxA promoter (Takahashi et al., 1998). The resulting “mutant” embryos contained a large number of cells, most likely entoderm cells that became translocated to notochord cells. The ability to generate these “notochord cell-enriched” embryos led to the identification of a significant number of notochord-expressed Brachyury target genes (Hotta et al., 1999, 2000; Takahashi et al., 1999). Additional experiments by other laboratories have demonstrated that embryonic phenotypes may also be produced by the ectopic expression of transmembrane receptors (Corbo et al., 1998) and dishevelled (Keys et al., 2002).

Our development of an electroporator, designed specifically for use with marine embryos, provides several significant improvements for generating transgenic ascidian embryos over commercially available electroporator devices. Our electroporators incorporate low-value timing resistors, in parallel to the cuvette, designed specifically for generating transgenic marine embryos. In commercial devices, the resistance of the electroporator circuit will vary depending on the exact amounts of seawater, eggs, DNA, and mannitol solutions placed in the cuvette. Because our new designs use timing resistors, the resistance of the DNA/egg/seawater solution is much larger than the timing resistor, so the total resistance in this electrical circuit is approximately equal to the timing resistor; thus, slight variations in the compositions of the solution are minimized. To our knowledge, commercially available machines are not equipped with equivalent timing resistor values. Because we have minimized the variation in our electroporator, pulse conditions are extremely consistent from experiment to experiment as demonstrated in Figure 2. Our electroporator is inexpensive and easy to build and should allow many laboratories without commercial electroporation units to create transgenic ascidians.

In addition to designing and building an electroporator designed for use with marine embryos, we systematically optimized the electroporation parameters with the goal of obtaining large numbers of morphologically normal transgenic embryos while minimizing the level of mosaic transgene expression. Our results show that, by varying the applied voltage, the amount of DNA used and the electroporator settings, we could produce transgenic ascidian embryos in which (1) 80–90% of the input eggs produced morphologically normal tadpole larvae, (2) nearly 100% of these larvae expressed the GFP reporter gene, and (3) mosaic expression was minimized such that, in a typical experiment, the transgene was correctly expressed in over 80% of the expected cells. Although difficult to quantify, the time required to fertilize and to dechorionate eggs also plays an important role in producing transgenic embryos. By using temperature control throughout all aspects of the procedure, embryo survival and transgene expression is increased. Because our electroporator produces consistent pulses and we have optimized the remaining parameters, we can routinely produce transgenic embryos with predictable patterns of transgene expression.

We performed several types of experiments to demonstrate the usefulness of generating embryos with predictable patterns of transgene expression. First, we examined the expression of a notochord-specific transgene with regard to the embryonic cell lineage. We demonstrated that the expression pattern of the Brachyury:GFP reporter gene follows the expected expression pattern predicted from the well-documented notochord cell lineage (Nishida and Satoh, 1983, 1985; Nishida, 1987), consistent with the original observations of Brachyury expression (Corbo et al., 1997b). Second, we demonstrated that, when the two different Brachyury transgenes are coelectroporated, both transgenes are expressed in exactly the same cells in > 95% of the resulting embryos. It is unclear currently if transgenes become integrated early into the embryonic genome like in sea urchin embryos (Flytzanis et al., 1985), if the transgenes recombine into large extrachromosomal arrays like in C. elegans as a result of microinjection (Stinchcomb et al., 1985; Mello et al., 1991), or if they integrate at low copy numbers as in C. elegans when using microparticle bombardment (Praitis et al., 2001). Electroporation may be used to generate stable transgenic ascidians (Matsumoto et al., 2000) and some of the predicted DNA aggregates, thus, are integrating into the genomes of a small number of embryos.

To investigate when transgenes become stably “expressed” in the ascidian embryo, we coelectroporated the Brachyury:RFP transgene together with the muscle-specific HrM:GFP transgene and monitored fluorescent protein expression. Both muscle and notochord cells share early cell lineages, and because coelectroporated transgenes are always expressed in the same cells, we expected to see different patterns of GFP and RFP expression that would be indicative of when these transgenes became stably expressed. Our observations are consistent with transgenes becoming stably “expressed” in one or more blastomeres sometime during the first few cleavages and it is likely that there is also selective loss of transgenes from different blastomeres as embryogenesis proceeds. Our ability to predictably generate transgenic ascidian embryos becomes extremely useful and important when the expressed transgenes are expected to produce an embryonic phenotype. Depending on the outcome desired, embryos could be produced with a range of transgene-induced different phenotypes or embryos could be produced in which maximal transgene expression is obtained.

