Introduction

Primary Goal of Thesis

The goal of my PhD thesis was to gain a greater understanding of the cell movements that shape the early development of the vertebrate body plan and to work out the molecular regulation of these cell movements. I was particularly interested in determining the structure of information flow in biological systems. I did not simply want to discover how one gene or signal controlled one particular cell movement, but rather to gain insight into how information is encoded and executed during early morphogenesis. Towards this end, I focused my efforts on studying two classical systems for studying early cell movements: the avian neural crest and the frog axial mesoderm during gastrulation. The avian neural crest provides a model for the migration of individual cells through a developing tissue and the axial mesoderm provides a model for the coordinated movement of a tightly connected epithelial sheet of cells.

Structure of Thesis

The opening chapter of the thesis will discuss the goals of my research, the reasons for selecting each model system, the methodology employed, the major techniques developed, and the major biological conclusions resulting from each aspect of my work. It will continue with a more philosophical reflection on why certain types of experiments provided more biological insights than others and conclude with an outline of my future plans for related research.

Cell Movements

I choose to work on the molecular control of cell movements for several reasons; first among them being a simple fascination with the degree of autonomy and information processing power that even these seemingly simple systems possess. It is simply fascinating to watch a cell explore its environment, interact with its neighbors, and shape an embryo. On a more practical level, cell movements are an important thing to study as they are the primary means by which the body plan of early vertebrate embryos is established. A very early embryo does not look like a miniature adult, it looks like a ball of cells. That ball of cells needs to quickly and precisely execute a dramatic series of cell movements to build the basic shape of the organism. Recent large-scale efforts (e.g., the Human Genome Project) have been tremendously successful in enumerating the components of biological systems: the challenge is now to understand how the components interact within dynamic systems. Finally, now is an opportune time to study the nature and molecular control of cell movements because the confluence of fluorescent labeling techniques (e.g., green fluorescent protein, GFP) and commercial laser scanning microscopes makes it possible to begin to follow these events, as they happen, in cells, tissues, and embryos [1].

Choice of Model Systems

The avian neural crest was selected as a model system for cell migration because of the rich embryological literature on its origins, induction, and

migration pathways (reviewed in [2-5]). It is also an experimentally tractable system as the premigratory neural crest is readily accessible and easily labeled via dye injection, electroporation [6, 7], and viral infection [8]. Additionally, as I was interested in studying cell migration in a vertebrate model system, the neural crest was a good choice as it is a shared derived characteristic of vertebrate embryos, and is believed to be evolutionarily critical to the diversification and success of the vertebrates [4, 9]. Finally, there was a strong history at Caltech and in the Fraser Lab of studying the neural crest, and significant progress had recently been made in understanding the control of neural crest cell migration [10-12].

The frog axial mesoderm was selected as a model for epithelial sheet movements for the rich literature describing its cell movements, recently reviewed in [13-15]. Additionally, the frog *Xenopus laevis* is experimentally convenient as it is routine to produce large numbers of embryos, the embryos develop approximately synchronously, and the tissues of the frog are readily amenable to explant culture. Critically for the success of our experiments, the explanted dorsal marginal zone of a gastrulating frog appears to execute its normal convergent extension cell movements autonomously, even when isolated from the rest of the embryo [16].

Methodology

The basic approach that I took was to develop imaging techniques to visualize cellular movements, document the normal biology of the system, then perturb

some potentially important molecular signal and observe the consequences. One aspect of developmental biology that I find intellectually satisfying and experimentally demanding is the necessity to consider dynamics and information flow across many length and timescales. Molecular recognition and signaling can take place on any timescale from nanoseconds through minutes. Cell behavior has observable dynamics from milliseconds through days. The development of the organism can take days, but the body plan, at least in amphibians, is established in the first few hours. It is very difficult *a priori* to determine on which timescales a given process is occurring. Even if a given range of cell behavior persists for only a few minutes, such as the formation of bottle cells in early gastrulation in the frog, it is quite difficult to determine when the crucial information exchange occurred and committed the organism to that decision.

