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A Small Molecule Screen on Zebrafish Embryos Identifies Pathways Vital to Hypaxial Muscle Precursor Migration

Teresa Easterbrooks
University of Maine, teresa.easterbrooks@maine.edu

A SMALL MOLECULE SCREEN ON ZEBRAFISH EMBRYOS IDENTIFIES PATHWAYS VITAL TO HYPAXIAL MUSCLE PRECURSOR MIGRATION

By

Teresa Easterbrooks

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Dr. Jared Talbot, Assistant Professor of Biological Sciences, Advisor

Dr. Clarissa Henry, Professor of Biological Sciences

Dr. Julie Gosse, Associate Professor of Biochemistry

A SMALL MOLECULE SCREEN ON ZEBRAFISH EMBRYOS IDENTIFIES MULTIPLE PATHWAYS VITAL TO HYPAXIAL MUSCLE PRECURSOR MIGRATION

By Teresa Easterbrooks

Thesis Advisor: Dr. Jared Talbot

An Abstract of the Thesis/Dissertation Presented
in Partial Fulfillment of the Requirements for the
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Hypaxial muscles form through long-range migration of muscle precursor cells (MMPs) from the somites. In zebrafish, the MMPs migrate in three compact streams to generate four muscles - the sternohyoideus muscle, homologous to mammalian neck and tongue; the posterior hypaxial muscle; and the two pectoral fin muscles, homologous to mammalian limbs. Several factors, such as Six1/4, are known to promote this migration; however, many aspects of guidance, pathing, and modulation of these streams are still unknown. To fill this gap, we conducted a small-molecule screen. This pharmacological approach allows us to identify molecules that cause pronounced changes in the normal pattern of MMP migration. Using a transgenic zebrafish line, *six1b:lyn-GFP*, we are able to visualize MMPs during development under treatment with pools of bioactive molecules and observe their effects. Fish are treated from 24 hours post fertilization (hpf), prior to migration, through 48 hpf, when the migrating cells begin to specify and generate muscle fibers. So far, we have tested over 800 small molecules and identified a dozen that cause promising effects. The screen has successfully generated new hypotheses concerning new cues - estrogen signaling, cholesterol synthesis - and new roles for known factors in development of these muscles - retinoic acid. In this thesis, I report the overall results of the small molecule screen and the new models developed using our findings.

ACKNOWLEDGEMENTS

I would like to acknowledge the journey & all the mentors that have led to the extremely full and well-rounded graduate experience I've had. In the fall of 2019, I joined the Graduate School of Biomedical Science and Engineering (GSBSE) at the University of Maine, and began my rotations, with Ron Korstanje, Jared Talbot, and Melissa Maginnis. I thank Ron Korstanje for inviting me to his lab and Ron, his lab, and the staff at JAX for the experience that year. After I left the GSBSE program, Jared Talbot invited me back to his lab as a master's student, where I began this thesis work. Under Jared's mentorship, I've grown as a scientist, a writer, a teacher, a mentor, and a person.

I would like to additionally acknowledge the resources, facilities, and support that have enabled me to do this specific thesis research. Mark Nilan and the rest of the UMaine Fish Facility for excellent care of our fish; the Henry Lab, for support in microscopy. Finally, this work could not have been done without the support of many students in the Talbot lab, especially those who participated in the screen:

Kaitlyn Bear (Jan '20 - May '21)	Sean Eggermann (Oct '21 - Feb '23)	Angelina White (Feb '23 - current)
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Additionally, the Developmental Biology (Bio336) classes from Fall 2020, 2021, and 2022 participated in the screen as part of research integration in the lab. Altogether, these students have assisted with testing several molecules, and contributed to multiple findings - Dominic Crowley identified an estrogen molecule, diethylstilbestrol as part of the Fall 2020 class, and Sara Loiselle identified (+) JQ-1 as part of the Fall 2021 class screen.

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CHAPTER 1

INTRODUCTION

In both embryos and adult vertebrates, long-range migration is vital to muscle health and formation. In adults, this process is key to muscle growth and regeneration (Berberoglu et al., 2017; Di Rocco et al., 2015; Velders and Diel, 2013). During embryonic development, long-range migration produces muscles in many body-regions, from head (tongue and hypobranchials) to toe (hindlimb) (Figure 1.1). This set of hypaxial muscles are formed from migratory muscle precursors (MMPs) that originate in the trunk and migrate out to become muscles such as the neck, tongue, chest, diaphragm, and limbs (Vasyutina and Birchmeier, 2006). This skeletal muscle precursor migration is found in jawless fish, where the hypobranchial muscle mirrors the tongue muscle development in jawed vertebrates (Kusakabe and Kuratani, 2005). Parallel studies from zebrafish, xenopus, chick, and mouse demonstrate similarities in migration patterns as well as many common genetic factors, such as *Six* family genes, regulating this migration (Dietrich et al., 1998; Grifone et al., 2005; Grifone et al., 2007; Haines et al., 2004; Martin and Harland, 2001; Talbot et al., 2019). In addition to homologies through evolution, MMP migration also shares both genes and physical properties with another type of long-range movement, tumor metastasis. Similarly to MMP migration, metastasis requires an initial epithelial-mesenchymal

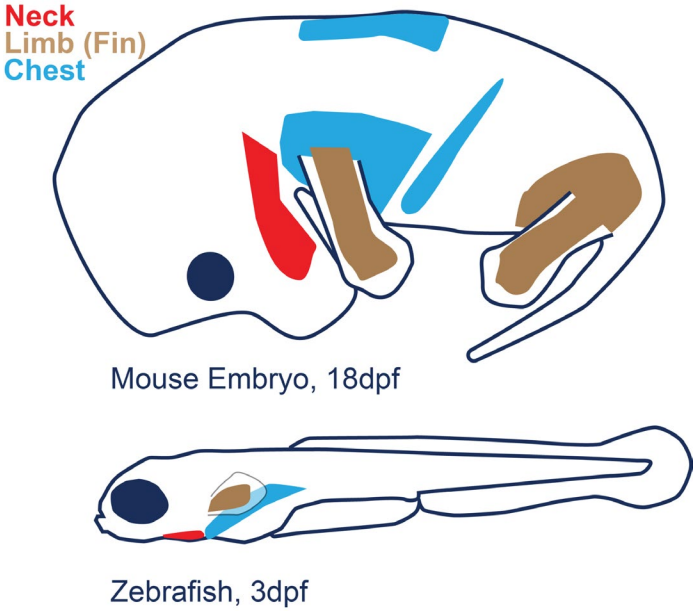


Figure 1.1
Homology of hypaxial muscles between mouse and zebrafish. The zebrafish sternohyoideus muscle (SHM, red) is homologous to the neck and tongue in the mouse; the fin (brown) is homologous to the limbs of the mouse; and the posterior hypaxial muscle (PHM, blue) is comparable to the chest and diaphragm in the mouse.

transition, followed by delamination from a base structure (in this case, a tumor instead of a somite) and migration as a mesenchymal cell before re-differentiating (into a tumor or a muscle cell) (Di Zazzo et al., 2019; Farabaugh et al., 2012; Yu et al., 2009). In addition, known MMP genes such as *Six1*, *Lbx1, Met,* and *Pax,* are all known oncogenes as well (Blevins et al., 2015; Farabaugh et al., 2012; Hsu et al., 2022; Kahsay et al., 2022; Yu et al., 2009). With these parallels, relevant cancer research can add to our background for generating new hypotheses from the screen, and new discoveries about long range migration via the MMPs may also add to the cancer metastasis field.

Across vertebrates, this hypaxial muscle formation begins with the somite – an epithelial structure whose derivatives, the sclerotome and the dermomyotome, give rise to vertebrae and skeletal musculature respectively (Tajbakhsh and Spörle, 1998; Vasyutina and Birchmeier, 2006). The dermomyotome contributes to multiple muscle groups, from the dorsal side generating deep back muscle, to the ventral dermomyotome being the source of the MMPs needed for these long-range sourced muscles (Denetclaw et al., 1997; Dietrich, 1999; Kahane et al., 1998). Cells from the ventrolateral lip of the dermomyotome undergo an epithelial-mesenchymal transition and delaminate before migrating (Buckingham et al., 2003; Dietrich, 1999; HAINES and CURRIE, 2001; Kalcheim, 2016). *Six 1/4* targets *Lbx1, Pax3,* and *Met* are expressed in this dermomyotomal lip and required for hypaxial muscle formation in the mouse (Brohmann et al., 2000; Buckingham et al., 2003; Grifone et al., 2005; Relaix et al., 2003; Vasyutina and Birchmeier, 2006). The gene *Lbx1* is required for muscle precursor migration and proliferation, resulting in tongue muscle size in mice, geniohyoideus muscle in *Xenopus*, and limb bud population across species (Brohmann et al., 2000; Dietrich, 1999; Gross et al., 2000; Martin and Harland, 2001; Masselink et al., 2017; Schäfer and Braun, 1999). This limb bud is specified by retinoic acid (RA), which recruits MMPs to populate the limb and induces *sonic hedgehog* to pattern the limb's axes, in teleosts (a group of most ray-finned fishes, including the zebrafish, medaka, and pearlfish), mouse, and chick (Cunningham and Duester, 2015; Helms et al., 1994; Kin Ting Kam et al.,

2012; Letelier et al., 2018; Mok et al., 2014; Tani-Matsuhana et al., 2018). *Pax* family genes are also involved in precursor migration in mouse, chick, and zebrafish, particularly in the limb and maintaining the dermomyotomal lip (Dietrich, 1999; Grifone et al., 2005; Mennerich et al., 1998; Minchin et al., 2013; Nord et al., 2022; Relaix et al., 2003; Relaix et al., 2005). This requirement is somewhat matched in zebrafish, where *pax3a* and *pax3b* function is partially compensated for by *pax7a/b*, such that the *pax3a;pax3b;pax7a;pax7b* compound mutant lacks hypaxial muscles (Nord et al., 2022). Met*,* a cell surface receptor, and its ligand Hgf, is essential for epithelial to mesenchymal transition in muscle precursor migration in mammal, chick, and fish (Birchmeier and Gherardi, 1998; Brohmann et al., 2000; Christ and Brand-Saberi, 2002; Dietrich, 1999; Masselink et al., 2017; Talbot et al., 2019). However, like in the *Pax* family, the effect of *Met* mutation is subtler in zebrafish than mouse, suggesting that other key players may be able to compensate in its absence (Talbot et al., 2019). These differences suggest that there are still major pathways in hypaxial MMP migration that are undiscovered. Additionally, while we know genes like *Six1/4, Pax3, Met,* and *Lbx1* that are required for cell motility, migration, and proliferation, and we know some mechanics of the pathway of migration via fate mapping and timelapse, pathways that control many aspects of migration such as specific guidance along the route and prevention of overgrowth remain unclear. As the MMP specification and migration is well-conserved, the lessons gained from zebrafish hypaxial muscles should provide insights into the development of homologous tissues in mammals.

