CHARACTERIZATION OF ADULT STEM CELL MIGRATION IN THE PLANARIAN *SCHMIDTEA MEDITERRANEA*

by

Otto Charles Guedelhoefer IV

A dissertation submitted to the faculty of
The University of Utah
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Neurobiology and Anatomy

University of Utah

August 2011

The University of Utah Graduate School

STATEMENT OF DISSERTATION APPROVAL

The dissertation of **Otto Charles Guedelhoefer IV**

has been approved by the following supervisory committee members:

Alejandro Sánchez Alvarado	, Chair	**5/25/2011** Date Approved
Charles Murtaugh	, Member	**5/25/2011** Date Approved
Shannon Odelberg	, Member	**5/25/2011** Date Approved
Gerald Spangrude	, Member	**5/25/2011** Date Approved
Monica Vetter	, Member	**5/25/2011** Date Approved

and by **Monica Vetter**, Chair of the Department of **Neurobiology and Anatomy**

and by Charles A. Wight, Dean of The Graduate School.

ABSTRACT

The migration of stem cells is a fundamental aspect of developmental biology that is directly relevant to regenerative medicine. Specifically, the signals that direct migratory stem cells to the necessary location could be used therapeutically to recruit transplanted or endogenous stem cells. Additionally, given the similarities between stem cell mobilization and cancer cell metastasis, the factors that induce mobilization may provide new anti-metastasis drug targets. We have learned much about the factors involved in adult stem cell migration from vertebrates; however, given the deep anatomical positions of most vertebrate stem cell populations, observing migration has proven difficult. Thus there remain many unanswered questions as to how stem cells migrate in response to both tissue homeostasis and regeneration that must be investigated in a simpler, more accessible system.

We established the planaria, *Schmidtea mediterranea* as a model system for studying stem cell migration, which required the development of methodology for observing planarian stem cell movements *in vivo*. Therefore we evaluated tissue transplantation, partial irradiation, and cell transplantation for this purpose. After modification, we found tissue transplantation, the gold standard assay for stem cell function in other systems, and partial irradiation, an underused method for local stem cell ablation, to be effective methods for interrogating stem cell behaviors.

Using these methods, we found that stem cell migration in planaria is a dynamic process. Although they constantly produce migratory progeny, planarian stem cells are nonmigratory during tissue homeostasis and require wounding to become mobile. Transplanted stem cells are capable of rescuing lethally irradiated hosts and migratory stem cells are collectively totipotent, similar to the rest of the stem cell population. We characterized the repopulation of the stem cell compartment during rescue and evaluated the movement of the transplanted stem cells in relationship to their progeny. Finally, multiple wounding events showed how stem cells integrate multiple wound signals during stem cell recruitment.

The work presented here establishes planaria as a model system for studying stem cell migration and defines two useful assays for investigating stem cell function and behavior. This work will inform future studies aimed at discovering stem cell mobilization and recruitment factors.

For Toby and Quin

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENTS

I first need to thank Dr. Andy Fire, without whose suggestion I would have never considered heading out to Utah to study flatworms. In fact, without his guidance and encouragement I may not have gone to graduate school at all.

Secondly, I owe a large debt to Alejandro, for taking me into his lab, setting me up with a fascinating project, setting me loose, and setting me straight when I veered off course. I feel very lucky to have a mentor that cares equally for my happiness inside the lab and out. His admiration for scientists of centuries past and his passion for cutting edge discovery are truly inspiring.

I am also very grateful to all of the members of the Sánchez Laboratory both past and present, but especially those that gave me a particularly hard time – namely Bret, Jason, Jochen, and Kyle, for thickening my scientific skin. Thanks to Carrie for being my sounding board. Jim and Yvonne for taking care of all of all the stuff no one else would do, and Alessandro for always knowing just when I needed a coffee. Additionally I appreciate Diane for the endless supplies of big fat planaria for transplantation and plenty of laughs.

My project would not have been possible if not for the groundwork laid down by George Eisenhoffer. George has blazed a trail for me in more ways than one and has always been there to offer advice or at least condolences. If imitation is the highest form of flattery, then I guess I'm his biggest fan.

My thesis committee, Charlie, Jerry, Shannon, and Monica, are incredibly helpful, understanding, and supportive. I'm thankful that their fingerprints are all over the work presented in this dissertation. Their input helped shape not only my research but my development as a scientist. That goes for other members of the Neurobiology and Anatomy Department as well, especially Rich Dorsky and Chi-Bin Chen who are both great teachers in and out of the classroom.

I'm also grateful for my classmates who went through the trenches with me. We had a lot of fun too. Thanks to JP and Derrick for introducing me to all things Utah. I greatly appreciated the expertly guided tours of Alta, Moab, Zion, Havasupai, the Unitas, and many other places. To all the other school and non-school friends who I'm not going to attempt to name in my sleep deprived state – thank you for the good times and here's to more to come.

I want to also thank my parents for not calling me crazy (at least not to my face) when I left a good job in Baltimore to move out to Utah and go back to school. I have always felt their support. I'm also extremely appreciative of my three wonderful sisters, Heidi, Laura, and Carla who all helped out in their own ways, whether offering emergency Adobe Illustrator help, driving the support vehicle in Durango, or sending me a regular supply of Kona coffee and Macadamia nuts.

Finally, Rachel, my wife who wrangled our twin infant boys while I was engrossed in putting many years of research into writing— She's an amazing woman who does too many things for me to name, but most of all she gives me reason to smile everyday even when the skies are dark and stormy.

CHAPTER 1

OVERVIEW

Introduction

The great majority of study within the field of developmental biology has focused on embryonic development. Indeed, the process through which a single fertilized egg forms into a multicellular embryo is fascinating and has taught us much about the regulation of cell proliferation, differentiation, and morphogenesis. Relatively little attention, however, has been paid to the processes that maintain form and function of an organism once it has reached its adult shape. These processes are tissue homeostasis, the maintenance of tissue during normal turnover, and regeneration, the repair of damaged or diseased tissue.

Humans shed and replenish approximately 50 to 70 billion cells every day which over the course of a year equates to losing and replacing a mass of cells roughly equivalent to our bodyweights (Reed, 1999). To further complicate this feat, the cells of our various tissues turn over at very different rates: on the range of days to years in most tissues (Pellettieri and Sánchez Alvarado, 2007) and some that may never turn over, lasting chronologically as long as we do (Spalding et al., 2005). Therefore, tissues made up of differently renewing cell types or two connected tissues that turnover at different rates must constantly integrate recently born cells with the existing structures. All of this must be carefully orchestrated such that the individual tissues and the organism as a whole do not dramatically change morphology on a day to day basis. We know that these processes take place but we know very little about how they are accomplished.

Regeneration is the more dramatic cousin of tissue homeostasis. Whereas tissue homeostasis replenishes dying cells in a slow and nearly invisible way, regeneration replaces whole portions of the animal often quickly, rebuilding new tissue out from

where there was none only days before. The problem of integration of old and new tissue also exists during the process of regeneration, but is localized to the wound site. Also, in a manner no less impressive than tissue homeostasis, during regeneration massive numbers of cells are often produced and differentiated into brand new tissues that, at times, eventually become larger than the preexisting tissue from which they were derived (Kellogg, 1904).

The processes of tissue homeostasis and regeneration have a few very important similarities and differences. Conceptually, they are most different in how the processes are initiated. Whereas cells lost to tissue turnover die at predictable rates and possibly even predictable locations, the cells lost to injury cannot be predicted. Thus regeneration inherently requires signaling from the wound to initiate the process whereas tissue homeostasis may not require a similar initiation signal. The two processes are most similar in that in many cases they both utilize adult stem cell populations to accomplish their impressive charge. In fact, most cycling tissues that have been thoroughly investigated have been found to contain a required resident stem cell population (Raff, 2003). This is true for most regenerative tissues, with the possible exception of salamander limb regeneration, which instead may dedifferentiate its existing cells to a stem-cell-like state before regeneration can take place (Kragl et al., 2009). Therefore, by better understanding the different manners in which stem cells function during both tissue homeostasis and regeneration we can hope to define signals that stem cells use to respond differently to everyday tissue loss and sudden debilitating injury.

This chapter will serve to describe the current state of adult stem cell biology as it pertains to regeneration and tissue homeostasis. Additionally, it will provide a brief

summary of adult stem cell migration and an explanation of why it is an important event in regeneration. Furthermore, I will explain our rationale for studying the processes of stem cell migration in planaria while providing background on planaria as a model system for developmental biology. Finally, the historical context of planarian stem cell migration will be covered in order to orient the reader to what is known and what theories have been presented by previous researchers in the field.

Adult Stem Cells

Stem cells by definition are any cell that can both self-renew and differentiate into one or more cell types (Alonso and Fuchs, 2003; Raff, 2003). Adult stem cells are therefore any cell that fulfills those criteria while existing within a differentiated tissue of an adult organism, and they are then named for their tissue of origin. This rather loose definition has led to the identification of many adult stem cell populations in mammalian tissues including bone marrow, epidermis, hair follicle, intestine, brain, and many more (Raff, 2003). Although they all fit the criteria to be called adult stem cells, cells from these disparate populations often have very different characteristics. For instance, the hematopoietic stem cells (HSCs) of the bone marrow are largely quiescent, only rarely activating to produce progeny (Cheng et al., 2000), whereas intestinal stem cells rapidly proliferate to keep up with the 5 day turnover time of the intestinal epithelia (Blanpain et al., 2007). Additionally, some adult stem cells are hugely multipotent, for instance HSCs which produce over 10 cell types; whereas others, like muscle satellite cells or neural stem cells, produce relatively few types of differentiated progeny (Tajbakhsh, 2003; Temple, 2001). Notwithstanding the huge amount of information we have gained from

the mammalian stem cell systems, there is nonetheless an obvious need for a simpler model system for studying adult stem cells (Sánchez Alvarado, 2004).

As previously discussed, the processes of tissue homeostasis and regeneration are inherently linked by their use of stem cells, however within a given tissue the ability to regenerate is not necessarily predictive of the ability to maintain tissue homeostasis (Rando, 2006). For instance, in mammals, muscle tissue is readily regenerated following injury, but muscle satellite cells are largely quiescent during muscle tissue homeostasis (Collins et al., 2005). If muscle satellite cells can produce the necessary progeny during regeneration, why then do they not function during tissue homeostasis? It would appear that the deficiency lies not within the stem cell population, but in the signaling events that accompany tissue homeostasis and wounding. These processes, especially as they pertain to both tissue homeostasis and regeneration in a given stem cell population, remain poorly understood.

Stem Cell Migration

Besides the required events of self-renewal and multipotent differentiation, stem cells perform many other processes that are inherently necessary for tissue homeostasis and regeneration. Because adult stem cells normally reside within a niche, migration out of the niche (mobilization) before differentiating is required to maintain the stem cell compartment. Migration of stem cells serves other purposes besides simply moving stem cells out of the niche before they differentiate. In two murine epithelial structures, the lining of the stomach and the epidermis surrounding the hair follicle, stem cell migration is required to move stem cell progeny to sites of tissue turnover or regeneration

respectively, where they are needed to replace missing cells or structures (Barker et al., 2010). Also, although why the process takes place remains unknown, HSCs appear to occasionally leave the bone marrow, enter into the blood stream, and reenter the bone marrow under normal homeostatic conditions (Abkowitz et al., 2003; Mendez-Ferrer et al., 2008). Whether this process is necessary for normal function of HSCs remains under debate, nonetheless without the preprogrammed homing capabilities of HSCs, bone marrow transplantations, as they are routinely done today, may not have been possible (Lapidot et al., 2005).

Migration appears to be a common characteristic of not only adult stem cells but embryonic stem cells as well (Laird et al., 2008). Any persistent movement, either singly or as a group, of cells in the early embryo that self-renew and eventually differentiate into various cell types is by definition a stem cell migration, even though many of these stem cell populations do not persist in the adult. One beautiful example of this is the zebrafish lateral line primordium migration as it deposits neuromasts along the anterior-posterior axis of the fish (Aman and Piotrowski, 2008). As this group of undifferentiated cells moves not only do they self-renew to maintain their numbers in the primordium, but also differentiate and deposit structures composed of multiple cell types (Aman and Piotrowski, 2008).

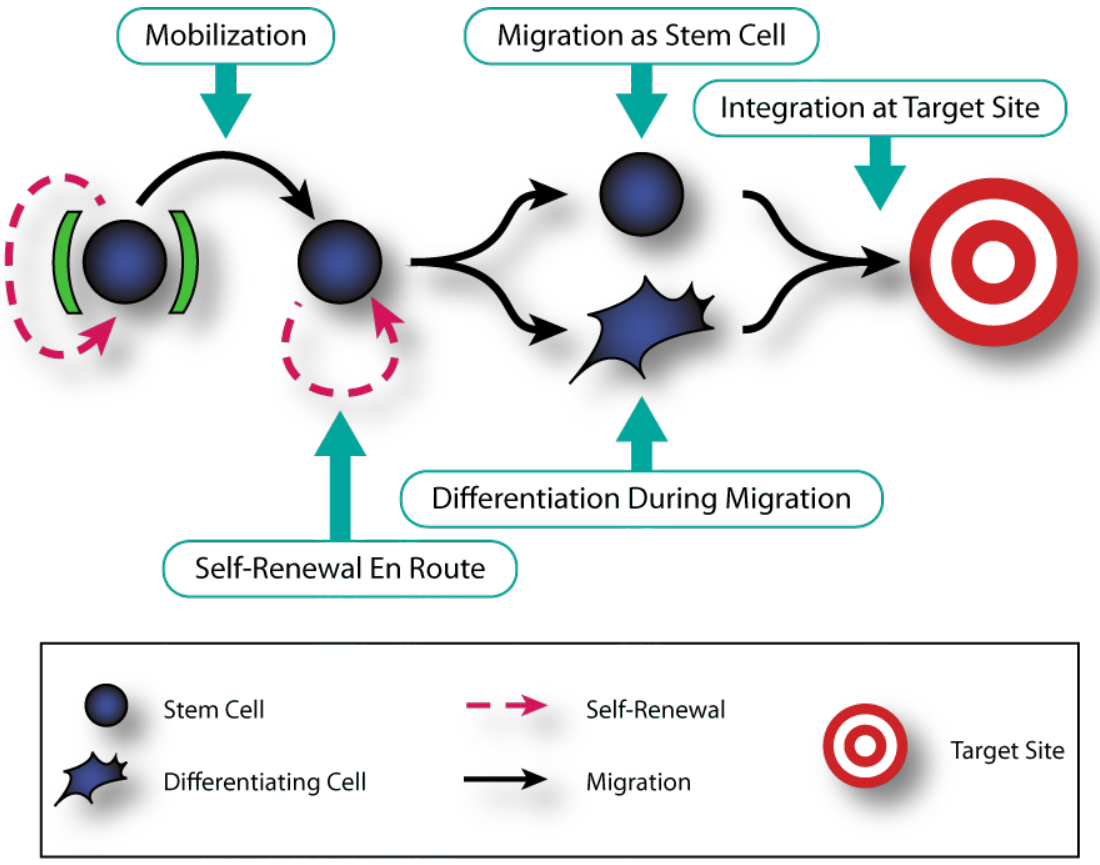
These kinds of clear and well studied embryonic stem cell migrations do not exist for adult stem cell populations, largely because many of the best studied relevant adult stem cell populations are inaccessibly contained within the mammalian body plan. For instance, we know from studies using histological sectioning and genetic manipulations that the immediate progeny of intestinal stem cells are displaced from the niche but we

have been unable to observe this process *in vivo* to determine how this migration takes place (Barker et al., 2010). Similarly, we know that HSCs are capable of migrating into and out of the bone marrow both during repopulation following bone marrow transplantation and during normal homeostatic conditions (Abkowitz et al., 2003; Chute, 2006; Mendez-Ferrer et al., 2008), but we have been unable to observe the process *in vivo* without the use of highly technical and low resolution imaging techniques (Niemeyer et al., 2010).

Nonetheless, the hematopoietic system has been our best source of knowledge about factors that control adult stem cell migrations. HSC migration is largely dependent on chemokines, namely CXCR4 and CXCL12 (a.k.a. SDF-1), to control the mobilization of HSCs out of the bone marrow niche and capture of circulating HSCs out of the bloodstream during niche homing (Wright et al., 2002). Integrins and other adhesion molecules have been implicated in the migratory processes, but the chemokines still remain the most heavily studied key players (Mendez-Ferrer and Frenette, 2007; Wagers et al., 2002). Interestingly, the very same chemokine receptor-ligand pair also controls the embryonic migration of the zebrafish lateral line already described (Aman and Piotrowski, 2010). The CXCR4/CXCL12 axis appears to be a robust migration signaling scheme that has been co-opted many times throughout vertebrate evolution (Raz and Mahabaleshwar, 2009), and it therefore remains to be seen what molecules control stem cell migration in animals whose divergence predates the evolution of chemokines.

Regardless of the molecules controlling adult stem cell migration, there are a number of hypothetical events that migrating stem cells either must or could undergo (Fig. 1.1). Mobilization, the first of these events, has already been discussed and

Figure 1.1. Predicted Events in Stem Sell Migration. Most stem cells are believed to reside in a niche (green parentheses), where they self-renew. The initiating event of stem cell migration is mobilization out of the niche. Mobilization can be pharmacologically induced in some stem cell systems (Chen et al., 2006) and may be akin to the epithelial-to-mesenchymal transition that metastasizing cancer cells undergo (Tse and Kalluri, 2007). Once the stem cell is mobile, it may or may not self-renew. Some theories state that positioning within the niche is required for stem cell self-renewal (Morrison and Spradling, 2008), a hypothesis that can be tested in a stem cell migration system. As the stem cell migration continues, the cell may migrate as a stem cell, maintaining its stem cell characteristics and functions, or it may being to differentiate, losing its stem cell characteristics and taking on those of committed progeny. Regardless of whether the cell migrates as a stem cell or committed progeny, near the end of the migration the cell integrates into the target site which may be a tissue undergoing turnover or a regenerative blastema. These events outline processes that have either been previously observed or predicted in other stem cell systems and are presented here to illustrate the observable events that would be contained within a gold standard *in vivo* stem cell migration system.


Mobilization
Migration as Stem Cell
Integration at Target Site
Differentiation During Migration
Self-Renewal En Route
Stem Cell
Differentiating Cell
Self-Renewal
Migration
Target Site

generally refers to the stem cell leaving its niche environment. The second event, self-renewal en route, may or may not take place but, if observed, could help us understand whether stem cell self-renewal is extrinsically governed by the niche or intrinsically controlled by stem cells themselves regardless of their microenvironment (Leatherman and Dinardo, 2008; Wu et al., 2009). Next, the stem cell may migrate as a stem cell, retaining its stem cell characteristics like HSCs that are induced to circulate (Abkowitz et al., 2003; Mendez-Ferrer et al., 2008), or it may begin to differentiate as it migrates, giving support to the hypothesis that stem cell migrations are required for correct differentiation in some systems (Aguirre et al., 2010). The final event of an adult stem cell migration would be integration into the target tissue, whether the target is the position of a dying cell being lost to tissue turnover or a regeneration blastema being built prior to differentiating into a new structure. The ideal adult stem cell migration model system would allow observation and manipulation of all of these events *in vivo*.

The Planarian *Schmidtea mediterranea*

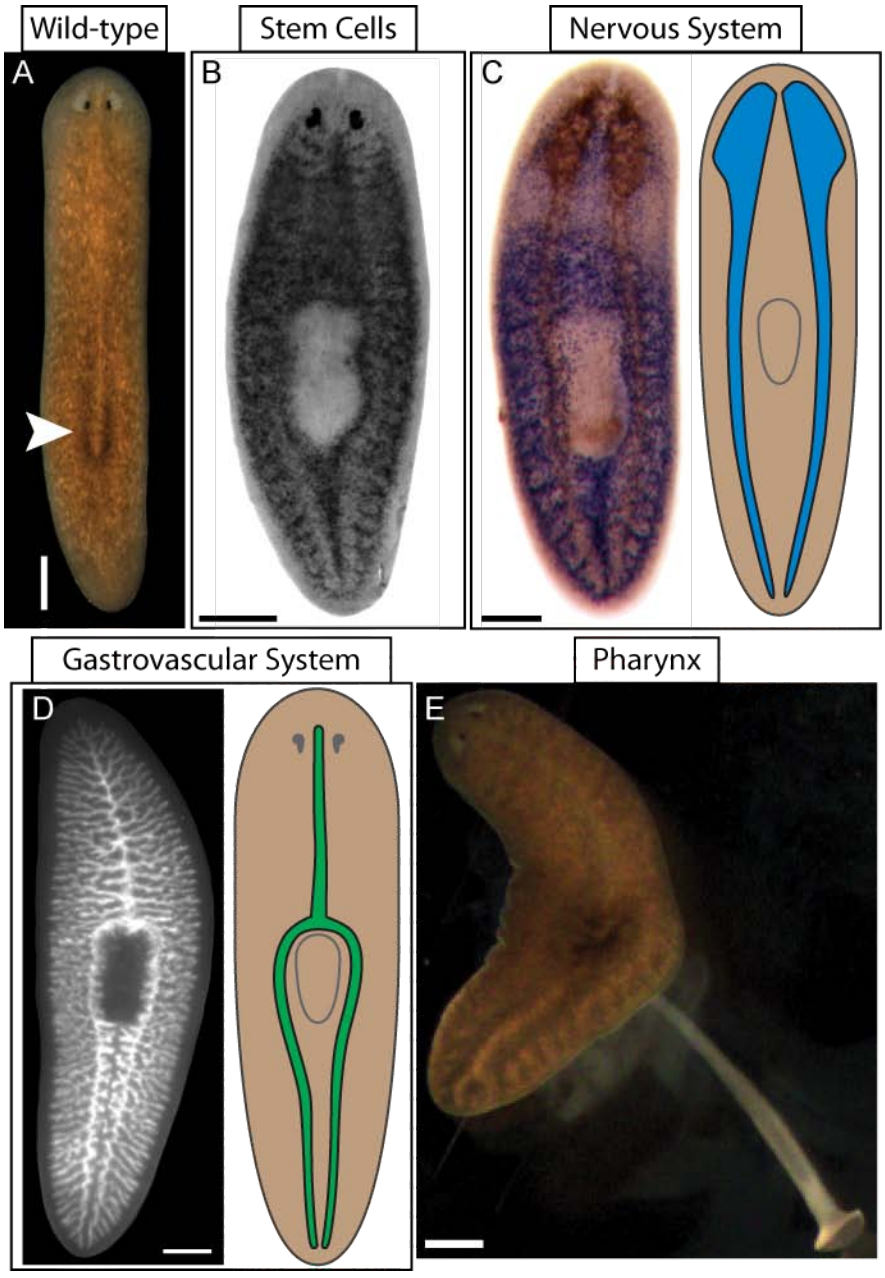
Planaria are primarily free-living flatworms that have successfully populated many freshwater, marine, and even terrestrial geographic niches throughout the planet, a testament to their adaptability (Beauchamp, 1932; Johns and Boag, 2003; Vila-Farré et al., 2010). The majority of developmental biological work has been performed on freshwater planaria due to their accessibility and rapid regeneration. As members of the platyhelminthes, planaria are now accepted to belong to the lophotrochozoa, an understudied group of organisms that may be less derived and more closely related to the last common ancestor of bilaterians than the more rigorously studied ecdysozoa

(Tessmar-Raible and Arendt, 2003). This favorable evolutionary placement increases the likelihood that molecular regulatory mechanisms uncovered in planaria will be directly comparable to those in vertebrates (Pearson and Sánchez Alvarado, 2008).

Planarian regeneration has been studied for over a century. Their ability to perform this fascinating phenomenon has been attributed a cell type called neoblasts, which has since been shown to be the stem cell population that is directly responsible for regeneration (Morgan, 1901; Newmark and Sánchez Alvarado, 2000; Randolph, 1892; Shibata et al., 1999). The asexual planaria body plan has an enticing mix of complexity and simplicity, they are triploblastic and contain a central nervous system yet they consist of only one body opening and are not outwardly segmented (Fig. 1.2) (Newmark and Sánchez Alvarado, 2002). Our chosen species, *Schmidtea mediterranea*, has a stable diploid genome and exists in two biotypes, asexual and sexual (Saló and Baguñà, 1985).

Planarian stem cells are distributed throughout the body plan with the notable exceptions of the area anterior to the photoreceptors and the pharynx proper (Fig. 1.2) (Reddien et al., 2005b), which are the only two structures that neither regenerate nor survive when separated from the rest of the animal (Morgan, 1900). The entire stem cell compartment appears to be continually mitotically active, indicating a lack of both a transit amplifying population and a quiescent subpopulation of stem cells (Newmark and Sánchez Alvarado, 2000). The constant mitotic state and high rate of turnover of planarian stem cells is further confirmed by the ability to ablate the entire population with one dose of ionizing radiation (Bardeen and Baetjer, 1904; Hayashi et al., 2006). Though the majority of cell lineages emanating from the stem cell population have yet to be worked out, a number of progeny have been identified (Eisenhoffer et al., 2008). These

Figure 1.2. Planarian Anatomy. (A) The asexual biotype planarian *Schmidtea mediterranea* showing outwardly observable anatomical features, namely the obvious photoreceptors (anterior is up) and the outline of the pharyngeal pouch (arrowhead). (B) The broadly distributed stem cell compartment can be identified by RNA whole-mount in situ hybridization (WISH) for any of a number of stem cell markers (in this case *Smed-piwi-1, shown in grayscale, dark = signal*). (C) The planarian nervous system consists of two anterior lobes and two nerve cords that run the longitudinal axis of the planaria (show in brown by WISH for *Smed-pc2* with *Smed-piwi-1* for reference in blue, and schematized on the right). (D) The highly branched planarian gastrovascular system can be easily visualized by feeding animals dye (here shown by feeding CellTracker Orange™ CMTMR (Invitrogen), fixing, and re-labeling with anti-tetramethylrhodamine), and consists of one main anterior branch and two posterior branches that bifurcate at the anterior extreme of the pharynx (green, schematic). (E) The pharynx (white protrusion, shown in a still image taken from a movie of a planaria feeding on brine shrimp extract), is a muscular tube and the only opening to the planarian gastrovascular system, thus it is responsible for food intake and expulsion of waste. The protonephridia system (not pictured, situated near the distal tips of the gastrovascular branches) is responsible for planarian osmoregulation. Scale bars are 500 μm.


Wild-type
Stem Cells
Nervous System
A
B
C
Gastrovascular System
Pharynx
D
E

progeny which reside in various locations throughout the animal and vary in their generational distance from the stem cells have proven very useful in defining the mechanism of stem cell function in subsequent studies (Pearson and Sánchez Alvarado, 2010; Wenemoser and Reddien, 2010).

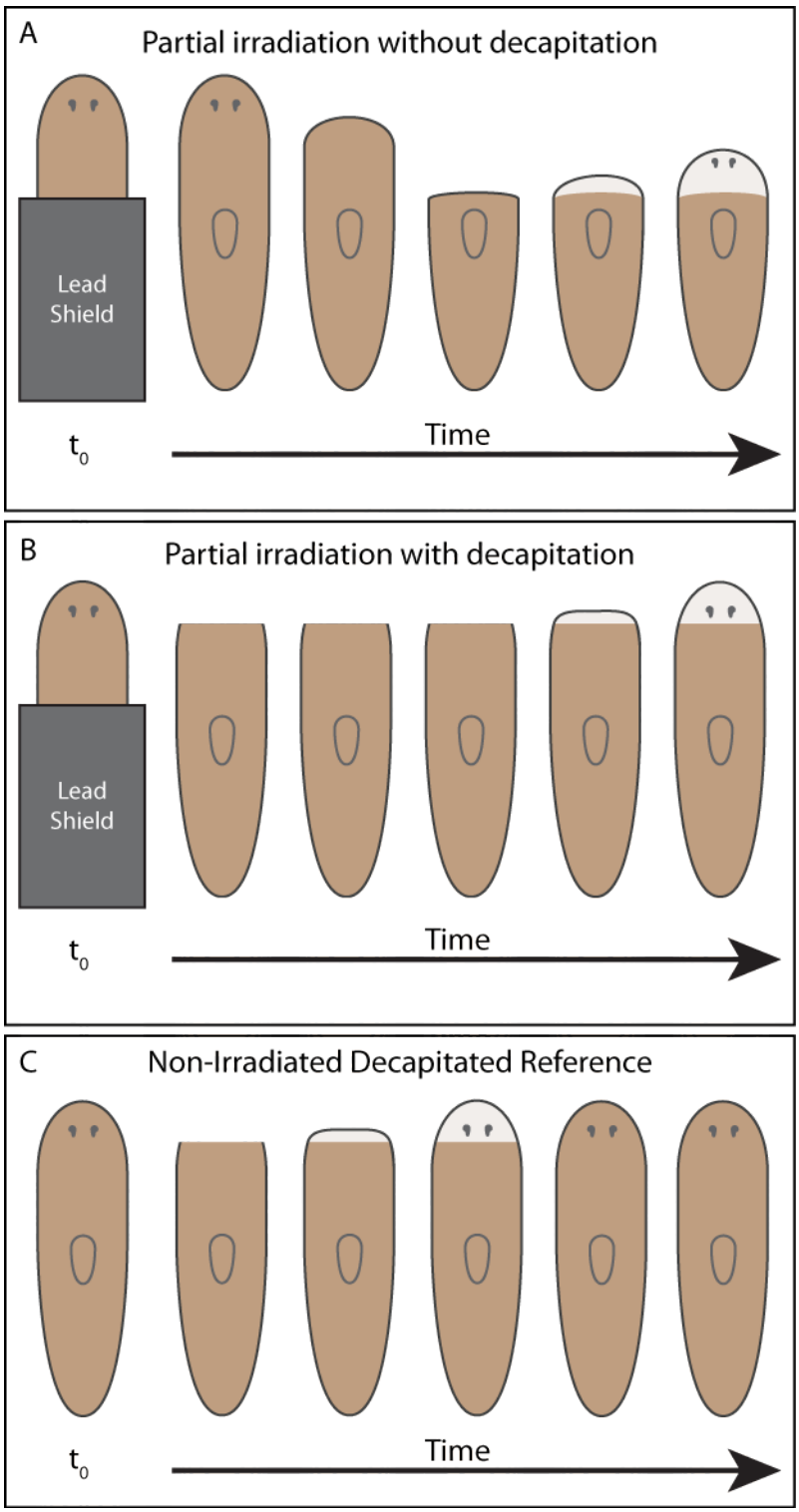
When amputated, planaria mount a rapid response consisting of two bursts of proliferation, one systemic and one local, followed by blastema formation and eventual differentiation of the blastema into the missing structures, all within about one week (Saló and Baguñà, 1984; Wenemoser and Reddien, 2010). During the regenerative response, existing tissue is also remodeled, through coordinated cell death (Pellettieri et al., 2010), such that the final anatomical scale and proportion of the animal is reestablished in both fragments regardless of where the animal was cut (Morgan, 1900). The ability of this massive response to happen equally quickly regardless of where the animal is cut likely depends heavily on the wide distribution of the stem cell population but may also require robust mechanisms for the recruitment of stem cells. The building of a blastema out from the body of the animal inherently requires migration of stem cell progeny into the newly forming structure, and indeed this has been observed (Newmark and Sánchez Alvarado, 2000). One might expect therefore that stem cells would also be recruited to the wound site to assist in producing progeny or replace differentiating stem cells during regeneration, as has been observed in other organisms (Abkowitz et al., 2003; Barker et al., 2010; Wilson et al., 2008). However, adult stem cell recruitment and migration has yet to be directly observed in planaria.