The manner that I chose to address this challenge was to attempt to build cell behavioral assays capable of analyzing cell movements across several length and timescales. Rather than choose to study cell migration over hours and read out a single cellular decision at a handful of fixed time points, I attempted to experimentally observe and manipulate neural crest cell migration and frog gastrulation through a linked series of assays ranging from very short-term cell behavior through consequences of cell behavior read out over hours, through the long-term effects of molecular perturbations on avian and amphibian development over many hours through days.

I had originally hoped to study the role of Eph/ephrin signaling on neural crest migration in a mechanistic manner from its immediate early effects on cytoskeletal and membrane dynamics *in vitro* through a characterization of the intermediate cell migration decisions made by trunk neural crest cells aberrantly misexpressing ephrin ligands as they migrated through the somite, through the visualization of the entire migratory neural crest population and its derivatives in whole embryos over time as a function of ephrin disruptions.

Similarly, with the frog axial mesoderm, we understood that the coordination of motility within the axial mesoderm would require long-range, fast communication and hypothesized that calcium signaling would be ideally suited to these requirements. We therefore examined short timescale calcium fluctuations with fluorescent techniques, examined the consequences of disrupted calcium signaling in explanted tissues and whole embryos, and systematically analyzed the normal events of gastrulation in whole embryos using novel microscopic techniques developed for the purpose.

Technical Accomplishments

Overview

My thesis work concentrated on the development and application of fluorescent microscopic techniques for the purpose of visualizing cell movements *in vitro* and *in vivo* and the consequences in whole embryos of disrupting these cell

movements. During the course of my thesis work, I developed new methodology in three areas: microscopy and optics, culture and imaging of cell and embryos, and assay development. The first half of each experimental paradigm within this thesis was to visualize the normal behavior of the system, using some type of microscopic imaging technique. This description provided the means to understand what to expect as the normal state(s) of the system. To make progress in understanding the regulation of the cell movements, it is necessary to interfere with the normal molecular signaling processes that might govern the cell movements. The most technically advanced, and risky, aspects of my thesis dealt with developing systematic ways to challenge cell behavior. I present the major technical highlights from each of these sections below, and the details are presented in the relevant body chapters of the thesis.

Microscopy

Each of the chapters in this thesis focuses on visualizing some aspect of embryonic development with a light microscope. Over the course of my thesis, I visualized membrane and cytoskeletal dynamics with green fluorescent protein (GFP) fusions for the neural crest cultures (Chapter 2), visualized nuclear and mitochondrial organelle dynamics with neural crest and mouse fibroblast cultures (Chapter 2 and Appendix 1, respectively), visualized intercellular calcium dynamics within explants of gastrulating frog tissue (Chapter 4), and visualized whole embryos at subcellular resolution to study the relationship between cell polarity and tissue and embryo structure (Chapters 5 and 6). Most of this work

involved learning and applying fairly standard high performance microscopic techniques. Two of these projects required developing more original methods.

As described in detail in Chapter 2, we needed to visualize cytoskeletal dynamics within neural crest cells with relatively high temporal resolution (i.e., several scans per minute) over relatively long periods (i.e., several to many hours). The essential difficulty is that fluorescent labels are readily destroyed in the process of imaging. We made two changes that made successful imaging possible. First, we tested a broad range of Zeiss objective lenses to determine which gave the best light throughput for confocal imaging. Beyond the obvious increase in imaging performance from high numerical aperture objectives, we observed a significant benefit to using relatively simple, less optically corrected lenses, such as the Zeiss Fluar 20x lens. The more significant change was to design a custom set of filters and dichroics from Chroma Corporation. We designed and Chroma fabricated several competing types of primary dichroics and emission filters. After careful testing we selected the best components and integrated them into our Zeiss 310, 410, and Pascal confocal microscopes. For my neural crest cytoskeletal imaging experiments I observed a tenfold increase in sensitivity on the Zeiss 410 confocal. I was able to use this increased sensitivity to image at lower laser intensities and thereby dramatically reduce photobleaching. For many experiments, we were able to observe such a dramatic reduction in photobleaching that it became essentially undetectable. We interpreted this to mean that there is an experimentally achievable threshold within cells below

which there is effectively no photobleaching and above which damage is constantly and cumulatively being done.

