Investigation of the Role of Hyaluronan in Neural Crest Cell Migration, Adhesions, and Morphology

Kathryn Mykyten

Georgia Institute of Technology

First Reader; Jennifer Curtis, PhD

Second Reader; Shuyi Nie, PhD

Special acknowledgements to Shlomi Cohen, PhD candidate, for assistance and guidance in this project.

Table of Contents

1. Abstract

Xenopus laevis neural crest cells are a common and useful model for studying cell migration in many contexts, including developmental biology, cancer, and other basic science. This study examines the role hyaluronan (also known as hyaluronic acid or hyaluronate), an extracellular glycosaminoglycan, plays in regulating neural crest cell migration-a critically understudied molecule in this context. Neural crest cell explants were taken from fertilized *Xenopus* embryos injected with HAS1 and/or HAS2 mRNAs or HAS1 and/or HAS2 translation-blocking morpholinos. The effect of these gain-of-function and loss-of-function injections on hyaluronan secretion was verified by the introduction of a fluorescent reporter, ssGFPG1. The effects of the subsequent gain or loss of function on neural crest migration was investigated via an explant spreading assay. It is expected that HAS upregulation speeds neural crest migration by reducing cell-substrate focal adhesions, though it is also possible that an increase in these adhesions could increase neural crest cell migration speeds. Regardless, any results would further elucidate the role the hyaluronan glycocalyx plays in mediating or disrupting cell adhesions and its impact on cell migration on the whole.

2. Introduction

Neural crest cells (NCCs) are a particular population of multipotent cells found in the developing embryos of most vertebrates. They are fated to become any of several mature structures, including certain cranial nerves, facial cartilage, melanocytes, and more.[1,2,3,6] In order for adequate development, these cells must collectively migrate to the correct locations on the developing embryo before differentiating into other stem cell types [1, 2, 3]. Failure to migrate properly can result in deformation and other birth defects.[2, 3] Furthermore, research surrounding NCC's has been of some interest to oncological fields, due to the wide array of fates the cells may achieve-some of which may become rather aggressive types of cancers.[2]

Surrounding certain NCCs is a structure known as the extracellular matrix (ECM), a network of various molecules composed largely of hyaluronan (HA), as well as fibronectin, laminin, and collagen I & IV, and others.[6, 7] The ECM provides support and structure to the cells it surrounds; it has also been implicated in microenvironment regulation and cell signaling.[6, 7] Cells are tethered to the ECM via integrin-mediated focal adhesions, which connect the cell cytoskeleton

with molecules in the external environment.[4, 6] The ECM notably impacts long term migration, not simply providing support to cell shape but creating permissive, non-permissive, or fully impermeable environments for cells to navigate and a substrate upon which to do so.[6]

Hyaluronan is a glycosaminoglycan made of repeating disaccharide units, which makes up a considerable portion of the ECM.[(6,7)] HA is synthesized at the cell surface by transmembrane hyaluronan synthases, so that it projects outwards, away from the cell surface.[8] The overexpression of hyaluronan has been associated with several biological events, including infection, inflammation, and cancer.[8] In fact, some studies have shown that HA-rich pericellular microenvironments assist in tumor metastasis, a migration-related process.[8] However, whether or not hyaluronan contributes to a more permissive, nonpermissive, or impermeable ECM environment is far from well-studied in NCCs, despite its role as a prominent constituent of the ECM.[6,7] Furthermore, while minimal study has been done on the impacts of HA on migration, these studies are outdated and were performed upon substrates that were not necessarily conducive to migration.[9, 10] Though one of these studies had determined that HA does not impact migration speeds, we believe these results could be contested by studies informed by new information on the ECM and updated methods that better imitate the NCC microenvironment.[9, 10]

The factors involved in this directed migration are well studied from a signaling standpoint, but investigation into biophysical factors is still lacking. A better understanding of the basic science behind NCC migration may guide future research not only in embryology, but potentially oncology and other disease-related fields. We intend to investigate the role of the hyaluronan glycocalyx in preventing or facilitating NCC migration by characterizing NCC explants overexpressing HA, and then performing object-removal assays to measure several migration-related parameters during HA-overexpressing CNCC collective monolayer migration. We expect that HA-overexpressing NCC monolayers will have larger gaps between cells and migrate more slowly than NCC monolayers expressing HA at typical levels.

