Stem Cell Migration in the Early Stages of Regeneration in Hofstenia miamia.

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Accessibility
Stem Cell Migration in the Early Stages of Regeneration in *Hofstenia miamia*.

Victoria Beinhart

A Thesis in the Field of Biology
for the Degree of Master of Liberal Arts in Extension Studies

Harvard University
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Abstract

The process of whole-body regeneration is a phenomenon seen broadly throughout animal species, and comparisons between the physiological mechanisms involved in the regenerative process between distantly-related organisms can shed light on the evolution of different processes in regeneration and development. The mobilization of cell types involved in tissue growth and replacement following amputation is an important aspect of regeneration across multiple species. The model organism *Hofstenia miamia* is an acoel worm capable of whole-body regeneration, with a stem cell population involved in tissue replacement following tissue amputation. The cellular and molecular response to injury, the reestablishment of body polarity and the development of regenerated structures have been studied previously in this organism, but little is known about the mechanisms of cell migration during regeneration.

Single-cell RNA-sequencing data and fluorescent in-situ hybridization were used to evaluate the mechanics of cell migration during the early stages of regeneration in *Hofstenia*. As was expected, the cell cluster assumed by previous research to represent neoblasts in these animals appeared to be the sole cell type migrating towards the wound site, while other progenitor types either did not appear to express motility-related genes in the dataset, or did not migrate long distances to the wound site to repopulate missing tissues. Most of the genes associated with migration in other species, namely planarians, that were investigated during this research (such as homologues for planarian *snail-1, zeb-1* and *mmp*) did not appear to be highly upregulated during regeneration; however,
the wound response gene *follistatin* was expressed in neoblasts which may have been migrating, and two integrins- *integrin α7* and *integrin β6*- were strongly expressed following amputation in cells surrounding neoblasts, potentially playing a role in neoblast motility.
Acknowledgments

Many thanks to Dr. Mansi Srivastava and the Srivastava lab for their invaluable support, kindness, and instruction. Thanks also to Dr. James Morris for his assistance with this thesis.
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Chapter I.

Introduction

Regeneration Across Animal Species

Regeneration, the ability to replace lost or damaged tissue, is found within diverse branches of the animal kingdom. This capability has a clear evolutionary advantage: enabling an organism to recover from injury that may otherwise prevent it from feeding, reproduction, or avoiding environmental hazard or predation (Bely, 2010). While the capacity to regenerate some tissue is common, a more restricted but highly-diverse collection of animals are capable of whole-body regeneration, which allows the recovery of complex tissues and organs following the removal of a significant portion of the animal’s body (Bely, 2010).

Organisms capable of significant tissue regeneration are represented throughout Bilateria. Within vertebrates, salamanders such as the axolotl are commonly-studied examples, capable of regenerating limbs, tails, and even significant portions of internal organs including the liver and heart (Li et al, 2015). Other species may have more limited regenerative capabilities- mice and some species of *Xenopus* are capable of limited regeneration, especially in neonatal animals. Regeneration is also evident in invertebrate animals- different levels of regenerative capacity exist in hydra, echinoderm, annelid, and many other metazoan families (Li et al, 2015). Common molecular pathways involved in regeneration between distantly-related animals regulating the initiation and organization of regenerative events suggests that this capacity may be analogous, and basal metazoans may also have been capable of regrowing lost tissues (Fields & Levin, 2020). The
presence of whole-body regeneration in highly diverse clades further suggests that the last common ancestor of bilaterians may have been capable of regenerating all tissues in the event of fragmentation.

On a molecular level, regeneration shares many aspects in common with developmental pathways (Jansson et al, 2015). Both involve the relatively rapid division of immature cells, similar molecular signals instructing tissue identity and positioning, and the process of cellular differentiation and maturation. This overlap suggests the potential of a last common ancestor to modern bilaterians that used the precursors to these pathways for regeneration. Therefore, studying regeneration in diverse modern animals may help elucidate the evolution of modern animal regeneration at the molecular resolution.

Model systems for studying regeneration

The acoel worm *Hofstenia miamia* is a marine animal capable of whole-body regeneration and serves as a novel model organism for regenerative mechanisms. As an evolutionary outgroup within the Bilateria clade (Figure 1), acoels are believed to have diverged from other bilaterian lineages some 550 million years ago, making them an important organism in studying the evolutionary history of development and regeneration (Ruiz-Trillo et al, 1999).

In terms of gross morphology, *Hofstenia* has a strong resemblance to the planarian flatworm genus *Schmidtea*, despite the two species having diverged early in bilaterian evolution. Parallels between the mechanisms of regeneration between these two species may indicate regenerative strategies developed by basal bilaterians, and lost in non-regenerating branches. However, many specific regenerative mechanisms have been
studied in *Schmidtea* that have not yet been investigated in *Hofstenia* (Reddien & Alvarado, 2004); demonstrating which shared mechanisms persist in these distantly-related organisms may help resolve when specific regenerative pathways evolved.

*Schmidtea* regeneration is mediated by Piwi-expressing (Piwi+) stem cells called neoblasts, and similar Piwi-expressing cells are found in *Hofstenia* as well. Neoblasts, the only proliferative cells in these animals, were found to be destroyed when the animals were subjected to high levels of radiation; the eradication of these cells in turn eliminated the ability of the planarian to regenerate. While the Piwi+ cell population in planarians referred to as neoblasts was found to be relatively diverse in subsequent transcriptional cell sorting (Van Wolfswinkel et al, 2014), a specific population of neoblast cells expressing cell-surface protein Tetraspanin-1 were identified as likely pluripotent stem cells and termed “clonogenic neoblasts,” or cNeoblasts (Zeng et al, 2018). Replacing a single cNeoblast in an irradiated planarian was found to replace the Piwi+ cell population in the animal, and recover its ability to regenerate following amputation (Wagner et al, 2011; Zeng et al, 2018); thus, these cells are considered to be the sole contributors to tissue regeneration following amputation. Neoblast cells in *Hofstenia* are presumed to play a similarly important role, though their precise behavior following injury requires further study.
Injury Response and Cell Mobilization

While much of the early stages of regeneration in Hofstenia remains unknown, chromatin accessibility evaluation studies have illuminated some of the genes involved early in the regeneration process. Cells of multiple tissue types, including muscle, epidermal and neural cells at the site of the injury, upregulate expression immediately after amputation of an ortholog of Early Growth Response gene, egr, a transcription factor required for the regeneration response (Gehrke et al, 2017). Animals inhibited through RNA interference (RNAi) from expressing egr are unable to regrow amputated tissue (Gehrke et al, 2017). Egr, in turn, upregulates other wound-induced genes, such as runt (a transcription factor important to promote heterogeneity in cell populations within
planarian blastema) (Wenemoser et al, 2012), follistatin (a component of a signalling pathway required for body patterning and missing tissue response in planaria) (Tehwari et al, 2018) and nrg-1 (identified in planarian regeneration as important for the differentiation of gut tissues) (Barberan et al, 2016).