Zebrafish traits make them an ideal system to rapidly identify signaling pathways that influence vertebrate body plan formation. Firstly, they develop externally, with each female laying hundreds of eggs, and transparently, and so their development from single cell stage through formation of whole bodily systems can be observed in brightfield on a dissecting scope level over the course of days (Kimmel et al., 1995). The zebrafish genome has been mapped, techniques have allowed for creation of transgenic reporter lines, and a host of genetic tools are available to delve into specific genes (Hwang et

al., 2013; Meng et al., 1999; Talbot and Amacher, 2014; Woods et al., 2000; Zon and Peterson, 2005). These have made zebrafish exciting model organisms since they gained prominence in the field in 1996 with a set of genetic screens that revealed many genes key to vertebrate development, in a way that had been previously restricted to invertebrates or plants (Driever et al., 1996; Eisen, 1996; Haffter et al., 1996; Patton and Zon, 2001). After the success of these initial zebrafish genetic screens, chemical screening joined the discovery toolbox. Chemical screens are an efficient method for identifying previously unknown candidates to pursue, and have been used for over 20 years to form hypotheses across many disciplines (Jimenez et al., 2016; Kim et al., 2022; Lescouzères et al., 2023; Nishiya et al., 2014; Oehlers et al., 2017; Taylor et al., 2010; Wiley et al., 2017).

Zebrafish high-throughput screens fall into three major categories: screens of gross morphology, behavior, and cell state. A gross morphological screen is one where simple bright-field microscopy is used to identify phenotypes of interest, like the original Tubingen screen (Haffter et al., 1996). In a striking recent example, Nishiya et al performed a screen where the output was either presence or absence of eyes in the embryo (Nishiya et al., 2014). Behavioral screens use the movement/response of the zebrafish as their phenotype - this includes movement presence vs absence, type of movement, change in response to stimuli, and/or seizure activity, to name a few(Wiley et al., 2017). Finally, and most applicable to this work, are cell state screens. This group uses assays such as in-situ hybridization, immunolabel, or transgenic reporters, to visualize specific cells and score them. In this screen, zebrafish carrying the *six1b:lyn-GFP;mylpfa-mCherry* transgenes, which label the migratory muscle precursor cells in green under the *six1b* promoter, a known MMP marker, and differentiated fast muscle in red under the *mylpfa* promoter, a fast-muscle specific regulatory light chain protein, allow for specific phenotype scoring of the migration patterns of the labeled cells - for example, whether the cells are migrating normally, have a stream reduction, or a change in guidance (Figure 1.2) (Ignatius et al., 2012; Talbot et al., 2019). This cell state scoring method allows for more precise screening of just cells of interest,

making it ideal for answering questions about a particular population. Although our priority is to find molecules that affect MMP migration, we also kept an eye to gross morphology and behavior during our screen, to augment these cellular findings.

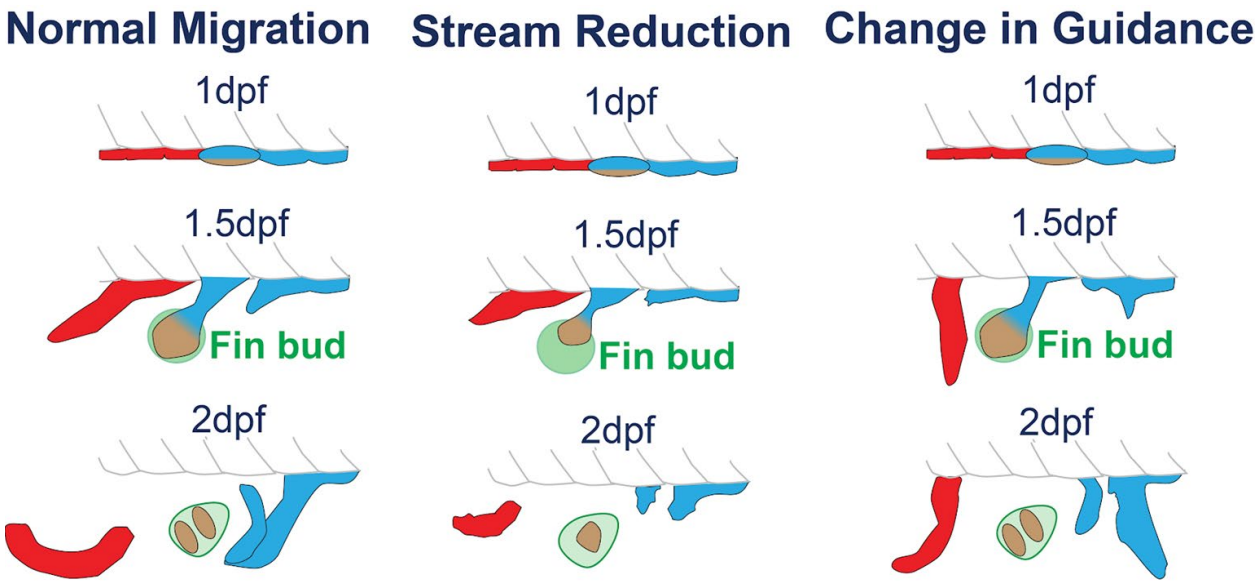


Figure 1.2. Classes of phenotypes sought in the screen. Compared to normal migration, molecules of interest may cause a stream reduction or a change in stream guidance

In this study, we perform a small molecule screen, to identify critical regulators of hypaxial muscle development in zebrafish. We tested 894 molecules and found 12 of these to pursue. Our work suggests previously unexplored critical roles for estrogen receptor signaling, cholesterol synthesis, and retinoic acid function in the migration of hypaxial muscle precursors. Each of these pathways had already been implicated in muscle development, but the specific roles identified for hypaxial muscle precursor migration is new. These results demonstrate that, indeed, there are still unknown cues to uncover that control hypaxial muscle precursor migration, and that small molecule screening is an efficient technique to find them.

CHAPTER 2

MATERIALS AND METHODS

2.1 Zebrafish maintenance, husbandry, and strains

Zebrafish are maintained using standard maintenance and husbandry procedures as previously described (Westerfield, 2007). All animal protocols are approved by the University of Maine Institutional Animal Care and Use Committee [A2022-09-05]. Experiments were performed in embryos obtained from cross of zebrafish carrying the BAC transgene *TgBAC (six1b:LY-GFP)oz5Tg* (henceforth: *six1b:lyn-GFP*), alongside *Tg (mylpfa:mCherry)cz327Tg* (henceforth: *mylpfa:mCherry*), maintained on the AB background (Ignatius et al., 2012; Talbot et al., 2019).

2.2 Small molecule screening

This screening protocol was performed over the course of three days of live embryo work, followed by imaging; starting from collection of *six1b:lyn-GFP;mylpfa:mCherry* embryos at every hour to ensure equivalent staging within a clutch. Embryos were then sorted for viability and staged according to established series (Figure 2.1) (Kimmel et al., 1995). In preparation for small molecule treatment, green fluorescent embryos were manually dechorionated using forceps (2.1). At 24 hours post fertilization (hpf), 8-15 embryos were transferred to 12-well plates, and soaked in either 0.2% DMSO (negative control) or 10 µM of bioactive compound in 2 mL of facility water for 24 hours (Figure 2.1). At 48 hpf, embryos were observed under a Leica M165 fluorescent dissecting microscope, equipped with a K5 cMOS camera, using the GFP filter. Image settings were held constant, with 500 ms exposure time and 8x zoom prior to fixation in 4% paraformaldehyde in phosphate buffered saline (Figure 2.1). This dissecting microscope was used for initial observation and preliminary imaging of fixed embryos to identify molecules that cause a disproportionate change in MMPs compared to overall development.