Historical Perspective on Neoblast Migration

The idea that some planarian cell type might be assisting in regeneration by migrating to wound sites is not new. In fact, in some of the first reported regenerations experiments performed in planaria, Morgan argued against the migration of cells from the old half into the regenerating half of longitudinally bisected animals, instead preferring the movement of "food substances" from the old tissue into the new tissue as the explanation for the corresponding decrease in size of the old half (Morgan, 1900). The migratory nature of the stem cell population has remained in debate since. A number of histological studies suggested that the stem cells had a migratory appearance, even going as far as to dub them "wanderzellen" or wandering cells (Steinmann, 1925; Stevens, 1901). However, besides their migratory appearance in sections, there were no data to support the conclusion that these cells were recruited to wound sites nor migrated at all.

The first functional test of this theory came by the means of partial irradiation studies, where the posterior halves of animals were shielded with lead and the anterior halves were irradiated (Dubois, 1949). Dubois observed regression of the anterior irradiated tissue in those animals that were left intact, but saw rescue of the irradiated tissue when the animals were amputated near the anterior end within the irradiated tissue (Fig. 1.3). Amazingly, the amputated partially irradiated animals regenerated although the rate of regeneration was delayed and the length of the delay was proportional to the length of the irradiated tissue (Fig. 1.3). This was interpreted to indicate that the stem cells were migrating from the shielded portion through the irradiated tissue, rescuing it along the way, to reach the wound and begin formation of the blastema (Dubois, 1949). Although indirect, these studies were the earliest best indication that planarian stem cells

Figure 1.3. Schematic of Dubois's Observations of Partially Irradiated Animals with or without Subsequent Amputation. (A) When the posterior half of planaria were shielded with lead prior to x-ray irradiation the anterior tissue was observed to regress back to the boundary between the irradiated and shielded tissue at which point an unpigmented blastema forms and the animal begins to regenerate. (B) On the other hand, when animals were decapitated following the same partial irradiation performed in (A), tissue regression was not observed and the remaining irradiated anterior tissue was rescued. The decapitated partially irradiated animals regenerated heads (B) however regeneration was significantly delayed as compared to unirradiated decapitated controls (C). Dubios found the length of the delay before regeneration to be directly proportional to the length of the remaining irradiated anterior tissue, and thus concluded that stem cells were migrating from the posterior shielded tissue to reach the amputation plane. (Dubois, 1949).

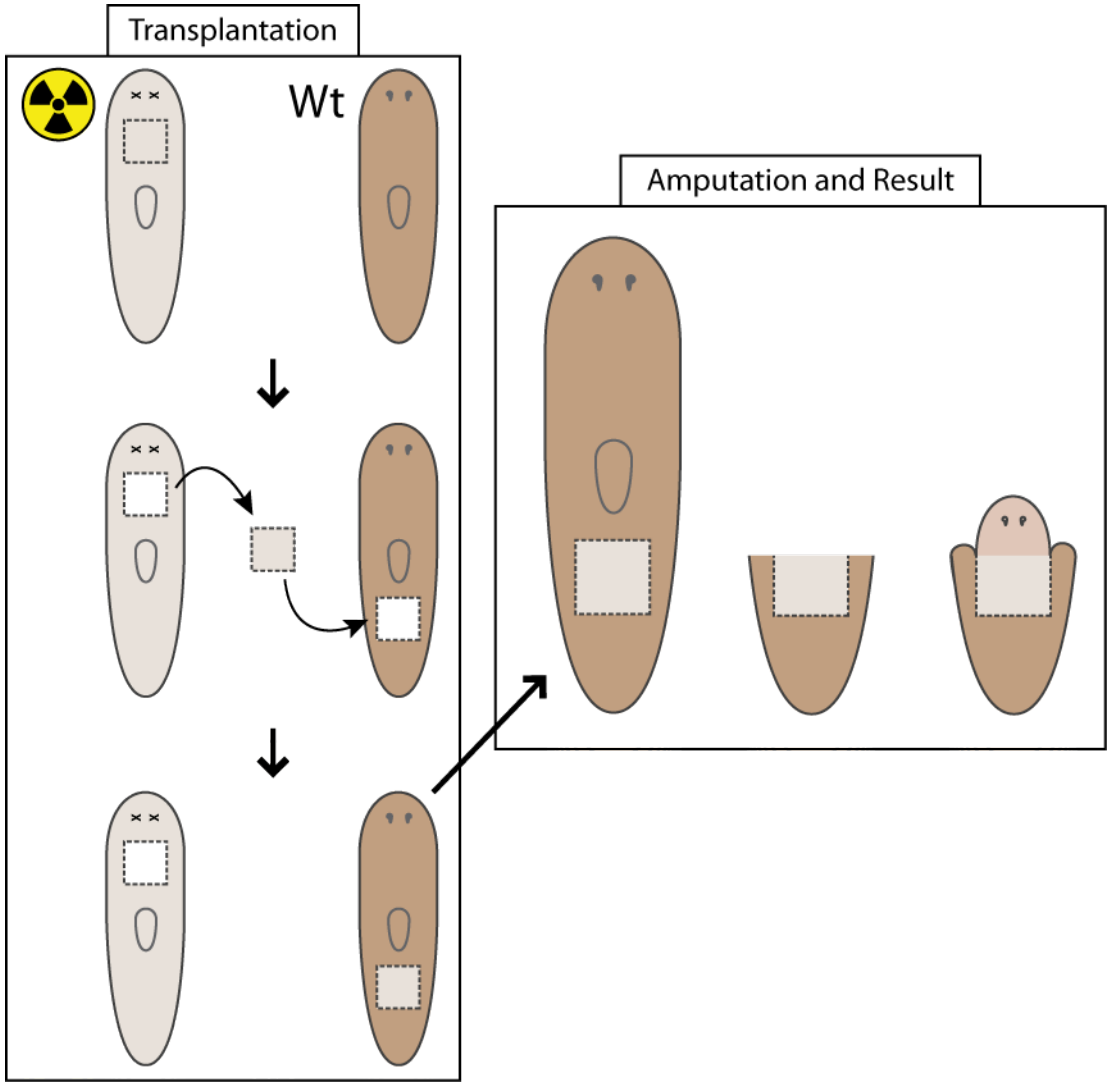

A
Partial irradiation without decapitation
Lead Shield
t_0
Time
B
Partial irradiation with decapitation
Lead Shield
t_0
Time
C
Non-Irradiated Decapitated Reference
t_0
Time

may actually be recruited to distant wounds.

Corresponding work supporting this conclusion was performed using *Dendrocoelum lacteum*, a planarian species incapable of head regeneration from any tissue posterior to its pharynx, in which an irradiated anterior graft was placed in the tail of a nonirradiated worm and a subsequent lateral amputation was performed through the graft and host tissue (Fig. 1.4) (Stéphan-Dubois, 1961). The isolated fragment regenerated a head from the amputation within the irradiated graft, suggesting that nonirradiated stem cells from the host tissue migrated into the irradiated tissue and anterior to the wound site (Fig. 1.4). Again these data are indirect evidence of the conclusion, but the migration of stem cells into the irradiated tissue is by far the most parsimonious explanation.

The migratory nature of planarian stem cells remained largely accepted by the field following Dubois' initial experiments (Brøndsted, 1969) until Flickinger, using ^{14}C-labeled grafts transplanted into a nonlabeled hosts, failed to detect graft derived cells in the blastema (Flickinger, 1964). Performing a similar transplantation experiment, but using chromosomal differences between different planarian biotypes for cell type markers, Saló concluded that the movement of the transplanted stem cells was due only to proliferation driven passive spreading and not active migration (Saló and Baguñà, 1985). On the other hand, the same group reported rescue of lethally irradiated planaria by focal cell injection, a process that would necessitate migration of the injected cells in order to repopulate the stem cell compartment of the host (Baguñà et al., 1989). Even more perplexing, again using cross-biotype transplantations and mitosis as a marker to investigate the relative regenerative contribution of stem cells near or far from the wound,

Figure 1.4. Schematic Representation of Dubois's Transplantation Experiments in *Dendrocoelum lacteum*. *D. lacteum* are incapable of regenerating heads from lateral amputations posterior to the pharynx. Therefore Dubois irradiated donor *D. lacteum* and transplanted irradiated anterior tissue into the posterior regions of wild-type *D. lacteum*. Once the transplantations were complete, Dubios amputated laterally through the graft and followed the tail section that now contained an amputated anterior piece of irradiated tissue within a nonirradiated tail. A head was observed to regenerate from the cut surface of the irradiated anterior tissue. Thus Dubois concluded that the stem cells present in the surrounding tail tissue migrated into the irradiated anterior tissue to allow the head to regenerate. Therefore, Dubios reasoned, the tail tissue provided the stem cells necessary for regeneration and the anterior tissue provided the signals necessary for head formation (Stéphan-Dubois, 1961).


Transplantation
Wt
Amputation and Result

Saló reported that cells near the wound "spread" more than cells far from the wound (Saló and Baguñà, 1989). Therefore, although Dubois's partial irradiation experiments had yet to be revisited, a mounting mass of evidence suggesting that planarian stem cells do not actively migrate was beginning to accumulate. From as far back as Morgan's initial observations of the source of blastemal tissue, many of the right experiments were performed to address the question of whether planarian stem cells migrate in response to wounding, however the lack of a reliable stem cell marker to directly evaluate cell position greatly handicapped the endeavor.

Research Summary

Building off the previous but conflicting work, we set out to answer the question of whether planarian stem cells migrate in response to wounding. This dissertation will therefore describe our development of tools necessary for studying stem cell migration in planaria and the subsequent characterization of adult stem cell migration and recruitment we performed with those tools. In Chapter 2 we describe our renewal of both tissue transplantation and partial irradiation techniques from the classical literature. The validation and optimization of these techniques is discussed along with the initial testing of the transplantation technique in conjunction with RNA interference.

Chapter 3 comprises the characterization of stem cell migration that was performed using the techniques described in Chapter 2 combined with molecular markers for following cell movements. Briefly, we found that planarian stem cells do not inherently migrate into irradiated tissue, but require a wounding event to induce migratory behaviors. Additionally, the process of repopulation of irradiated host tissues

was evaluated by following both the stem cell and stem cell progeny, and functionally testing of the potency of the repopulating stem cells. Finally, wounding was found to induce stem cell recruitment, or directed migration towards a target, while changing the relationships between stem cells and progeny. In Chapter 4 an attempt was made to reconcile our data with the previous work outlined in the introduction as well as to evaluate not only the significance of our findings for the greater stem cell biology field, but also the possibilities the newly developed techniques present for the future of planarian research.

.

CHAPTER 2

METHODS FOR INVESTIGATING CELL REPOPULATION AND MIGRATION IN THE PLANARIAN *SCHMIDTEA MEDITERRANEA*

Credits

This chapter consists of technical methodology for the improvement of planarian transplantation and partial irradiation techniques which in part, along with the accompanying protocol in Appendix A, will be submitted for video publication through the Journal of Visualized Experiments (www.jove.com). Both the transplantation and partial irradiation techniques are largely based off of procedural descriptions from the classical literature (Dubois, 1949; Santos, 1929). Alejandro and I conceived of all the technical advances and I alone performed all of the experiments with one exception. The initial testing of transplantation in conjunction with RNAi feeding was a collaboration with Dr. Jochen Rink, a postdoctoral fellow in our laboratory who identified and cloned the planarian *Sid-1* homologs.

The work described could not have been accomplished without the helpful input of both past and present members of the Sánchez Laboratory. Especially, Dr. Alessandro Rossi, whose experience from previous attempts at partial irradiation proved crucial to the development of our new radiation shield design. Otto Guedelhoefer was supported by an NIH Developmental Biology Training Grant (5T32 HD07491) and Dr. Alejandro Sánchez Alvarado is a Howard Hughes Medical Institute Investigator.

Abstract

Planaria contain a large population of highly potent and continually proliferative stem cells that are dispersed throughout their body mesenchyme. This stem cell population is directly responsible for the amazing regenerative powers and the less studied incredible tissue homeostasis capabilities of planaria. In order to study the

migration of planarian stem cells during tissue homeostasis and regeneration we required a method to visualize and functionally isolate a small group of stem cells within tissue that was otherwise devoid of stem cells. The ideal method would allow us to evaluate cell movements *in vivo* over time as well as subject the animal to additional injury and observe changes in cell movements. Three possible mechanisms for creating the desired scenario existed in the literature: tissue transplantation, partial irradiation, and cell transplantation. We evaluated all three methods and determined that, with optimization, tissue transplantation and partial irradiation would provide us with the necessary tools to directly study planarian stem cell migration *in vivo*. Additionally, it became clear that these methods could also be used as assays combined with RNAi screening to test for gene involvement in stem cell migration, and thus aspects pertaining to this goal were tested as well.

Introduction

In order to investigate the *in vivo* repopulation and migration of stem cells within an organism that contains stem cells throughout its body plan, methods were needed for isolating a population of stem cells that could be observed within an otherwise stem-cell-devoid environment. Three possible methods to create this situation were represented in the planarian literature: tissue transplantation (Santos, 1929), partial irradiation (Dubois, 1949), and cell injection (Baguñà et al., 1989). To create a stem-cell-devoid area in which neoblasts movements would be analyzed, all three of these techniques would take advantage of the ability of ionizing radiation to ablate planarian stem cells (Bardeen and Baetjer, 1904; Hayashi et al., 2006).

As outlined in Chapter 1, the migratory nature of planaria stem cells was very much an open question when these investigations were begun; however, previous work in our lab had shown that indeed stem cell progeny migrate and that those migrations contribute to regeneration (Newmark and Sánchez Alvarado, 2000). Briefly, a short pulse of bromodeoxyuridine was used to label a subset of stem cells which were followed as they accumulated over time in the area anterior to the photoreceptors and anterior regeneration blastemas (Newmark and Sánchez Alvarado, 2000), two tissues known to be devoid of stem cells (Reddien et al., 2005b), suggesting that although the labeled cells had migrated into those areas, they were unlikely to have done so while maintaining their stem cell characteristics. Because the labeling scheme labeled stem cells and lacked additional stem cell or progeny markers, it was impossible to determine if all of the observed migrations took place within the progeny populations, therefore leaving open the formal possibility that the stem cells themselves contributed to the migrations. On the other hand, if planarian stem cells were found to be nonmigratory as some evidence suggested (Flickinger, 1964; Saló and Baguñà, 1985), the planned method development would have nonetheless proven useful for further characterizing stem cell progeny migration, which was also under-studied.

Planarian tissue transplantation was both the oldest and most recently used technique that we investigated. Lillian Morgan pioneered its use in aquatic planaria for studies pertaining to the inductive patterning capacity of various sized pieces of tissue (Morgan, 1906). The technique was greatly refined by Santos, as he investigated the source of the planaria "organizer" (Santos, 1929), and performed largely as he described by others (Miller, 1938; Okada and Sugino, 1937; Stéphan-Dubois, 1961) including work

done recently (Kato et al., 1999; Saito et al., 2003; Saló and Baguñà, 1985; Saló and Baguñà, 1989). Although effective at some low rate, the Santos technique was laborious, and because the graft was either cut out by hand or punched out using hand pulled glass pipettes, it was also highly variable, making consistent, quantifiable experimentation very difficult. Thus tissue transplantation had remained an under-used technique.

Partial irradiation, though simpler technically than transplantation, had been even less utilized. Only the French School under Wolff and Dubois reported using partial irradiation in planaria (Dubois, 1949; Kolmayer and Stéphan-Dubois, 1960; Stéphan-Dubois and Lender, 1956; Wolff and Dubois, 1948). The relative unpopularity of partial irradiation as compared to transplantation was likely due to the questions researchers were attempting to ask. Transplantation could be used more effectively to address questions of tissue induction, the popular topic of the first half of the last century, whereas partial irradiation was better suited for addressing questions pertaining to stem cell function which only became a popular topic recently. Furthermore, the additional information as to the migratory nature of planarian stem cells that could be gained using partial irradiation were limited without the development of cell type markers.

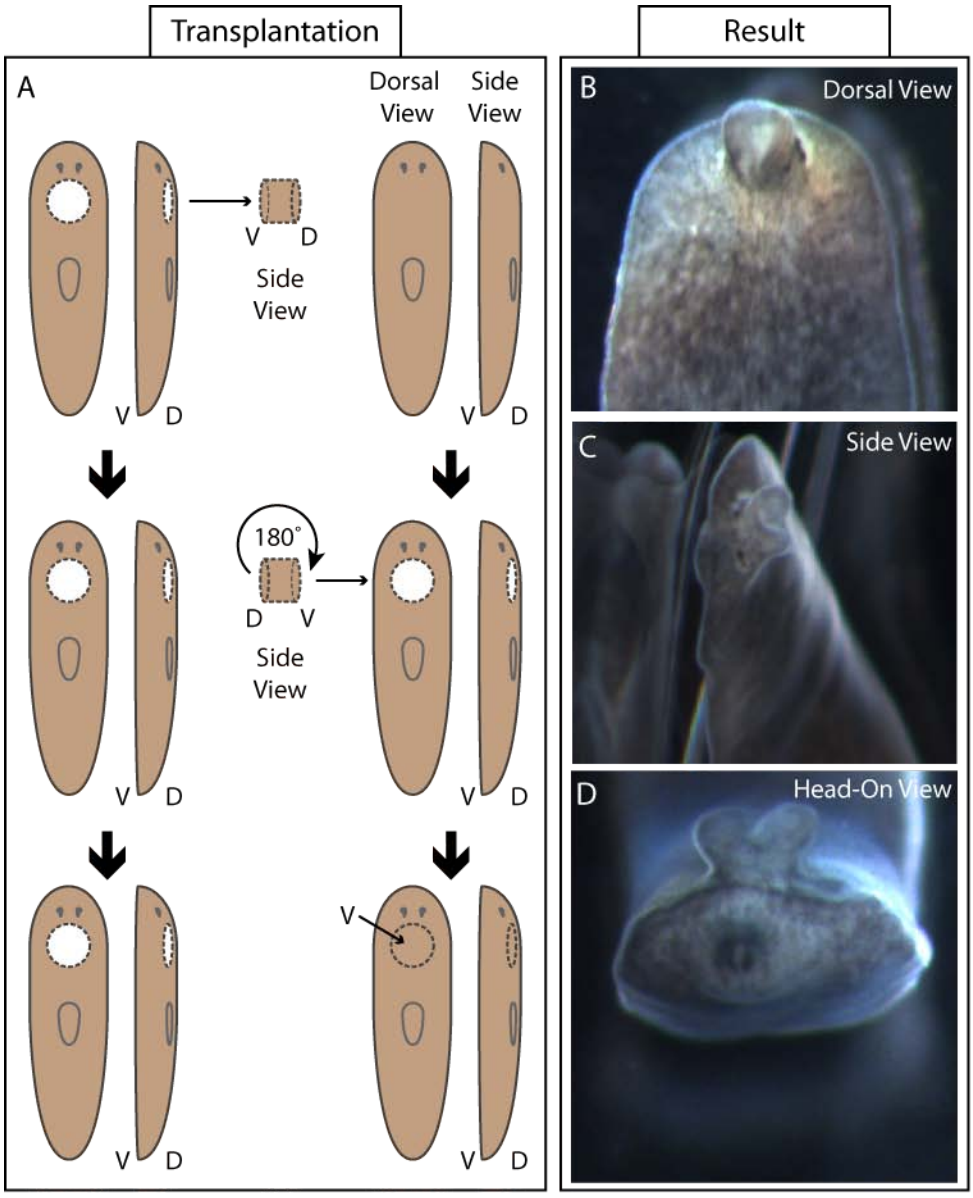
The final technique that could be used to situate planarian stem cells within an irradiated animal, cell injection, had only been reported once in the literature (Baguñà et al., 1989). Members of our laboratory have attempted to replicate this technique as published without success.

Results

Initial attempts at tissue transplantation within the sexual biotype of *Schmidtea mediterranea* or large asexual biotype animals were performed using the Santos method as well as could be reproduced from the text (see Materials and Methods). A successful take rate of ~21% (48/234) was achieved over 14 separate trials spread out over a period of over 1.5 years. In comparison, when capillary tubes of the same size were used to both extract the graft tissue and create the hole in the host (see Materials and Methods) instead of hand pulled glass pipettes the transplant success rate rose to 30% (6/20, two separate trials). Likewise, when size matched capillary tubes (the interior diameter of the tube used to extract the graft equals the exterior diameter of the tube used to create the hole in the host) were used the success rate further increased to ~81% (25/31, three separate trials), and continued use of this method over 3 years has resulted in a steady success rate of greater than 90% (general observation).

Previous data indicated that inductive affects from misoriented grafted tissue can result in tissue remodeling and/or tissue outgrowth (Kato et al., 1999; Santos, 1931). We therefore performed transplantations where the graft tissue was intentionally misoriented and scored for obvious gross morphology changes. As other groups had reported with a different planarian species (Kato et al., 1999), we found that dorsal-ventral inversion resulted in a high frequency of tissue outgrowth (Fig. 2.1). We did not, however, observe any tissue outgrowths from partial (45°-135°) or full (180°) rotation of the graft relative to the anterior-posterior axis in *S. mediterranea* as others, studying a different species, have also reported (Kato et al., 1999). Therefore, although all attempts were made to maintain proper anterior-posterior orientation of the graft, we concluded that small

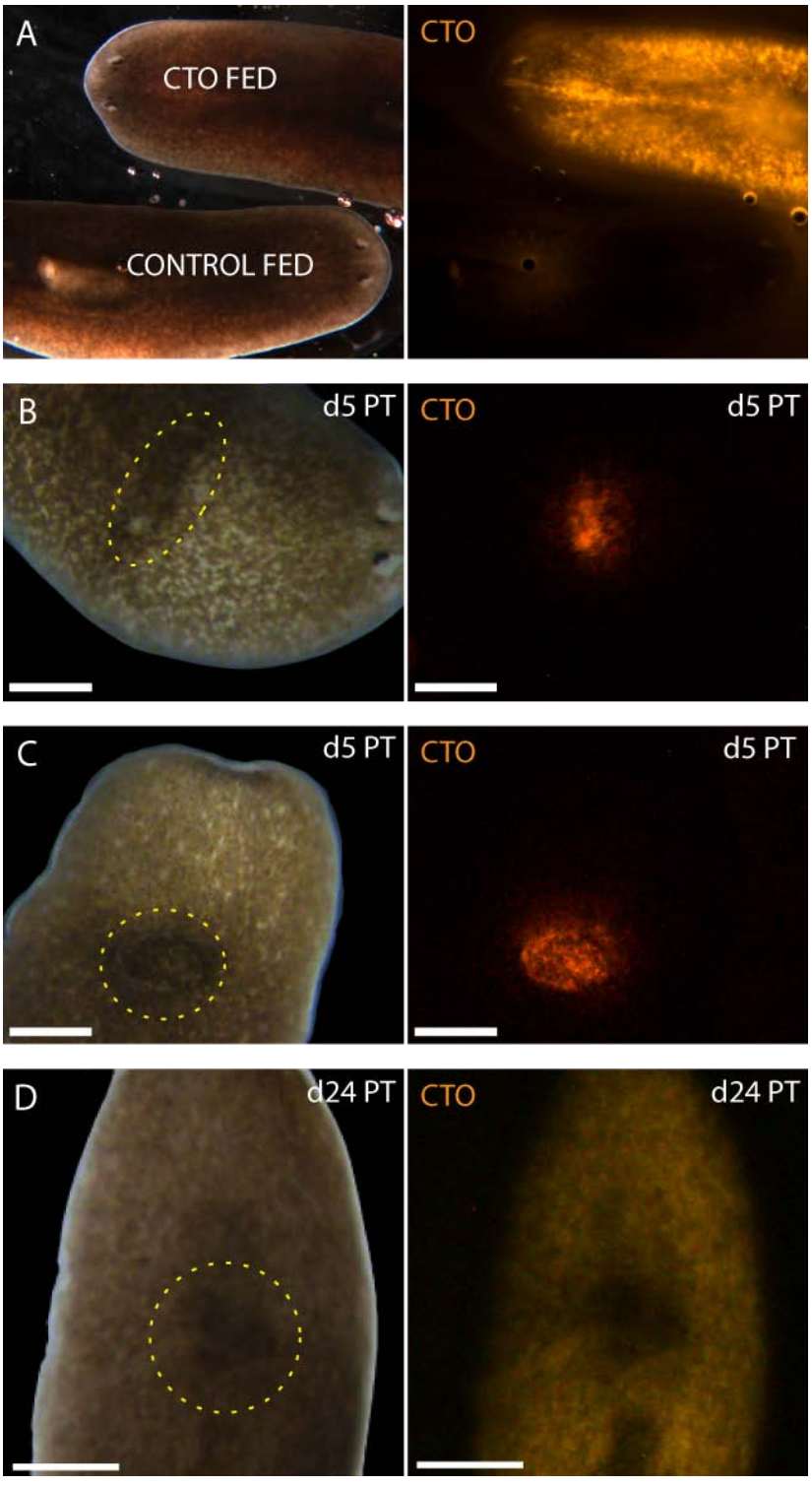
Fig. 2.1. Dorsal-Ventral Graft Misorientation Causes Tissue Outgrowth. In order to test the affects of graft misorientation in our transplantation procedure we purposefully inverted grafts along their dorsal-ventral axis. (A) The procedure is schematized, showing both dorsal and side views of both the donor and the recipient animals. The cylindrical shaped graft is removed from the donor, rotated 180° to invert the dorsal (D) and ventral (V) surfaces, and placed within a prepared hole in the recipient, resulting in the ventral surface of the graft being confluent with the dorsal surface of the recipient. (B-D) As others have observed in other species (Kato et al., 1999; Santos, 1931), this type of transplantation also results in tubular shaped tissue outgrowths in *S. mediterranea*. Whereas, intentional anterior-posterior misorientation did not produce outgrowths in *S. mediterranea* as has also be observed in other planarian species (Kato et al., 1999).


Transplantation
Result
A
Dorsal View
Side View
V
D
Side View
180°
B
Dorsal View
C
Side View
D
Head-On View

deviations from the correct positioning of the graft were unlikely to affect further analysis. Likewise, we tested the influence of graft location on downstream assays of cell migration and found no obvious difference between grafts placed anterior or posterior to the pharynx (these data are presented in Chapter 3).

Following the optimization of the tissue transplantation method, we next sought to determine to what extent the grafted tissue is maintained within the host. Planaria efficiently remodel existing tissue to maintain scale and proportion (Morgan, 1900). For instance, a trunk fragment, produced by head and tail amputation, rapidly reduces the existing pharynx, which after amputation is much too large for the fragment (Gurley et al., 2010). Based on this ability, the possibility remained that the graft tissue would be rapidly remodeled following transplantation, thus complicating analysis of migration of cells out of the graft. We performed tissue tracking studies using CellTracker^{TM} Orange CMTMR, a commercially available dye that is reportedly cell permeable until the molecule undergoes a thiol reaction in the cytoplasm, rendering it cell impermeable, and has been used for long-term cell tracking previously (Borgstrom et al., 1998; Borgstrom et al., 1995). Labeled tissue was transplanted into nonlabeled hosts which revealed the graft shortly after transplantation (Fig. 2.2), but the graft became difficult to detect over time. To test whether the loss of visible labeling in the graft tissue was due to tissue remodeling or dispersion of the label, we performed the converse experiment, ubiquitously labeling hosts and grafting nonlabeled tissue. The unlabeled graft remained readily identifiable at up to 24 days posttransplantation(Fig. 2.2), indicating that the label was not diffusing considerably and remodeling did not appear to be taking place to any extent that would interfere with analysis within our much earlier time window.

Fig. 2.2. Dye Labeling Reveals that Graft Tissue Is Maintained Following Transplantation. (A) Brightfield and corresponding epifluorescence images of a CellTracker™ Orange CMTMR (CTO) fed animal adjacent to a control-fed (liver mixture without CTO added) animal, showing the efficacy of the labeling method and the lack of significant background in the control. (B) Five days posttransplantation (d5 PT) the graft (yellow ellipse) from a CTO labeled animal that was transplanted into a wild-type host remained visible by epifluorescence. (C) Labeled graft tissue remained visible in regenerating (3 days post decapitation) animals as well. (D) Wild-type unlabeled graft tissue could be clearly seen under epifluorescence 24 days posttransplantation (d24 PT) into CTO-labeled hosts, indicating that the graft tissue is not significantly remodeled during this time period. Scale bars are 500 μm.


A
CTO FED
CONTROL FED
CTO
B
d5 PT
CTO
d5 PT
C
d5 PT
CTO
d5 PT
D
d24 PT
CTO
d24 PT

An enticing aspect of tissue transplantation versus other methods of isolating stem cells within irradiated hosts, was the theoretical possibility of testing the cell autonomous and non-autonomous requirement of various cell migration candidate genes by either donor or host RNAi treatment respectively. RNAi is an effective method for gene knockdown in planaria (Newmark et al., 2003); however many aspects of the mechanisms of whole animal gene knockdown, including the amplification and spread of the knockdown signal, remain unclear. The ability to deliver RNAi by feeding suggested that silencing was disseminated (Newmark et al., 2003). We therefore sought to determine to what extent RNAi knockdown within a donor or host spreads among adjacent tissues following transplantation. We began by attempting to rescue with wild-type tissue grafts, host animals that had been treated with *Smed-piwi-2* RNAi, which has been shown to be strongly lethal because of autonomous disruption of stem cell function (Reddien et al., 2005b). We observed 0% (0/18) rescue of the RNAi treated hosts, whereas 100% (16/16) of control lethally irradiated hosts were rescued by transplantation of wild-type tissue. These data indicated that the effect of the RNAi treatment was able to spread into the grafted tissue, thus rendering the transplanted neoblasts incapable of rescuing the host. Surprisingly however, when grafts derived from *Smed-piwi-2* RNAi or *Smed-cdc73* RNAi, a lethal gene perturbation with more rapid phenotype progression than *Smed-piwi-2* RNAi, treated donors were transplanted into wild-type hosts 100% (24/24) of the animals survived long-term. Together, these data indicated that although the RNAi treatment was capable of spreading from an RNAi treated host to affect transplanted neoblasts, it was not capable of spreading from an RNAi treated graft to affect the entire neoblast population of a wild-type animal. Since the effect of RNAi

knockdown is known to be transient in planaria (Pineda et al., 2000), as it is in other organisms (Timmons et al., 2001), we attributed the inability of a *Smed-piwi-2* or *Smed-dcd73* RNAi treated graft to kill a naïve animal to a lack of effective RNAi amplification and dispersal within the permissive time window of RNAi knockdown.

