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Molecular Mechanisms behind Germline Stem Cell Parasitism

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Molecular, Cellular and Developmental Biology

by

Megan Kailani Fentress

Committee in charge:

Professor Anthony De Tomaso, Chair

Professor Carolina Arias

Professor Kathleen Foltz

Professor Denise J. Montell

June 2020

THE DISSERTATION OF

Megan Kailani Fentress, M.S.

Is approved

APPROVED:

_____

Anthony De Tomaso, Ph.D.

Committee Chair

_____

Carolina Arias, Ph.D.

_____

Kathleen Foltz, Ph.D.

_____

Denise J. Montell, Ph.D.

June 2020

DEDICATION

I would like to dedicate this thesis to my wonderful mom, Debra, whose help and sacrifice has made this possible. She gave me the strength to follow my dreams and taught me the grace to leave space for magic in my life.

I would also like to dedicate this to my grandparents, Marie and Randall Fentress. While they are no longer with us I know that they would be proud. The stubbornness and perseverant attitudes were passed down in full.

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VITA OF MEGAN KAILANI FENTRESS

June 2020

EDUCATION

Associate of Arts in Biology Highest Honors. Ventura College. Spring 2010

Bachelor of Arts in Biology. University of California Santa Barbara. Spring 2013

Master of Science in Genes and Development, Biomedical Science. University of Texas Graduate School of Biomedical Sciences/MD Anderson Cancer Center. July 2015.

Doctor of Philosophy in Molecular, Cellular and Developmental Biology. University of California, Santa Barbara. June 2020 (Expected)

PUBLICATIONS

Fentress, M.K., Chen, X., Gladden, A.B. (In Review). "Local Changes in Tension Initiate Epidermal Stem Cell Niche Development."

Dimarco Hewitt, P.R., Chen, X., Fentress, M.K., & Gladden, A.B. (Revised and Resubmitted). "Regulation of Adherens Junction Mediated Cell Adhesion by the Tyrosine Phosphatase, PTPN14."

PROFESSIONAL EMPLOYMENT

Research

2012 Scientist aboard 2012 NMFS Aleutian Islands Bottom Trawl Survey
National Oceanic and Atmospheric Administration (NOAA)

2011-2013 Undergraduate Researcher, Kuris Lab of Ecological Parasitology. UC Santa Barbara

2013-2015 Graduate Student, Genes and Development. MD Anderson Cancer Center and UT Houston Health Science Center
2015-2020 Graduate Student, Molecular, Cellular and Developmental Biology. UC Santa Barbara

Teaching

2015-2020 Teaching Assistant for Molecular, Cellular and Developmental Biology

2016-2018 Teaching Associate, Freshman Summer Start Program, "Introduction to Life Science Research." UC Santa Barbara

2016 Teaching Associate, Molecular, Cellular and Developmental Biology, "Research Immersion in Molecular Biosciences. UC Santa Barbara

2016-2017 Guest Lecturer, Ecology and Evolution, Freshman Summer Start Program, UCSB Scholars Program. UC Santa Barbara

AWARDS

Fellowships

Jane Altman Memorial Fellowship. Fall 2019
University of California President's Dissertation Fellowship. 2018-2019
The Doreen J. Putrah Cancer Research Foundation Conference Fellowships. July 2017

Awards

UCSB Teaching Initiatives distinguished graduate student partner who serves students beyond the classroom. 2017

Pre-Candidacy First Place Research Achievement Award. 2014
Genes and Development Graduate Program Student Retreat Research Poster First Place Award. 2014. *"The Role of Merlin in Regeneration and Stem Cell Proliferation."*

Worster Award for Undergraduate Research. Summer 2011. "Pathology and life history of the cephalopod kidney parasite, *Dicyemennea californica*, in *Octopus bimaculoides*."

FIELDS OF STUDY

Major Field: Molecular, Cellular and Developmental Biology
Studies in Biomedicine with Dr. Andrew Gladden
Studies in Parasitology with Professor Armand Kuris

ABSTRACT

MOLECULAR MECHANISMS BEHIND GERMLINE STEM CELL PARASITISM

By Megan Kailani Fentress, M.S.

Cell competition (CC) is a universal, quality control process found across the taxonomic spectrum in which neighboring or interacting cells compare their relative fitness (e.g., growth rates.) In this process the “winner” cells contribute to the further development of the organism or tissue, while suboptimal cells, or “losers,” are outcompeted. This process has been studied in organisms ranging from fungi to humans, and has both developmental and disease relevance. For example, during cancer development, neoplastic cells can function as super-competitors in a tissue, outcompeting wild-type cells and driving tumor growth. Although there is intense interest, the molecular mechanisms that underlie this process are not well understood. One potential candidate pathway shown to be involved in tumorigenesis is the Notch Pathway. Recent studies have shown that differentially elevated expression of the Notch ligand Jagged is associated with heightened invasiveness and motility of cancer cells as well as increased tumor development. Here we utilize a novel *in vivo* system that allows us to study individual stem cells during a migration and niche lodgment process. The unique biology of the colonial tunicate, *Botryllus schlosseri*, has germline stem cells (GSCs) with genetically determined competitive phenotypes; when GSCs of two individuals are mixed, one genotype will outcompete the other, solely contributing to the germline of subsequent generations. Termed *Germ Cell Parasitism* (GCP)

this clonal dominance is heritable, stable, and reproducible, providing a tractable system in which to study cell competition.

Here we show a potential mechanism for GCP and cell competition in which GCP is partially due to differential homing of winners and losers to the germline niche and partially due to direct interaction between winner/loser cells and with the niche itself. We show that up-regulation of Delta and Jagged occurs in the winner GSCs, providing further support for the role of the Notch pathway in the process. Moreover, live-imaging of transplanted GSCs from winner/loser genotypes reveal that winner and loser GSCs both arrive in the developing germline niche, further suggesting a role for direct interaction between winner/loser GSCs within the niche. Based on these results, we propose that the basis of GSC competition resides in a combination of variation in homing ability and cell competition for niche occupancy controlled by differential expression of the Notch Pathway components.

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CHAPTER 1: INTRODUCTION AND BACKGROUND

1.1 INTRODUCTION

Cell competition (CC) is a phenomenon seen across a grand array of taxa and cell processes. From basic development to the seeding of tissues, cell competition plays a role in multiple processes on a multitude of biological scales.[1–8] While seen in normal and healthy development and homeostasis, cell competition also has a role in cancer.[4,5,9] Cancer stem cells are cells within a tissue that have gained an aberrant stem cell phenotype, essentially out-competing the "normal" cells around them and eventually form tumors and metastases.[10–14] As cell competition has such diverse functions, the mechanisms behind a cell's ability to outcompete its neighbor are likely to be equally as diverse. Understanding how this occurs under normal circumstances is paramount in understanding how it occurs during cancer and would greatly increase our ability to create treatment options specific to the winner cancer stem cell, increasing efficacy and specificity of the treatment itself and subsequently the quality of life of those afflicted.

We are utilizing the unique biology of the model organism *Botryllus schlosseri* (forthwith *Botryllus*), a colonial tunicate, to study the potential molecular mechanisms behind stem cell competition. *Botryllus* have a unique biology where germline stem cells (GSCs) have genetically determined competitive phenotypes.[15–19] These competitive GSCs provide a model for investigating the mechanisms of cell competition. Due to the fact that *Botryllus* grow through asexual reproduction this genetically determined competitive ability is stable throughout the genetically identical individuals and can be passed on

through subsequent generation, and thus the competitive abilities of these cells are *directly* subjected to natural selection,

1.2 *BOTRYLLUS* AS A MODEL ORGANISM

Botryllus is a colonial tunicate of the subphylum Urochordata or Tunicata, exhibiting a vertebrate-like notochord during the early tadpole stage of its lifecycle. Sexual reproduction results in a mobile tadpole that eventually settles onto a substrate and metamorphoses into a sessile, filter feeding animal. Characteristically, the notochord is absorbed. Following metamorphosis, *Botryllus* colonies grow through a seven-day asexual reproductive cycle. In this cycle the entirety of the animal, both somatic and germline, are regenerated through asexual budding creating a colony of genetically identical animals. The colony itself is surrounded by an extracellular matrix called the tunic and all individuals are interconnected within the tunic through the sharing of both an extracorporeal vasculature as well as shared blood (Figure 1A, 1B). During the process of asexual budding there are three generations of the animal present, the adult zooid and two generations of growing buds (primary and secondary) (Figure1A). As the asexually produced, clonal buds develop, germline stem cells from the adult zooid migrate to the developing germline niche of the clones through the shared vasculature. The developing buds reach a critical size and absorb the material of the adult zooid, becoming themselves the new adults with their own primary and secondary buds. This process takes around 7 days in lab reared colonies given ideal conditions. The timing is sensitive to seasonal changes in water temperature; regeneration can take multiple weeks under low temperatures, disruptions in water flow or nutritional fluctuations.

The cells migrating to the secondary buds are termed GSCs (Germline Stem Cells). Previous work has found that they have genetically determined competitive phenotypes for niche occupancy both in the field and in the lab[15,17,20].

In the field, individual colonies grow on rocks and docks, with multiple colonies settling in small areas. Inevitably, separate colonies will come into contact with each other. When this occurs the animals physically touch the outer protrusions from their vasculature called ampullae. These ampullae, will either fuse, creating a shared network of vasculature, or a rejection event will occur. Fusion or rejection is dependent on allorecognition of the highly polymorphic FuHC gene[16]. This process has been extensively studied in this lab.

Following fusion the extracorporeal vasculature is then shared between colonies, as is the blood flow (Figure 1B). Thus, as either colony undergoes the budding process, the GSC's from both colonies are migrating through the shared vasculature and settling into the available niches. In some cases the migrating germ cells from one genotype will settle in both genotypes niches, occupying the entirety of niches available and thereby taking out the fitness (ability to perpetuate the genome) of the other genotype completely. This process is called Germ Cell Parasitism (GCP) (Figure 1C). We are able to utilize this unique biology within the lab using known, genetically distinct genotypes that we can introduce to each other, selecting for those that are able to fuse (Figure 1C) and form predictable Winner/Loser Pairs.

Similar to what happens in a normal fusion event, when GSCs isolated from one genotype are injected into another, the two cell populations compete for

germline niche occupancy and subsequent development (Figure 1B). Following this injection, there are two potential outcomes, either the developing buds and subsequent adults will have the germline genotype of the host and/or injected GSC's in a stochastic manner, or one genotype will entirely outcompete the other, and only the winning genotype will contribute to the germline of subsequent asexual generations. The GCP process by which one genotype completely outcompetes the other is a repeatable, stable, and heritable trait.