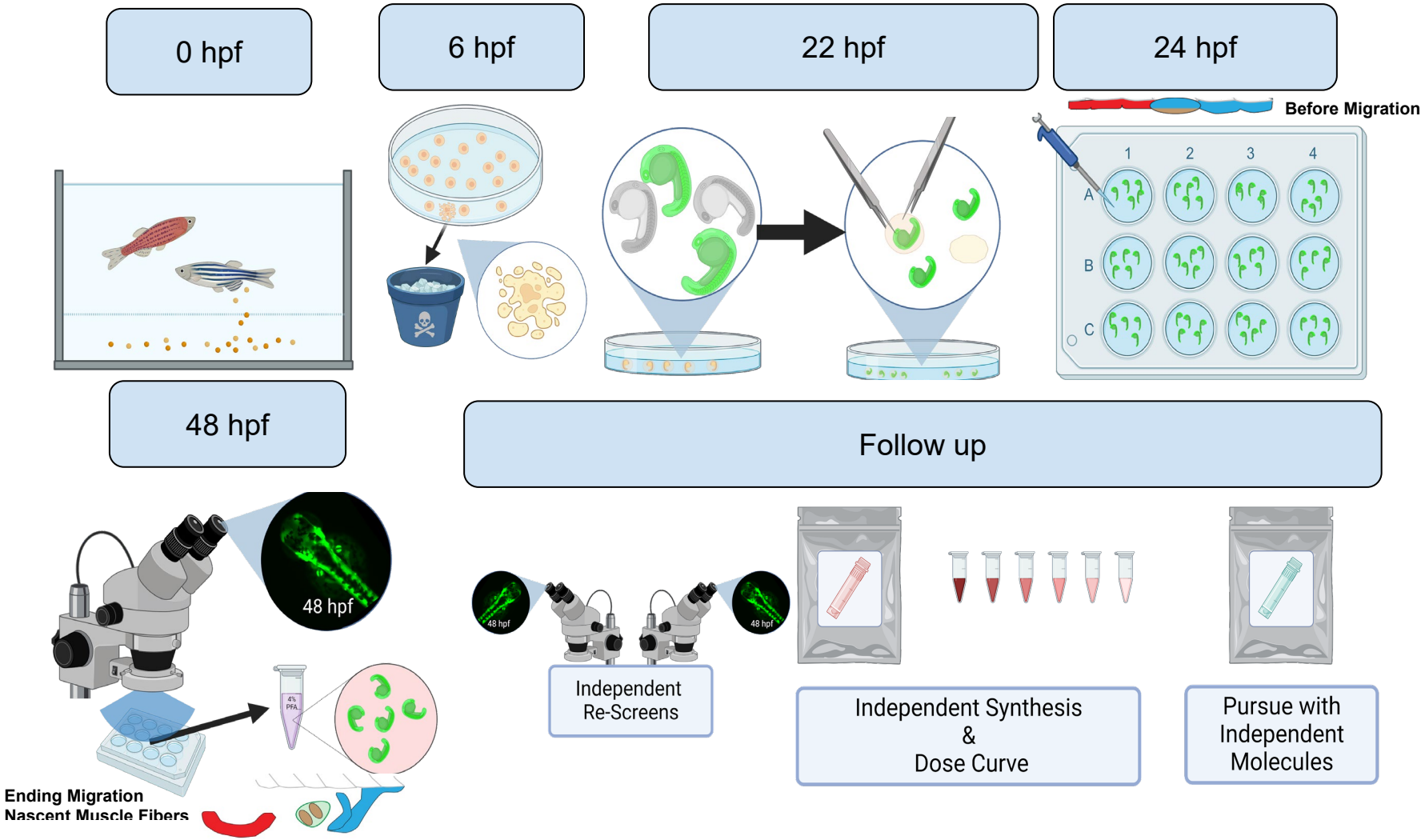


Figure 2.1 Workflow of this small molecule screen. On the day *six1b:lyn-GFP* eggs are collected from breeding tanks (0 hpf), they are sorted for fertility (6 hpf) and allowed to develop overnight. The next day, the embryos are sorted for GFP expression, and GFP+ embryos are dechorionated (22 hpf). At the start of migration (24 hpf), the dechorionated embryos are placed into a 12-well plate and the water is treated with the small molecule. When the precursors have migrated to their endpoint and are beginning to show nascent muscle fibers, the fish are observed live under a fluorescence dissecting microscope before being fixed in 4% PFA (48 hpf).

For this screen we utilized two compound libraries: the Pharmakon Collection from MicroSource Discovery (hereafter, "the Pharmakon library"), and Tocriscreen 2.0 Micro from BioTechne (Cat 7152) ("the Tocriscreen library"). To evaluate the phenotypes resulting from treatment, multiple screeners look at the embryos (Figure 2.1). The screen is run using well numbers as labels, to blind screeners to the molecule being used. MMP streams are then evaluated for a few key phenotypes - generally, stream reduction compared to normal migration or a change in guidance of the streams (Figure 1.2), though any deviation from normal migration is noted, and acute toxicity (e.g. lethal effects or signs of necrosis) is evaluated at multiple time points, including an immediate toxicity check at 25 hpf, followed by noting lethality at 36 hpf and 48 hpf observations.

2.3 Confirmation and pathway analysis

After dissecting imaging identifies compounds of interest, the fish treated with these compounds are imaged using a confocal Sp8 microscope for a higher resolution view of the cells. Strong candidates are then tested using an independent synthesis of the small molecule. During these independent repeat experiments, we also perform dose curves. Only compounds that repeatedly induce MMP-specific phenotypes are considered for further analysis. Pathways of interest are pursued further, using compounds targeting the same pathway either from one of our libraries or purchased independently. Gene expression patterns from literature and public databases are also consulted to evaluate the likelihood of a pathway being involved in MMP migration.

2.4 A baton-pass system for screening experiments

A large part of my master's work has been the training, building, and overseeing of a team of students that work with me on the screen. In my time in the lab, I have had eight mentees at different points that I trained on the screen. In addition to general lab training, and specific methods for the

week's experiments, I also mentored these students in how to research. We have regular (generally, weekly) meetings to discuss the rationale for our work, relevant literature, and plans. I work with students during and between experiments on their note-taking skills to ensure that they have appropriate skills and that experiments are documented appropriately. Because of the key role of these students and of my development as a mentor in my work on the screen, I will be highlighting students who contributed particularly to experiments in the appropriate results section.

2.5 Data compilation and analysis

A.

	ID	Pooled?	Searchable Wells	Observations	Lab Notebook
318	T_7G4	No	T_7G4	Severe fin defect/delay	2:121-124
319	T_7G5	No	T_7G5	Normal	2:121-124
320	T_7G6	No	T_7G6	Normal	2:121-124

Teresa · Jared · Bri/Sarah · Holly · Willow · Allie · Sean · Michael · Angelina · Lauren

B.

=concatenate("T_",J2,L2,M2)

Lookup	Cat.No	Compound name	Target Class	Primary Target*	Primary Actio	Brief Description	Batch	Data Sheet	Plate Number	Plate Location	Row	Column
T_1A2	0189	DNQX	Ion Channels	AMPA Receptors	Antagonist	Selective non-NMDA iGluR antagonist	14	Datasheet_0189	1	A02	A	2
T_1A3	0190	CNQX	Ion Channels	AMPA Receptors	Antagonist	Potent and selective non-NMDA iGluR antagonist	34	Datasheet_0190	1	A03	A	3
T_1A4	0223	Kynurenic acid	Ion Channels	Non-selective Ionotropic Glutamate	Antagonist	Broad spectrum glutamatergic antagonist	58	Datasheet_0223	1	A04	A	4
T_1A5	0237	7-Chlorokynurenic acid	Transporters	Glutamate Transporters	Inhibitor	Potent competitive inhibitor of L-glutamate uptake	6	Datasheet_0237	1	A05	A	5
T_1A6	0286	5,7-Dichlorokynurenic acid	Ion Channels	NMDA Receptors	Antagonist	Potent NMDA antagonist; acts at glycine site	6	Datasheet_0286	1	A06	A	6
T_1A7	0357	N-Acetyltryptamine	Enzymes	Other Transferases	Substrate	Substrate for serotonin N-acetyl transferase	2	Datasheet_0357	1	A07	A	7
T_1A8	0369	W-7 hydrochloride	Cell biology	Calcium Binding Protein	Antagonist	Calmodulin antagonist. Inhibits myosin light chain kinase	8	Datasheet_0369	1	A08	A	8
T_1A9	0373	NBQX	Ion Channels	AMPA Receptors	Antagonist	Potent AMPA antagonist; more selective than CNQX (Cat.	16	Datasheet_0373	1	A09	A	9
T_1A10	0415	Ro 20-1724	Enzymes	Phosphodiesterases	Inhibitor	PDE4 inhibitor	4	Datasheet_0415	1	A10	A	10
T_1A11	0421	GBR 12909 dihydrochloride	Transporters	Dopamine Transporters	Inhibitor	Selective DA uptake inhibitor; also σ ligand	5	Datasheet_0421	1	A11	A	11
T_1B2	0431	ML 9 hydrochloride	Kinase	Myosin Light Chain Kinase	Inhibitor	Myosin light chain kinase inhibitor	5	Datasheet_0431	1	B02	B	2
T_1B3	0456	Chlormezanone	Ion Channels	GABAA Receptors	Benzodiazepine	Positive allosteric modulator of benzodiazepine site	4	Datasheet_0456	1	B03	B	3
T_1B4	0478	Flurofamide	Enzymes	Other Hydrolases	Inhibitor	Urease inhibitor	1	Datasheet_0478	1	B04	B	4
T_1B5	0523	4F 4PP oxalate	7-TM Receptors	5-HT2A Receptors	Antagonist	Selective 5-HT2A antagonist	3	Datasheet_0523	1	B05	B	5
T_1B6	0541	Fasudil hydrochloride	Kinase	Rho-kinases	Inhibitor	Inhibitor of cyclic nucleotide dependent- and Rho-kinases	3	Datasheet_0541	1	B06	B	6

C.

=if(AND(isblank(G2)),if(and(isblank(B2),isblank(C2),isblank(D2),isblank(E2),isblank(F2)),iferror(1/0),filter(Master!E2:L,search(B2,Master!F2:F),search(C2,Master!E2:E),search(D2,Master!G2:G

	Library	Well	Molecule	Information	Screened	Observations	Notable?	Toxic?
Search By	tocris			retinoic acid receptor	Yes			
12 Results								

Matching Wells	Library	Molecule	Target Class	Target	Information	Screened?	Observations
T_14D6	Tocris	Ly 2955303	Nuclear Receptors	Retinoic Acid Receptors	Retinoic Acid ReceptorsAntagonist High affinity and selective RARγ antagonist	Yes	Teresa: Pooled: Dead/Missing? Toxic |, |,
T_1D4	Tocris	Am 580	Nuclear Receptors	Retinoic Acid Receptors	Retinoic Acid ReceptorsAgonist Retinoic acid analog; RARα agonist	Yes	Teresa: Pooled: 1 dead, 2 with severe reduction in streams and overall posterior shift | | Jared: Pooled: !! Note SHM is there, thin and tucked; pharyngeal arch ~36hpf w/o jaw; | | Allie: Pooled: 2 of 2 fish very abnormal, reversed development (direction wise), SMH tu Pooled: Live: 2 of 3 fish reverse structure, SMH tucked under head, no fin present Pooled: Live: 3 of 4 fish duplicated from previous week, 1 of 4 fish dead, fin present, cu Live: 3 of 3 fish abnormal, gradiently different, lack of phiringeal arch development, thin Pooled: 2 of 3 very blotchy fin develoment, 1 of 3 reverse development, 3 of 3 SHM tuc 2 of 2 fish complete reverse development, fin muscle present |Yes ,Yes,,Yes,,Yes |,,,,

Figure 2.2 A database used to query screen data. Observations are entered independently by individual screeners (A), which are then combined with data provided by each library (B), leading to searchable data that compiles all observations with known targets (C)

In order to compile observations, I created a spreadsheet-database using Google Sheets. Individual observers each have a tab in the spreadsheet, to which they add their observations for screen molecules (by library & coordinate) summarized from their lab notes, as well as references to which notebook contains further notes and sketches (Figure 2.2A). This allows the spreadsheet to automatically compile all the observations for a given molecule on the “Master” tab, making every result easily searchable on the customized search tab (Figure 2.2C). This spreadsheet also contains the reference material from the manufacturers of both libraries, as well as space for additional notes from lab members regarding molecule targets, allowing for quick reference of potentially interesting molecules (Figure 2.2B).

CHAPTER 3

SUMMARY OF RESULTS

3.1 Selection of screening library and dosage

The Talbot lab began this screen in January of 2020, using the Pharmakon library, with a dosage of 20 µM, to match the mean screening dose in a recent review (Wiley et al., 2017). Before I joined the lab in Fall 2021, they had tested 208 candidate molecules. Because I draw conclusions from both the parts of the screen I took part in and those that came before, I am including these 208 in this thesis and applying appropriate credit to the people who did this early work.