Experiments in *C. elegans* have identified a membrane channel, called systemic RNA interference defective 1 (SID-1), which is necessary for RNAi dissemination through tissues (Winston et al., 2002). We have identified in the genome and cloned three *Sid-1* homologs from planaria. As is the case in *C. elegans* (Winston et al., 2002), individual disruption of these genes did not produce any obvious gross phenotypes (Jochen Rink, personal communication). Attempted rescue of *Smed-piwi-2* RNAi treated hosts by transplantation with *Smed-Sid-1A* RNAi treated grafts failed to protect the transplanted cells and rescue the host.

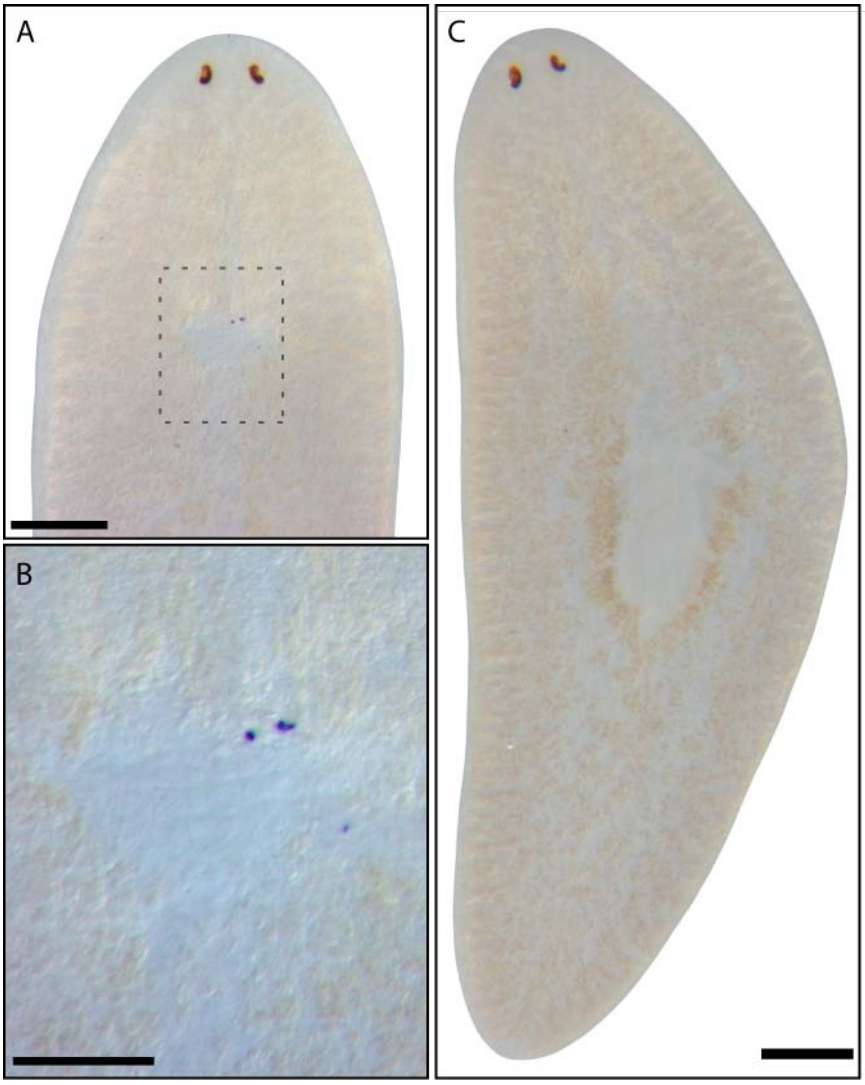
The spreading of RNAi treatment between donor and host cells decreased the likelihood that cell autonomous and noncell autonomous mechanisms of candidate genes could be tested using transplantation. We therefore also investigated the possibility of using partial irradiation to establish a situation where a population of stem cells is adjacent to stem cell-devoid tissue. Partial irradiation was the most technically simple of the three methods we investigated, the methodology is therefore presented with the data collected in Chapter 3. Our most significant advance on the technique as described by Dubois (Dubois, 1949) was the design of a lead shield that allowed for the simultaneous irradiation of both anterior and posterior portions of planaria (also presented in Chapter 3), thus allowing for the testing of stem cell migration in more than one direction.

Cell transplantation was the final method we tested for the creation of planaria with stem cells situated adjacent to an area completely devoid of stem cells. This technique presented the unique advantage of allowing us to isolate, and therefore test, planarian stem cells or even subpopulations therein prior to transplantation. We used dissociation and cell size enrichment to create a cell suspension enriched for planarian stem cells (Baguñà et al., 1989), mixed the cell suspension with artificial cell matrix, and transplanted the cell loaded matrix into lethally irradiated animals. None of the transplanted animals from four individual trials survived long-term (N = 50). RNA whole mount in situ hybridization (WISH) for the stem cell marker *Smed-piwi-1* in a cohort of animals shortly after cell transplantation did however detect a very small number of cells at the graft site (Fig. 2.3) which was never observed in controls transplanted with artificial matrix alone (Fig. 2.3). The small number of *Smed-piwi-1* positive cells detected in the host following transplantation indicated that either the cell delivery method was ineffective or that stem cells were being delivered but some aspect of the procedure was causing the great majority of transplanted cells to stop expressing characteristic stem cell genes.

Discussion

We have investigated three separate techniques for positioning healthy planarian stem cells near an area of stem-cell-devoid tissue, namely tissue transplantation, partial irradiation, and cell transplantation. All three techniques had potential benefits and drawbacks. Tissue transplantation had the benefit of maintaining the stem cells in their natural tissue environment, thus decreasing the likelihood of observing artifactual cell

Figure 2.3. Cell Transplantation Is an Inefficient Method for Localizing Stem Cells within an Irradiated Host. We utilized the cell transplantation method described in the text and evaluated the presence of *Smed-piwi-1* positive stem cells in the host following transplantation. (A) Only rarely did we observe any positive cells within the host following transplantation of dissociated cells into irradiated host animals, (B) which could be seen located in the area of transplantation in the zoomed image (of the boxed area in A). (C) However, *Smed-piwi-1* cells were never observed in animals grafted with artificial cell matrix alone. These data together indicate that although effective, cell transplantation is a highly inefficient method for transplanting stem cells in planaria.


A
B
C

behaviors; on the other hand, the process was highly variable and tedious. Partial irradiation was the least variable and most easily scaled-up technique; however, it was impossible to retroactively identify the boundary between the shielded and irradiated tissue, which would make measuring cell movements difficult. Finally, although cell transplantation promised isolated analysis of the stem cells alone, previous work done in our lab indicated cell transplantation could not effectively deliver enough healthy stem cells for analysis.

After testing and attempting to optimize all of these methods it became clear that tissue transplantation would be the most effective method for studying stem cell migration in planaria. This was primarily because we were able to minimize the largest potential drawbacks of the technique by using standardized capillary tubes to limit variability and by increasing the transplantation efficiency. Presumably, practice played a role in the increased success rate over time; however, the dramatic shift in transplantation success following protocol optimization (primarily the introduction of size matched capillary tubes) was indeed causative. That such a seemingly simple change led to such a technical advance is striking.

A number of potentially valuable methods that could be used in conjunction with tissue transplantation were also introduced during the optimization and testing of the new protocol. Live cell labeling of either donor or host tissue proved useful for determining to what extent the graft tissue remained intact (Fig. 2.2). This technique was abandoned for further use in studying stem cell migration because we were unable to determine if the dye was labeling stem cells and live imaging proved prohibitively difficult. Now that the

basics of stem cell migration have been characterized in this dissertation, revisiting live cell labeling and imaging with this technique is certainly justified.

Based on the evidence of RNAi spreading presented here, we conclude that cell autonomous and cell non-autonomous testing by the use of differentially RNAi treated host and donor tissue cannot be done effectively without further characterization of RNAi transport mechanisms. It remains possible that functional redundancy between the three planarian Sid-1 homologs renders individual knockdown ineffective and this has yet to be tested. Additionally, the intrinsic difficultly of RNAi knockdown of RNAi machinery components necessitates further validation of any evidence supporting a block in RNAi signal spreading through Sid-1 targeting. On the other hand, effective spreading of RNAi knockdown may indicate that only host RNAi treatment is necessary for initial large-scale screening purposes in a transplantation assay.

Our initial partial irradiation experiments supported the use of the technique for studying stem cell migration, particularly in a wounding free context. However, our inability to retrospectively determine the location of the boundary between the shielded and irradiated tissue necessitated either invention of new quantification methods of migration or initial characterization of baseline migration in the transplantation context, where the host graft boundary is clear. Both of these alternative methods were employed and the resulting partial irradiation migration data are presented in Chapter 3.

Cell transplantation was the only one of the three methods that was not further studied. The preliminary experiments indicated that too few cells were delivered by this method to produce enough data that would allow for generalizable conclusions. Although effectively done in the HSC system (Kiel et al., 2005), separation of the stem

cells from their niche and subsequent processing *in vitro* seemed likely to produce confounding and unnatural changes in the cells, especially considering what little is known about isolation and culture of freshwater invertebrate cells (Quinn et al., 2009).

Conclusion

Of the three methods investigated we were able to improve and validate two, tissue transplantation and partial irradiation, for further use in characterizing planarian stem cell repopulation and migration. In our hands, the tissue transplantation protocol of Santos was optimized to increase the successful transplantation rate from ~20% to over 90%, while increasing consistency in graft size across experiments. Additional experiments indicated that small deviations in graft orientation and positioning as well as tissue remodeling of the graft were unlikely to confound our future analyses. Furthermore, RNAi gene knockdown was shown to work effectively in the transplantation context, though RNAi spreading limited our ability to study cell autonomous and noncell autonomous functions of target genes. Overall, the newly optimized tissue transplantation protocol presented here is likely to be an effective method for studying the movements of stem cells and their progeny *in vivo*.

Materials and Methods

Experimental Animals

Except where noted, all experiments were performed on the CIW4 asexual biotype clonal line of the species *Schmidtea mediterranea*. Animals were kept as described previously (Reddien et al., 2005a) with the following exceptions. For initial

transplantation experiments a stock of animals kept at 10°C were used because the lower temperature promotes increased growth prior to autonomous fission (Child, 1910), thus promoting a larger final size. Using these animals however produced many caveats within our experiments due to the known effect of temperature on the rate of regeneration (Morgan, 1901) and the tendency for these animals to fission once they reach room temperature (data not shown). We therefore produced large (> 1 cm in length) animals by simply overfeeding, two to three feeds per week as compared to one feed every week or every other week. These larger animals had an increased frequency of fission as compared normally fed stocks, but fissioning appeared to be stochastic and therefore less of a caveat than fissioning coincident with manipulation.

γ Irradiation

To produce lethally irradiated animals for transplantation, large asexuals (either kept at 10°C or overfed) were given a 100 Gy dose (17.7 min., ~565 rad/min) of gamma irradiation within a J.L. Shepherd and Associates model 30, 6,000 Ci self-shielded Cs^{137} irradiator and used for experimentation 24 hours thereafter.

Tissue Transplantation

The Santos method for planarian tissue transplantation consisted of anesthetizing a 10-22 mm long *Planaria dorotocephala* or *Planaria Maculata* (now referred to as *Dugesia dorotocephala* and *Dugesia tigrina* respectively (Kenk, 1989)) host using 0.01M chloretone (~0.2% w/v) and then cutting a 0.5 mm diameter circular graft from the donor by means of pulled glass pipette and transferring the graft using the pipette into a

prepared hole of nearly equal size in the host (Santos, 1929; Santos, 1931). Santos also used grafts of various shapes (triangular, rectangular, etc.) cut by hand, but these types of grafts were not attempted in this study because of inherently high levels of variability in both size and shape. Completed transplantations were allowed to heal for 12-24 hours in only slightly moistened dishes and many animals were decapitated to decrease motility and increase transplantation success (Santos, 1931).

Our improved method of transplantation continued to utilize 0.2% chloretone (w/v in planaria water) as the preferred anesthetic with the addition of chilling the solution on ice before use. A detailed protocol of our method is attached as Appendix A; therefore only the major advancements from the Santos method will be outlined here. Transplantations were performed on damp filter paper and a cold plate in order to not only increase the effectiveness of the anesthetic but also promote tissue survival after injury. The use of size matched thin-wall capillary tubes to create a graft (0.75 mm inner diameter capillary, catalog# 30-30-0 FHC, Bowdoin, ME) and a receiving hole (0.7 mm outer diameter capillary, catalog# 30-50-8 FHC, Bowdoin, ME) in the host of equal diameter, greatly improved the reproducibility of this technique such that transplantations from experiment to experiment contained grafts of equal size. The necessity of speed, i.e., limiting the amount of time the receiving hole is exposed before the graft is inserted was absolutely critical to improving the efficacy of these tissue transplantations. The use of a cold recovery chamber following transplantation (see Appendix A) also increased the rate of successful grafting.

Whole Animal Dye Labeling

Cytoplasmic dye (CellTracker™ Orange (CTO) CMTMR, Invitrogen, Carlsbad, CA) was delivered by feeding or micro injection to host or donor worms. For feeding, 10 mM CTO in DMSO was diluted to 400 μM with 1:1 mixture of planaria water and homogenized calf liver, which was added as a paste to the bottom of a 10 cm petri dish containing planaria in planaria water. Worms were allowed to feed for 1 hour, then food was removed and the dish was rinsed. For injection, 10 mM CTO in DMSO was diluted to 100 μM with PBS and injected into the gut with 6-8 pulses of 50 nL using a Drummond Nanoject mounted on micromanipulator.

RNAi Feeding

Feedings of *Smed-piwi-2* (Reddien et al., 2005b) and *Smed-cdc73* (Kang, 2009) were performed as previously described for other gene targets (Gurley et al., 2008).

Cloning of Planarian SID-1 Homologs

Planarian SID-1 homologs were identified in the planarian genome and cloned as previously described (Gurley et al., 2008).

X-ray Partial Irradiation and Shield Design

See Chapter 3 for x-ray methods and lead shield design considerations.

Cell Dissociation

Cell dissociation was performed by finely chopping planaria in ice cold calcium and magnesium free media (CMF) then rocking the resulting solution with 1-2 U per mL trypsin added in a 15 mL Falcon tube for 1 hr with agitation once every twenty minutes. The solution was centrifuged at 500 x g and resuspended in 20 mL CMF. The cell suspension was then serially filtered through 53 μm and 20 μm nylon mesh. Following serial filtration, the cell suspension was concentrated by centrifugation, counted on a hemacytometer, and used directly.

Seeding of Artificial Matrix and Transplantation

Concentrated, stem cell enriched dissociated cells (~30 million) were resuspended in one hundred microliters of Gelin-S™ (thiol-modified gelatin) to which one hundred microliters of Glycosil™ (thiol-modified hyaluronan) were added. Fifty microliters of Extralink™ (PEGDA) were added to begin the cross-linking of the artificial matrix (Extracel™, Gylcosan Biosystems, Inc., Salt Lake City, UT). Transplantations of the cell seeded artificial matrix were done by either pipetting the still liquid solution into a hole made in a planaria with a capillary tube as described for tissue transplantation, or a small fraction of the solidified matrix was transferred into a hole made in the host using forceps. The Extracel™ solutions were a kind gift from Dr. Glen Prestwich. Transplanted animals were allowed to recover overnight in a recovery chamber as described for tissue transplantation (see Appendix A).

RNA Whole-Mount In Situ Hybridization (WISH)

WISH was performed largely as described (Pearson et al., 2009) with the changes for optimization in large worms as presented in Chapter 3.

Microscopy and Imaging

All images were captured on a Zeiss SteREO Lumar.V12 with 1.5x objective using a Zeiss AxioCam HRc digital camera and Zeiss AxioVision software. Brightfield images were illuminated with both transmitted and reflected white light. Fluorescent images of CTO were captured using a Cy3 filter. Multichannel live images were captured using multidimensional acquisition to image both channels as rapidly as possible; however, the two channels were still slightly misaligned due to movement of the live planaria. Images were adjusted for optimal brightness and contrast. No gamma adjustments were made on fluorescent images. Cropping and staging was performed using Adobe Photoshop.

CHAPTER 3

WOUND INDUCED ADULT STEM CELL RECRUITMENT IN THE PLANARIAN *SCHMIDTEA MEDITERRANEA*

Credits

This chapter consists of work that was prepared for publication and will be submitted to the journal *Cell Stem Cell* and thus conforms to the journal's style instructions and formatting.

I owe a special debt to Alejandro and Dr. Phil Newmark who have amassed an amazing library of classical planarian and regeneration literature. I would like to additionally thank Dr. Phil Newmark for sharing his planarian karyotyping protocol. None of this work could have been completed without the help, support, and constructive criticism of all Sánchez Laboratory members past and present. However, I am particularly grateful to Sarah Elliott, Dr. Li-Chun Cheng, Dr. Alessandro Rossi, Dr. Kyle Gurley, and Dr. Bret Pearson for their helpful comments during the formulation of this manuscript. I would also like to thank Diane Downhour for caring for my planaria while I was out writing.

. Alejandro and I conceived of and I performed all of the experiments presented here. Otto Guedelhoefer was supported by an NIH Developmental Biology Training Grant (5T32 HD07491) and Dr. Alejandro Sánchez Alvarado is a Howard Hughes Medical Institute Investigator.

Summary

How adult stem cell populations are recruited is a fundamental question of stem cell biology. Mobilization of stem cells out of their niche and correct migration to a site of tissue turnover or injury, whether this occurs before or after the stem cell begins to differentiate, is an absolute requirement for proper tissue homeostasis and regeneration.

However, we understand little about the mechanisms that control this process, primarily because the best studied vertebrate adult stem cell systems do not lend themselves easily to *in vivo* observation of stem cell migration. Here we show that planarian stem cells are stationary during tissue homeostasis but are directionally recruited during regeneration, and are even able to integrate recruitment signals from multiple wounds. Our characterization of the mobilization and migration of planarian stem cells sets the stage for discovery of core stem cell recruitment factors relevant to adult stem cell therapeutics and cancer metastasis.

Introduction

During embryonic development tissue morphogenesis occurs in a predictable pattern that deviates little between individuals within a species. Thus, embryonic cell migration and proliferation have evolved such that tissue progenitor cells arrive at correct locations and with sufficient numbers as tissues form *de novo*. During regeneration, however, what tissues will require repair and rebuilding cannot be predicted due to the inherently random nature of injury. Although recruitment of progenitor cells to the regenerating wound site seems therefore likely, such mechanisms are poorly understood (Daley and Scadden, 2008).

In terms of human health, understanding the signals that stem and progenitor cells use to navigate within an organism is of the utmost importance as we move forward with stem cell transplantation therapies (Karp and Leng Teo, 2009). Such therapies have existed for nearly half a century in the form of bone marrow transplantation (Congdon, 1971), and a recent surge in potential therapies has accompanied the discovery of new

mammalian adult stem cell populations (Blanpain et al., 2004; Pittenger et al., 1999; Rietze et al., 2001; Sherwood et al., 2004). Stem cell therapies have quickly advanced to clinical trials regardless of our poor understanding of what factors control the movement of stem cells injected into a patient. A better grasp of how adult stem cell migration is controlled would serve not only to improve the efficacy of these therapies, but may also provide novel approaches to combat cancer metastasis given the stem-cell-like nature of cancer progenitors (Reya et al., 2001; Visvader and Lindeman, 2008).

In mammals, factors involved in homeostatic migration of hematopoietic stem cells (HSCs) and wound response migration of muscle satellite cells have been identified (Laird et al., 2008). However, direct *in vivo* observation of the migratory processes of adult stem cells in these systems is limited by the opacity and thickness of mammalian tissues, requiring highly technical imaging techniques that are hampered by low resolution (Kim et al., 2007). Study of adult stem cell migration in vertebrates is further complicated by their complex anatomy and varied regenerative potential. There is a clear need for a simpler animal model for investigating adult stem cell migration that possesses efficient regeneration and can be easily manipulated and imaged.

The planarian *Schmidtea mediterranea* is uniquely suited to investigation of adult stem cell migration due to its remarkable powers of regeneration and relatively simple body plan (for review of the advantages of studying regeneration in planaria see (Newmark and Sánchez Alvarado, 2002)). Planarian regeneration of any missing or damaged body part is made possible by a population of collectively totipotent adult stem cells called neoblasts (Randolph, 1892). The idea that a regenerative cell might migrate from distant sites to a wound during regeneration dates back over a century (Brøndsted,

1969; Lehnert, 1891; Morgan, 1900). Exposure to ionizing radiation abolishes the planarian's ability to regenerate by specifically ablating neoblasts (Bardeen and Baetjer, 1904; Hayashi et al., 2006). Taking advantage of this fact, as well as the inability of x-rays to penetrate lead, Dubois performed partial irradiation experiments which indirectly demonstrated that neoblasts may migrate through irradiated tissue to reach a distant wound (Dubois, 1949). Until the work presented here, Dubois's partial irradiation technique had yet to be revisited.

As in other organisms, transplantation was initially used in planaria to address the morphogenic potential of various tissues (Morgan, 1906; Rand and Browne, 1926; Santos, 1929), but has since been underutilized as an experimental technique due to its labor intensiveness and variability. The small number of studies that have used transplantation to attempt to determine if stem cells migrate in response to wounding produced conflicting results largely because they lacked reliable stem cell markers (Saló and Baguñà, 1985; Stéphan-Dubois and Lender, 1956). This study demonstrates that, with minor technical adjustments and the addition of modern molecular markers, planarian tissue transplantation and partial irradiation can be powerful and efficient techniques for evaluating the movements of stem cells and their progeny. Additionally, for the first time in any animal outside of vertebrates, we describe the rescue of stem cell deficient adults by stem cell transplantation.

Here we study the dynamics of repopulation as transplanted stem cells invade lethally irradiated host tissue, restoring host regeneration. We investigate stem cell proliferation and differentiation to determine how long-term organism survival and previously described processes of tissue homeostasis are restored (Eisenhoffer et al.,

2008). The following work establishes planaria as an effective model system for studying the underlying mechanisms of stem cell recruitment.

Results

Stem Cells Migrate Minimally in the Absence of a Wound

In order to study adult stem cell mobilization and recruitment in planaria we needed to first establish the baseline level of planarian stem cell migration in intact animals. The use of ionizing radiation to ablate planarian stem cells is well established (Hayashi et al., 2006; Reddien et al., 2005b). We therefore developed a method of irradiating anterior and posterior portions of planaria while protecting consistently sized (~3 mm wide) portions of the animals' mid-sections using identical custom manufactured lead shields (Fig. 3.1, see Experimental Procedures for full description). Due to the geometry of the shields (Fig. 3.2) and their ability to effectively attenuate x-rays, we hoped to produce planaria that had areas devoid of stem cells both anteriorly and posteriorly with a band of healthy stem cells medially.

With this new method we could investigate whether stem cells from the shielded portion migrate into the irradiated portions in the absence of a wound. X-ray irradiated tissue is known to degenerate and eventually disintegrate in planaria (Bardeen and Baetjer, 1904). We first partially irradiated a cohort of animals, placing the shield as close to the middle of the anterior-posterior axis as possible, and analyzed the gross morphology of the animals over time (Fig. 3.1B-E). We observed that although the animals initially looked normal (Fig. 3.1B), they began to display characteristics common in lethally irradiated animals (Reddien et al., 2005b) such as head regression (Fig. 3.1C

Figure 3.1. Stem Cells Do Not Migrate in Intact Partially Irradiated Planaria. (A) Partial irradiation is performed using a lead shield (gray) placed between the anesthetized worm and the x-ray source such that the anterior and posterior (see inset) receive lethal doses (~30 Gy) of unblocked x-rays (green lines) and the mid-section receives a minimal dose because of efficient shielding (98% theoretical at 6 mm thick) by the lead (see Experimental Procedures for details). (B-E) Live images of partially irradiated worms at 7, 10, 14, and 21 days postirradiation (dpi). Although variable, partially irradiated planaria appear normal at 7 dpi (B), begin to show head regression (C', arrowhead) at 10 dpi (C), show complete head regression (D', arrowhead) at 14 dpi (D), and eventually regenerate their regressed heads (E) complete with photoreceptors (E', arrowheads). Whether tissue regression does not take place in the posterior or whether it is simply undetectable because of a lack of posterior anatomical features to use as reference points remains unclear. (F) Whole mount *in situ* hybridization (WISH) for stem cell marker (*Smed-piwi-1*) in nonirradiated planaria shows that the stem cells are distributed throughout the animal, with the exceptions of the area anterior to the photoreceptors and the pharynx. (G) WISH for *Smed-piwi-1* in partially irradiated animals reveals the absence of stem cells in the unshielded anterior and posterior regions and the presence of a healthy stem cell population medially at 3dpi (arrows indicate boundaries between irradiated and shielded tissue). (G-J) A WISH time course labeling stem cells at 3, 5, 7, and 9 dpi shows that the pattern seen at 3 dpi remains largely unchanged over the time course, though the slight variability in shield placement is evident (animals to scale). By 9 dpi (J) some head regression is beginning to become visible even in stained and fixed specimens. (K) Measuring the length from the anterior to posterior boundary (defined by

the most anterior and posterior cells) in 3 (N = 9), 5 (N = 9), 7 (N = 8), and 9 dpi (N = 7) animals revealed that no significant (NS*, $p<0.1$, one-way ANOVA pairwise comparison) amount of migration into the irradiated regions is taking place during the time course. Anterior is up. Scale bars are all 500 μm (except C', D', and E', which are 200 μm).

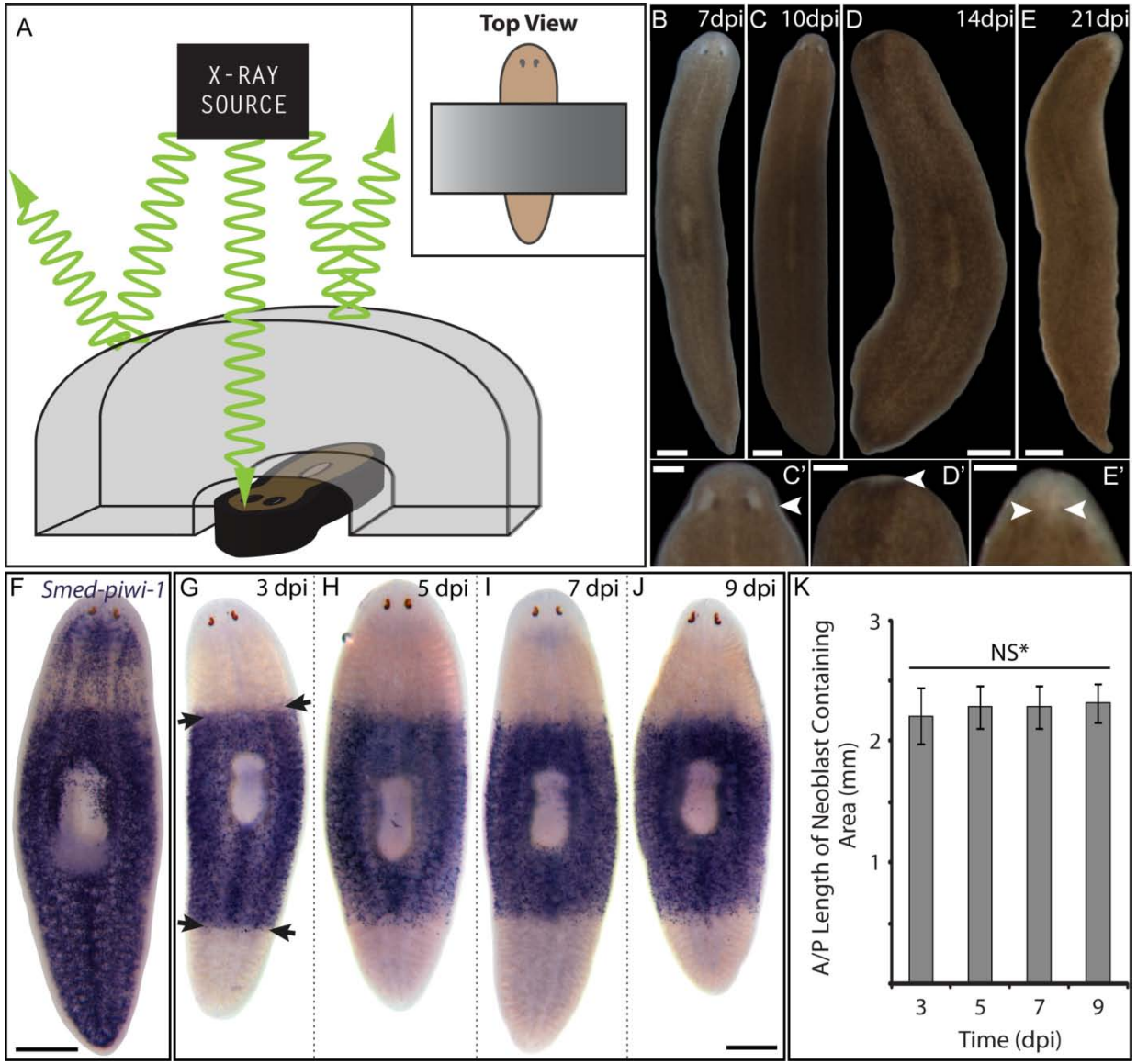
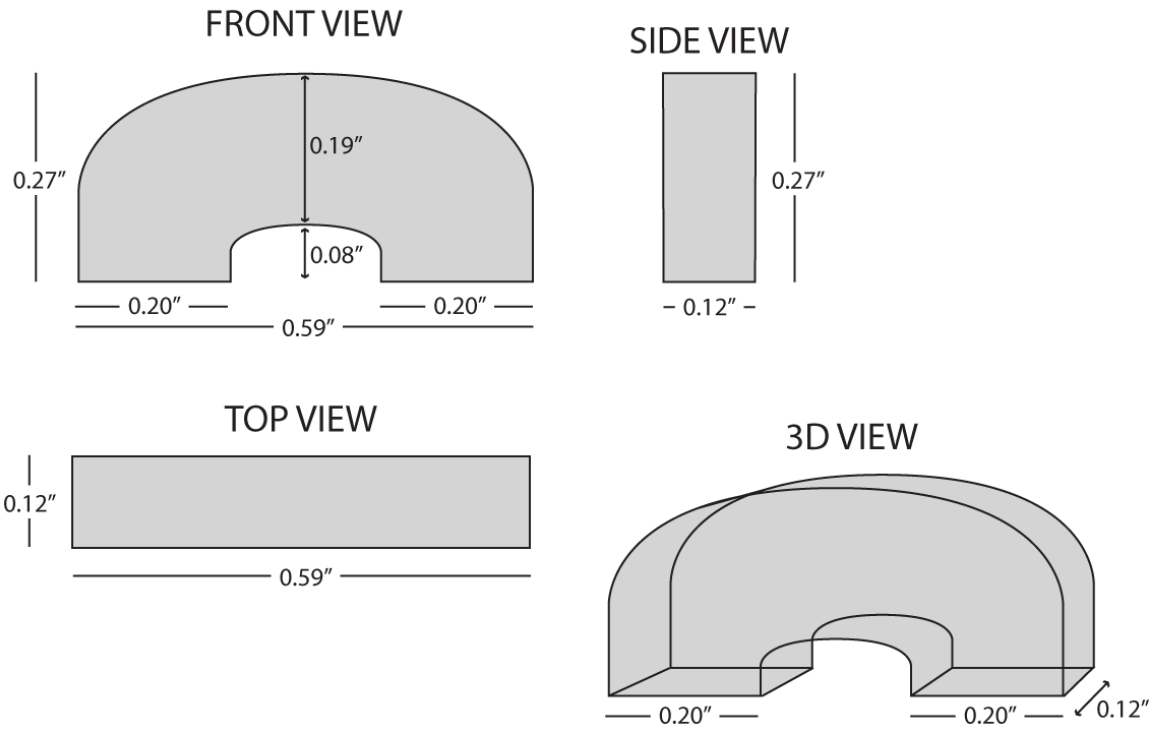

A
X-RAY SOURCE
Top View
B 7dpi
C 10dpi
D 14dpi
E 21dpi
C'
D'
E'
F Smed-piwi-1
G 3 dpi
H 5 dpi
I 7 dpi
J 9 dpi
K
NS*
A/P Length of Neoblast Containing Area (mm)
Time (dpi)
3
5
7
9
1
2
3

Figure 3.2. Design and Geometry of the Radiation Shield. The diagram shows all four views (front, side, top, and 3D rendered) of the vomitorium, or arch, shaped lead shield with included measurements in inches. The shields are free standing. The design was submitted as shown for fabrication out of pure lead and 30 identical units were made.