3. Literature Review; Overview of Neural Crest Cell Function and Migration

2.1 Neural Crest Cell Overview

Neural crest cells (NCCs) are a particular population of transient, multipotent cells found in the developing embryos of most amniote vertebrates.[1,3,4,8] They are fated to become any of various differentiated cell types, including melanocytes (pigment cells in the skin), the majority of the peripheral nervous system, cranial nerve I (the olfactory nerve), endocrinal cells, and some mesenchymes (which then beget the cranial skeleton and other connective tissue), among others.[1,8] However, NCCs themselves may be divided into multiple subpopulations dependent on their location in the developing neural tube.[1,3] For example, cranial neural crest cells emerge from the hindbrain (rhombencephalon) and possess particular characteristics, especially certain migration behaviors.[3] One should note that this does not mean that cranial neural crest cells are necessarily destined to become part of the cranium.

Of particular interest to the researchers is the biophysical picture of NCC migration. NCCs tend to migrate along discrete pathways, migrating as multiple streams, and failure to migrate properly can result in significant facial deformation and other birth defects.[3,8] Extant questions about the NCC microenvironment, single cell versus collective dynamics, and the lack of consensus on the underlying causes of NCC migratory patterns reflect the continued need for more research into NCC migration.[3,4,6,8,10]

2.2 Neural Crest Cell Migration

We may separate the initiation of NCC migration into three phases: changes in the molecular environment that induce the epithelial-to-mesenchymal transition (EMT), the EMT itself resulting in delamination from the basal epithelium, and departure from the neural tube.[3,8] Cranial NCCs will generally migrate as a series of streams during embryonic development.[3,8] These cranial NCC streams may refer to wide streams of cells, or thinner, more chain-like streams; there is conflicting information available.[3,8]

On a more detailed level, NCC migration is largely accomplished by filopodial and lamellipodial projections.[3,8,10] What causes the formation of these projections is yet unclear. A possible explanation is that contact inhibition is responsible; when one cell contacts another's membrane, it may begin migrating away from it, be unaffected, or migrate towards it-there are contradictory conclusions between studies [See source 3 for more detail]. It is possible that there is a missing chemical or mechanical link determining how NCCs react to contact with other cells; it may also be possible that these are differences between animal species or subpopulations of the neural

crest.[3] Some research has shown that disruption of cell-cell contact leads to alteration of migration streams and the development of "bridges" of cells between them.[3] Variance between species should be noted, however, as it has been recently established that migrating chick NCCs do not rely on contact inhibition during migration.[4]

The means in which contact inhibition itself is accomplished is unclear; multiple possibilities exist. For example, the idea of cell "nudging" is a phenomenon in which NCCs exert some mechanical force on other NCCs, which causes cell blebbing on the opposite side of the impacted cell, ultimately leading to filopodia formation and thus migration.[3] Another idea is that cell-cell contact could cause an internal signaling pathway in the cells, which would then affect their behavior, or that cells may be highly dependent on their microenvironment for chemotaxis and ligand-receptor guidance cues.[3] If these ligand-receptor pairs are on cell membranes, there may be a way to integrate cell-cell contact inhibition with the latter guidance cue theory, as well as include the role of signaling in the cell microenvironment.

2.3 The Influence of the ECM in Collective Cell Migration

While some review papers have framed NCC migration as "loosely connected, [and] individually migrating"[3], other, more recent reviews consider NCCs as a prime example of collective cell migration, despite their loose connections.[7, 8] It is important to understand that NCCs as a group having undergone an epithelial-to-mesenchymal transition and are indeed qualified as mesenchymal cells; such loose connections are expected.[7] There is a form of collective cell migration that is particular to mesenchymal cells, where there is collective directional migration, but leader cells often detach from the tissue and migrate forward seemingly independently, leaving a chemical trail for other cells to follow.[7] Despite the name, it is apparent that in at least some species, NCCs favor the more wholly collective migration stratagem, though this is not the case in chick embryos.[7]