I began my work by re-evaluating the screening method. The Pharmakon library was selected because its compounds were FDA approved, and because it was inexpensive. However, it targeted many pathways that are not expressed in eukaryotes and initial screening using this library showed that 12% of molecules were lethal to the larvae. Three molecules were identified as being of possible interest in the initial Pharmakon screen - Mequinol, Estradiol, and Diethylstilbestrol. Of these three, only the effects of Mequinol had been confirmed or explored. When my work began, Dr. Talbot and I re-evaluated available library options, and found that the Tocriscreen library was better annotated for the pathways targeted, as well as using fewer molecules (1280 vs 1760) to target a broader spectrum of pathways. At the start of my master's project, I tested the last thirty candidate molecules from the Pharmakon library that targeted cell signaling pathways of interest from the Pharmakon library, leading to the identification of Genistein as a MMP regulator. Thereafter, all our work has used the Tocriscreen library. The concentration of the small molecules was determined by prior literature, as well as by preliminary work in the laboratory. In zebrafish screens, reported treatment concentrations range from 1-100 µm (Wiley et al., 2017). In preliminary screening, the concentration of 20 µm was used for 238 molecules and was found to have a high rate of toxicity (~12%). As well as evaluating the library as a whole, Dr. Talbot and I also checked the literature on screen dosage, noting that while the average

reported tested concentration is 20 µm, the most frequent is 10 µm (Wiley et al., 2017). We adjusted our concentration to 10µM, the mode, and switched to the new Tocriscreen library, and thereafter found a lethality rate of only 1%.

3.2 Summary of findings from the small molecule screen

In total, the Talbot lab has screened 894 molecules were screened across two libraries (Figure 3.1, blue); and I have led screening for 678 of these after joining the lab. We identified twelve compounds as 'hits' that alter muscle precursor migration without toxicity or developmental delay (Fig 3.1, red). Eight of these were found using unbiased screening, and four were identified during subsequent tests to confirm interesting pathways suggested by the first 8 (Asterisk in Table 3.1). Additionally, we ordered compounds outside of the two libraries during further pathway investigation (Asterisk in Table 3.1, *).

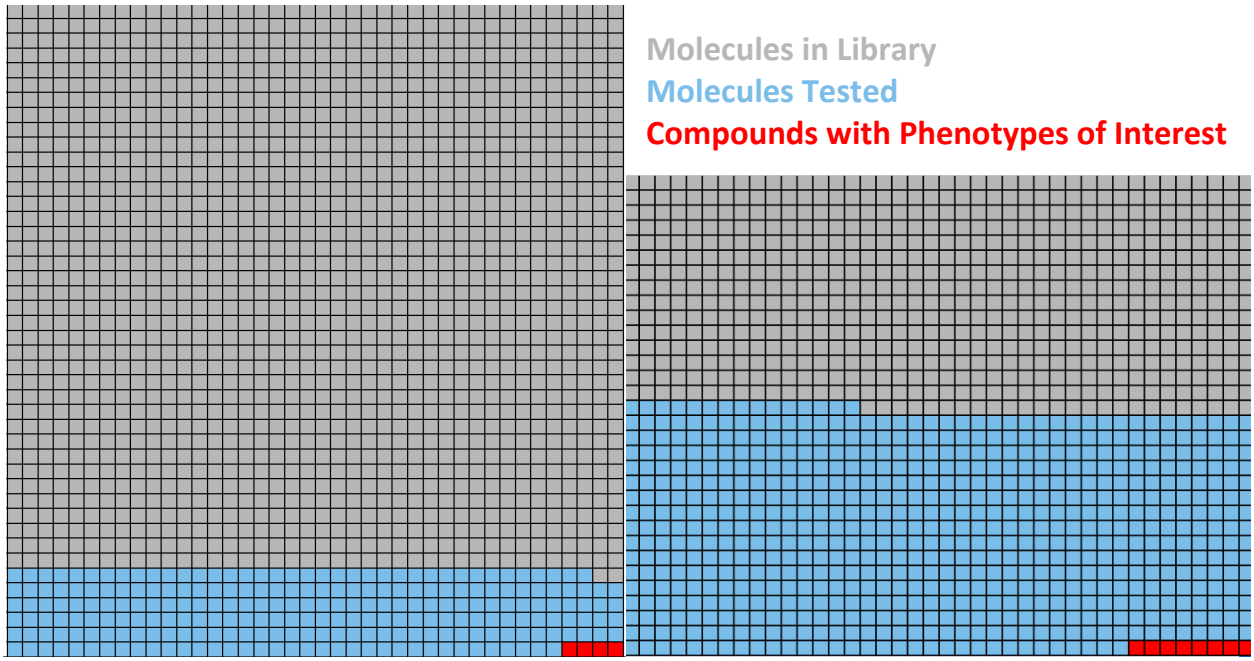


Figure 3.1. Summary of 894 small molecules screened across two libraries. Total molecules in each library (gray), screened compounds (blue), and molecules of interest (red).

The Tocriscreen library contains, by their annotations, molecules targeting 362 pathways. Of these 362, we have screened molecules that target 247 of these pathways. Our twelve molecules of interest target five of these pathways - Estrogen Receptor (4, or 33%), Retinoic Acid Receptor (3, 25%), Hmg-CoA reductase (2, 15%), Tyrosinase (1, 8%), Bromodomains (1, 8%), and Other (1, 8% - targets Rab27a).

Table 3.1. Small Molecules identified as MMP regulators, with abbreviations, and targets.

Molecule	Abbreviation	Target/Activity
Genistein	Genistein	Estrogen Receptor β Agonist
Estradiol	Estradiol	Estrogen Receptor Agonist
Diethylstilbestrol	DES	Estrogen Receptor Agonist
FERb 033*	FERb 033	Estrogen Receptor β Agonist
AM580	AM580	Retinoic Acid Receptor α Agonist
ER50891*	ER50891	Retinoic Acid Receptor α Antagonist
4-Diethylaminobenzaldehyde**	DEAB	Aldh1a2 inhibitor
R115866**	Talarozole	Cyp26 inhibitor
Pitavastatin	n/a	HMG CoA Reductase inhibitor
Simvastatin*	n/a	HMG CoA Reductase inhibitor
Nexinhib20	Nexinhib	Rab27a inhibitor
(+)JQ-1	(+)JQ-1	Bromodomain Inhibitor
*: Pulled from the screen library to test a pathway **: Not in library - hypothesis selected based on library findings		

The remaining confirmed candidate molecules are (+)JQ-1, targeting Bromodomains; Mequinol, targeting Tyrosinase; and Nexinhib20, targeting *Rab27a*. These three compounds each caused unique phenotypes, however they each have attributes that kept us reserved during initial pursuit. (+)JQ-1 is a

bromodomain inhibitor that was of interest because it reduced cohesion in the migrating MMPs; however, the active dosage that caused this phenotype turned out to be close to a dose that causes lethality, so we were concerned that the phenotype of interest may be related to these indirect toxicity effects.

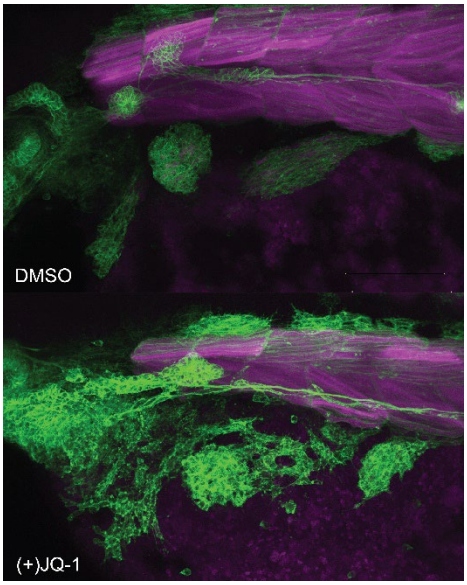


Figure 3.2 Bromodomain Inhibitor (+) JQ-1 causes disarray of MMP streams.

Prior to my arrival, screening identified Mequinol as an MMP-migration blocker; work by Kaitlyn Bear suggests that Mequinol may act via a cell-autonomous toxicity mechanism, making it a compound of lesser interest for understanding MMP function. Nexinhib20 is still preliminary and is being validated currently.

3.3 Steps needed to complete the small molecule screen

Future directions for the screen as a whole include finishing the remaining 623 molecules in the Tocriscreen library and pursuing further molecules that come up. At a 1% hit rate, we would expect to find six more molecules. In the past six months, I have set up 69 breeding tanks at 13 different setups and collected 1309 eggs, an average of only 19 eggs/breeding tank, when we expect a few hundred per female. This has impacted our screening rate tremendously; at peak fecundity, when we were able to screen 160 molecules in a week, the remaining Tocriscreen molecules would have taken under a month. Additionally, pursuing the 'in-progress' molecules, such as Nexinhib20, will round out the screen. Finally, adding quantitative results to the qualitative observations will improve the reporting; for example, using ImageJ to measure the sizes of the MMP streams, gaps between them and reference structures, and migration angles could all contribute to our understanding of the effects of these small molecules.

CHAPTER 4

CHOLESTEROL SYNTHESIS IS NECESSARY FOR MUSCLE PRECURSOR MIGRATION

4.1 Small molecules target the rate-limiting step of cholesterol synthesis

Two molecules with potent effects on MMP development, pitavastatin and simvastatin, target 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase), a crucial and rate-limiting enzyme that controls cholesterol synthesis (Alberts, 2008; Kajinami et al., 2003). Treatment of embryos with these molecules (Figure 4.1 B, C) causes reduction in MMP stream size. Even after the treatment, the vector-angle of cell movement appears normal, suggesting that active cholesterol synthesis is not needed for MMPs to interpret directionality cues. Instead, we see loss of stream cohesion, especially in the posterior streams (Figure 4.1B, C) and a change in cell shape. Normally migrating MMP cells (Figure 4.1A') are elongated and extending filopodia and lamellipodia to maintain migration (Fairchild and Barna, 2014; Sanders et al., 2013; Talbot et al., 2019). However, in statin-treated cells (Figure 4.1B', C') the cells are much rounder, and we do not see these normal extensions.