Many considerations were taken into account during the design. Due to the highly penetrating nature of gamma rays and therefore the unreasonable thickness of lead that would be required to effectively shield any portion of the animal (> 6 cm for 99% theoretical attenuation), we instead used x-ray irradiation for the ablation of planarian stem cells, an effective and well used technique (Dubois, 1949; Hayashi et al., 2006; Steele and Lange, 1976). Reanalysis of published empirical values for the transmittance of 325 kV x-ray beams through various thicknesses of lead (Miller and Kennedy, 1955) revealed that 6 mm thick lead would allow for ~99% hypothetical shielding and 4.5 mm would allow for ~97.5%. Thus we designed a 4.8 mm thick arch shaped shield, which were custom manufactured (Alpha Systems Corp., Bluffdale, UT) for use in a 320 kV X-RAD 320 Biological Irradiator (PXi Precision X-Ray, North Branford, CT).

Planarian Irradiation Shield
"The Vomitorium"
Design for fabrication - Jan. 11, 2010
Pure lead, roughly to scale (1" :: 0.197"), all measurements in approximate inches



and C') and complete head resorption (Fig. 3.1D and D'). This regression of irradiated tissues in partially irradiated animals has been described previously (Dubois, 1949). However, contrary to Dubois, the irradiated tissue did not regress to the level of shielding before head regeneration was initiated; instead, animals were observed to regenerate their heads (Fig. 3.1E-E') from small blastemas at various times following irradiation but before full regression of the irradiated tissue. Subsequently, partially irradiated animals survived long-term (N = 8) whereas the same dose of irradiation proved lethal to unshielded animals (N = 5). Except for differences in the level of tissue regression, which could be due to species differences, these changes in gross morphology largely agree with the finding of Dubois that irradiated anterior tissue in partially irradiated animals regresses back toward the boundary between the irradiated and shielded tissue.

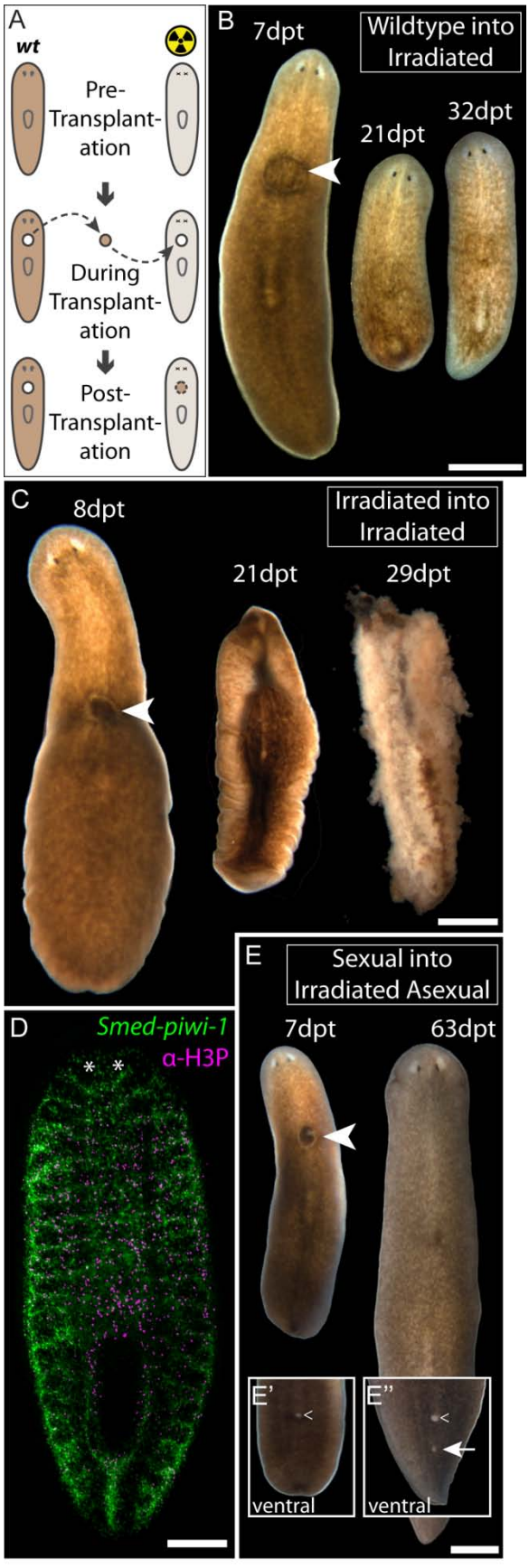
We next looked directly at the positions of the stem cells by RNA whole mount *in situ* hybridization (WISH) for the stem cell marker *Smed-piwi-1* (Reddien et al., 2005b) (Fig. 3.1F) in partially irradiated animals and found well defined borders between the irradiated and shielded tissue (Fig. 3.1G, arrows) with areas devoid of stem cells both anteriorly and posteriorly and a band of *Smed-piwi-1* positive stem cells medially as predicted. Observing partially irradiated animals by this method through 9 days postirradiation (dpi), revealed no qualitative changes in the size or shape of the band of stem cells (Fig. 3.1G-J), thus indicating a lack of stem cell migration in the absence of wounding. Quantitative measurements of the stem cell band length along the anterior-posterior axis revealed no significant change across all four time points (Fig. 3.1K, $p > 0.1$, one-way pairwise ANOVA), further supporting the conclusion that stem cells do not undergo substantial migration into the irradiated regions. This surprising lack of

migration following partial irradiation, indicating a lack of wound signaling from irradiation alone, was in contrast to the large amount of cell death observed in irradiated tissue (Pellettieri et al., 2010), a dichotomy that warrants further investigation.

Transplantation Rescues Tissue Homeostasis in Lethally Irradiated Planaria

In order to better study stem cell recruitment and migration we developed an efficacious and highly reproducible method of tissue transplantation whereby a cylindrical core of tissue of consistent size is taken from a healthy donor worm and grafted into a size matched hole made in a lethally irradiated host (Fig. 3.3, see also Experimental Procedures). When healthy tissue was successfully grafted into lethally irradiated planaria the hosts were rescued (Fig. 3.3B). In fact, using this newly optimized protocol, whenever a healthy tissue graft fully adhered to a lethally irradiated host the host animal was rescued ($N > 100$). Although rescued animals decreased in size over the length of the experiment due to starvation induced allometric scaling (Oviedo et al., 2003), long-term follow-up revealed that rescued animals show no difference in mortality as compared to stock animals (unpublished data). Conversely, transplantation of grafts from lethally irradiated donors into separate lethally irradiated hosts (Fig. 3.3C) had no influence on animal survival, resulting in head regression, ventral curling, and eventual lysis. Furthermore, endpoint analysis of transplantation rescued animals by WISH for *Smed-piwi-1* coupled with immunostaining for mitotic activity (Fig. 3.3D), showed that the stem cell compartment is eventually repopulated indistinguishably from that of wild-type worms (compare to Fig. 3.1F).

Figure 3.3. Transplantation Rescues Tissue Homeostasis in Lethally Irradiated Hosts. (A) Transplantation scheme where a cylindrical core of tissue is taken from a wild-type donor and grafted into a size matched hole made in the lethally irradiated (100 Gy) recipient (see Experimental Procedures). (B) Live images of a lethally irradiated donor grafted with wild-type tissue at 7, 21, and 32 days posttransplantation (dpt). The transplantation site can be clearly seen (B, arrowhead) at 7 days. Animals do not show head regression, ventral curling, or lysis throughout the time course. (C) Live images of a lethally irradiated donor grafted with lethally irradiated tissue at 8, 21, and 29 dpt. The transplantation site can be clearly seen (C, arrowhead) at 8 dpt. All animals display head regression and ventral curling (C, 21d, ventral view) and later total lysis (C, 29dpt). (D) Fluorescent WISH of stem cells (*Smed-piwi-1*) and immunostaining of mitotic cells (antiphosphohistone H3) in a 32 dpt animal (as in (B) 32dpt) is indistinguishable from wild-type animals (* denote photoreceptors) (Newmark and Sánchez Alvarado, 2000; Reddien et al., 2005b). (E) Live images of a lethally irradiated asexual biotype donor grafted with wild-type sexual biotype tissue at 7 and 63 dpt. The darker pigmentation of the sexual graft (E, arrowhead) is clearly visible against the lighter pigmentation of the asexual host at 7 dpt. Ventral view at 7 days (E') clearly shows the lack of a gonopore in the tissue posterior of the pharynx (< denotes pharyngeal opening). By 63 dpt the whole animal displays the darker sexual pigmentation and has developed a clearly visible gonopore (E'', arrow). The apparently sexualized animals are indeed functionally sexualized, displayed by their ability to lay cocoons (not shown). Anterior is up. Scale bars are 1 mm (B,C, and E) and 500 μm (D).

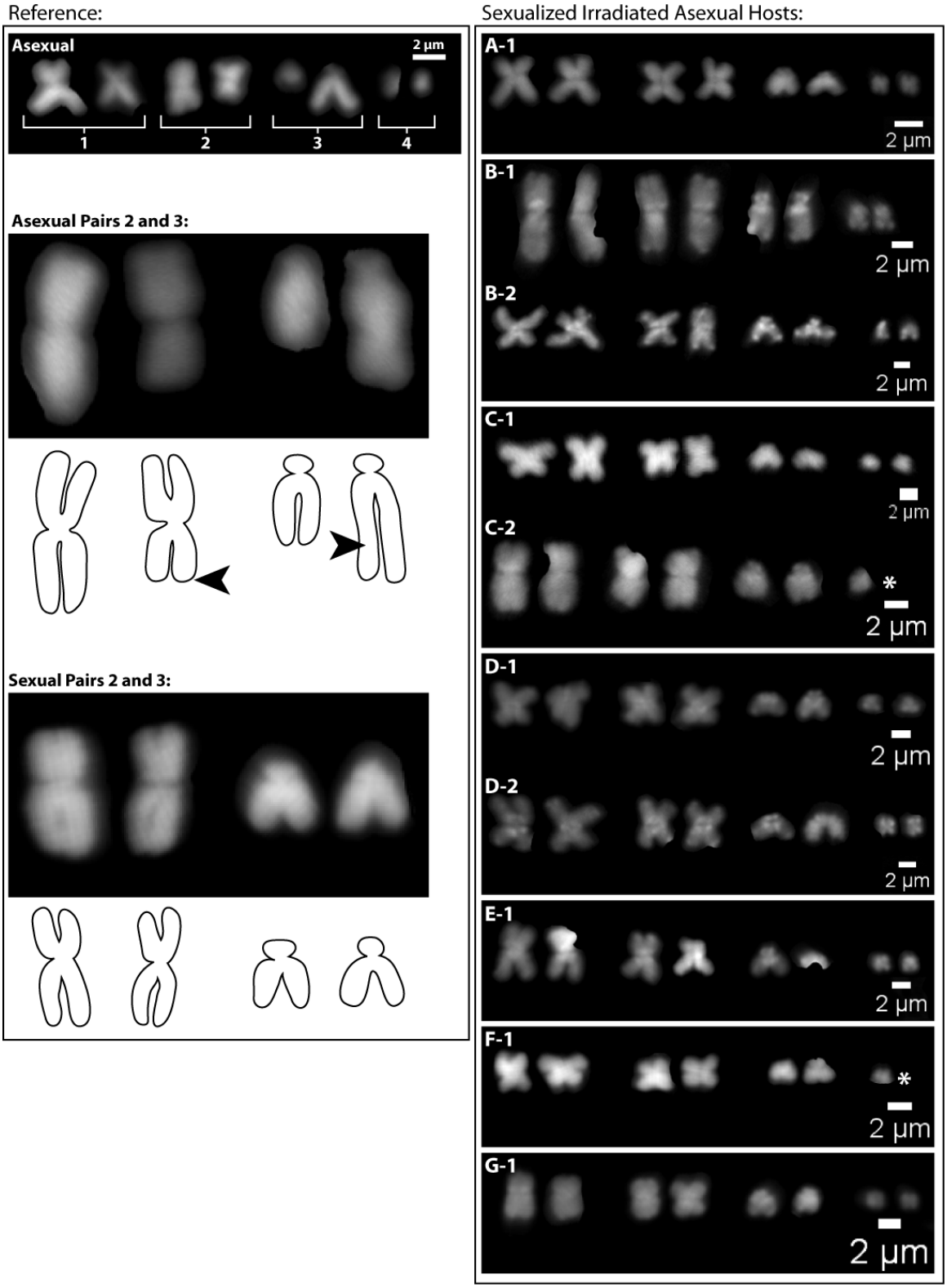

A
wt
Pre-
Transplant-
ation
During
Transplant-
ation
Post-
Transplant-
ation
B
7dpt
Wildtype into
Irradiated
21dpt
32dpt
C
8dpt
Irradiated into
Irradiated
21dpt
29dpt
D
Smed-piwi-1
α-H3P
E
Sexual into
Irradiated Asexual
7dpt
63dpt
E'
E''
ventral
ventral

Rescue with Sexual Strain Stem Cells Sexualizes

Lethally Irradiated Asexual Hosts

To investigate the relative contribution to long-term tissue homeostasis of the irradiated host cells versus the transplanted healthy cells, we attempted to rescue lethally irradiated asexual planaria with healthy tissue grafts from the genetically distinct sexual biotype. The asexual and sexual biotypes of *S. mediterranea* are characterized not only by differences in reproduction, pigmentation, maximal size, and anatomical structures, but also by a large chromosomal translocation present only in the asexual (Newmark and Sánchez Alvarado, 2002; Saló and Baguñà, 1985). One week following transplantation, the irradiated asexual host tissue and sexual graft tissue remain distinguishable by differences in pigmentation (Fig. 3.3E, 7 days posttransplantation (dpt), arrowhead) and the ventral surface of grafted animals showed no signs of sexual organs indicated by the absence of a gonopore (Fig. 3.3E'). After two months of tissue turnover however, rescued animals clearly displayed sexual biotype pigmentation (Fig. 3.3E, 63 dpt) as well as gonopores (Fig. 3.3E'', arrow). Not only did these animals appear to have taken on the sexual biotype by their physical characteristics but they also were functionally sexualized (9/9) evidenced by their ability to lay egg capsules. Furthermore, karyotyping of these sexualized animals revealed only the sexual karyotype and the asexual specific translocation was never observed (Fig. 3.4). The sexualization described is in contrast to the complete lack of development of any sexual characteristics throughout the length of the experiment in nonirradiated asexuals that also received sexual grafts (9/9), suggesting that sexualization is not due to some dominate sexualization factor but rather cell replacement. These data together, combined with what we know about the high rate

Figure 3.4. Asexual Host Animals Sexualized By Irradiation and Transplantation of Healthy Sexual Host Tissue Eventually Display the Sexual Karyotype. Sexualized animals were karyotyped at long-term follow-up (> 1 year posttransplantation) as described in Experimental Procedures. Reference asexual and sexual karyotypes are shown on the left, focusing on the second and third pair of chromosomes which are used for identification and consist of two identical sets in the sexual animal and four different chromosomes in the asexual due to a large translocation (indicated by arrowheads) (Newmark and Sánchez Alvarado, 2002). The karyotypes of all remaining sexualized animals (7 of 9, two animals were lost to inadvertent desiccation) are shown on the right (A-G). Karyotypes of all individual animals are consistent with the sexual reference (each letter represents an individual animal and numbers represent extra replicates). Occasionally, because of their small size, the second chromosome of the fourth pair could not be found (*), but this chromosome is not essential for identification. These data indicate that the predominate, if not only, cell type present in these animals is derived from the sexual graft.


Reference:
Asexual
2 µm
1
2
3
4
Asexual Pairs 2 and 3:
Sexual Pairs 2 and 3:
Sexualized Irradiated Asexual Hosts:
A-1
2 µm
B-1
2 µm
B-2
2 µm
C-1
2 µm
C-2
*
2 µm
D-1
2 µm
D-2
2 µm
E-1
2 µm
F-1
*
2 µm
G-1
2 µm

of planaria tissue turnover (Kang, 2009), suggest that the sexual biotype stem cells contained within the graft eventually reconstituted all of the existing irradiated asexual host tissues through invasion and tissue turnover.

Invading Transplanted Stem Cells Remain Mitotically Active in Host Tissues

The rescue of lethally irradiated host tissue by healthy tissue grafts suggested that stem cells were migrating out of the graft and repopulating the empty stem cell compartment within the host. This inference was also based on previous work, which showed that the type of injury caused during transplantation initiated local expression of a wound response molecule (Petersen and Reddien, 2009). Furthermore, we found that transplantation induces a local increase in mitotic activity near the graft at 3 dpt (Fig. 3.5), a well described characteristic wound response in planaria (Baguñà, 1975; Wenemoser and Reddien, 2010). We therefore hypothesized that the wound introduced during transplantation would cause the migration of stem cells out of the graft and into the irradiated host tissue.

Based on previous work, we knew that 24 hours following irradiation planaria are devoid of detectable stem cells (Eisenhoffer et al., 2008). Therefore, to determine if repopulation was indeed taking place we looked directly at the position of all stem cells within the transplantation rescued animals (Fig. 3.6). Concordantly, at 2 dpt we observed that the irradiated host lacked S*med-piwi-1* positive stem cells except for those contained within the nonirradiated graft and a very small number that had migrated into the host tissue (Fig. 3.6A). By 3 dpt a considerable number of *Smed-piwi-1* positive stem cells

Figure 3.5. Tissue Disruption Inherent in Transplantation Initiates a Wound Response. The planarian wound response is characterized in part by a well studied local increase in mitotic activity 48-72 hours following wounding (Saló and Baguñà, 1984; Wenemoser and Reddien, 2010). In order to determine if the simple act of tissue transplantation is initiating wound response mechanisms, we grafted wild-type tissue into wild-type hosts using our described transplantation procedure (diagram) and immunostained for anti-phosphohistone H3 (α-H3P) 3 days posttransplantation (dpt). Low autofluorescence was used to identify the boundary between the graft and host tissue (yellow ellipse). Indeed, a qualitatively obvious local increase in mitotic activity was present local to the graft as compared to the level of mitosis in the rest of the animal (merged image). This data, with other findings (Petersen and Reddien, 2009), indicated that the tissue disruption caused during transplantation was displaying characteristics of an active wound. Immunostaining and imaging were performed as described in Experimental Procedures.

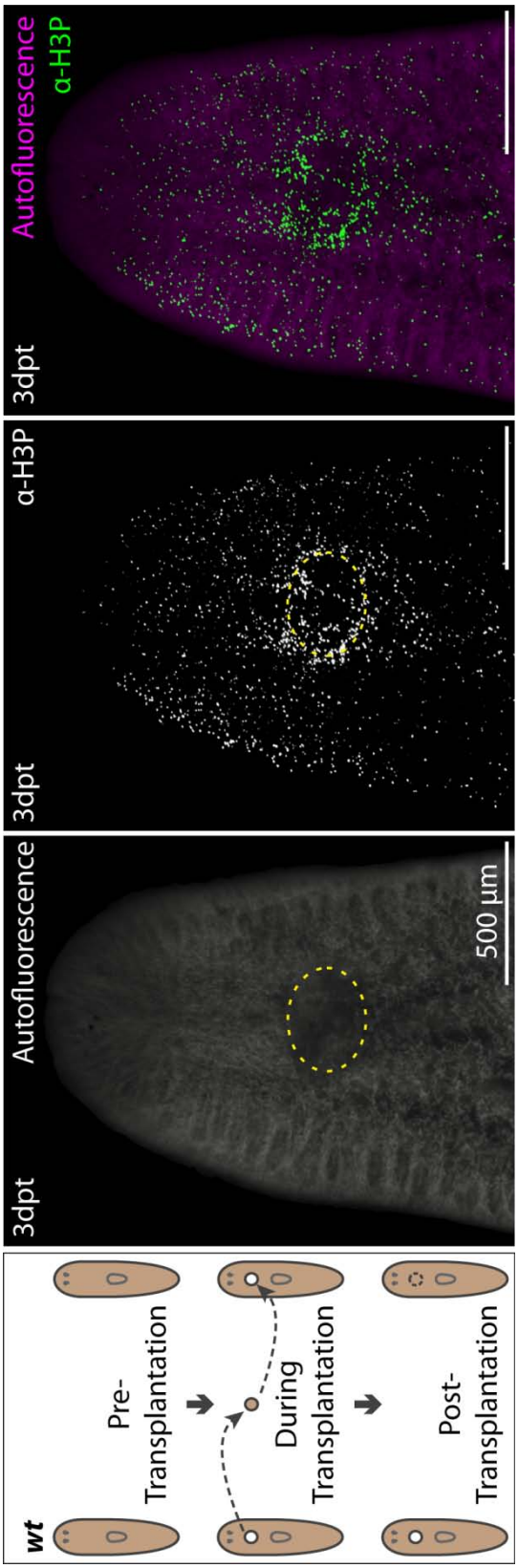
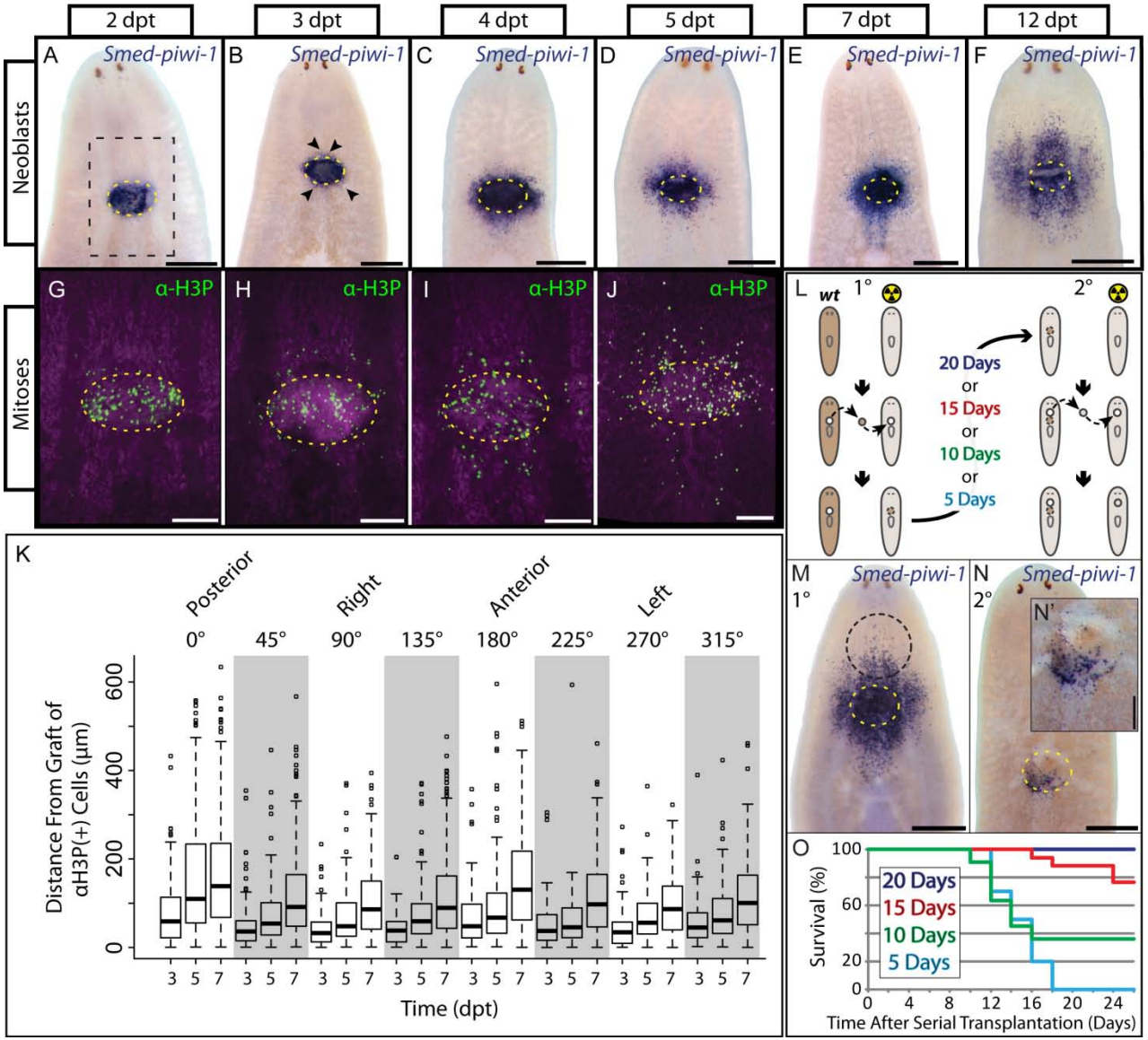

wt
Pre-
Transplantation
During
Transplantation
Post-
Transplantation
3dpt
Autofluorescence
500 µm
3dpt
α-H3P
3dpt
Autofluorescence
α-H3P

Figure 3.6. Mitotically Active Stem Cells Progressively Repopulate Lethally Irradiated Hosts. (A-F) Identifying stem cells by WISH (*smed-piwi-1*) in lethally irradiated hosts grafted with wild-type tissue at 2, 3, 4, 5, 7, and 12 dpt. The graft-host boundaries are identified (yellow ellipses) showing very few stem cells within host tissues at 2 dpt (A) and slightly more at 3 dpt (B, arrowheads) with progressively more invasion by 4 (C), 5 (D), and 7 dpt (E). By 12 dpt (F) stem cells have spread from the graft to the lateral edges of the host. (G-H) Immunostaining for mitoses (antiphosphohistone H3, green) in lethally irradiated hosts showing the general region around the wild-type graft (box in (A)) in separate specimens at 2, 3, 4, and 5 dpt. Autofluorescence (G-J, magenta, see Experimental Procedures) was used for landmarking as the graft-host boundary and the ventral nerve cords clearly lack autofluorescence. At 2 dpt (G) few H3P(+) cells are seen outside the graft. More H3P(+) cells are seen progressively further from the graft within host tissues at 3 (H), 4 (I), and 5 (J) dpt, corresponding with the above stem cell population progression (Fig. 3.6A-D). (K) Quantification of the distances of H3P(+) cells from the graft-host boundary divided into 45° directional bins encompassing the entire animal (inset) measured over 7 dpt, showing similar repopulation in all directions with a slight longitudinal bias (see Experimental Procedures quantification details). (L) Serial transplantation scheme, showing the primary transplantation performed as described in Fig. 3.3A, the various lengths of time allowed to pass between primary and secondary transplantations (5-20 days), and the secondary transplantation that involves grafting tissue from the area anterior to the primary graft into a second lethally irradiated host. (M) WISH for stem cell marker (*smed-piwi-1*) 10 days following primary transplantation, indicating the

primary graft-host boundary (yellow ellipse) and the approximate location where tissue is removed for secondary transplantation (black circle). (N) Serially transplanted stem cell are labeled (*smed-piwi-1*) 3 days following secondary transplantation in a graft taken 10 days following primary transplantation (as shown in (M)), indicating the graft-host boundary (yellow ellipse). A magnified view of the graft location (N') clearly shows the single cell resolution of this technique that makes quantification of cell numbers possible (animals received varied numbers of cells upon secondary transplantation, an animal representing near the median is shown). (O) Survival curves of serial transplantations where 20 (blue, N = 12), 15 (red, N = 17), 10 (green, N = 11), or 5 days (cyan, N = 10) were allowed to pass between primary and secondary transplantations. Scale bars are 500 μm (A-F and M-N) and 200 μm (G-J and N').

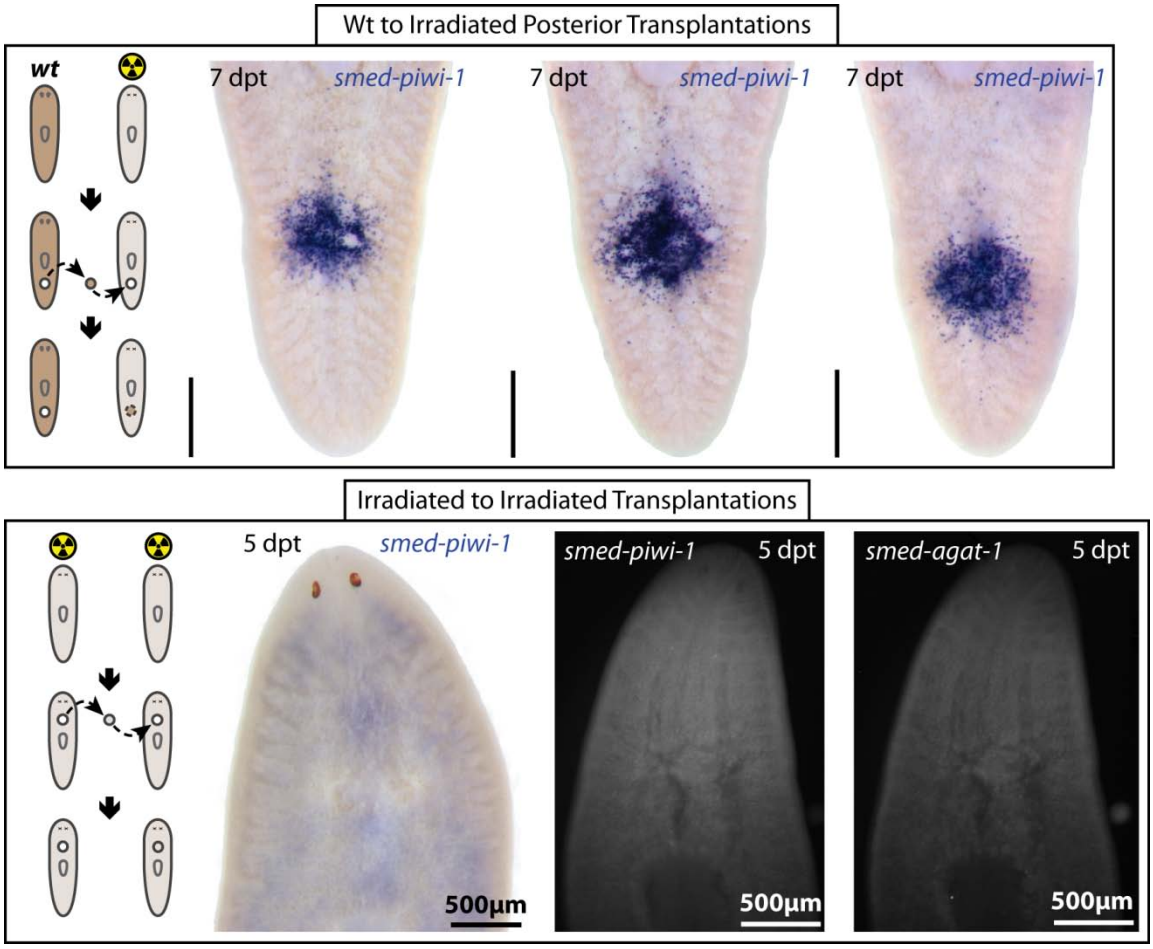

2 dpt
3 dpt
4 dpt
5 dpt
7 dpt
12 dpt
Neoblasts
Mitoses
A Smed-piwi-1
B Smed-piwi-1
C Smed-piwi-1
D Smed-piwi-1
E Smed-piwi-1
F Smed-piwi-1
G α-H3P
H α-H3P
I α-H3P
J α-H3P
K
Posterior
Right
Anterior
Left
0°
45°
90°
135°
180°
225°
270°
315°
Distance From Graft of αH3P(+) Cells (μm)
0
200
400
600
3 5 7
Time (dpt)
L wt 1°
2°
20 Days
or
15 Days
or
10 Days
or
5 Days
M Smed-piwi-1
1°
N Smed-piwi-1
2°
N'
O
Survival (%)
100
60
20
0
20 Days
15 Days
10 Days
5 Days
0 4 8 12 16 20 24
Time After Serial Transplantation (Days)

had appeared within the host (Fig. 3.6B, arrowheads). Observing successive days following transplantation, we concluded that stem cells were progressively repopulating the host tissue adjacent to the graft (Fig. 3.6C-F). Altering transplantation location did not appear to affect repopulation (Fig. 3.7). Predictably, in control irradiated grafts transplanted into irradiated hosts, we did not detect any cells that spontaneously expressed *Smed-piwi-1*, nor any newly made stem cell progeny (Fig. 3.7).

