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Investigating the Mechanisms of Neural Stem Cell Mechanotransduction: The Roles of Angiomotin and Hyperglycemia

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Publication Date
2019

Peer reviewed|Thesis/dissertation

Investigating the Mechanisms of Neural Stem Cell Mechanotransduction: The Roles of Angiomotin and Hyperglycemia

by

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A dissertation submitted in partial satisfaction of the

requirements for the degree of

Joint Doctor of Philosophy
with the University of California, San Francisco

in

Bioengineering

in the

Graduate Division

of the

University of California, Berkeley

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Professor Sanjay Kumar, Co-chair
Professor David V. Schaffer, Co-chair
Professor Todd McDevitt
Professor Andrew Dillin

Spring 2019

Abstract

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Neural stem cells (NSCs) are remarkable mediators of structural plasticity in the adult mammalian brain and play important roles in physiological and pathophysiological neurobiology. Ongoing research has uncovered that mechanical cues can potently regulate NSC behavior. For example, soft substrates promote the neuronal differentiation of NSCs by suppressing cytoskeletal contractility and perturbation of the same pathway influences neurogenesis *in vivo*. However, the mechanisms that link cytoskeletal signaling to transcriptional regulation and govern stiffness-dependent NSC fate commitment are not fully understood. Additionally, studies have shown that dysfunction of hippocampal NSCs may underlie the cognitive deficits linked to diabetes. This may be due to the dysregulation of cellular energetics, which is tightly regulated in stem cells and influences cytoskeletal dynamics. Therefore, the effect of hyperglycemia on NSC mechanotransductive signaling warrants further study.

In this dissertation, we show that angiomotin (AMOT) is critical for mechanotransduction in NSCs. Loss of AMOT inhibited neurogenesis on soft substrates while also severing the functional tie between reduced myosin contractility and increased neurogenesis. We found that AMOT is critically regulated by its phosphorylation and localization and promotes neurogenesis by inhibiting yes-associated protein (YAP) and upregulating β-catenin activity. Additionally, we found that hyperglycemia suppresses NSC growth in culture and that NSC mechanosensitive differentiation can be regulated by glucose concentration. Specifically, activation of 5'adenosine monophosphate-activated protein kinase (AMPK) either by lowering the glucose concentration or metformin treatment rescued neurogenesis on stiff substrates while high glucose or compound C (AMPK inhibitor) treatment suppressed neurogenesis. Therefore, hyperglycemia may have a direct

influence on how NSCs sense and respond to their physical microenvironment through an AMPK-dependent mechanism.

These findings significantly add to our understanding of how NSCs sense, integrate, and respond to biophysical inputs and how those pathways are influenced by glucose dysregulation. Further elucidation of these mechanisms has the power to inform how NSCs are regulated in health and disease while also advancing how these cells are utilized in regenerative medicine approaches in the nervous system.

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Acknowledgements

I'd like to first thank my thesis committee co-chairs and scientific advisors, Sanjay Kumar and David Schaffer, for their never-ending support since my first day in lab. I will never take for granted the amazing opportunity to develop professional and personal relationships with world-class research minds like yours. I am constantly floored by your abilities to absorb, condense, combine, re-imagine, and communicate complex ideas and hope to emulate those skills in my own work. The opportunities that I've gained in both of your labs through the years have been formative in my development as a researcher and independent thinker.

I also would like to thank the remaining members of my qualifying exam and dissertation committees for offering their time and vast expertise – Professor Mohammad Mofrad, Professor Daniel Fletcher, Professor Todd McDevitt, and Professor Andrew Dillin. Your guidance and insights were instrumental in directing (and re-directing) my thesis work.

Like any long-term position, it's often those that you work around that can make or break your experience. In my case, I was lucky enough to have entered graduate school with The Greatest Cohort of students with whom I have experienced young, blind enthusiasm (we knew nothing in 2013), struggles, triumphs, the realities of physical aging, and everything in between. I am also supremely grateful to have shared time with the dozens of past and present members of the Kumar and Schaffer labs over the past 5+ years. Michael Kang, Jasmine Hughes, George Lin, Elena Kassianidou, Jessica Lee, Badri Ananthanarayanan, Andrew Rape, Sophie Wong, Yushan Kim, Joseph Chen, Jieung Baek, Jason Coombs, Carmela Rianna, Emily Carvalho, Stacey Lee, Ruoxing Lei, Andy Lopez, Eric Qiao, Kayla Wolf, Benjamin Epstein, Thomas Gaj, John Chen, Jorge Santiago-Ortiz, Gonçalo Rodrigues, Prajit Limsirichai, Alyssa Rosenbloom, Dawn Spelke, David Ojala, Anasuya Ramasubramanian, Sebastian Rammensee, Andrew Bremer, Riya Muckom, Sabrina Sun, Chris Barnes, Sarah Yang, Nicole Repina, Kaz Lewis, Christina Fuentes, Olivia Scheideler, Maroof Adil, Rocio Sampayo, Josh Zimmermann, Kira Mosher, Chun Yang – thank you for your support, being amazing scientists and people, and endless baked goods/Hot Cheetos. It has been a joy working with all of you. I'd also like to particularly thank Noem Ramey and Kelsey Springer for the infinite things you do to keep the lab not only a functional and safe but fun place to work.

I also owe my literal life and this amazing one that I can even dare to pursue to my parents, Sang and Kay Kang. Thank you a thousand times over for your endless love, support, understanding, and prayer – I could not be here without you. Thank you also to my younger brother, Peter Kang, who is infinitely cooler, more creative, thoughtful, and skilled than me. I can't wait to see what is in store for you.

Finally, I'd like to thank my amazing wife Lillian, who unexpectedly became the greatest part of my PhD experience by a significant margin. I know for a fact that I could not have completed this without you walking with me through the lows and highs. Thank you for constantly supporting me with unending patience and love like only you can.

Chapter 1 : The influence of mechanical cues and mechanotransductive signaling on neural stem cell behavior

Parts of this chapter are excerpted with permission from Elsevier, from the article "Novel biomaterials to study neural stem cell mechanobiology and improve cell-replacement therapies", by Phillip Kang, Sanjay Kumar, and David Schaffer in *Current Opinion in Biomedical Engineering*, 4: 13-20, 2017.

1.1 Introduction

Neural stem cells (NSCs) have been implicated as key cellular effectors of adult neuroplasticity and have been explored as potential cell-replacement sources in therapies for neurodegenerative disease[1,2,3]. In postnatal mammals, NSCs are confined to two specific regions, the subventricular zone (SVZ) of the lateral ventricles and subgranular zone (SGZ) of the hippocampal dentate gyrus[4]. NSCs within the SGZ are of particular interest as they play critical roles in learning, memory formation, behavioral and mood regulation, and disease pathology[5,6,7,8]. Accordingly, there has been an interest in precisely elucidating the extracellular signaling inputs and intracellular molecular pathways that regulate NSC behavior, with the goal of enhancing our basic understanding of NSC physiology and uncovering novel means to reliably control NSC function in regenerative medicine applications.

Within their endogenous neurogenic niches, NSCs receive an array of microenvironmental biochemical and biophysical cues that tightly control their behavior (Fig. 1.1). A number of potent soluble and cell-cell biochemical signals that regulate NSCs have been identified, including: Wnt3, which promotes hippocampal neurogenesis; Notch, which helps maintain NSCs in their stem-like state; and astrocyte-presented ephrins, which inhibit NSC growth[9,10,11,12,13]. However, mechanical inputs such as substrate stiffness are being increasingly recognized as important regulators of NSC self-renewal and fate commitment as well. For example, we have previously reported that substrate stiffness strongly biases neuronal vs. astrocytic differentiation[14]. Interestingly, investigations by our group and others have revealed that there are stiffness gradients between the various regions of the hippocampal neurogenic niche, highlighting the potential importance of mechanotransduction *in vivo.* However, while it is clear that these mechanical cues impact NSCs, the intracellular mechanisms that link extracellular stiffness to gene regulation remain incompletely understood.

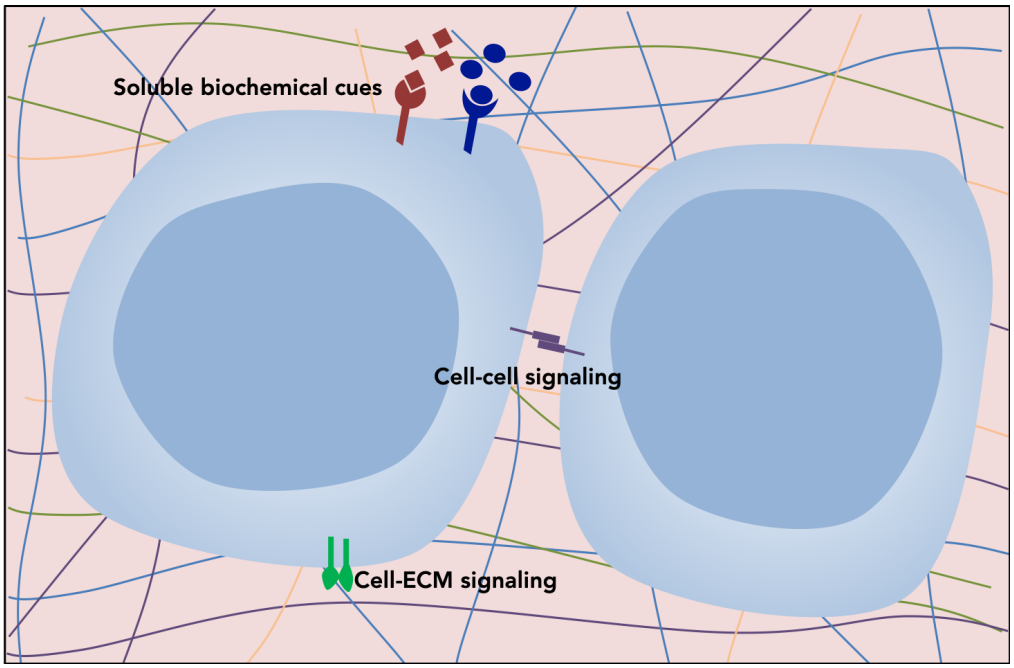

Figure 1.1 Cells are regulated by a variety of microenvironmental cues.
Stem cells, like other cell types, receive a myriad of inputs that control their behavior. These signals include soluble factors that may be transported into the cell or interact with specific receptors, signals via interactions with neighboring cells, and biophysical signals via integrin-ECM interactions, amongst others.

The motivation to more precisely understand these pathways is more than academic; mechanistic insight can significantly improve our understanding of NSC neurobiology and inform the successful use of NSCs in tissue engineering applications[15]. Luckily, numerous studies have highlighted some crucial signaling events that govern mechanosensitive NSC behavior. For example, our group has previously reported that cellular contractile forces mediated by the Rho family GTPases RhoA and Cdc42 strongly modulate mechanosensitive fate commitment in cultured hippocampal NSCs[16]. Importantly, we demonstrated that modulating RhoA activity can influence hippocampal neurogenesis *in vivo* in the same manner. We later showed that stiff matrices increase levels of Yes-Associated Protein (YAP), which inhibits the pro-neurogenic transcription factor and Wnt pathway effector β-catenin to suppress neurogenesis[17]. However, the mechanisms that link cytoskeletal mechanics to changes in the activity of transcriptional regulators such as YAP remains a major gap in our understanding of stem cell mechanotransduction.

During differentiation, stem cells undergo a series of biochemical and functional transformations from unspecialized, self-renewing cells into specialized, terminally differentiated cells. Early in this process, transcriptional and proteomic changes cause the differentiating stem cells to become "fate committed" after which they are developmentally restricted to a particular cellular fate[18]. Therefore, fate commitment represents the first deterministic fork in multipotent

or pluripotent stem cell differentiation and the influence of signal cues before or within that time window are particularly important to understand. Fittingly, studies in mesenchymal stem cells (MSCs) have shown that these cells display a type of "mechanical memory" whereby cellular behavior is influenced by the duration and relative difference in stiffness of earlier culture conditions[19,20]. In our studies, we have found that NSCs display similar behavior such that earlier "pulses" of stiffness inputs influence end-point differentiation and that differences in YAP protein expression on soft or stiff substrates are maximized within a similar time window (Rammensee et al., 2017). Therefore, understanding the signal cascades that are induced by stiffness inputs at earlier timepoints is particularly important to elucidating how stiffness influences stem cell fate commitment.

1.2 The significance of adult neurogenesis and the therapeutic potential of NSCs

1.2.1 Overview of adult neurogenesis

The discovery that neurogenesis occurs in the adult mammalian brain shook the long-held dogma that neurodevelopment in the central nervous system ceases after the early postnatal development period [21,22,23]. In organisms such as rodents and non-human primates, the scientific community has generally accepted adult neurogenesis as a bona fide phenomenon. Evidence has also strongly suggested that neurogenesis occurs and persists throughout adulthood in humans as well[24,25,26], but it is worth noting that the topic is still debated within the field[27,28]. Regardless of the animal system, many aspects of the adult neural stem cell (NSC) population and the physiology of neurogenesis is largely conserved. As discussed previously, adult mammalian neurogenesis occurs in two specific regions of the brain, the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus. NSCs of the SVZ migrate along the rostral migratory stream (RMS) to the olfactory bulb, where they continue differentiating into various olfactory neurons. Hippocampal NSCs differentiate into amplifying neural progenitors that generate new granular neurons within the dentate gyrus and integrate into the existing neural circuitry. *In vivo*, NSCs and their progeny are tightly controlled throughout all phases of neurogenesis by their surrounding microenvironmental niche, which includes the NSCs, the newly generated immature neurons, glial cells, endothelial cells, immune cells, and the extracellular matrix[29]. This sensitivity of NSCs to niche-derived extracellular cues are also preserved to a certain extent *in vitro*. Importantly, NSCs and early neural progenitors (so-called "Type 2" cells) are responsive to physiological stimuli such as exercise, environmental enrichment, seizures, sleep, and stress[30], further highlighting the complex nature of structural neuroplasticity in the hippocampus.

1.2.2 The functional significance of hippocampal neurogenesis

While adult neurogenesis occurs in two distinct regions, much of the experimental effort and scientific discussion to date has focused on hippocampal neurogenesis, in no small part because of the functional implications of neuroplasticity in this region[31]. Even before the discovery of postnatal neurogenesis, the hippocampus was known to play a vital role in several important functions including learning, memory, and spatial navigation[32]. Its importance is further highlighted by increasing evidence that hippocampal dysfunction is implicated in several neurodegenerative and psychiatric disorders such as age-related Alzheimer's disease (AD), depression, and anxiety[33,34].

Continued neurogenesis within such a critical region of the brain has driven researchers to further study its functional significance[6]. Although there is still much to left to uncover, accumulating evidence has led to a predominant hypothesis that neurogenesis aids in "pattern separation"[31,35,36]. In other words, newly generated neurons within the hippocampus maintain and improve how mammals distinguish between highly similar experiences or events, and studies have shown that ablating neurogenesis by various methods in rodents damages this ability in certain behavioral tasks[37,38,39,40,41,42]. Although the causative link between changes to cell-level activity in the hippocampus and behavioral improvement is not yet firmly established, the existing evidence has led the field to reach general consensus that neurogenesis does play a functional role in some forms of learning and memory in adult mammals[43].

1.2.3 Biomaterial platforms to study NSCs *in vitro* and potential for tissue engineering and regenerative medicine

While the study of NSCs *in vivo* is vital to understanding their role and significance, the development of isolation and long-term culture methods have enabled much more extensive investigation into their behavior and the mechanisms that govern them. In addition, parallel and subsequent innovations within the biomaterials field have enabled further study of how NSCs respond to extracellular biophysical cues. For example, we and other groups have used various polymeric substrates with tunable stiffness to investigate mechanosensitive cellular behavior[14,16,17,44]. These studies have revealed novel mechanistic insights and cellular phenomena such as the YAP-dependent suppression of neurogenesis on stiffer substrates. Further innovations such as the development of materials with a continuous stiffness gradient, platforms capable of controlled stretching, and substrates with reversibly tunable stiffnesses have propelled even further study into the intricate signal inputs and signaling mechanisms that can underlie the biophysical regulation of NSCs[15] (Fig. 1.2). These biomaterial platforms are an invaluable tool in the quest to better model and map how NSCs sense, integrate, and respond to mechanical cues that they may receive within their *in vivo* niches.

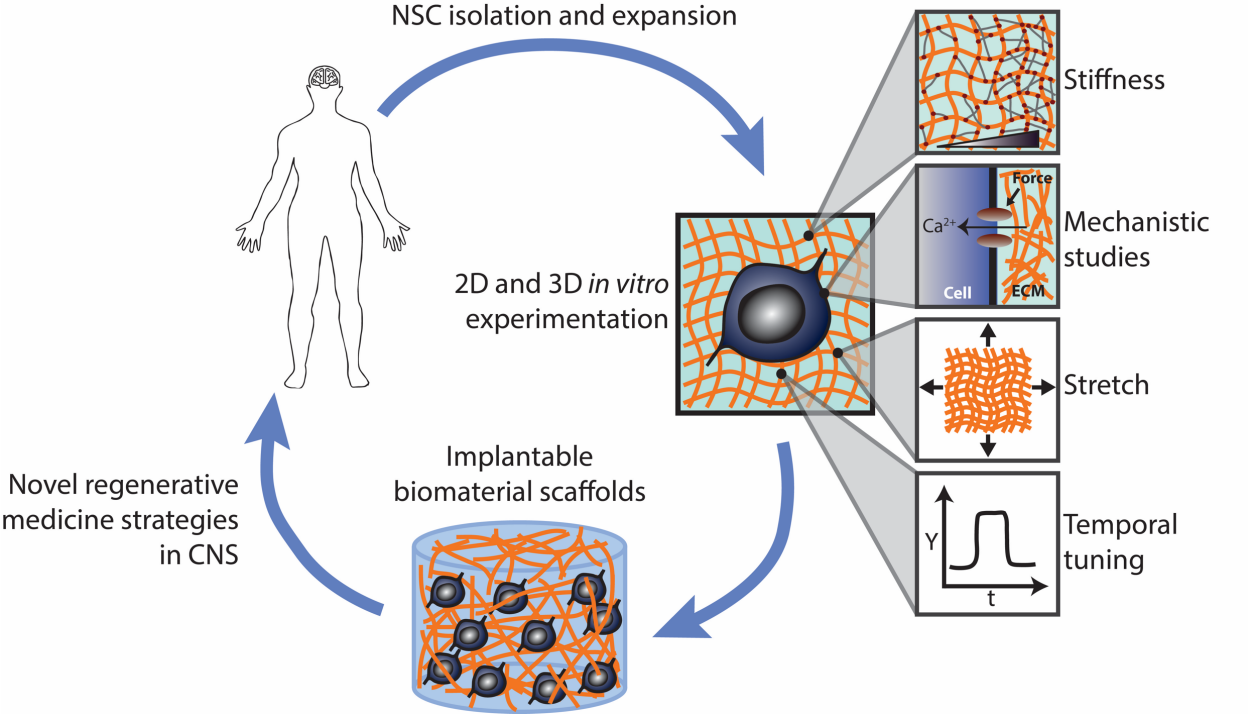

Figure 1.2 Understanding the influence of biomechanical inputs on NSC behavior can inform tissue engineering approaches.

The development of NSC isolation and *in vitro* culture methods as well as innovations in biomaterials have enabled valuable insight into NSC behavior. These findings can then be applied to develop advanced regenerative medicine approaches to address damage or degeneration in the CNS.

In addition to enabling *in vitro* investigation of mechanobiology, biomaterials have also been harnessed as scaffolds to address common challenges in stem cell regenerative medicine such as inefficient cellular expansion and differentiation, widespread death of transplanted cells, and limited homing to or retention in the desired site[45]. Scaffolds have a distinct advantage over the injection of dissociated "bolus" cell suspensions since it is possible to engineer "synthetic microenvironments" that support NSC survival and differentiation upon transplantation. Although scaffolds have shown promise in improving the engraftment of NSCs into the CNS, increased understanding of the mechanical effects of these scaffolds is again needed to enable precise tuning of NSC behavior[46].

In a recent example, we have reported that a rationally-designed 3D HA scaffold could support the maturation and implantation of fragile midbrain dopaminergic (mDA) neurons differentiated from hESCs[47]. The HA scaffold offered distinct advantages over culturing cells in 2D or injecting a dissociated cell suspension, including brain-mimetic mechanical tuning (350 Pa), conjugation of chemical factors that promote cellular adhesion and neurite extension (RGD and heparin), and physical protection during injection. In general, NSC mechanobiology in such

translatable contexts has not yet been exhaustively studied, but this recent finding demonstrates exciting potential for materials-based approaches to replacing lost neural populations and aiding in functional recovery. Importantly, the stiffness used in this study was designed to be within a range that was shown to be neurogenic in previous studies, exhibiting that basic research into NSC mechanobiology can directly inform the effective design of materials for translational tissue engineering applications.

1.3 Angiomotin (AMOT) as a potential mechanistic link between actin cytoskeleton and YAP in NSCs

1.3.1 AMOT background

We have previously reported that cellular contractile forces mediated by the Rho family GTPases RhoA and Cdc42 strongly modulate mechanosensitive fate commitment in hippocampal NSCs[16]. We later showed that stiff matrices increase levels of Yes-Associated Protein (YAP), which acts inhibits the pro-neurogenic transcription factor and Wnt pathway effector β-catenin[17]. However, the key signaling events that link cytoskeletal mechanics to changes in the activity of transcriptional regulators such as YAP have remained unclear. Angiomotin (AMOT) binds both actin and YAP and is thus a promising candidate protein to bridge this signaling gap[48,49,50,51].

Angiomotin (AMOT) was originally discovered as an angiostatin-binding protein[52] and belongs to a family of proteins that includes angiomotin-like 1 (AMOTL1) and angiomotin-like 2 (AMOTL2). The angiomotins share a similar protein structure that includes a coiled-coil domain near the N-terminus and a C-terminal PDZ-binding motif (Fig. 1.3). Functionally, AMOT was found to promote angiogenesis and endothelial tube formation[52], which motivated initial studies exploring AMOT as a potential therapeutic target in cancer. Early mechanistic studies also revealed that AMOT can bind to F-actin and participate in cytoskeletal remodeling and actin bundling[53,54]. This association with the actin cytoskeleton can consequently influence cellular behavior, as subsequent studies demonstrated that AMOT can promote dendritic spine maturation and cell migration[55,56,57]. AMOTL1 and AMOTL2 may also participate in these functions as they contain similar binding domains, but this has not yet been tested rigorously in every context and likely varies between tissues and cell types. Therefore, AMOT may be an important molecule to study in the context of stiffness-sensitive behavior in NSCs, which involves dynamic cytoskeletal remodeling and signaling activity. Interestingly, various reports have shown that the AMOT family proteins have variable expression throughout different tissues, can have redundant or unique roles, and can bind together as a functional complex[58,59,60,61]. Therefore, while studies have suggested that the angiomotin family of proteins are influential in cellular behavior, there is still much to be understood about their function and role.