The other main microscopic innovation in this thesis is the application of surface imaging microscopy (SIM) to early embryonic samples. Early in graduate school it became clear that there was a level of anatomy and three dimensional context that was critically necessary to understand neural crest cell migration and frog gastrulation that was inaccessible by traditional confocal microscopic means. In collaboration with Resolution Sciences Corporation, we adapted an existing microscopic techniques, surface imaging microscopy, to enable high resolution high contrast imaging of large thick biological specimens. The results of this collaboration are reported in Chapters 5 and 6. We had originally hoped to use SIM to visualize the entire population of migratory neural crest cells in normal chick embryos and embryos with different disruptions in Eph signaling, using fluorescent antibodies or GFP. We were unable to reliably detect these specific probes with SIM though and so our focus shifted to visualizing the frog gastrula.

Culture and Labeling of Cells

A perquisite of any imaging-based assay is the ability to successfully reproduce some aspect of the sample's normal physiology or development on the microscope stage. This effort typically takes two parts: introducing contrast to enable visualization of the structure or process of interest, then culturing that sample on the microscope stage. As I will discuss at further length in Chapter 2, I refined culture methods for quail neural tube explants to reliably produce

disperse cultures of healthy cells in serum free media. I then tested a variety of GFP fusion proteins for their ability to label structures within primary neural crest cells, specifically the actin cytoskeleton, the nucleus, and the plasma membrane and identified good labels for each. Finally, it was necessary to build imaging chambers for culturing neural crest cells on custom fabricated glass substrates both in conventional incubators and on the microscope stage. Examples of the most useful fusion proteins and culture chambers will be presented in Chapter 2.

This knowledge of labeling and culture techniques laid the technical foundation for the imaging of calcium waves in frog explants (Chapter 4) and mitochondrial structure and dynamics (Appendix 1). Though we were studying very different underlying biological processes the technical aspects of the experiments were quite similar.

Assay Development

The core of my efforts to understand the molecular regulation of cell movements was to observe cell behavior, challenge that cell behavior with a molecular perturbation, then observe the resulting effects of the perturbation. This represents a very standard experimental design. We were somewhat more innovative in the choice of molecular perturbations. For the neural crest cell migration experiments I developed two fundamentally different assays for challenging migrating neural crest cells in vitro. The first involved the creation of patterns of proteins on glass substrates. By observing neural crest cells

interacting with fixed spatial patterns of proteins on glass substrates we wanted to document the kinetics of the cell behavioral response to a repulsive boundary, in a way that was similar to the repulsive boundary these cells encountered within the caudal half of the somite during normal migration. The second assay was more specifically focused on the timing of the normal response of a neural crest cell to ephrin-B ligand. It involved coating glass beads with ephrin ligand and then presenting those beads to migrating neural crest cells using optical tweezers. We bound the proteins tightly to the beads so that the stimulus would not begin until the cell was in contact with the bead; the bead was not a diffusing source of protein. Using this assay, we documented the timing of the response of the beads, compared the effect of single beads to clusters of beads, compared the effect of presentation to different regions of the cell, and compared the effects of presenting the beads to isolated cells or to cells in contact with other neural crest cells.

For the frog gastrulation project, I developed labeling approaches to visualize calcium dynamics in living frog dorsal marginal zone explants, then we imaged the calcium dynamics and associated cell movements in the presence and absence of pharmaceutical inhibitors of calcium release. Once it was clear that there was a requirement for calcium signaling in gastrulation, I began applying surface imaging microscopy to systematically documenting the events of frog gastrulation and to visualizing the polarity of cells within different tissues as a function of developmental stage and Disheveled signaling activity.

Biological Conclusions

Neural Crest Cell Migration

The dynamics of neural crest cells migrating alone on patterned glass substrates was simply too variable to enable strong conclusions about the effect of ephrin signaling upon them. Some cells were repulsed by contact with ephrin-B regions and some seemed to prefer it; many did not appear to notice the difference. A more detailed discussion of the reasons for this are presented in Chapter 2.