Of particular interest to us is the role of the extracellular matrix in collective cell migration. Neural crest cells interact with the constituents of the ECM in multiple facets of migration, including the epithelial-to-mesenchymal transition, route establishment, and responsiveness to the environment.[4] The ECM notably impacts long term migration, not simply providing support to cell shape but creating permissive, non-permissive, or fully inhibitory environments for cells to navigate and a substrate upon which to do so.[4] Permissive environments tend to be delineated

as supporting strong cell adhesions and migration, and non-permissive environments result in weak cell adhesions and but still enable migration.[4] Inhibitory environments may more directly or fully prevent migration.[4] The permissiveness of an environment is determined by its composition: fibronectin, laminin, and some types of collagen make for a more permissive environment; integrin-mediated adhesions to these molecules are a mode of cell interaction with the ECM.[4, 7] Fully inhibitory molecules include aggrecan and versican, though the overall permissiveness associate with any molecule may be context-dependent.[4]

Of interest to us is hyaluronan (HA, hyaluronic acid), a polysaccharide glycosaminoglycan made of repeating D-glucuronic acid and N-acetyl-D-glucosamine units, is a prominent constituent of the extracellular matrix (ECM) and pericellular matrix.[2, 6] Hyaluronan is synthesized by transmembrane proteins at the cell surface called hyaluronan synthases; the HA synthesized varies in size, ranging anywhere from a few repeating units to over 25,000 repeats, and projects out of the cell into the ECM.[2, 6] The ECM is also comprised of other proteins, including but not limited to fibronectin, laminin, and collagen I & IV.[4] Cells are tethered to the ECM via focal adhesions, which connect the cell cytoskeleton with molecules in the external environment.[4] These adhesions to the ECM are generally integrin-mediated, whereas the cell-cell adhesions found in contact inhibition are cadherin-mediated.[4, 6, 7, 8] Whether hyaluronan is a generally permissive, nonpermissive, or inhibitory molecule remains to be seen, despite its presence as the "backbone" of the ECM. The interaction of HA with cell-cell adhesions has yet to be discussed; given the bulk of HA molecules in the ECM, it is possible that a dense ECM could prevent contact inhibition by creating a physical barrier to adhesion formation.

Migrating NCCs may deploy their own substrates and several proteases to break down the external ECM, in order to alter these environments before and during migration, known as ECM remodeling.[4] This remodeling is necessary before the EMT stage of migration initiation, though not solely responsible for it-cadherins and other molecular signals play a role as well.[4, 7, 8] The ECM also traps molecules in the cells' microenvironments, thus exerting some influence on the chemical gradients surrounding migrating cells.[4] Much of this further implicates the role of the ECM and its constituents into migration, warranting further study into these phenomena.

2.4 Further Research and Gaps in the Literature

While research has supported the roles of various transmembrane proteins, such as integrins and cadherins, in cell migration (including NCC migration), there is a marked lack of research into the role HA may play in migration. Extensive research is available on how HA acts as a signaling molecule overall, but not nearly as much has been elucidated about its role in permissive or nonpermissive environments during NCC migration.[2, 4] While some recent research into hyaluronan signaling exists, the biophysical research that implicates ECM hyaluronan concentration in migration is uncommon, and it appears that the subject has largely been left behind.[5, 9] This has given researchers with relatively few sources of information about HA and the developing nervous system, despite the ubiquity of the molecule. The resulting study will focus on the possibility that HA promotes a nonpermissive environment by generating a physical barrier between cells; in other words, a thick glycocalyx would prevent cell-cell and cell-substrate contact, reducing the efficiency of cell migration.

3. Methods

3.1 Embryo Fertilization and Injection/Explant Preparation

Xenopus laevis oocytes were retrieved and fertilized with the testes taken from male *Xenopus laevis*. The fertilized and de-jellied embryos were kept at 19^{o} C, in .01x MMR (Marc's Modified Ringer's Solution) until dissection. During the two-cell stage of *Xenopus laevis* development, the embryos were injected with various combinations of HAS1 and HAS2 encoding mRNA sequences, as well as with a secretion sequence-tagged GFP-fused versican G1 domain (ssGFPG1) mRNA (*See Table 1*). This designer molecule labels hyaluronan in the glycocalyx indirectly, after secretion outside of the cell enables the fluorescent G1 domain to bind extracellular HA-a similar sequence lacking a G1 domain coding region was developed as a negative control (ssGFP). Other cohorts were injected with ssGFPG1 and designer morpholinos; oligonucleotides complementary to HAS1 or HAS2 mRNA sequences, downregulating their translation into proteins.