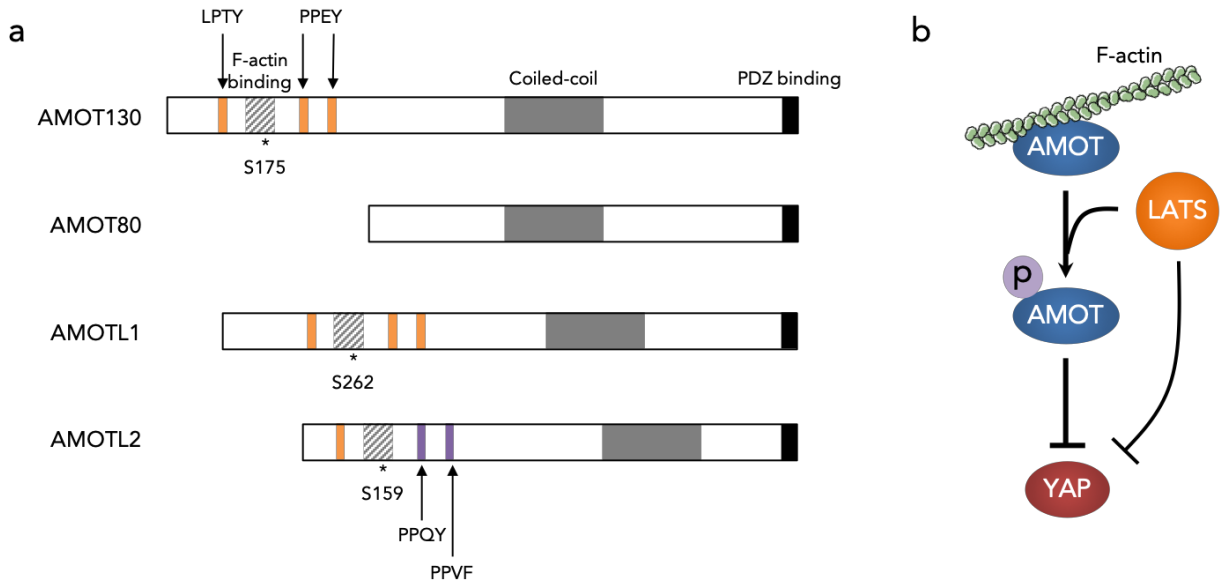

Figure 1.3 AMOT family proteins have high sequence homology and functional binding domains that link F-actin and Hippo signaling.
(a) The AMOT family proteins including AMOT's two splice isoforms all have conserved PDZ binding motifs, coiled-coil domains, F-actin binding domains, YAP binding motifs (L/PPxY/F), and LATS phosphorylation sites (HXRXXS*). (b) AMOT's phosphorylation by LATS inhibits its F-actin binding and promotes inhibition of YAP.

1.3.2 AMOT's potential mechanistic role in NSC mechanotransduction

AMOT is expressed as two splice isoforms, the full-length p130 protein, which contains an N-terminal extension, and the truncated p80 protein (Fig. 1.3). Within this N-terminal region, AMOT contains three L/PPxY motifs that facilitate its interaction with YAP's WW domains. This binding interaction has thrust AMOT into the spotlight as a potential upstream regulator of YAP, and multiple studies have now reported that AMOT can inhibit YAP via a direct binding mechanism[49,58,62]. However, several recent reports have shown that AMOT can also promote YAP activity in the nucleus and may also act as a scaffold that binds to multiple other proteins including YAP as a complex to facilitate signaling[63,64,65]. Therefore, the significance of AMOT's localization and the nature of its interaction with YAP is important to understand within each cellular context.

AMOT130's N-terminal extension also contains a recognition site for large tumor suppressor kinase (LATS), which is a part of the Hippo pathway kinase cascade and canonically inhibits YAP via phosphorylation. Notably, LATS phosphorylation of the S175 residue in AMOT130 is known to inhibit AMOT's F-actin binding activity. Taking a hint from the location of the functional F-actin binding domain and L/PPxY motifs (Fig. 1.3), groups have shown that F-actin and YAP can compete for binding to AMOT and that the AMOT-actin and AMOT-YAP

interactions can be mutually exclusive[50,66,67]. The functional consequence of this signaling axis is that phosphorylated AMOT, which is freed from F-actin binding, demonstrates enhanced YAP binding and inhibition of YAP activity (Fig. 1.3). In this way, AMOT has been shown to link F-actin to YAP regulation and serve as a parallel mechanism by which LATS regulates YAP activity in several non-stem cell contexts. However, the relevance of this signaling pathway has not yet been explored in stem cells or in stiffness-conscious platforms.

1.4 Diabetes, hyperglycemia, and cellular energetics in NSCs

The significance of NSCs and adult neurogenesis is perhaps highlighted best by examining conditions where their function is damaged. Already, studies have shown that dysfunction or damage to hippocampal neurogenesis is implicated in the pathophysiology of age-related neurodegenerative disease, cognitive deficits following traumatic brain injury, major depressive disorders (MDD), and epilepsy[68]. More recently, the chronic effects of another disease are being increasingly attributed to damage to the hippocampus: diabetes mellitus[69]. While the increasing survival and quality of life of diabetic patients is a testament to advancements in treatment and healthcare practices, it has revealed that diabetes can also have negative effects on other tissues and functions that were previously underappreciated, including the brain[70].

Since the 1990s, epidemiological studies have shown that cognitive decline and dementia are more common in patients with diabetes[71,72]. The true cause(s) of this link have been difficult to determine, as diabetes leads to a host of complications that may damage brain tissue, such as microvascular damage, dysglycemia, and dyslipidemia. However, studies have repeatedly shown that persistent hyperglycemia can lead to reduced performance in neurocognitive behavioral tests[73], implicating that dysregulated systemic glucose levels may be a major causative factor in hippocampus-dependent cognitive decline in diabetes. Further supporting this hypothesis, studies have shown that hyperglycemia can directly impair hippocampal NSCs and newly generated neurons[74,75].

While the effect of diabetes-related hyperglycemia on neurogenesis seems clear, the mechanisms that cause these deleterious effects are still largely unknown. Glucose serves as the body's main metabolic substrate, and cells of all tissues utilize it as a primary starting point to ultimately generate intracellular adenosine triphosphate (ATP), the essential molecular energy currency within the cell[76]. Glucose metabolism also creates a myriad of intermediate biochemical products that are involved in other biosynthetic and signaling pathways. For example, glycolysis generates acetyl-CoA, which can then be used in the synthesis of fatty acids or used as a substrate for histone acetylation, a key epigenetic modification that can influence gene expression, in addition to continuing onto the Krebs cycle[77]. Therefore, when the body's tight regulation of serum glucose concentration is disrupted in diabetes, a multitude of intracellular

signaling events may be influenced and studies are still needed to understand how specific cell populations respond to this hyperglycemic signal within their niche[78].

The regulation of cellular energetics is particularly important in stem cells, which have characteristic metabolic programming that drive and maintain their proper state[79]. For example, pluripotent and various tissue-specific multipotent stem cells including NSCs have been found to preferably generate ATP via glycolysis compared to the more efficient oxidative phosphorylation pathway, which is potentially related to their hypoxic niches[80]. During differentiation, the cells then shift their metabolic mechanism through a process called "metabolic reprogramming" such that their differentiated progeny preferably generate ATP via oxidative phosphorylation[81]. If we superimpose this paradigm onto *in vitro* differentiation of NSCs, this shift in cellular energetics also coincides with when cells are sensing and integrating stiffness cues to tune their fate commitment[17]. Indeed, we have shown that variable RhoA signaling directs mechanosensitive differentiation of NSCs and it is well-known that the cytoskeletal remodeling that RhoA induces is an energy-consuming process[16]. Therefore, mechanotransduction and stiffness-sensitive differentiation may be intimately linked to cellular energetics. In this way, hyperglycemia may dysregulate this well-orchestrated metabolic maintenance and reprogramming, which would disrupt the function and state of NSCs.

As it turns out, cells have a "master sensor" of cellular energy state that allows them to adapt to changes in their energy demands or nutrient availability and may therefore be a key component to the cellular response to hyperglycemia: AMP-activated protein kinase (AMPK)[82]. As its name suggests, AMPK is activated in high relative AMP/ATP levels, or low energy states. Upon activation, AMPK regulates numerous downstream targets that ultimately redirect cellular pathways towards ATP generation and decreased ATP consumption. AMPK is highly conserved across various species and cell types and has been shown to have significant roles in regulating stem cell proliferation and differentiation[83,84,85,86]. In general, AMPK activation seems to be a necessary signal to maintain pluripotency and preserve stemness, which has been explored *in vivo* and in tissue engineering contexts[87,88,89]. Additionally, AMPK activation has been shown to induce cytoskeletal reorganization and regulate various cellular processes via the GTPase Rac1[90,91]. Therefore, a possible mechanism for diabetes-related impacts on NSCs may involve the suppression AMPK in hyperglycemic conditions, which could inhibit NSC maintenance, influence cellular biomechanics, and impede neurogenesis.

1.5 Discussion and scope of dissertation

Tissue-specific adult stem cells are a remarkable mechanism that the body uses to regenerate cells to replace those that become damaged or dysfunctional to sustain proper tissue function. This useful "repair and replace" process occurs in many tissues of the body including bone marrow, blood, skeletal muscle, intestinal epithelia, dental pulp, liver, pancreas, and cornea.

However, the discovery of adult neural stem cells within the mammalian brain was not just a significant biological finding – it had the potential to shift a significant dogma of developmental neurobiology, which was that the generation of new neurons was largely completed following the early postnatal period. The excitement of this discovery and its potential implications drove a wave of studies to further study the characteristics and functional significance of adult neurogenesis. These and ongoing work have shown that neurogenesis occurs in two specific niches of the brain, including the SGZ of the hippocampal dentate gyrus. These newly generated neurons have been shown to functionally integrate into the existing hippocampal trisynaptic circuit and studies in rodents have shown that dysfunction of neurogenesis impairs performance on particular behavioral tasks. Although research is still needed to fully deconvolve the role of neurogenesis and structural plasticity in hippocampal function, it is clear that the persistent NSC population enables important neural processing in this significant brain region.

In addition to playing a role *in vivo*, NSCs also represent a promising resource for novel tissue engineering applications and regenerative medicine approaches. With increasing innovations for improved cell culture expansion, biomaterial scaffolding, directed differentiation, implantation, and post-implantation survival, NSCs and NSC-derived cells could be a viable treatment for disease and injury in the CNS.

However, understanding the precise intracellular mechanisms that sense inputs of all kinds and direct cellular behavior is still a major challenge to understanding NSC function *in vivo* and *in vitro*. The field has made promising advancements on this front and we now know a considerable amount about how NSCs maintain their population and differentiate into specialized cells. However, there are countless other discoveries that remain uncovered. The influence of biophysical cues is a relatively newer direction that has already yielded many significant findings that have expanded what we understand about NSC behavior. Another thrust is understanding how NSCs contribute to the pathophysiology of certain diseases that implicate hippocampal dysfunction, such as diabetes and hyperglycemia-related complications. In both of these research areas, we lack significant knowledge about the mechanisms that translate a complex myriad of cues, including ECM stiffness and glucose concentration, into signaling cascades that influence NSC proliferation and neurogenic differentiation. Towards addressing this need, we have investigated the importance of the protein angiomotin (AMOT) in converting stiffness-sensitive cytoskeletal dynamics to the regulation of transcription factors that can direct differentiation, yes-associated protein (YAP) and β-catenin. Our findings from these studies are discussed in Chapters 2 and 3 of this dissertation. In Chapter 4, we discuss our efforts to study how hyperglycemia affects NSC function *in vitro* and the specific role that AMP-activated protein kinase (AMPK) plays in integrating cellular energetic state with mechanosensitive differentiation.

Chapter 2 : Angiomotin is a stiffness-sensitive regulator of mechanosensitive differentiation in neural stem cells

Parts of this chapter are excerpted with permission from Elsevier, from the article "Novel biomaterials to study neural stem cell mechanobiology and improve cell-replacement therapies", by Phillip Kang, Sanjay Kumar, and David Schaffer in *Current Opinion in Biomedical Engineering*, 4: 13-20, 2017.

2.1 Abstract

Neural stem cells (NSCs) are remarkably sensitive to stiffness inputs when differentiating *in vitro*. Soft substrates that are more reminiscent of their native tissue of origin promote robust neurogenesis, while stiff substrates suppress neurogenesis and support increased astrogliogenesis when NSCs are given the same differentiation cues. Better understanding of this fundamental cellular phenomenon of how NSCs sense, process, and respond to their physical microenvironment is critical for reliably using these cells in tissue engineering or regenerative medicine applications and understanding normal and aberrant NSC behavior *in vivo*. A major gap in our current understanding of the mechanotransductive signaling pathway is how stiffness-sensitive changes in cytoskeletal dynamics lead to transcription-level regulation that ultimately govern neural stem cell fate commitment. Towards addressing this need, we investigated the importance of Angiomotin (AMOT), a protein with the capability to bind both F-actin and YAP, a mechanosensitive transcriptional co-activator. When AMOT expression was ablated with CRISPR/Cas-9 or shRNA systems, neurogenesis on soft substrates was attenuated compared to controls. Interestingly, while overexpression of wild-type AMOT displayed no effect, overexpression of a phospho-mimetic mutant variant (S175E) of AMOT that is unable to bind to F-actin or a mutant that lacked the essential actin-binding region (ΔAB) rescued neurogenesis on stiff substrates. Consistent with this finding, endogenous pAMOT, but not total AMOT, was enriched in differentiating NSCs cultured on soft substrates. Diffuse cytoplasmic localization of AMOT, which has also been shown to indicate pAMOT, was also increased on soft substrates. Together, these findings support that AMOT is a stiffness-sensitive pro-neurogenic signaling factor in NSCs that is regulated by its phosphorylation state and localization.

2.2 Introduction

Neural stem cells (NSCs) have been established as important mediators and effectors of plasticity, learning, and memory in the adult nervous system[1] and are envisioned as a potential source for transplantation in neurodegenerative diseases[2,92,93]. NSCs reside in two specific

regions of the adult mammalian brain, the subventricular zone (SVZ) of the lateral ventricles and subgranular zone (SGZ) within the dentate gyrus of the hippocampus[1]. Hippocampal neural stem cells in particular have been indicated to play critical roles in learning, memory formation, behavioral regulation, and disease pathology, important processes that motivate a deeper basic understanding of NSC behavior[94]. Since the landmark discovery of these cells in mammals[95], the development of long-term NSC culture methods have enabled basic investigations of their behavioral regulation as well as exploration of their therapeutic potential to treat neurodegenerative disease, traumatic brain injury (TBI), spinal cord injury (SCI), and stroke[96].

NSCs, like other stem cells, are tightly regulated by the extracellular microenvironment within their resident tissues, collectively termed the stem cell niche[97]. Previous research has revealed that biochemical cues present in the niche can strikingly direct NSC behavior *in vitro* and *in vivo*[9,98]. However, biophysical and specifically mechanical cues have been more recently implicated as a potentially important but relatively poorly understood signal input for NSCs[99]. Early *in vitro* work showed that softer 2D substrates that more closely emulate brain tissue stiffness (< 1 kPa) promote neuronal differentiation of NSCs, whereas stiffer substrates (> 1 kPa) suppress neurogenesis[14,100], of strong potential interest given the presence of tissue stiffness gradients within the hippocampus[101]. These earlier findings implicate exciting new discoveries into how NSCs are influenced by their mechanical microenvironments as they propagate and differentiate. However, the mechanisms that govern this behavior are still unclear, and further mechanistic studies will provide greater understanding of how cells' extracellular signal inputs or their intracellular mechanisms change with conditions such as disease, age, or injury.

Within this field, biomaterials have not only played a role in enabling *in* vitro investigation of mechanobiology, but have also been harnessed as scaffolds to address common challenges in stem cell regenerative medicine such as inefficient expansion and differentiation, widespread death of transplanted cells, and limited homing to or retention in the desired site[45]. Scaffolds have a distinct advantage over the injection of dissociated "bolus" cell suspensions since it is possible to engineer "synthetic microenvironments" that support NSC survival and differentiation upon transplantation. Although scaffolds have shown promise in improving the engraftment of NSCs into the central nervous system (CNS), increased understanding of the mechanical effects of these scaffolds is again needed to enable precise tuning of NSC behavior[46].

Therefore, it is clear that we not only need to understand which mechanical cues can influence NSC behavior and their outcomes, but how cells sense, process, and respond to those cues through intracellular pathways. We have previously reported that cellular contractile forces mediated by the Rho family GTPases RhoA and Cdc42 strongly modulate mechanosensitive fate commitment in hippocampal NSCs[16]. We later showed that stiff matrices increase levels of Yes-Associated Protein (YAP), which acts within a temporally restricted 12-36 h window to inhibit

the pro-neurogenic transcription factor and Wnt pathway effector β-catenin[17]. However, the key signaling events that link cytoskeletal mechanics to changes in the activity of transcriptional regulators such as YAP have remained unclear.

Angiomotin (AMOT) binds both actin and YAP and is thus a candidate protein to bridge this signaling gap[48,49,50]. Importantly, AMOT's actin binding activity is known to be inhibited by large tumor suppressor kinase (LATS) phosphorylation, which could thereby regulate AMOT's interactions with YAP[62,102]. Indeed, recent studies have confirmed that phospho-mimetic or non-actin-binding mutants of AMOT display enhanced YAP binding and inhibition of YAP activity, demonstrating that the AMOT-YAP interaction is influenced by the actin cytoskeleton and that AMOT phosphorylation is a potentially functionally important consequence of the Hippo pathway kinase cascade[50,66, 67,103]. Interestingly, while most initial studies indicated that AMOT inhibits YAP, several recent reports have shown that AMOT can also promote YAP activity, particularly in the nucleus[63,104,105,106]. Therefore, we sought to explore the potential role of AMOT as a mechanosensitive regulator of NSC fate commitment capable of bridging Rho and YAP signaling. To do this, we ablated AMOT expression in NSCs by both shRNA-mediated knockdown and CRISPR/Cas 9-mediated knockout to test if it was necessary for stiffness-sensitive neurogenesis. Furthermore, we overexpressed wild-type and various mutants of AMOT in NSCs that allowed us to further explore the mechanistic consequences of AMOT in NSC fate commitment. Specifically, the AMOT mutants used in this study enabled us to probe the significance of AMOT-actin binding and AMOT phosphorylation.

2.3 Materials and methods

2.3.1 Polyacrylamide gel synthesis and functionalization

Polyacrylamide-bis precursor solutions were made for each stiffness by mixing various concentrations of acrylamide monomer and bis-acrylamide crosslinker (Bio-Rad). Solution compositions to achieve various final polymerized stiffnesses were as follows: 0.2 kPa = 3% acrylamide + 0.04% bis, 0.5 kPa = 3% acrylamide + 0.1% bis, 72 kPa = 10% acrylamide + 0.3% bis[16]. Polyacrylamide gels were synthesized on 19- or 25-mm glass coverslips with 0.1% TEMED + 1% Ammonium Persulfate. Polyacrylamide gels were then functionalized with laminin conjugation via sulfo-SANPAH (Thermo-Fisher) to facilitate cell attachment.

2.3.2 Cell culture

Adult rat hippocampal NSCs were cultured in DMEM/F12 with N2 supplement (Life Technologies) on tissue-culture polystyrene plates that had been coated with poly-ornithine and laminin. Growth conditions for NSCs included 20 ng/mL FGF-2 whereas mixed differentiation conditions included 1% FBS + 1 μM retinoic acid. For full differentiation experiments on

polyacrylamide substrates, NSCs were first seeded onto the gels in growth media for 16-18 hours before the coverslips were then transferred into new wells with mixed differentiation media. The NSCs were allowed to differentiate for 6 days with 50% media changes every 2 days before fixation.

2.3.3 Constructs and antibodies

shAMOT, shCntrl, sgAMOT, and sgAMOT oligonucleotide inserts were obtained from Elim Biopharmaceuticals. shRNA inserts were designed with AgeI and EcoRI-based overhangs and cloned into the pLKO.1 vector, a gift from David Root (Addgene plasmid # 10878). sgRNA inserts were designed with vector-specific BsmBI-based overhangs and cloned into the lentiCRISPR v2 vector, a gift from Feng Zhang (Addgene plasmid # 52961). pcDNA4 plasmids with cDNA encoding WT, ΔAB, S175E, and mYB AMOT were a generous gift from D. McCollum (University of Massachusetts Medical School, Worcester, MA). The AMOT cDNA sequences were PCR-amplified, digested with SfiI and PmeI, and cloned into the pCLPIT vector. S175A and S175E + mYB AMOT cDNA sequences were generated using the QuikChange Site-Directed Mutagenesis system (Agilent Technologies) before also being digested and cloned into the pCLPIT vector.

Primary antibodies and dilutions used were as follows: Tuj1 (1:750; BioLegend 801201), AMOT (1:1,000 for western blotting, 1:250 for immunocytochemistry; Bethyl Laboratories A303-303A), c-Myc (1:250; Santa Cruz Biotechnology A-14), pAMOT (1:1,000 for western blotting, 1:250 for immunocytochemistry; EMD Millipore ABS1045), β-actin (1:100,000; Sigma-Aldrich A1978), GAPDH (1:1,000; Sigma-Aldrich G8795)

2.3.4 siRNA and sgRNA sequences

siRNA targeting rat *amot* mRNA (gagaaagccatgaggaaca) and a scramble control (gatgcatgttgatagacgtaa) were designed using the online tool Dharmacon siDesign. sgRNA targeting the rat *amot* genomic locus (gatggatgctacgagaagg) and a scramble control (gcactaccagagctaactca) were designed using the online tool E-CRISP.

2.3.5 Viral packaging and transduction

CLPIT vectors encoding the various human AMOT cDNA were packaged as retroviruses via calcium phosphate-based transfection into HEK 293T cells (10 μg transfer vector + 6 μg pCMV gag-pol + 4 μg pcDNA3 IVS VSV-G per 10 cm diameter plate). Supernatants were collected 48 and 72 hours post-transfection and pooled before filtration and ultracentrifugation to purify the viral particles. Vectors encoding shRNA or sgRNA were were similarly packaged into lentiviruses (10 μg transfer vector + 7.5 μg psPAX2 + 2.5 μg pMD2.G per 10 cm diameter plate)

before viral purification. Cells were transduced in all cases at an MOI of 1-2 as calculated by puromycin resistance titer and selected with 0.6 μg/mL puromycin for at least 4 days[109].