Much is still to be determined about the behavior of neoblasts during Hofstenia regeneration. While they seem vital for regeneration and appear distributed through the acoel body plan in much the same way as seen in planarian flatworms, it has not yet been demonstrated whether these cells in Hofstenia are functionally identical to their equivalents in planarians. It is not yet known if these cells represent a single population of pluripotent cells, or a more diverse population of more restricted progenitor cells. It’s unknown if pluripotent neoblast cells are specifically responsible for fully generating all amputated tissue during regeneration or if multiple cell types contribute. Little is known about how these cells respond to wound signalling and migrate to wound sites.

In planarians, neoblast cells tend not to migrate significantly in homeostasis; even if a partially-shielded animal is exposed to radiation that ablates neoblasts from large patches of its body, Piwi+ cells are not seen to migrate into those irradiated areas to replace them (Gueldelhoefer & Alvarado, 2012). Following an amputation, however, neoblasts are mitotically activated and mobilized, and are actively recruited to the wound site (Gueldelhoefer & Alvarado, 2012).

Early research in Hofstenia found gene expression of piwi-1 localized in cells distributed throughout the body within mesenchymal tissue in a similar distribution pattern as found in planarian neoblasts, and BrdU staining and labeling with the mitotic marker H3P verified these cells were actively dividing at homeostasis (Srivastava et al,
Inhibiting *piwi-1* expression via RNAi prevented regeneration following amputation (Srivastava et al, 2014) demonstrating that in *Hofstenia*, as in *Schmidtea*, Piwi+ cells are necessary for regeneration. However, how these cells respond to chemical signals following injury to migrate to the wound site and contribute to regenerating tissue is unknown. Which cells migrate in regenerating *Hofstenia*, and by which mechanism, has not previously been explored.

Motility in other cell populations is accomplished through a range of mechanisms, but all migrating cells must interact with their environment in order to move. In planarians, the upregulation of the transcription of several proteins shortly after amputation is associated with stem cell mobilization, including myosins, actin-bound proteins, dynactins and tubulins (Rossi et al, 2007). Changes in levels of these proteins correspond with changes in the cell’s cytoskeleton, which corresponds with changes in motility in many cell lineages (Yourek et al, 2014). Of these diverse proteins, integrins are a particularly relevant group. Integrins are a broad, evolutionarily-conserved family of proteins strongly associated with adhesion of a cell to either other neighboring cells or the extra-cellular matrix (ECM) (Wehrle-Haller, 2013). The adhesion of the tip of a cell protrusion, such as a filopodia, with the surrounding environment, via integrins and their receptors, precedes the movement of these cells. Once anchored to its environment, the cell then uses cytoskeletal protein conformational changes to crawl forward towards these attachment points (Wehrle-Haller, 2013).

In planarians, multiple genes are associated with neoblast motility following wounding. These include the transcription factors *snail-1, snail-2* and *zeb-1*, the interference of which impacts cell migration following wounding in *Schmidtea* (Abnave 2014).
et al, 2017), and the matrix metalloproteinase \textit{mmpa}, of which RNAi has been found to delay blastema growth in \textit{Schmidtea} while allowing neoblast proliferation to continue unaffected. This indicates that the localization of these dividing cells to the wound site is impacted, suggesting a role of matrix metalloproteases in cell migration during regeneration (Isolani et al, 2013).

**Single-Cell RNA Sequencing**

While previous research on related processes in other animals is a useful resource when investigating cell migration and regeneration within \textit{Hofstenia}, other tools are available to directly investigate gene regulation and transcriptomic changes specifically within our model organism, and generate candidate genes for different processes of interest in an unbiased and computational manner. Single-cell RNA sequencing (ScRNA-seq) is a technique through which the transcriptome of different cell populations within an organism can be examined (Svensson et al, 2018). These data can also be examined for changes in the transcriptome of cell populations over time, such as during embryonic development or regeneration, and examined for the expression of proteins associated with specific functions (Svensson et al, 2018).

Analysis of ScRNA-seq data using Seurat illuminated the transcriptome of many distinct and statistically-significant cell populations in \textit{Hofstenia}, including multiple populations of somatic tissues such as muscle, digestive tissues, and neural populations. It has also found multiple neoblast-like and immature progenitor cell populations at homeostasis in hatchling, juvenile and adult worms. The data provided by these computation methods of analysis are information-rich and, once generated, ScRNA-seq data can be easily accessed and mined to formulate hypotheses or answer questions
regarding cell populations and transcriptomic activity at different stages in an organism’s life history.

Illuminating the role of each of these cell type populations in homeostasis and regeneration will allow us to more clearly understand regeneration within this organism, and compare it to regenerative mechanisms in other animals throughout bilateria. These techniques allowed for the exploration of transcriptomic changes during regeneration, and the exploration of potential migration-related genes within Hofstenia.

Specific Aims

First, I aimed to establish the degree to which single-cell RNA seq data previously generated of Hofstenia miamia remains robust and consistent when examined using slightly changed frameworks, in order to establish the boundaries of reliability in these data. I also evaluated the consistency of manual analysis of data produced by visual examination, versus the computational calculations produced programmatically.