Congruent with the loss of *Smed-piwi-1* expression, lethally irradiated planaria are also completely devoid of mitotic activity after 24 hours (Reddien et al., 2005a). Therefore, we looked at the distribution of mitotic activity at corresponding time points to determine if the stem cells repopulating the host are mitotically active (Fig. 3.6G-J). Similar to what was observed by WISH, at 2 dpt the majority of mitotic activity was contained within the graft tissue and only a small number of mitotically active cells were seen in the host (Fig. 3.6G). However, at subsequent time points an increasing number of mitotic cells were observed further outside the graft and within host tissue (Fig. 3.6H-J). From both the WISH data and analysis of mitotic activity it appeared that stem cell repopulation of host tissue progressed in a generally radial manner with a minor longitudinal bias. Indeed, quantification of the distance of mitotic cells from the graft-host boundary at various time points following transplantation confirmed that stem cell repopulation progressed nearly equally in all directions with a slight increase anteriorly and posteriorly (Fig. 3.6K). Although small, the observed directional bias is more consistent with an active directional cell migration mechanism than with random passive movement due solely to proliferation.

Figure 3.7. Transplantation Control Experiments. Experiments were preformed to test whether migration was dependent on the graft location and whether transplantation alone induces reemergence of stem cell marker expression in irradiated tissue. Wild-type to irradiated transplantations where posterior tissue was taken from a wild-type donor and grafted into the posterior of a lethally irradiated host (top diagram) as was done as described for anterior transplantations. Animals were then WISH labeled for the stem cell marker *Smed-piwi-1* 7 days posttransplantation (dpt) and the grafts within the tails were imaged (top panel). Three replicate individual animals all showed levels of migration comparable to anteriorly grafted animals of the corresponding time point (compare to Fig. 3.6E). Minor differences may exist but the overall radial migration appears similar, suggesting that graft location does not appear to influence gross radial repopulation.

Additionally, irradiated to irradiated anterior transplantations were performed (bottom diagram) and the animals were labeled by WISH for *Smed-piwi-1* and the late progeny marker *Smed-agat-1* at 5 dpt (bottom panel). A lack of any staining for either colorimetric or fluorescent detection of the stem cell marker or the progeny marker was observed. These data indicated that all stem cells and late progeny that were present prior to transplantation were no longer detectable 6 days postirradiation and that transplantation does not induce irradiated tissue to spontaneously produce stem cells or late progeny by dedifferentiation. Transplantations and WISH were performed as described in Experimental Procedures.


Wt to Irradiated Posterior Transplantations
wt
7 dpt
smed-piwi-1
7 dpt
smed-piwi-1
7 dpt
smed-piwi-1
Irradiated to Irradiated Transplantations
5 dpt
smed-piwi-1
500µm
smed-piwi-1
5 dpt
500µm
smed-agat-1
5 dpt
500µm

Migrating Stem Cells Are Highly Potent and Capable of Rescuing Additional Lethally Irradiated Hosts

Having observed repopulation of *Smed-piwi-1* positive stem cells and mitotic activity in irradiated hosts rescued with healthy tissue grafts, we next asked whether the migrating stem cells are functionally equivalent to the stem cells that remain in the graft. To test this we used serial transplantations where, following a primary graft of healthy tissue into a lethally irradiated host as previously described (Fig. 3.1A), the now grafted host is reused as a donor and tissue taken from a region anterior to the primary graft is transplanted into a naïve irradiated worm (Fig. 3.6L). The tissue used for the secondary transplantation therefore contained those cells that have left the primary graft and migrated into the irradiated primary host (Fig. 3.6M). WISH for *Smed-piwi-1* in serially transplanted animals revealed that indeed stem cells are successfully grafted into the final host using this novel method (Fig. 3.6N).

Scoring for survival of serially transplanted animals revealed that grafts containing migrating stem cells are capable of rescuing lethally irradiated hosts (Fig. 3.6O). However, depending on the amount of time allowed to pass between the primary and secondary transplantations, different rates of survival were observed. For instance, no (0/10) animals survived when only 5 days were allowed to pass between the primary and secondary transplantations, whereas when the time between transplantations was increased to 10 and 15 days then ~36% (4/11) and ~77% (13/17) of the final hosts survived, respectively. We presume that increased time between transplantations is correlated with increased survival because as time passes after the primary transplantation more stem cells repopulate the area that is then taken for the secondary

graft. Ergo, if the repopulating stem cells are functionally equivalent to those contained in the primary graft, given enough time between transplantations, the host tissue should be sufficiently repopulated such that all serially transplanted animals are rescued, as is the case with primary transplantations. Indeed, when the time between primary and secondary transplantation was further increased to 20 days, 100% (12/12) of animals survived.

The Positional Relationships between the Stem Cell and the Progeny Populations Are Reestablished during Repopulation

Having observed the pattern of stem cell repopulation following transplantation, we next asked if stem cell progeny repopulation followed similar patterns. After irradiation, loss of early (at 2 dpi) and late stem cell progeny (at 4 dpi) quickly follows loss of the stem cell population (at 1 dpi) (Eisenhoffer et al., 2008), and altering the relationship between stem cells and their progeny has lethal consequences in planaria (Pearson and Sánchez Alvarado, 2010). We therefore expected that reestablishment of normal stem cell-progeny relationships would be crucial to the rescue of tissue homeostasis and long-term survival. Additionally, characterization of stem cell progeny repopulation would provide additional reference points against which underlying stem cell migrations can be evaluated.

In intact planaria the stem cells reside proximal to the gut near the core of the animal, early progeny inhabit a layer distal to the stem cell zone, and late progeny exist in a layer distal to the early progeny (Eisenhoffer et al., 2008) (Fig.3.8A). During tissue

homeostasis, stem cell progeny progress from the stem cell zone distally, turning off expression of stem cell markers and turning on corresponding progeny markers as they transition through each zone. We therefore looked, following transplantation, at the expression domains of two routinely used progeny markers, *Smed-NB.21.11e* and *Smed-AGAT-1*, known to identify early and late stem cell progeny respectively (Fig. 3.8). Because our earliest time point (4 dpt, 5 dpi) occurs after *Smed-NB.21.11e* and *Smed-AGAT-1* progeny are absent following irradiation (4 dpi), we can assume that host expression of these markers is gone and that the progeny identified are derived from the graft tissue.

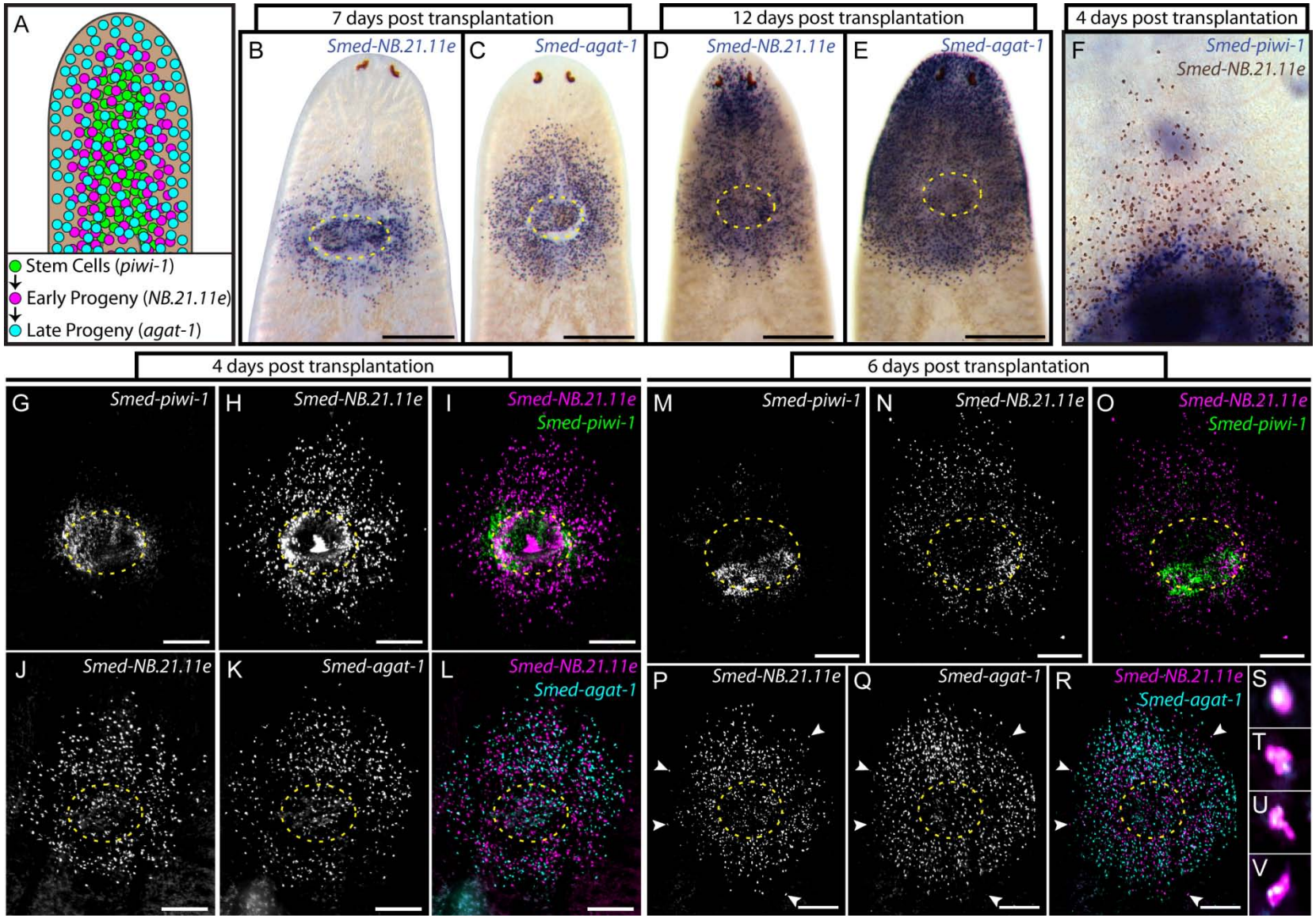
We found that both the early progeny (Fig. 3.8B,D,H,N) and the late progeny (Fig. 3.8C,E,K,Q) progress radially out of the graft in a manner characteristic of the repopulating stem cells. We therefore sought to determine if the positional relationships are reestablished in a lethally irradiated host rescued by transplantation. Similar to the positional relationship between early and late progeny in intact worms, the *Smed-NB.21.11e* zone of expression (Fig. 3.8B) appeared smaller than the *Smed-AGAT-1* zone of expression (Fig. 3.8C) at 7 dpt in most animals. The more expanded late progeny zone became more obvious when *Smed-NB.21.11e* and *Smed-AGAT-1* animals were compared at 12 dpt (Fig. 3.8D-E), when expression of early and late progeny markers in the anterior third of rescued animals largely resembled wild-type (Eisenhoffer et al., 2008). Colorimetric staining for both *Smed-piwi-1* and *Smed-NB.21.11e* in the same animal (Fig. 3.8F) revealed that the wild-type relationship between stem cells and their early progeny may already be reestablished at this early time point (4 dpt).

For the purposes of this study, shortly after transplantation, the graft acts as a

Figure 3.8. Repopulation Reestablishes the Normal Stem Cell-Progeny Positional Relationships

(A) Schematic showing the nested positional relationships between the stem cell, early progeny, and late progeny lineage during normal tissue homeostasis (Eisenhoffer et al., 2008). WISH for the early progeny marker (*Smed-NB.21.11e*) at 7 dpt (B) reveals a zone of expression that appears smaller than the late progeny marker (*Smed-AGAT-1*) zone at 7 dpt (C) in independent transplantation rescued animals, suggesting that the early to late progeny positional relationship is reestablished by this time point (yellow ellipses indicate the graft location). The positional relationship between the early (D) and late progeny (E) is fully recovered in the anterior ends of 12 dpt animals as they appear very similar to wild-type. (F) Extended focal plane zoomed image of the area anterior to the graft in a transplantation rescued animal 4 dpt stained for stem cells and early progeny shows the early progeny cells extended further anteriorly than the stem cells. (G-I) Double fluorescent WISH showing the entire smaller stem cell domain (G) as compared to the early progeny domain (H) at 4 dpt, confirming that the stem cell to early progeny positional relationship is reestablished at this time point. (J-L) Double WISH for the early (J) and late (K) progeny domains at 4 dpt showing their largely overlapping domains and lack of normal positional relationship. (M-O) Staining for stem cells (M) and early progeny (N) at 6 dpt shows that the reestablished relationship is maintained. (P-R) Staining for early (P) and late progeny (Q) at 6 dpt reveals a slightly smaller early progeny zone as compared to the late progeny zone. The merged image (R) shows that *Smed-AGAT-1* late progeny cells are predominately the most distal cells with the exception of a small number of *Smed-NB.21.11e* expressing cells (arrowheads). Closer

analysis of these most distal *Smed-NB.21.11e* expressing cells revealed that they also express *Smed-Agat-1* at low levels (S-V), suggesting that these cells may be transitioning from early to late progeny. Anterior is up. Fluorescent images are z-projections from confocal stacks (G-R, except S-V which are single slices). (F) Extended focal plane rendering done using CombineZP, 200x mag. Scale bars are 500 μm (B-E) and 200 μm (G-R).


A
Stem Cells (piwi-1)
Early Progeny (NB.21.11e)
Late Progeny (agat-1)
7 days post transplantation
B Smed-NB.21.11e
C Smed-agat-1
12 days post transplantation
D Smed-NB.21.11e
E Smed-agat-1
4 days post transplantation
F Smed-piwi-1
Smed-NB.21.11e
4 days post transplantation
G Smed-piwi-1
H Smed-NB.21.11e
I Smed-NB.21.11e
Smed-piwi-1
J Smed-NB.21.11e
K Smed-agat-1
L Smed-NB.21.11e
Smed-agat-1
6 days post transplantation
M Smed-piwi-1
N Smed-NB.21.11e
O Smed-NB.21.11e
Smed-piwi-1
P Smed-NB.21.11e
Q Smed-agat-1
R Smed-NB.21.11e
Smed-agat-1
S
T
U
V

point source of stem cells and progeny, as all cell types share the same general location. Therefore, we sought to determine at what point during repopulation the wild-type positional relationships between the stem cells and their progeny are reestablished. Double fluorescent WISH for *Smed-piwi-1* and *Smed-NB.21.11e*, even as early as 4 dpt (Fig. 3.8G-I), showed that the stem cells reside in a zone much smaller than the early progeny zone, suggesting that already the general positional relationship between the stem cells and their early progeny is reestablished. This is, however, in contrast to the similar zones of expression for *Smed-NB.21.11e* and *Smed-AGAT-1* in animals also at 4 dpt (Fig. 3.8J-L), in which case the positional relationship between these early and late progeny had yet to be reestablished. Not until 6 dpt did the late progeny zone of expression begin to exceed that of the early progeny (Fig. 3.8P-R). Although some *Smed-NB.21.11e* expressing cells appear near the distal edges of the *Smed-AGAT-1* zone (Fig. 3.8R, arrowheads), closer inspection revealed that these cells also lowly expressed *Smed-AGAT-1* (Fig. 3.8S-V), suggesting that the most distal early progeny cells were in the process of transitioning into late progeny. Co-staining for stem cells and early progeny also at 6 dpt revealed that their reestablished relationship is maintained at this time point (Fig. 3.8M-O). These data combined indicated that, within a week of transplantation, repopulating stem cells and their progeny reestablish positional relationships that are characteristic of those observed during normal tissue homeostasis in an intact planaria. Furthermore, although we did not independently follow the migration of stem cell progeny that exist in the graft at the time of transplantation, we inferred from their short (2 day) life-span (Eisenhoffer et al., 2008) that repopulation of the host

progeny compartments was largely due to daughter cells produced by the transplanted stem cells.

We cannot definitively say what amount of the observed progeny migration was due to movement of the progeny themselves or movement of the stem cells prior to becoming progeny. However, the high rate of turnover of stem cell progeny (Eisenhoffer et al., 2008) and the relatively small migration of stem cell progeny in the absence of stem cells (Fig. 3.9) led us to conclude that the repopulation dynamics of the stem cell progeny was at least partly due to the underlying movements of the stem cells themselves. Also, the distance to which the zones of stem cell progeny extend out beyond the stem cell zone (Fig. 3.8G-I and 3.8M-O) is in the range of the known migratory capabilities of stem cell progeny already described (Newmark and Sánchez Alvarado, 2000).

Tissue Transplantation Rescues Regeneration

Lethally irradiated planaria are known to correspondingly lose their ability to regenerate with the loss of mitotically active stem cells (Bardeen and Baetjer, 1904; Hayashi et al., 2006). Having described the process of stem cell repopulation of rescued hosts, we sought to test whether regeneration is also restored in lethally irradiated hosts grafted with healthy tissue. Regeneration was assayed by performing transplantations as described and decapitating the grafted animals (Fig. 3.10A) then scoring for the appearance of blastemas and new photoreceptors in the remaining posterior fragments. First, we evaluated the effect of transplantation itself on head regeneration in nonirradiated planaria by grafting healthy tissue into healthy hosts. Nonirradiated hosts

Figure 3.9. Transplantation and Sequential Irradiation Experiments Reveal Minimal Migration of Stem Cell Progeny Alone. Transplantations of wild-type tissue into lethally irradiated hosts were performed as previously described and the animals were subjected to an additional irradiation to ablate the transplanted stem cells and leave the non-proliferative progeny (Experimental Schematic). The experimental timing was performed as diagramed (IR = irradiation, Trans. = transplant, Amp. = amputation, Fix = fixation for staining). Also, the predicted presence of stem cells (blue bars), early progeny (green bars), and late progeny (red bars) based on their known survival following irradiation (Eisenhoffer et al., 2008) was diagramed. Therefore, by design, the stem cells should be nearly gone by the time of amputation, leaving only progeny to respond to wounding. WISH for the late progeny marker (*Smed-agat-1*) in sequentially irradiated animals shows little difference between intact and amputated animals regardless of whether they display a large (top 2 panels) or small (bottom 2 panels) number of persisting progeny. These data indicate that the progeny alone do not migrate substantially more in response to amputation as compared to intact controls within the period of time that they exist following irradiation. Therefore, combined with their known short lifespan (Eisenhoffer et al., 2008), we predict that the progeny contribution to the overall observed migration, especially in response to amputation, is minimal. WISH, tissue transplantation, and γ-irradiation were performed as described in Experimental Procedures. Scale bars are 500 μm.

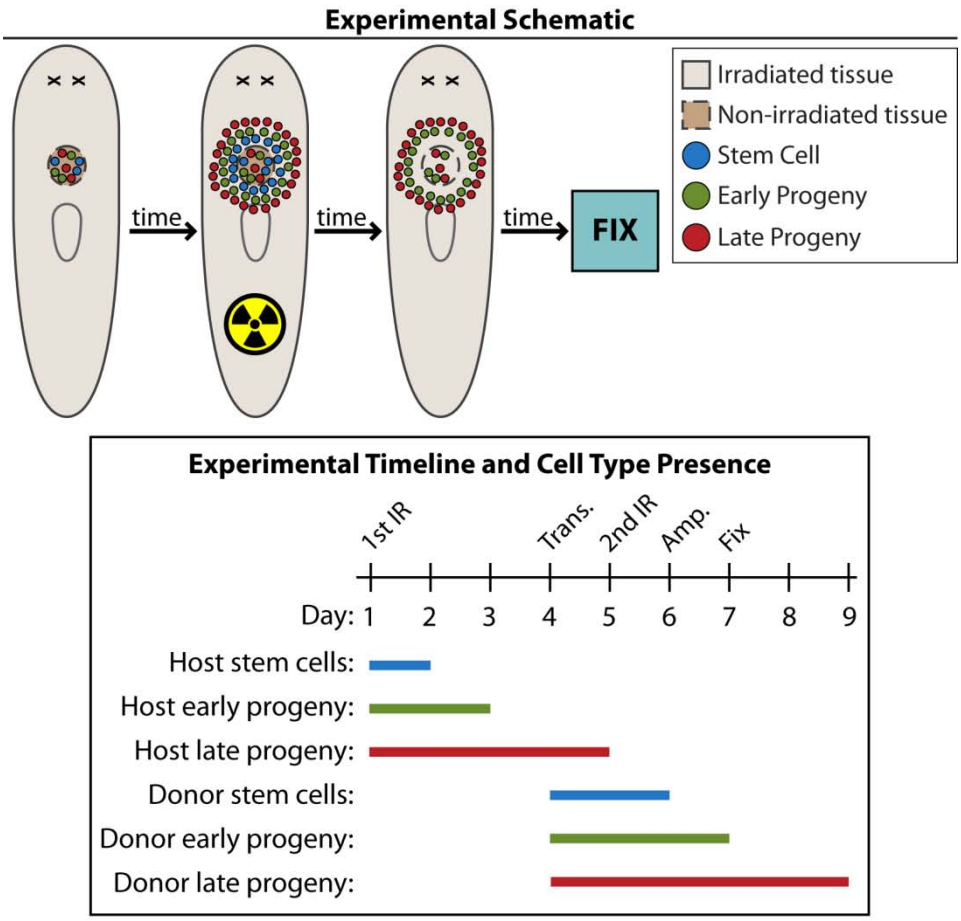
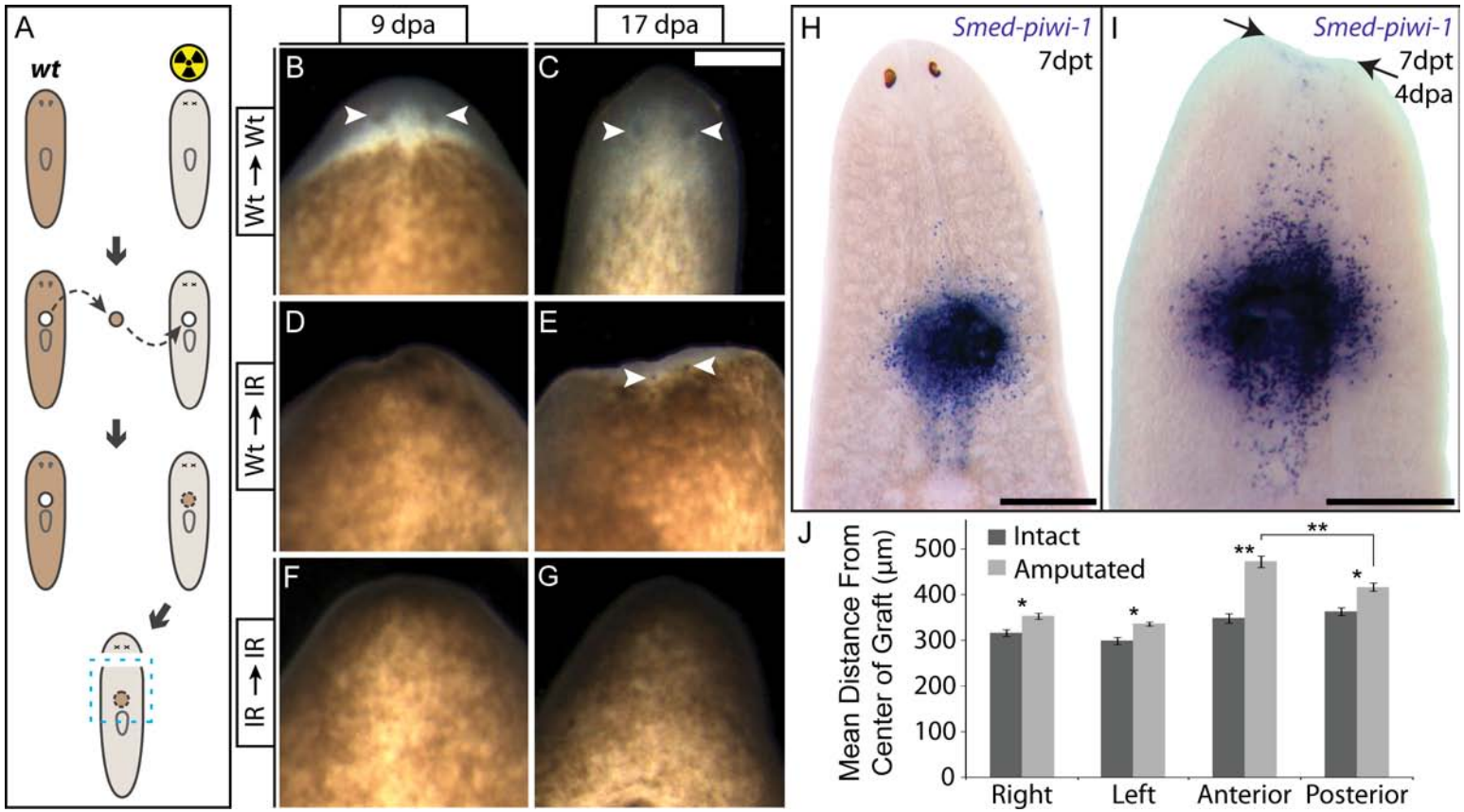

Experimental Schematic
time
time
time
FIX
Irradiated tissue
Non-irradiated tissue
Stem Cell
Early Progeny
Late Progeny
Experimental Timeline and Cell Type Presence
1st IR
Trans.
2nd IR
Amp.
Fix
Day: 1 2 3 4 5 6 7 8 9
Host stem cells:
Host early progeny:
Host late progeny:
Donor stem cells:
Donor early progeny:
Donor late progeny:
Intact
Amputated
Sequentially Irradiated
3dpt
Smed-agat-1
3dpt
Smed-agat-1
3dpt
Smed-agat-1
3dpt
Smed-agat-1

Figure 3.10. Transplantation Rescues Regeneration and Additional Wounding Increases Stem Cell Migration. (A) Transplantation and amputation scheme showing irradiations and transplantations as previously diagramed followed by amputation 2-3 days following grafting. (B-C) Live images of a decapitated nonirradiated graft (wt) into a nonirradiated host (wt) control transplantation, showing a well formed anterior blastema at 9 days postamputation (dpa) (B, arrowheads indicate newly forming photoreceptors) and a completely reformed head at 17 dpa (G, arrowheads indicate regenerated photoreceptors). (D-E) Decapitated lethally irradiated host (IR) grafted with nonirradiated tissue (wt) shows no blastema at 9 dpa (D) and a small blastema at 17 dpa (E, arrowheads indicate newly forming photoreceptors), clearly showing a regeneration delay as compared to the positive control (B-C). (F-G) Negative control decapitated lethally irradiated host (IR) grafted with lethally irradiated tissue (IR) show no regeneration at 9 (F) or 17 dpa (G). (H) WISH for stem cell marker (*smed-piwi-1*) in 7 dpt otherwise intact animal (reproduced from Fig. 2E for reference). Compared to a non-decapitated animal (H) the migration out of the graft of the stem cell population identified by WISH in a 7 dpt and 4 dpa animal (I) is increased and predominately shifted towards the anterior with a small number of stem cells present at the amputation plane (arrows). (J) The impact of amputation (I for example) on the mean distance of stem cells from the center of the graft was quantitated in four directions (right, left, anterior, and posterior) and compared to non-amputated controls (H for example) in animals 7 dpt (see Experimental Procedures for quantification method). The greatest significant (**, $p < 0.002$, Wilcoxon rank sum test) increase following amputation was seen in the anterior direction. However significant (*, $0.01 < p < 0.02$, Wilcoxon rank sum test) increases

were seen in all other directions as well. Anterior is up. Scale bars are 500 μm. Error bars represent SEM.


A
wt
9 dpa
17 dpa
Wt → Wt
Wt → IR
IR → IR
B
C
D
E
F
G
H
Smed-piwi-1
7dpt
I
Smed-piwi-1
7dpt
4dpa
J
Mean Distance From Center of Graft (µm)
Intact
Amputated
500
400
300
200
100
0
Right
Left
Anterior
Posterior
*
**

regenerated heads along the same progression as amputated wild-type animals, forming a blastema containing regenerated photoreceptors by 9 dpa (Fig. 3.10B) and a complete pigmented head by 17 dpa (Fig. 3.10C). Interestingly, irradiated hosts grafted with healthy tissue did regenerate heads following amputation although the rate of regeneration was greatly delayed in comparison to nonirradiated controls (Fig. 3.10D-E). As hypothesized previously from related work (Dubois, 1949), we also attributed the observed delay in blastema formation to the time necessary for healthy stem cells in the graft to migrate through the irradiated host tissue and reach the amputation plane. As expected, irradiated hosts grafted with irradiated tissue did not regenerate heads following amputation (Fig. 3.10F-G).

Wounding Increases Stem Cell Migration

The functional recovery of regeneration in transplantation rescued animals led us to ask whether the migration of repopulating stem cells is altered by amputation. Therefore, we directly compared stem cell migration in rescued animals that were allowed to heal for 3 dpt and then either left intact or decapitated (Fig. 3.10A). Qualitatively, stem cells in 7 dpt decapitated animals appeared to have increased their migration relative to their intact cohorts (Fig. 3.10I vs. 3.10H). In fact, in 7 dpt decapitated animals stem cells were seen as far anterior as the amputation plane (Fig. 3.10I, arrows), having migrated greater than 500 microns from the graft in as few as 4 dpa.