2.3.6 Western blotting

Cell lysates were collected in RIPA buffer + 1% sodium molybdate, 3% sodium fluoride, and 1X Halt Protease/Phosphatase inhibitor cocktail (Thermo Fisher). 1-10 μg total protein was loaded per well of a precast 4-12% gradient polyacrylamide gel cassette. Within each experiment, the total amount of protein loaded for each sample was normalized using BCA protein quantification. Gel electrophoresis and protein transfer onto nitrocellulose membranes were carried out using a Life Technologies Mini Gel Tank. Antibody solutions and blocking was carried out with LI-COR blocking buffer before membrane imaging on a LI-COR Odyssey Blot Imager.

2.3.7 Immunofluorescence staining and imaging

Cells were fixed in 4% paraformaldehyde (Alfa Aesar) for 10 minutes before washing in PBS and permeabilization in 5% goat serum + 0.5% Triton X-100 (Sigma-Aldrich) for 10 minutes. Permeabilized cells were blocked in 5% goat serum for 1 hour at room temperature before immunostaining. Primary and secondary antibody solutions were also made in 5% goat serum.

Epifluorescent images were taken using a Nikon Eclipse Ti Microscope, Hamamatsu Photonics K.K. C10600-10B-H camera, 10X objective lens, and native NIS-Elements AR 5.02.00 software. Samples were submerged in PBS during image acquisition. Nuclei were labeled with a DAPI stain (Sigma-Aldrich) and Tuj1 was labeled with a 488 dye-conjugated secondary antibody (Thermo-Fisher). Confocal images were taken using a Prairie Technologies 2-photon and confocal microscope, QuantEM 512SC camera, 60X objective lens, and native Prairie View software. Samples were submerged in PBS during image acquisition. Nuclei were labeled with a DAPI stain, F-actin was labeled with an Alexa Fluor 546 Phalloidin (Thermo-Fisher), and other targets were labeled with either a 488 or 633 dye-conjugated secondary antibody. All image processing and analysis was carried out in the free ImageJ software.

2.3.8 EdU proliferation assay

EdU assays were conducted according to manufacturer protocol and recommendations (Abcam). All cell lines analyzed by EdU assay were cultured in typical proliferation conditions (20 ng/mL FGF-2) for 24 hours on laminin-coated tissue culture polystyrene wellplates prior to a 4-hour pulse of EdU at a concentration of 10 μM. The cells were then fixed in 4%

paraformaldehyde and permeabilized prior to incubation with a fluorescently labeled probe for EdU. Labeled samples were then imaged on an epifluorescent microscope (Section 2.3.7).

2.4 Results

2.4.1 Investigating Angiomotin (AMOT) localization in neural stem cells (NSCs) compared to other cell types

AMOT has been reported as a YAP regulator and an F-actin-binding protein in several cellular contexts and functional motif mapping and experimental evidence has suggested that those binding events are mutually exclusive[49,50]. Therefore, AMOT's regulation of YAP may be sensitive to cytoskeletal dynamics within stem cells, which would be a novel stiffness-sensitive YAP regulatory mechanism during fate commitment. However, AMOT has been reported to participate in a wide variety of other signaling events and cellular contexts, including cadherin-based migration[107], polarity-sensitive Hippo signaling[67], dendritic spine maturation[55], and transcriptional regulation[63]. These diverse reports implicate that AMOT may display a variety of localization and functional differences depending on cell type and context. Indeed, there has been a discussion the field regarding whether AMOT should be categorized as a tumor suppressor or an oncogene as evidence exists for both[108].

Therefore, in an initial study we sought to examine AMOT localization in a range of cell types as a clue into how AMOT may be signaling in NSCs and how generalizable that mechanism may be. We observed a strikingly wide range of localization patterns for AMOT across the different cell types. In human embryonic kidney (HEK) 293T cells, AMOT is preferentially found in the cytoplasm outside of the nucleus or at cell-cell junctions and also co-localizes strongly with F-actin (Fig 2.1a). In human adipose-derived mesenchymal stem cells (hMSCs), AMOT is found in distinct puncta in the nucleus and also along the prominent stress fibers (Fig 2.1c). In human malignant glioblastoma cells (U251s), AMOT was expressed in a more pan-cellular fashion (Fig 2.1b). In rat hippocampal NSCs, AMOT displayed an interesting mix of localization as it could be found distinctly in the nucleus and in the cytoplasm of proliferating and differentiating cells, and also along cortical F-actin in proliferating cells (Fig 2.1d-e). Therefore, we hypothesized that AMOT may have a functional role in both the cytoplasm and nucleus and that this may underlie its mechanistic impact in NSCs.

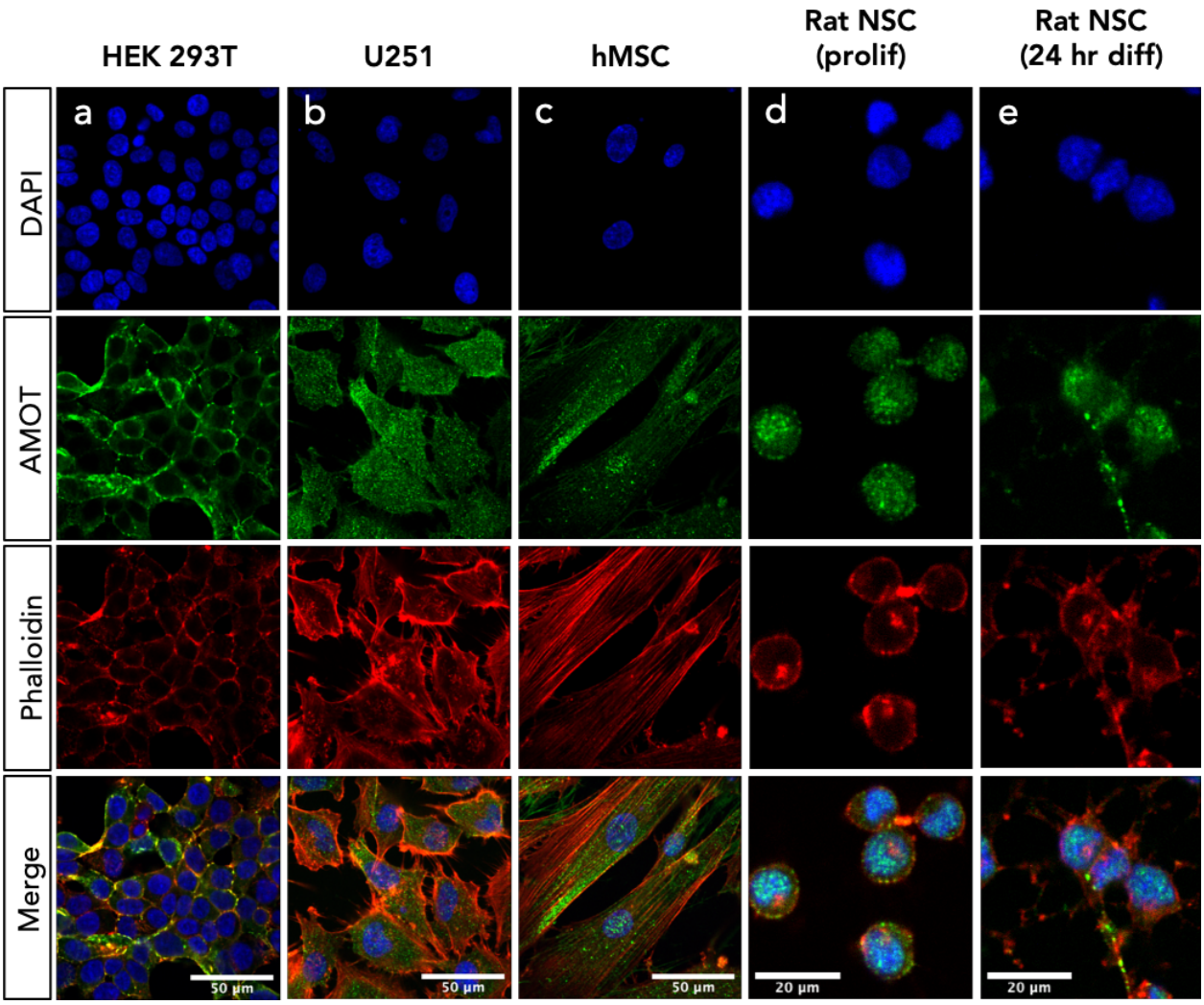

Figure 2.1 AMOT displays a wide range of localization patterns in various cell types.
All cells were cultured for 24 hours in their respective media conditions on tissue culture polystyrene prior to fixation and staining for nuclei (blue), AMOT (green), or F-actin (red). NSCs were cultured in both proliferative (20 ngl/mL FGF-2) or mixed differentiation conditions (1 % FBS + 1 µM retinoic acid). Bar = 50 µm for HEK 293T, U251, and hMSC, 20 µm for rat NSCs.

2.4.2 AMOT loss-of-function and stiffness-sensitive neurogenesis

To investigate the potential importance of AMOT in stiffness-sensitive NSC differentiation, we eliminated functional expression of the AMOT gene with targeted short hairpin RNA (shAMOT) or single guide RNA (sgAMOT) + Cas 9-mediated silencing (Fig. 2.1a). Both approaches utilized lentiviral delivery of constructs encoding the requisite silencing machinery. We also created lentiviral constructs encoding non-targeting scrambled sequences as controls for both shRNA-mediated knockdown and CRISPR/Cas 9-mediated knockout. We

infected NSCs at a multiplicity of infection (MOI) of 1, which minimizes the potential off-target effects of multiple infections of a single cell while allowing for a majority of the cells to be infected with a single copy of the silencing machinery[109]. We established stable shAMOT and sgAMOT cell lines by puromycin treatment for 4 days and screened for successful ablation of AMOT protein expression prior to downstream experimentation. To test stiffness-sensitive differentiation, we differentiated shAMOT and sgAMOT NSCs along with their controls on either soft (0.2 kPa) or stiff (72 kPa) polyacrylamide gels under media conditions that support both neuronal and astrocytic differentiation (1 μM Retinoic Acid + 1% FBS) and immunostained for the neuronal marker Tuj1, which demonstrated that shAMOT and sgAMOT cells exhibited ~40% reduced neurogenesis compared to their controls (Fig. 2.1b,c). In particular, loss of AMOT reduced neurogenesis on soft gels to levels similar to what we observed for naive and control cells on stiff substrates (Fig. 2.1c). These results implicate AMOT as an important effector of proper neurogenic differentiation of hippocampal NSCs and a potential mediator of robust neurogenesis under mechanical conditions that more closely mimic the mechanical properties of the *in vivo* niche.

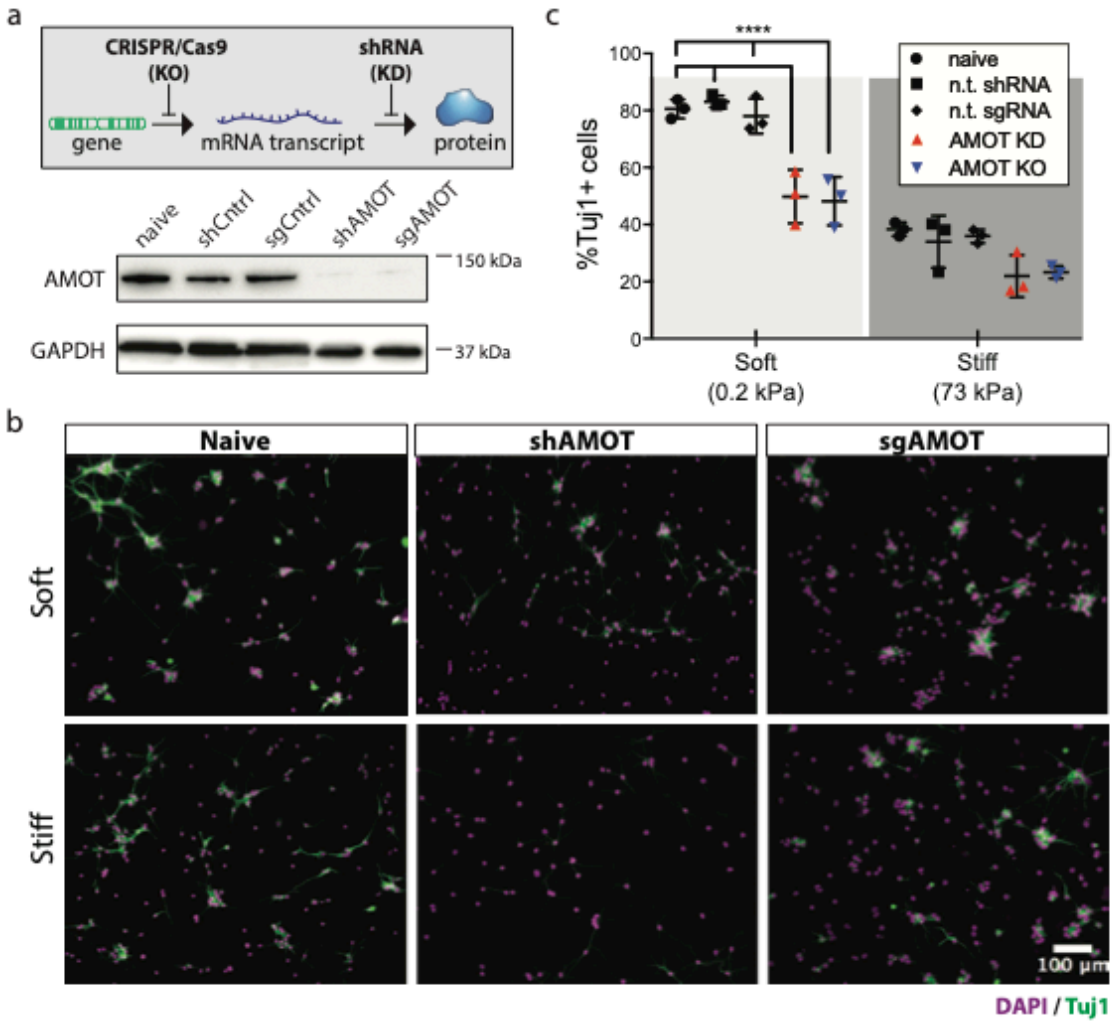

Figure 2.2 AMOT is necessary for high neurogenesis on soft substrates.
(a) Western blot showing protein depletion and gene KO of AMOT in NSCs using shRNA and CRISPR/Cas 9. (b) Representative immunofluorescence images of naïve, AMOT KD (shAMOT) and AMOT KO (sgAMOT) NSCs after culture in mixed differentiation conditions (1 μM retinoic acid + 1% FBS) on soft (0.2 kPa) or stiff (73 kPa) substrates. Cells were fixed and stained for DAPI (magenta) and Tuj1 (green), a neuronal marker. (c) Quantification of neurogenesis was measured by the percentage of Tuj1+ cells after 6 days of differentiation. Error bars represent SD (n=3 gels). Bar = 100 μm. ****$p<0.0002$ by two-way ANOVA followed by Tukey's post-hoc test.

2.4.3 Overexpression of wild-type and mutant variants of AMOT

To further examine whether AMOT is a pro-neurogenic factor in NSCs, we next overexpressed AMOT variants and conducted differentiation experiments analogous to those with the shAMOT and sgAMOT cells. We hypothesized that F-actin binding may critically regulate AMOT function, as previous studies have described a competitive binding mechanism between AMOT binding to F-actin vs. YAP[50,110]. Although the degree of F-actin polymerization and cytoskeletal dynamics in general could influence this balance, previous studies have shown that phosphorylation by LATS at serine-175 (S175) within the F-actin binding region potently inhibits AMOT's ability to bind F-actin and promotes the AMOT-YAP interaction[66]. To examine the importance of the phosphorylation state and F-actin binding on AMOT's impact on NSC behavior, we generated cell lines overexpressing phospho-null (S175A) or phospho-mimetic (S175E) AMOT, as well as another AMOT variant with a deletion of 10 AA necessary for functional F-actin binding (ΔAB) that includes S175 (Fig. 2.2a). The cDNA were variants of AMOT's full-length p130 isoform, which contains the functional F-actin binding domain and S175 residue, which are not present in the truncated p80 isoform.

Strikingly, while overexpression of wild-type (WT) or S175A AMOT did not rescue neurogenesis on stiff substrates, ΔAB and S175E AMOT increased the fraction of Tuj1+ cells on stiff gels by more than 1.5-fold compared to Venus YFP controls (Fig. 2.2b). By comparison, there was no significant difference between the various cell lines on soft substrates, indicating that the levels of neurogenesis seen in the controls apparently approach the maximum NSC neurogenic capacity in the conditions used. Consistent with AMOT's reported F-actin binding capabilities and the functional mapping of the regions responsible for facilitating the F-actin interaction, exogenous S175A and WT AMOT displayed co-localization with actin-based structures, whereas ΔAB and S175E AMOT did not, validating both AMOT's F-actin binding capacity and the effect of S175 phosphorylation on AMOT-F-actin binding in NSCs (Fig. 2.2c). Interestingly, further examination of the localization of the overexpressed AMOT variants revealed that ΔAB and S175E AMOT were significantly enriched in the cytoplasm (Fig. 2.2c), consistent with a recent report showing that phosphorylated AMOT is preferentially localized to cytoplasm and plasma membrane, whereas unphosphorylated AMOT localizes to the nucleus[103].

Together, these results strongly indicate that AMOT is not only pro-neurogenic in NSCs, but that this effect is significantly regulated by its S175 phosphorylation state and localization. Since LATS itself has been shown to be negatively regulated by actin assembly[111,112,113], these results are consistent with a signaling axis in which increased cytoskeletal signaling in response to biophysical inputs have an inhibitory effect on AMOT's activity by inhibiting LATS and increasing unphosphorylated AMOT.

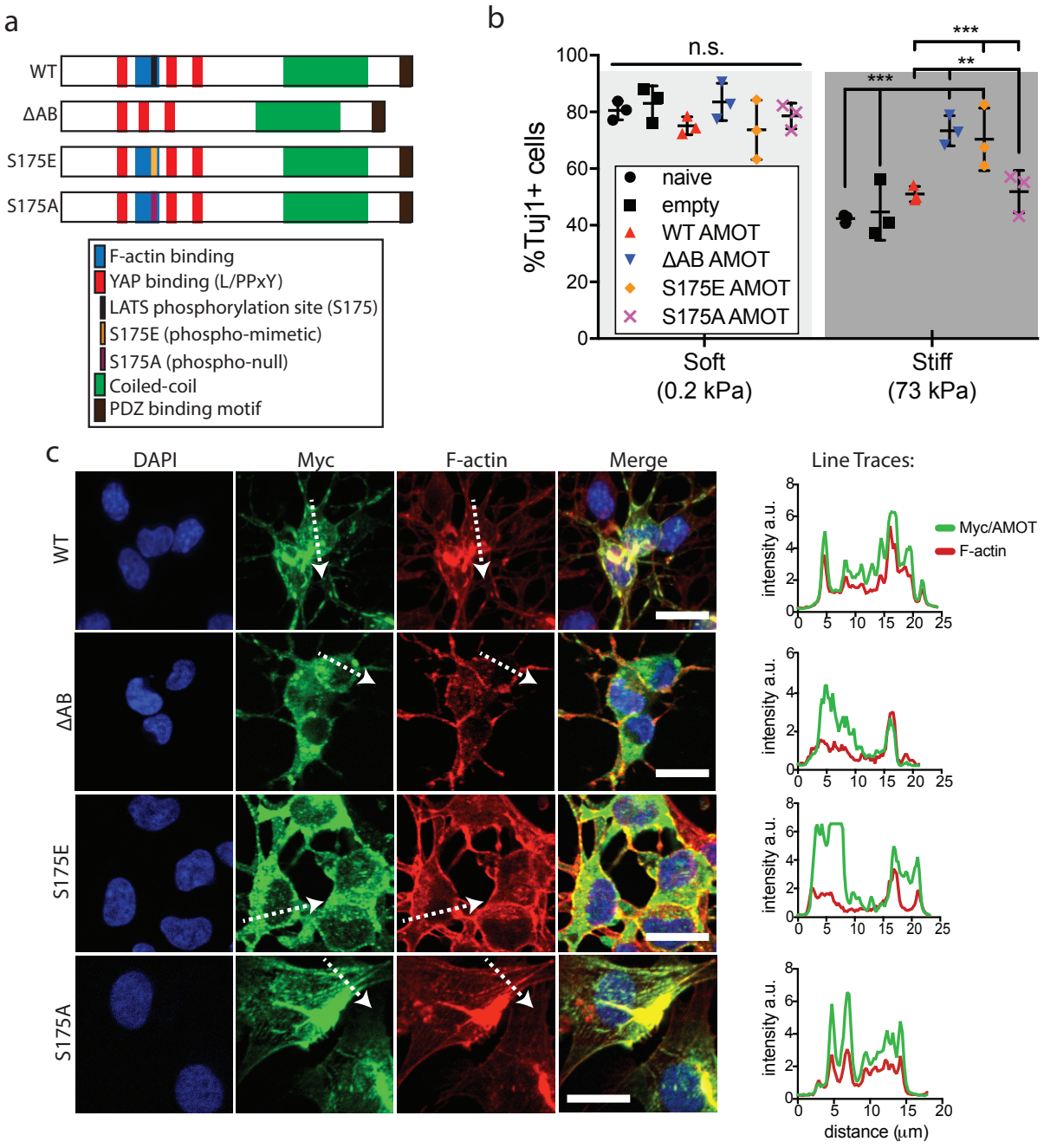

Figure 2.3 AMOT phosphorylation and actin binding regulate its neurogenic effect.
(a) Schematic drawings depicting protein sequence of AMOT variants that were cloned into retroviral plasmids before being packaged and used to generate stable AMOT overexpression NSC cell lines. (b) Quantification of neurogenesis was measured by the percentage of Tuj1+ cells after 6 days of differentiation. Error bars represent SD (n=3 gels). (c) Left: Representative immunofluorescence images of WT, ΔAB, S175E, and S175A AMOT overexpression NSCs after 24 hours of differentiation on stiff substrates. Myc antibody detects a C-terminal epitope tag only present on exogenous AMOT. Right: Plotted intensity line traces from the Myc/AMOT and F-actin channels correlating to white dotted arrows shown on the left. Bars = 20 μm. **$p<0.005$, ***$p<0.001$ by two-way ANOVA followed by Tukey's post-hoc test.