Then, by exploring in-depth ScRNA-seq data in Hofstenia both in homeostasis and during regeneration, I explored the expression of migration-associated genes in neoblast and progenitor cell populations, in hopes of highlighting prospective migratory populations during regeneration. I used gene ontology (GO) to select for genes associated with cellular motility among piwi+ neoblast and progenitor cells. This allowed me to narrow down the prospective migratory cell populations to neoblasts and epidermal progenitor cells. I investigated the behavior of these candidate populations at different stages of regeneration, focusing on changes in distribution of expression of specific cell markers through the early stages of regeneration.
Finally, I investigated both upregulated genes within prospective migratory cell populations derived through analysis of single-cell data between 0 and 6 hours post amputation, and genes previously indicated as being important components of cell migration in other regenerating organisms, in order to shed light on the mechanisms by which *Hofstenia* stem cells migrate to the wound site and contribute to regenerating tissue. Genes which served as markers differentiating a population of neoblast or progenitor cells from the same population at different an earlier timepoint during early regeneration were selected computationally, and both they and candidates selected from the literature were evaluated to determine expression pattern changes during early regeneration.
Chapter II.

Methods and Materials

Aim 1

Single-cell RNA-seq data, previously acquired by the lab, were used to verify the reliability of Seurat data analysis and clustering. Single-cell data were acquired by macerating whole worms into single-cell suspensions, which were then analyzed by Bauer core. Matrix files were generated which retained the mRNA transcripts detected and levels at which each transcript was present within each cell of the animal. While matrices had been generated for multiple developmental and regenerative stages for *Hofstenia*, the data set used for this aim was restricted to whole late-juvenile stage worms. This stage was selected due to the use of late juvenile worms for fluorescent in-situ hybridization (FISH) analysis, particularly during regeneration studies. Late juvenile worms are small enough to be easily imaged using confocal microscopy while being large enough to amputate.

Previous computational analysis on *Hofstenia* single-cell data had been performed using version 2 of the Seurat program from the Satija lab. At the time of the initiation of this project, version 3 of the same program was in the process of being released, generating a need to assess the consistency of the program’s principal component clustering analysis. Data from whole late-juvenile stage worms were imported into versions 2 and 3 of Seurat and converted into Seurat objects. For the dataset in each version, cells were excluded if they contained too few genes (n < 200) or too many (n >
to exclude damaged cells and doublets. The datasets were log normalized, and the
data were scaled using Seurat’s native ScaleData function.

Principal component analysis was performed on both datasets, and a Jackstraw
plot was created to ensure PCs used were statistically significant for the data. For both the
version 2 and version 3 analysis, 20 PCs were significant (p = 0.05) and were used to find
Nearest Neighbors and cluster the cells. Umap clustering was performed for both
datasets. The resulting Umap generated by Seurat version 2 had 16 clusters, compared to
20 generated by version 3.

The top 50 most significant markers were generated using Seurat’s native
FindMarkers call for each of the clusters. Most clusters for the original version 2 dataset
were identified by cell type using gene markers previously identified in the lab, and
validated as relating to specific tissue types in the animal. A short program was written to
iterate through the markers for each cluster in the version 3 dataset and compare them to
the top 50 marker lists for each cluster from version 2. The end result of this produced a
list of represented version 2 cluster markers for each cluster in the version 3 dataset, and
a number of marker genes yielded by version 3 that was not represented at all within the
50 most significant markers in the version 2 umap. This information was used to match
version 3 clusters to corresponding version 2 clusters, as well as providing a number of
matching genes between the clusters and a number of genes not overlapping between the
versions.

The consistency of markers for tissue types computationally generated as the
“best” for each cluster was also investigated, with direct comparisons of the top ten gene
markers generated using Seurat’s FindMarkers command performed between
corresponding clusters yielded in Seurat version 2 and Seurat version 3. Markers were then also analyzed by eye- a short program was written to generate visual umap projections of each of the top 50 markers of each cluster, which were each visually inspected. Signal strength, signal presence throughout the cluster of interest, and signal absence in all other clusters, were used as criteria to determine a “good” potential marker for cell type, and the top markers, for a maximum of ten, for each cluster in each version were identified visually. For some clusters, fewer than ten markers appeared on visual analysis to be robust markers of the cluster. As PCA differentiates some cell clusters by an absence of gene expression, this was not overly surprising, and for these clusters fewer than ten markers were selected. These results were then compared to determine if cell type markers that were generated computationally or identified by eye were more consistently maintained between the two program versions.

For all future single-cell RNA seq analysis, Seurat version 3 was used.

Aim 2

To identify cell types expressing migratory genes during early regeneration, a single-cell RNA seq dataset generated from macerated worm head and tail fragments 6 hours past amputation was imported, normalized and analyzed via principal component analysis as discussed above. Neoblasts and progenitor cells were the focus of this assay as it was presumed these cells were responsible for replacing amputated tissue. Cells expressing the *Hofstenia piwi* homologue *piwi-1* to a log-normal low threshold of 2.5 (based on average positive expression across the 6hpa dataset) were subsetted into a new dataset. Umap reduction was performed on the *piwi+* data, yielding a Umap with 9 clusters.
Seurat’s FindMarkers function was used to generate the top 50 markers for each cluster in the piwi-1+ data, which were compared to experimentally-validated markers of known tissue types in the animal. Additionally, gene ontological analysis was performed using the David Bioinformatics Database to locate genes associated with cell migration and motility.

To visualize cell migration in cut worms, 60 juvenile worms were starved for approximately one week, then bisected into head and tail fragments. Fragments were fixed in paraformaldehyde at either 0, 6, or 24 hours post-amputation (20 worms in 40 fragments per timepoint). in situ hybridization was carried out as per lab protocol (Srivastava et al, 2014). The fragments were bleached, fixed again in paraformaldehyde, then incubated for two hours in prehybridization at 72 degrees before being hybridized overnight with digoxigenin (DIG) probes for each marker gene (5 heads and five tails per gene per timepoint). The worm fragments were then cleaned and incubated for at least two hours in a blocking solution, before being incubated overnight in anti-DIG antibody solution. The fragments were then washed again, developed in a rhodamine solution, cleaned for a final time, then mounted on slides and imaged using a confocal microscope.