More insight into neural crest cell migration was gained by focal application of ephrin coated beads (Chapter 3). Since the protein was bound strongly to the beads, I was able to very tightly control the timing of neural crest exposure to ephrin stimulus. The typical response to ephrin coated bead is a local collapse of lamellapodia, not filopodia, and an associated global increase in protrusive activity. This response is fast (1-5 minutes), implying that it can occur without recourse to transcription or translation. This response is also in sharp contrast to that observed in previous studies [10, 12], as previous authors reported a 20-40 minute delay in response, followed by a global collapse of all cellular projections. The nature of the reaction I observe is a reorganization of the polarity of the cell, accomplished by local collapse of lamellapodia, but leaving retraction fibers, and a globally increased protrusive activity. Interestingly, cells remain sensitive to multiple stimulations over a short (i.e., 1 minute) timescale. We observed

reductions in the strength of the cellular reaction to an ephrin stimulation when the cell in question was in contact with neighboring cells, as well as weaker reactions when the bead was presented to the lateral surface of the cell rather than its leading edge. Finally contact with multiple beads elicited a more severe reaction from cells than did a single bead. All of the noted differences were significant when compared to beads coated in Protein-G instead. The background level of protrusive cellular activity was high though, complicating the analysis, as detailed further in Chapter 3.

Frog Gastrulation

In Chapter 5, I present the results of our efforts to understand the mechanisms by which large numbers of cells coordinate their motility during gastrulation. We hypothesized that calcium waves could provide a means for cells in the dorsal mesoderm to coordinate their cell movements during gastrulation. We looked for and observed long-distance, intercellular propagating waves of calcium within the dorsal mesoderm. These waves were typical in the dorsal mesoderm, and specific to it. We did not observe waves in explants of animal cap ectoderm or ventral mesoderm. Inhibition of calcium release, using the drug thapsigargin, prevented elongation of dorsal marginal zone explants, and blocked the elongation of the prospective notochord domain within whole embryos. This effect appeared to be a direct effect on morphogenesis, as the fate specification of dorsal marginal zone explants treated with thapsigargin remained normal, as assayed by molecular marker analysis.

Based on this increased knowledge of the molecular control of gastrulation cell movements, as well as parallel work done on the role of Wnt signaling in the dorsal mesoderm [17], I decided to take a more systematic approach to the evaluation of phenotypes within frog embryos. Since the frog embryo is more than a millimeter thick and highly opaque at gastrula stages, confocal and two photon microscopy are unable to observe deep events within the living embryo. To gain a deeper understanding of the structure of the *Xenopus* gastrula, and the relative phenotypic consequences of different disruptions of gastrulation, we used a novel form of fluorescence microscopy, surface imaging microscopy (SIM), as discussed in Chapter 6. We imaged a full developmental time series of *Xenopus* development, from blastula through early neurula stages. To take best advantage of these digital datasets, we have developed computational methods to normalize each of these embryos to a common set of coordinates, based on the blastocoel, blastopore, and the dorsal midline. We then extracted surfaces from these volumes and documented the characteristic events of gastrulation: tissue separation, archenteron elongation, and mesendoderm migration, and blastopore closure. We have also begun to compare and contrast these processes in normal embryos and those in which the dorsal mesoderm overexpresses a mutant form of the Disheveled protein, Xdd1 [17, 18].

Conclusions and Way Forward

Relative O/I Merits of Different Approaches

As I have reflected on the relative information content of different categories of experiments, several ideas have emerged. The underlying theme to all my biologically informative experiments is that the (sub)cellular behavior that I was interested in understanding was autonomously present in the explanted sample on which I performed my experiments. My job as an experimentalist was simply to keep the cell or tissue alive and healthy and it would then perform its characteristic behavior, whether this was a calcium wave propagating through a dorsal marginal zone explant, or mitochondrial fusion and transport in a fibroblast, or large scale morphogenetic movements in the frog gastrula. The key to these experiments is that I could reproducibly prepare samples that would exhibit similar behaviors, and differences could be inferred to be chiefly due to natural variation.