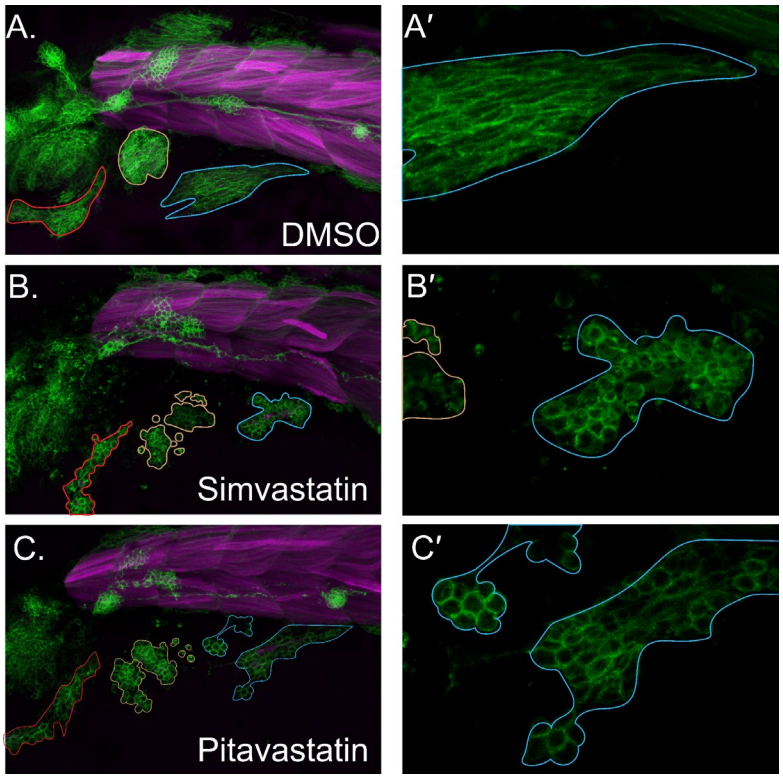


Figure 4.1 Images of statin-treated embryos. *Six1b:lyn-GFP* (green) and *mylpfa:mCherry* (magenta) expression in 48 hpf embryos (A-C). Treatment with statin class molecules causes defects in cell morphology and migration. When cholesterol synthesis is blocked, the cells change shape and become rounder, and migration is impeded. The shape, both of the streams (A, B, C) and of the individual cells (A', B', C') are altered in statin treatment (B, C) compared to control (A)

4.2 Functions of cholesterol in the cell membrane

Cholesterol is a crucial component of the cell membrane. Cholesterol is also necessary for the proper processing of *sonic hedgehog* (*shh*) in the membrane and its responsive genes, and can rescue the associated defects in models of fetal alcohol spectrum disorder (FASD) and lead to Shh signal transduction (Li et al., 2007). Fitting most of its structure into the lipid bilayer, with only small end groups interfacing with the external environment, the interaction of cholesterol with sphingomyelin and other membrane components regulate fluidity and flexibility of the membrane (de Oliveira Andrade, 2016). Additionally, cholesterol is often a major component of membrane rafts (though not always necessary), which are 10-200 nm dynamic structures of proteins and lipids within a membrane, and have a variety of functions depending on their composition (Pike, 2006; Simons and Ehehalt, 2002). These rafts are associated with connections between the cell membrane and cytoskeleton proteins such as actin and tubulin, as well as reorganization of the cytoskeleton (Chichili and Rodgers, 2009; de Oliveira Andrade, 2016). Cholesterol-rich membrane rafts are specifically associated with interactions with the actin cytoskeleton on the leading edge of migrating cells (Mañes and Martínez-A, 2004). Membrane rafts have been associated with regulating the cell cycle, membrane trafficking, and fusion, as well as cytoskeleton interaction and membrane polarization, all of which are important processes to migrating muscle precursors among other developing structures (de Oliveira Andrade, 2016; Nebl et al., 2002; Scarpa and Mayor, 2016; Solnica-Krezel and Eaton, 2003).

4.3 Statin treatment inhibits somite development and muscle maintenance

In somite formation, simvastatin treatment, causes disruption in somite structure - cell shrinking with greater space between, with anterior somites losing their larger size compared to posterior - as well as effects on adhesive structures including laminin and micro & intermediate filaments (Campos et al., 2015). Reduction of HMG-CoA, by statin treatment or by knockdown, causes atrogin-1 mediated

damage to myofiber structure and alignment via post transcriptional processing defects (Campos et al., 2015; Cao et al., 2009; Hanai et al., 2007). Limb girdle myopathy, which can be induced by either mutation in HMGCR or statin treatment, in both mouse and human has been shown to be treatable by mevalonolactone, suggesting a possible downstream player in statin treatment (Yogev et al., 2023). Downstream of HMGCR are two different players that could be contributing. While the default descriptor of statins are cholesterol synthesis inhibitors, HMGCR also supports Rho/Ras GTPases via geranylgeranyl-pyrophosphate (GGPP), which have important roles in early development (Eisa-Beygi et al., 2014).

4.4 Model and future directions

The change in cell morphology suggests that, despite their EMT-associated ability to separate from the somite and begin migrating, these cells may not fully be adopting a mesenchymal and migratory cell type. This may be connected to other known mechanisms of MMP migration, as stream cohesion affects are also seen in partial *six1/4* loss of function, and treatment with an Shh antagonist reduces cell count and causes issues with fin muscle separation (Talbot et al., 2019).During the time frame when the precursors are migrating, the zebrafish is still developing its liver, which will later (4 dpf) produce cholesterol that is distributed by the bloodstream, and therefore cells aren't receiving cholesterol via the blood; only what can be made in each cell (Tao and Peng, 2009). Inhibiting

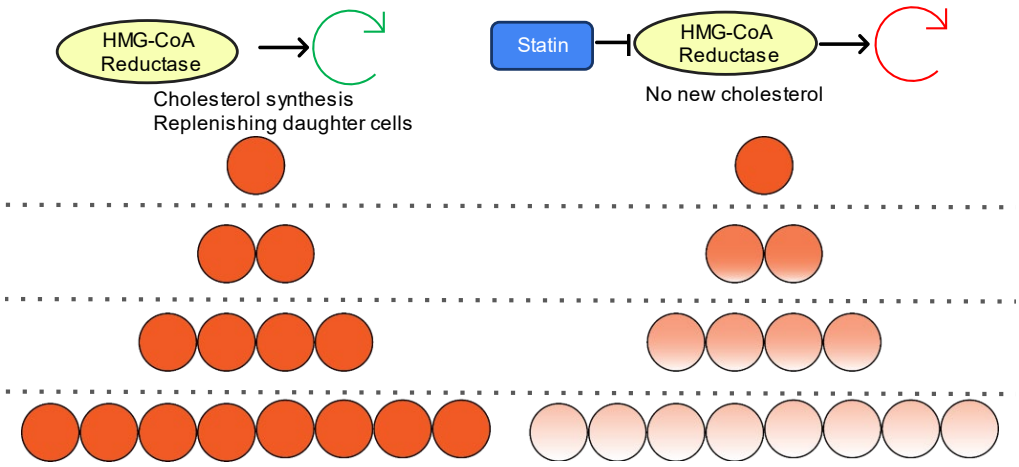


Figure 4.2. A model of cholesterol in migratory cell morphology. Under statin treatment, generations of proliferating cells may have diluted amounts of cholesterol, leading to problems in cell morphology and membrane composition.

cholesterol synthesis in this timeframe (24-48 hpf) would leave each cell in the embryo with the amount of cholesterol it had already produced for that time. However, cells that are actively proliferating during this inhibition, such as the MMPs, are unable to create more cholesterol to replenish individual cell stores, and instead divide one cell's cholesterol amongst the daughter cells, leading to a dilution effect on cholesterol amount per cell. Considering the strong role of cholesterol synthesis in aspects of migration associated with the membrane - polarity, fluidity, and interactions with the cytoskeleton - I hypothesize that cholesterol is required for cell membranes to adopt aspects of this mesenchymal migratory character.

In the immediate future, using time lapse imaging on embryos treated with these compounds could give insight into how these cells are able to move with such a different cell shape and morphology. Additionally, looking past our usual screen time frame to 72 hpf would let us see if and how the MMPs recover, and how if at all their specification to muscle cells is altered. To confirm the specific pathway involved, cholesterol can be directly measured in the embryos, and addition of cholesterol directly can attempt to rescue migration defects and determine if cholesterol synthesis inhibition is the cause of the changes in MMPs. Especially this early in development, while cholesterol is also absorbed directly from the yolk, alternative targets for HMGCR are worth exploring (Sant and Timme-Laragy, 2018). So alternatively, attempted rescue with GGPP, the downstream product of HMG-CoA reductase that support Rho/Ras GTPases, could specifically implicate the Rho/Ras pathway in MMP migration (Eisa-Beygi et al., 2014). Further investigation by membrane composition assays would more directly test the hypothesis that membrane polarity and composition changes are the cause of the difference in morphology and migration.

4.5 Acknowledgements for Chapter 4

This work investigating these statin molecules and the cholesterol synthesis pathway was a primary focus for Lauren Dumont as a Summer 2023 project.

CHAPTER 5

ESTROGEN RECEPTOR β BLOCKS MMP MIGRATION

5.1 Small molecules activate estrogen receptors

Estrogen receptor activators were the first multi-molecule pathway implicated in the screen, with a correlation between selectivity for ERβ and degree of reduction in migration. Mammals have two ancestral estrogen receptors, alpha (Erα) and beta (Erβ). In zebrafish, there are three estrogen receptors because of the teleost genome duplication and subsequent reduction: in the ERα subtype, zebrafish have esr1; in the ERβ subtype, zebrafish have two receptors, esr2a and esr2b (Menuet et al., 2002) (Menuet et al., 2002). The discovery of Genistein, an ERβ agonist, suggested that estrogen receptor signaling may be the pathway of interest. To support this proposal, I returned to possible earlier screen results Estradiol and Diethylstilbestrol (DES), which are estrogen mimics, and confirmed them using independent syntheses. While Estradiol and DES are general estrogen receptor agonists, Genistein is known to have strong preference for ERβ. Because of the association between selectivity for ERβ and reduction in stream size (Figure 5.1), as well as the known expression of *esr2b*, one of the zebrafish homologues of ERβ, being the most promising for overlap with the muscle precursors, we tested FERb 033, which has a strong selectivity for ERβ (Lee et al., 2012; Thisse and Thisse, 2008).

Compared to DMSO (Figure 5.1), activating estrogen receptors leads to reduced migration. Generally, the SHM remains connected to the anterior somites instead of migrating further out and around the pharyngeal arches as in DMSO; the fin moves less far from the trunk, does not split as normal, and is often still connected to the somites via the cells that usually by 48 hpf have connected to form the PHM. Even when that separation has occurred, such as in Estradiol and DES treatment (5.1B,C), the PHM does not form one mass from the two streams that form it as it does in DMSO, instead showing a gap or two separate streams. Compared to DMSO, estrogen mimics Estradiol and Diethylstilbestrol

(DES), as well as estrogen receptor agonists Genistein and FERb 033, all lead to reduced migration of the muscle precursors.