Having seen qualitative changes in stem cell migration following amputation, we sought to quantitate these changes, specifically focusing on whether the apparent increase

is global or specific to certain portions of the migrating stem cell population. We therefore measured the mean distance of stem cells from the center of the graft within 45 degree bins in four directions (anterior, posterior, right, and left) radiating out from the graft in both intact and decapitated animals (Fig. 3.10J). As predicted from qualitative observations, the decapitated animals showed a highly significant increase ($p < 0.002$) in the distance traveled by anteriorly directed stem cells as compared to anteriorly directed stem cell in intact transplantations (Fig. 3.10J, Anterior). Surprisingly, significant increases ($0.01 < p < 0.02$) in the mean distance of stem cells from the center of the graft following decapitation were also found in the posterior, right, and left directions although less so than in the anterior direction (Fig. 3.10J). In support of these findings, analysis of the position of mitoses, as previously described, also revealed an increase in anterior migration following anterior amputation as compared to intact animals and an increase in anterior migration was not observed following posterior amputation alone (Fig. 3.11). These data together suggest that subsequent decapitation of transplantation rescued animals induced not only a global increase in stem cell migration, but also an even greater specific increase in stem cell recruitment towards the anterior wound.

Planarian Stem Cells Are Directionally Recruited to Wounds

Although the transplantation method is highly effective for stem cell repopulation and functional analyses, stem cell recruitment studies are complicated by the presence of the wound caused during transplantation. Therefore, we utilized our newly developed partial irradiation technique to determine whether stem cells respond to wounding with a directional migration as some of the transplantation evidence suggests. We performed

Figure 3.11. Migrating Mitotic Stem Cells Respond Directionally to Anterior Amputation. Transplantations of wild-type tissue into lethally irradiated animals were performed as previously described. Two days following transplantation animals were left intact, amputated anteriorly, amputated posteriorly, or amputated both anteriorly and posteriorly and allowed to regenerate for 5 days. Animals were fixed 7 days post-transplantation and immunostained for anti-phosphohistone H3 (α-H3P) as shown in Fig. 3.6. The mean distance of α-H3P(+) stem cells from the graft in the anterior and posterior direction was measured as described in Experimental Procedures. A significant increase in anterior migration as compared to intact controls was seen when animals were amputated anteriorly either alone or together with posterior amputation. No significant increase in anterior migration as compared to intact controls was seen when animals were amputated posteriorly only. A significant increase in posterior migration as compared to intact controls was seen when animals were amputated both posteriorly and anteriorly, but not when animals were amputated posteriorly alone. These data suggest that amputation both causes a global increase in migration during repopulation and that stem cells respond directionally to anterior amputation. However, significant noise in these data, likely due to the presence of the additional wound inherent in the transplantation, argues for further analysis using the partial irradiation scheme, where wounding can be more tightly controlled. P-values are One Way ANOVA.

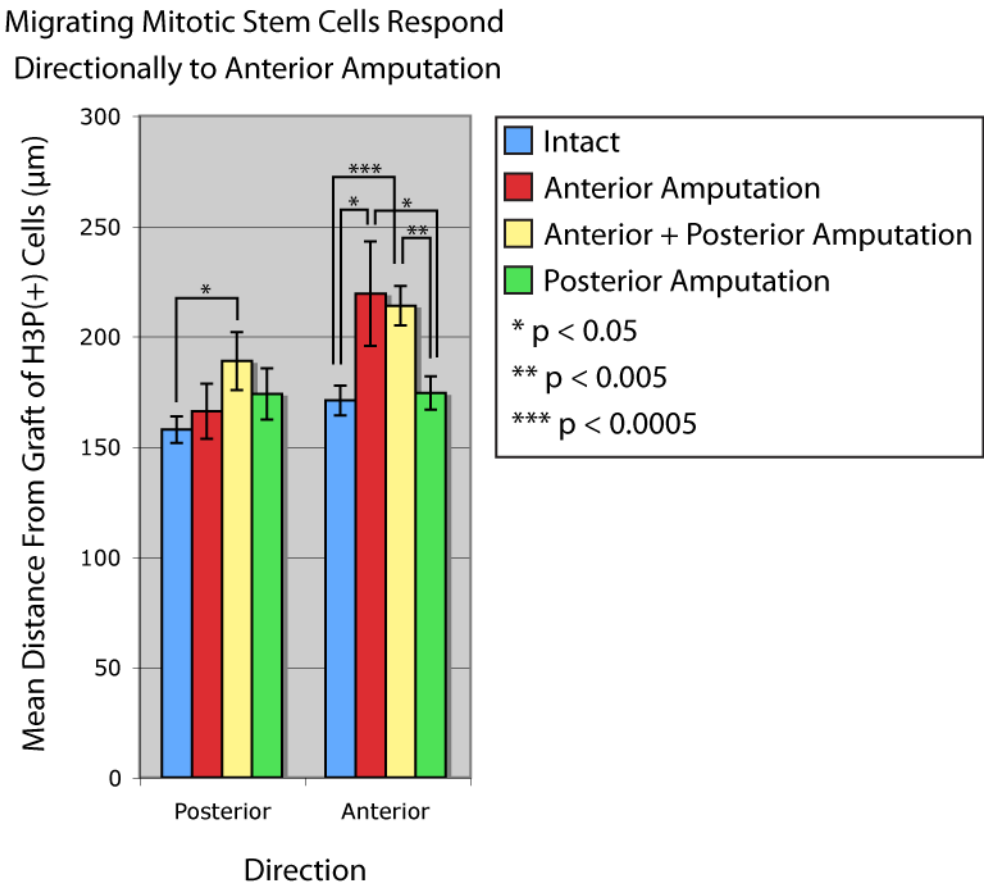
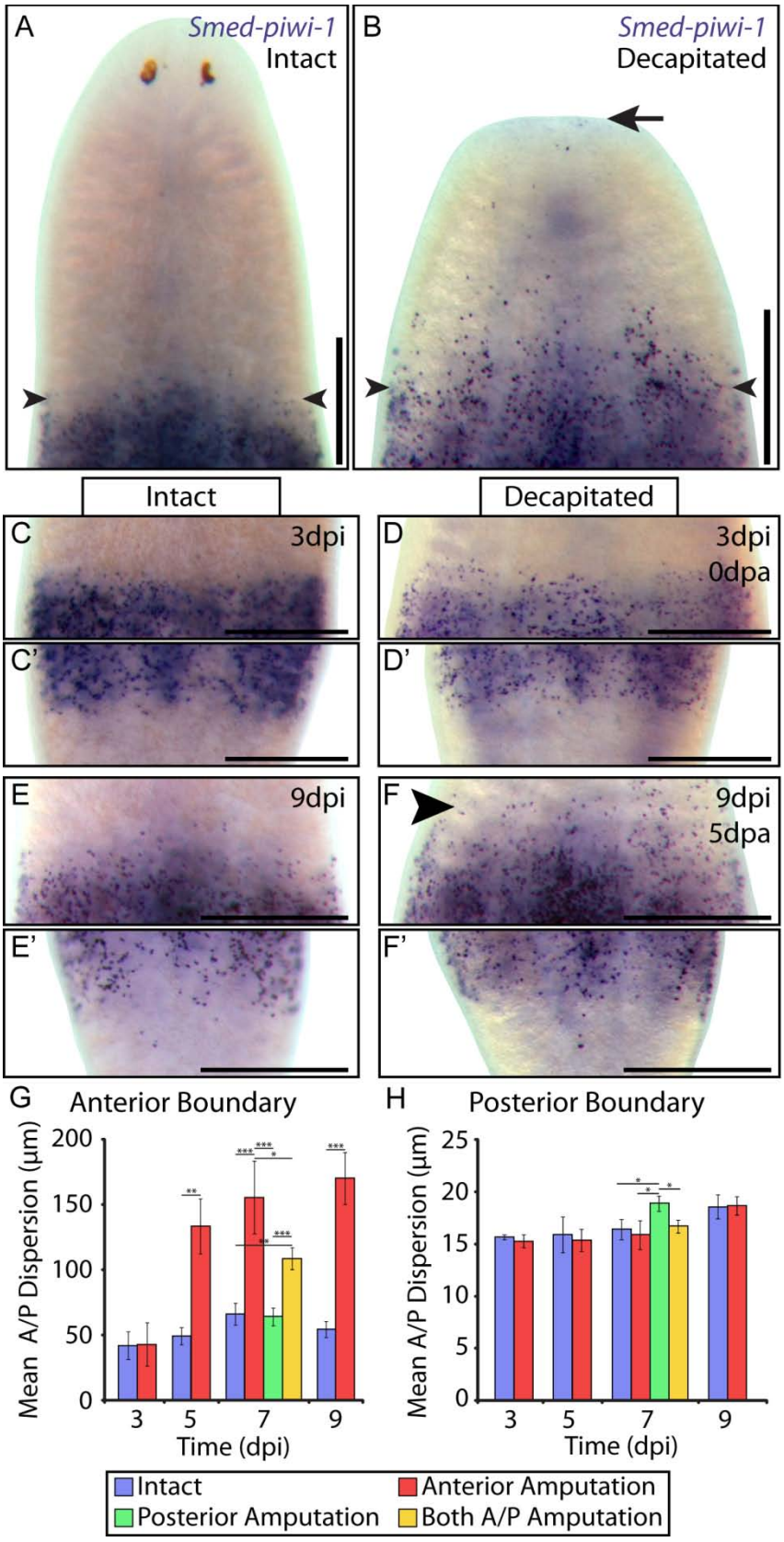

Migrating Mitotic Stem Cells Respond
Directionally to Anterior Amputation
Mean Distance From Graft of H3P(+) Cells (µm)
300
250
200
150
100
50
0
Posterior
Anterior
Direction
Intact
Anterior Amputation
Anterior + Posterior Amputation
Posterior Amputation
* p < 0.05
** p < 0.005
*** p < 0.0005

Figure 3.12. Following Amputation Stem Cells Are Recruited Towards Wounds

(A) The anterior irradiated portion of a partially irradiated animal stained for stems by WISH reveals the clean boundary (arrowheads) present even at 9 dpi and the complete lack of stem cells anterior to the boundary. (B) Following decapitation (5 dpa) WISH reveals dispearse stem cells anterior to the estimated boundary (arrowheads) and some as far anterior as the amputation plane (arrow) in a partially irradiated at 9 dpi. Magnified images of the anterior (C-F) and corresponding posterior (C'-F') stem cell boundaries of intact and decapitated partially irradiated animals at 3 and 9 dpi show the varied amounts of stem cell dispersion at those boundaries. Anterior (C) and posterior (C') boundaries of intact partially irradiated animals at 3dpi show clean boundaries with little dispersion. Anterior (D) and Posterior (D') boundaries of 3dpi animals decapitated only hours before fixation also show clean boundaries with low levels of dispersion. Intact animal boundaries at 9 dpi (E and E') show slightly more dispersion of stem cells at the boundaries compared to intact animals at 3 dpi (C and C') though the irradiated regions remain largely devoid of stem cells (see A for reference). (F) The anterior boundary of a decapitated (5dpa) 9 dpi partially irradiated animal shows large amounts of dispersion with stem cells present far anterior of the boundary. This is in contrast to the posterior boundary of the same animal (F') which shows comparative levels of dispersion to its intact counterpart (E'). A/P dispersion was quantified (see Experimental Procedures for details) at anterior (G) and posterior (H) boundaries in intact, anteriorly amputated (decapitated), posteriorly amputated, or both anteriorly and posteriorly amputated animals at 3, 5, 7, and 9 dpi, corresponding to 0, 2, 4, and 6 dpa respectively (i.e. animals

were amputated at 3 dpi). See text for analysis. Significance: *** $p<0.001$, ** $p<0.01$, * $p<0.05$, one-sided Wilcoxon rank sum test. Error bars are SEM. All scale bars are 500 μm.


A
Smed-piwi-1
Intact
B
Smed-piwi-1
Decapitated
Intact
Decapitated
C
3dpi
D
3dpi
0dpa
C'
D'
E
9dpi
F
9dpi
5dpa
E'
F'
G
Anterior Boundary
Mean A/P Dispersion (µm)
200
150
100
50
0
3
5
7
9
Time (dpi)
H
Posterior Boundary
Mean A/P Dispersion (µm)
25
20
15
10
5
0
3
5
7
9
Time (dpi)
Intact
Anterior Amputation
Posterior Amputation
Both A/P Amputation

partial irradiations as previously described and illustrated in Fig. 3.1G, at which point the animals were amputated. Staining for stem cells in decapitated partially irradiated animals clearly showed stem cells in the anterior irradiated tissue at 5 dpa (Fig. 3.12B). This is in contrast to the complete lack of stem cells present in the anterior irradiated tissue in intact controls (Fig. 3.12A). The anterior (Fig. 3.12C) and posterior (Fig. 3.12C') boundaries between the stem-cell-occupied shielded tissue and the stem-cell-devoid irradiated tissue in intact animals was clearly discernable at 3 dpi. Likewise, the anterior (Fig. 3.12D) and posterior (Fig. 3.12D') boundaries of planaria decapitated at 3 dpi and fixed immediately following decapitation were also clearly discernable, indicating that the physical act of amputation itself did not directly affect the observed position of the stem cells within planaria. Although by 9 dpi the stem cells at the boundaries in intact planaria (Fig. 3.12E-E') were slightly more dispersed than those at 3 dpi (Fig. 3.12C-C'), the most anterior and posterior regions remained devoid of stem cells. Conversely, corresponding 9 dpi decapitated animals showed noteworthy scattering of stem cells at the anterior boundary and numbers of stem cells dispersed throughout the irradiated anterior region (Fig. 3.12F), thus obscuring the boundary. Strikingly, the posterior boundaries of the very same decapitated animals remained defined (Fig. 3.12F') and comparable to intact controls (Fig. 3.12E'). The apparent stem cell response at anterior boundaries and lack of stem cell response at posterior boundaries following decapitation suggested that stem cells may be directionally recruited by the anterior wound.

These observations suggested that stem cell dispersion (calculated as the mean standard error in the relative longitudinal location of the 50 most anterior cells, see

Experimental Methods for details) at anterior and posterior boundaries could be used as a means of measuring directional recruitment within the boundary stem cell populations. We therefore set about measuring anterior boundary dispersion in partially irradiated animals that had been left intact or subjected to anterior, posterior, or both amputations. We found that at 3 dpi, the day of amputation, there was no significant difference between intact and amputated animals (Fig. 3.12G-H). However, when animals were fixed at 2, 4, or 6 days following anterior amputation the anterior boundaries showed large increases in dispersion compared to corresponding intact animals (Fig. 3.12G, 5, 7, 9 dpi red vs. blue). Interestingly, anterior boundaries of animals amputated posteriorly showed levels of dispersion equivalent to intact controls (Fig. 3.12G, 7dpi green vs. blue), indicating that the stem cells at the anterior boundary migrate anteriorly in response to an anterior wound but do not migrate anteriorly after a posterior wound. Furthermore, animals amputated both anteriorly and posteriorly showed dispersion at their anterior boundaries that was significantly greater than intact controls (Fig. 3.12G, yellow vs. blue) but also significantly less than animals amputated only anteriorly (Fig. 3.12G, 7dpi yellow vs. red). This result may indicate competition for stem cells by opposing wounds and further supports the existence of wound directed stem cell migration.

We next repeated our analysis of boundary dispersion on posterior boundaries in the same animals and found largely congruent results. Predictably, stem cell dispersion at posterior boundaries was significantly increased by posterior amputation as compared to intact controls (Fig. 3.12H, 7dpi green vs. blue) and anteriorly amputated animals (Fig. 3.12H, 7 dpi green vs. red), indicating that stem cells migrate posteriorly in response to a

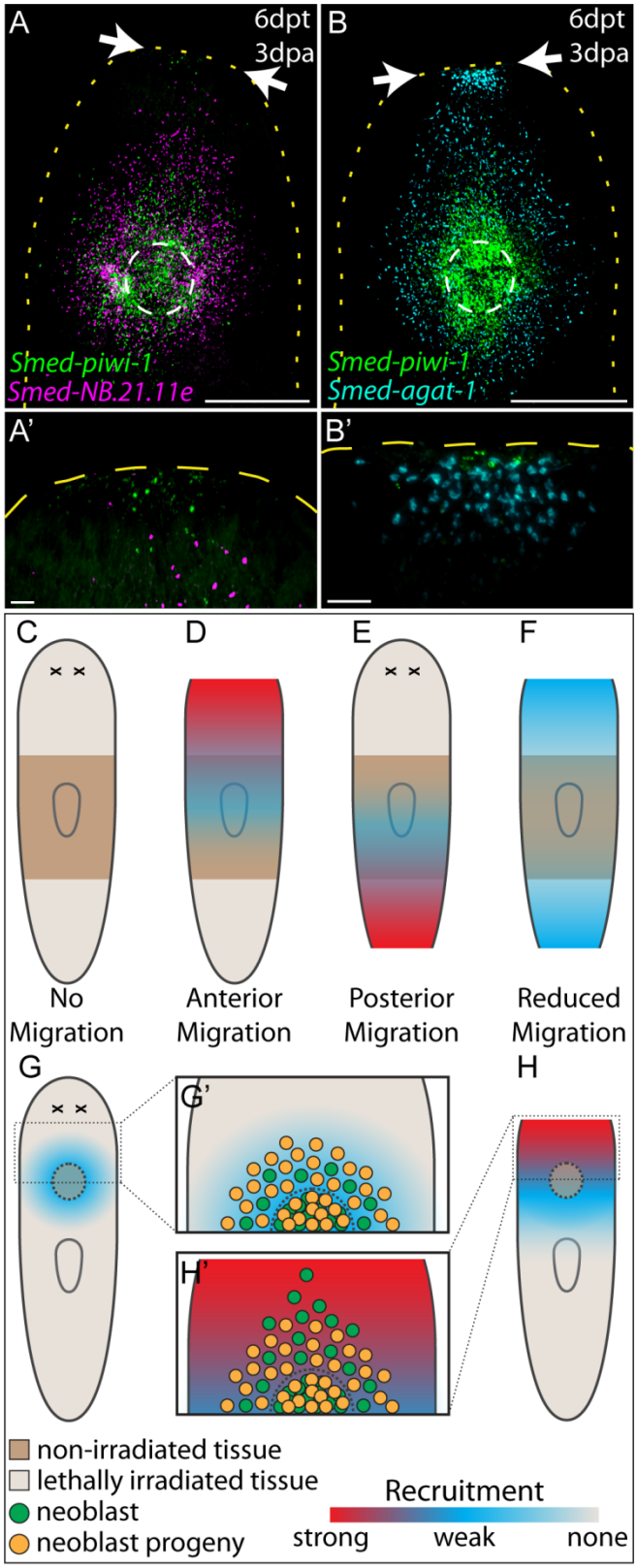
posterior wound but do not migrate posteriorly following an anterior wound. Additionally, we saw no significant difference in stem cell dispersion at the posterior boundaries between intact controls and anteriorly amputated animals at any time point following partial irradiation (Fig. 3.12H). Surprisingly, we did not see a significant increase in dispersion at the posterior boundary of animals amputated both posteriorly and anteriorly as compared to intact controls (Fig. 3.12H, 7 dpi yellow vs. blue). This could be due to a lack of sensitivity in the measurement because of the inherently lower levels of dispersion at posterior boundaries (compare y-axes in Fig. 3.12G and H) or it could reflect a real biological difference between the ability of anterior and posterior wounds to recruit stem cells. Either way, these data together further strengthen our conclusion that stem cells are recruited, i.e., migrate in response to wounding in a directional manner.

Wounding Spurs Stem Cells to Migrate Beyond Their Progeny

After establishing the directional recruitment of stem cells and the general characteristics of stem cell and progeny repopulation, we next sought to determine what influence increased stem cell recruitment might have on the relationships between stem cells and progeny. To this purpose, transplantations of healthy tissue grafts into lethally irradiated worms followed by decapitation were performed as previously described (see Fig. 3.10A). The relationships between the stem cells and their progeny were evaluated following the transplantation and subsequent amputation (Fig. 3.13A-B) and compared to what was seen during repopulation in otherwise intact animals (Fig. 3.8). Strikingly, co-staining for stem cells and their early progeny revealed a dramatic shift in the relationship

Figure 3.13. Wounding Induced Directional Recruitment Alters the Relationship between Stem Cells and their Progeny. (A) Fluorescent WISH for stem cells (*smed-piwi-1*) and early progeny (*smed-NB.21.11e*) after transplantation rescue and decapitation, showing the graft (white circle) and amputation plane (arrows) at 6 dpt and 3 dpa (compare to Fig. 4O, intact 6 dpt). The stem cell population is continuous from the graft to the amputation plane and in the magnified view of the amputation plane (A') stem cells are clearly present at the wound site anterior to the early progeny. (B) Fluorescent WISH for stem cells (*smed-piwi-1*) and late progeny (*smed-AGAT-1*) at 6 dpt and 3 dpa, again showing a continuous stem cell population from the graft (while circle) to the amputation plane (arrows) (compare to Fig. 4R, intact 6 dpt). (C') Magnified view shows stem cells slightly anterior to late progeny and a large mass of late progeny just posterior to the amputation site. For reference the edges of the animals are identified (yellow dashed lines). Magnified confocal projections (B' and C') obtained separately from and contain less z-depth than corresponding overview images (B and C).
(C) In the absence of a wound, stem cells are not induced to migrate even in a partially irradiated animal that contains large areas devoid of mitotic stem cells. (D) Anterior amputation in a partially irradiated animal triggers a hypothetical wound signal that strongly recruits stem cells from the nonirradiated tissue to migrate through the irradiated tissue to reach the anterior wound. (E) Likewise, posterior amputation in a partially irradiated animal also triggers a hypothetical wound signal that recruits stem cells. In neither the anterior (D) nor posterior (E) amputation do stem cells migrate into the non-amputated irradiated region, suggesting that wounding does not induce random migration but rather specific directed recruitment. (F) Combined anterior and posterior amputation

in partially irradiated animals results in weakened recruitment to both wounds as diametrically opposed hypothetical wound signals compete for stem cells. (G) Competing wound signals would theoretically also exist in a circular wound like that introduced during transplantation, resulting in weak recruitment, here referred to as repopulation, of stem cells into the irradiated host tissue. (G') Weak recruitment allows dividing and differentiating stem cells to produce progeny as they slowly invade host tissues. (H) Anterior amputation of a repopulating transplanted animal results in a summation of wound signals that shifts the recruitment anteriorly. (H') This new strong anterior recruitment either accelerates the anterior migration of stem cells or induces anteriorly migrating stem cells to repress their production of progeny such that stem cells are observed outside of the progeny zones that normally contain them during repopulation (A-A'). The demonstrated ability of stem cells to accelerate their migration, repress progeny production, and/or integrate multiple wound signals make this avenue of investigation not only biologically fascinating but also highly medically relevant. Scale bars are 500 μm (A-B) and 50 μm (A'-B').

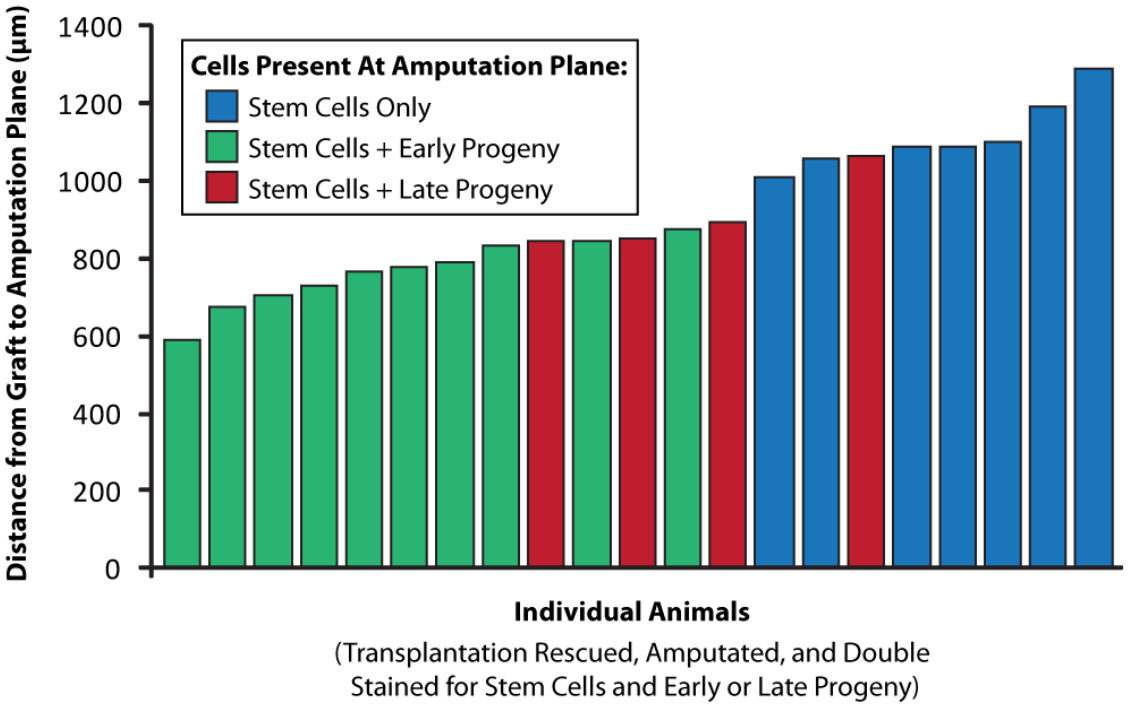

A
6dpt
3dpa
Smed-piwi-1
Smed-NB.21.11e
B
6dpt
3dpa
Smed-piwi-1
Smed-agat-1
A'
B'
C
D
E
F
No Migration
Anterior Migration
Posterior Migration
Reduced Migration
G
G'
H
H'
non-irradiated tissue
lethally irradiated tissue
neoblast
neoblast progeny
Recruitment
strong
weak
none

between these two cell populations, as stem cells were observed to exceed the anterior limit of the early progeny zone (Fig. 3.13A). Additionally, closer examination of the amputation plane (Fig. 3.13A arrows, 3.13A') revealed considerable numbers of stem cells present at the wound, clearly anterior to the most anterior early progeny. Interestingly, stem cells can be seen evenly spread throughout the area between the graft and the amputation plane (Fig. 3.13A) suggesting that although altered, the migration is continuous. Continuity in the spread of stem cells from the graft to the amputation plane was always seen, including in replicates where the distance from the graft to the amputation plane is shorter (Fig. 3.13B). Dense groups of late progeny at the amputation plane were also seen in these shorter distance replicates (Fig. 3.13B arrows) and closer inspection again revealed stem cells at the amputation plane that were at least as anterior as the most anterior late progeny (Fig. 3.13B').

These data together suggested that stem cells not only migrate out beyond the zone of early progeny but also serve as a new concentrated source of progeny once they reach the amputation plane. A time course approach proved insufficient to isolate the appearance of the early progeny at the amputation plane from the appearance of the late progeny likely because of the close temporal relationship between the early and late progeny (Eisenhoffer et al., 2008). Further complicating matters, controlling for the amount of time following amputation did not prove sufficient to separate the appearance of stem cells and the individual progeny at the amputation site because of the variability inherent in the distance between the graft and the amputation plane. Nonetheless, the data suggested that for a given time point, the animals with a longer distance between the graft and the wound appeared more likely to have only stem cells present at the

Figure 3.14. Following Amputation in Transplantation Rescued Animals, Stem Cells Are the First to Reach the Wound Site. We took advantage of the variation in the distance between the amputation plane and the graft to increase the resolution of our time point analysis of the arrival of cell types at the amputation plane. From double WISH staining for stem cells and progeny (as shown in Fig. 3.13A-B), we observed three situations at the wound site: stem cells present alone, stem cells present with early progeny, and stem cells present with late progeny. By categorizing all of the imaged amputation planes, measuring the distance from the graft to the amputation plane, and ranking the categorized data based on the measured distance, a trend emerged. We saw a close correlation between amputation planes that contained stem cells only and a far distance from the amputation plane to graft. Therefore, when the stem cells have a longer distance to travel they are more likely to be alone at the amputation plane at the time of fixation (for a given time point). These data suggest that during wound directed migration, the stem cells arrive at the amputation plane first and are followed by the progeny, which appear either by slower migration or are produced by the stem cells already present at the amputation plane.

No animals with short distances (< 800 μm) between the amputation plane and the graft were stained for the late progeny marker, thus giving the incorrect appearance that late progeny and stem cells do not appear together in short graft to amputation distance animals. This situation has indeed been observed in separate experiments.


Distance from Graft to Amputation Plane (µm)
1400
1200
1000
800
600
400
200
0
Cells Present At Amputation Plane:
Stem Cells Only
Stem Cells + Early Progeny
Stem Cells + Late Progeny
Individual Animals
(Transplantation Rescued, Amputated, and Double Stained for Stem Cells and Early or Late Progeny)

amputation plane (Fig. 3.14). Indeed, the mean ranks of the distance from the graft to the wound were significantly different between those animals with only stem cells at the amputation plane and those with both stem cells and progeny ($p < 0.0005$, Kruskal-Wallis rank sum test). This trend led us to conclude that the stem cells were first to reach the wound and the progeny appeared second, either by migration or differentiation. Regardless, these results clearly demonstrated that the stem cell-progeny relationships that are reestablished during repopulation, become further altered by additional wounding. This process further supports the existence of an active and highly dynamic mechanism for recruiting stem cells following wounding in planaria.

Discussion

Stem Cell Ablation Alone Does Not Induce Recruitment

Whether ablation of stem cells, generally across animal taxa, results in recruitment of repopulating stem cells or other responses has remained an open question (Kai and Spradling, 2003; Zhong et al., 2002). The fact that stem cells were not seen to migrate into the irradiated portion of intact partially irradiated animals lead us to conclude that stem cell ablation alone does not induce stem cell recruitment in planaria (Fig. 3.13C). Only after partially irradiated animals were amputated were stem cells recruited into the irradiated tissue (Fig. 3.13D-F). In fact, the absence of both a mitotic response (Baguñà, 1975; Wenemoser and Reddien, 2010) and expression of known wound induced molecules in irradiated planaria (Gurley et al., 2010; Petersen and Reddien, 2009), strongly argue that irradiation alone does not induce a wound response.

Planaria certainly have cellular responses to irradiation (Pellettieri et al., 2010), but stem cell recruitment does not appear to be one of them.

Tissue Breech Initiates Stem Cell Recruitment

The absolute necessity of a breech in tissue integrity to initiate a wound response is consistent not only with our partial irradiation data but also the data of Dubois that is now over half a century old (Dubois, 1949). It has been argued that, in order to initiate a wound response, the breech in tissue integrity must result in the interaction of dorsal and ventral tissue (Kato et al., 1999). Our data for repopulation after transplantation, where dorsal and ventral zones of tissue do not interact, along with the work of others (Wenemoser and Reddien, 2010) suggest that dorsal-ventral tissue interaction is not necessary for mitotic and migratory wound responses. However, our work has shown that stem cell recruitment can be spurred by transplantation alone which is contrary to the conclusion of Wenemoser and Reddien that, "neoblast migration [sic] is induced by tissue absence rather than by injury *per se*" because the graft is of equal or greater size than the tissue removed from the host, resulting in no net tissue loss (Wenemoser and Reddien, 2010). These disparate conclusions could be due simply to the fact that our observations were done in very different locations within the animal, theirs being anterior to the photoreceptors and ours being the main body, or it remains possible that some other yet to be studied event is the true initiator of stem cell recruitment. Consistent with ours and previous work, the joining of two foreign tissues may be the initiating event that induces stem cell recruitment, as the only injury to not induce wound signaling is when

previously confluent tissues are rejoined in the case of an incision that is allowed the heal (Wenemoser and Reddien, 2010).