Between stages 15-18, prepared embryos were dissected and NCC explants were plated on a fibronectin coated glass bottomed dish containing DFA+ (Danilchick's for Amy Solution). Explants were considered adhered 2 hours after dissection. This protocol was followed for all

explants used in experiments. Each experiment was performed on a given explant, and triplicate datasets for each condition were gathered.

	HAS1	**HAS2**	**ssGFPG1**
A	+	+	+
B		+	+
C	+		+
D	-	-	+
E	-		+
F		-	+
Positive Control			+
Negative control			ssGFP only

Table 1. Experimental Conditions. The experimental conditions all following tests will be run on is displayed above. "+" indicates upregulation by mRNA injection; "-" indicates downregulation by morpholino injection. Spaces left blank indicate that the given protein was left at endogenous levels. ssGFP refers to a signaling sequence tagged GFP molecule that lacks the G1 domain that binds hyaluronan.

3.2 Migration Assay

In order to quantify NC explant migration, a brightfield microscopy-based migration assay was developed. This assay measures the changes in the area of the explant over time, in order to better understand how neural crest tissues migrate in high-hyaluronan conditions, rather than focusing on single cell behavior. A brightfield image of each explant was taken twice per day for two days (specific timepoints; 3 PM, 6 PM, 9 AM, 5 PM) for a total of four images of each explant. These images were then analyzed using custom MATLAB code that automatically

selects the borders of the spreading explant and quantifies the explant area for each image. The changes in the explant area will be used to calculate the speed of migration, and some macroscopic phenomena (such as the emergence of polarization or changes in migration direction) should be measurable.

3.3 Live Glycocalyx Labeling

We characterized single cell glycocalyces via the injection of ssGFPG1, a custom secretion sequence green fluorescent protein fused versican G1 domain. Once again, this labeled the hyaluronan glycocalyx by signaling the cell to secrete a construct of a HA-binding domain fused to a fluorescent protein, allowing for visualization as it binds to the glycocalyx. Single, isolated cells from experimental condition and control explants with labeled HA were then imaged via live confocal microscopy. Control injections were done, including an ssGFPG1-only injection and a negative control injection of an ssGFP protein lacking the G1 domain fusion.

3.4 Adhesion and Morphology Labeling

After live confocal imaging, explants were fixed with 4% formaldehyde, washed three times in PBS, permeabilized with .1% Triton-X, washed thrice in PBS again, and blocked using 10% goat serum. Fixation ensures the stability of samples while preventing decay or alteration over time, and a blocking step prevents nonspecific binding of secondary antibodies. These explants were then incubated with anti-vinculin/metavinculin primary antibodies (1:50, Developmental Studies Hybridoma Bank VN 3-14) overnight. The following morning, non-bound primary antibodies were washed off, and the explants were incubated with a goat anti-mouse secondary antibody (1:500, ThermoFisher A-21422) for an hour, then washed three times in PBS. Each labeled sample was imaged via confocal microscopy to examine the distribution of cell-substrate adhesions throughout the explant, as well as migrating cell morphology. After the adhesion imaging was completed, explants were incubated with CellTracker™ Red CMTPX Dye (ThermoFisher C34552) for half an hour, then imaged via confocal microscopy again to visualize both single cell and monolayer morphology.

3.5 Analysis and Statistical Methods

During confocal imaging, the quantified fluorescence of the GFP (green), the cytosol dye (red), the secondary antibodies (red) were defined as the number of fluorescent pixels per field-of-view

image in their respective color channels. For morphological quantification, we located the center of the nucleus in each cell and determined the average area of a given cell, as well its number of filipodia/lamellipodia and the average reach of each from the nucleus to its longest point in each image. Furthermore, the number of cells per field of view were counted and cell density calculated for migrating explants. Pre-analysis image processing was done in Fiji-ImageJ and other analysis done by custom MATLAB code.[11, 12]

For each experiment (Adhesion Quantification, Glycocalyx Quantification, etc.), a one-way ANOVA was run between quantifications of each condition, with Tukey post-hoc tests to distinguish any significant groups. This was done using JMP-15.[13]