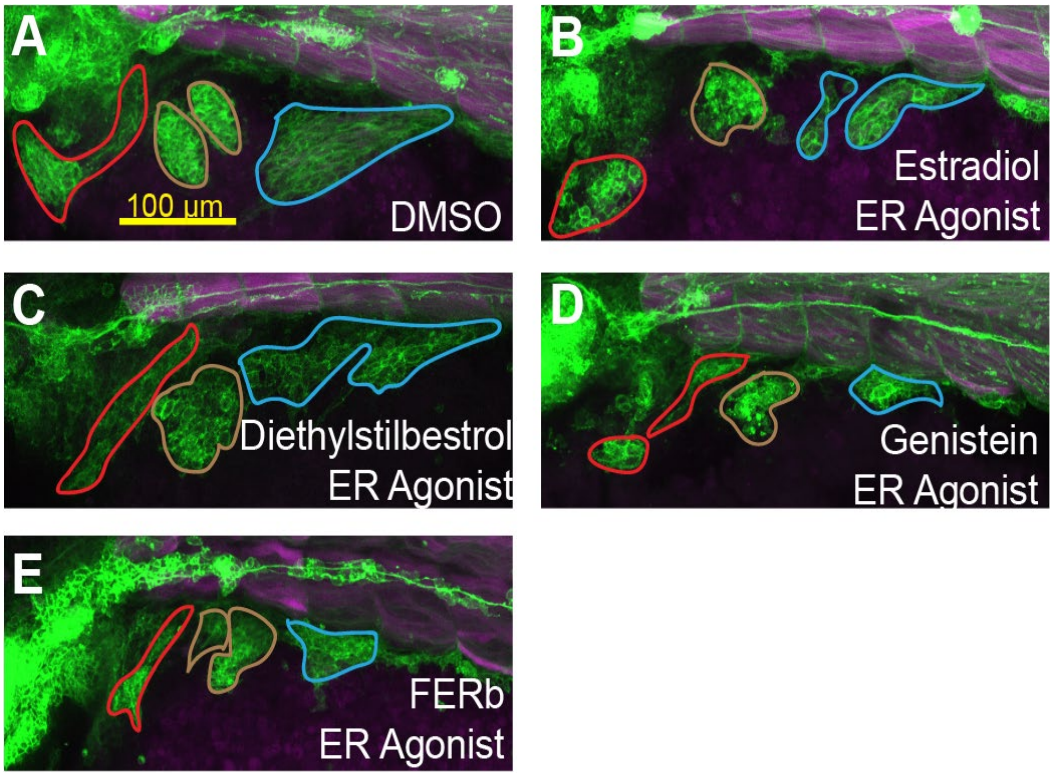


Figure 5.1. Estrogen Receptor β activation reduces muscle precursor migration. Compared to DMSO (A), treated fish have less migration across all three streams, as well as a lack of fin splitting into abductor and adductor. This effect is more severe in molecules selective for estrogen receptor β (D, Genistein; E, FERb 033 (FERb)) compared to those selective for estrogen receptor α (B, Estradiol; C, Diethylstilbestrol)

5.2 Estrogen Receptor β signaling in muscle migration

In addition to development, long range migration is used for muscle regeneration. In this realm, estrogens are needed in adult mammalian (mouse and human) bone and skeletal muscle for maintenance and regeneration (Lai et al., 2016; Seko et al., 2020). However, in tendons and ligaments, estrogen has a counterintuitive effect and decreases performance and increases injury (Chidi-Ogbolu and Baar, 2019). Estrogen's effects on developing muscle are less well studied. In human myoblasts, Estradiol was shown to reduce Pax3/Pax7 expression in a time-dependent manner (Hangul et al., 2021).

5.3 Estrogen signaling and metastasis, a parallel process to MMP migration

The proposed involvement of estrogen signaling in MMP migration highlights a similarity between this embryonic migratory process and the disease-causing cell migration of metastasis. Because of the parallels between these processes, existing research on metastasis may inform us about potential targets of ERβ signaling that could also be players in MMP migration. In cultured breast cancer cells, and in human breast cancer patients, a subtype lacking estrogen receptor expressions (and thus estrogen signaling) is considered 'unfavorable' because ER signaling blocks cell migration and proliferation (Dixcy Jaba Sheeba et al., 2016; Thomas and Gustafsson, 2011; Yuan et al., 2021). Notably, the homeobox gene *LBX1*, a key factor in MMP migration, was overexpressed in these tumor cells (Yu et al., 2009). In prostate cancer, ERα and ERβ have been shown to work against each other, with ERα inducing EMT, while ERβ blocks this EMT, similar to anti-estrogen cancer drugs (Di Zazzo et al., 2019). This aligns with our findings that ERβ reduces MMP migration, a process also dependent on EMT. ERβ signaling has been shown to inhibit tumor progression, opposing ERα, in cancer progression (Di Zazzo et al., 2019). Specifically, ERβ contains a phosphotyrosine switch that ERα does not; phosphorylation of this switch (human: Y36) is required for the anti-tumor/migration-repressing activity of ERβ (Yuan et al., 2014; Yuan et al., 2021). While the mechanism is not fully known, when phosphorylated, ERβ inhibits cell growth, the cell cycle, and epithelial to mesenchymal transition, all of which are crucial to not only cancer metastasis but also to muscle precursor migration (Mak et al., 2010; Thomas and Gustafsson, 2011; Thomas et al., 2012).

Interestingly, this phosphorylation site on ERβ has connections to Six1, which is a homeodomain protein vital to activating muscle precursor migration in both mouse and zebrafish (Grifone et al., 2005; Talbot et al., 2019). Six1 forms a complex with its cofactor Eya2 (Six1-Eya2) in order to act, as the Eya family provides activation domains in these complexes that Six1 lacks (Patrick et al., 2013). Eya2 in this Six1-Eya2 complex dephosphorylates the phosphotyrosine switch of ERβ (Grifone et al., 2007; Ohto et

al., 1999; Simpson, 2014; Yuan et al., 2014). This Six1-Eya2 complex is found in both developmental and cancer contexts (Blevins et al., 2015; Farabaugh et al., 2012; Grifone et al., 2007; Ohto et al., 1999; Patrick et al., 2013; Xu et al., 2019). Furthermore, through a collaboration with Heide Ford's lab (University of Colorado, Anschutz Medical Campus), we learned that human SIX1 protein 1 binds directly to the promoter of human Estrogen Receptor β gene, hinting at a possible direct connection between prior Six1 discoveries and our ERβ results (Hsu et al., 2022).

5.4 Model and future directions

Our results in activating ERβ pharmacologically, where streams are reduced and look quite similar to the inhibition of streams seen in a Six1/4 mutant, combined with data from a collaborator showing that Six1, a known activator of muscle precursor migration, binds to the ERβ promoter in human cancer, suggest that they may be components of the same pathway (Figure 5.2). In this model, the Six1-Eya complex dephosphorylates the phosphotyrosine switch in ERβ, repressing ERβ activity. ERβ inhibition allows for cell proliferation and epithelial-mesenchymal transition that are activated by Six1 through other pathways such as TGFβ (Blevins et al., 2015; Farabaugh et al., 2012; Zhou et al., 2020). We hypothesize that ERβ inhibits migration of muscle precursor cells through similar mechanisms as found in cancer metastasis, such as EMT inhibition and that this inhibition is repressed by the Six-Eya complex. To test this hypothesis, we have done some preliminary immunolabel work with EMT markers ZO-1, β-catenin, and Vimentin; however the ensuing label was very faint (not shown) and the pattern difficult to interpret so further testing is needed (Battaglia et al., 2018; Chernoivanenko et al., 2013; Hangul et al., 2021; Polette et al., 2007; Xuan et al., 2020).

Alternatively, in addition to known-ligand estrogen receptors, orphan estrogen related receptors also show promise as potential targets of our estrogen related molecules. These are receptors that have no known ligands but show similarity to estrogen receptors. Of these, zebrafish have five;

esrra, esrrb, esrrga, esrrgb, esrrdr (Bardet et al., 2004). ERRα has been shown to have a twofold effect on migration dynamics - firstly, it modulates actin polymerization via the RhoA-ROCK pathway, and secondly, it modulates cell adhesion via MAP4K4 (Tribollet et al., 2022). These would be good targets for additional pathway testing.

This finding marks the first identified inhibitory cue for these precursor cells - wherein activating these receptors blocks migration. Previously, identified genes such as *six1/4, lbx, met,* and *pax3/7*, were all activating cues. This gives us an exciting avenue to explore, because while *esr2* may have been implicated in cell migrations in other systems and contexts, this is a new and unique role for estrogen receptors in terms of muscle precursor migration.

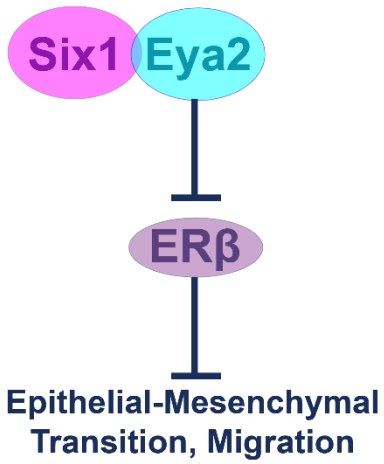


Figure 5.2 A proposed model of Estrogen Receptor β signaling on muscle precursor migration. In this model, ERβ acts as an inhibitory cue, blocking EMT and cell proliferation needed for migration, opposed by Six1.

5.5 Acknowledgements for Chapter 5

Dr. Talbot and the Fall 2020 Dev Bio class (particularly Dominic Crowley) initially flagged the Estradiol and DES molecules for further analysis. Sean Eggermann participated in FERb 033 testing.

CHAPTER 6

RETINOIC ACID MAY HAVE A ROLE IN GUIDING MUSCLE PRECURSORS

6.1 Introduction to the Retinoic Acid signaling pathway

All-trans retinoic acid (RA) is derived from Vitamin A, or retinol. In retinoic acid producing cells, retinol dehydrogenases (RDH) convert retinol to retinaldehyde, which is then processed by retinaldehyde dehydrogenases (RADH) into 11-cis-retinaldehyde and all-trans-retinoic acid (RA) (Figure 6.1). RA is then released, and either is degraded by cytochrome proteins such as Cyp26 or acts through paracrine signaling, being taken in and transported to the cell nucleus (Thatcher and Isoherranen, 2009). In the nucleus, nuclear Retinoic Acid Receptors (RAR) bind with Retinoid X Receptors (RXR) into heterodimers, which bind to retinoic acid response elements (RARE) and either repress or activate transcription when RA binds to the RAR portion of the heterodimer (Cunningham and Duester, 2015).