Stem Cells Are Recruited Towards Wounds
and Can Integrate Many Wound Signals

We attribute the slow repopulation of irradiated host tissue by transplanted stem cells to the wounding caused by the transplantation itself. One would expect a circular wound caused by inserting a circular graft to recruit cells from all directions and indeed we observed a corresponding radial migration of stem cells out of the wound (Fig. 3.13G). The geometry of a circular wound inherently establishes opposite wound signals in all directions, resulting in 360 degrees of diametrically opposed recruitment. This may well be the reason we see modest stem cell migration following transplantation, such that the stem cells are outrun by their progeny during repopulation (Fig. 3.13G') much like they exist during normal tissue homeostasis (Fig. 3.8A) (Eisenhoffer et al., 2008). Consequently, when anterior recruitment is activated upon decapitation the resulting product of wound signals results in either accelerated stem cell migration anteriorly or repressed differentiation in anteriorly migrating stem cells, such that stem cells are now capable of outrunning their progeny (Fig. 3.13H-H').

The complex interplay between wounding caused during transplantation and subsequent amputation has been largely ignored in previous work, which may have led in part to the conflicting conclusion that stem cells do not actively migrate (Flickinger, 1964; Saló and Baguñà, 1985). The work presented here complements a previous bromodeoxyuridine labeling study which challenged the standing hypothesis that stem

cell migration was due solely to proliferation-driven passive spreading (Newmark and Sánchez Alvarado, 2000). It is now clear that stem cells themselves actively migrate and are capable of integrating multiple wound signals to reach their required location.

A simpler example of competing wound signals is seen when recruitment in a partially irradiated animal amputated both anteriorly and posteriorly (Fig. 3.13F) is compared to recruitment in a partially irradiated animal amputated anteriorly only (Fig. 3.13D). We observed less anterior recruitment in doubly amputated worms and we hypothesize that this is due to the presence of a competing wound signal originating from the posterior wound. The fact that stem cells still migrate in the presence of opposing wound signals argues that either the net anterior and posterior signals are inherently unequal, resulting in one overpowering the other, or that the physical properties of the two migratory paths differ in some way, making it easier for cells to migrate in one direction than the other. This finding that stem cells appear to be capable of integrating multiple wound signals may partially explain the planarian's ability to regenerate from not only a myriad of different wounds but also an many combinations of multiple wounds as well.

What Is the Potency of Planarian Stem Cells?

Previous studies did not test whether the migrating stem cells were functionally equivalent to nonmigratory stem cells; thus the possibility remained that the migrating stem cells were in fact limited progenitors rather than multipotent stem cells (Dubois, 1949; Newmark and Sánchez Alvarado, 2000; Saló and Baguñà, 1985; Saló and Baguñà, 1989). For example, the bromodeoxyuridine labeled cells observed migrating into the

area anterior to the photoreceptors in intact animals were likely limited potency progenitors and not multipotent stem cells because mitotic stem cells are known not to reside in that location (Newmark and Sánchez Alvarado, 2000) and when separated from the body of the animal that tissue is known not to regenerate nor survive (Morgan, 1898). The serial transplantation experiments presented here directly tested, for the first time, the functional potency of the migrating cells because the serially transplanted tissue was taken from an area separate from the original graft. The fact that a portion of the animals that received serial transplantations survived long-term indicated that the migrating cells are *collectively* totipotent and, considering the small number of cells contained within the serial grafts, increases the likelihood that they may in fact be *individually* totipotent.

The gold standard test for adult stem cell potency is the rescue or reconstitution of an entire organ from a single cell. For instance, the regeneration of the lethally irradiated murine hematopoietic system is possible with transplantation of a single HSC, suggesting not only that the true stem cell of the hematopoietic system has been identified but also that HSCs are extremely potent (Osawa et al., 1996). Rescue of this sort is also made possible by keeping the mice in sterile laboratory conditions, subverting the mouse's immediate need for immune cells, and providing a temporary source of progenitors in a competitive repopulation scenario.

Rescue of a lethally irradiated planaria is a different matter because the extremely high rate of tissue turnover normally present throughout the worm is further exacerbated by the irradiation necessary to ablate stem cells (Eisenhoffer et al., 2008; Pellettieri and Sánchez Alvarado, 2007). This high rate of turnover necessitates incredible levels of proliferation if one transplanted stem cell is to rescue an entire worm. Furthermore,

because a transient amplifying population does not appear to exist in planaria (Newmark and Sánchez Alvarado, 2000), we cannot provide progenitors to temporarily produce cells necessary for survival while a transplanted stem cell amplifies itself. These hurdles may be overcome by future technical advances; nonetheless, we used our serial transplantation method to estimate the minimal number of stem cells needed to rescue a lethally irradiated worm in our context. Aided by single cell resolution WISH, we counted the number of smed-piwi-1 positive cells shortly following serial transplantation in a cohort (N = 6) of a larger population of specimens that were scored for survival (N = 17). Using both the counts and the survival data we were able to make the conservative estimate that ~578 cells (~100 cells/mm^2) are required to rescue a lethally irradiated host in this context (see Experimental Procedures for calculation). This represents a 19-27 fold decrease from the previous estimate, which was based on rescue of lethally irradiated planaria of a different species using a cell injection technique that has yet to be independently replicated (Baguñà et al., 1989). Since we know that the migrating stem cells are mitotically active and likely self-renewing from the time of the transplantation until fixation, we can safely assume that the true number of transplanted stem cells is in fact less than the measured amount (~168 cells, based on an average cell cycle time of ~21 hours (Kang and Sánchez Alvarado, 2009)). Furthermore, it remains likely that this number could be reduced by additional experiments specifically designed for the purpose which use an optimized radiation dose and/or reduce the size of the host to be rescued. Regardless, the ability of a few hundred cells to rescue an animal consisting of millions of cells within weeks clearly demonstrates the remarkable potency of migrating planarian stem cells.

Are Stem Cells Recruited to Wounds in Nonirradiated Animals?

Limited by available technology, we were only able to evaluate stem cell migration through irradiated tissues where the native stem cells have been ablated. The fact that stem cells are not activated to migrate following partial irradiation alone (Fig. 3.1) (Dubois, 1949) suggests that the act of irradiating does nothing to induce migratory behavior in these cells. There is, on the other hand, precedent from mouse that stem cell recruitment through irradiated tissue differs from recruitment through nonirradiated tissue (Takada et al., 1971). Previous work using chimeric transplantations between the sexual and asexual forms of S. mediterranea, as done in our study, suggested that although there may indeed be increased migration through irradiated tissues there nonetheless appears to be invasion of graft cells into host tissues whether or not the host has been irradiated (Saló and Baguñà, 1985). Saló, however, did not possess a cell cycle independent stem cell marker and instead used only mitoses as indicators of stem cell position, meaning that the reported difference in migration through irradiated vs. nonirradiated tissues may in fact have been a difference in rates of mitosis instead. Ergo, the reported data would be consistent with proliferation having been induced in invading stem cells by the irradiated environment (Otsuka and Meistrich, 1993) or with proliferation having been repressed in invading stem cells due to the presence of host stem cells in the nonirradiated tissue (Kai and Spradling, 2003). Future identification of constant molecular differences between the sexual and asexual biotypes that can be used as reliable tissue origin markers or the development of stable transgenics will provide us with the tools necessary to understand how the general conclusions in this study relate to stem cell recruitment and migration in nonirradiated animals.

What Is the Wound Signal?

Nearly half a century passed between the proposal of "wound hormones" (or wound signals) as substances necessary for a damaged plant to regenerate and the actual purification of the first plant wound hormone (Bonner and English, 1937; Wiesner, 1892). Likewise, the existence of planarian wound signals as substances that emanate from physical injuries, triggering stem cell proliferation and migration has long been postulated, but little evidence has been gathered to support their existence even today (Stéphan-Dubois and Lender, 1956). Thus, this study set forth to characterize the effects of wound signals, if they indeed exist, and develop assays that could be reliably used for planarian wound signal discovery.

Since the inception of the planarian wound signal concept, researchers have speculated as to its source and identity unaware that the hypothetical substance that they were referring to would become a hotly sought after adult stem cell recruitment factor (Chen et al., 2011). The best studied examples of adult stem cell recruitment factors are the chemokines of the mammalian hematopoietic system, having been shown to be essential in the homing and outward migration of HSCs (for review see (Lapidot et al., 2005)). The importance of chemokines in HSC migration cannot be overstated and their dominance of nearly half of the vertebrate stem cell migration literature speaks to the focus of the field on this single group of proteins (95 of 209 references, Pubmed Search ("stem cell"[title] AND (migration[title] OR recruitment[title] OR homing[title])) AND (SDF* OR CXCR* OR CXCL* OR CCR* OR cytokine OR chemokine OR "stromal cell-derived factor"). Such a bias begs the question, what other factors, protein or otherwise, control the migration of stem cells as they are directed towards an

unpredictable wound site through what must be very complex environments? Planaria are uniquely suited to address this question as they diverged evolutionarily prior to the emergence of chemokines (S. mediterranea genome lacks detectable homologs of chemokine receptors and ligands) (DeVries et al., 2006). Therefore, the characterization of recruitment and assays presented here combined with developed methods of large scale RNAi screening (Reddien et al., 2005a) should allow for identification of underlying and evolutionarily ancient stem cell migration factors.

Experimental Procedures

Planaria

Asexual *Schmidtea mediterranea* of the CIW4 clonal line (Sánchez Alvarado et al., 2002) were maintained in 1X Montjuïch salts (Cebria and Newmark, 2005) at 20°C and fed homogenized calf liver two to three times per week. Hermaphroditic (sexual) animals of the CIWsx2 clonal line were maintained in dechlorinated tap water with supplemental salts (Newmark and Sánchez Alvarado, 2000) and fed once per week. All animals used were > 1 cm in length and starved for 1 week prior to manipulation.

Immobilization

For partial irradiation and transplantation animals were anesthetized by 5-10 min. treatment in 0.2% chloretone (w/v in 1X Montjuïch salts) chilled on ice.

X-ray Partial Irradiation

Immobilized animals were arranged on 0.2% chloretone soaked filter paper in a 15 mm square petri dish on ice for the duration. Identical custom manufactured lead shields (Fig. 3.2, Alpha Systems Corp., Bluffdale, UT) were centered along each animal's longitudinal axis. The animals were positioned at 30 cm from the cathode tube within an XRAD-320 Biological Irradiator (Percision X-Ray, North Branford, CT) and a dose of ~30 Gy (3.6 min. at 320 kV 10 mA, no hardening filter) was delivered.

RNA Whole-Mount In Situ Hybridization (WISH)

Animals were killed, fixed, and processed as previously described (Pearson et al., 2009) with the following adjustments for staining in large animals. No nutation/rocking was used. N-acetylcysteine concentration was increased to 10% and animals were fixed for 30 min. An additional 10 min. 1% SDS (in PBS) treatment was performed after fixation. Proteinase K treatment was performed at 37°C for 20 min. Hybridization solution contained 50% de-ionized formamide, 5x SSC, 1x Denhardts, 1mg/mL yeast torula RNA, 100 µg/mL heparin, 0.05% Triton-TX, 0.05% Tween-20, 50 mM DTT, 5% dextran sulfate, and 1% SDS. Digoxigenin-labeled riboprobes were used for all single color specimens and dinitrophenol-labeled riboprobes were added for double color specimens. 5% PVA was added to NBT/BCIP development buffer. Development with DAB was performed in 5% PVA with 2x ImmPACT™ DAB chromogen (Vector Labs), 0.01% H_2O_2 in ImmPACT™ DAB diluent for 30 min. NBT/BCIP only developed specimens were mounted in 80% glycerol and all others were mounted in BABB (1:2 benzyl alcohol and benzyl benzoate).

γ-irradiation

Whole animal 100 Gy γ-irradiation was preformed as previously described (Eisenhoffer et al., 2008).

Tissue Transplantation

See Appendix A for complete protocol. For serial transplantations the included protocol was simply repeated for the 2° transplantation using previously grafted animals as the tissue donors and tissue anterior to the primary graft as the 2° graft.

Immunostaining

Animals were killed, fixed, reduced, and permeabilized as described for WISH. WISH was performed first where immunostaining was performed in combination. Where immunostaining was performed alone, animals were then treated for 30 min. in 3% formamide, 6% H_2O_2, in PBST and bleached overnight in 6% H_2O_2 in PBST. Animals were washed with PBST then blocked in 1% BSA (Sigma) + 1% horse serum (Sigma) or 1% goat serum (Sigma) for 4 hours. Incubation with anti-phosphohistone H3 (α-H3P, Millipore) at 1:300 was performed O/N at RT in block. α-H3P was detected with either 1:100 anti-rabbit-Igg (Zymax) and subsequent tyramide amplification as described (Reddien et al., 2005a) or 1:300 anti-rabbit-Alexa555 (Invitrogen). Specimines were mounted in either 80% glycerol with Vectashield™ or BABB.

Karyotyping

Animals were placed in 0.05% colchicine O/N and fixed for 15 min. in 3:1 ethanol and acetic acid. Animals were then treated for 2 min. in 1N HCl at RT then 6 min. at 60°C. HCl was replaced with acetic orcein for 15 min. Animals were then treated for 5 min. each in 60% acetic acid then 1:1:1 lactic acid, acetic acid, and water. Specimines were placed on a Superforst Plus glass slide (VWR), squashed with a siliconized coverslip (Hampton Research), and allowed to settle O/N at 4°C. Slides were frozen in liquid N_2 and coverslips removed with a razorblade then submerged in -20°C ethanol. Slides were warmed to RT, air dried, stained with 0.5 μg/mL propidium iodide for 30 min., rinsed with water, and mounted in Vectashield™.

Image Acquisition and Manipulation

Live animal and NBT/BCIP developed WISH images were captured on a Zeiss SteRIO Lumar v.12 with an Axiocam HRc. Except for NBT/BCIP and DAB double image captured on Zeiss AxioVert and extended focal distance rendering performed using CombineZP software. Images were auto-scaled and color and gamma adjustments were made using Zeiss AxioVision v.3-4.1. Levels were adjusted and backgrounds cropped in exported TIFF files using Adobe Photoshop.

Fluorescent images were captured on a Zeiss LSM 5 LIVE confocal microscope using RealTime automation for image tiling and concatenation/stitching. Lack of specimen autofluorescence when excited with a 488 nm λ laser was used to identify the graft-host boundary in transplantation experiments (as shown in Fig. 3.6G-J) All

fluorescent images are z-projections except those noted as single slices (Fig. 3.8S-V). Scaled LSM files were level adjusted, channel mixed, and cropped using NIH ImageJ.

Image Analysis and Quantification

All image quantization was performed using NIH ImageJ. The longitudinal length of the cell bands of intact partially irradiated animals was measured from the most anterior cell to the most posterior cell along the midline. A ventral and dorsal measurement was made for each animal and the mean values for each time point were computed (Fig. 3.1K).

The distance of H3P(+) foci from the graft boundary (Fig. 3.6K) was determined using *Cell Counter* to define the quardinate positions of the graft-host boundary and all H3P(+) foci. The resulting XY positions were radialized and combined into 45° bins centered on the pictured axes (Fig. 3.6K). The boundary position for each bin was subtracted from the measured radian distance of each cell and resulting negative values (those cells within the graft) were excluded. The raw data was imported into R and the boxpot show (Fig. 3.6K) was generated.

The mean distance from the center of the graft for NBT/BCIP WISH specimens (Fig. 3.10J) was calculated by estimating the center of the graft and polar transforming around that point (*Polar Transformer*, ImageJ plugin). The XY positions of all cells that could be individually resolved on the resulting image were determined using *Cell Counter*. Then the data was grouped into 45° bins centered on the anterior, posterior, right and left axes and the mean for each bin was calculated.

Dispersion as it pertains to cells at the boundary between the shielded and irradiated tissue in partially irradiated animals was calculated as the mean standard error in the relative longitudinal location of the 50 most anterior (at anterior boundaries) or posterior (at posterior boundaries) cells. This measurement took into account the relative movement of a larger portion of the cell population than simply measuring the most anterior and most posterior cell to define the cell band length (as done in Fig. 3.1K), thus the results better represent the behavior of the population as a whole. The image analysis procedure will be described for anterior boundaries and is equivalent for posterior boundaries. The relative XY positions of the 50 most anterior cells in oriented and scaled images were determined using *Cell Counter*. The data were compiled, the X positions discarded, and the SEM for all 50 Y positions for each boundary was computed. The mean of the SEM values for each experimental group was then calculated and graphed (Fig. 3.12G).

The calculation of the minimal number of cells needed for rescue was based on 36% survival of the larger population from which the cohort was taken. We counted the number of cells present from ventral and dorsal views of the transplant site in the entire cohort. The mean was then calculated and the values tested for normal distribution ($\bar{x}$ = 456, p = 0.7, Shapiro-Wilk normality test). These data combined allowed estimation of the theoretical number of cells present in animals at the 64^{th} percentile threshold of a normally distributed population.

CHAPTER 4

DISCUSSION

Summary

In this dissertation we set out to characterize stem cell migration in planaria during both tissue homeostasis and regeneration. This goal required very specific tools for creating a situation where stem cell movements could be observed. We therefore revived and refined tissue transplantation and partial irradiation, two methods with different strengths and weaknesses. The known and predicted weaknesses of these tools were evaluated prior to implementation and, where possible, their impact on the study at hand was reduced. For instance, the existing method of tissue transplantation was known to be highly inefficient and therefore we improved the efficiency. Also, the existing method for partial irradiation was known to only allow analysis of migration in one direction and so was altered to allow for bidirectional analysis. Once the techniques were sufficiently improved we were able to address our original broad question: are planarian stem cells recruited in response to wounding?

We used partial irradiation in the absence of injury to show that planarian stem cells do not migrate significantly during tissue homeostasis nor does stem cell ablation induce a migratory response. We then used transplantation to show that stem cells are capable of repopulating an irradiated host worm and that the repopulating cells reestablish normal positional relationships with their progeny within the host. From our new understanding of the repopulation scenario, we were able to visualize changes in repopulation following additional wounding, indicating that the stem cells were responding to wounding. We saw increased migration following amputation, especially in the direction of the amputation. Additionally, we saw changes in the positional relationships between the stem cells and their progeny, indicating that the stem cells may

be migrating faster or repressing their differentiation programs as they moved towards the new wound. We then returned to the partial irradiation assay to show that indeed stem cells are recruited to wounds in this scenario.

How Can We Reconcile Our Findings with the Contradictory Earlier Work?

We have shown definitively that, at least in the contexts we devised, planarian stem cells migrate. This was the same conclusion Dubois came to over 50 years ago, though she did not have the ability to look at the stem cells directly. More recent work laid doubt on the migratory nature of stem cells (reviewed in Chapter 1). How can we reconcile our findings with contradictory earlier work?

First, Flickinger used the absence of ^{14}C labeled donor tissue within the blastema of a wild-type worm grafted with a ^{14}C-labeled graft to conclude that migration was not taking place (Flickinger, 1964). Notably, Flickinger did not irradiate his host worm, thus we cannot refute his conclusion, as all of our data showing stem cell migration involves migration through irradiated tissue and the inherent dynamics of migration in an irradiated versus nonirradiated context may be different. However, Flickinger also only looked as far as four days following grafting and three days following amputation, which our data indicate that, even if the stem cells were migrating rapidly, very few would have reached the blastema in that amount of time. Thus his results are not surprising whether or not stem cells migrate in the nonirradiated context.

Saló and Baguñá used a number of indirect methods, namely chromosomal differences, nuclear size, and mitosis to follow stem cell migration after grafting and

concluded that migration was not taking place and that cell movements were rather due to proliferation driven passive spreading (Saló and Baguñà, 1985; Saló and Baguñà, 1989). Their transplantation technique, grafting of a large rectangle of tissue (Saló and Baguñà, 1985), would have introduced a significant amount of wounding in the graft location, which likely induced similar diametrically opposed wound signals we presume exist in our circular grafts. As we presented here, diametrically opposed wounds appear to result in slow migration, which could explain the slow migration they reported. Based on our data, it is surprising that they did not report an increase in anterior migration of mitotic cells following an anterior amputation (Saló and Baguñà, 1985). Performing their analysis in sections and lacking a direct stem cell marker may have caused them to miss the relatively small population of cells that we observed being recruited to anterior wounds in our whole-mount analysis of amputated transplantation rescued animals. In fact, in the later analysis Saló and Baguñá reported some small contribution of stem cells to the blastema from over 500 μm away (Saló and Baguñà, 1989). Based on their limited tools for detecting the stem cell population, this observation could correspond to the migratory stem cells we observed in our work.

What Did We Learn About Planarian Stem Cells and General Stem Cell Biology?

Can Stem Cell Migration Be Studied in Planaria?

In Chapter 1 we outlined theoretical events a migrating stem cell either must or could display to be considered a good model system for stem cell migration research. Those events were mobilization, self-renewal en route, migration while maintaining stem

cell characteristics, differentiation while migrating, and integration into the target site. To varying degrees of certainty, we have observed all of these events in our system. We saw planarian stem cells mobilize out of a graft to repopulate host tissue and that those repopulating stem cells were capable of self-renewal because they rescued irradiated hosts upon serial transplantation. We observed repopulation of both the stem cell compartment and two separate stem cell progeny compartments with patterns highly representative of the stem cell repopulation pattern. This scenario indicates that migration of the stem cells while retaining stem cell characteristic is indeed taking place and that differentiation during migration may also be represented. Finally, following wounding, we observed stem cells migrating and appearing at the wound site suggesting their integration into a target tissue. We have not been able, however, to detect stem cell integration into a target site during tissue turnover. This may be simply because this is a rare event to observe or because during tissue homeostasis the stem cells are largely stationary as our partial irradiation data suggest. Nonetheless, observation of these events not only supports the conclusion that planarian stem cells are a good model system for studying stem cell migration but also informs us greatly about the biological capabilities of this remarkable cell population.

Should Stem Cell Migration Be Studied in Planaria?

Now that we have established that stem cell migration *can* be studied in planaria, the next obvious question becomes, *should* we do so? We have argued that simpler model systems are needed to study tissue turnover and regeneration (Pellettieri and Sánchez Alvarado, 2007; Sánchez Alvarado, 2004). Clearly, planaria are a simpler

system than vertebrates for these studies and few organisms display such robust regeneration. However, is planarian stem cell migration sufficiently complex such that lessons learned will be applicable to our general understanding of regeneration and, equally important, to human health? The dynamic nature in which the stem cells in this study were seen to progress from a nonmigratory state to a mobile one, and to one where multiple directional cues are being integrated, suggests that indeed the process is sufficiently complex to be relevant.

The relatively close relationship between planaria and humans, as compared to some more mainstream model systems, has been discussed elsewhere (Pearson and Sánchez Alvarado, 2008). The difficulties in studying adult stem cell migration in vertebrates do not inherently justify studying stem cell migration in planaria. The work presented here, however, shows that many cellular events that are relevant to general biological interest and human health are readily displayed in this system. For instance, the mobilization of planarian stem cells may be akin to an epithelial-to-mesenchymal transition that are hotly investigated topics of both developmental biology, in the mobilization of neural crest cells (Rinon et al., 2011), and medical research, in the form of cancer metastasis (Tse and Kalluri, 2007). Additionally, how migration affects the differentiation of cells is largely unknown and of direct importance to human health (Laird et al., 2008). With the groundwork laid by this analysis and the tools we have in hand, we can immediately ask interesting and important questions about how migration affects cell fate as well.

How Important Is the Niche?

From this analysis it appears that although planarian stem cells are not inherently migratory they can be induced to migrate. This may represent a general characteristic of adult stem cells where, although the stem cells reside and are stationary in the niche, positioning in the niche is not a *de facto* requirement for maintenance of stem cell characteristics. This may in fact be the case for HSCs, which are observed displaying their stem cell characteristics, including the ability to functionally reconstitute an irradiated host, while in circulation away from their bone marrow niche (Abkowitz et al., 2003). Indeed, we observed similar behaviors—proliferation of the migrating stem cell population and functional recovery of irradiated hosts transplanted with migrating stem cells (Fig. 3.6). Although the planarian stem cell niche has yet to be identified, the HSC data would indicate that stem cells can exist outside of the niche (Abkowitz et al., 2003; Mendez-Ferrer et al., 2008). Additionally, recent evidence indicates that glial cells, which often exist and function outside the described neurogenic niches, may in fact be a stem cell population as they are known to responding to injury and facilitate repair (Robel et al., 2011). These behaviors beg the question, how important is the niche? Data, including our own, supporting the migration of stem cells that maintain their stem cell characteristics, suggest that either the niche is not a necessary component of a stem cell system or there exists a subclass of stem cells which can, at least temporarily, remain fully functional outside of the niche. At the very least, exit from the niche does appear to render a stem cell incapable of self-renewal nor unable to properly differentiate. However, because the repopulating stem cells in our study may be repositioned within a yet-to-be-identified planarian stem cell niche, whether return to the niche is a requirement

for continued self-renewal remains to be determined. The presented hypothesis suggesting minimal importance for the niche is only one interpretation of a small amount of data from a much larger field of data supporting an important role for the stem cell niche (Jones and Wagers, 2008; Morrison and Spradling, 2008; Voog and Jones, 2010).

How Are the Observed Stem Cell Behaviors during Tissue Homeostasis, Repopulation, and Regeneration Related?

As discussed in Chapter 1, there are inherent differences in tissue renewal during tissue homeostasis and tissue regeneration even though, in many cases, these two processes utilize the same stem cell populations. The lack of migration in intact partially irradiated animals shown in this study suggests that planarian stem cells do not migrate in the absence of a wound signal. Previous work performed in our lab also indicated that the stem cell population is relatively stationary during tissue homeostasis and the majority of cell movement in the intact animal is the responsibility of committed progenitors (Eisenhoffer et al., 2008). These data support a model where dying cells outside of the stem cell zone do not signal to stem cells to migrate but instead either signal progeny to migrate and replace the dying cell or the progeny are preprogrammed to replace the dying cell whether or not they are signaled to do so. Whether progeny recruitment takes place during tissue homeostasis is still very much an open question; however, our work does not support stem cell recruitment in this context. Thus similar to what has been observed in the intestinal epithelia (Barker et al., 2010), planarian stem cells appear to reside in a single location and continually produce migratory daughter cells that maintain the rapidly cycling tissue. It remains possible that planarian stem cells

migrate substantially within the stem cell compartment during tissue homeostasis; however, the work done thus far has not allowed us to evaluate this prospect.

On the other hand, as we have shown here, the stationary character of planarian stem cells changes dramatically when the animal is wounded. Our data suggest that not only do the stem cells become highly migratory but they also may even repress or at least adjust the timing of their differentiation programs during wound directed migration. Such a change in cell behavior implies a signaling event. Just as stem cells of the hair follicle migrate minimally during tissue homeostasis, they can be activated by nearby wounding to migrate significantly further distances (Barker et al., 2010). Our work shows that planarian stem cells similarly react to a wounding event, mobilized out of their sedentary homeostatic state by some yet unknown signal to migrate long distances.

How tissue homeostasis and regeneration combine to produce the observed repopulation after transplantation is a more difficult question. Our hypothesis is that the wounding caused during transplantation induces mobilization of the stem cell population. Just as stem cells are recruited to the wound edge following amputation, so too are the stem cells within the graft recruited to the wound following transplantation. However, because tissue exists beyond the wound in the transplantation case the cells are capable of continuing their migration; whereas in the normal scenario once migrating cells reach an amputation plane they cannot physically continue. This process of continued migration, without a clear directional wound signal, may be more akin to what is observed during tissue homeostasis, as the relationships between the stem cell population and the progeny appear very similar in the two contexts.

An alternative explanation may be that the repopulating stem cells continue migrating simply because they are unable to stop. Evolutionarily this makes sense because, it is believed that regeneration arose or was maintained in organisms that were subject to high levels of predation (Baumiller and Gahn, 2004; Bely and Nyberg, 2010), thus the mechanisms controlling regeneration related stem cell migration would have evolved primarily under selective pressure in an amputation scenario. Therefore, no mechanism would have been required to stop the stem cells once they reached the amputation plane because it was, after all, a dead end, and thus no evolutionary pressure would have existed to select for such a mechanism. This broad conjecture is further complicated by the observed unequal recruitment by opposing wounds. If the transplantation site is indeed acting as 360° of diametrically opposed wound signals emanating in all directions from the circular wound, then the slow migration observed during repopulation could more simply be the resulting summation of many different wound signals, resulting in recruitment directed away from the graft. Grafting half of an irradiated animal to half of a wild-type animal (which necessitated grafting two free halves of planaria together, a technique we have unsuccessfully attempted but is nonetheless reported in the classical literature (Morgan, 1906)) and observing whether stem cells migrate across the wound site would begin to clarify this poorly understood and complicated situation.

Nevertheless, the differences in stem cell migration described in intact, amputated, and grafted animals suggested that not only are wound signals necessary for recruitment but complicated mechanisms for integrating multiple wound signals must also exist. This may be extrapolated to the differences between tissue homeostasis and

regeneration in that the different processes may utilize different migration signals, thus allowing the very same stem cell population to respond to both processes simultaneously and in very different ways.

The Hypothetical Wound Signal

As discussed in Chapter 3, the mounting evidence for the existence of a hypothetical wound signal has accumulated over a long period of time; however, we have made no progress in actually identifying such a wound signal. We set out with this line of investigation with exactly that purpose and soon realized that in order to identify any wound signal we first needed to better understand the processes involved in stem cell migration. Now that the processes of planarian stem cell migration have been better defined, and we have optimized assays for testing the function of putative wound signals, we are poised for an attempt at wound signal identification.

One can envision many different methods for identifying planarian wound signals. The most simple and arguably most laborious method, would be to test the necessity of candidate genes known to be important in other systems or unbiasedly screening the whole genome for defects in wounding induced stem cell migration. A more direct method for wound signal identification may be to compare the transcriptomes, by either microarray or RNAseq, of intact irradiated tissue and amputated irradiated tissue, because our work has shown that wounds within irradiated tissue are capable of recruiting stem cells. This approach would still necessitate the testing of candidate genes in a migration assay derived from our work, but would have the added benefits of being unbiased and not requiring testing of every gene in the genome. It

remains possible that the wound signal is not transcriptionally regulated however, and thus would not be detected in transcriptome analysis. If this is the case, proteomics could be employed if the wound signal is proteinaceous in nature but posttranscriptionally regulated. If the wound signal is, on the other hand, not a protein, a large scale small molecule screen utilizing the higher throughput partial irradiation method could be employed for identification. Regardless, these advanced methods available to us today will hopefully speed the discovery of the planarian stem cell recruitment signal.