2.4.4 Proliferation in AMOT KD/KO and AMOT OE NSCs

While the experiments presented above provide strong evidence that AMOT has a strong effect on stiffness-sensitive neurogenesis, we sought to rule out the possibility that perturbing AMOT expression provided a selective rather than instructive cue for NSC behavior. In particular, we tested if AMOT knockdown (KD), knockout (KO), or overexpression (OE) affected NSC proliferative capacity, which could select for cells that are more likely to differentiate into a particular fate. Indeed, studies have reported that AMOT can promote or inhibit cell proliferation in various cell types and *in vivo*. To test for proliferation in NSCs, we conducted an EdU assay, which measures the fraction of cells that are actively undergoing DNA synthesis and is a reliable indicator of cell proliferation. We found that shAMOT (KD) NSCs, sgAMOT (KO) NSCs, the various AMOT OE cell lines, and the relevant control NSCs all exhibited an EdU+ fraction of approximately 40% after a 4 hr pulse of EdU, indicating that the same relative number of cells were proliferating within that time window for all cell lines. Therefore, AMOT loss- or gain-of-function do not seem to affect NSC proliferation in cell culture. While formally testing the effects on apoptosis would be needed to rule out that selective mechanism as well, we have not observed any notable cell death during long-term culture or experimentation with any of the cell lines. Combined with the impacts on neurogenesis discussed in Sections 2.4.2 and 2.4.3, these results strongly implicate that AMOT has an instructive effect on NSC fate commitment.

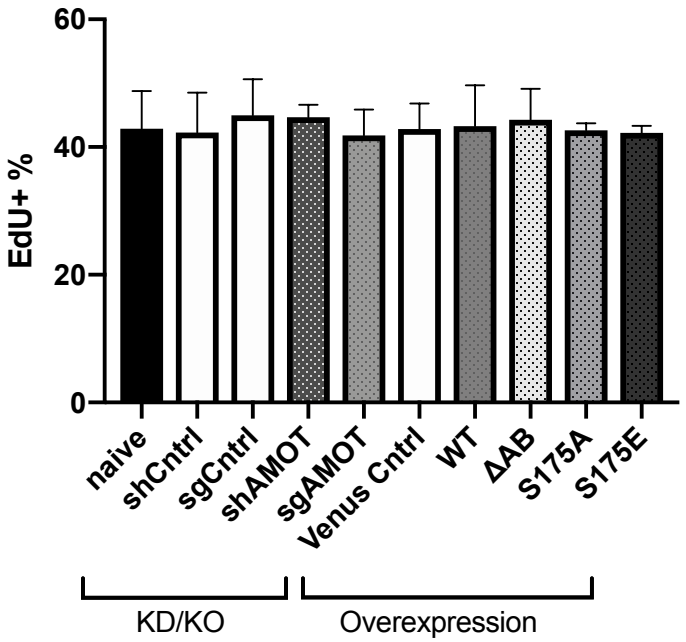

Figure 2.4 AMOT perturbations do not impact NSC proliferation.
Each cell line was cultured for 24 hours in renewal conditions (20 ng/mL FGF-2) prior to the addition of 10 μM EdU for 4 hours. Cells were then fixed in 4% PFA and EdU was detected by a specific 488 fluorescent probe. DAPI was also added to the wells to calculate the total number of cells and the EdU+ fraction. At least 500 cells were analyzed per cell line. Error bars represent SD (n = 3 biological replicates).

2.4.5 Effect of stiffness on AMOT phosphorylation and localization

To examine if stiffness could influence the localization and degree of endogenous pAMOT in a manner consistent with observations of overexpressed phosho-mimetic (S175E) AMOT, we immunostained pAMOT in NSCs that had been differentiated on soft and stiff substrates. Importantly, the cells were fixed after 18 hours of differentiation, which is within the 12-36 hour timeframe in which NSC fate commitment is sensitive to extracellular stiffness input that we have previously described[17]. We observed that endogenous pAMOT is preferentially localized within the cytoplasm in differentiating NSCs, and that the ratio of nuclear/cytoplasmic (N/C) pAMOT was consistent between soft and stiff substrates (Fig. 2.5a-b). However, total pan-cellular pAMOT was elevated in cells differentiated on soft substrates (Fig. 2.5c).

Because we found that AMOT's phosphorylation state to be a key regulator of its impact on neurogenesis from the overexpression studies (Section 2.4.2) and that pAMOT showed preferential localization to the cytoplasm, we next tested if stiffness impacted overall AMOT localization to see if the patterns we observe are consistent with this mechanism. We cultured NSCs for 24 hours in mixed differentiation conditions on soft (500 Pa) or stiff (72 kPa)

substrates prior to fixing and staining for AMOT. We found that cells on soft substrates displayed increased cytoplasmic total AMOT compared to cells on stiff substrates (Fig. 2.5d-e), which would be consistent with increased pAMOT levels on the softer, more neurogenic substrates. We were intrigued by the prominent nuclear localization of AMOT on stiff substrates, which we observed in this experiment and consistently throughout our studies. Because AMOT has two known splice isoforms that have been shown in some studies to have distinct functional roles, we wondered if one isoform was overrepresented in the nucleus to account for the localization patterns we observed. Therefore, we collected sub-fractionated cytoplasmic and nuclear lysates from cells differentiating on stiff substrates to examine the isoform composition in each compartment. Strikingly, we found that nuclear AMOT was almost exclusively the full-length AMOT130 isoform and that both isoforms could be found in the cytoplasmic fraction (Fig. 2.5e). Because AMOT130 contains an N-terminal extension that is truncated in the shorter AMOT80 protein, this implies that the nuclear translocation and function of AMOT130 may be governed by AMOT functionality in that region, which includes the YAP and F-actin binding motifs as well as the LATS phosphorylation site.

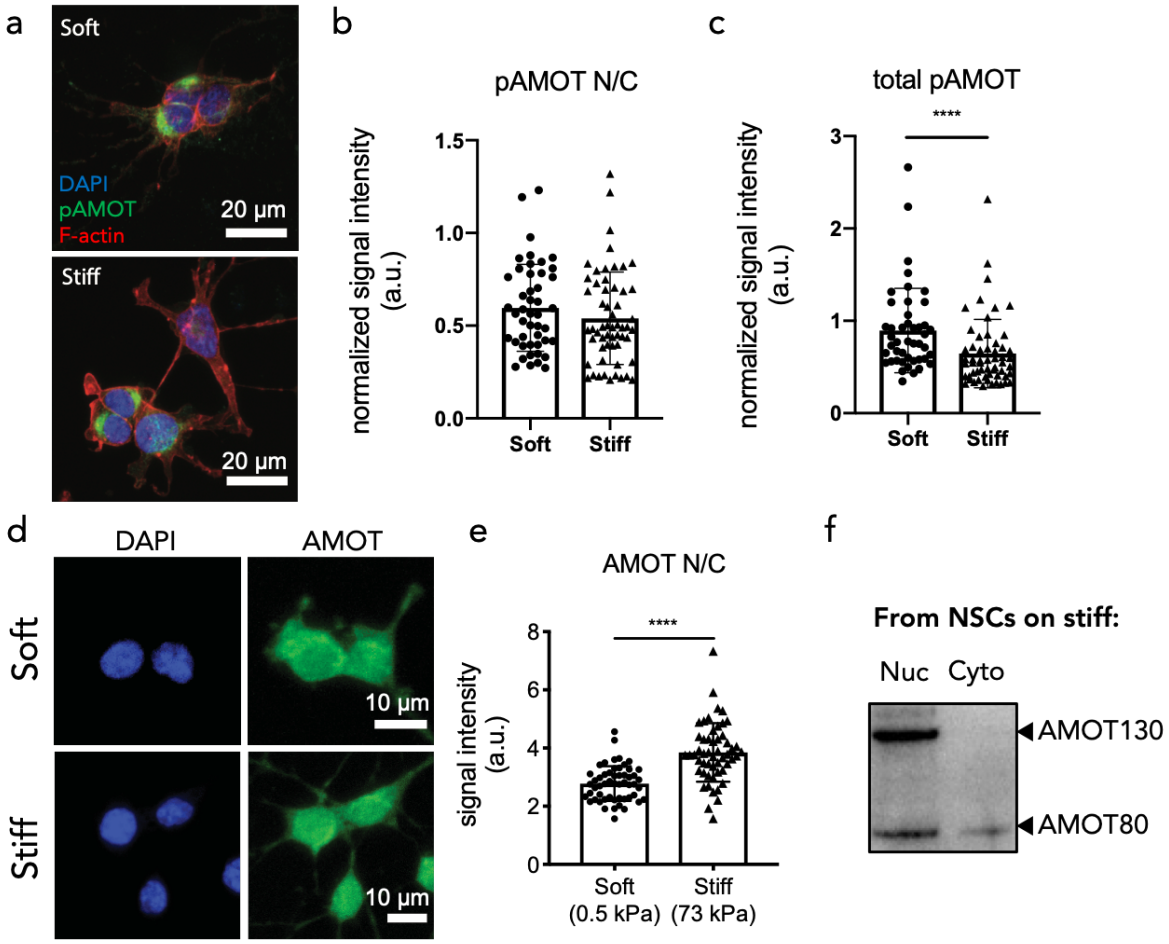

Figure 2.5 Stiffness regulates AMOT phosphorylation and localization.
(a) Representative images of pAMOT staining in differentiating NSCs. Cells were fixed and stained for endogenous pAMOT (green), nuclei (blue), and F-actin (red) after 18 hours of differentiation on soft (500 Pa) or stiff (73 kPa) substrates in mixed differentiation conditions (1 μM retinoic acid + 1% FBS). (b-c) Quantification of nuclear/cytoplasmic or total pAMOT staining intensity from images. n = 46 cells for soft, 57 cells for stiff. (d) Representative images of total AMOT staining in differentiating NSCs. Cells were fixed and stained for endogenous total AMOT (green), and nuclei (blue) after 24 hours of differentiation on soft (500 Pa) or stiff (73 kPa) substrates in mixed differentiation conditions (1 μM retinoic acid + 1% FBS). (f) Western blot for full-length 130 kDa and truncated 80 kDa AMOT isoforms in subfractionated nuclear or cytoplasmic lysates collected from NSCs differentiating on stiff (73 kPa) substrates for 24 hours. Error bars represent SD. ****$p<0.005$ by Mann-Whitney nonparametric t-test.

2.4.6 Functional consequences of nuclear AMOT localization

To further interrogate the importance of AMOT localization on its activity, we engineered a plasmid to express WT AMOT tagged with a C-terminal SV40 NLS sequence to target the overexpressed AMOT to the nucleus as well as a Venus YFP fluorescent tag to track its intracellular localization. We then packaged that plasmid into retroviral particles before transducing NSCs. Importantly, we overexpressed the WT AMOT-NLS-YFP construct on an AMOT-null background by using the shAMOT NSCs that we had generated and characterized previously, which prevents confounding the effects of the ectopic and endogenous AMOT (Fig 2.2). We hypothesized that since endogenous pAMOT and overexpressed phospho-mimetic S175E AMOT were cytoplasmically localized and promoted neurogenesis, forced nuclear localization of AMOT may further suppress neurogenesis. This would provide causative evidence for the influence of AMOT localization on neurogenic outcomes and provide insight into the functional consequences of the enriched nuclear AMOT we observed on stiff substrates. Unfortunately, we were unable to get a significant number of cells to successfully translocate the ectopic AMOT-YFP to the nucleus. However, those that we did find were strikingly Tuj1-negative, an effect that is visually accentuated by their neighboring cells, which are largely Tuj1-positive and display cytoplasmic AMOT-YFP (Fig. 2.6a-c). Indeed, after quantification we found that only a mere 20% of the cells (3/15) with nuclear AMOT-YFP differentiated into neurons. In contrast, cells with cytoplasmic AMOT-YFP resulted in 89% Tuj1+ cells (98/110). Notably, cells that displayed negligible AMOT-YFP signal resulted in 28% neurogenesis (10/36), which was strikingly consistent with what we had observed with the shAMOT NSCs previously (Fig. 2.2c). These results suggest that nuclear AMOT actively inhibits neurogenesis, potentially even to a greater degree than when AMOT is ablated via KD. Further, our findings in this experiment reinforce our previously discovered correlation between AMOT localization and neurogenesis (Fig. 2.3b-c) with more causative results. Together, these data strongly suggest that AMOT localization has a critical impact on its influence on neurogenesis.

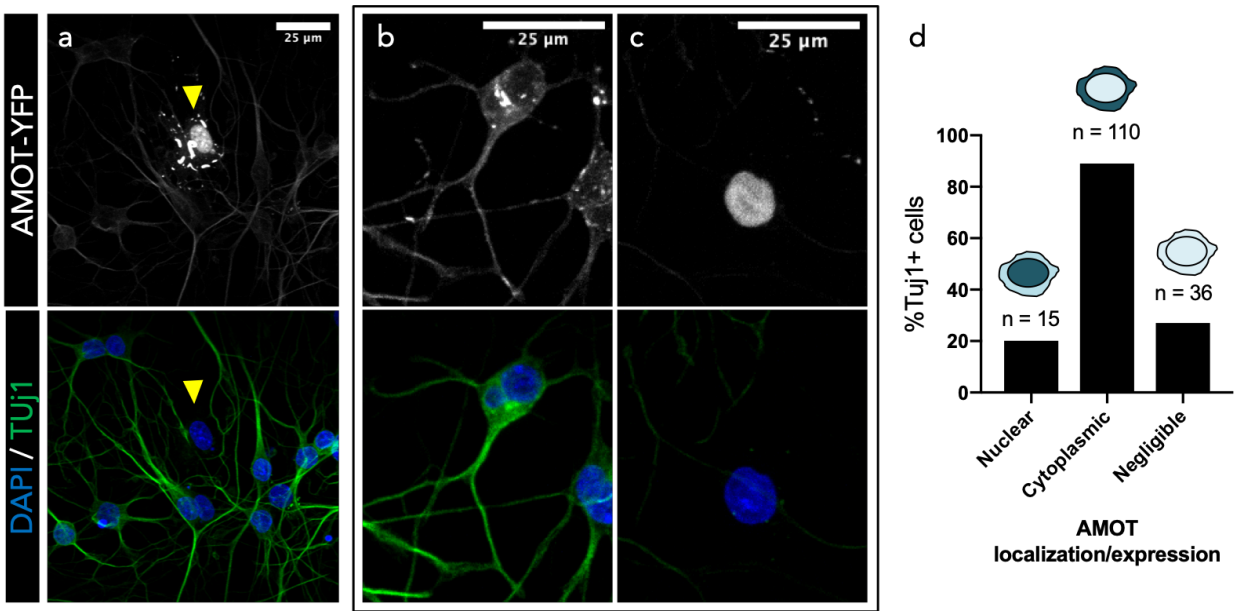

Figure 2.6 Localization of overexpressed AMOT strongly impacts neurogenic outcomes. (a) An NSC showing nuclear AMOT-YFP signal (yellow arrowhead) and its corresponding Tuj1 signal compared to its neighboring cells. Zoomed-in representative images of an NSC with (b) cytoplasmic AMOT-YFP or (c) nuclear AMOT-YFP. shAMOT NSCs were transduced with a WT-AMOT-NLS-YFP construct for 24 hours prior to differentiation initiation. Cells were differentiated for 6 days in mixed differentiation conditions (1 µM retinoic acid + 1% FBS) prior to fixing and staining for Tuj1 (green) and nuclei (blue). AMOT-YFP fluorescent signal was detected without staining with FITC filter. (d) Quantification of Tuj1+ positive cells categorized by observed AMOT-YFP localization. n=15 cells for nuclear, 110 for cytoplasmic, and 36 for negligible.

2.4.7 AMOT expression and phosphorylation within the early mechanosensitive fate commitment window

While these results strongly suggest the importance of AMOT phosphorylation, we sought to more thoroughly test if the degree of AMOT phosphorylation is regulated by stiffness inputs during NSC differentiation. Additionally, because we observed increased pAMOT staining on soft substrates after 18 hours of differentiation, we were motivated to further investigate AMOT phosphorylation with greater temporal resolution in this previously described mechanosensitive window. Therefore, we next conducted western blots with lysates collected from cells cultured on soft or stiff substrates at various time increments within that temporal window and beyond. Excitingly, we observed that while total AMOT levels did not vary with stiffness at any tested timepoint (Fig. 2.7a, c), the fraction of pAMOT was higher at 0, 12 and 36 hours of differentiation on soft substrates (Fig. 2.7a-b). Interestingly, this difference dissipates by the 48 and 72-hour timepoints. Combined with our previous results showing that total pAMOT staining is elevated on soft substrates, these data together show that substrate stiffness impacts AMOT phosphorylation specifically rather than total AMOT levels during early NSC fate commitment.

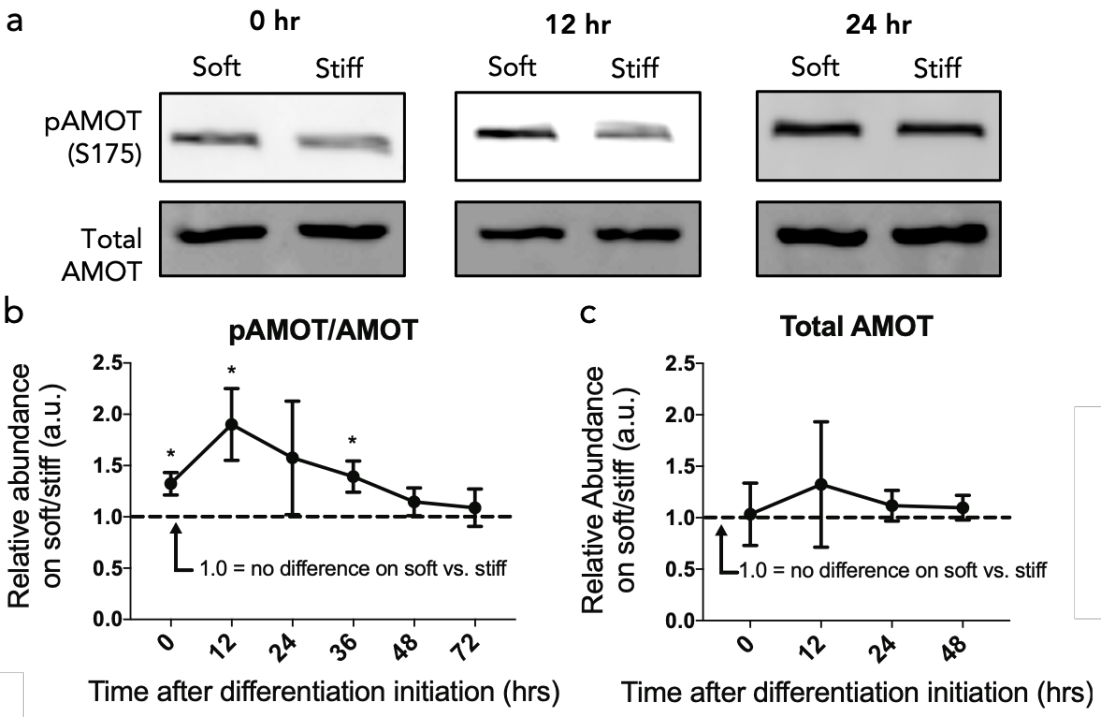

Figure 2.7 AMOT phosphorylation is enhanced during early differentiation on soft substrates. (a) Representative western blots of pAMOT and total AMOT in NSCs differentiating on soft (500 Pa) or stiff (73 kPa) substrates at 0, 12, and 24 hours post-differentiation initiation. Cells were cultured in mixed differentiation conditions (1 μM retinoic acid + 1% FBS). Quantification of the ratio of (b) pAMOT/AMOT or (c) total AMOT on soft vs. stiff at each timepoint where a ratio of 1 indicates equivalent detection on soft and stiff at that timepoint. Error bars represent SD (n = 3 biological replicates). *p<0.05 by one-sample t-test compared to hypothetical value of 1.0.

2.5 Discussion

Recently, it has become clear that extracellular stiffness and other biophysical cues are key inputs for stem cell regulation, but the intracellular mechanisms that transduce these signals remain incompletely elucidated. Furthermore, our findings that YAP influences mechanosensitive differentiation in NSCs through a non-canonical interaction with β-catenin[18] motivated us to more closely examine mechanisms regulating YAP. To address these questions, we investigated AMOT, which has been shown to link F-actin and YAP in prior studies that did not study stem cells or cellular mechanics[50,66]. Specifically, we interrogated the importance of AMOT in stiffness-sensitive neurogenesis, the biochemical and functional consequences of AMOT phosphorylation, and the temporal tuning of pAMOT during early fate commitment. The results presented here describe a critical role for AMOT, whose ablation leads to a striking loss of neurogenesis on soft substrates. Furthermore, overexpression of ΔAB AMOT, a mutant

lacking the actin-binding domain, or phosho-mimetic (S175E) AMOT, which is functionally inhibited from binding actin, rescued neurogenesis on stiff substrates while overexpression of WT or S175A did not.

Previous studies have shown that mesenchymal stem cells can display "mechanical memory" where earlier presentation of mechanical cues can influence long-term cell behavior[17,20,97,114]. Further exploring this concept, we discovered that NSCs are sensitive to stiffness inputs only within an early, relatively narrow time window of differentiation in culture (12-36 hours). Therefore, intracellular signaling events that ultimately influence fate commitment should also occur within a similar time frame. For example, YAP levels in NSCs differentiating on stiff substrates are higher compared to NSCs on soft substrates, and this difference is maximized between 24-48 hours[17]. Strikingly consistent with this timing, we found that AMOT phosphorylation was most increased 12 and 36 hours after differentiation initiation on soft substrates vs. stiff substrates. Previous work has shown that AMOT phosphorylated at the S175 residue can display enhanced YAP binding and antagonism upon being freed from F-actin[50]. Therefore, the approaches we employed in this work and its findings further delineate the intricate mechanisms by which stiffness input can direct NSC fate commitment through an AMOT-YAP signaling axis, providing crucial insights into the continuous cascade of mechanotransductive events within stem cells.

2.7 Acknowledgements

We kindly thank Dr. Dannel McCollum (University of Massachusetts Medical School, Worcester, MA) for sharing his lab's pCDNA constructs encoding WT, S175E, and S175A AMOT. We also thank Qianhe Zhang (University of California, Berkeley, Berkeley, CA) for her experimental assistance. P.H.K, S.K., and D.V.S. gratefully acknowledge grant support from the National Institutes of Health (grant #5R01NS074831: Mechanisms of Neural Stem Cell Mechanoregulation), awarded to S. K. and D.V. S..