While GCP is a known phenomenon, the molecular mechanisms behind this parasitism-like interaction are unknown. These competitive GSCs are a powerful model for investigating the mechanisms of cell competition from a stem cell phenotype because competitive abilities of these cells are *directly* inherited and undergo natural selection.

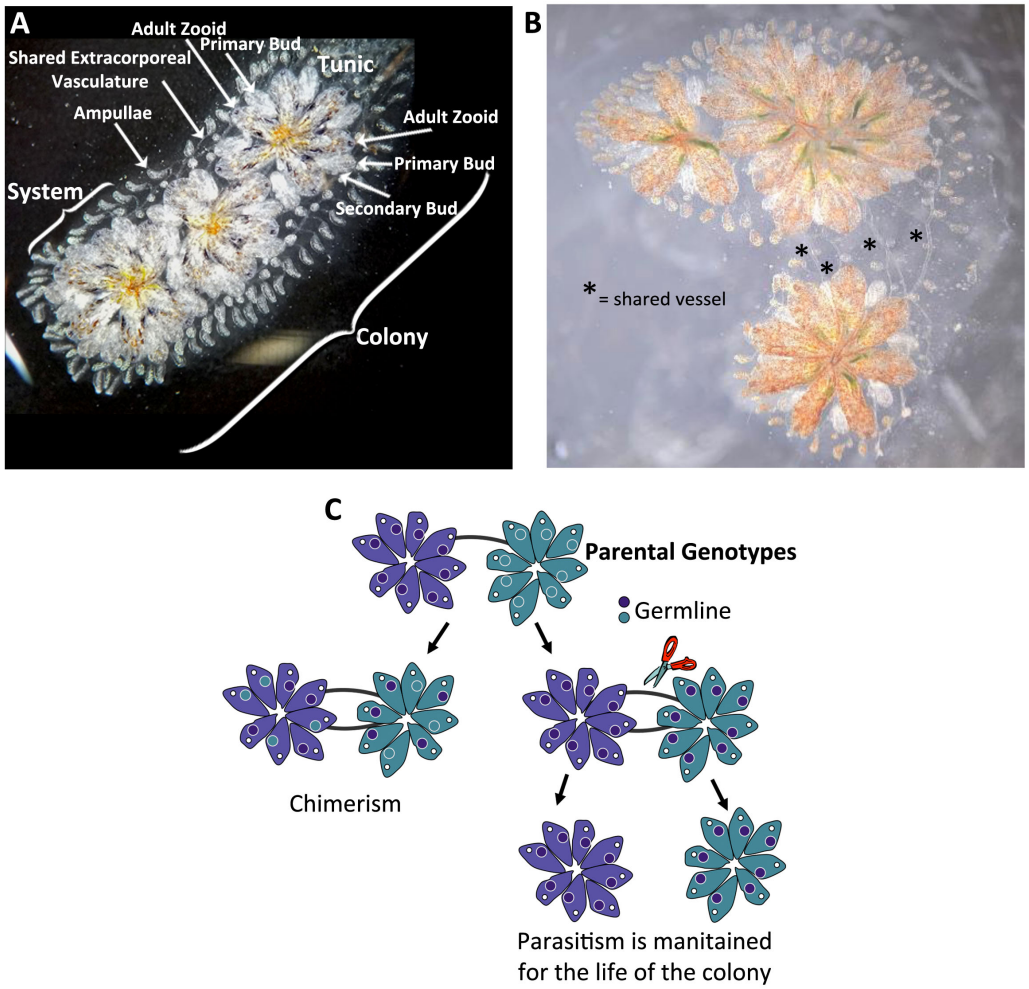


Figure 1. Botryllus schlosseri morphology and Germline Stem Cell Parasitism (A) Ventral view of a Botryllus schlosseri colony. The colony is the combination of several rosette shaped systems composed of adult animals (zooids). Individual animals are connected by a shared extracorporeal vasculature, which ends in bulb like extensions called ampullae. Each zooid has asexually developing primary buds and secondary buds. During the asexual budding process, new buds form during a weekly regeneration process and germline stem cells (GSC) migrate into the germline niche in the developing buds. **(B)** Ventral view of colonies sharing extracorporeal vessels (indicated by *). **(C)** Schematic showing the possible outcomes of an ampullary fusion event. Germ cells destined for the developing secondary bud germline niche migrate through the extracorporeal vasculature and contribute to the developing bud. Fusion of related individuals results in mobile progenitors of each genotype moving between animals. Initially mixed, the GSCs of the two individuals may be maintained (chimerism) or one genotype will outcompete the other, solely contributing to the germline of subsequent generations. The latter, termed Germ Cell Parasitism (GCP) is explained by a predictable clonal dominance that is heritable, stable and reproducible.

1.3 CELL COMPETITION

Cell competition is a phenomenon seen in a variety of contexts, from tissue development where cells compete for tissue composition through identification and removal of damaged or stressed cells, to stem cell niche occupancy, where stem cells vie for access to growth factors such as p53 and adhesion to the niche.[2,21–25] Observed in organisms ranging from social amoebae to humans, cell competition is likely an evolutionary conserved process important for both development and homeostasis and provides insight into how genetically clonal tissues arise from genetically heterogeneous cell populations during organ development[3,26–28].

Cell competition is dependent on a cells' ability to interact with surrounding cells and the mechanism is distinct from cell-autonomous regulation and extrinsic apoptotic death pathways. Instead, an intrinsic comparison between neighboring cells through direct interaction results in the recognition of more-fit "Winner" cells out-competing and even influencing apoptotic pathways in the out-competed or "Loser" cells. "Winner" cells are able to out-compete through increased utilization of some limiting resource such as growth rates, survival factors and translation of various proteins[7,29,30]; for example those involved in ribosomal biogenesis[2]. The response to this comparison varies between organisms and tissues involved though elimination of the sub-optimal cells is common.

The ability to eliminate less competent or sub-optimal cells is important during development, as seen in studies of Drosophila development and in the mammalian epidermis where increased expression of genes involved with

cellular growth and proliferation in winner cells results in the winner cell inducing apoptotic pathways in loser cells[14,31,32]. Another method of elimination of less-fit cells has recently been uncovered - the engulfment of loser cells by the winner cells themselves in development, tissue homeostasis and in the tumor environment[3]. This internal selection process ensures that abnormal or mutated cells are eliminated and the fittest cells comprise a tissue.

As in most cellular processes aberration in the pathway can result in various pathologies including cancer. When genes involved in cell competition are overexpressed a resulting super-competitor can be created[14]. These super-competitors outcompete and out-proliferate wild-type cells. For example in Drosophila, overexpression of micro-RNA 8 and EGFR promotes disruptions in cytokinesis and tumor metastasis through triggering engulfment of neighboring loser cells[14]. In mice it has been shown that super-competitors can outgrow normal cells during embryogenesis[33] and in Drosophila it was found that cells with a depletion of APC (a tumor suppressor) promotes adenomas through elimination of surrounding wild-type cells[4].

While it is clear that cell competition plays a role in cancer progression the field remains complicated, as there are situations where cell competition acts as a tumor suppressor. For example, T lymphocytes are constantly cycling, with younger cells outcompeting old cells. When this process is lacking, T cell acute lymphoblastic leukemia arises[2]. And in epidermal cell culture, mutated cells overexpressing polarity proteins are extruded from the surrounding cells through rearrangement of the actin cytoskeleton[34–36].

While common throughout multicellular eukaryotes, the molecular mechanism of cell competition is not well understood. This stems from its complex, dual role in both cancer formation and suppression, and in how difficult it is to measure temporally and spatially. The outcome of cell competition is clear during development – a tissue or organ is formed and is genetically homogeneous; however, the *process* of the competition itself is unseen. Another challenge in this field is the measurement of cell fitness. While “winner” cells outcompete “loser” cells in one context, in a different context the role might be reversed. That is, relative fitness is relative to the situation. Thus, teasing apart the mechanism behind cell competition would greatly increase our understanding of its role in aberrant cells, promoting tumor formation and increased proliferation.

1.4 Notch Pathway in Cancer and Cell Competition

The notch pathway is a key, evolutionarily conserved signaling pathway involved in multiple processes including cell communication, cell-cell contact, cell fate, development and differentiation as well as stem cell renewal and homeostasis[37–39].

Notch signaling in mammals consists of several Type 1 transmembrane proteins, the Notch receptor and several ligands, Jagged 1 (JAG1), Jagged 2 (JAG2), and the Delta/Delta like family Delta-like 1,3 and 4 (DLL1, DLL3, DLL4). Interaction of the Notch receptor with the ligands on a neighboring cell induce cleavage of the Notch receptor intracellular domain (NCID) and it's subsequent movement to the nucleus where it associations with transcription factor complexes inducing activation of target genes.

The ligands Delta and Jagged also undergo intracellular cleavage and in fact can compete with Notch for interaction with γ-secretase, the protease responsible for the cleavage[40]. When release by cleavage, the ligands' intracellular domain then form transcription factor complexes to initiate downstream gene transcription with target genes including those involved in angiogenesis, smooth muscle adhesion, cell proliferation, and cell migration. For example Jagged interacts directly with the transcription factor AP-1[40] whose target genes include those involved in organogenesis, embryogenesis, bone formation and maintenance and cell proliferation, among many others[41,42]. With such a broad function, it is no surprise that AP-1 has been shown to be important in many cancer types, in particular breast cancer[43].

As with many developmental processes, the Notch pathway is implicated in cancer development and progression[10,44–47]. JAG2 in particular is found to be overexpressed in multiple cancer types including: Breast Cancer, where overexpression is an indicator for poor prognosis and marker of high metastatic risk through JAG2 activation of uPA[48]; Colorectal Cancer, Endometrial Cancer, Gastric Cancer, Head and Neck Cancer, Hepatocellular Carcinoma, Non-small Cell Lung Cancer, Ovarian Cancer, Pancreatic Cancer, Prostate Cancer, Renal Cancer, Leukamia, Burkitt's Lymphoma, Hedgkin Lymphoma and Multiple Myeloma[49–53]. In all cancer types where JAG2 expression is increased, there is an association with aggressive type tumors[54], poor prognosis and malignancy.

JAG2 has been found to promote cancer cell survival through multiple processes depending on the cancer, from inhibition of apoptosis and increased cell proliferation[55–57], to increased migratory ability to decreased susceptibility to chemotherapy. Yet another way that JAG2 promotes cancer survival is its ability to increase angiogenesis within the tumor itself[58,59]. It has been suggested that JAG2 in angiogenic tip cells upregulates VEGF3, thereby promoting angiogenesis[1]. Further supporting JAG2's role in angiogenesis, mutations in the JAG2 gene in humans results in Alagille Syndrome, characterized by irregularities in the cardiovascular system, skeletal system, and liver function[59].