4. Preliminary Results

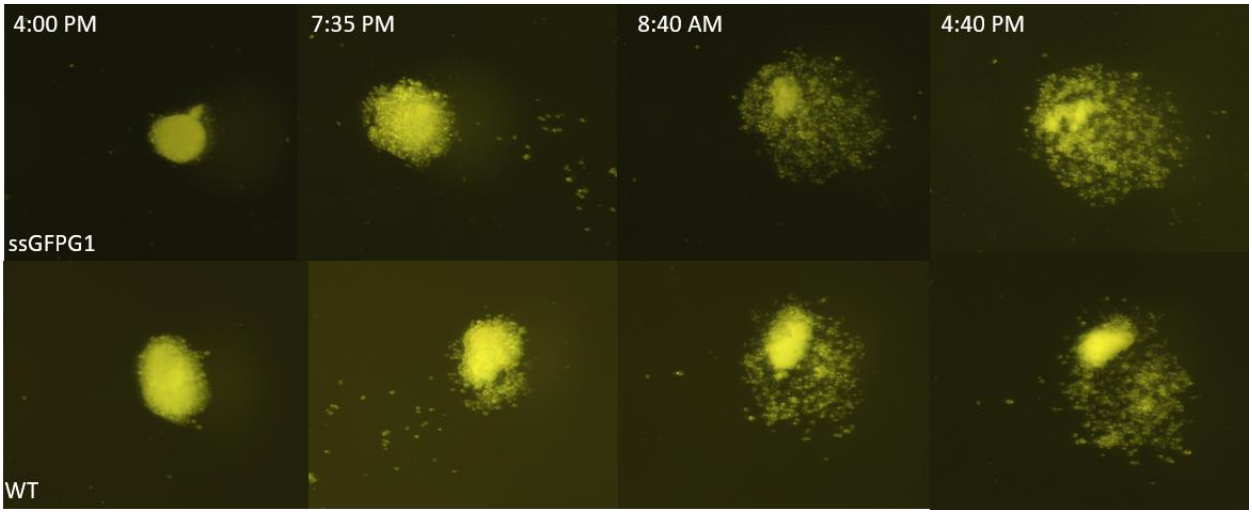

Figure 1. Neural crest cell migration in hyaluronan synthase overexpressing and downregulating explants. Embryos pictured above were either injected with ssGFPG1 alone (Row 1) or noninjected (Row 2) and were dissected between stage 15-16. Images were taken approximately 2 (Column 1), 4.5 (column 2), 19 (Column 3) and 27 hours post dissection (Column 4). It appears that the NCC explants display directional migration, with the establishment of this direction appearing between 4.5-19 hours after plating. No statistical analyses have been run as of yet.