Chapter 3 : Angiomotin as a link between Rho-ROCK and non-canonical YAP/β-catenin signaling

3.1 Abstract

Previous work in our group and others have shown that cytoskeletal dynamics directed by Rho GTPases and the activity of mechanosensitive transcription factors can orchestrate stiffness-sensitive fate commitment in stem cells. However, how those two nodes of signaling events are connected is still an open question in many contexts. In neural stem cells (NSCs), we have shown that YAP impacts neurogenesis by inhibiting β-catenin and not through its transcriptional regulatory activity in association with TEAD transcription factors in the nucleus. Therefore, the stiffness-sensitive upstream regulation of YAP activity is still unclear in NSCs. We sought to investigate if AMOT could bridge this signaling gap and regulate YAP in a Rho signaling-dependent manner. Treatment with various small molecule inhibitors of ROCK and other RhoA downstream effectors revealed that AMOT subcellular localization is sensitive to this stiffness-influenced pathway, suggesting that AMOT is downstream of RhoA signaling. Further, we showed that AMOT is necessary for the rescue of neurogenesis on stiff substrates upon inhibition of ROCK and myosin II. Significantly, we found that mutation of AMOT's YAP-binding motifs reversed the neurogenic effect of a phospho-mimetic (S175E) mutation in overexpressed AMOT, suggesting that AMOT's influence on neurogenesis is YAP-dependent. We further found that overexpression of S175E AMOT inhibits YAP and promotes β-catenin activity by western blotting and a luciferase reporter of β-catenin-based transcription, further supporting that AMOT promotes neurogenesis as an upstream inhibitor of YAP.

3.2 Introduction

In addition to soluble or anchored biochemical cues, cells are also capable of responding to the mechanical properties of their surroundings such as the stiffness, topography, porosity, and viscoelasticity[15,115,116,117]. This process of sensing extracellular mechanical cues and converting them into intracellular biochemical signals that elicit biological changes within the cell is often referred to as mechanotransduction. The field has demonstrated that mechanical cues can direct a myriad of cellular processes including cell migration (durotaxis), changes to cell morphology and size, proliferation, and differentiation[118,119,120]. Because stem cells are characterized by their ability to self-renew through proliferation and differentiate into one or more functional cell types, understanding how mechanical cues can direct those processes is critical to understanding stem cell biology.

Cells can adjust their own intrinsic mechanical properties in response to mechanical cues from their extracellular microenvironment. For example, our group has previously shown that

NSCs cultured on stiffer substrates are significantly stiffer compared to those cultured on softer substrates, demonstrating NSCs' propensity for "mechanoadaptation"[16]. These responsive changes are largely accomplished through changes in the cellular cytoskeleton, which serves a wide range of functions within the cell, including establishing the physical infrastructure and generating tension[121,122]. The Rho family of GTPases are group of small G-proteins that play central roles in regulating actin dynamics and are therefore critical in this phenomenon. Rho GTPases are molecular switches that actively signal to their downstream targets when they are GTP-bound but are inactive in their GDP-bound state[123]. Accordingly, our previous work has shown that the Rho GTPases RhoA and Cdc42, but not Rac, are more activated when NSCs are cultured on stiff substrates compared to softer substrates. Furthermore, overexpression of constitutively active (CA) or dominant negative (DN) mutants of RhoA and Cdc42 prevent NSCs from properly adjusting their mechanics to stiffness cues. Wild-type NSCs display mechanosensitive differentiation whereby soft substrates that more closely mimic the stiffness of their endogenous niche promote strong neurogenesis while stiff substrates suppress neurogenic differentiation and promote astrogenesis. Importantly, overexpression of CA RhoA or Cdc42 suppresses neurogenesis on soft substrates while DN RhoA and Cdc42 rescue neurogenesis on stiff substrates[8]. Therefore, Rho GTPases and the cytoskeletal processes they control are important signaling events that underlie mechanosensitive differentiation in NSCs.

However, the precise mechanism by which Rho GTPases impact mechanosensitive neurogenesis remains unclear. Other groups have shown that cytoskeletal tension, the degree of F-actin polymerization, and stress fiber formation are important cellular responses that can dictate mechanotransduction in stem cells[124,125,126]. Because RhoA is most directly associated with stress fiber formation and actomyosin contractility and our prior work has demonstrated its importance in NSCs specifically, RhoA is a prime candidate for further mechanistic study. RhoA signals to a number of downstream effector proteins, notably Rho-associated protein kinase (ROCK), which increases myosin contractility by promoting phosphorylated myosin light chain (MLC) and also promotes F-actin polymerization indirectly by promoting LIM kinase-based inhibition of cofilin, which disassembles actin filaments. Diaphanous-related formins (DRFs) are another major class of RhoA effector proteins and also promote actin polymerization at the growing barbed ends of filaments[127].

Ultimately, fate commitment in stem cells is directed by changes in transcriptional programming towards the desired cell lineage[128,129,130]. In the case of mechanosensitive differentiation, extracellular biophysical cues must be translated into biochemical signaling that impacts the transcriptional machinery or expression of specific genes. In multiple stem cell contexts including NSCs, Yes-Associated Protein (YAP) and the related protein transcriptional co-activator with PDZ-binding motif (TAZ) have been reported as exciting mechanosensitive transcriptional regulators that can direct stem cell lineage commitment[17,131,132]. Canonically, YAP is active within the nucleus where, upon association with the TEAD family of transcription

factors, it upregulates pro-proliferative and pro-survival genes. Phosphorylation of YAP via the serine/threonine large tumor suppressor kinase (LATS) of the Hippo signaling pathway in mammals targets YAP for binding to 14-3-3 proteins, which anchor YAP to the cytoplasm and prevent its nuclear translocation while also promoting its degradation[133]. Therefore, YAP's relative nuclear vs. cytoplasmic localization has been commonly used in the literature as an indirect indicator of YAP activity. In mesenchymal stem cells (MSCs), stiffer substrates promote YAP nuclear localization to drive increased osteogenesis while suppressing adipogenic differentiation compared to soft substrates[131]. Further studies have shown that YAP acts as a stiffness-sensitive switch that can oscillate between active and inactive states based on cytoskeletal tension and dynamics, directly connecting YAP to the cytoskeleton[134,135,136]. However, we have shown that in NSCs, YAP's interaction with the TEAD proteins is not functionally necessary. While YAP's abundance does increase in NSCs differentiated on stiff substrates, we have observed that YAP localization does not significantly shift with varying stiffnesses. Further investigation revealed that YAP's antagonism of β-catenin was the mechanism for YAP-based suppression of neurogenesis[17]. Therefore, there may be unique upstream regulators of YAP activity in NSCs that help orchestrate mechanosensitive differentiation.

Angiomotin (AMOT)-based signaling is a is a potential downstream consequence of Rho-ROCK signaling that can direct mechanosensitive differentiation in NSCs in a YAP-dependent manner. First, AMOT can bind to F-actin, which can sequester AMOT from interacting with its own downstream targets, including YAP[49,62 ,102]. Indeed, previous studies have shown that AMOT's activity can be controlled by a competitive binding axis between F-actin-AMOT and YAP-AMOT[50]. Since F-actin polymerization is a stiffness-sensitive consequence of RhoA signaling, it is possible that AMOT could also be regulated by the actin cytoskeleton in NSCs by this mechanism. Furthermore, AMOT's F-actin binding is inhibited by phosphorylation at its S175 residue by LATS[28]. Previous studies have shown that AMOT can directly impact YAP activity[51,137,138] , while others have pointed to the importance of LATS-based regulation of AMOT[49,50,139]. Therefore, AMOT may be an important parallel mechanism of YAP inhibition either by Hippo-dependent or -independent mechanisms. In NSCs, our previous work showing that overexpression of phospho-mimetic (S175E) but not phospho-null (S175A) or wild-type AMOT rescues neurogenesis on stiff substrates suggests that phosphorylation is a critical regulator of AMOT's activity in directing mechanosensitive differentiation (Chapter 2).

In this study, we sought to understand if AMOT was a signaling bridge in between Rho-ROCK signaling and YAP, which would connect mechanosensitive cytoskeletal dynamics to the non-canonical YAP/β-catenin signaling that we have observed in NSCs. To do this, we employed various small molecule inhibitors of ROCK, formins, myosin II, and F-actin to interrogate if those perturbations affected AMOT's stiffness-sensitive variable localization.

Additionally, we have reported in the past that inhibiting various cytoskeletal elements rescues neurogenesis on stiff substrates. Therefore, we tested if AMOT was a necessary signaling component for that outcome, which would further implicate AMOT downstream of Rho-ROCK signaling. Furthermore, we wanted to test if AMOT promoted neurogenesis by regulating YAP activity. Previous studies have discovered that AMOT has two PPxY and one LPxY motif that facilitate its direct binding to YAP's WW domains[49]. This interaction can be inhibited by mutating the three tyrosine (Y) residues in the L/PPxY motifs to alanines (A). Therefore, we created YAP binding-deficient mutations in the S175E AMOT mutant, which we previously showed rescued neurogenesis on stiff substrates (Chapter 2). We then measured the differentiation of this S175E + mYB (YAB-binding mutant) AMOT on soft and stiff substrates. To further measure if AMOT inhibits YAP activity and the potential functional consequences, we performed western blots to determine the degree of YAP and β-catenin activation as well as a luciferase reporter assay of β-catenin transcriptional activity. These studies enabled us to investigate if AMOT was mechanistically downstream of Rho-ROCK and upstream of YAP/β-catenin to regulate mechanosensitive differentiation in NSCs.

3.3 Materials and methods

3.3.1 Polyacrylamide gel synthesis and functionalization

Polyacrylamide-bis precursor solutions were made for each stiffness by mixing various concentrations of acrylamide monomer and bis-acrylamide crosslinker (Bio-Rad). Solution compositions to achieve various final polymerized stiffnesses were as follows: 0.2 kPa = 3% acrylamide + 0.04% bis, 0.5 kPa = 3% acrylamide + 0.1% bis, 72 kPa = 10% acrylamide + 0.3% bis[16]. Polyacrylamide gels were synthesized on 19- or 25-mm glass coverslips with 0.1% TEMED + 1% Ammonium Persulfate. Polyacrylamide gels were then functionalized with laminin conjugation via sulfo-SANPAH (Thermo-Fisher) to facilitate cell attachment.

3.3.2 Cell culture and inhibitor treatment

Adult rat hippocampal NSCs were cultured in DMEM/F12 with N2 supplement (Life Technologies) on tissue-culture polystyrene plates that had been coated with poly-ornithine and laminin. Growth conditions for NSCs included 20 ng/mL FGF-2 whereas mixed differentiation conditions included 1% FBS + 1 μM retinoic acid. For full differentiation experiments on polyacrylamide substrates, NSCs were first seeded onto the gels in growth media for 16-18 hours before the coverslips were then transferred into new wells with mixed differentiation media. The NSCs were allowed to differentiate for 6 days with 50% media changes every 2 days before fixation.

The concentrations of inhibitors used were as follows: 10 μM Y-27632 (ROCK inhibitor), 10 μM SMIFH2 (formin inhibitor), 1 μM blebbistatin (myosin II inhibitor), and 1 μM Latrunculin A (F-actin inhibitor). In full differentiation experiments, inhibitors were added along with differentiation media for the first 48 hrs, after which differentiation media without inhibitors was used. In experiments shorter than 48 hrs, inhibitors were also added with the differentiation media and left for the duration of the experiment.

3.3.3 Constructs and antibodies

pcDNA4 plasmids with cDNA encoding WT, ΔAB, S175E, and mYB AMOT were a generous gift from D. McCollum (University of Massachusetts Medical School, Worcester, MA). The AMOT cDNA sequences were PCR-amplified, digested with SfiI and PmeI, and cloned into the pCLPIT vector. S175A and S175E + mYB AMOT cDNA sequences were generated using the QuikChange Site-Directed Mutagenesis system (Agilent Technologies) before also being digested and cloned into the pCLPIT vector.

Primary antibodies and dilutions used were as follows: Tuj1 (1:750; BioLegend 801201), AMOT (1:1,000 for western blotting, 1:250 for immunocytochemistry; Bethyl Laboratories A303-303A), c-Myc (1:250; Santa Cruz Biotechnology A-14), pAMOT (1:1,000 for western blotting, 1:250 for immunocytochemistry; EMD Millipore ABS1045), YAP (1:1,000; Cell Signaling Technologies 4912S), pYAP (1:1,000; Cell Signaling Technologies 4911S), β-catenin (1:1,000 Cell Signaling Technologies 9562S), non-phospho β-catenin (1:1,000; Cell signaling Technologies 8814S), β-actin (1:100,000; Sigma-Aldrich A1978), GAPDH (1:1,000; Sigma-Aldrich G8795)

3.3.4 Viral packaging and transduction

CLPIT vectors encoding the various human AMOT cDNA were packaged as retroviruses via calcium phosphate-based transfection into HEK 293T cells (10 μg transfer vector + 6 μg pCMV gag-pol + 4 μg pcDNA3 IVS VSV-G per 10 cm diameter plate). Supernatants were collected 48 and 72 hours post-transfection and pooled before filtration and ultracentrifugation to purify the viral particles. Cells were transduced in all cases at an MOI of 1-2 as calculated by puromycin resistance titer and selected with 0.6 μg/mL puromycin for at least 4 days[109].

3.3.5 Western blotting

Cell lysates were collected in RIPA buffer + 1% sodium molybdate, 3% sodium fluoride, and 1X Halt Protease/Phosphatase inhibitor cocktail (Thermo Fisher). 1-10 μg total protein was loaded per well of a precast 4-12% gradient polyacrylamide gel cassette. Within each experiment, the total amount of protein loaded for each sample was normalized using BCA

protein quantification. Gel electrophoresis and protein transfer onto nitrocellulose membranes were carried out using a Life Technologies Mini Gel Tank. Antibody solutions and blocking was carried out with LI-COR blocking buffer before membrane imaging on a LI-COR Odyssey Blot Imager.

3.3.6 Immunofluorescence staining and imaging

Cells were fixed in 4% paraformaldehyde (Alfa Aesar) for 10 minutes before washing in PBS and permeabilization in 5% goat serum + 0.5% Triton X-100 (Sigma-Aldrich) for 10 minutes. Permeabilized cells were blocked in 5% goat serum for 1 hour at room temperature before immunostaining. Primary and secondary antibody solutions were also made in 5% goat serum.

Epifluorescent images were taken using a Nikon Eclipse Ti Microscope, Hamamatsu Photonics K.K. C10600-10B-H camera, 10X objective lens, and native NIS-Elements AR 5.02.00 software. Samples were submerged in PBS during image acquisition. Nuclei were labeled with a DAPI stain (Sigma-Aldrich) and Tuj1 was labeled with a 488 dye-conjugated secondary antibody (Thermo-Fisher). Confocal images were taken using a Prairie Technologies 2-photon and confocal microscope, QuantEM 512SC camera, 60X objective lens, and native Prairie View software. Samples were submerged in PBS during image acquisition. Nuclei were labeled with a DAPI stain, F-actin was labeled with an Alexa Fluor 546 Phalloidin (Thermo-Fisher), and other targets were labeled with either a 488 or 633 dye-conjugated secondary antibody. Super-resolution images were taken using Structured Illumination Microscopy (SIM). Cells were cultured on laminin-coated glass coverslips and were secured to microscope slides prior to imaging after fixation and staining. Nuclei were labeled with a DAPI stain, F-actin was labeled with an Alexa Fluor 546 Phalloidin (Thermo-Fisher), and AMOT was labeled with a 488 dye-conjugated secondary antibody. All image processing and analysis was carried out in the free ImageJ software.

3.3.7 Luciferase assay

AMOT S175A, S175E, and S175E + mYB cells expressing ectopic AMOT to similar levels were further transduced with a lentiviral construct encoding a 7xTFP TFC/LEF luciferase reporter, which is responsive to β-catenin-TCF/LEF-based transcription. Lysates were collected after 24 hours of differentiation in mixed conditions on stiff (73 kPa) substrates using the provided lysis buffer from the Luciferase Assay System kit (Promega). The Luciferase Assay was carried out according to the manufacturer's protocol in a 96-well plate format and measurements were taken with a plate-reading luminometer.

3.4 Results

3.4.1 Super-resolution microscopy of AMOT and F-actin in NSCs

While other reports[48,50] and our own studies (Fig. 2.1) have shown that AMOT can clearly localize to actin-based fibers in other cell types such as endothelial cells, HEK 293Ts, and mesenchymal stem cells (MSCs), we have observed that the mammalian NSCs that we use in our studies do not show the prominent stress fiber-like structures that are often displayed in these and other cell types when imaged with conventional microscopy methods such as epifluorescence or confocal microscopy. Therefore, we wondered if super-resolution microscopy would enable us to image the interior actin cytoskeleton with greater clarity to see if AMOT also localizes to F-actin in NSCs. We cultured NSCs on laminin-coated glass coverslips and differentiated them for 24 hours prior to fixation and staining. Using Structured Illumination Microscopy (SIM), we found that AMOT displays a strikingly fiber-like pattern that is undetectable with confocal microscopy (Fig. 3.1). Therefore, it is plausible that AMOT's activity can be regulated through its interaction with the actin cytoskeleton.

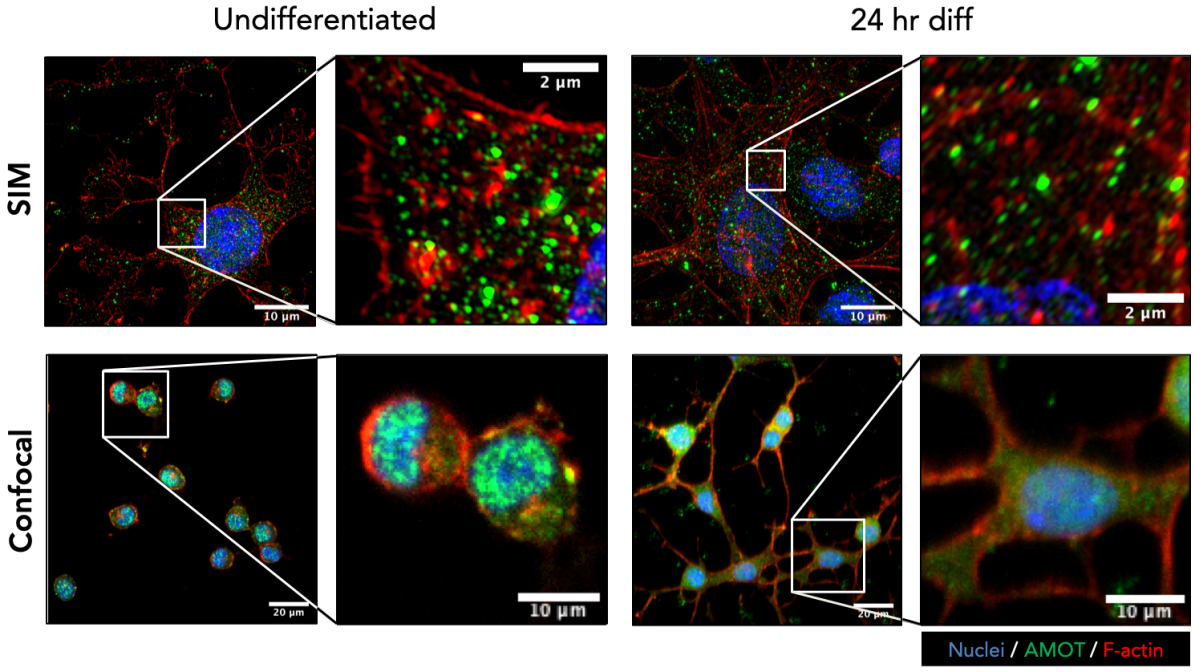

Figure 3.1 AMOT displays fiber-like pattern when observed with super-resolution microscopy. Structured Illumination Microscopy (SIM, top) reveals more intricate cytoskeletal structure with greater resolution. SIM also reveals AMOT displaying a clear fiber-like pattern after 24 hours of differentiation that is not observed under confocal microscopy. Cells were cultured for in 20 ng/mL FGF-2 for undifferentiated samples or for 24 hours in mixed differentiation conditions (1 µM retinoic acid + 1% FBS) on laminin-coated glass (for SIM) or tissue culture plastic (for confocal). Cells were fixed in 4% PFA and stained with DAPI for nuclei (blue), AMOT (green), and phalloidin for F-actin (red).

3.4.2 The effect of inhibiting RhoA's downstream cytoskeletal effectors on AMOT

Rho GTPases have been reported to mediate many cellular processes including cytoskeletal remodeling, proliferation, migration, and differentiation through their modulation of the actin cytoskeleton[16,140,141,142,143,144]. In NSCs, we have shown that the Rho GTPases RhoA and Cdc42 are activated in response to increased substrate stiffness, and that constitutive activation of these proteins reduces neurogenesis *in vitro* and *in vivo*[16]. Therefore, we tested if AMOT is regulated by Rho GTPase signaling and potentially responsible for the influence of Rho GTPase activity on stiffness-sensitive NSC fate commitment.

To investigate functional contributions of Rho GTPase signaling, we used inhibitors for ROCK (Y-27632), formins (SMIFH2), and myosin II (blebbistatin), which all lie downstream of Rho activation (Fig. 3.2c). As shown in Chapter 2 (Fig. 2.5), NSCs undergoing differentiation on stiff substrates display enrichment of AMOT in the nucleus. However, treatment with any of the inhibitors for the first 24 hours of differentiation reduced the degree of nuclear AMOT localization and resulted in a subcellular distribution similar to that of cells cultured on soft substrates (Fig. 3.2a-b). Therefore, stiffness-induced myosin contractility mediated by Rho influences AMOT subcellular localization during NSC fate commitment.

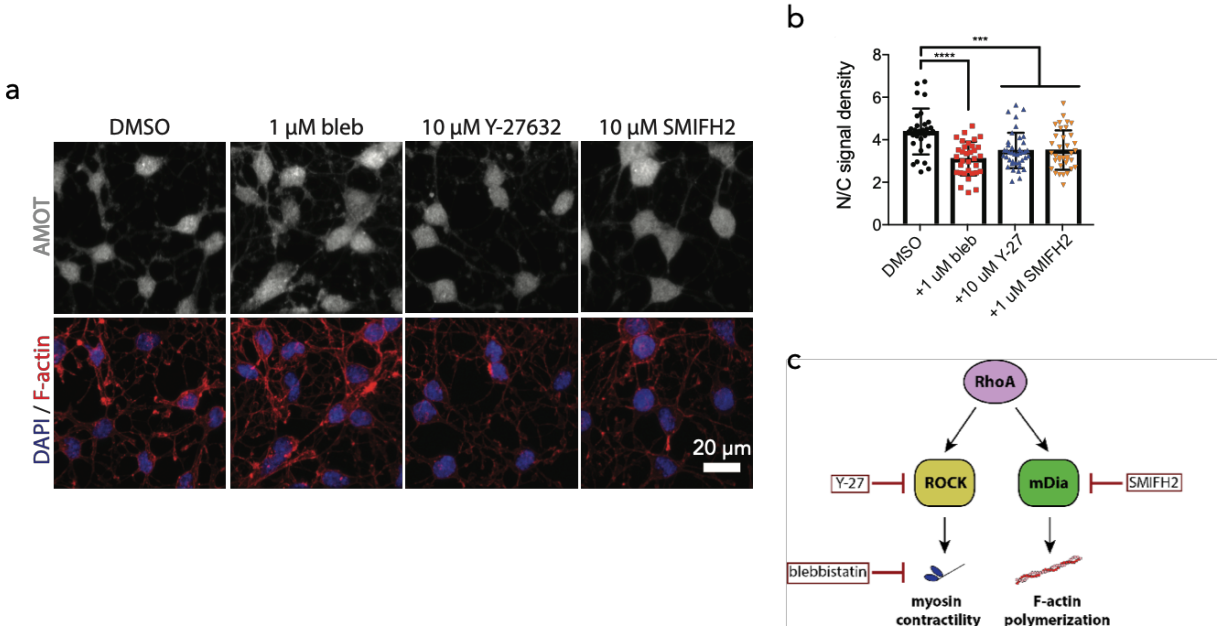

Figure 3.2 Subcellular AMOT localization is influenced by RhoA signaling components.
(a) Representative images of NSCs that have been differentiated for 24 hours in mixed differentiation media conditions (1μM retinoic acid + 1% FBS) with indicated inhibitors or DMSO control added. Cells were fixed and stained for AMOT (greyscale), nuclei (blue), and F-actin (red). (b) Quantification of AMOT's nuclear/cytoplasmic (N/C) ratio in the various conditions. $n \geq 45$ cells in all conditions. ***$p<0.001$, ****$p<0.0005$ by one-way ANOVA followed by Tukey's post-hoc test. (c) Diagram depicting the inhibitors used and their respective targets.