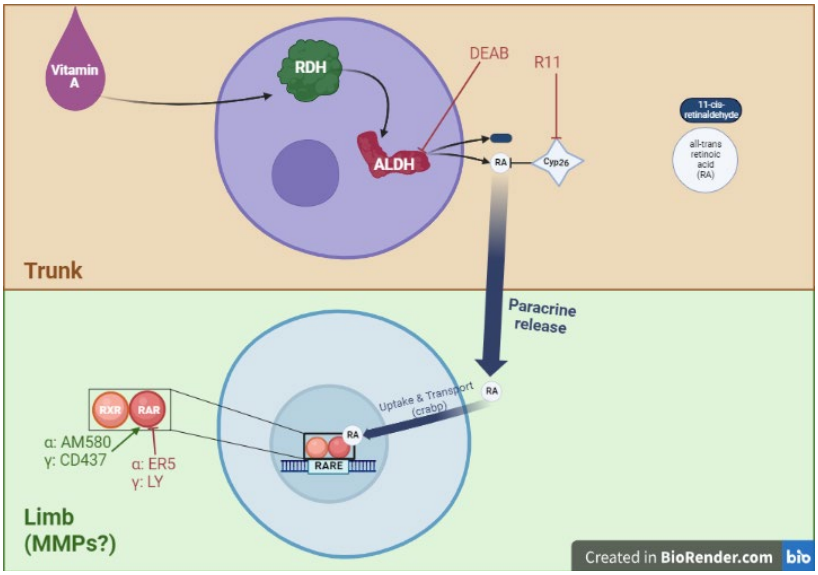


Figure 6.1 Retinoic acid is synthesized from Vitamin A and transported into the nucleus to interact with retinoic acid receptors. Vitamin A is broken down into all-trans Retinoic Acid (RA) by RDH & ALDH (the target of the small molecule DEAB). RA is then either degraded by Cyp26 (the target of the small molecule Talarozole) or released in a paracrine fashion from the RA producing cells in the trunk out into the limb region where the MMPs are. Once taken into a cell, RA is taken up into the nucleus where it interacts with retinoic acid receptors (RAR) that are in heterodimers with retinoid x receptors (RXR) and bound to retinoic acid response elements (RARE) which are transcriptionally regulated by retinoic acid. The α and γ versions of these receptors are modulated by our small molecules.

Mammals & chicks have three retinoic acid receptors – RARα, RARβ, and RARγ. In contrast, zebrafish have four; in the lineage, RARβ was deleted from the zebrafish genome before the teleost genome duplication, leading to *raraa*, *rarab*, *rarga*, and *rargb* in the zebrafish (Hale et al., 2006). Of the two orthologs to human RARα, *raraa* and rarab, published expression data appears to support activity of rarab in relevant regions of the developing embryo (Hale et al., 2006; Thisse and Thisse, 2008; Waxman and Yelon, 2007) (Figure 6.2).

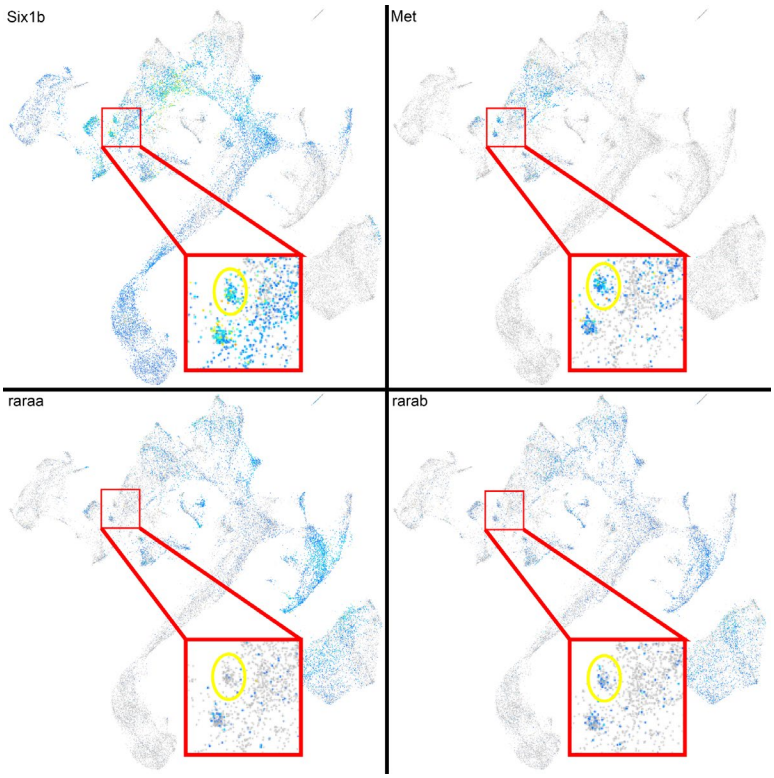


Figure 6.2 Expression of RARα compared to known MMP markers. Retinoic Acid Receptor α orthologs *raraa* and *rarab* (primarily *rarab*) are expressed in the same population of cells as known MMP markers *Six1b* and *Met*

6.2 Retinoic acid pathway modulators influence hypaxial muscle precursor movements

Our small molecules target different steps of this pathway. DEAB is an ALDH inhibitor, blocking RA synthesis, while Talarozole inhibits Cyp26 activity - by blocking the degradation of RA, Talarozole has the opposite effect of DEAB, leading to higher endogenous RA present (Figure 6.1) (Grandel and Brand, 2011; Morgan et al., 2015; Pavez Lorié et al., 2009; Thatcher and Isoherranen, 2009; White et al., 1997). Two of our small molecules, AM580 and ER50891, target RARα, agonizing and antagonizing, respectively

(Kagechika et al., 1988; Wang et al., 2020). After our initial discovery of AM580 in the screen, we used this assortment of molecules to assess the pathway in multiple steps and directions. Two further molecules, targeting RARγ, were also tested. However, both CD437 and Ly 2955303, the agonist and antagonist, proved toxic to the fish. While Ly 2955303 was toxic within an hour of treatment, CD437 survived past 36 hpf but was dead at 48 hpf. While in the initial 36 hpf observation, CD437 was marked as potentially interesting; however, the general toxicity confounds this preliminary interest, and both are excluded from pursuit. Because RARα activation caused specific and potent MMP migration defects and RARγ activation simply kills the fish, we chose to focus on the alpha receptor for subsequent analysis.

Compared to control (Figure 6.3A), when retinoic acid signaling is activated by an RARα agonist (Figure 6.3C) or by the Cyp26 inhibitor Talarozole (Figure 6.3E), the SHM does not move as far away from the fin bud (orange rectangle), the fin is smaller and does not split as fully, and the PHM invades the usual region (orange triangle) of no cells outlined by the fin, trunk, and PHM. In contrast, when RA signaling is reduced by RARα antagonist (Figure 6.3D,F), the gap between the fin and the SHM is increased (orange rectangle), and while cells are not invading the orange triangle region laid out

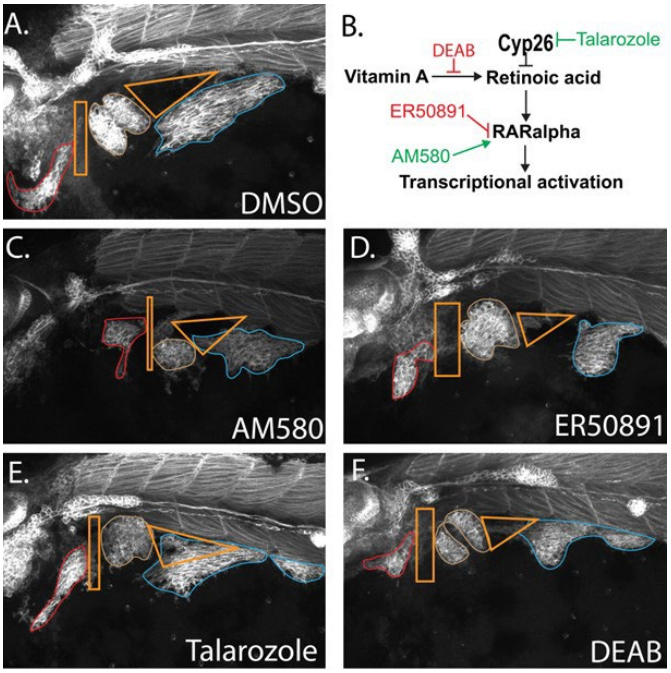


Figure 6.3 Retinoic Acid controls muscle precursor migration through RARα. When retinoic acid signaling is upregulated by blocking degradation of RA (Talarazole) or by RARα agonist (AM580) - the SHM fails to migrate away from the fin; the PHM does not migrate in its usual path, instead staying near to the trunk and filling in the usual 'gap' between the PHM and the trunk posterior to the fin. Conversely, when RARα is antagonized (ER), or when total RA is reduced (DEAB), the PHM does not progress toward the fin, and instead moves away, the fin splits more as well.

previously, the PHM also does not form the usual leg of the triangle, as this stream moves out at a much more perpendicular angle to the trunk than in control. While RA has been previously shown to be necessary for fin formation, in our embryos treated with RARα modulators, the fin bud is populated by MMPs but the streams are altered (Figure 6.3), suggesting that RA is playing a new role in the hypaxial muscle development.

6.3 Model and future directions

Based on this pathway analysis and ensuing phenotypes, I hypothesize that Retinoic acid in the developing hypaxial muscles, instead of being ubiquitously expressed across all three streams, may instead be a fin-focused attractant cue via RARα. When RARα is activated, either by inhibition of RA degradation (Talarozole) or by direct agonist (AM580), the SHM has reduced migration compared to DMSO (Figure 6.3C,E), suggesting that attractant cues from the fin region may be blocking the normal path of migration that would draw cells further from the area. Additionally, the fin fails to fully separate into two distinct masses in the RARα activated fish, which could again be attributed to an over-attractant/over adhesive role of retinoic acid. In contrast, when RARα is inhibited by the antagonist ER50891, the angle of both the SHM and PHM shift away from the fin region and more perpendicular to the trunk (Figure 6.3D), which suggests that without an attractant cue, other directional cues are unbalanced and pull the path of the streams away from the fin region. In another foil to RARα activation, in both DEAB and ER50891 treated fish (RARα inhibited), the fin does have a strong separation into the abductor and adductor muscle precursors, which would be consistent with a

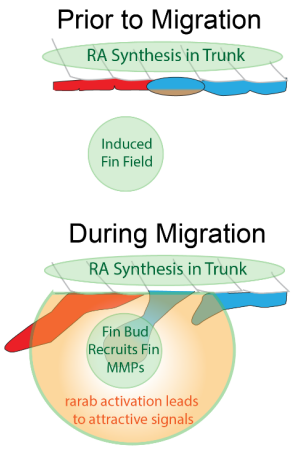


Figure 6.4 A model for how Retinoic Acid signaling may influence muscle precursor migration. Prior to migration, RA induces the fin field, which later recruits fin MMPs. Additionally, during migration, we propose that the fin region also uses RA to, via RARα signaling, emit an attractive cue that changes the path of the muscle precursors in all streams.

reduction in attractive signals holding the cells together (Figure 6.3D, F). This could give a molecular basis for the well-established role of the fin in muscle precursor migration.