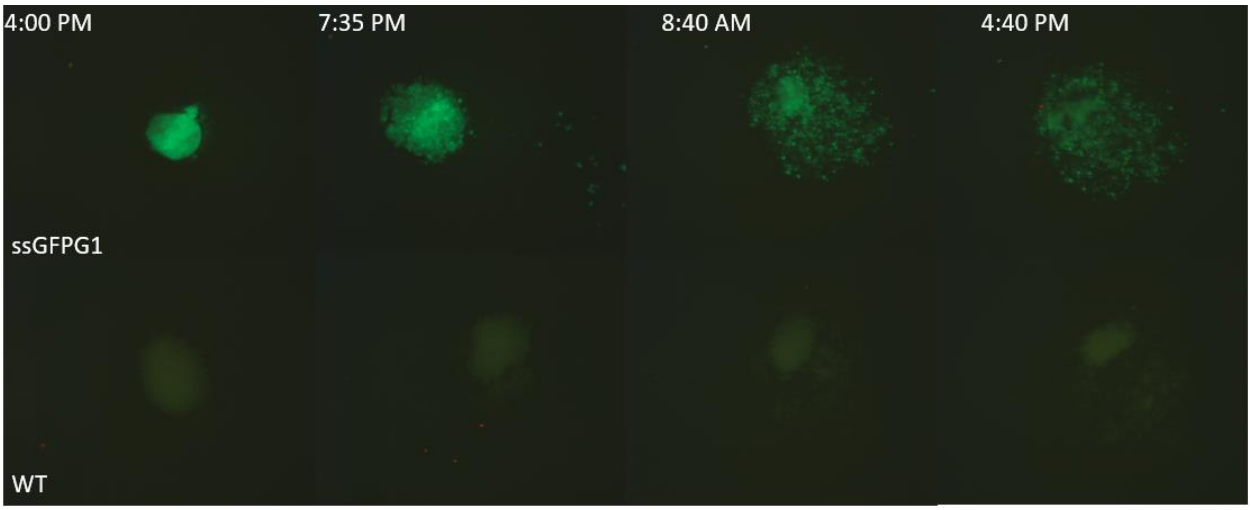

Figure 2. Rudimentary evidence of ssGFPG1 labeling of the hyaluronan glycocalyx. Embryos pictured above were either injected with ssGFPG1 alone (Row 1) or noninjected (Row 2) and were dissected between stage 15-16. Images were taken approximately 2 (Column 1), 4.5 (column 2), 19 (Column 3) and 27 hours post dissection (Column 4). The ssGFPG1 injected embryos are displaying GFP fluorescence, as compared to autofluorescence in the wild-type explants, demonstrating that the reporter molecule is effective to some extent.

4. Discussion

Currently, the lack of experimental data means that there are no key results to report. As such, we will summarize what we expect to find in support of our hypothesis.

Glycocalyx Quantification

Ideally, we would like to see evidence that HAS1 and HAS2 mRNA injections both increase the thickness of the hyaluronan (HA) glycocalyx, and that morpholino injection reduces HA glycocalyx thickness (in terms of both length and density), especially below and between cells. Our assay may result in a more specific understanding of where these higher levels can be seen-it is not guaranteed that HAS will be distributed evenly around the cell surface and may be impacted by the presence of other cells or tissues. Furthermore, it is possible that upregulation of either HAS1 or HAS2 will result in different levels of HA production or different types of HA distribution between each

condition, further elucidating the differences in expression and function of each type. Any alterations in distribution are likely to be related to cells' utilization of HA to control migration by forming or relieving adhesions to other cells.

Migration, Adhesions and Morphology

Conclusions about HA up/downregulation on NCC migration cannot be drawn in a vacuum-each part of this study relies on the others for general conclusions. After demonstrating the HA is indeed upregulated, then we can see its' effects on migration speeds and trajectories, and then look for underlying causes in adhesions. While there is a clear picture of what we expect to see (HAS upregulation leading to thick glycocalyces, slower migration speeds and fewer adhesions, and thus concluding that HA is nonpermissive), these are naturally not guaranteed. Given the size of this experiment, we have chosen not to speculate on alternative results due to the sheer number that may present.

Our expected results would be slow explant expansion/migration speeds concurrent with thick glycocalyces, a low number of focal adhesions (FA's), and little podia projection in experimental conditions with HAS upregulation. The opposite is expected in HAS downregulating conditions; fast migration, lots of FA's, and increased podia projection compared to controls. Observing these sets of results would be consistent with our prediction that HA is a nonpermissive ECM molecule, on the theory that focal adhesions necessary for migration are interrupted by HA-mediated tension between the cell and the substrate. While a demonstration of this theory is not present in this study, this would present enough basic science to warrant further investigation into this phenomenon.

Limitations and Future Work

Our studies on this topic will continue once laboratory *Xenopus laevis* are capable of consistently producing healthy eggs and sperm for embryo generation. Up to this point, the viability of the embryos has been inconsistent, often resulting in death or mutated development, which may drastically affect our results. As such, these results would not necessarily reflect the typical neural crest migration observed in healthy cells, limiting

our ability to fully understand the role of HA. Furthermore, the COVID-19 pandemic has severely limited productivity in the lab and has lead to a variety of scheduling restraints. As such, our protocols are still being optimized to ensure our results are consistent and reflect the true underpinnings of neural crest migration. At the time of submission, experiments to test the immunofluorescence protocol for labeling adhesions is underway, involving the use of fixed wild-type explants and controls labeled with e-cadherin antibodies to test the efficacy of a protocol with vinculin primary antibodies.