Once the natural variability was understood, I could then perturb some aspect of the molecular machinery, and attribute any observed differences in cell behavior to effects of the perturbation. The more biophysical approaches sought to create the conditions under which the neural crest cells would respond uniformly to inherently artificial stimuli, such as photolithographed patterns of ephrin protein. I failed to appreciate at the time that there were very few truly autonomous cell behaviors of a neural crest cell in culture, and as such it is difficult to know *a priori* what to expect a neural crest cell to do in response to an artificial stimulus.

The only autonomous, repeatable cell behaviors that I observed in my neural crest cultures were delamination from the neural tube and outgrowth from the neural tube. Even these proved highly variable once I looked carefully at the details. I was seeking to build an assay with single cell predictability, where there would be, at least statistically, a consistent observable cellular response to my imposed ephrin stimulation. As a consequence, all of my neural crest cell migration guidance experiments had a high threshold to surpass before they could begin to produce biologically relevant information. First I needed to successfully engineer the physical and chemical conditions for presentation of the molecular guidance cue. In each case I succeeded in doing this, with several different substrate pattern generation techniques (described in detail in Chapter 2), as well as focal presentation of beads using optical tweezers (Chapter 3). The problem is that in each case this was merely the prelude to the experiment. I couldn't begin to do developmental biology in any of these paradigms, until I established that the neural crest cells had a highly reproducible reaction to our artificial stimulus. They didn't.

A reproducible reaction implies two separate things. First, it implies that the background level of cell behavior in the absence of intentionally imposed stimulus is low. This is not the case. Neural crest cells, left to their own devices, are incredibly dynamic and are in a constant state of extension and retraction, sampling their environment and making contact with other cells.

Second it implies that the cells will behave similarly to each other, from dish to dish, and day to day. Again with neural crest cells this is not the case. There is considerable variation in cell size, cell shape, and degree of activity. This makes it very difficult to interpret whether a given difference in cell reaction is due to a constant reaction to a varying stimulus or to varying reactions to the same stimulus. Once the cell being studied is in contact with another neural crest cell it is nearly impossible to parse apart what part of its action is due to the cell and what due to the substrate or bead. In my substrate experiments, I observed a strong community effect, where cells in extensive contact with each other rarely observed the imposed boundaries, but isolated cells could respond more readily. In the optical tweezers experiments the bead similarly evoked a less dramatic reaction in cells with extensive cell-cell contact.

Normal Neural Crest Cells

The cells that were most interpretable in both substrate and tweezers experiments were isolated highly polarized cells. They are also highly unusual and highly unstable cells. Neural crest cells seem to have a strong affinity for each other even in culture. Pioneer cells far from the main outgrowth are quite uncommon. Recent *in vivo* time-lapse data [19-21] implies strongly that the functional migration unit for a neural crest cell is not a single neural crest cell, but rather that they typically migrate in chains or small groups of cells.

Simplest Functional Unit

I am still a strong believer in reductionist science. I don't believe that all genes or proteins or cells are equivalently important to a given process. I still believe that the most direct way to test if a given component is necessary is to pharmaceutically break that component or genetically remove it and then see if the process proceeds normally. I would now place more emphasis on the functional part of the phrase 'simplest functional unit' than I did at the onset of graduate school. It is experimentally convenient to have a simpler system. It is necessary that the system still retains the essential features of its *in vivo* behavior.

By focusing so completely on experimentally imposed migration guidance choices, I was, in effect, using neural crest cells as expensive, hard to manipulate, and short lived fibroblasts. To take best advantage of their properties as a migratory system I would have needed to carry more of the system into culture, as has recently been done [22, 23] to study neural crest cell migration and muscle cell precursor migration. A better starting point for studying neural crest cell migration would be the trunk explant [24] or the intact embryo. The imaging and culture approaches that I developed for examining neural crest cell migration *in vitro* would be best applied to the analysis of a phenotype that has already been observed *in vivo*, rather than an assay for critical migration components, that all then need to be tested for *in vivo* relevance.

Therein lies the strength of the frog experiments that I have finished. By starting with the dorsal marginal zone explant, we were able to observe cellular calcium release events and correlate those with cellular movements, link those movements and their regulation to the behavior of the tissue as a whole and then demonstrate the necessity of calcium signaling for the successful completion of gastrulation. Similarly, by employing high resolution imaging techniques such as SIM to study the effects of Disheveled disruptions that we already know are important to the development of the organism [17, 25], we gain deeper insights into the details of a process that we already know is important.