In summary, we have demonstrated that our electroporator has several significant improvements over commercially available machines. We have optimized the electroporation parameters and demonstrated that mosaic transgene expression follows expected cell lineage patterns. Our ability to generate transgenic embryos with
predictable patterns of transgene expression will prove extremely useful in future experiments. Although we and others have shown that electroporation can create transgenic embryos from a variety of ascidian species, attempts to use electroporation with Halocynthia roretzi, a Japanese ascidian, have been unsuccessful thus far (cited in Di Gregorio and Levine, 2002). All of the ascidians in which electroporation has been successful produce eggs of approximately the same diameter ~150 μm. We suspect, based on our optimization experiments, that the large size of Halocynthia eggs (~400 μm) will require significant modification of electroporation parameters, including the dimensions of the cuvette and applied voltage. Similarly, marine invertebrates with much smaller diameter eggs, such as sea urchin embryos (~80 μm), will require different procedural optimizations. With properly optimized conditions, our electroporator design should be valuable for generating transgenic embryos from a wide variety of marine invertebrates.

EXPERIMENTAL PROCEDURES

Construction of Electroporators

The electroporators used in this study were specifically designed for creating transgenic ascidian embryos using low direct current (DC) voltages. These devices may be assembled by anyone familiar with simple soldering techniques, and design schematics for both devices are shown in Figure 1. Electronic components were purchased from Digi-key Corporation ( Thief River Falls, MN), Newark InOne (Chicago, IL) and local electronic component vendors. The adjustable device (Fig. 1A) has an internal power supply and is best suited for general electroporation studies. The fixed device (Fig. 1B) requires an external power supply, such as a standard laboratory gel electrophoresis power supply, and is useful for outputting a consistent, nonadjustable pulse. In our laboratory, this electroporator consistently produces embryos that express transgenes with approximately 50% mosaic expression. Cuvette holders, constructed from pieces of 1-cm-thick acrylic, were fitted with copper electrodes and connected to the pulse output of the electroporator. Time constants for electroporator pulse discharges were measured using a Hitachi Model VC-6025 storage oscilloscope.

Embryo Culturing

Adult ascidians were collected in various marinas located in San Diego County, CA. (Ciona intestinalis, C. savignyi, Styela plicata) or in Woods Hole, MA (Ascidia aspera) and were maintained in either a closed, recirculating sea water system or in a sea table under constant lighting to induce gamete production. Gametes from two or more individuals were surgically removed and mixed, and fertilization was allowed to commence for 2 min at 18°C. Sperm was washed away from the eggs by passing the eggs through several changes of filtered sea water, and the embryos were cultured at 18°C on cooled copper plates (Whittaker, 1973).

Electroporation Procedure

A detailed electroporation and culturing protocol, slightly modified from the original procedure (Corbo et al., 1997b), is published elsewhere (Zeller, 2004). A brief description of this procedure is as follows. Gelatin-coated dishes are used throughout all stages of the dechorionation procedure, which is performed at 18°C. After fertilization, eggs were dechorionated by incubation in a solution of 1% thioglycolic acid (sodium salt, Sigma) and 0.05% protease (Type XIV, Sigma) in pH ~9.5 seawater for 5–7 min. After the chorions were removed, the eggs were washed through 4 changes of 0.45 μM filtered seawater. Approximately 300 μl of seawater/eggs were added to a microfuge tube containing plasmid DNA (25–100 μg) in 500 μl 0.77 M mannitol. After briefly mixing with a glass Pasteur pipet, the egg/DNA mixture was transferred to an electroporation cuvette (0.4 cm electrode gap) and electroporated. Electroporated eggs were removed from the cuvette and gently spread across the bottom of a gelatin-coated 60-mm dish. Transgenic embryos were reared in 0.45 μM filtered sea water at 18°C containing 0.1mM ethylenediaminetetraacetic acid and antibiotics (10 U penicillin and 10 μg of streptomycin per ml of sea water).

Transgene Constructs

The Brachyury transgenes used in this study were constructed using the pSP72-1.27 parent vector and include approximately 3.5 kb of cis-regulatory domain and the first two amino acids of the Brachyury coding sequence (Corbo et al., 1997b). A GFP variant based on the native Aequa sequence (Zeller et al., in press) was fused in-frame with the 3.5-kb Brachyury cis-regulatory domain to create the GFP reporter (Brachyury::GFP). The same cis-regulatory domain was also fused in-frame with the coding sequence for native DsRed (Clontech) to make the RFP transgene (Brachyury::RFP). The Halocynthia roretzi muscle actin–GFP reporter (BrM::GFP), used to express GFP in muscle cells has been described elsewhere (Corbo et al., 1997b). Fluorescent protein expression was observed on a Zeiss Axiosplan 2e Imaging microscope equipped with a Nikon Coolpix 995 digital camera.

ACKNOWLEDGMENTS

We thank Dr. Greg Harris for providing access to the oscilloscope used in these studies and Dr. Constantine Tsoukas for use of the Bio-Rad Gene Pulser I. M.J.V. is a recipient of a Minority Biomedical Research Support Scholarship from the National Institutes of Health (NIH/NIAMS SDSA MBRS Program 5R25GM58907). R.W.Z. was funded by San Diego State University, the San Diego State University Foundation, and a CSUPERB Faculty Seed Grant.

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