Aim 3

To identify gene candidates, in an unbiased manner, involved in cell migration during regeneration, data sets for single-cell RNA seq data for adult worms at 0 and 6 hours post amputation were integrated using “anchor” aligned canonical correlation analysis as described by Stuart et al, 2020. Umap reduction was performed on the resulting integrated dataset as previously described.
Expression of *piwi-1* was used as a metric for potential neoblast cells that may be beginning to express migratory genes between the 0 and 6 hour timepoints. Six cell clusters were identified as significantly expressing *piwi-1*. The FindMarkers function was used to identify differentially expressed genes within those clusters between the two timepoints, and each of these genes were visualized by projecting expression onto the integrated Umaps.

In total, six genes were found to be differentially expressed within *piwi-1*+ clusters between these two timepoints. Of these, two genes were found to be differentially expressed in neoblasts that have been of interest previously in *Hofstenia* research- *follistatin* (Gehrke et al, 2017) and *wnt-3* (Ramirez et al, 2020). The other genes yielded by this approach were primarily small genes of unknown function.

In an alternative approach, gene candidates were selected from previous literature regarding cell migration during development and regeneration. Homologues for these genes were determined by BLAST using Genious software. 9 genes in total were used for this study: *follistatin*, *wnt-3*, *mmp1*, *paxillin*, *scrt1* (homologue for planarian *snail-1* gene), *integrin β3*, *integrin β6*, *integrin α6*, and *integrin α7*.

As above, juvenile worms were selected and starved. 36 animals were fixed and bleached whole, while the 108 were cut, and the head and tail fragments were fixed at 0, 6 and 24 hours post amputation and then bleached as in Aim 2.

Whole animals and fragments were incubated in prehybridization solution before being hybridized with a fluorescein *piwi-1* probe and a DIG probe of one of the above nine genes (four head and four tail fragments per gene per timepoint). After being hybridized overnight, the worms and fragments were washed, incubated in blocking
solution, then treated overnight in anti-DIG antibody solution. Animals and cut fragments were then developed in rhodamine, washed, and incubated again in blocking solution. They were then treated overnight in anti-fluorescein antibodies, washed, and developed in Fluor dye. Worms were then washed and mounted onto slides. Imaging was done using a confocal microscope.

Table 1. Gene transcripts as annotated within the *H. miamia* transcriptome and as referenced for the purposes of this manuscript.

<table>
<thead>
<tr>
<th>Transcriptome Gene IDs Used</th>
<th>Gene ID</th>
<th>Gene Name</th>
<th>Source</th>
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<tr>
<td>98043523-piw11-2</td>
<td><em>piwi-1</em></td>
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<td>Srivastava et al, 2014</td>
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<td>Neoblast marker</td>
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<td>Neoblast Marker</td>
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<tr>
<td>98058295-fmr1n-2</td>
<td><em>fmr1n</em></td>
<td></td>
<td>Epidermal progenitor marker</td>
</tr>
<tr>
<td>98005757-ogt1</td>
<td><em>ogt1</em></td>
<td></td>
<td>Epidermal progenitor marker</td>
</tr>
<tr>
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<td><em>Integrin β3</em></td>
<td></td>
<td>Best blast hit</td>
</tr>
<tr>
<td>98113087-ibt6</td>
<td><em>Integrin β6</em></td>
<td></td>
<td>Best blast hit</td>
</tr>
<tr>
<td>98022379-ita6</td>
<td><em>Integrin α6</em></td>
<td></td>
<td>Best blast hit</td>
</tr>
<tr>
<td>98032164-ita7</td>
<td><em>Integrin α7</em></td>
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<td>Best blast hit</td>
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<td><em>Schmidtea mmpa</em> homolog</td>
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<tr>
<td>98045879-paxi</td>
<td><em>paxillin</em></td>
<td></td>
<td>Best blast hit</td>
</tr>
<tr>
<td>98038855-scrt1</td>
<td><em>snail</em></td>
<td></td>
<td><em>Schmidtea snail1</em> and <em>Schmidtea snail2</em> homolog - best blast hit for both</td>
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<td>wnt-3</td>
<td>Srivastava et al, 2014</td>
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</table>
Chapter III.

Results

Evaluation of Single-Cell RNA-seq Data

To establish consistency within computational analysis of the *Hofstenia miamia* transcriptome, umap reduction was performed on a late juvenile single-cell RNA seq dataset using the same parameters for normalization, scaling, and principal component analysis in versions 2 and 3 of the Satija lab’s Seurat program.

Major Cell Type Clusters Consistent Between Program Versions

While there were initial differences immediately recognized, the umap projections of the data also had multiple apparent consistencies (Figure 2). The newer version of Seurat yielded a umap with a higher number clusters (20, compared to 16 clusters in the older version), but the general organization of the cell types remained similar. Both umaps had a central cluster (labeled Cluster 0 in both) that expressed genetic markers of neoblast cells in *Hofstenia*, such as *piwi-1*, *h10* and *ryr1*. This central cluster branched outward into nearby clusters of cells expressing markers of progenitor cell types, such as endodermal and neural progenitor cells. Progenitor cells also were visualized as expressing mid-to-high levels of *piwi-1*, while also expressing transcripts specific for their intermediate tissue type, such as *sox* genes in neural progenitors. Clusters further from the center tended to be somatic, fully-differentiated tissue types, such as muscle cells, identified as expressing *tropomyosin* and other markers (Raz et al, 2017).
The top 50 generated cell type markers for each cluster in both reductions were generated and compared to determine overlap between markers selected within the two versions (Figure 3). Of 20 clusters generated by the version 3 analysis, 12 clusters overlapped with a single corresponding version 2 analysis cluster, sharing more than 40 out of 50 top markers. These clusters corresponded primarily with better understood and delineated tissue types, such as neoblasts (cluster 0), neural (in the version 3 umap, represented by cluster 8), pharyngeal epidermis (cluster 19), endodermal (cluster 15), mesenchymal (cluster 4) and muscle cells (cluster 5) and their progenitor types (ie, Cluster 17 contains cells expressing genes associated with muscle progenitors in Hofstenia, while Cluster 2 appears to contain epidermal progenitor cells). Clusters with relatively low overlap between versions represent either currently unknown biology of the animal, potentially subsets of known cell types previously not separated from larger clusters in the older version, or may represent computational noise.