3.4.3 The necessity of AMOT on RhoA-based regulation of neurogenesis

Inhibition of ROCK, formins, and myosin are all expected to convert cells into low-tension states that are reminiscent of cells cultured on soft substrates, and application of these inhibitors often phenocopies effects of soft or confined substrates on stem cell differentiation[120,124]. Appropriately, we have previously shown that treatment with blebbistatin and Y-27632 rescues neurogenesis on stiff substrates[16]. To test if AMOT was responsible for this effect, we differentiated AMOT KD (shAMOT) or non-targeting scramble cells on stiff substrates for 6 days before fixing and staining for neuronal markers. Indeed, we found that while blebbistatin and Y-27632 treatment both rescued neurogenesis in scramble control cells, shAMOT cells were unresponsive to inhibitor treatment (Fig. 3.3b-c). In addition, consistent with previous reports, we observed that blebbistatin and Y-27632 treatment influenced cellular morphology in scramble cells, which displayed increased neurite length and decreased neurite branching when compared to DMSO treatment controls[112,145](Fig. 3.3a). Interestingly, AMOT KD desensitized neurite length and branching to these inhibitors (Fig. 3.3a), suggesting that AMOT may not only be influenced by cytoskeletal dynamics but may be an active participant in Rho/ROCK- dependent cytoskeletal remodeling.

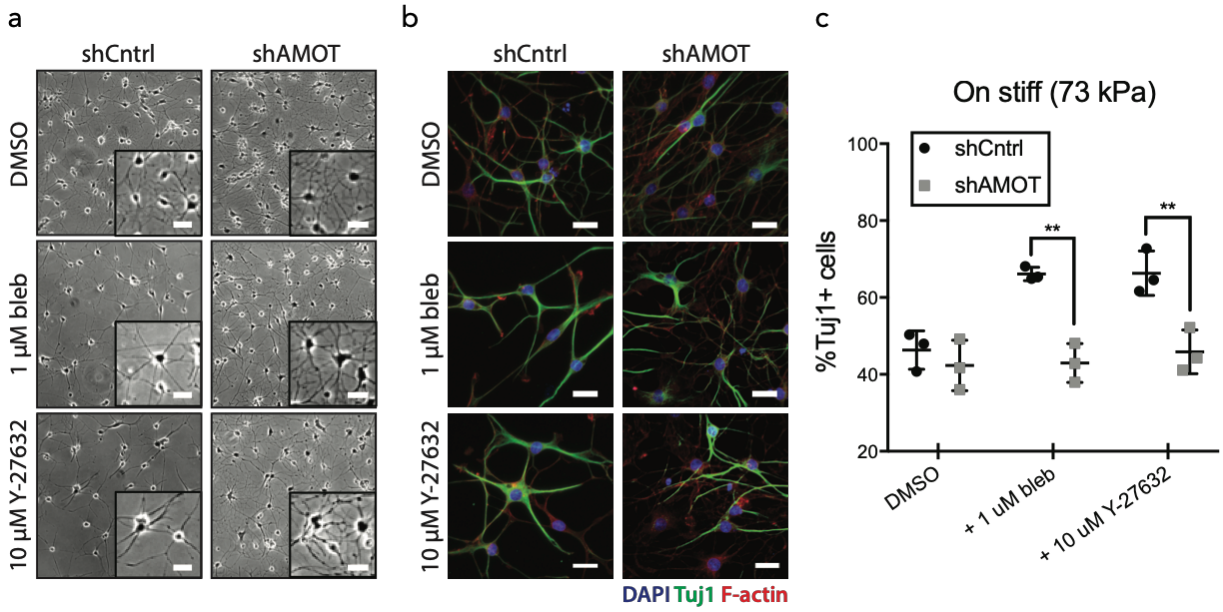

Figure 3.3 AMOT is necessary for neurogenesis that is sensitive to cytoskeletal perturbation. (a) Representative brightfield images of cells after 6 days of differentiation in mixed differentiation conditions (1µM retinoic acid + 1% FBS) on stiff substrates (73 kPa) and in the presence of 1 µM blebbistatin, 10 µM of Y-27632, or DMSO control. Insets are zoomed-in portions from larger ROI that they are embedded within. (b) Representative immunostaining images after differentiation. Cells were fixed in 4% PFA and stained for nuclei (blue), Tuj1 (green), a neuronal marker, and F-actin (red). (c) Quantification of Tuj1+ cells after differentiation. **p<0.005 by two-way ANOVA followed by Tukey's post-hoc test.

3.4.4 Testing if AMOT influences neurogenesis in a YAP-dependent manner

Multiple reports have shown that YAP is a crucial molecular rheostat within cells and can direct stem cell self-renewal and differentiation in a stiffness-sensitive manner[131,146,147]. We have previously shown that while YAP is increased in differentiating NSCs on stiff substrates, its effect is mediated by its interaction with β-catenin rather than its canonical downstream effectors, the TEAD transcription factors[16]. Studies in other cell systems have indicated that AMOT can inhibit YAP via direct binding, and that this inhibition is enhanced when AMOT is phosphorylated[49,50]. However, no studies to-date have experimentally investigated whether the AMOT-YAP interaction is important in mechanotransductive contexts, though it has been hypothesized[148]. Therefore, we examined if AMOT's pro-neurogenic effect is regulated by substrate stiffness through its phosphorylation and ability to impact YAP and/or β-catenin activity.

AMOT is known to bind to YAP's WW domains through three L/PPxY motifs near its N-terminus. Therefore, we generated an AMOT variant with a phospho-mimetic S175E mutation as well as mutations in its three L/PPxY motifs (S175E + mYB) to determine if an interaction with YAP is essential for S175E AMOT's pro-neurogenic function. Strikingly, overexpressed S175E + mYB AMOT completely lacked the capacity to rescue neurogenesis on a stiff substrate (Fig. 3.4a-b). This finding strongly suggests that AMOT, and phosphorylated AMOT in particular, influences NSC fate commitment in a YAP-dependent manner.

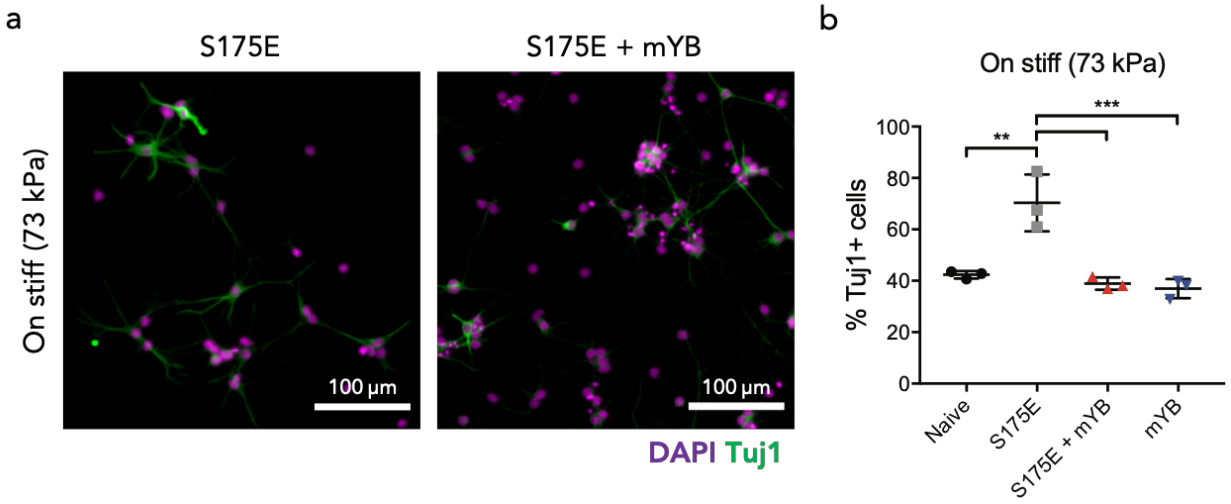

Figure 3.4 AMOT's YAP binding capability is necessary for its neurogenic effect.
(a) Representative immunofluorescence images of cells that were differentiated for 6 days in mixed differentiation conditions (1µM retinoic acid + 1% FBS) on stiff substrates (73 kPa) prior to fixing in 4% PFA and staining for nuclei (magenta) and Tuj1 (green), a neuronal marker. S175E = NSCs overexpressing phosho-mimetic AMOT, S175E + mYB = NSCs overexpressing AMOT with the phospho-mimetic mutation and additional mutations of its three L/PPxY YAP-binding motifs ("mYB" = YAP-binding mutant). (b) Quantification of Tuj1+ cells after differentiation. mYB = YAP-binding mutant. **p<0.005, ***p<0.001 by one-way ANOVA followed by Tukey's post-hoc test.

3.4.5 AMOT's effect on YAP and β-catenin activity

While the result above indicated that S175E AMOT must bind YAP to promote neurogenesis, we sought to further test if AMOT had direct functional consequences on YAP and β-catenin activity downstream. Because phosphorylation inactivates both YAP and β-catenin, we used western blotting to measure the relative inactive or active fractions of both proteins upon AMOT overexpression. We collected lysates from NSCs overexpressing S175E, S175E + mYB (YAP-binding mutant), or S175A AMOT after 24 hours of differentiation on stiff substrates (73 kPa) to analyze the activity of YAP and β-catenin. We found that S175E cells displayed increased YAP phosphorylation, a marker for YAP inactivation, and active (unphosphorylated) β-catenin by western blotting compared to S175E + mYB cells (Fig. 3.5a-c). Next, we wanted to more directly test if the changes in β-catenin phosphorylation functionally impacted its transcriptional activity. To do this, we generated cells carrying a 7xTFP luciferase reporter for β-catenin/TCF/LEF transcriptional activity and then overexpressed the various AMOT mutant variants in that reporter cell line (Fig. 3.5d). Notably, the cells were selected by antibiotic resistance after transduction with the reporter construct but not after transduction with the AMOT expression constructs. However, the NSCs were infected at the same estimated multiplicity of infection per AMOT variant to control for differences in AMOT expression and experiments were conducted within 16 hours after transduction. After 24 hours of differentiation on stiff substrates, we collected and analyzed lysates for β-catenin/TCF/LEF-driven luciferase expression. Strikingly, we found that β-catenin/TCF/LEF activity was higher in S175E cells than in S175A or S175E + mYB cells (Fig. 3.5e), which is consistent with the western blotting results (Fig. 3.5b-c). Taken together, these experiments indicate that AMOT phosphorylation promotes neurogenesis by inhibiting YAP and promoting β-catenin activity.

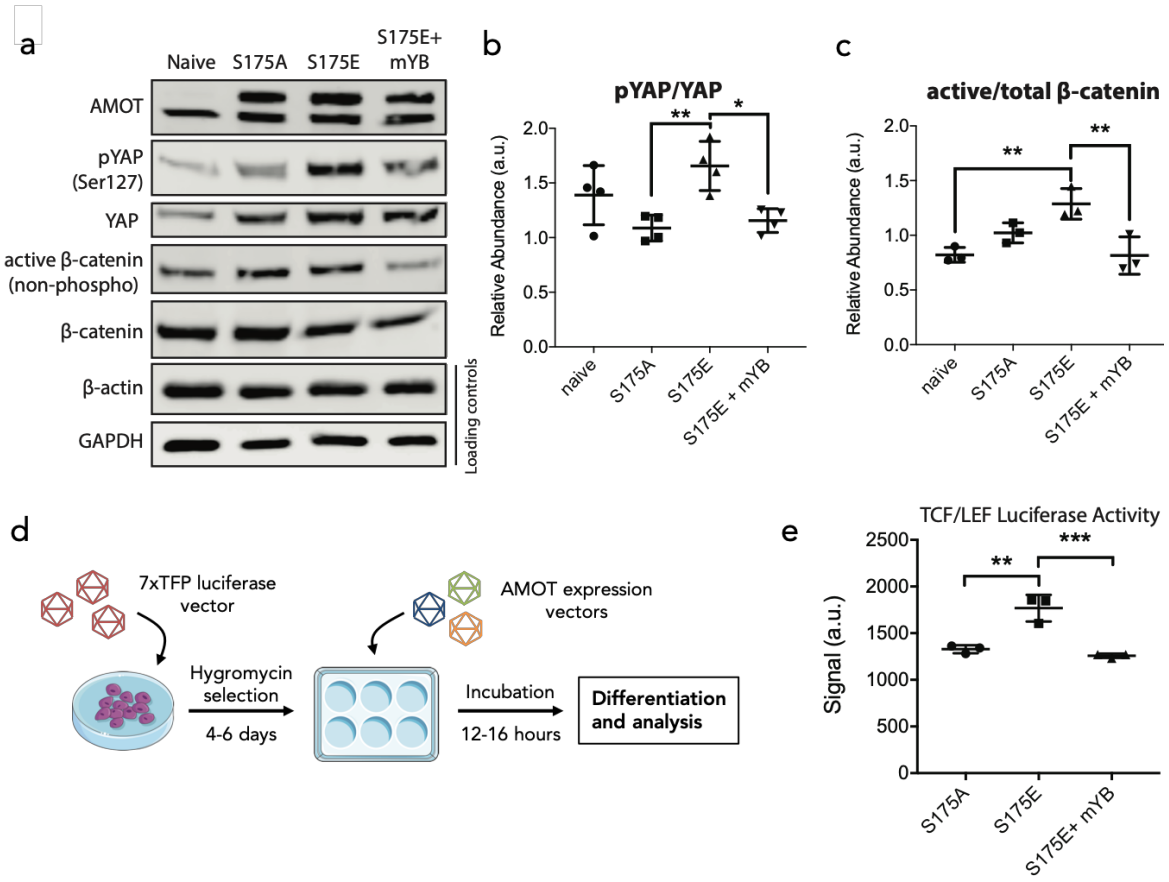

Figure 3.5 Phosphorylated AMOT promotes neurogenesis by inhibiting YAP and promoting β-catenin activity.

(a) Representative western blots from lysates collected from NSCs overexpressing phospho-null (S175A), phospho-mimetic (S175E), or phospho-mimetic + YAP non-binding (S175E + mYB) AMOT. Lysates were collected after 24 hours of differentiation on stiff substrates and analyzed for total YAP, pYAP (inactive), total β-catenin, non phospho-β-catenin (active), and loading controls. (b-c) Quantification of western blot bands normalized to both GAPDH and β-actin (n = 3). (d) Diagram depicting strategy for TCF/LEF luciferase reporter cell line development and experimentation. (e) Quantification of luminescence signal from cells acutely overexpressing S175A, S175E, or S175E + mYB AMOT in addition to stably expressing the 7xTFP luciferase reporter construct. Cells were differentiated for 24 hours on stiff substrates prior to lysate collection (n = 3). *p<0.05, **p<0.005, ***p<0.001 by one-way ANOVA followed by Tukey's post-hoc test.

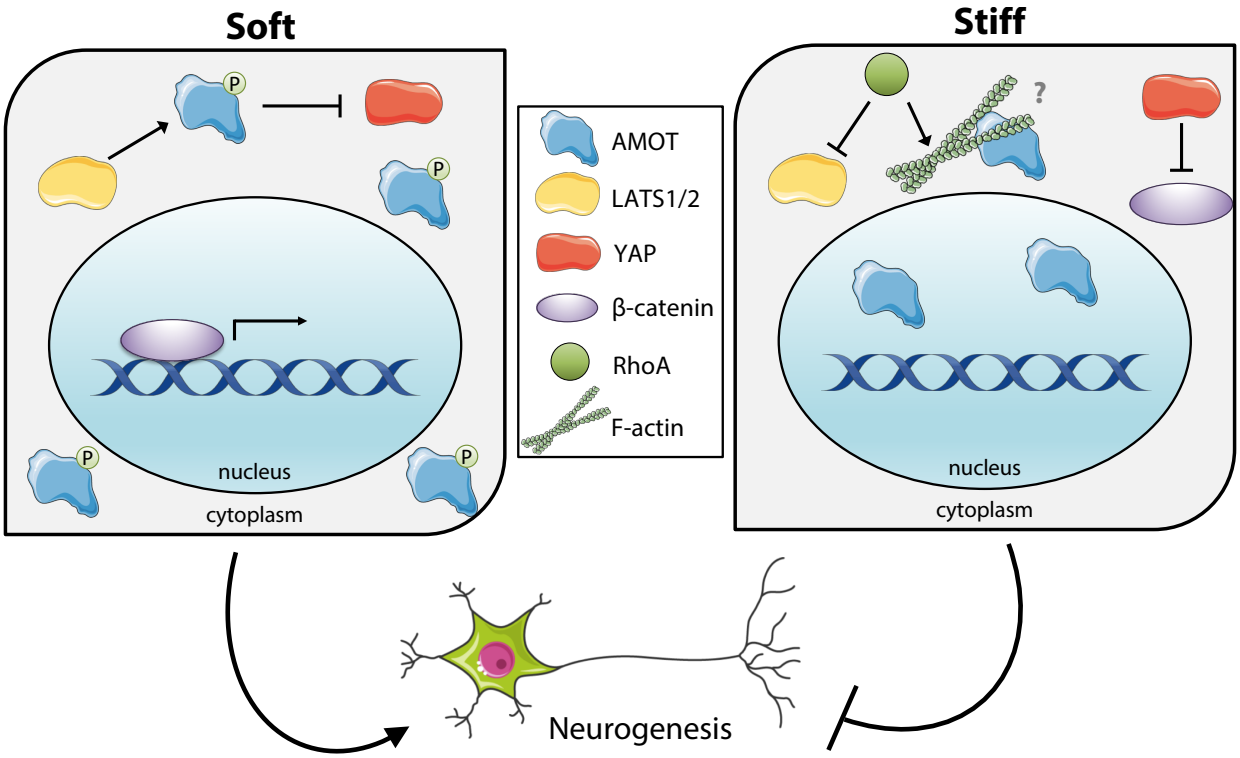

Figure 3.6 Proposed mechanism for AMOT's influence on stiffness-sensitive neurogenesis on NSCs.
The mechanoregulation of AMOT phosphorylation influences AMOT's localization and regulation of YAP, ultimately driving or inhibiting neurogenesis.

3.5 Discussion

It has been well established that cytoskeletal changes are an integral cellular response to extracellular mechanical cues and that Rho GTPases play a critical role in this process. In addition to its canonical effect of promoting cytoskeletal (re)organization and intracellular tension generation, the small GTPase RhoA has a myriad of described and yet-undescribed downstream effectors that can impact a wide range of cellular functions. In the context of stem cells, RhoA has been demonstrated to orchestrate mechanosensitive differentiation such that culture on soft or stiff matrices lead to varying fate commitment outcomes for populations of stem cells. While some studies have been able to directly link various aspects of cytoskeletal signaling, such as actin processing factors or the ratio of globular actin monomers (G-actin) to filamentous polymeric actin (F-actin), to the regulation of transcription factors that can direct fate commitment, a similar system has not yet been described in neural stem cells, which are of significant therapeutic interest. Therefore, we investigated if Angiomotin (AMOT) is regulated by RhoA signaling and is an important pathway component in NSC mechanosensitive fate commitment.

In the previous chapter, we showed that AMOT was necessary for robust neurogenesis on soft substrates and that AMOT's phosphorylation and subcellular localization could regulate AMOT's impact on neurogenesis in a stiffness-sensitive manner. Here, we demonstrated that various downstream effectors of RhoA signaling, namely ROCK, formins, and myosin II can influence AMOT localization, suggesting that RhoA mediates the impact of stiffness on AMOT regulation. Furthermore, our work (Chapter 2) and others have shown that endogenous pAMOT or phospho-mimetic exogenous AMOT preferentially localize to the cytoplasm[50,62], so the localization of AMOT seems to be a critical element of its activity. Initially, this seemed counter-intuitive as AMOT phosphorylation at S175 has been demonstrated to robustly inhibit AMOT's interaction with F-actin[67]. Therefore, one might expect pAMOT to not be enriched in the cytoplasm, which is abundant in cytoskeletal actin fibers. Through preliminary exploration of this interesting result, we found that there is a putative bipartite nuclear localization signal (NLS) of moderate predicted strength that includes the S175 residue. Therefore, it is possible that phosphorylation of that serine residue could disrupt AMOT nuclear transport. In summary, in NSCs, RhoA regulates AMOT localization, which seems intimately linked with and possibly regulated by its phosphorylation, which ultimately regulates its functional impact on stem cell fate commitment.

Most studies to date have reported that AMOT inhibits YAP through direct binding, which apparently sequesters YAP in the cytoplasm to prevent YAP's transcriptional impact in the nucleus. However, one study has reported that in hepatic epithelial cells, AMOT promotes YAP nuclear translocation by sterically blocking its interaction with LATS in the cytoplasm and then forms a complex with YAP-TEAD within the nucleus to co-regulate target gene transcription[63]. These seemingly conflicting reports highlight that while AMOT clearly interacts with YAP, the nature of this interaction is particularly important and highly context-dependent. Through the use of phospho-mimetic and YAP non-binding mutants of AMOT, we found that in NSCs, AMOT inhibits YAP in the cytoplasm by promoting YAP phosphorylation and that this interaction is enhanced by AMOT's own phosphorylation. The importance of AMOT phosphorylation on its binding with YAP is consistent with previous reports[66], and our findings are consistent with a model where AMOT may act as a scaffolding protein to promote YAP phosphorylation by LATS while AMOT is itself phosphorylated. Overall, the role of the canonical Hippo pathway in stiffness-sensitive YAP regulation remains unclear. In our study, AMOT phosphorylation by LATS at S175 enhanced AMOT's inhibition of YAP and promotion of neurogenesis, indicating that AMOT's effects are Hippo-dependent. Interestingly, a recent study showed that the Ras family GTPase RAP2 inhibits YAP/TAZ in a stiffness- sensitive manner by promoting LATS activity[149], which could potentially be upstream of AMOT in NSCs.