While accepted as critical in cell processes during development, homeostasis and cancer progression, Notch's role in cell competition has not been widely studied. In experiments using a Drosophila model for tumor formation and expression, the apical basal polarity and proliferation gene *scribble*

mutants were found to undergo apoptosis when surrounded by WT cells[60]. Mutant scribble cells are unable to form stable spot adherens junctions with surrounding cells, resulting in a hyper-proliferative state brought on by a lack of proper interaction with surrounding cells. Researchers utilized GAL-4 dependent transgenes to produce *scrib* mutants surrounded by wild-type cells and found that scribble mutant cells were eliminated through JNK mediated apoptosis. They then induced ectopic expression of oncogenic Notch which they found rescued the scribble mutant cells and apoptosis did not occur, resulting in tumor formation[60]. This highlights a scenario where mutant cells are out-competed through induced apoptosis and tumor formation does not occur, yet with expression of oncogenic Notch, the mutant cells end up outcompeting the surrounding wild-type cells and tumor formation is initiated.

Much research has focused on identifying specific Notch targets for the therapeutic intervention of cancer. Pan-Notch inhibitors can have severe global effects, as Notch is an important signaling pathway in general homeostasis, tissue stem cell maintenance, both in the resident stem cell and within the stem cell niches. Alternatively, Notch ligand-specific functions that are separate from the canonical Notch pathway have become increasingly appealing for targeted cancer therapies. Notch ligands are a particularly interesting option as they have more restricted functions in homeostasis, meaning that targeting them would have less widespread side effects for patients. Jagged also serves as a mediator between the Notch pathway and other critical pathways such as MAPK, VEGF and Wnt[45,49,58], which makes it a great target for multiple types of cancers when

these pathways become mutated and promote a cancer phenotype. Identifying the role of these ligands in cell competition will further our understanding of their function in cell communication and broaden our knowledge of how to create more specific therapies for cancer progression and malignancy.

CHAPTER 2: HYPOTHESIS AND SPECIFIC AIMS

HYPOTHESIS:

My overarching hypothesis is that the basis of germline stem cell (GSC) competition resides in a combination of variation in homing ability and cell competition for niche occupancy controlled by differential expression of the Notch Pathway components. I hypothesized that the predicted GSCs winners and losers would show characteristic differences in several in vitro and in vivo assays as well as in gene expression patterns, particularly in the Notch pathway components. To explore this relationship, I first had to establish the system for tracking GSCs and monitoring their ability to migrate using reproducible winner and loser pairings. I then tested specific aspects of the molecular mechanism of competition in an effort to develop a model that explains GSC competition in the context of our general understanding of cell competition. The Specific Aims were:

Aim1: Identify migration and niche interaction differences between winner and loser germline stem cells *in vivo and in vitro*. Through identification and comparison of winner-loser migration patterns a better understanding of the physical process involved can be ascertained. As cell behavior *in vitro* is often different *in vivo* comparison of GSC presence at the developing niche may strengthen or refute data from *in vitro* migration assays.

(a) Identify and maintain true winner - loser animal pairs; (b) isolate GSCs from known winner/loser pairs using FACs; (c) perform comparative, in vitro quantitative migration assays on isolated GSCs; and (d) carry out in vivo microinjection and tracking of GSCs to the developing niche.

Aim2: Characterize molecular mechanisms driving GSC competitive phenotypes.

(a) Ascertain differences in expression characteristics between winner and loser GSCs using transcriptomics; utilizing a tunicate winner-loser transcriptone candidate genes will be identified and assayed using qRT-PCR; (b) perturb molecular pathways identified as having differential expression through chemical inhibition or activation.

CHAPTER 3: MATERIALS AND METHODS

Animal husbandry, identification of fusion-rejection, and genotyping

B. schlosseri colonies used in this study were lab-cultivated strains, spawned from animals collected in Santa Barbara, CA, and cultured in laboratory conditions at 18–20 °C. Animals are reared in 5 liter tanks supplemented with food in suspension daily, and food is not limiting. Collections were performed at only one local harbor, the Santa Barbara Harbor (Longitude -119.6887448 and Latitude 34.407), which is owned by the City of Santa Barbara and performed under the authority of the California Department of Fish and Game. These collections did not involve any endangered or protected species. Fusion assays were performed on animals of comparable size that were isolated and placed in direct contact on glass slides. Pairs were checked under a dissection microscope 24 and 48 hours later for fusion of vasculature or rejection. At the 48hr mark all pairs were either fused or had gone through a rejection response. Fused animals were maintained as above in a separate tank. To genotype specific pairs, samples of a colony were placed in 70% ethanol followed by dissection of testes. gDNA extraction using the Nucleospin DNA XS kit was performed, followed by PCR (HotStarTaq, Qiagen) of microsatellites to identify individuals.

Cell Sorting

Cell Sorting was performed as previously used in the lab to isolate circulating germ cells from the animal[61]. Genetically identical, stage-matched animals were pooled, and a single-cell suspension was generated by mechanical dissociation. Whole animals were minced and passed sequentially through 70µm

and 40μm cell strainers in ice-cold sorting buffer (filtered sea-water with 2% horse serum and 50mM EDTA). FACS was performed using a FACSAria (BD Biosciences) cell sorter. Samples were gated with two previously identified markers for Botryllus germ cells, aldehyde dehydrogenases+/integrin alpha 6+as previously described[61]. Analysis was performed using FACSDiva software (BD Biosciences). Cells were sorted using a 70μm nozzle and collected into sorting buffer.

When FACS was not utilized whole animals were minced and passed sequentially through 70μm, 40μm, and 0.22μm cell strainers in ice-cold sorting buffer (filtered sea-water with 2% horse serum and 50mM EDTA). qRT-PCR was performed on isolated cells compared to those FAC sorted and no expressional difference was found ($p<0.001$, $n=3$).

Quantitative RT–PCR

Sorted cells were pelleted at 700Xg for 10 min, and RNA was extracted using the Nucleospin RNA XS kit (Macherey Nagel), which included a DNAse treatment step. RNA was reverse transcribed into cDNA using random primers (Life Technologies) and Superscript II Reverse Transcriptase (Life Technologies). Quantitative RT-PCR was performed using a LightCycler 480 II (Roche) and LightCycler DNA Master SYBR Green I detection (Roche) according to the manufacturer's instructions. The thermocycling profile was 5 min at 95 °C, followed by 45 cycles of 95 °C for 10 s, 60 °C for 10 s. The specificity of each primer pair was determined by BLAST analysis (to human, *Ciona* and *Botryllus*

genomes), by melting curve analysis and gel electrophoresis of the PCR product. To control for amplification of genomic DNA, 'no RT'-controls were used. Primer pairs were analyzed for amplification efficiency using calibration dilution curves. All genes included in the analysis had cycle threshold (CT) values <35. Relative gene expression analysis was per- formed using the $2^{-\Delta\Delta CT}$ Method. The CT of the target gene was normalized to the CT of the reference gene *elongation factor 1 alpha (EF1α)*: $\Delta C_T = C_{T(target)} - C_{T\ (EF1\alpha)}$. To calculate the normalized expression ratio, the ΔCT of the test sample (IA6-positive cells) was first normalized to the ΔCT of the calibrator sample (IA6-negative cells): $\Delta\Delta C_T = \Delta C_{T(IA6\text{-}positive)} - \Delta C_{T(IA6\text{-}negative)}$. Second, the expression ratio was calculated: $2^{-\Delta\Delta CT}$=Normalized expression ratio. The result obtained is the fold increase (or decrease) of the target gene in the test samples relative to IA6-negative cells. Each qPCR was performed at least three times on cells from independent sorting experiments, and each gene (Table 1) was analyzed in duplicate in each run. The ΔCT between the target gene and EF1alpha was first calculated for each replicate and then averaged across replicates. The average ΔCT for each target gene was then used to calculate the ΔΔCT as described above. Data are expressed as averages of the normalized expression ratio (fold change). Standard deviations were calculated for each average normalized expression ratio. Statistical analysis was performed using a paired, two-sided Student's t-test. **P<0.05.

Cell Tracking

GSCs were isolated as above from Winner and Loser animals, and

labeled with either cmdii (Loser) (Cell Tracker™, Thermo Fisher Scientific) or Syto59 (Winner) (Cyto™ Red Fluorescent Nucleic Acid Stain, Thermo Fisher Scientific.) Loser genotype colonies were microinjected with the labeled cells into the blood stream, 24 hours later colonies were imaged using an Olympus FLV1000S Spectral Laser Scanning Confocal at 20X.

Transwell migration assay

Transwell filters with 8μm pore size inserted in a 24-well plate (Corning) were coated with laminin over night at 4 °C and briefly air dried before adding 50,000 sorted cells, resuspended in 100 ml filtered sea water with 10% DMEM, 1% FBS/1mL DMEM, penstrep (1:500) and Primocin (1:500). The bottom of the well contained filtered sea water with 10% DMEM/1% FBS, Hoechst 33342 and S1P (2μM or 0.2μM), or Phorbol 12-myristate 13 acetate (PMA) where applicable. After 4hr incubation at room temperature images were taken at three random locations at the bottom well. Average cell coverage was analyzed using "cell count" in FIJI software. All assays were performed in triplicates with cells from three independent sorts. Statistical analysis was performed using a paired, two-sided Student's t-test.

Small-molecule inhibitor treatment.

Isolated cells were incubated for 2 hours in 30 ml of sea water containing 10μM U0126, 10μM Ly294002 (Cell Signaling), 40ng/ml Hepatocyte Growth

Factor (Sigma Aldrich) or 5μM FR180204 (Tocris). Controls were incubated in sea water without inhibitors or with vehicle (0.1% ethanol or 0.001% dimethylsulphoxide). For each treatment, three genetically identical colonies were treated simultaneously. Transwell Migration Assays and Quantitative RT-PCR was performed as described above and data are reported as averages from all experiments. Statistical analysis was performed using a paired, two-sided Student's t-test (***$p<0.001$).

Microinjection and *In vivo* GSC Tracking

GSCs were isolated as above from Winner and Loser animals, diluted to 10,000cells/μL buffer and incubated with either CellTracker™ CM-DiI Dye or Syto59. Colonies of the desired genotype were microinjected with 10,000 labeled cells into the blood stream and 24 hours later colonies were imaged using an Olympus FLV1000S Spectral Laser Scanning Confocal microscope.