Program Generated Markers More Consistent Than Visually Selected Genes

It was also of interest to discern whether the program’s FindMarkers function reliably called transcripts that could most predictably be used as markers in benchwork experiments as top results, when compared to transcripts that appeared to be restricted to specific clusters when evaluated by eye via projection onto the Umap. To determine whether computationally determined markers or visually determined markers were more robust, the top 10 genetic markers generated computationally for each cluster in Seurat version 2 were compared to the corresponding cluster in version 3 (Figure 4). Additionally, each of the top 50 markers for each cluster were projected onto umaps images, and the images that best appeared to show expression strongly and exclusively in
the cluster of interest were selected, with a maximum of ten for each cluster. Visually-selected transcripts for each cluster in Seurat version 2 were compared to that cluster’s corresponding cluster in version 3 (Figure 5).

It was found that computationally-generated markers were more consistent between versions than those selected by eye for every “pair” of corresponding clusters. This may be due to human error, as differences between color intensity representing transcript level in different cells may be hard to parse visually. It may also be a result of the process of generating a umap, in which each rendered dot represents a single cell, and in which some dots are drawn overlapping due to similarities between the cells in gene expression. This can obscure some important information and make visual determination of marker validity difficult to ascertain. In either case, it was determined that the raw data generated by Seurat’s FindMarker function provided more robust and presumably reliable transcripts of interest than visual inspection of transcript projection on a umap.
Figure 2. Single-Cell Data Visualization. Comparison of umaps produced by Version 2 (left) and Version 3 (right) of Seurat using the Hofstenia miamia late juvenile dataset. Both projections have a central cluster expressing genes associated with neoblast cells, with projections representing progenitor and somatic cell types.

Figure 3. Overlapping markers between program versions. A visualization for overlapping top 50 genetic markers for different clusters generated in Versions 2 and 3 of Seurat. Each horizontal bar represents a cluster in the version 3 umap reduction of the late juvenile dataset. Each bar is composed of segments representing a number of genes within the version 3 cluster that are also represented in a specific cluster in the version 2 reduction. The color of each segment represents the version 2 cluster within which those genes appear, and the length of the segment represents the proportion of genes occurring within that segment. The white number within the segment shows how many genes are represented by that segment. If genes within the version 3 cluster are not found within any clusters of the version 2 reduction, the number of those genes are given in black to the right of the bar. Clusters representing well-explored cell types with known markers,
such as version 3’s Cluster 0 (neoblasts), Cluster 5 (muscle), and Cluster 19 (pharyngeal epidermis) are robust, sharing a majority of markers between the two protocols. However, less well-defined clusters also exist.

Seurat v3 - Late Juvenile - Compared to Seurat v2 - Late Juvenile

Top 10 markers generated by FindMarkers command

Figure 4. Comparison of program-generated markers. Top ten markers generated computationally by Seurat’s native FindMarkers call, compared between corresponding clusters in versions 2 (pink) and 3 (blue). 16 out of 20 total version 3 clusters shared at least 70% of genetic markers between the version 3 cluster and a corresponding version.
Seurat v3 - Late Juvenile - Compared to Seurat v2 - Late Juvenile

Top markers selected by eye, maximum of 10 per cluster

Figure 5. Comparison of visually-selected markers. The top 50 markers for each cluster in versions 2 and 3 of Seurat were generated, projected onto umap projections, and
visually assessed for strength of signal, expression throughout a cluster, and restriction of expression to the cluster of interest. The top ten markers selected for each cluster by eye were then compared between versions. Significantly less consistency was seen between versions 2 and 3 when markers were selected by eye. Only one cluster from version 3 shared 70% of its top ten markers with a corresponding version 2 cluster and 8 clusters- 6 from version 3, and 2 from version 2, did not share any overlap at all with a corresponding cluster.

Identification of Motile Cells During Regeneration

Following tissue loss in animals capable of whole-body regeneration, the surviving tissue must be capable of some degree of remodeling and regrowth to obtain important structures, like lost muscle, digestive structures, and even brain tissue. In many studied species, this involves a stem cell population that migrates from its homeostatic niche to the site of tissue loss, and multiplies. It is assumed that cells which are considered to be neoblasts in Hofstenia, currently conceptualized as pluripotent stem cells and maintained in the mesenchyme of the animal, migrate to the wound site in the event of amputation. However, it is unknown whether these cells are the only cells that participate in tissue regeneration, as previously discussed in planarians, or whether multiple types of multipotent progenitor cells may migrate and contribute, either by contributing differentiating progeny, or by dedifferentiation as seen in animals such as the axolotl. Establishing a specific subset of cells which are involved in cell migration in response to amputation may help clarify and verify aspects of the mechanics of acoel regeneration.
Figure 6. Gene transcription during early regeneration. Umap reduction of the ScRNA-seq dataset of adult worm head and tail fragments 6 hours post-amputation - at the likely initiation of neoblast migration.

A dataset of single-cell RNA seq data of adult worms 6 hours post-amputation was used to evaluate which cells were expressing transcripts associated with cell migration and motility. The dataset was normalized and scaled, then PCA and umap reduction were performed on the normalized data. As it is believed that cells contributing to regenerated tissue express the piwi homologue piwi-1 in Hofstenia, piwi+ cells were subsetted out of the dataset, and PCA and umap reduction were then performed on the piwi+ cell subset (Figure 7).
Figure 7. Neoblast and progenitor cell populations express genes associated with different functions during early regeneration. *Umap reduction of piwi-l+ cells subsetted from the 6hpa regeneration dataset with most significant gene ontology terms of the top 300 markers for each cluster. Of the clusters containing genes associated with cell motility, only the neoblast cluster (cluster 4) and the epidermal progenitor cluster (cluster 0) were selected for FISH analysis. Clusters 6 and 2 expressed genes related to cell migration, but appeared to consist of gamete progenitor cells, and were thus disregarded.