3.6 Acknowledgements

We kindly thank Dr. Dannel McCollum (University of Massachusetts Medical School, Worcester, MA) for sharing his lab's pCDNA constructs encoding S175E and S175A AMOT. We also thank Qianhe Zhang (University of California, Berkeley, Berkeley, CA) for her efforts in the Structured Illumination Microscopy imaging. P.H.K, S.K., and D.V.S. gratefully acknowledge grant support from the National Institutes of Health (grant #5R01NS074831: Mechanisms of Neural Stem Cell Mechanoregulation), awarded to S. K. and D.V. S. and from the Siebel Scholarship, awarded to P.H.K..

Chapter 4 : Exploring the influence of extracellular glucose and cellular energetics in regulating neural stem cell mechanotransduction

4.1 Abstract

Recent studies have shown that diabetes is linked to cognitive deficits and neurodegenerative diseases in the brain in addition to the well-documented damage to the eyes, kidneys, and peripheral nerves. Intriguingly, functions that are attributed to the hippocampus and its resident neural stem cell population are particularly impacted by diabetes and hyperglycemia. Indeed, some *in vitro* and *in vivo* studies have shown that diabetes can be detrimental to NSC viability or proliferation. However, the precise mechanisms that describe how hyperglycemia influences NSC behavior are still largely unknown. In our study, we delved further into how hyperglycemia alters NSC growth and differentiation in response to biophysical cues. Importantly, we developed a low glucose culture protocol that enabled us to study a physiologically relevant range of glucose concentrations, since common culture media already contain super-diabetic glucose levels. We found that NSC growth is suppressed by hyperglycemia in a dose-dependent manner, which may be due to alterations in cell cycle kinetics. Further, we found that activation of 5'adenosine monophosphate-activated protein kinase (AMPK) either by lowering the glucose concentration or metformin treatment rescued neurogenesis on stiff substrates while high glucose or compound C (AMPK inhibitor) treatment suppressed neurogenesis. Therefore, hyperglycemia may have a direct influence on how NSCs sense and respond to their physical microenvironment through an AMPK-dependent mechanism.

4.2 Introduction

Diabetes mellitus is a chronic disease characterized by hyperglycemia, or elevated blood glucose levels. Although the term describes a group of conditions, there are two principal classifications used that are based on the physiological cause of the disease[150,151,152]. Type 1 diabetes (T1D) refers to cases where the pancreatic beta cells are lost, leading to insufficient insulin production and subsequent increased blood sugar[153]. Commonly, the destruction of beta cells in T1D is due to aberrant autoimmune reactivity targeting the beta cells[154,155]. Type 2 diabetes (T2D), on the other hand, is characterized by insulin resistance or reduced insulin sensitivity in the target tissues of the body and is commonly related to lifestyle and dietary habits[156,157]. In a report from the US Centers for Disease Control and Prevention (CDC) in 2017, more than 30 million Americans suffer from diabetes, with over 90% of those with Type 2[158]. Furthermore, other studies have projected that this number will continue to increase due to both an increase in the number of diagnosed cases as well as improved survival among individuals with diabetes[159,160,161]. Because the prevalence and risk factors associated with diabetes are much

higher in the elderly[162], the current major health burden of diabetes-related care will only become of greater societal importance.

The dysregulation of blood sugar in diabetes can lead to a host of related complications that adversely affect individuals' health. Historically, the resulting long-term risk to cardiovascular health and damage to tissues such as the eyes, kidneys, and peripheral nerves have been most well-noted, studied, and treated[163]. However, over the decades since the discovery of recombinant insulin in the 1920s as a treatment[164], improved disease management and patient survival have revealed that complications from diabetes can also impact other tissues, including the brain[69]. Type 2 diabetes in particular has been suspected of having a link with an increased risk of cognitive deficit, dementia, and Alzheimer's Disease. Indeed, epidemiological studies dating back to the 1990s have reported that patients with T2D have a statistically increased risk for dementia[71,72]. Since then, further studies have shown that diabetes and hyperglycemia are intimately associated with cognitive dysfunction, the onset of Alzheimer's Disease, and reduced neurogenesis in more controlled *in vitro* and *in vivo* experiments[75,165,166,167,168,169,170]. However, there is still much unknown about the specific mechanisms by which hyperglycemia or other diabetes-associated conditions can impact the brain and cognitive function.

Intriguingly, some studies have highlighted an association between diabetes and deficits in learning and memory in animal models[171,172]. Because hippocampal neurogenesis is known to play a significant role in these processes, neural stem cells (NSCs) that reside within the hippocampal dentate gyrus may be a critical cellular population to study in the context of diabetes and hyperglycemia. Consistent with this hypothesis, previous studies using various mouse models of T1D and T2D have demonstrated a reduction in hippocampal cell proliferation and neurogenesis compared to control mice[74,173,174] , suggesting that cognitive impairment in diabetes may be caused by deficits in NSC-driven neurogenesis and neuroplasticity.

There are several mechanisms by which diabetes and hyperglycemia can have an impact on NSCs. Glucose plays a critical role in human physiology as the main monomeric carbohydrate used for cellular respiration and ATP generation within cells. In general, energetics has been increasingly appreciated to play a critical role in both the proliferation and differentiation of stem cells. Illustratively, there are distinguishable metabolic signatures or profiles depending on the potency of the cell; while stem cells tend to generate much of their intracellular energy via the less efficient glycolytic pathway, more lineage-restricted progenitor cells or terminally differentiated cells generate much of their energy from the oxidative phosphorylation (OxPhos) mechanism (Fig. 4.1)[79,81]. During differentiation, this so-called "metabolic re-programming" is a critical step that enables the proper development of the specialized cell[175,176,177]. Furthermore, various metabolic intermediates are also important for other signaling pathways that can influence cell behavior such as histone methylation and

acetylation, the mTOR pathway, the generation of reactive oxygen species (ROS), and the MAPK/ERK pathway, amongst many others[178]. Therefore, hyperglycemia could alter glucose metabolic pathways and their off-shoots that are tuned to drive and reinforce NSC behavior during both proliferation and differentiation.

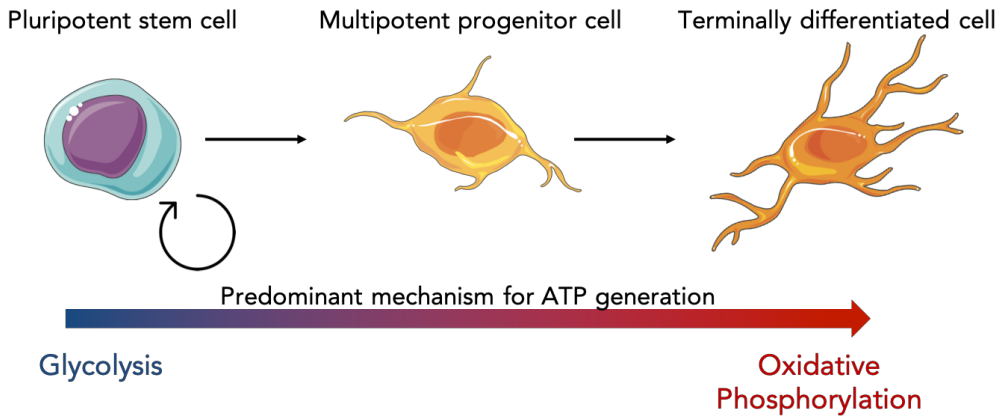

Figure 4.1 Metabolic reprogramming during stem cell differentiation.
In general, as stem cells undergo differentiation and become more specialized, a shift in energetic needs is linked with a shift in the primary mechanism for energy generation from the glycolytic process of glucose metabolism to electron transport chain-linked oxidative phosphorylation.

We have discussed previously that cytoskeletal re-organization and tension generation is a key mechanosensitive response in cells, including NSCs, that underlie signaling cascades that orchestrate cellular behavior. Furthermore, the dynamic modulation of actomyosin contractility is an energy consuming process that is ATP-dependent and therefore sensitive to cellular energetics. As such, alterations in glucose concentration and cellular metabolism may influence mechanosensitive behavior in NSCs through mechanisms that affect cytoskeletal signaling. For example, cellular migration and cancer metastasis, which require dynamic cytoskeletal activity, have been shown by others to be sensitive to glucose concentration and metabolism[179,180].

To understand the role of glucose and the direct impact of hyperglycemia on cells, *in vitro* studies have a distinct utility since the concentration of glucose can be altered simply by adjusting the composition of the culture media. In these kinds of experiments, the physiological relevance of the glucose concentrations being tested is an important consideration in the study's design and execution. Clinically, diabetes or prediabetes is diagnosed by testing an individual's plasma glucose concentrations after either a 12-hour period with no food (fasting glucose) or 2 hours after ingestion of a known glucose solution (glucose tolerance). As shown in the table below, a fasting blood glucose concentration at or above 7 mM or a 2-hr glucose tolerance test concentration above 11.1 mM is clinically diagnosed as diabetes. Notably, typical culture protocols for most cell types employ media such as Dulbecco's Modified Eagle Media (DMEM)

or DMEM/Ham's F-12, which contain 25 mM and 17.5 mM of glucose, respectively, which are above even diabetic concentrations of blood glucose. While these levels of glucose enable robust cell culture that has been invaluable for a wide range of *in vitro* studies, mindful consideration of the basal glucose concentration is an important starting point for studying the impact of hyperglycemia on cell behavior specifically.

Table 4.1 Clinical guidelines for diagnosing diabetes and prediabetes, based on WHO recommendations. Fasting glucose and glucose tolerance tests are assayed by venous plasma glucose measurements.

	Fasting glucose			**2 hr glucose tolerance test**	
Condition	**mg/dL**	**mM**		**mg/dL**	**mM**
Normal	< 110	< 6.1	**and**	<140	< 7.8
Impaired fasting glucose	110 – 125	6.1 – 6.9	**and**	<140	< 7 .8
Impaired glucose tolerance	< 126	< 7	**and**	140 – 200	7.8 – 11.1
Diabetes	≥ 126	≥ 7	**or**	≥ 200	≥ 11.1

Therefore, we sought to utilize *in vitro* culture platforms to investigate the impact of hyperglycemia on NSC behavior. In initial experiments, we used typical DMEM/F-12 (Life Technologies) containing 17.5 mM glucose as our basal media and added additional glucose to subject the cells to hyperglycemic conditions. However, in our efforts to test more physiologically relevant glucose concentrations, we then developed a platform using another DMEM/F12 basal media (Biowest) that contained no initial glucose, which gave us design freedom over what concentrations were tested. Using this media, we established a "priming" approach to re-acclimate NSCs to a lower, more physiologically relevant glucose concentration that gave us a wider parameter space of concentrations to test. Following low glucose priming, we then tested NSC growth and stiffness-sensitive differentiation in low or high glucose concentrations. We also explored the importance of AMPK activation, which is directly sensitive to cellular energetic state, by utilizing a pharmacological inhibitor (compound C) and activator (metformin) of AMPK and measuring the changes to glucose-sensitive, stiffness-directed differentiation of NSCs.

4.3 Materials and methods

3.3.1 Polyacrylamide gel synthesis and functionalization

Polyacrylamide-bis precursor solutions were made for each stiffness by mixing various concentrations of acrylamide monomer and bis-acrylamide crosslinker (Bio-Rad). Solution compositions to achieve various final polymerized stiffnesses were as follows: 0.2 kPa = 3% acrylamide + 0.04% bis, 0.5 kPa = 3% acrylamide + 0.1% bis, 72 kPa = 10% acrylamide + 0.3% bis (Keung et al., 2011). Polyacrylamide gels were synthesized on 19- or 25-mm glass coverslips

with 0.1% TEMED + 1% Ammonium Persulfate. Polyacrylamide gels were then functionalized with laminin conjugation via sulfo-SANPAH (Thermo-Fisher) to facilitate cell attachment.

3.3.2 Cell culture and pharmacologic treatment

Long-term culture of adult rat hippocampal NSCs was done in DMEM/F12 with N2 supplement (Life Technologies) on tissue-culture polystyrene plates that had been coated with poly-ornithine and laminin. Growth conditions for NSCs included 20 ng/mL FGF-2 whereas mixed differentiation conditions included 1% FBS + 1 μM retinoic acid. For low glucose priming, cells were grown for two days prior to experimentation start in DMEM/F-12 containing 5 mM glucose and 20 ng/mL FGF-2. This low glucose basal medium was created using another DMEM/F12 (Biowest) that has no initial glucose, HEPES, or L-glutamine but is otherwise chemically identical to the Life Technologies DMEM/F-12. The Biowest medium was then supplemented with HEPES, L-glutamine, and N2 supplement. A 250 mM glucose stock solution was made in the basal media and used to achieve the final desired concentrations of glucose. For full differentiation experiments on polyacrylamide substrates, NSCs were first seeded onto the gels in growth media for 16-18 hours before the coverslips were then transferred into new wells with mixed differentiation media. The NSCs were allowed to differentiate for 6 days with 50% media changes every 2 days before fixation.

Metformin was added to the media at a final concentration of 5 mM and Compound C was added at a final concentration of 10 uM in experiments that included either small molecule. In full differentiation experiments, the small molecules were added along with differentiation media for the first 48 hrs, after which differentiation media without the small molecules was used. In experiments shorter than 48 hrs, the pharmacologic agents were also added with the differentiation media and left for the duration of the experiment.

3.3.3 Cell growth studies

NSCs were seeded in four separate wells of a laminin-coated 12-well plate at a density of 10,000 cells/cm^2 for each glucose concentration tested and cultured in 1 or 20 ng/mL FGF-2. After each subsequent day, one well of each condition was washed once with PBS and then the total number of cells in each well was counted by hemacytometer and recorded. The well plate was then returned to the incubator until the last day of the experiment. Relative population expansion was calculated for each condition by dividing the number of cells by the number of cells at day 1. The experiment was repeated for a total number of three biological replicates per condition.

3.3.4 Antibodies

Primary antibodies and dilutions used were as follows: YAP (1:250; Cell Signaling Technologies 4912S), Ki67 (1:500; Abcam ab15580), Tuj1 (1:750; BioLegend 801201), GFAP (1:1,000; Abcam ab4674), AMPK (1:1,000; Cell Signaling Technologies 2532S), pAMPK (1:1,000; Cell Signaling Technologies 2535S)

3.3.5 Immunofluorescence staining and imaging

Cells were fixed in 4% paraformaldehyde (Alfa Aesar) for 10 minutes before washing in PBS and permeabilization in 5% goat serum + 0.5% Triton X-100 (Sigma-Aldrich) for 10 minutes. Permeabilized cells were blocked in 5% goat serum for 1 hour at room temperature before immunostaining. Primary and secondary antibody solutions were also made in 5% goat serum.

Epifluorescence images were taken using a Nikon Eclipse Ti Microscope, Hamamatsu Photonics K.K. C10600-10B-H camera, 10X objective lens, and native NIS-Elements AR 5.02.00 software. Samples were submerged in PBS during image acquisition. Nuclei were labeled with a DAPI stain (Sigma-Aldrich) and Tuj1 was labeled with a 488 dye-conjugated secondary antibody (Thermo-Fisher). Confocal images were taken using a Prairie Technologies 2-photon and confocal microscope, QuantEM 512SC camera, 60X objective lens, and native Prairie View software. Samples were submerged in PBS during image acquisition. Nuclei were labeled with a DAPI stain, F-actin was labeled with an Alexa Fluor 546 Phalloidin (Thermo-Fisher), and other targets were labeled with either a 488 or 633 dye-conjugated secondary antibody.

4.4 Results

4.4.1 NSC morphology and YAP localization in elevated glucose concentrations

In an initial set of experiments, we sought to examine if hyperglycemia could impact NSC mechanosensing, which would suggest that stiffness-sensitive differentiation in NSCs could be influenced by aberrantly high glucose concentrations such as those experienced in diabetic individuals. To test this, we investigated cellular morphology and intracellular YAP localization, which are both indicative read-outs of stiffness-sensitive behavior[135,181]. We found that some NSCs subjected to hyperglycemic conditions (25 and 50 mM) relative to their normal culture conditions (17.5 mM) displayed striking cellular hypertrophy under both proliferative and differentiation conditions (Fig. 4.2). This observation is consistent with what has been reported previously in other cell types[182]. Notably, we observed the development of prominent stress fibers during differentiation in elevated glucose conditions, which we normally do not observe in NSCs. Furthermore, we also observed that hypertrophic cells displayed a striking nuclear enrichment of YAP during early differentiation, which has been reported as a stiffness-induced

translocation that can direct stem cell fate commitment[131]. Notably, we have not observed clear nuclear localization of YAP on stiff substrates in NSCs under typical culture conditions and have reported that YAP suppresses neurogenesis in response to stiffness via a non-canonical antagonism of β-catenin[17]. However, aberrant hyperglycemia-induced hypertrophy in NSCs seems to drive nuclear YAP localization, which is known to be sensitive to cell spreading and intracellular force generation[135]. This suggests that hyperglycemia could have an influence on cytoskeletal organization and critical downstream signaling events in NSCs.

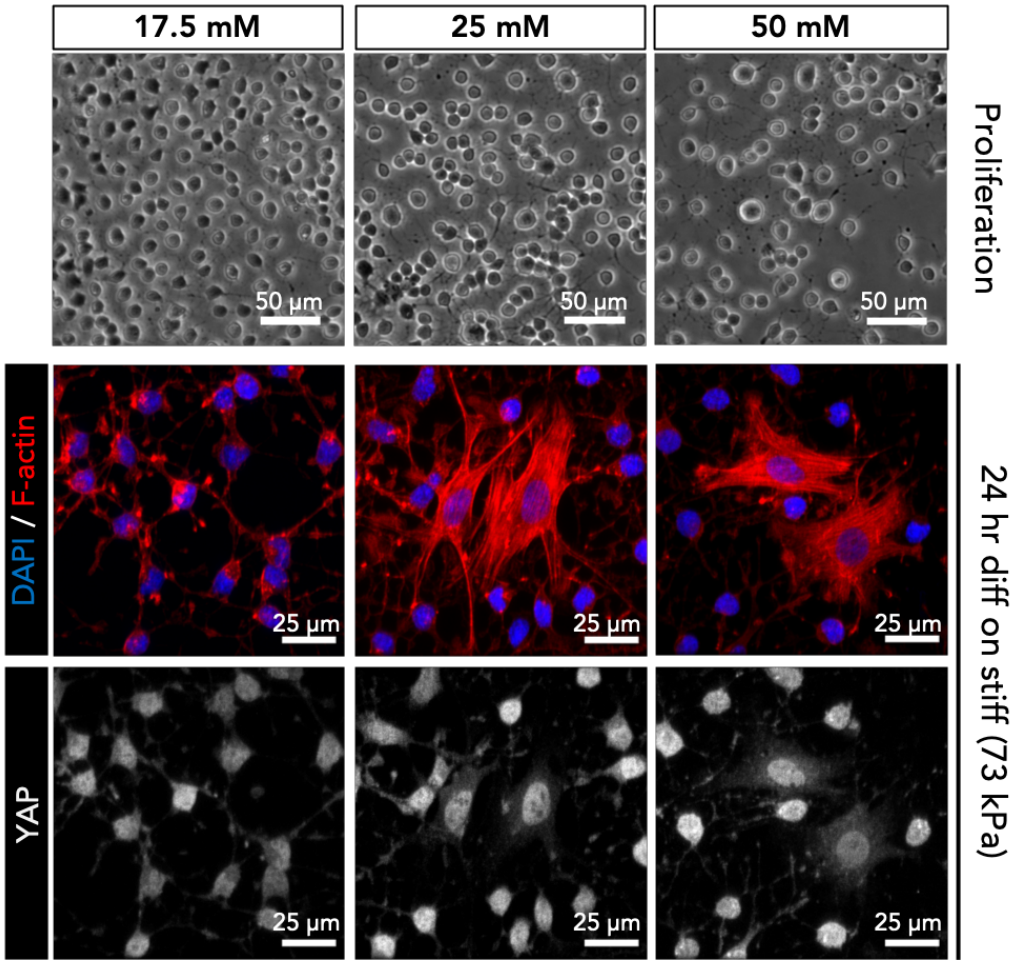

Figure 4.2 Elevated glucose indicues NSC hypertrophy and YAP nuclear localization. (top) NSCs were cultured in renewal conditions (20 ng/mL FGF-2) in either the basal DMEM/F-12 media (17.5 mM glucose) or with additional glucose added to subject them to hyperglycemia (25 or 50 mM glucose). Cells were observed under brightfield microscopy after 48 hours in culture. (bottom) NSCs were seeded onto stiff substrates (73 kPa) and differentiated for 24 hours in mixed differentiation conditions (1μM retinoic acid + 1% FBS) in shown basal or elevated glucose concentrations prior to fixation in 4% PFA and staining for nuclei (blue), F-actin (red), and YAP (greyscale).

4.4.2 NSC growth in elevated glucose concentrations

After our initial observations that hyperglycemia could impact NSC morphology and YAP localization, we then sought to more thoroughly test how hyperglycemia may affect NSC behavior, namely proliferation, cell growth, and differentiation. To measure cell growth, we monitored total cell populations in 20 ng/mL FGF-2 and either 17.5 mM (basal), 25 mM, or 50 mM glucose over 4 days in culture. We observed that the relative increase in cell number over time was subdued with increasing glucose in a dose-dependent manner, suggesting that hyperglycemia has a negative effect on cell growth (Fig. 4.3a). However, statistical analysis revealed that those differences were not significant at any time point. Given the trends we observed in this experiment, we wondered if the FGF-2 concentration that we used provided a strong proliferative cue that dampened the anti-growth effects of the hyperglycemia. Therefore, we reduced the concentration of FGF-2 to 1 ng/mL, which we have observed to support lowered levels of proliferation and minimal apoptosis and repeated the experiment. Strikingly, the dose-dependent growth-suppressive effect of the hyperglycemia remained consistent but was exacerbated in the lowered FGF-2 (Fig. 4.3b), leading to a significant difference in relative total cell number between the 17.5 mM and 50 mM conditions after 3 days in culture.