Table 1	qPCR Primers
ef1-α F	CGTGGTCATTGGCCACGTAGA
ef1-α R	ATGAAATCACGATGACCGGGA
VASA F	GGCGGATTTAGCGATGATGAG
VASA R	TTCCCCCATAGCGACTGTTAGAC
Notch 2 F	CGGCATTACCTGCGAACAGA
Notch 2 R	AATCTGGTTCACGCATTCGC
Notch 4 F	CGCGGAGCCTATGAGGATTT
Notch 4 R	TTGAGCAGGCAACTGGAACA
Delta 1 F	GGCTGTTTCCCTCGGCTATT
Delta 1 R	ACCCCTAACCCCTTACACGA
Jagged 1 F	CCTGCGAACGCAACATTGAT
Jagged 1 R	CCATCTTCGCACAGGCAATG
Jagged 2 F	AGGCAAAAACTGCAGCGAAC
Jagged 2 R	CGGCTGTTCACGCAATCATC
RFP 121 F	AAGAATGTTTCCGTCGCCGT
RFP 121R	ATGCCTGCACGAGAAACAA
RFP 121-2 F	TTGTTTCTCGTGCAAGGCAT
RFP 121-2 R	TCGTAACAGCGCCTGTGATA
AIF3 F	GGCTGCGTGTGTTCCATTTT
AIF3 R	GGCCACAACTTCCTCACCTT
AIF3-2 F	TGCGATCATGAAAGAGGCGA
AIF3-2 R	GATTGGTATGGCGCAGCAAG
cPLA2 F	GCAAGAGGGGACCTTAACCC
cPLA2 R	ACACCCGATGCTTGCTTACA
Alox 5 LF	AGCGGAAACGAAGACGAGTT
Alox 5 R	GGTTTTGGTTCAACCTCCGC

CHAPTER 4. RESULTS

4.1 IDENTIFICATION OF WINNER/LOSER PAIRS USING MICROSATELLITE GENOTYPING

Germ cell parasitism is found both in wild-type field animals as well as those grown in the lab (see Chapter 1). In the field, large colonies of genetically identical animals will often encounter other colonies on substrates. When this occurs, the tips of the vasculature of each colony will touch and either a rejection or fusion of rejection event will occur[16–18,62–64]. During the asexual reproduction phase following a fusion event, the GSCs in each colony migrate through the shared vasculature to the developing buds. GSCs from both animals will reach animals from both colonies (Figure 1B), resulting in chimerism. However, some fused colonies will result in germ cell parasitism, where only one genotype GSC remains and this parasitism is long-lived and robust in the lab and field.

To identify colonies that will fuse and result in GCP, random lab reared colonies were assayed for genotype specific markers. Two genetically distinct zooids were placed on a glass slide so that they were touching. A fusion event showed a connection of the vasculature resulting in shared blood flow between both animals. Animals from each parental genotype were then analyzed for colony specific microsatellites through dissection of testes tissue and PCR of microsatellites. A total of 10 microsatellites were analyzed and genotypes were associated with combinations of microsatellite markers. Each parental genotype was identified for differences in microsatellite markers compared to the other parental genotype. Following a fusion testes were assayed for microsatellites that differed between the Parental genotypes, allowing for identification of parasitism, the microsatellite markers from only one parent were present, or a

chimeric colony, where both parental microsatellite markers were present. (Figure 2A). Once the pairwise fusion tests were complete and genotypes were identified, this allowed for us to identify winner GSCs (WG) and loser GSCs (LG) in fused individuals.

Fused Winner genotype and Loser Genotype animals results in WG in the germline

Two months following fusion, ten testes were isolated from subsequent colonies and genotypic microsatellites were detected using PCR. For every fifteen pairs of successful fusions, one pair would have complete winner parasitism of loser animals, while the majority of animals were chimeric for both parental genotypes in the testes (Figure 2B; refer also to Figure 1B in Chapter 1). In this case, a combination of microsatellite markers were shared with both parental genotypes. Far less frequent were those with true parasitism, where only one parent's microsatellite markers were present. Testes from fusion colonies were analyzed once a month for the remainder of the project to ensure continued parasitism or chimerism. Over the course of 4 years no change was observed.

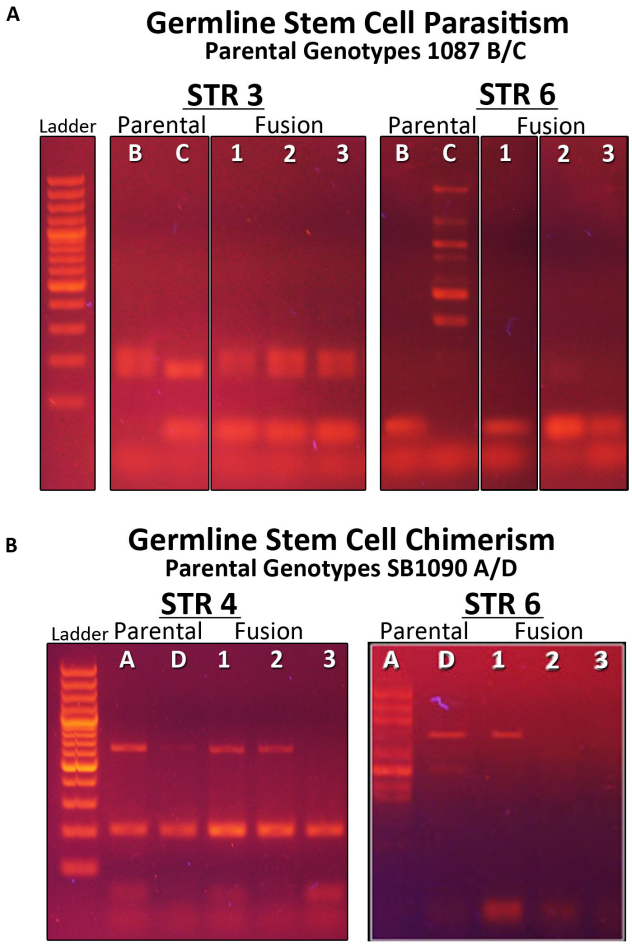


Figure 2. Microsatellite (STR) PCR results identify genotype specific chimerism and parasitism (A) Germline Stem Cell Parasitism. Parasitism is indicated in fusion of genotypes 1087 B/C showing genotype 1087B as the Winner Genotype. PCR of STR 3/6 from fusion animals match those of Parental Genotype B due to presence of smaller banding from STR 3 and absence of bands from STR 6 in fused animals.**(B)** Germline Stem Cell Chimerism. Chimerism is indicated in the fusion of Genotypes SB1090 A/D. PCR of STR 4/6 from fusion animals are a combination of both parental genotypes. as indicated by presence of the smaller band at STR 4 from Parental Genotype A in fusion animals 1 and 2. Absence of bands at STR6 in fusion animals 1 and 3 more resembles parental genotype D with faint bands in fusion animal 2 and 3.

4.2 Germline Stem Cells home to the developing niche

Winner and Loser GSCs migrate to the niche

The ability of GCP to occur could be explained by at least two broad physiological processes. Either the Winner GSCs are better at migrating to the developing niche or the Winner GSC are better competitors at the niche itself. To address this question, we designed an experiment to monitor whether GSCs of both genotypes appeared in the niche. Known Winner and Loser GSCs were isolated, differentially labeled, and injected into a loser animal.

GSCs were FACS isolated from early stage winners and losers based on aldehyde dehydrogenases/ integrin alpha-6 (ALDH/IGA6) expression, as previously described[61]. Isolated Winner GSCs were labeled with CellTracker™ CM-DiI Dye and Loser GSCs were labeled with Syto59. Labeled cells were diluted into 10,000cells/μL and 1μL was then injected into Loser Genotype animals using a glass micropipette. Following a 24hr window the recipient animal's secondary buds were imaged using a spinning disc confocal microscope (Figure 3A-I). Interestingly, both WG and LG GSCs were consistently found in the secondary buds. However, the WG cells numbered 1.9X more than LG (Figure 3J; p=0.0179).

The presence of both Loser GSCs and Winner GSCs in the developing niches introduces the possibility that the GSCs could be interacting with each other directly and the Winner GSCs are outcompeting the Loser GSCs at the niche, or that the GSCs have differential interaction with the niche itself allowing them to outcompete the loser GSCs for niche occupancy. While only a snapshot

of the entire window of migration, given the equivalent number of injected cells these data suggest that the ability of Winner GSCs to take over the germline in a colony is predominantly due to migration abilities, but with the possibility to be partially due to interaction at the niche. We thus next sought to identify if there is differential migration potential between GSCs in Winner versus Loser genotypes.

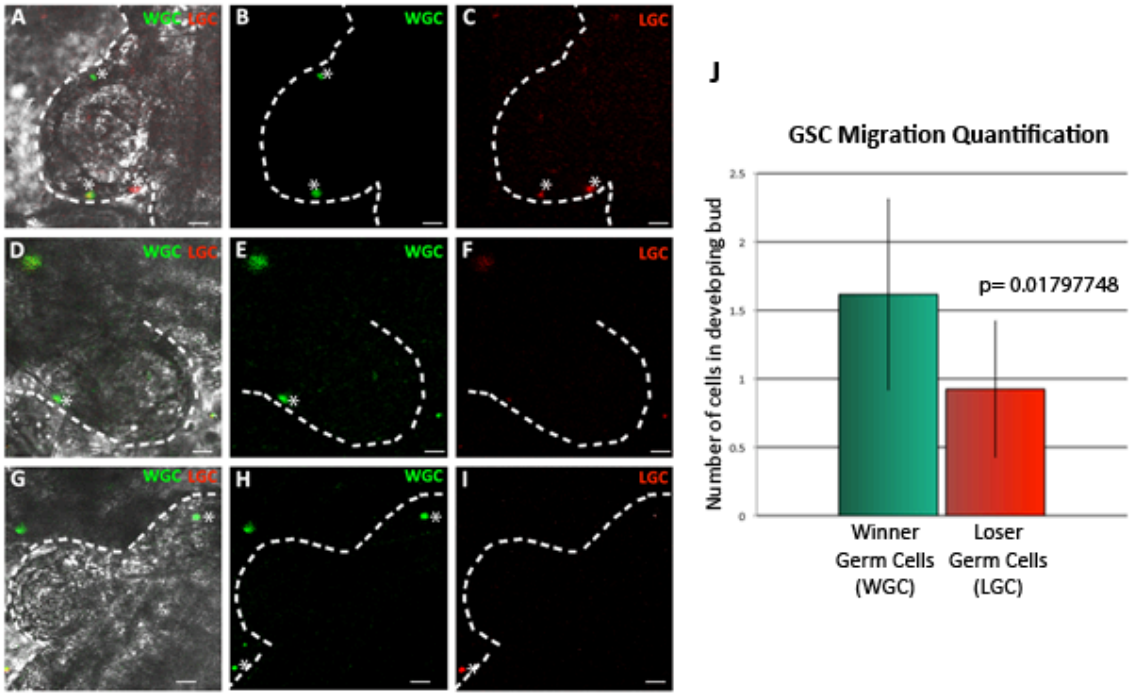


Figure 3: Isolated winner and loser germline stem cells migrate to the primordial germline niche during secondary bud development. (A-I) FACS isolated cells were stained, Winner Germline Cells (WGC) were labeled with CellTracker™ CM-DiI Dye (green) and Loser GSCs were labeled with Syto59 (red). Confocal microscopy shows WGCs migrating to developing buds **(A,B,D,E,G,H)** and LGSc in **(A,C,D,F,G,I)** 12 hours post-injection. **(J)** Quantification reveals that an WGCs are present in the developing niche 1.9X more often, $p<0.05$ by students t-test. Indicating that both winner and loser genotyped cells are capable of migrating to the developing germline niche. n=13