Of nine clusters in the piwi+ 6hpa regeneration dataset, four clusters showed transcripts associated with cell migration via gene ontology terms. One cluster expressed transcripts related to the gelsosin-villain complex, an important component of podosome formation and thus cell motility. This cluster, however, also expressed genes associated with meiosis and spermatogenesis, and the cluster itself was located close to gamete cells in the umap representation, implying that these cells were likely gametic progenitor cells and therefore less likely to be contributing to regeneration. A nearby cluster expressed high levels of fibronectin (associated with integrin complexes, interaction with the

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extracellular matrix, and migration) and metalloproteases, but its location near the previous cluster on the umap and ambiguous identity made it appear likely that these cells were also precursors to gamete cells in the regenerating worms.

A cluster (Figure 7, cluster 0) was identified to have transcripts associated with focal adhesion, cell-to-cell adhesion, and interaction with the extracellular matrix, all cellular functions associated with migration (Wehle-Harler, 2013). This cluster also expressed markers associated in ongoing work in the lab with epidermal progenitors, such as urom and pax9. While epidermal progenitors are understood to migrate within the epidermis of many metazoans, it is still possible that the transcripts seen within this cluster may imply a long-range migration specific to the regeneration process.

Additionally, the cluster that appeared to correspond to neoblast cells due to expression of neoblast makers such as h10, h2ax2, ryr1 and other markers, also expressed transcripts associated with cell adhesion to the ECM, some of which have been associated with migration, such as actin gamma (Shum et al, 2011).

Due to the low likelihood that gamete progenitors contribute to regenerating tissue, neoblasts and epidermal progenitors appeared to be the best prospective candidate cell types for migration towards the wound site and contribution to tissue replacement during regeneration.
Figure 8. Gene selection for FISH analysis. *Projections of the four selected marker genes intended to label the two clusters of interest.* Neoblast genes s28a1 and nano1 and epidermal progenitor genes ogt1 and fmr1n-2 were selected due to previous FISH studies showing good performance in marking desired cell types.

Visualization of cell migration in-vivo, while ideal, was not tenable within the available timeframe and with lab resources readily available. A regeneration timecourse fluorescent in-situ hybridization (FISH) was therefore carried out to visualize changes in the distribution of neoblast and epidermal progenitor cells at 0, 6 and 24 hpa in late juvenile animals. It was expected that non-migratory cell populations would retain similar distribution patterns for the entire timecourse, while migratory populations would shift distribution towards the wound site, with signal in the intact portion of the fragment decreasing, and signal increasing along the borders of the cut edge (Figure 9).
Figure 9. Illustration of cellular migration over time. Schematic of desired results showing a clear concentration of fluorescent signal at the wound site of an amputated animal over a 24-hour period. A loss of signal in areas further from the wound and simultaneous increase in signal at the site of tissue regeneration would support the hypothesis of cell movement to contribute to regenerated tissue.

Evaluation of cell migration by this method was not as precise as desired, and changes in signal across the fragment were particularly difficult to visualize in the head.
fragments of all animals in the study. Due to the distribution of both neoblast and epidermal progenitor cells beginning just behind the head region in intact animals, discerning between migration, expansion due to proliferation, and positional changes due to slight differences in fragment size was difficult. Results, however, appeared more distinct in tail fragments. Neoblast cells labeled by s28al and nano1 (Figures 10, 11) both change significantly in distribution within the tail fragment, losing signal strength near the caudal tip and maintaining expression near the wound site. This was more noticeable with s28al+ cells, which demonstrated a more restricted distribution at 6hpa and appeared to maintain a higher concentration at the most anterior tip of tail fragments at 24hpa. The nano1+ cells, in contrast, displayed a weaker change in signal distribution, but still showed a loss in signal strength at the more caudal region that became more predominant at 24hpa. Overall loss in signal strength of both s28al and nano1 at 6hpa and 24hpa may be due to transcriptional changes, apoptosis, or differentiation at different stages in regeneration.

Epidermal progenitor cells labeled by expression of fmn1r did not change distribution or signal strength within tail fragments in any noticeable way (Figure 12). Overall loss of signal strength at 24hpa was noticeable, but seemed generalized and did not correspond to a change in concentration of these cells in any specific location of the fragment. It is therefore unlikely that this change relates to migration of these cells. Fluorescent labeling of ogt1+ cells did not develop appropriately at all timepoints and was therefore not considered for this study.
Figure 10. Neoblast time course (1). Change in distribution of s28a1+ neoblast cells during regeneration. Changes in signal concentration are difficult to discern in head fragments (left, all three timepoints). Cells are initially distributed throughout the tail fragment (right, all three timepoints) in a pattern typically seen with neoblasts within the
organism. After the 0hpa timepoint, a generalized reduction in overall signal is seen, potentially corresponding with apoptosis. However, a relatively high concentration remains at the wound site, especially at the 6hpa timepoint.
Figure 11. Neoblast time course (2). Cells expressing nano1 in early regeneration. As seen in Figure 9, little change is seen in cell concentration in the head fragments (left); however, changes are more evident in the tails (right). Signal appears relatively widespread at 0hpa, but shifts to the left, towards the wound site, in subsequent timepoints. This is most noticeable in the 24hpa timepoint. It is unknown if this corresponds to proliferation or migration, but supports the hypothesis that neoblasts migrate to contribute to regenerated tissue.
Figure 12. Epidermal progenitor time course. Cells expression \( fmrln \) through regeneration. These cells, epidermal progenitors, appear to be distributed regularly throughout the tail fragments (right) at all three timepoints, with no significant shift in concentration as seen with the neoblast markers in Figures 9 and 10. An overall decrease
in signal is noted at 24hpa, but it appears uniform and does not lead to a significant concentration of signal at the wound site.

These data support the hypothesis of a set of neoblast cells which respond, on wounding, migrate to the site of tissue loss and contribute to regeneration.

Analysis of Candidate Migration Genes During Regeneration

To elucidate the mechanisms involved in neoblast migration during *Hofstenia* regeneration, I generated a list of candidate regulators based on previously published studies of migration genes in other animals. The role of integrins in cell motility was a point of focus due to the conserved core functions of these proteins across metazoans (Wehrle-Haller, 2013). Other genes were selected due to their interactions with integrins during cell migration in other organisms (*matrix metalloproteinase 1* (*mmp1*), *paxillin*) (Isolani et al, 2013) or due to their expression being required for cell migration during regeneration in planarians (*snail* proteins, *zinc finger E-box binding homeobox 1* (*zeb1*)) (Abnave et al, 2017). For these genes, the blast function of the program Genious was used to find homologues within the *Hofstenia* genome, which were then used to generate probes for FISH experiments. Many of these genes yielded low or non-specific signal in uncut control worms and in the regeneration time course.