The investigation of HA as a “permissive”, “nonpermissive”, or “inhibitory” molecule in the ECM is the primary goal of this study. However, due to practical, methodological, and other types of limitations, this question cannot be completely answered by this study. In the future, there should be replications of this study using various migration substrates, such as collagen or HA itself, to see if the general conclusions of our study can be replicated. There are areas of our methods that are promising, such as the use of secreted probes to label the HA glycocalyx, but this does not guarantee complete labeling of the structure. Despite this, we do expect the number of labeled molecules to scale with the amount of HA produced by the cells themselves. With hyaluronan synthase being a transmembrane protein and successful labeling to some degree, a proof-of-concept is established regardless. While spreading assays are reasonably common, future work should also track the migration trajectories of single cells in order to fully determine the effects on single cell as well as coordinated, macroscopic neural crest cell migration.

mRNA injections are a method in the public eye recently, and thus this study may be useful to those interested in the efficacy of these injections from a more genetics-focused perspective. In the future, researchers may wish to replicate this study using CRISPR-Cas9 knockouts as well, rather than morpholinos. Furthermore, the influence of HA upregulation of NCC cell differentiation may be an interesting direction to take the topic.

Conclusions

Should we find our expected results and our hypothesis is supported, there may be greater implications for neural crest migration on the whole. The establishment of hyaluronan as

a permissive, nonpermissive, or inhibitory molecule would not only contribute to the bigger picture of NCC migration, but potentially give insight into birth defects or cancer behaviors. Furthermore, such findings may have implications for the lab environment; perhaps the introduction of HA matrices as substrates for further migration studies. Regardless, even should the findings not support our hypothesis, they will have elucidated further the role of HA in neural crest migration behavior, as well as its impacts on cell morphology and adhesions.

Works Cited;

1. Dupin, E., & Sommer, L. (2012). Neural crest progenitors and stem cells: from early development to adulthood. Developmental biology, 366(1), 83-95. doi.org/10.1016/j.ydbio.2012.02.0357

2. Heldin, P., Lin, C. Y., Kolliopoulos, C., Chen, Y. H., & Skandalis, S. S. (2019). Regulation of hyaluronan biosynthesis and clinical impact of excessive hyaluronan production. *Matrix Biology*, *78*, 100-117. doi.org/10.1016/j.matbio.2018.01.017

3. Kulesa, P. M., Bailey, C. M., Kasemeier-Kulesa, J. C., & McLennan, R. (2010). Cranial neural crest migration: new rules for an old road. *Developmental biology*, *344*(2), 543-554. doi.org/10.1016/j.ydbio.2010.04.010
4. Leonard, C. E., & Taneyhill, L. A. (2020, April). The road best traveled: neural crest migration upon the extracellular matrix. In *Seminars in cell & developmental biology* (Vol. 100, pp. 177-185). Academic Press. doi.org/10.1016/j.semcdb.2019.10.013

5. Luckenbill-Edds, L., & Carrington, J. L. (1988). Effect of hyaluronic acid on the emergence of neural crest cells from the neural tube of the quail, Coturnix coturnix japonica. *Cell and tissue research*, *252*(3), 573-579. doi.org/10.1007/BF00216644

6. Preston, M., & Sherman, L. S. (2012). Neural stem cell niches: critical roles for the hyaluronan-based extracellular matrix in neural stem cell proliferation and differentiation. *Frontiers in bioscience (Scholar edition)*, *3*, 1165.

7. Scarpa, E., & Mayor, R. (2016). Collective cell migration in development. *Journal of Cell Biology*, *212*(2), 143-155. doi.org/10.1083/jcb.201508047

8. Szabó, A., & Mayor, R. (2018). Mechanisms of neural crest migration. *Annual review of genetics*, *52*, 43-63. doi.org/10.1146/annurev-genet-120417-031559

9. Tucker, R. P., & Erickson, C. A. (1984). Morphology and behavior of quail neural crest cells in artificial three-dimensional extracellular matrices. *Developmental biology*, *104*(2), 390-405. doi.org/10.1016/0012-1606(84)90094-0

10. Yamada, K. M., Collins, J. W., Cruz Walma, D. A., Doyle, A. D., Morales, S. G., Lu, J., ... & Wang, S. (2019). Extracellular matrix dynamics in cell migration, invasion and tissue morphogenesis. *International Journal of Experimental Pathology*, *100*(3), 144-152. doi.org/10.1111/iep.12329

11. Schindelin, J.; Arganda-Carreras, I. & Frise, E. et al. (2012), "Fiji: an open-source platform for biological-image analysis", Nature methods 9(7): 676-682, PMID 22743772, doi.org/10.1038/nmeth.2019

12. MATLAB. (2010). *version 9.9 (R2020b)*. Natick, Massachusetts: The MathWorks Inc.

13. JMP®, Version 15. SAS Institute Inc., Cary, NC, 1989-2019.