4.3 WINNER GSCS HAVE INCREASED MIGRATION CAPABILITIES COMPARED TO LOSER GSCS

Increased migration in winner cells

It has been previously shown that the Sphingosine-1-phosphate receptor 1 (S1P) is highly expressed on ALDH/IA6+ GSCs and in transwell migration assays, GSCs respond to S1P with chemotactic migration in a dose dependent manner[61]. To identify whether Winner GSCs and Loser GSCs respond differently to a chemoattractant, thereby altering migratory abilities, varying concentrations of S1P (2μM, 0.2μM) were added to the bottom well of a transwell, serving as a chemoattractant. The migratory activity of Winner Genotype (WG) and Loser Genotype (LG) GSCs to S1P was compared to their migration in control wells with Phorbol 12-myristate 13 acetate (PMA) as a negative control stimulant

In all conditions, the migration of WG GSCs was higher than the LG GSCs. Both genotypes revealed a dose-dependency, with an optimal migration score in a S1P concentration of 2μM compared to a concentration of 0.2μM as well as in the unstimulated control (as seen before). Migration levels with the use of PMA were comparable to those in unstimulated control.

The results from this experiment indicate that in both winner and loser GSCs, S1P induces chemotaxis. However, winner-GSCs possess an intrinsically higher migratory activity compared to loser cells, even in unstimulated controls (Figure 4). It is important to note that LG GSCs responded in a similar pattern to those of the WG albeit at a lower number. In sum, GSCs from both genotypes are able to respond to migratory stimulation, but the winner genotype has

increased migratory ability and response to migratory stimulation. These data support the hypothesis that migration capability is a parameter of GCP. We next sought to identify potential pathways involved.

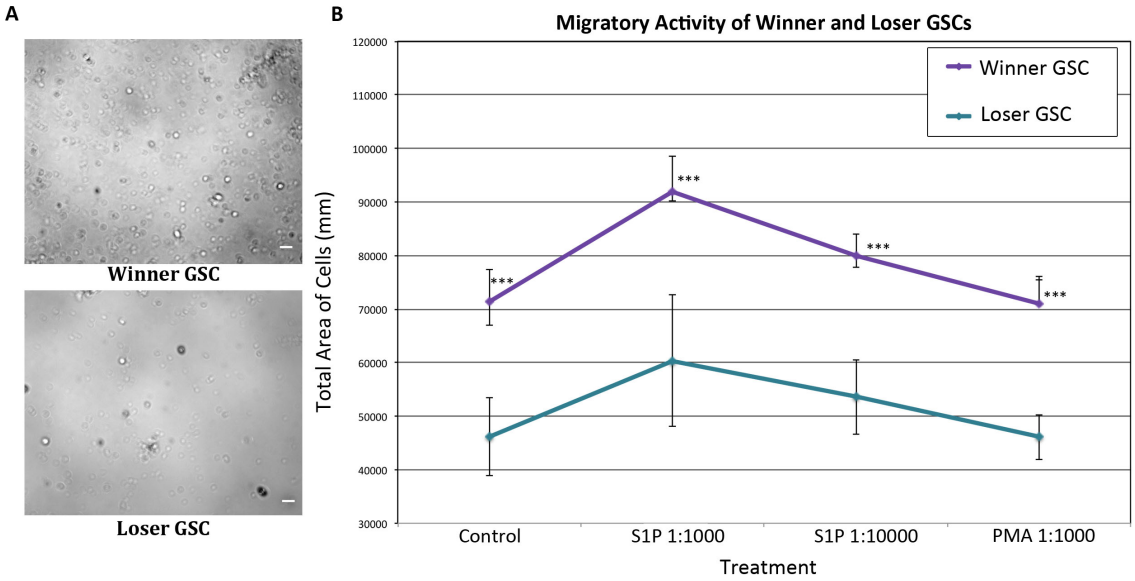


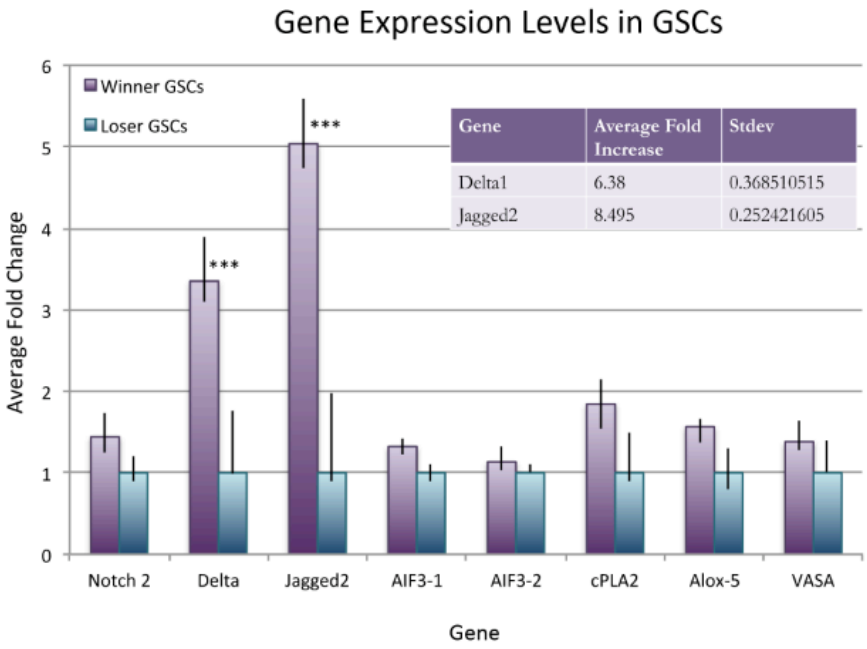
Figure 4: Winner Germline Stem Cells have increased migration capabilities compared to Loser Germline Stem Cells. (A) Images of winner (Top) and loser (Bottom) GSCs after 4hrs of migration in control treatment. SB=20µm. (B) Plot of total area of cells following 4hrs of migration, Winner GSCs (purple) Loser GSCs (Blue) with the following conditions; Control, sphingosine-1-phosphate concentration of 1:1000 and 1:10000, positive control with known migration stimulant Phorbol 12-myristate 13-acetate (PMA) at a concentration of 1:1000. ***p<0.001. between WGCs and LGCs at indicated treatment. n=30

4.4 NOTCH PATHWAY IS UPREGULATED IN WINNER PHENOTYPE

Jagged indicated in migration of *Botryllus* germline stem cells.

To identify candidate pathways involved in GCP, a publically available lab Winner/Loser expression assay from a relative tunicate, *Ciona*, Supplemental Table 1) was used to determine genes whose expression differed in winner and loser cells. Following identification of candidate genes quantitative real-time PCR was performed on FACS isolated WG and LG GSCs. Due to potential differences between tunicate species multiple genes were chosen as candidates including (Table 1). We focused on two genes, which were found to be differentially expressed ($p<0.001$), between Winner GSCs and Loser GSCs, Jagged 2 (JAG2), which showed an average fold increase of 8.459X and Delta, which had an average fold increase of 6.38X (Figure 5). Interestingly, the upregulation of these two Notch receptor ligands did not coincide with an increase in the Notch receptor itself (Figure 5).

Jagged and Delta are both indicated in multiple stem cell processes[44,53,55,59,65] , and increased Jagged expression in particular is seen in multiple cancers such as breast cancer, colon cancer and glioblastoma [50,54,66,67] as well as being associated with aggressive and invasive tumor phenotypes[38,50,52,54,58]. Due to its importance in tumor invasiveness and chemoresistance, we chose to focus on the JAG2 ligand for this study.



Gene	Average Fold Increase	Stdev
Delta1	6.38	0.368510515
Jagged2	8.495	0.252421605

Figure 5. Jagged2 and Delta are upregulated in Winner GSCs. Q RT-PCR analysis on isolated GSCs from Winner and Loser animals. Relative quantification was performed using the $2^{-\Delta\Delta CT}$-method with ef1a as the control gene. Data expressed as averages of relative fold change, normalized to loser animals. SDEVs were calculated for each average expression ratio (n=9). ***p<0.001 using Student's t-test.

Treatment of HGF results in increased Jagged2 expression and alteration in migratory ability

To begin understanding if the Notch pathway is important in the increased migration of Winner germline stem cells seen in this study, *in vitro* migration assays were performed under several conditions. We first aimed to identify the specific Notch pathway involved in GSC migration. It has been previously shown that multiple Notch pathway-associated growth factors have an effect on stem cell migration and proliferation, with Hepatocyte Growth Factor (HGF) being indicated in Jagged expression[50]. Previous studies have shown that alteration in the Notch pathway via HGF effects Jagged expression and alters invasiveness of tumors[50,65,68].

Following dose dependency and viability assays Isolated GSCs were incubated in 10ng HGF for one hour and then subjected to *in vitro* migration and expression analysis. HGF treatment of GSCs resulted in increased expression of JAG2 in both cell populations as seen in qRT-PCR (Figure 6A). Loser GSCs had increased JAG2 expression levels ($p<0.001$) and were indistinguishable from untreated Winner GSCs, ($p<0.001$) (Figure 6A). Interestingly HGF-treated Winner GSCs only had a slight increase in JAG2 expression, suggesting a threshold of Jagged expression and function within the cells.

Transwell migration assays showed that Loser GSCs had a 1.5% increase in migratory ability, including response to stimulant S1P at the preferred dilution of 1:1000 ($p<0.001$) thereby rescuing the Loser GSC phenotype to the Winner GSC phenotype. There was no statistical difference between Winner GSC

migration levels and HGF-treated Loser GSCs (Figure 6B). Similarly to the mild expression increase in Jagged 2 following HGF treatment, Winner GSCs treated with HGF had virtually no increase migratory capabilities, strengthening the model of a threshold of Jagged2 and migration ability that was already achieved in the W GSCs. This could in part be explained through the interaction of pathways activated by HGF and the Notch pathway creating a self-regulating activation loop. Further study into other pathways involved in GSP would shed light on the apparent upper limit of JAG2 expression in GSCs.