Unbiased candidates for genes involved in cell migration were generated using Seurat. First, an integrated dataset was created by combining the 0hpa and 6hpa regeneration datasets using anchor-point CCA reduction. Then, clusters within the resulting umap with high relative *piwi* expression were noted, and compared for differential gene expression between the two timepoints (Figure 13). This analysis
yielded several proteins with increased expression following the first initial hours of regeneration. While many were relatively unexplored proteins of unknown function, two of the proteins, *follistatin* and *wnt-3* have been previously investigated as being involved in other aspects of wound signalling and regeneration. While the functions of the less understood genes may be interesting in later studies, it was decided to investigate the role of *follistatin* and *wnt-3* in neoblast cells during early regeneration.

No significant expression was noted of the *Hofstenia snail* homologue, *zeb1*, *paxillin*, *mmp1*, or most of the integrins looked at in this study beyond background signal that did not change significantly between timepoints.

In whole, uncut worms, no noticeable expression was noted of any integrin. However, immediately after cutting, at the earliest post-amputation timepoint, *integrin β6* (itb6) was expressed with strong signal in large, mesenchymal cells in the head fragments only (Figure 14). These cells appeared to continue expressing itb6, with some expression tapering off towards the 24hpa timepoint. *Integrin α7*, while not being evident as early as the 0hpa timepoint, was clearly expressed in similar large, mesenchymal cells, also in the head fragments only, at the 6hpa timepoint (Figure 15). At 24hpa, this expression had also waned.
Figure 13. Change in expression of selected genes over time. Merged datasets of Hofstenia miamia adults at 0hpa and 6hpa post-amputation regeneration data showing changes between timepoints of notable genes. A) The merged umap reduction showing projected piwi-1 expression in six notable clusters. These clusters were then evaluated for changes in gene expression between the timepoints. B,C) Two integrin candidates, ita7 and itb6, showed slight changes in expression in the central neoblast cluster when 0hpa(left) was compared to 6hpa(right), though, while both showed significant changes in expression during regeneration, neither showed upregulation in piwi+ cells during the FISH analysis. D, E) follistatin and wnt-3 were both candidates generated by unbiased investigation of the merged dataset, with significant upregulation of both genes in piwi+ cells at 6hpa compared to 0hpa.

No coexpression was noted between itb6/ita7 and piwi, though the integrin-expressing cells appeared to be located physically close to neoblast cells in the head fragments. Though co-expression between the integrins was not looked at in this study, the behavior of integrins forming from alpha and beta subunits and the pattern of
expression of these two genes does indicate that they may be coexpressed. The rapid change in integrin expression in these cells immediately and consistently after wounding, and their proximity to neoblast cells, raises the possibility that neighboring cells in the mesenchyme may contribute to neoblast migration, through cell-cell adhesion and potentially coordinated and collective movement.
Figure 14. Integrin β6. Time course FISH of piwi-1 (whole worm: blue, cut worms: green) and integrin β6 (whole worm: purple, cut worms: red). In whole worms, no signal was seen beyond background in any of the animals imaged. However, starting just after wounding, signal was seen in large, mesenchymal cells located physically close to
neoblasts in the head segments only. These cells did not appear to express piwi-1. No signal was seen beyond background in the tail fragments at any point.
Figure 15. Integrin α7. Time course FISH of piwi-1 (whole worm: blue, cut worms: green) and integrin α7 (whole worm: purple, cut worms: red). In whole worms, no signal was seen beyond background in any of the animals imaged. However, at 6hpa, signal was seen in large, mesenchymal cells located physically close to neoblasts in the head segments only, similar in shape and location to those labeled by itb6 in Figure 13. These cells did not appear to express piwi-1. Signal was greatly reduced or gone by 24hpa. No signal was seen beyond background in the tail fragments at any point.
Figure 16. Wnt-3. Time course FISH of piwi-1 (whole worm: blue, cut worms: green) and wnt-3 (whole worm: purple, cut worms: red). In whole worms, signal was restricted primarily to small cells at the caudal tip of the animal. Shortly after wounding, signal appears more widespread, appearing at the wound site in head fragments. In head
fragments only, coexpression with piwi-1 appears at 6hpa and more strongly at 24hpa, though the nature and behavior of those cells is difficult to discern and there is no clear sign that wnt-3 is playing a role in neoblast migration.
Figure 17. Follistatin. Time course FISH of piwi-1 (whole worm: blue, cut worms: green) and follistatin (whole worm: purple, cut worms: red). In whole worms, follistatin is expressed in non-piwi+ cells along the muscle wall. Following amputation, seen starting in the 6hpa fragments and as previously shown by Gerkhe et al 2019, follistatin is expressed at the wound site of both head and tail fragments. In 6hpa and 24hpa fragments, coexpression with piwi-1 is shown (white arrows, yellow signal). Unlike wnt-1, cells expression both piwi-1 and follistatin appear both close to the wound site and slightly further towards the anterior end of head fragments and posterior end of tail fragments. Neoblasts may, therefore, be expressing follistatin during migration.

The role of wnt-3 in regeneration has been a topic of interest previously in the lab, and its role in establishing anterior-posterior polarity in Hofstenia as in other organisms following amputation has been previously investigated (Ramirez et al, 2020). Whether it is expressed in migrating cells or has any role in cell migration, specifically, is unknown.

In uncut worms, wnt-3 is expressed in muscular cells at the tail end of the animal (Ramirez et al, 2020). Immediately following amputation, wnt-3 was visible in the muscle cells of the tail fragment but relatively absent from the head fragment (Figure 16). At 6hpa, muscle cells near the wound site of the head fragment were expressing wnt-3, but no coexpression was seen until 24hpa, where small sites of potential coexpression were visible, only in the head fragment and mostly concentrated near the wound site. Neoblast cells further from the wound site and migrating to contribute to regeneration were not expressing wnt-3. While there does seem to be a change in expression of wnt-3 in piwi+ cells during regeneration as predicted by the dataset, wnt-3 does not seem to be directly involved in cell migration.