Although not directly part of our goals at the beginning of this study, our work did teach us some important characteristics of the hypothetical planarian stem cell recruitment signal. First, because the stem cells are recruited to wounds made in irradiated tissue, the signal is inherently produced by a differentiated cell type that persists following irradiation, thus ruling out the stem cells and possibly rapidly lost early progeny. Second, the signal is likely either highly diffusible or transported long distances as our work and the work of Dubois shows that it was able to act at distances over 500 μm (Dubois, 1949). This action over long distances does not necessarily rule out proteins, as very little migration is seen within 2 days following wounding, enough time for transcriptional up regulation, protein production, and diffusion, transport or cell to cell signaling to propagate the signal. Finally, the signal does not appear to be induced by irradiation, because migration was not seen in intact partially irradiated animals. Since irradiation is known to induce large amounts of apoptosis in planaria (Pellettieri et al., 2010), we can infer that the signal is therefore apoptosis independent, which is contrary to one hypothesis that apoptotic cells signal and induce tissue repair mechanisms (Pellettieri and Sánchez Alvarado, 2007). These features of the hypothetical stem cell

recruitment signal could be used to guide the culling of candidate gene or molecule lists prior to functional testing in a migration assay.

Conclusions

We therefore conclude that indeed our newly optimized methods of tissue transplantation and partial irradiation can successfully be used to study planarian stem cell migration and recruitment. Also, we have been able to retrospectively reconcile our findings with much of the previous contentious literature that suggested a lack of stem cell migration in planaria. We have also shown that, due to sufficiently complex and easily recognizable stem cell migration events, planaria are a good model system for studying stem cell migration. Additionally, because of their separate stem cell behaviors during tissue homeostasis and regeneration, planaria appear to be a good system for studying the separate molecular control of these different processes. The observed strong stem cell recruitment following injury combined with molecular genetic tools like RNAi set the stage for the discovery of stem cell recruitment factors. However, we have shown that not only are planaria likely to allow the identification of such factors but they will also provide a platform for which to test the complex molecular interactions and cell biological consequences of modulating those factor *in vivo*. These conclusions together will hopefully further our understanding of stem cell biology and lead to advances in the treatment of stem cell disorders and better control of stem cell therapies in humans.

APPENDIX

PROTOCOL: TISSUE TRANSPLANTATION BY PLUG GRAFT

This appendix consists of our optimized tissue transplantation method with the improvements detailed in Chapter 1, included here for completeness and ease of duplication.

Planarian Transplantation by Plug Graft

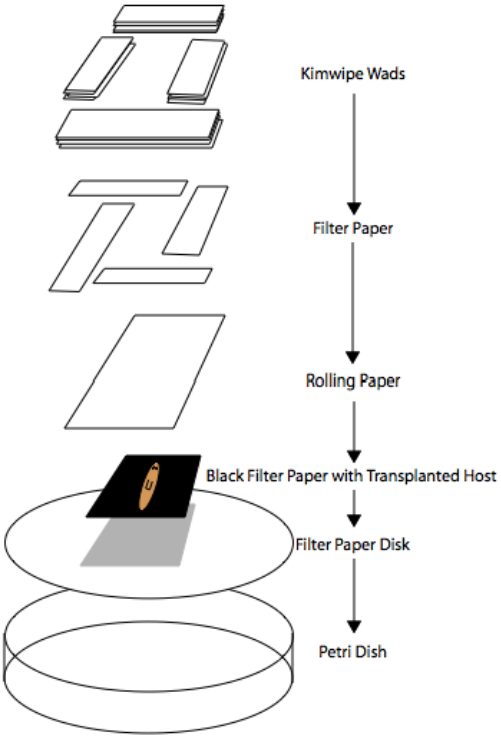
Otto Guedelhoefer, Alejandro Sánchez Alvarado Laboratory, The Department of Neurobiology & Anatomy, University of Utah, Salt Lake City, Utah – otto@neuro.utah.edu
Adapted from: Santos, F.V. (1931) Studies on transplantation in planaria. *Phys Zool* **4**, 111-164.
See also: Kato et. al., Development, 1999.
Updated: May 15, 2011

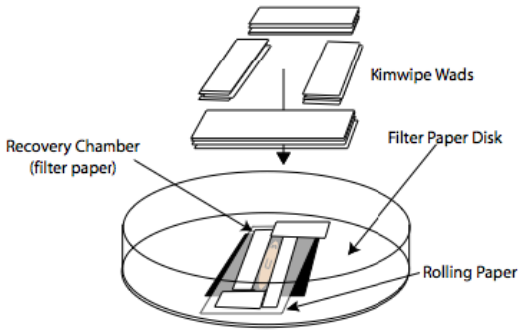
Materials:
Large (> 1 cm in length) planaria (*S. mediterranea* sexual strain, asexual strain grown at 10°C, or extremely well fed asexual worms (2-3 feedings per week for > 1 month))
Capillary tubes interior diameter (0.75 mm) and exterior diameter (0.7 mm) size matched and flame bent at 90° angle (FHC catalog #'s 30-30-0 and 30-50-8)
Observation scope
Peltier cooler plate
Parafilm
Kimwipe
Black filter paper (cut into rectangles ~2.5 cm x 1.5 cm) (Schleicher & Schuell, Ref. No. 10310809)
Whatman #2 filter paper (small disks ~6 cm in diameter) (Cat. No. 1002 055)
Whatman #3 filter paper (cut into rectangles ~2 cm x 0.5 cm) (Cat. No. 1003 185)
Kimwipe (folded and cut into wads ~3 cm x 0.5 cm x 4 ply) (Kimberly-Clark Cat. No. 34155)
Cigarette rolling paper (cut into rectangles ~3 cm x 2 cm) (Zig-Zag, original, with gum-strip removed)
10 cm^2 (medium size) Petri dishes (Greiner bio-one Cat. No. 628161)
Forceps (FST by DUMONT, INOX #5, two pair)

Solutions:
1X planarian water
Chloretone solution: 0.2% chloretone diluted in planarian water (can be made from 1% stock in DI H_2O, store at 4°C, shelf-life ≤ 2 months)
Holtfreter's solution (see attached sheet)
Casein (Sigma C3400) saturated Holtfreter's solution

Procedure:
1) Cool all solutions on ice.
2) Cool Peltier plate (at 3-4 V).
3) Wet the filter paper that lines the recovery dish with ice cold Holtfleter's solution and place the dish on ice (you may wish to prepare all recovery dishes for this set of transplants in this way at this time).
4) Staple a folded Kimwipe to a square of Parafilm, place it on the cooled Peltier plate with the parafilm side down, and wet the Kimwipe with planarian water.
5) Place two small rectangles of black filter paper onto the damp Kimwipe.
6) Soak the donor and host worms in chilled to RT chloretone solution for 5-10 min or until the worms become motionless.
7) Remove the worms from the chloretone solution and rinse them briefly in Holtfreter's solution.
8) Remove the worms from the Holtfreter's solution and place each worm on a separate rectangle of black filter paper.

9) Using a 0.75 mm inner diameter capillary tube remove the graft plug from the donor and place it on an out of the way portion of the host.[1]
10) Using a 0.7 mm outer diameter capillary tube remove a plug from the host and using forceps position the graft into the hole that is left behind.
11) Transfer the transplanted host on its black filter paper rectangle into the recovery dish.
12) Wet a piece of rolling paper with casein saturated Holtfreter's solution and place it on top of the transplanted host as diagramed below.
13) Soak four pieces of filter paper in casein saturated Holtfleter's solution and encase the transplanted host as diagramed below.
14) Soak four wads of cut Kimwipe in casein saturated Holtfleter's solution and lay them over the filter paper from step 13. Replace lid and put the petri dish on ice.
15) Transfer the donor worm into planarian water to recover, heal, and regenerate.
16) When all transplants are completed, place the transplanted worms into a 10°C incubator overnight.
17) The following morning, taking care not to disturb the graft, uncover the worm and transfer it (on its black filter paper) to a petri dish filled with planaria water.
18) Either allow the worm to dislodge itself from the filter paper or gently remove it with forceps.
19) Change the planarian water once every 2 days.





[1] If graft material gets stuck in the capillary tube, dislodge with forceps.

Modified Holtfreter's Solution
NaCl 3.5 g
$NaHCO_3$ 0.2 g
KCl 0.05 g
$MgSO_4$ stock solution 333 µl
$CaCl_2$ stock solution 333 µl
DI H_20 1 liter

Check pH; should be between 7 and 7.5.

$MgSO_4$ stock solution
$MgSO_4$ 300 g
DI H_20 500 ml

$CaCl_2$ stock solution
$CaCl_2$ 150 g
DI H_20 500 ml

REFERENCES

Abkowitz, J.L., Robinson, A.E., Kale, S., Long, M.W., and Chen, J. (2003). Mobilization of hematopoietic stem cells during homeostasis and after cytokine exposure. Blood *102*, 1249-1253.

Aguirre, A., Rubio, M.E., and Gallo, V. (2010). Notch and EGFR pathway interaction regulates neural stem cell number and self-renewal. Nature *467*, 323-327.

Alonso, L., and Fuchs, E. (2003). Stem cells of the skin epithelium. Proc Natl Acad Sci U S A *100 Suppl 1*, 11830-11835.

Aman, A., and Piotrowski, T. (2008). Wnt/beta-catenin and Fgf signaling control collective cell migration by restricting chemokine receptor expression. Dev Cell *15*, 749-761.

Aman, A., and Piotrowski, T. (2010). Cell migration during morphogenesis. Dev Biol *341*, 20-33.

Baguñà, J. (1975). Mitosis in the intact and regenerating planarian Dugesia mediterranea n.sp. II. Mitotic studies during regeneration, and a possible mechanism of blastema formation. J Exp Zool *195*, 65-80.

Baguñà, J., Saló, E., and Auladell, C. (1989). Regeneration and pattern formation in planarians. III. Evidence that neoblasts are totipotent stem cells and the source of blastema cells. Development *107*, 77- 86.

Bardeen, C., and Baetjer, F. (1904). The inhibitive action of the Roentgen rays on regeneration in planarians. J Exp Zoöl *1*, 191-195.

Barker, N., Bartfeld, S., and Clevers, H. (2010). Tissue-resident adult stem cell populations of rapidly self-renewing organs. Cell Stem Cell *7*, 656-670.

Baumiller, T.K., and Gahn, F.J. (2004). Testing predator-driven evolution with Paleozoic crinoid arm regeneration. Science *305*, 1453-1455.

Beauchamp, R. (1932). Some ecological factors and their influence on competition between stream and lake-living triclads. Journal of Animal Ecology *1*, 175-190.

Bely, A.E., and Nyberg, K.G. (2010). Evolution of animal regeneration: re-emergence of a field. Trends Ecol Evol *25*, 161-170.

Blanpain, C., Horsley, V., and Fuchs, E. (2007). Epithelial stem cells: turning over new leaves. Cell *128*, 445-458.

Blanpain, C., Lowry, W.E., Geoghegan, A., Polak, L., and Fuchs, E. (2004). Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. Cell *118*, 635-648.

Bonner, J., and English, J., Jr. (1937). Purification of traumatin, a plant wound hormone. Science *86*, 352-353.

Borgstrom, P., Bourdon, M.A., Hillan, K.J., Sriramarao, P., and Ferrara, N. (1998). Neutralizing anti-vascular endothelial growth factor antibody completely inhibits angiogenesis and growth of human prostate carcinoma micro tumors in vivo. Prostate *35*, 1-10.

Borgstrom, P., Torres Filho, I.P., and Hartley-Asp, B. (1995). Inhibition of angiogenesis and metastases of the Lewis-lung cell carcinoma by the quinoline-3-carboxamide, Linomide. Anticancer Res *15*, 719-728.

Brøndsted, H. (1969). Planarian Regeneration (Pergamon Press).

Cebria, F., and Newmark, P.A. (2005). Planarian homologs of netrin and netrin receptor are required for proper regeneration of the central nervous system and the maintenance of nervous system architecture. Development *132*, 3691-3703.

Chen, F.M., Wu, L.A., Zhang, M., Zhang, R., and Sun, H.H. (2011). Homing of endogenous stem/progenitor cells for in situ tissue regeneration: Promises, strategies, and translational perspectives. Biomaterials.

Chen, J., Larochelle, A., Fricker, S., Bridger, G., Dunbar, C.E., and Abkowitz, J.L. (2006). Mobilization as a preparative regimen for hematopoietic stem cell transplantation. Blood *107*, 3764-3771.

Cheng, T., Rodrigues, N., Shen, H., Yang, Y., Dombkowski, D., Sykes, M., and Scadden, D.T. (2000). Hematopoietic stem cell quiescence maintained by p21cip1/waf1. Science *287*, 1804-1808.

Child, C. (1910). Physiological isolation of parts and fission in planaria. Archiv für Entwicklungsmechanik der Organismen *30*, 159-205.

Chute, J.P. (2006). Stem cell homing. Curr Opin Hematol *13*, 399-406.

Collins, C.A., Olsen, I., Zammit, P.S., Heslop, L., Petrie, A., Partridge, T.A., and Morgan, J.E. (2005). Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. Cell *122*, 289-301.

Congdon, C.C. (1971). Bone marrow transplantation. Science *171*, 1116-1124.

Daley, G.Q., and Scadden, D.T. (2008). Prospects for stem cell-based therapy. Cell *132*, 544-548.

DeVries, M.E., Kelvin, A.A., Xu, L., Ran, L., Robinson, J., and Kelvin, D.J. (2006). Defining the origins and evolution of the chemokine/chemokine receptor system. J Immunol *176*, 401-415.

Dubois, F. (1949). Contribution á l 'ètude de la migration des cellules de règènèration chez les Planaires dulcicoles. Bull Biol Fr Belg *83*, 213-283.

Eisenhoffer, G.T., Kang, H., and Sánchez Alvarado, A. (2008). Molecular analysis of stem cells and their descendants during cell turnover and regeneration in the planarian Schmidtea mediterranea. Cell Stem Cell *3*, 327-339.

Flickinger, R. (1964). Isotopic evidence for a local origin of blastema cells in regenerating planarians. Exp Cell Res *34*, 403-406.

Gurley, K.A., Elliott, S.A., Simakov, O., Schmidt, H.A., Holstein, T.W., and Sánchez Alvarado, A. (2010). Expression of secreted Wnt pathway components reveals unexpected complexity of the planarian amputation response. Dev Biol *347*, 24-39.

Gurley, K.A., Rink, J.C., and Sánchez Alvarado, A. (2008). Beta-catenin defines head versus tail identity during planarian regeneration and homeostasis. Science *319*, 323-327.

Hayashi, T., Asami, M., Higuchi, S., Shibata, N., and Agata, K. (2006). Isolation of planarian X-ray-sensitive stem cells by fluorescence-activated cell sorting. Dev Growth Differ *48*, 371-380.

Johns, P.M., and Boag, B. (2003). The spread and distribution of terrestrial planarians (Turbellaria: Tricladida: Geoplanidae) within New Zealand. New Zealand Journal of Ecology *27*, 201-206.

Jones, D.L., and Wagers, A.J. (2008). No place like home: anatomy and function of the stem cell niche. Nat Rev Mol Cell Biol *9*, 11-21.

Kai, T., and Spradling, A. (2003). An empty Drosophila stem cell niche reactivates the proliferation of ectopic cells. Proc Natl Acad Sci U S A *100*, 4633-4638.

Kang, H. (2009). Cell cycle, fate, stem cells, and the planarian Schmidtea mediterranea. In Dept of Neurobiology and Anatomy (Salt Lake City, University of Utah), pp. 147.

Kang, H., and Sánchez Alvarado, A. (2009). Flow cytometry methods for the study of cell-cycle parameters of planarian stem cells. Dev Dyn *238*, 1111-1117.

Karp, J.M., and Leng Teo, G.S. (2009). Mesenchymal stem cell homing: the devil is in the details. Cell Stem Cell *4*, 206-216.

Kato, K., Orii, H., Watanabe, K., and Agata, K. (1999). The role of dorsoventral interaction in the onset of planarian regeneration. Development *126*, 1031-1040.

Kellogg, V.L. (1904). Restorative regeneration in nature of the starfish Linckia diplax (müller and troschel). Journal of Experimental Zoology *1*, 353-356.

Kenk, R. (1989). Revised list of the North American freswater palanarians (Platyhelminthes: tricladida: paludicola). Smithsonian Contributions to Zoology *476*, 1-10.

Kiel, M.J., Yilmaz, O.H., Iwashita, T., Terhorst, C., and Morrison, S.J. (2005). SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell *121*, 1109-1121.

Kim, D., Hong, K.S., and Song, J. (2007). The present status of cell tracking methods in animal models using magnetic resonance imaging technology. Mol Cells *23*, 132-137.

Kolmayer, S., and Stéphan-Dubois, F. (1960). Néoblastes et limitation du pouvoir de régénération céphalique chez la planaire Dendrocoelum lacteum. J Embryolexp Morph *8*, 376-386.

Kragl, M., Knapp, D., Nacu, E., Khattak, S., Maden, M., Epperlein, H.H., and Tanaka, E.M. (2009). Cells keep a memory of their tissue origin during axolotl limb regeneration. Nature *460*, 60-65.

Laird, D.J., von Andrian, U.H., and Wagers, A.J. (2008). Stem cell trafficking in tissue development, growth, and disease. Cell *132*, 612-630.

Lapidot, T., Dar, A., and Kollet, O. (2005). How do stem cells find their way home? Blood *106*, 1901-1910.

Leatherman, J.L., and Dinardo, S. (2008). Zfh-1 controls somatic stem cell self-renewal in the Drosophila testis and nonautonomously influences germline stem cell self-renewal. Cell Stem Cell *3*, 44-54.

Lehnert, G. (1891). Beobachtung an Landplanarien. Arch Naturgech *1*, 306-350.

Mendez-Ferrer, S., and Frenette, P.S. (2007). Hematopoietic stem cell trafficking: regulated adhesion and attraction to bone marrow microenvironment. Ann N Y Acad Sci *1116*, 392-413.

Mendez-Ferrer, S., Lucas, D., Battista, M., and Frenette, P.S. (2008). Haematopoietic stem cell release is regulated by circadian oscillations. Nature *452*, 442-447.

Miller, J. (1938). Studies on heteroplastic transplantation in triclads. I. cephalic grafts between Euplanaria dorotocephala and E. tigrina. Phys Zool *11*, 214-247.

Miller, W., and Kennedy, R.J. (1955). X-ray attenuation in lead, aluminum, and concrete in the range 275 to 525 kilovolts. Radiology *65*, 920-925.

Morgan, L. (1906). Regeneration of grafted pieces of planarians. J Exp Zoöl *3*, 269-294.

Morgan, T. (1898). Regneration in Planaria maculata. ScienceNew Series *7*, 196-197.

Morgan, T. (1900). Regeneration in planarians. Archiv Entwick Mech *10*, 58-119.

Morgan, T.H. (1901). Regeneration (New York, Macmillan Company).

Morrison, S.J., and Spradling, A.C. (2008). Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. Cell *132*, 598-611.

Newmark, P.A., Reddien, P.W., Cebria, F., and Sánchez Alvarado, A. (2003). Ingestion of bacterially expressed double-stranded RNA inhibits gene expression in planarians. Proc Natl Acad Sci U S A *100 Suppl 1*, 11861-11865.

Newmark, P.A., and Sánchez Alvarado, A. (2000). Bromodeoxyuridine specifically labels the regenerative stem cells of planarians. Dev Biol *220*, 142-153.

Newmark, P.A., and Sánchez Alvarado, A. (2002). Not your father's planarian: a classic model enters the era of functional genomics. Nat Rev Genet *3*, 210-219.

Niemeyer, M., Oostendorp, R.A., Kremer, M., Hippauf, S., Jacobs, V.R., Baurecht, H., Ludwig, G., Piontek, G., Bekker-Ruz, V., Timmer, S.*, et al.* (2010). Non-invasive tracking of human haemopoietic CD34(+) stem cells in vivo in immunodeficient mice by using magnetic resonance imaging. Eur Radiol *20*, 2184-2193.

Okada, Y., and Sugino, H. (1937). Transplantation Experiments in Planariagonocephala Duges. Zoological Institute, Kyoto Imperial University *7*, 373-439.

Osawa, M., Hanada, K., Hamada, H., and Nakauchi, H. (1996). Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science *273*, 242-245.

Otsuka, M., and Meistrich, M.L. (1993). Radiation-induced proliferation in contralateral unirradiated kidneys. Radiat Res *134*, 247-250.

Oviedo, N.J., Newmark, P.A., and Sánchez Alvarado, A. (2003). Allometric scaling and proportion regulation in the freshwater planarian Schmidtea mediterranea. Dev Dyn *226*, 326-333.

Pearson, B.J., Eisenhoffer, G.T., Gurley, K.A., Rink, J.C., Miller, D.E., and Sánchez Alvarado, A. (2009). Formaldehyde-based whole-mount in situ hybridization method for planarians. Dev Dyn *238*, 443-450.

Pearson, B.J., and Sánchez Alvarado, A. (2008). Regeneration, stem cells, and the evolution of tumor suppression. Cold Spring Harb Symp Quant Biol *73*, 565-572.

Pearson, B.J., and Sánchez Alvarado, A. (2010). A planarian p53 homolog regulates proliferation and self-renewal in adult stem cell lineages. Development *137*, 213-221.

Pellettieri, J., Fitzgerald, P., Watanabe, S., Mancuso, J., Green, D.R., and Sánchez Alvarado, A. (2010). Cell death and tissue remodeling in planarian regeneration. Dev Biol *338*, 76-85.

Pellettieri, J., and Sánchez Alvarado, A. (2007). Cell turnover and adult tissue homeostasis: from humans to planarians. Annu Rev Genet *41*, 83-105.

Petersen, C.P., and Reddien, P.W. (2009). A wound-induced Wnt expression program controls planarian regeneration polarity. Proc Natl Acad Sci U S A *106*, 17061-17066.

Pineda, D., Gonzalez, J., Callaerts, P., Ikeo, K., Gehring, W.J., and Saló, E. (2000). Searching for the prototypic eye genetic network: Sine oculis is essential for eye regeneration in planarians. Proc Natl Acad Sci U S A *97*, 4525-4529.

Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. (1999). Multilineage potential of adult human mesenchymal stem cells. Science *284*, 143-147.

Quinn, B., Costello, M.J., Dorange, G., Wilson, J.G., and Mothersill, C. (2009). Development of an in vitro culture method for cells and tissues from the zebra mussel (Dreissena polymorpha). Cytotechnology *59*, 121-134.

Raff, M. (2003). Adult stem cell plasticity: fact or artifact? Annu Rev Cell Dev Biol *19*, 1-22.

Rand, H., and Browne, A. (1926). Inhibition of regeneration in planarians by grafting: technique of grafting. Proc Natl Acad Sci U S A *12*, 575-581.

Rando, T.A. (2006). Stem cells, ageing and the quest for immortality. Nature *441*, 1080-1086.

Randolph, H. (1892). The regeneration of the tail in lumbriculus. J Morphol *7*, 317-344.

Raz, E., and Mahabaleshwar, H. (2009). Chemokine signaling in embryonic cell migration: a fisheye view. Development *136*, 1223-1229.

Reddien, P.W., Bermange, A.L., Murfitt, K.J., Jennings, J.R., and Sánchez Alvarado, A. (2005a). Identification of genes needed for regeneration, stem cell function, and tissue homeostasis by systematic gene perturbation in planaria. Dev Cell *8*, 635-649.

Reddien, P.W., Oviedo, N.J., Jennings, J.R., Jenkin, J.C., and Sánchez Alvarado, A. (2005b). SMEDWI-2 is a PIWI-like protein that regulates planarian stem cells. Science *310*, 1327-1330.

Reed, J.C. (1999). Dysregulation of apoptosis in cancer. J Clin Oncol *17*, 2941-2953.

Reya, T., Morrison, S.J., Clarke, M.F., and Weissman, I.L. (2001). Stem cells, cancer, and cancer stem cells. Nature *414*, 105-111.

Rietze, R.L., Valcanis, H., Brooker, G.F., Thomas, T., Voss, A.K., and Bartlett, P.F. (2001). Purification of a pluripotent neural stem cell from the adult mouse brain. Nature *412*, 736-739.

Rinon, A., Molchadsky, A., Nathan, E., Yovel, G., Rotter, V., Sarig, R., and Tzahor, E. (2011). p53 coordinates cranial neural crest cell growth and epithelial-mesenchymal transition/delamination processes. Development *138*, 1827-1838.

Robel, S., Berninger, B., and Gotz, M. (2011). The stem cell potential of glia: lessons from reactive gliosis. Nat Rev Neurosci *12*, 88-104.

Saito, Y., Koinuma, S., Watanabe, K., and Agata, K. (2003). Mediolateral intercalation in planarians revealed by grafting experiments. Dev Dyn *226*, 334-340.

Saló, E., and Baguñà, J. (1984). Regeneration and pattern formation in planarians. I. The pattern of mitosis in anterior and posterior regeneration in Dugesia (G) tigrina, and a new proposal for blastema formation. J Embryol Exp Morphol *83*, 63-80.

Saló, E., and Baguñà, J. (1985). Cell movement in intact and regenerating planarians. Quantitation using chromosomal, nuclear and cytoplasmic markers. J Embryol Exp Morphol *89*, 57-70.

Saló, E., and Baguñà, J. (1989). Regeneration and pattern formation in planarians II. Local origin and role of cell movements in blastema formation. Development *107*, 69-76.

Sánchez Alvarado, A. (2004). Regeneration and the need for simpler model organisms. Philos Trans R Soc Lond B Biol Sci *359*, 759-763.

Sánchez Alvarado, A., Newmark, P.A., Robb, S.M., and Juste, R. (2002). The Schmidtea mediterranea database as a molecular resource for studying platyhelminthes, stem cells and regeneration. Development *129*, 5659-5665.

Santos, F. (1929). Studies on transplantation in planaria. Biological Bulletin *57*, 188-197.

Santos, F. (1931). Studies on transplantation in planaria. Phys Zool *4*, 111-164.

Sherwood, R.I., Christensen, J.L., Conboy, I.M., Conboy, M.J., Rando, T.A., Weissman, I.L., and Wagers, A.J. (2004). Isolation of adult mouse myogenic progenitors: functional heterogeneity of cells within and engrafting skeletal muscle. Cell *119*, 543-554.

Shibata, N., Umesono, Y., Orii, H., Sakurai, T., Watanabe, K., and Agata, K. (1999). Expression of vasa(vas)-related genes in germline cells and totipotent somatic stem cells of planarians. Dev Biol *206*, 73-87.

Spalding, K.L., Bhardwaj, R.D., Buchholz, B.A., Druid, H., and Frisen, J. (2005). Retrospective birth dating of cells in humans. Cell *122*, 133-143.

Steele, V., and Lange, C. (1976). Effects of irradiation on stem cell response to differentiation inhibitors in the planarian Dugesia etrusca. Radiat Res *67*, 21-29.

Steinmann, P. (1925). Das verhalten der zellen und gewebe im regenerierenden tricladenkorper. Verh Naturf Ges Basel *36*, 133-162.

Stéphan-Dubois, F. (1961). Les cellules de régénération chez la planaire Dendrocoleum lacteum. Bulletin de la Société Zooologique de France *86*, 172-185.

Stéphan-Dubois, F., and Lender, T. (1956). Corrélations humorales dans la régénération de planaires paludicoles. Ann des Sc Nat, Zool *18*, 223-230.

Stevens, N. (1901). Notes on regeneration in Planaria Lugubris. Archiv Für Entwick *13*, 396-409.

Tajbakhsh, S. (2003). Stem cells to tissue: molecular, cellular and anatomical heterogeneity in skeletal muscle. Curr Opin Genet Dev *13*, 413-422.

Takada, A., Takada, Y., and Ambrus, J.L. (1971). Proliferation of donor spleen and bone-marrow cells in the spleens and bone marrows of unirradiated and irradiated adult mice. Proc Soc Exp Biol Med *136*, 222-226.

Temple, S. (2001). The development of neural stem cells. Nature *414*, 112-117.

Tessmar-Raible, K., and Arendt, D. (2003). Emerging systems: between vertebrates and arthropods, the Lophotrochozoa. Curr Opin Genet Dev *13*, 331-340.

Timmons, L., Court, D.L., and Fire, A. (2001). Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in Caenorhabditis elegans. Gene *263*, 103-112.

Tse, J.C., and Kalluri, R. (2007). Mechanisms of metastasis: epithelial-to-mesenchymal transition and contribution of tumor microenvironment. J Cell Biochem *101*, 816-829.

Vila-Farré, M., Sluys, R., D'Aniello, S., Cebrià, F., Ferrer, X., and Romero, R. (2010). Marine planarians (Platyhelminthes: Tricladida: Maricola) from the western Mediterranean Sea and the Cantabrian coast: new records, one new genus, and immunocytochemistry of the nervous system. Journal of the Marine Biological Association of the United Kingdom *90*, 409-422.

Visvader, J.E., and Lindeman, G.J. (2008). Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer *8*, 755-768.

Voog, J., and Jones, D.L. (2010). Stem cells and the niche: a dynamic duo. Cell Stem Cell *6*, 103-115.

Wagers, A.J., Allsopp, R.C., and Weissman, I.L. (2002). Changes in integrin expression are associated with altered homing properties of Lin(-/lo)Thy1.1(lo)Sca-1(+)c-kit(+) hematopoietic stem cells following mobilization by cyclophosphamide/granulocyte colony-stimulating factor. Exp Hematol *30*, 176-185.

Wenemoser, D., and Reddien, P.W. (2010). Planarian regeneration involves distinct stem cell responses to wounds and tissue absence. Dev Biol *344*, 979-991.

Wiesner, J. (1892). Die elementarstructur und das wachstum der lebenden substanz (A. Hölder).

Wilson, A., Laurenti, E., Oser, G., van der Wath, R.C., Blanco-Bose, W., Jaworski, M., Offner, S., Dunant, C.F., Eshkind, L., Bockamp, E.*, et al.* (2008). Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. Cell *135*, 1118-1129.

Winston, W.M., Molodowitch, C., and Hunter, C.P. (2002). Systemic RNAi in C. elegans requires the putative transmembrane protein SID-1. Science *295*, 2456-2459.

Wolff, E., and Dubois, F. (1948). Sur la migration des cellules de régénération chez les planaires. Revue Swisse Zool *55*, 218-227.

Wright, D.E., Bowman, E.P., Wagers, A.J., Butcher, E.C., and Weissman, I.L. (2002). Hematopoietic stem cells are uniquely selective in their migratory response to chemokines. J Exp Med *195*, 1145-1154.

Wu, Z., Luby-Phelps, K., Bugde, A., Molyneux, L.A., Denard, B., Li, W.H., Suel, G.M., and Garbers, D.L. (2009). Capacity for stochastic self-renewal and differentiation in mammalian spermatogonial stem cells. J Cell Biol *187*, 513-524.

Zhong, J.F., Zhan, Y., Anderson, W.F., and Zhao, Y. (2002). Murine hematopoietic stem cell distribution and proliferation in ablated and nonablated bone marrow transplantation. Blood *100*, 3521-3526.