Following induction of JAG2 expression and migratory ability by HGF we next explored the specifics of the pathway involved.

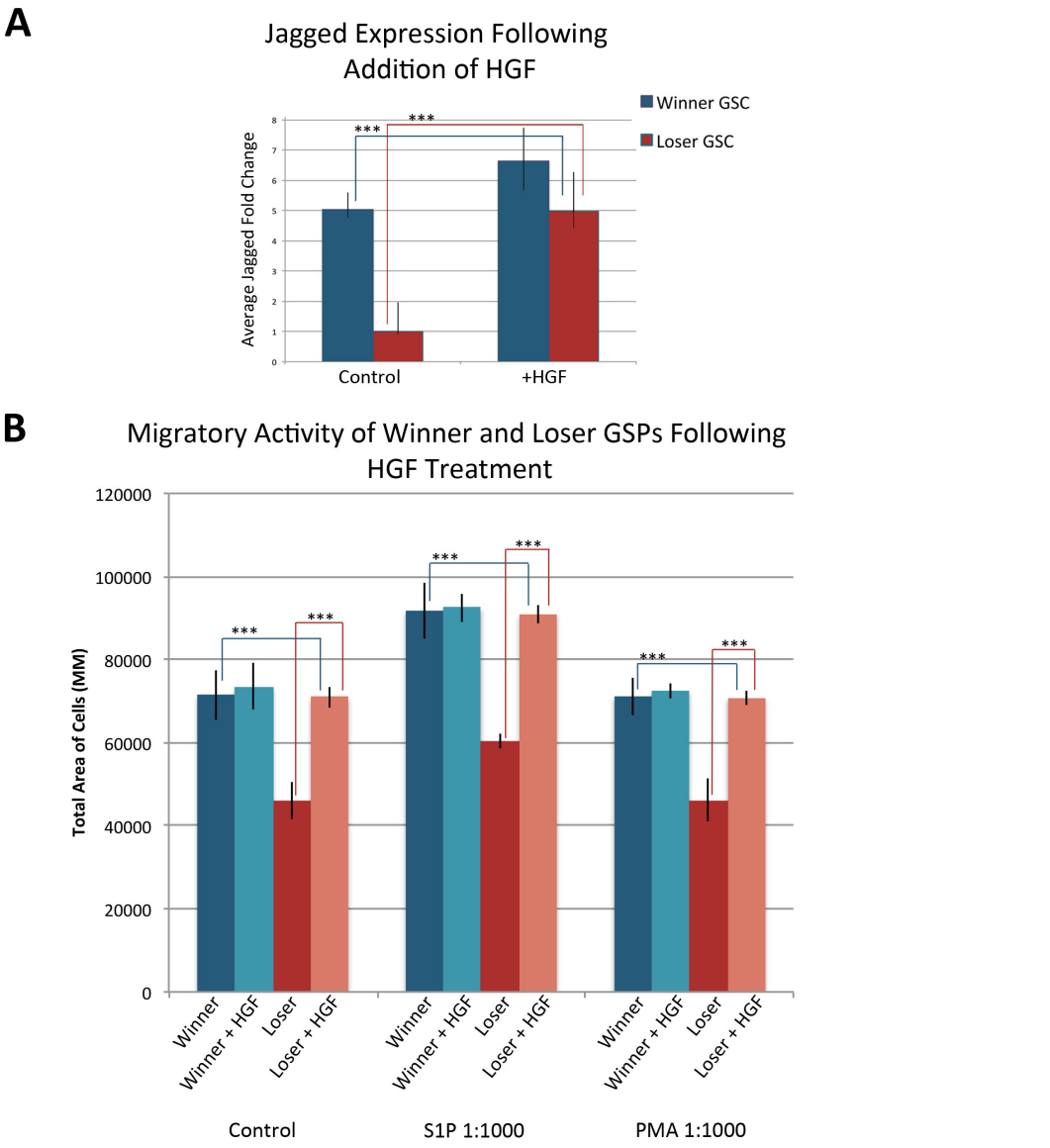


Figure 6. Increased Jagged2 expression from treatment with HGF Corresponds with Increased Migratory Ability. (A) Treatment with HGF of GSCs resulted in increased JAG2 expression shown from Q RT-PCR. Relative quantification was performed using the $2^{-\Delta\Delta CT}$-method with ef1a as the control gene. Data expressed as averages of relative fold change, normalized to loser animals. SDEVs were calculated for each average expression ratio (n=6). ***p<0.001 using Student's t-test. **(B)** Transwell migration assay of GSCs following HGF treatment. Addition of HGF to Loser GSCs resulting in increased migration to the level of Winner cells in unstimulated control wells, S1P chemoattractant wells and negative control PMA wells. n=6. ***p<0.001.

4.5 Molecular mechanism behind Winner GSC parasitism through MAPK interaction with the Notch Pathway

Perturbation of MAPK pathway results in changes of Jagged expression and migratory ability

HGF is known to activate the MAPK and Akt pathways[47,49,50,68,69], both of which are upstream of Jagged expression, thus we sought to identify which pathway may be involved in GSC migration. To do this we utilized the /MEK inhibitor U0126 and the AKT/P13 Kinase inhibitor Ly294002. Inhibiting the MAPK pathway using U0126 which blocks MEK1/2 has been shown to result in decreased Jagged expression in several systems, for example squamous cell carcinoma cells, HUVEC, and breast cancer cells[50,66,69,70]. Ly294003 inhibits AKT through P13Kinase and has been shown to attenuate Jagged expression[70] as well as mitigate activation of the AKT pathway following addition of HGF[68,71].

Following dose and viability assays a concentration of 10μM of U0126 for 1 hour was utilized. This concentration and time of incubation has been used in multiple cell assays within the field[72–74]. As seen in (Figure 7), inhibition of the MAPK pathway through addition of U0126 resulted in decreased migration of both winner and loser phenotypes. Jagged expression levels decreased up to three-fold in Winner GSCs, with Winner GSCs having similar expression levels to those of Loser GSCs (Figure 7A). As seen in HGF treatment on winner cells inhibition of MAPK in Loser cells decreased JAG2 expression only mildly. This supports the idea of a threshold of JAG2 expression as well as a required minimal expression level.

Following successful pharmacological inhibition of JAG2, transwell migration assays were performed. Remarkably, MAPK inhibition with UO126 resulted in the Winner GSCs migratory ability decreasing to the level of that of the Loser GSC migration levels ($p<0.001$) (Figure 7B). Migration levels of the Loser GSCs were not significantly lessened with MEK1/2 inhibition, suggesting a tightly regulated JAG2 expression paradigm.

Inhibition of Akt had virtually no affect on migration of either cell population (Figure 7A), nor on Jagged expression levels (Figure 7B), indicating that the MAPK pathway and not the AKT pathway is involved in winner phenotype migration.

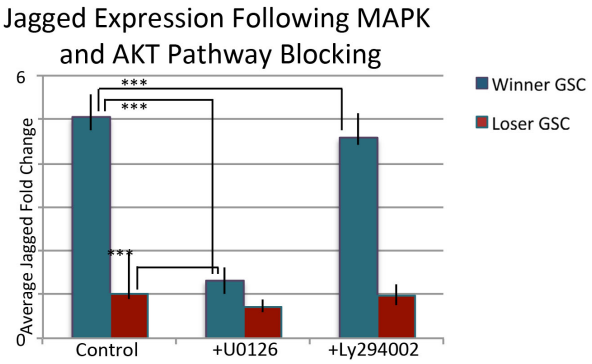
To further validate the finding that the MAPK pathway specifically affects Jagged expression and influences migratory ability, the selective ERK1/2 inhibitor FR180204 was utilized. FR180204 has been used in multiple cell types to inhibit the MAPK pathway through blocking of ERK1/2[75–77]. Inhibition with FR108204 resulted in a three-fold reduction in JAG2 expression, similarly to that of U0126 treatment as quantified with RT-PCR (Figure 8A) ($p<0.001$). As in U0126, addition of FR 108204 decreased migratory ability of Winner GSCs. Following ERK1/2 inhibition, Winner GSCs migration levels matched the level of untreated Loser GSCs (Figure 8B; $p< 0.001$).

These data support the potential connection between JAG2 and the MAPK pathway. When Loser GSCs were stimulated with HGF their JAG2 expression levels matched those of untreated Winner GSCs. Furthermore, following HGF treatment Loser GSCs gained migratory function, to levels that

phenocopied those of Winner GSCs. On the flip side, inhibition of the MAPK pathway through either MEK1/2 (U0126) or ERK1/2 (FR180204) in Winner GSCs resulted in not only a decrease of JAG2 expression, but also a decrease in migratory ability. Remarkably, the inhibited Winner GSCs phenocopied the JAG2 expression levels and migratory function of untreated Loser GSCs.

These data also suggest that regulation of JAG2 expression is very specific with a tightly controlled minimal and maximum level. Winner GSPs had very little response to stimulation of the MAPK pathway, either in actual expression levels of JAG2 or in migratory abilities. Conversely, inhibition of the MAPK pathway through either inhibition of MEK1/2 or ERK1/2 resulted in a minimal decrease in either JAG2 expression or migratory levels. Elucidation of the maintenance regulation of these pathways would greatly increase our understanding of germ cell parasitism and GSC migration.