The role of follistatin during Hofstenia regeneration has also been studied, as a key gene in the wound response pathway immediately following cutting. Like wnt-3, follistatin is primarily expressed in the muscle; it’s expression in neoblast cells during regeneration was relatively unknown.
In uncut worms, *follistatin* was expressed as previously described, in the muscle wall and especially in longitudinal muscle fibers (Figure 17). At 6hpa, however, strong expression is seen at the wound site, and coexpression with *piwi-1* appears to be visible in cells both directly at, and spreading slightly away from, the wound site. At 24hpa, coexpressing cells appear even further from the wound edge. Coexpression is seen in both head and tail fragments.

Whether *follistatin* is directly involved in cell migration is unclear; however, it appears to be expressed in neoblasts during the time frame where these cells are migrating towards the wound site, in neoblast cells which may be migrating based on their position in the animal. It is therefore possible that *follistatin* may be involved in facilitating neoblast movement during regeneration, and more research will need to be done to evaluate the precise role of this gene in neoblast motility.
Chapter IV.

Discussion

Single-cell RNA seq analysis is a valuable tool for understanding and utilizing transcriptomic data. The acoel worm *Hofstenia miamia* is a relatively novel model organism for the study of regeneration and development, with available single-cell transcriptome datasets for whole adult worms, as well as worms at varying stages of growth and regeneration. These datasets have been analyzed using principal-component analysis and umap reduction in the R program Seurat.

Evaluation of the consistency of results generated using this protocol found the cell type clusters and genetic markers of tissue types to be broadly consistent between versions of the program. This indicates that, for the most part, conclusions drawn from computational work using this ScRNA-seq data is fairly robust. The main tissue types identified through Seurat analysis, such as neoblasts, main somatic cell types such as digestive tissue and muscle cells, and progenitor cell lines, are consistent between analyses, and marker genes generated for these cell types are likely to be largely reliable. However, both versions produced additional clusters of largely unknown cell types, which tended to be less consistent between analyses. While these cells may very well depict real biology and should not be completely disregarded, comparisons between umaps generated in Version 3 of Seurat with its older predecessor show that genetic markers for these clusters are not persistent to the same degree as known tissue types.

This study highlights both the strengths of single-cell RNA seq data processing, in that gene transcripts associated with main tissue types are relatively reliable and easily
accessed, and potential weaknesses to be cautious of when using these protocols. Some error is to be expected when analyzing large quantities of data, and some small cell type clusters of enigmatic origin may not represent actual biology. Moreover, the graphics generated by Seurat may not be reliable for precise visual analysis, and researchers should be cautious when drawing specific conclusions from images drawn by the program. Computationally generated markers tend to be more consistent than visual conclusions derived from generated umap projection images.

Single-cell data and FISH were used to investigate mechanics of cell migration during the early stages of regeneration in *Hofstenia miamia*. While it was previously assumed that *piwi*+ stem cells were solely responsible for migrating to the wound site and propagating, replacing lost tissue, this had not been established as in other model organisms. Moreover, the mechanics of cell migration and transcription factors involved in this process are unknown.

Through a combination of single-cell RNA seq analysis and time-course, it was established that while both epidermal progenitor cells and neoblast cells express transcripts associated with cell migration, epidermal progenitor cells did not change distribution within the animal during the first 24 hours of regeneration. Motility-related genes expressed by these cells are more likely associated with short-distance migration rather than wound-induced, long-distance movement. Neoblasts, however, did appear to accumulate at higher concentration at the site of injury compared to more distal regions, where signal was lost during the timecourse. While there may be other factors involved with this change in signal distribution, including body-wide apoptosis and proliferation of cells near the wound site, these data do support the previously-supposed hypothesis that
neoblasts are the cell population responsible for responding to wound signalling and migrating to the wound site for restoring tissue lost following amputation.

Several genes were investigated to elucidate more clearly the mechanics of neoblast migration during *Hofstenia* migration, including several integrins, *follistatin*, and *wnt-3*, as well as transcription factors and metalloproteases previously found to be important for neoblast migration in planarian model systems. Looking for homologues of planarian migratory genes, however, did not yield results, and no notable expression was seen during regeneration.

As predicted by computational analysis, some expression of *follistatin* and *wnt-3* in *piwi*+ cells was visible at 6hpa and 24hpa, while completely absent in uncut and 0hpa cells. In *wnt-3* expressing cells, this expression would appear to have little to do with migration, as it occurs only in cells already physically close to the wound site. Cells expressing both *piwi* and *follistatin* appear to be more diffuse through the animal and may indicate migrating cells, though the precise role of *follistatin* in neoblast migration, if any, is still unclear.

Two integrin subunits, *itb6* and *ita7*, were strongly expressed in mesenchymal cells in head fragments during the first 24 hours following amputation, despite being absent in uncut worms. While these cells did not express *piwi* and were not neoblasts, they were located physically close to the neoblasts in the mesenchyme of the animal. In other animals, integrins are essential in cell-cell adhesion as well as cell-ECM adhesion and can aid in coordinated movement of cells as groups; it is possible that these large mesenchymal cells expressing integrins immediately following wounding associate with neoblasts and coordinate movement towards the wound site. The shape and location of
these integrin-expressing cells resemble fibroblasts, though the identity of the cells themselves were not investigated in the scope of this study. More research will need to be done to investigate the role of these cells, if any, in neoblast migration during regeneration, as well as the potential role of *follistatin* in migratory neoblast cells.
References


https://doi.org/10.1093/icb/icq118


https://doi.org/10.1007/BF00319372

https://doi.org/10.1016/j.celrep.2020.108098

https://doi.org/10.1038/s41467-017-01148-5
https://doi.org/10.1146/annurev.cellbio.20.010403.095114

https://doi.org/10.1002/wdev.321

https://doi.org/10.1186/gb-2007-8-4-r62


doi: 10.1242/dev.139774
https://doi.org/10.1016/j.cub.2014.03.042


https://doi.org/10.1016/j.celrep.2018.11.004