To pursue further this model of RARα, further molecules targeting RARα would confirm this pathway. Assays such as immunolabel for markers of adhesion would test for models of mechanisms for RARα as an adhesive signal. Preliminary work with RARγ agonist & antagonist from screen molecules had too high toxicity to mark as a molecule of interest, but also have documented off-targets such as DNA polymerase (Han et al., 2016), so building out the model of how the RARs interact could involve further digging into γ. In general, further work can find if this potential attractive cue through RARα can be confirmed, and either connected to or distinguished from the role of RA in forming the limb(Begemann et al., 2001; Talbot et al., 2019). Investigating this RA signaling as a molecular basis for the influence of the fin field on MMP migration could revitalize and add nuance to a pattern that's been known for decades (Christ and Brand-Saberi, 2002; Dietrich et al., 1998).

6.4 Acknowledgements for Chapter 6

This section compiles the results of multiple screens and independent repeats, performed in conjunction with a number of different undergraduates under my mentorship. I'd like to acknowledge Allie Myles, an undergraduate student on the screen who was key to the screen that identified AM580, including independently flagging that well and corroborating the phenotype. Angelina White and Lauren Dumont also carried out parts of the screen for that experiment. Finally, Anaïs De Fleurian, an IUT Quimper internship student from May 2023, who picked up the AM580 and associated RARα molecules as her project and who I mentored and assisted through experiments that led to the RA panel in Figure 6.3.

CHAPTER 7

FINAL MODEL AND DISCUSSION

The goal of this small molecule screen was to generate new hypotheses pertaining to hypaxial muscle precursor migration. Prior studies, while identifying a number of genes that are necessary for muscle precursor migration, still left many types of regulation of migration undiscovered. We approached this gap in knowledge with a broad approach, by testing major pathways for effects on the MMP's migration. There were many potential effects that could be found in this sort of work: activating cues, inhibitory cues, pathways that affect cell and stream identity, guidance cues, etc. Over the course of the 894 molecules tested during the screen thus far, we have generated three new hypotheses that each have a different type of effect, and which can teach us something new about this migration.

Firstly, our findings suggest that cholesterol synthesis is necessary for muscle precursor migration, potentially through altering membrane dynamics. When cholesterol synthesis is inhibited during migration (e.g., by simvastatin or pitavastatin treatment), we find that cell shape and morphology is altered. The apparent loss of cell numbers under treatment aligns with prior work showing reduced cell count across whole embryos in statin treatment (Campos et al., 2016). While statin treatment has been implicated in myopathy, our proposed role of cholesterol in these migratory muscle precursors is new (Campos et al., 2016; Yogev et al., 2023). Probing the connection between cholesterol's known roles in migratory structures such as filipodia and the specific migration defects found in MMPs under statin treatment may provide more insight into the mechanics of migration (Fairchild and Barna, 2014; Sanders et al., 2013). Finally, the time frame covered by MMP migration is not well-studied, leaving space for this work to potentially answer other questions about the role of statin treatment in development. For example, in humans there is known risk of miscarriage, and of limb deficiency, in fetuses exposed to statin treatment in utero (Edison and Muenke, 2004).

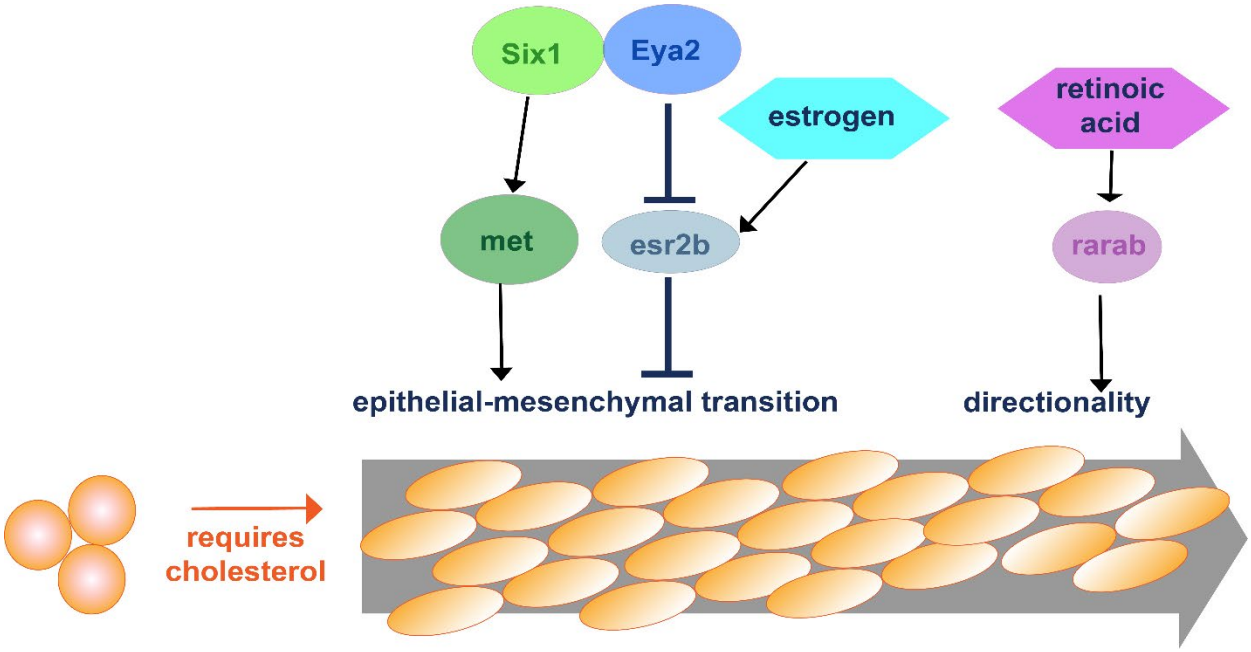
Our treatments with Estrogen Receptor β agonists demonstrate reduced migration. This is the first inhibitor cue found for MMP migration, wherein activating the receptor blocks migration. This finding is consistent with known roles for Estrogen Receptor β in cancer, where ERβ signaling blocks tumor metastasis, which has many parallels to MMP migration (Di Zazzo et al., 2019; Yuan et al., 2014; Yuan et al., 2021). Additionally, the connections between established MMP pathway *Six1* and *ERβ,* through *Eya2* and direct binding, suggest ways that this new cue may fit in to existing knowledge of migration, strengthen connections between metastasis and MMP migration, and help build out a stronger overall model of how pathways interact to control muscle precursor migration.

The screen also suggests new roles for an established cue – Retinoic Acid. Retinoic acid was previously known to be expressed in this region, and to have roles in limb patterning and induction (Begemann et al., 2001; Christ and Brand-Saberi, 2002; Dietrich et al., 1998; Mok et al., 2014). However, the way in which stream shape is altered in our embryos treated with RARα modulators is a new idea and contradicts the idea that retinoic acid's role in limb development and hypaxial muscle precursor migration is settled. Further investigation, including timelapse, that can determine the nature of this stream shape change may also indicate RARα as a guidance cue, directing the streams. This would be particularly exciting as one of the major goals at the outset of this small molecule screen was to identify guidance cues, as they are particularly unknown in this migration and are of interest.

This small molecule screen has successfully generated new and interesting hypotheses. If we continue to find molecules at our current rate, we expect six more candidates. However, we have noticed that the screen becomes more productive as it proceeds, because when finding new compounds are often confirmed and supported by a quick double-check of already tested molecules. The more 'hits' we find in a given pathway, the more confident we become in pursuing it. Our finding of a different type of cue, ERβ as an inhibitory cue, than was previously established, supports our rationale for this study, that there are more ways that the migration of these muscle precursor cells is controlled than are

currently known. Our findings regarding retinoic acid receptor signaling show a different way that cues are still undiscovered; though there are known roles for retinoic acid in limb development, these findings go beyond limb field induction and limb patterning and suggest that while the presence of RA in the region isn't new, there is another role for this RA signaling involved outside of the established ones(Cunningham and Duester, 2015; Grandel and Brand, 2011). Other results, such as cholesterol synthesis and preliminary pathways in bromodomains and Rab27a mediated exocytosis, demonstrate the strength of unbiased screening in finding pathways that would not have been early in a hypothesis-driven set of tests. Overall, the small molecule screen has not only resulted in new hypotheses to test and further elucidate pathways involved in muscle precursor migration but has also shown itself to be an efficient tool for finding these hypotheses and fleshing out the unknown ways that muscle precursors delaminate, migrate, and become this full set of hypaxial muscles.

Figure 7.1 Final Model. Combining these hypotheses, we come to a model wherein the *Six1/Eya2* complex activates EMT via *met*, while also inhibiting *esr2b*, which when activated by estrogen, inhibits EMT. Additionally, the cell shape changes necessary for migration require cholesterol, and *rarab* when activated by retinoic acid may play a role in MMP directionality.



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BIOGRAPHY OF THE AUTHOR

Teresa Easterbrooks was born November 18, 1996. She was raised in Winthrop, Maine and graduated from Winthrop High School in 2015. She attended Simmons University and graduated in 2019 with a Bachelor's degree in Biology with minors in Chemistry and Biostatistics. It was here that she first gained her love of working with zebrafish, having first been a lab assistant and then completing an undergraduate honor's thesis in the Engle Laboratory out of Boston Children's Hospital. In the program, she developed a technique of high throughput CRISPR screening of candidate genes for Duane Retraction Syndrome in zebrafish embryos. She returned to Maine and entered the GSBSE graduate program at the University of Maine in the fall of 2019, where she completed rotations in Ron Korstanje's lab, Jared Talbot's lab, and Melissa Maginnis's lab, before spending a year in the Korstanje lab as a PhD student researching kidney disease in a mouse model. In the fall of 2021, discerning a different path and returning to her first love in zebrafish research, she joined the Talbot lab as a Master's student, taking on the small molecule screen project. While doing research in the Talbot lab, she also had the opportunity to grow her teaching experience through teaching assistantships for Intro Biology (BIO 100), Histology (BIO 450), and Developmental Biology (BIO 336). She grew in her love and aptitude for mentoring while guiding eight undergraduate students in Talbot lab research experiences. Teresa considers this Master s program to be a tremendous period of growth and self-discovery. Teresa Easterbrooks is a candidate for the Master of Science degree in Biochemistry from the University of Maine in August 2023.