A



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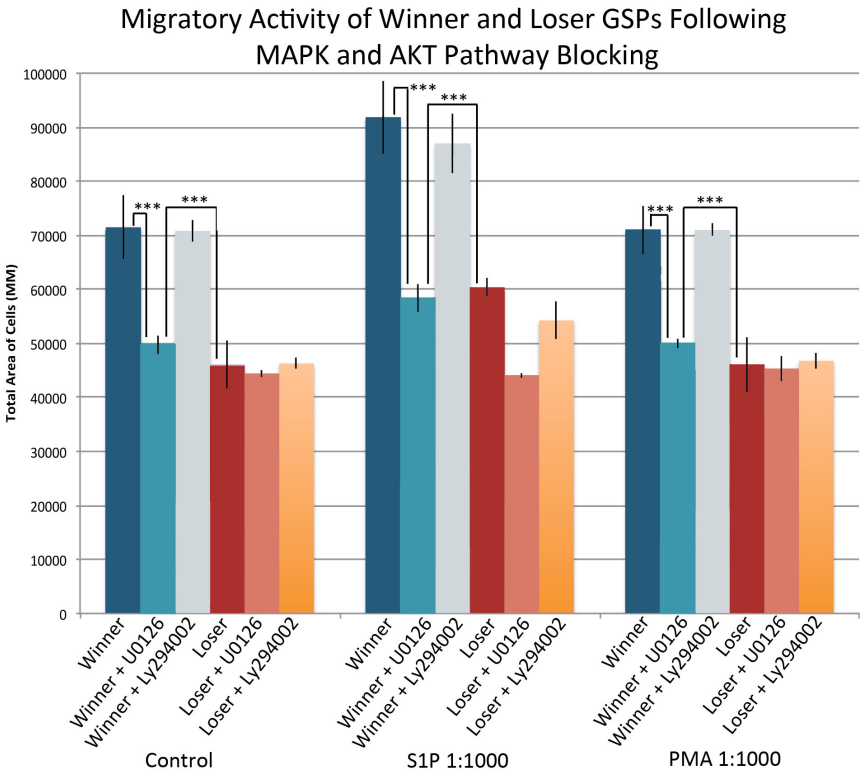

Figure 7. Decreased Jagged2 expression from treatment blocking MAPK pathway corresponds with decreased migratory ability. (A) Treatment with UO126 of GSCs resulted in decreased JAG2 expression shown from Q RT-PCR. Treatment with Ly2904002had no effect. Relative quantification was performed using the $2^{-\Delta\Delta CT}$ -method with ef1a as the control gene. Data expressed as averages of relative fold change, normalized to loser animals. SDEVs were calculated for each average expression ratio(n=6). ***p<0.001 using Student's t-test. **(B)** Transwell migration assay of GSCs following UO126 or Ly294002 treatment. Addition of UO126 to Winner GSCs results in decreased migration to the level of Loser cells in unstimulated control wells, S1P chemoattractant wells and negative control PMA wells. Treatment with Ly294002 had virtually no effect. n=27. ***p<0.001.

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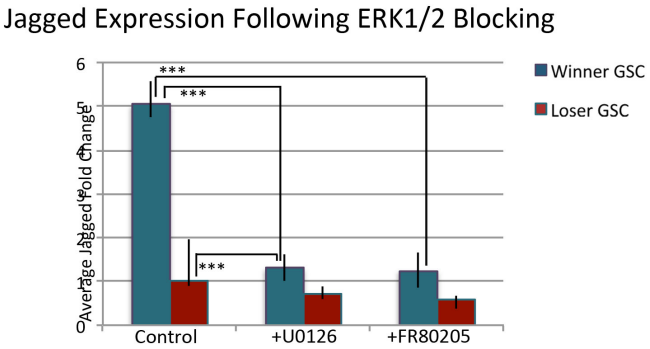

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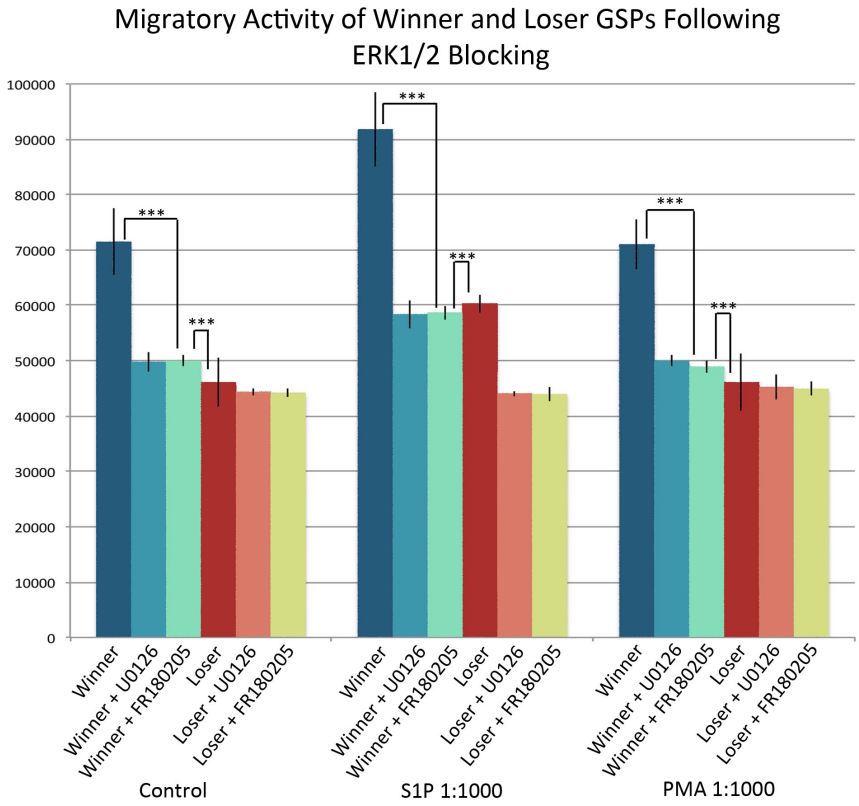

Figure 8. Treatment blocking ERK1/2 corresponds with decreased Jagged2 expression and decreased migratory ability. (A) Treatment with UO126 and FR180205, a selective ERK1/2 inhibitor, of GSCs resulted in decreased JAG2 expression shown from Q RT-PCR. Relative quantification was performed using the $2^{-\Delta\Delta CT}$-method with ef1a as the control gene. Data expressed as averages of relative fold change, normalized to loser animals. SDEVs were calculated for each average expression ratio (n=5). ***p<0.001 using Student's t-test. **(B)** Transwell migration assay of GSCs following UO126 or FR180205 treatment. Inhibition with FR180205 in Winner GSCs results in decreased migration to the level of Loser cells in unstimulated control wells, S1P chemoattractant wells and negative control PMA wells. n=15. ***p<0.001.

CHAPTER 5: DISCUSSION AND FUTURE DIRECTIONS

DISCUSSION

This study aimed at identifying the underlining mechanism behind germline stem cell parasitism in the model organism *Botryllus schlosseri*. In doing so, a potential link between cell competition, stem cells, and the Notch pathway was identified and explored. Mechanisms of cell competition are of the upmost importance as the phenomenon has a function in cancer, both during the formation of a tumor, but also propagation and in subsequent metastasis.

Studies suggest that tumors often develop from a single cell within a tissue[11]. This cell, termed a Cancer Stem Cell (CSC) is capable of either taking on a stem cell phenotype, or is already a stem cell, and in either case is able to bypass cell cycle regulatory signals and apoptotic queues[4,11], outcompeting its neighboring cells and going through increased proliferation[13,29]. Investigations into CSC's and their biology have led to a greater understanding of tumor formation and migration as well as give an explanation for the ability of some cancers to relapse. Part of the CSC phenotype is the ability to bypass self-death by damage and induced apoptotic pathways, thus giving the cell resistance to treatments such as chemotherapy and radiation[11,24,78].

In vitro migration assays showed that both winner and loser GSCs migrate towards chemoattractants in a similar pattern. While winner GSCs clearly had a higher migratory ability or increased movement speed the loser GSCs still migrated, indicating that the take-over of loser germline niches is partially due to migration ability. This was further illustrated by *in vivo* assays using fluorescently tagged germ cells. In these assays winner and loser germ cells were injected into

a loser animal and the developing niche was imaged. While there were more Winner GSC than Loser GSCs, both genotypes were found in the developing niches, supporting the possibility that direct cell competition between the winner and loser GSCs, either during migration or at the niche, is also contributing to GCP.

To begin to probe the molecular mechanism behind these differences, we evaluated expression of the Notch ligands Jagged and Delta. Both winner and loser GSCs express Jagged and Delta, with winner cells having an even greater increase than loser cells. As JAG2 had the most substantial difference, we focused on its role in GCP. Pharmacological blocking and enhancing was performed and migration ability was analyzed. Remarkably, inhibiting the ERK/MAPK pathway resulted in a decrease in migration of winner cells to the level of loser cells and this correlated with decreased Jag2 expression. This is likely a specific effect, as inhibition of the AKT pathway did not effect JAG2 expression or migration levels. Activation of the MAPK through HGF increased JAG2 levels of the loser GSCs to winner GSC levels and loser GSCs phenocopied migratory levels of winner cells.

The ERK/MAPK pathways are highly complex and interact with multiple pathways on different levels. For example, p38 of the MAPK pathway has several different isoforms, all of which respond differently to activation by upstream MAPK activators and have differential activation of downstream AP-1 regulation[79]. Indeed, although activated in general by Receptor Tyrosine Kinases, MAPK and AKT have diverse functions during development and homeostasis,

requiring a multitude of crosstalk between different pathway components and cellular environment[69]. The MAPK and Notch pathways also have a varied response to each other that depends on the cell and circumstance. During angiogenesis, for example, the effects on Jagged expression are dependent upon which MAPK pathway is involved. In endothelial cells, p38 MAPK inhibition results in an increase in VEGF/JAG2 angiogenic effects, while inhibition of p42/44 MAPK decreases VEGF angiogenic effects through Jagged[70]. Utilizing cell lines for myogenesis, it was found the activation of the Notch pathway suppressed p38 function[80], yet in breast cancer Notch and the Ras/MAPK pathways work in concert with each other to increase cell survival and maintenance of a stem cell state[66]. Elucidating the specific molecular mechanism in these complex pathways will help us understand the differences between winner cancer cells and loser wild-type cells associated with their MAPK/Notch interactions.

Within the context of cell competition, the MAPK pathway has been widely studied and a robust amount of data are available. Cells in a tissue with lower MAPK signaling are outcompeted by their neighboring MAPK-active members. Furthermore, MAPK is known to abrogate apoptotic signals that initiate from ligand induced cell death[13,81–83]. Quite interestingly it has also been shown that cells with a constitutively active (oncogenic) ectopic form of RAS can cause apoptosis in cells in the nearby vicinity[13,84,85], potentially through non-autonomous lateral induction, indicating that prolonged MAPK expression not only promotes cell survival but also induces cell destruction in nearby cells,

essentially creating a “hyper-winner.”

Notch also has a role in cell competition and cancer, although the mechanisms are not as well understood as those involving MAPK. Clearly with its involvement in basic cellular and developmental processes, from seeding an organ to cell migrations and cell cycle progression, the Notch pathway is involved in cell competition during normal development and homeostasis[37,39,86]. When looking at Notch and cancer, a variable role for Notch is prevalent. The majority of cancer types such as breast cancer, cervical cancer, colorectal cancer, head and neck cancer, ovarian cancer, gastric cancer, B-Acute and Chronic lymphocytic leukemia, Burkitt’s lymphoma, Multiple myeloma and many others, have increased expression of Notch ligands[46,52,54,58].

In laboratory experiments it has been observed that a decrease in Notch pathway components in these cell types results in a decrease in cancer cell proliferation and increases apoptotic outcomes[10,54,87–89]. However, some cancer types exhibit the opposite expression association and Notch has a tumor suppressor function in many carcinomas (squamous cell, small-cell lung, urothelial and Esophageal)[47,90–95].