Future Directions

I plan to continue the work described in Chapter 6 to explore the coupling of different morphogenetic processes during frog gastrulation. Inhibition of Dsh signaling in the dorsal mesoderm blocks convergent extension cell movements, but our preliminary data using SIM analysis suggests that the elongation of the developing gut, the archenteron, is fairly normal. I will be measuring the degree of coupling of blastopore closure to archenteron elongation, and dorsal mesodermal convergent extension, as a function of disruptions in calcium release and Wnt signaling.

My Role in Collaborative Projects

Much of my thesis work was done in close collaboration with a group of very talented and generous scientists. I owe them my deepest gratitude for assisting in my thinking and guiding different aspects of my experiments. For simplicity, I will acknowledge each project independently.

Confocal Optimization:

I worked with Michael Stanley of Chroma Corporation and Scott Fraser on the design of the components and Fritz Rohweder of Carl Zeiss Corporation in testing these dichroic mirrors and interference filters for the purpose of optimizing our Zeiss 410 laser scanning confocal microscope for imaging green fluorescent protein.

Substrate Patterning:

This project originated from previous work of Rusty Lansford in the Fraser Lab, and began under his mentorship. I had useful conversations with Grant Walkup regarding silane monolayers. I consulted with Michael Roukes, and was trained by members of his lab in the use of equipment for substrate fabrication. I am also indebted to Linda Griffith, of MIT, and Chris Chen and Srivatsan Raghavan, of Johns Hopkins, for valuable discussions of alternate patterning methodologies and for donating reagents.

Surface Imaging Microscopy:

The idea of applying surface imaging microscopy to imaging early embryos arose from discussions between myself and Russell Kerschmann, of Resolution Sciences Corporation and he donated instrument time at the company to the project. Helen McBride assisted in developing histological techniques for preserving early embryos through the processing steps. Mark Reddington, of Resolution Sciences Corporation, carried out the imaging at the company, on samples that I prepared and analyzed.

Frog Calcium:

This project emerged from discussions between myself, John Wallingford, Scott Fraser, and Richard Harland. The origins of the project predated my involvement, but I was involved in planning and carrying out the experiments from the first. I was chiefly responsible for developing labeling and imaging techniques to visualize the calcium dynamics. John did all of the microsurgical manipulations and in situ hybridizations. I was involved in the design and analysis of all of the experiments.

Digital Analysis of Frog Gastrulation

I originally developed the surface imaging microscopy approaches to visualize the avian neural crest in intact embryos. After many trials it became clear that SIM was extremely good at imaging bright small molecule dyes, and inefficient at imaging dim specific labels such as antibodies or green fluorescent protein. This

dichotomy emerged shortly after finishing the frog calcium project and I realized that the technique would be very well suited to imaging intact frog embryos and comparing normal embryos to experimentally altered embryos. I prepared the normal embryos and Mark Reddington imaged them at Resolution Sciences Corporation. To take best advantage of the full range of information present in these digital embryos I collaborated with J. Michael Tyszka to develop MatLab programs to digitally segment the embryos, and with John Wallingford to prepare embryos expressing mutant forms of the Disheveled protein. John Wallingford has also played a key advisory role in the project.

Mitochondrial Fusion:

One unexpected consequence of the confocal microscopy optimizations was that we had developed a strong capacity to visualize many fluorescent protein fusion proteins with little or no photobleaching. One of the tests that established this was done on a mitochondrial targeted yellow fluorescent protein. Through a stroke of serendipity a new professor, David Chan, arrived at Caltech shortly thereafter who was very actively studying the genetics of mitochondrial fusion, but lacked a means to visualize it. The culture, labeling, and imaging techniques that I had developed to examine neural crest cells proved ideally suited to visualizing mitochondrial fusion and so I collaborated with David and members of his lab to characterize the morphology and dynamics of mouse embryo fibroblasts from a variety of genetic backgrounds. This project is outside of the main theme of my thesis and is reported in Appendix 1.

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