Previous work has shown that a Jagged-Delta asymmetry is critical in the development of the sender/receive hybrid phenotype that promotes differential outcomes for neighboring cells[96]. When Notch is engaged through either the Jagged or Delta ligand the intracellular Notch domain (NICD) is cleaved and moves into the nucleus where it activates downstream target genes. Interestingly, NICD inhibits further expression of Delta and activates further

expression of Jagged. Thus a signaling circuit is created with a positive loop for Jagged and an inhibitory loop for Delta. This asymmetry between the Delta and Jagged ligands results in the Sender/Receiver phenotypes in neighbor cells, the Sender cell having high Delta, low Notch and the Receiver having low Delta and High Notch. Once Jagged levels in both cells becomes dominant a third phenotype is created, that of the Sender/Receiver Hybrid. This hybrid is often associated with cells that undergo a complete EMT transition and those that are Jagged dominant cells often maintain a partial EMT transition and are commonly referred to as Circulating Tumor Cells (CTCs)[96], though there are alternatives pathways that also result in CTCs such as direct repression of epithelial markers[97].

Notch's dynamic function during homeostasis of multiple tissue types and cell processes as well as its roles in cancer, creates a need for specification of mechanistic pieces of the pathway in cancer types in order to create therapeutic targets. Therapeutic designs to alter Notch signaling on a global level would have broad deleterious effects for healthy cells and tissues, not just the cancer cells. One potential answer to mitigate some side effects is targeting the specific ligands involved.

The data presented from this study suggest a role for the Notch pathway ligands in germline stem cell parasitism migration and cell competition. Association of migratory ability with winner germ cell phenotypes offered a tool for elucidating the molecular mechanism behind GCP through therapeutic treatment of isolated germ cells. While migration ability does not answer the

question fully, it is a remarkably robust assay for GCP outcomes, and when coupled with quantitative real-time PCR provides a snapshot of molecular pathways involved in the cell competition between winner and loser GSCs. While more research on this topic is needed to fully understand the mechanisms at hand, this work leads to a testable model, proposed below.

A Model for Germ Cell Parasitism

AP-1 is a transcription factor that interacts directly with the cleaved intracellular portion of JAG2, and has been shown to drive transcription of genes involved in VEGF, angiogenesis, cell proliferation, differentiation, and cell survival[42] . AP-1 has found to be upregulated in multiple cancer types such a breast cancer, glioblastomas, cell migration and metastasis[42,98]. It has also been found that ERK1/2-activated AP-1 expression through interaction with other MAPK pathway members[99,100].

In this study we have found that in *Botryllus* GSCs, pharmacological inhibition of the MAPK pathway greatly decreased migratory abilities, concomitant with a reduction in JAG2 expression. The inhibitors we used have been shown to inhibit AP-1 activity through specific inhibition of the MAPK pathway in other contexts [99,100], consistent with our findings. Other studies have also found that stimulation of endothelial cells with HGF initiated Jagged upregulation through the MAPK pathway and not AKT[50]. Furthermore, inhibition of the AKT pathway through introduction of Ly294002, a PI3k inhibitor, had no effect on GSC migratory ability. While the AKT pathway is known to be involved

in AP-1 promoter activity, it has been shown that activation of the MAPK pathway is sufficient to activate expression of AP-1 components as well as increase JAG2 expression, stimulation of AKT pathway does not always directly activate AP-1 promoter activity nor increase expression of JAG2[50,100]. Moreover, in HUVEC cells it was found that treatment with AKT pathway inhibitors did not alter Jagged expression but instead acted on the Notch pathway through Notch4[70].

Chemical inhibition of Jagged expression through the MAPK pathway points to a compelling potential interaction between the Notch pathway through JAG2 and MAPK; however, the specific interactions are yet unknown. It is possible that the downstream decrease in JAG2 expression through inhibition with U0126 and FR180204 is affecting not the Notch pathway, but Jagged2 in a Notch-independent manner. Indeed, Jagged has been shown to regulate AP-1 expression through cleaved Jagged intracellular domain interacting with transcriptional activating complex [40]. Unfortunately, there are technical barriers to to performing enzymatic and other biochemical assays in our model system, so the kinases and molecules directly involved are unknown. This leaves open several possibilities, one of which is that the inhibitors are acting on Jagged through regulation of the Notch receptor or the Delta ligand.

Direct studies on activation, suppression and interaction of specific proteins involved are needed in order to have a full understanding of the phenomenon seen here. While it is possible that JAG2 increase via HGF and decrease following treatment with both MEK and ERK inhibitors could be an off-

target effect, the parallel correlation cannot be overlooked. Further study into the effect on AP-1 activity would help detangle the potential pathway involved.

Based on our data and the described role of ERK1/2 on activation of AP-1 transcription and activity[72,74,99–101], I propose that Jagged2 acts downstream in the pathway where ERK1/2 activates both the AP-1 component and JAG2 expression, with subsequent binding of NICD JAG2 with AP-1. The complex of the intracellular cleaved portion of JAG2 with AP-1 promotes expression of genes regulating apoptosis-avoidance, proliferation and metastasis. Inhibition of the MAPK pathway through either MEK1/2 or ERK1/2 suppresses JAG2 expression thus inhibiting activity of AP-1 (Figure 9).

Recent strides in the fields of cell competition and cancer have furthered the potential of targeted Notch ligand therapy. For example, immunoregulated suppressor cells (Myeloid-derived suppressor cells (MDSC)) act as T-cell suppressors, inducing tumor growth. Pre-clinical trials in mice have found that Anti-JAG1/2 therapy using a IgG1-blocking antibody, CTX014, suppressed MDSCs and enhanced tumor-associated immune responses, thus, tumorigenicity was reduced[51]. This study highlights the potential impact of JAG2 targeting cancer therapies in reducing global toxicity by targeting cancer cells through aberrant Notch ligand, such as Jagged, expression.

The potential interaction found between GCP and the notch pathway provides a novel approach at tackling the role of integrating a specific Notch ligand with cell competition in cancer initiation and development. Therapies

targeted to the Notch ligands Jagged and Delta would provide treatment options with less off target effects and potentially greater results.

A

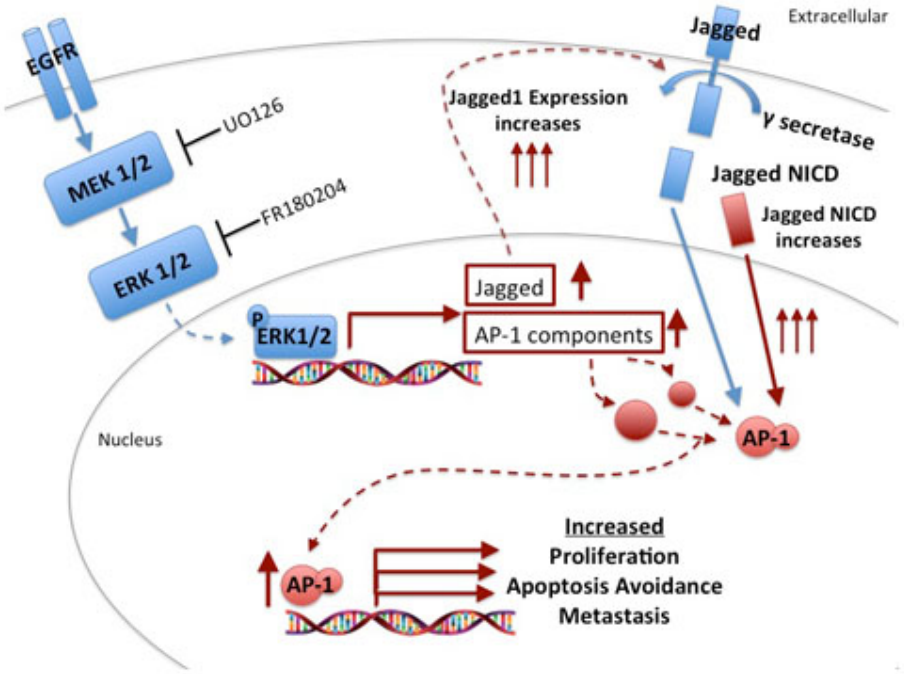

Figure 9. Proposed model for JAG2 and MAPK pathway interaction (A) Schematic interaction where MEK1/2 activates ERK1/1 resulting in activation of JAG2 and AP-1 component expression. JAG2 NICD interacts with AP-1 forming a complex that promotes expression of proliferation, motility and survival. Red pathway components represent points in the pathway that have the potential of causing cancer when aberrant signaling occurs. U0126 represses MEK1/2 while FR180204 represses ERK1/2. Inhibition by either results in decreased JAG2 expression, thus a decrease in available JAG2 NICD and a subsequent decrease in JAG2/AP-1 complex target genes.

FUTURE DIRECTIONS

This study suggests an association between Jagged and the winner phenotype GSC. Indeed, inhibition of Jagged through the MAPK pathway decreased migration of winner genotypes to that of loser genotypes, while activation through HGF increased loser migration abilities to the levels of the winner GSCs.

While Jagged was the primary focus of these studies, the Notch ligand Delta was also upregulated in GSCs, with Winner GSCs expressing more than the Loser GSCs. However, the level of difference was far higher in JAG2 expression, leading us to probe JAG2. One explanation for the differential expression levels between JAG2 and Delta are varying levels of functional robustness as is seen in Glioma cell lines. siRNA knockdowns of JAG and Delta-like-1 both had effects on proliferation and survival; however the Delta-like-1 had significantly higher effects than the JAG siRNA[57]. Additional experiments knocking down Delta specifically would shed light on whether the decrease in JAG specifically is responsible for the results seen. Also seen was a general maximum and minimal level of both JAG2 and migratory abilities. This suggests the possibility of interaction with another regulatory process being involved, either through Notch/Jagged or through MAPK.

Additionally, the model proposed relies on the idea that downstream interaction and activation of activity in AP-1 through Jagged2 is driving the winning phenotype. Along with identifying JAG2 vs Delta specific signaling further elucidation into whether AP-1 is the downstream player would greatly enhance

this study. Other potential players involved could be p38β, another MAPK associated protein that is associated with both JAG2 and AP-1[69,70,79,80]. The model presented excludes the interaction with p38β as it is generally not associated with MEK1/2, rather it is found involved in interaction with MEK3/6[69,79] and generally suppresses Notch signaling[70].

This work would be further validated through use of transgenic animals, identification of AP1 mRNA levels in isolated GSCs, knockdown of JAG2 to corroborate pharmacological inhibition and overexpression of JAG2 in Loser GSCs to identify the specific steps involved in GCP. Understanding the role of Jagged2 in GCP and its relationship to cell competition and cancer biology would shed light on specific, targeted treatments with decreased whole-body deleterious effects.

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