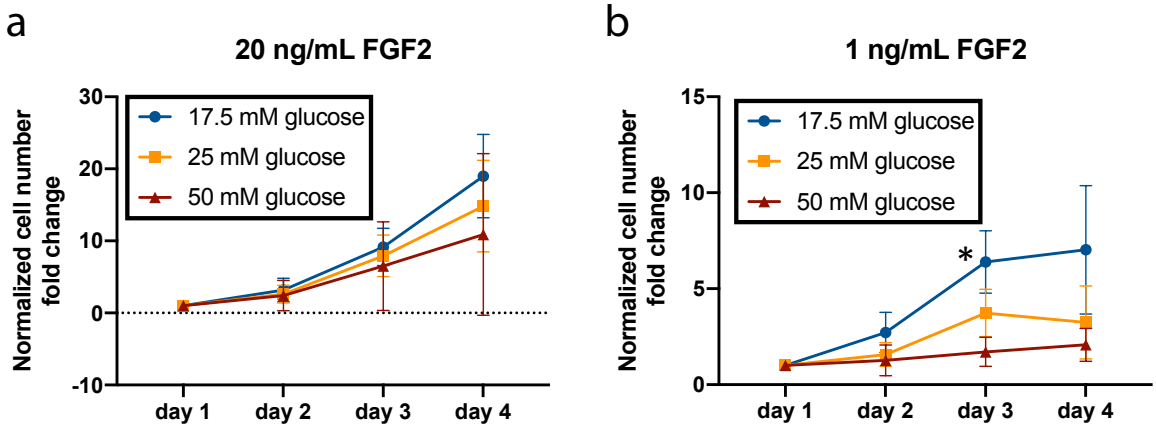

Figure 4.3 Hyperglycemia conditions reduce NSC growth.
(a) There is a dose-dependent trend that increasing glucose concentrations result in reduced cell growth over time in culture at 20 ng/mL FGF-2. (b) Reducing the proliferative FGF-2 cue exacerbates the effect seen in (a). Cells were cultured in separate wells for each day of data collection and were counted by Accutase treatment followed by hemacytometer analysis. *$p<0.05$ compared to 17.5 mM control condition, analyzed by two-way ANOVA followed by Tukey's post-hoc test.

4.4.3 NSC proliferation and cell cycle analysis in elevated glucose concentrations

We then tested if this lowered cell growth in NSCs was due to a shift in the population towards senescence and a reduction in proliferative capacity. To measure this, we stained the cells for Ki67, which is a known marker for cellular proliferation and is absent in quiescent cells. We observed that the proliferative capacity was markedly consistent (~85-93% Ki67+) among all conditions tested, which included 25 mM and 50 mM glucose as well as 25 mM and 50 mM mannose as an osmolarity control (Fig. 4.4a-b). Notably, this experiment was conducted at 5 ng/mL FGF-2. We also included control condition of 1 ng/mL FGF-2 at 17.5 mM, which displayed a reduced proliferative capacity (~72% Ki67+) as expected.

Hyperglycemia could also affect NSC population growth by slowing cell cycle progression, which has been shown previously in other stem cell contexts such as embryonic cardiac development[183]. To test this, we directly monitored cell cycle progression by developing an NSC cell line that stably expressed the Fluorescent Ubiquitination-based Cell Cycle Indicator (FUCCI) system, a commercially available tool for continuously tracking cell cycle state[184]. This sensor system relies upon the reciprocal oscillation of two E3 ubiquitin (Ub) ligase complexes, APC^{Cdh1} and SCF^{Skp2}, which target substrates for proteosomal degradation in a cell cycle-dependent manner. Two downstream targets of these complexes are Cdt1, a "licensing" and DNA replication factor that helps ensure that DNA replication only occurs once per cell cycle, and geminin, which inhibits Cdt1 activity (Fig. 4.4c). Cdt1 is most abundant in cells during the G1 phase of the cell cycle, while geminin accumulates in S/G2/M. Therefore, fluorescent tags on these two proteins enable the continuous read-out of cell cycle state in live cells. Using the FUCCI NSCs, we grew the cells in 17.5, 25, or 50 mM glucose in the presence of 1 or 20 ng/mL FGF-2 as proliferative cues. We observed interesting trends in both cases that helped shed light on our previous observations that hyperglycemia reduces cell growth (Fig. 4.3). Namely, the ratio of cells that were in S, G2, or M phase (geminin-GFP+) to those in G1 (Cdt1-RFP+) displayed a characteristic peak after 30-36 hours in 20 ng/mL FGF-2 and 17.5 mM glucose, which is consistent with doubling times that we have observed previously (Fig. 4.4d). However, this peak was delayed in a dose-dependent manner as the glucose concentration was increased to 25 and 50 mM. This would result in a lag in overall population proliferation, which may explain the reduction in cell growth over time that we observed in 20 ng/mL FGF-2 conditions (Fig. 4.3a). In comparison, when the FUCCI NSCs were grown in 1 ng/mL FGF-2 conditions, we observed an initial peak in the (S+G2+M)/G1 ratio at around 18 hours in all glucose concentrations but a dose-dependent reduction in the relative fraction of cells that were dividing. These results may also provide explanation for the more pronounced and earlier effects of hyperglycemia that we had observed in the lowered FGF-2 concentration previously (Fig. 4.3b). Overall, these results suggest that hyperglycemia may longitudinally influence cell cycle progression by multiple mechanisms to reduce NSC growth.

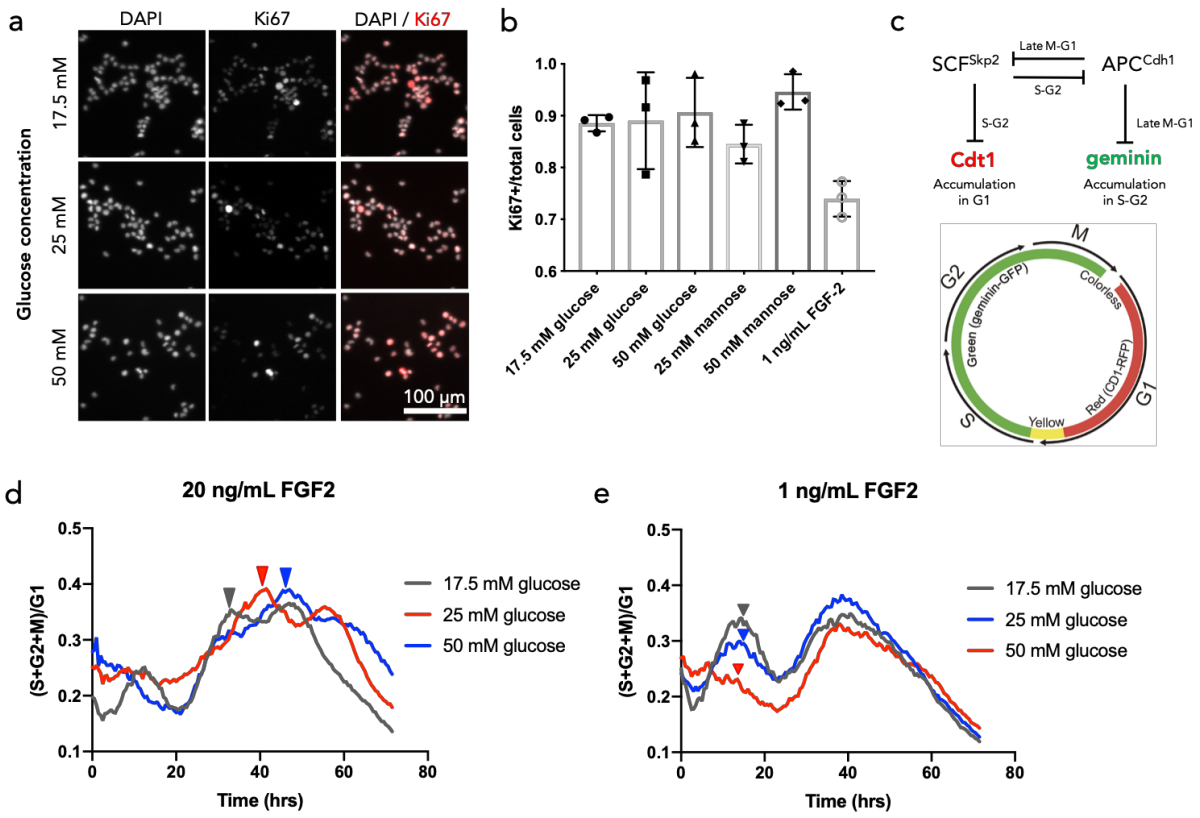

Figure 4.4 Hyperglycemia does not alter NSC proliferative potential but may influence cell cycle kinetics.

(a) Representative images of Ki67 staining in NSCs cultured in 17.5, 25, or 50 mM glucose. Cells were cultured for 24 hours in 5 ng/mL FGF-2 (or 1 ng/mL FGF-2 in the control) prior to fixing with 4% PFA and staining for nuclei (greyscale in merge) and Ki67 (red in merge). (b) Quantification of relative number of Ki67+ cells after culture in 17.5, 25, or 50 mM glucose as well as 25 and 50 mM mannose as a control (n = 3). (c) The FUCCI system is based on reciprocal and cell-cycle dependent activation of two E3 ubiquitin ligase complexes and their downstream targets (Cdt1 and geminin), which are tagged with red fluorescent protein (CDT1-RFP) and green fluorescent protein (geminin-GFP) to indicate cell cycle state. *Figures were adopted with permission from Elsevier*[184]. FUCCI NSCs were cultured in (d) 20 ng/mL or (e) 1 ng/mL FGF-2 and imaged with fluorescent and brightfield microscopy every 30 minutes for 71 hours. The ratio of GFP+ (S/G2/M) to RFP+ (G1) cells was plotted for each timepoint. Arrows point to analogous peaks of this ratio in each condition to highlight differences.

4.4.4 NSC differentiation in elevated glucose concentrations

In addition to measuring the effects of higher glucose levels on NSC proliferation, we also sought to investigate if hyperglycemia impacted fate commitment. In particular, we were encouraged by our previous observations that hyperglycemia induced cellular hypertrophy, cytoskeletal re-organization, and YAP nuclear translocation (Section 4.2.1) to test the impact of

hyperglycemia on stiffness-sensitive differentiation. We found that on stiff substrates, increasing the glucose concentration to 25 or 50 mM from a basal 17.5 mM decreased neurogenic differentiation by ~20% while astrogenesis was not significantly affected (Fig. 4.5). Notably, we could not collect data from cells differentiated on soft substrates as they were less strongly attached to the substrate and were washed away during the fixing and staining process. These results suggest that hyperglycemia suppresses neurogenic differentiation of NSCs even in the presence of other neurosuppressive cues, such as less compliant substrates.

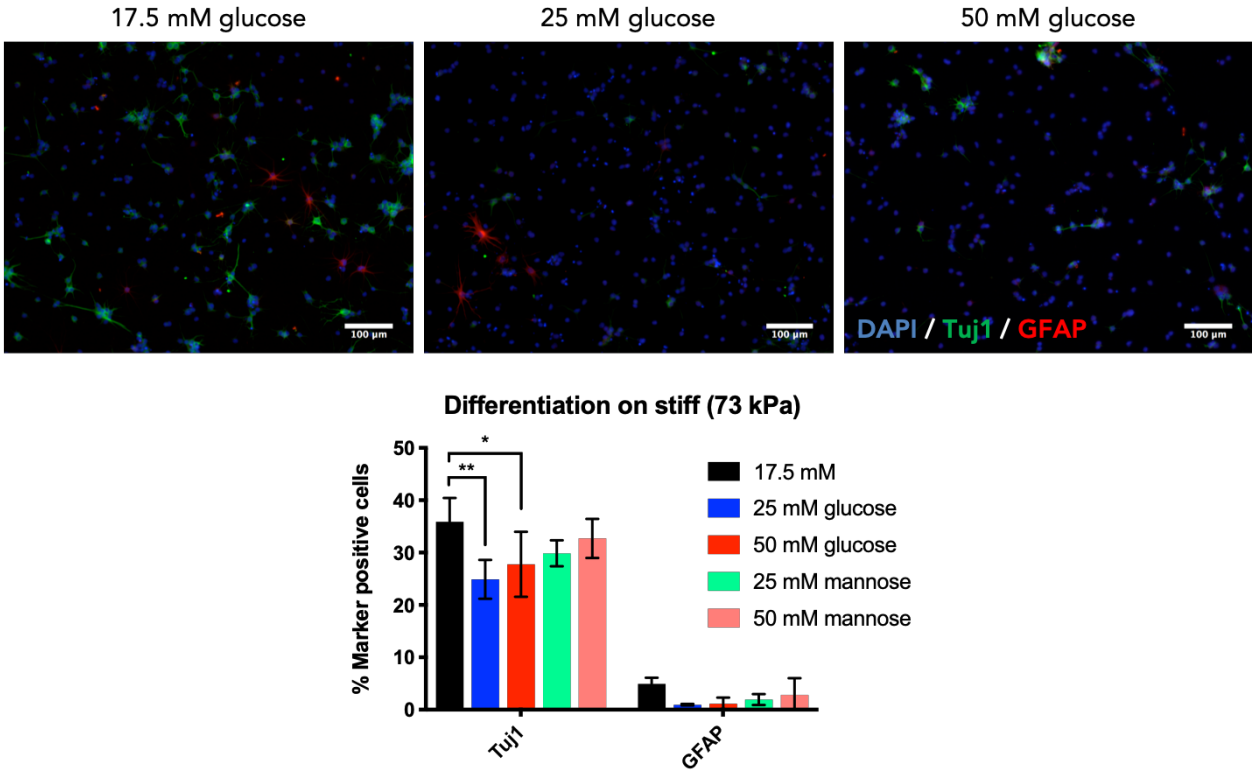

Figure 4.5 Hyperglycemia reduces neurogenesis on stiff substrates.
Representative images (top) and quantification (bottom) of NSCs that were differentiated for 6 days in mixed differentiation conditions (1 µM retinoic acid + 1% FBS) on stiff substrates (73 kPa). Cells were cultured in 17.5, 25, or 50 mM glucose as well as 25 or 50 mM mannose as an osmolarity control. After 6 days, cells were fixed in 4% PFA and stained for nuclei (blue), the neuronal marker Tuj1 (green), and the astrocytic marker GFAP (red). Bar = 100 µm, *$p<0.05$, **$p<0.005$ by two-way ANOVA followed by Tukey's post-hoc test.

4.4.5 Establishment of low glucose culture platform for NSCs

While these initial experiments provided useful insight into the impact of hyperglycemia on NSC behavior, the basal glucose levels were already above the clinical diabetic threshold (Table 4.1). Therefore, we sought to develop a culture platform that enabled us to use lower,

more physiologically relevant glucose concentrations. To do this, we purchased a DMEM/F-12 media (Manufacturer: Biowest) that does not contain any initial glucose, HEPES, or L-glutamine but is otherwise compositionally equivalent to the DMEM/F-12 we used previously (Manufacturer: Life Technologies). We subsequently supplemented the media with HEPES and L-glutamine such that the new basal media now was compositionally identical to what we had been using previously but without glucose. We then created a 250 mM stock solution of glucose using this media that we could dilute to create the desired final glucose concentration for experimentation.

Because the NSCs have been isolated and cultured in 17.5 mM glucose media since their isolation[185], we first sought to determine the best method to transfer the cells into a lower glucose concentration media for experimentation. As a read-out for the effect of glucose on cell behavior, we measured AMPK activity, which is activated via phosphorylation by various upstream kinases upon a shift in a cell's AMP/ATP and ADP/ATP ratios (Fig. 4.6a). Therefore, we would expect that decreasing glucose concentrations should increase AMPK activation due to a decrease in metabolic substrate, while increasing glucose concentrations should decrease AMPK activation. Initially, we saw that directly transferring the cells into glucose starvation conditions (0 mM) for 24 hours enhanced AMPK activation as expected (Fig. 4.6b). Additionally, direct transfer to low (4.5 mM) or elevated (15 mM) glucose concentrations displayed a dose-dependent inactivation of AMPK, or a decrease in pAMPK. However, this trend was highly non-linear and the difference in AMPK activation between 4.5 and 15 mM glucose conditions was relatively small compared to the difference between 4.5 and 0 mM (Fig. 4.6b). Because we wanted to establish a new low baseline glucose condition around 4.5 mM, we sought to achieve a greater difference in cell response between the 4.5 mM glucose condition and the 15-20 mM range.

To accomplish this, we decided to test a "priming" approach that may re-acclimate the cells to a low glucose condition prior to experimentation such that they would be re-sensitized to physiologically elevated glucose levels. We tested priming the NSCs in low glucose (5 mM) for either 24 or 48 hours prior to changing the media to high glucose or mannose (25 mM) for 24 hours prior to lysate collection (Fig. 4.6c). We found that priming the cells in low glucose for 48 hours increased baseline AMPK activation compared to 24-hour priming and that after both priming durations, subsequent treatment with 25 mM glucose decreased pAMPK as expected (Fig. 4.6d-e). Of note, 25 mM mannose also caused a decrease in AMPK activation, which was unexpected. Because there was a greater difference in AMPK activation between the low and high glucose in the 48-hour primed samples, we chose to use a 48-hour priming protocol in 5 mM glucose in further experiments.

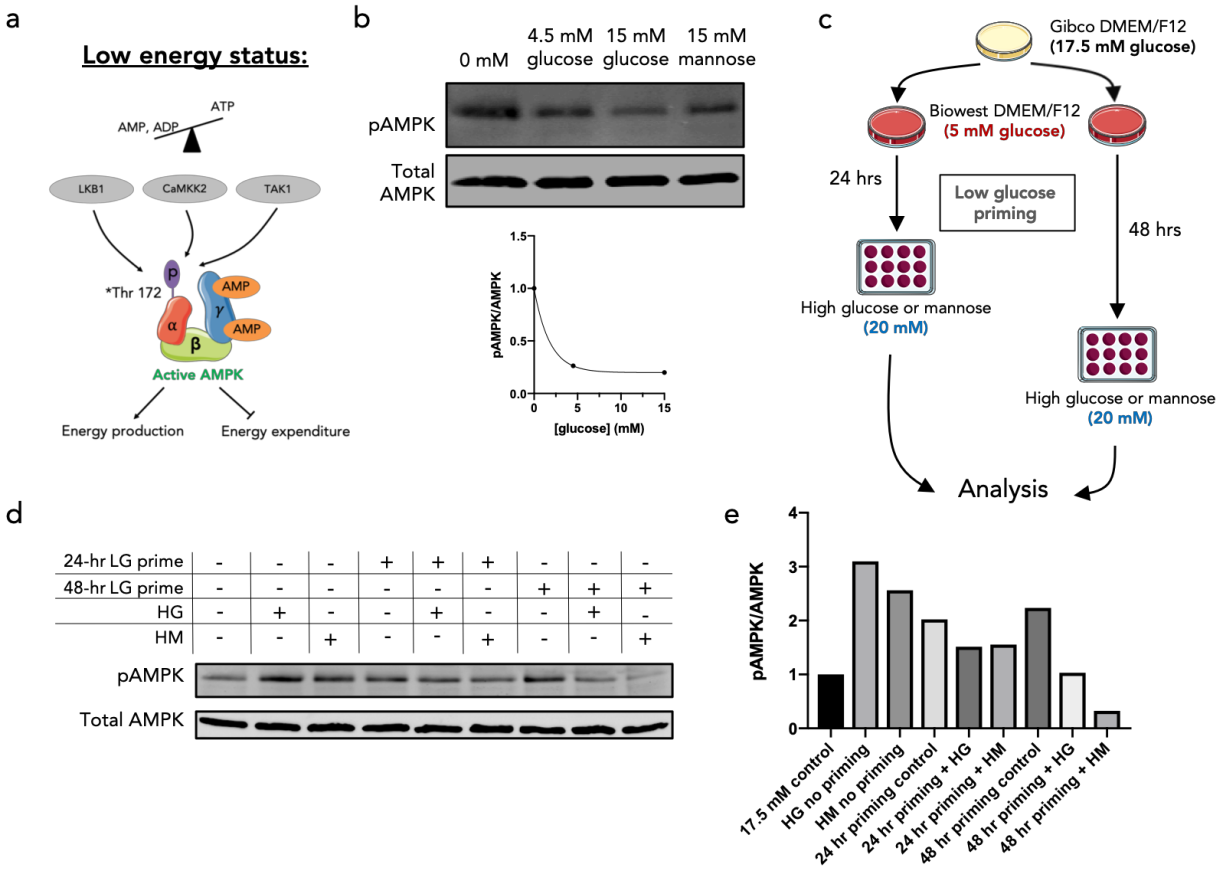

Figure 4.6 Establishment of low glucose priming protocol to sensitize NSCs to physiological range of glucose stimulation.

(a) Diagram depicting AMPK activation by lowered energy status within cells. AMPK directly senses the balance of ADP and AMP:ATP and is activated by upstream kinases in low ATP states. (b) Western blot and quantification of bands by densitometry from NSCs that were directly transferred to media containing 0, 4.5, or 15 mM of glucose as well as 15 mM mannose as an osmolarity control. pAMPK antibody is specific to phosphorylation at Thr172 residue. (c) Diagram depicting approach to "prime" NSCs in lowered glucose concentration (5 mM) prior to treatment with physiologically hyperglycemic conditions (20 mM). (d) Western blot of pAMPK and total AMPK after various priming and subsequent treatment conditions. (e) Quantification of bands from (d) via densitometry. HG = high glucose (20 mM), HM = high mannose (20 mM).

4.4.6 Differentiation of NSCs on soft vs. stiff after low glucose priming

After we developed our low glucose priming culture approach (Section 4.4.5), we next tested if NSC behavior was functionally sensitized to a physiological range of glucose concentrations. While the differentiation of NSCs is itself an important behavior to investigate with this approach, we also hypothesized that comparing differentiation on soft or stiff substrates may be particularly sensitive to variable glucose concentrations, since actomyosin contractility

and cytoskeletal re-assembly are energy-consuming processes[186]. Strikingly, we observed that after 48 hours of priming in low glucose (5 mM), continued culture in low glucose during differentiation resulted in high levels of neurogenesis regardless of substrate stiffness (Fig. 4.7a-b). In contrast, NSCs that were differentiated in higher glucose (20 mM) after low glucose priming displayed reduced neurogenesis on stiffer substrates (73 kPa) compared to those on soft substrates (500 Pa) (Fig. 4.7c-d). This is consistent with what we have reported in previous studies in which we found that stiffer substrates suppress neurogenesis[14,16]. Notably, those studies were conducted using media with a basal glucose concentration that is similar to the high glucose condition used in this study (17.5 mM vs. 20 mM). Therefore, low glucose priming does not seem to significantly alter NSC response to stiffness input at elevated glucose concentrations while enabling study at lower concentrations. This finding demonstrates that mechanosensitive differentiation by NSCs is itself sensitive to glucose concentrations. Further, these results suggest that there may be a stiffness-sensitive regime of glucose concentrations below which neurogenesis may be insensitive to stiffness input.

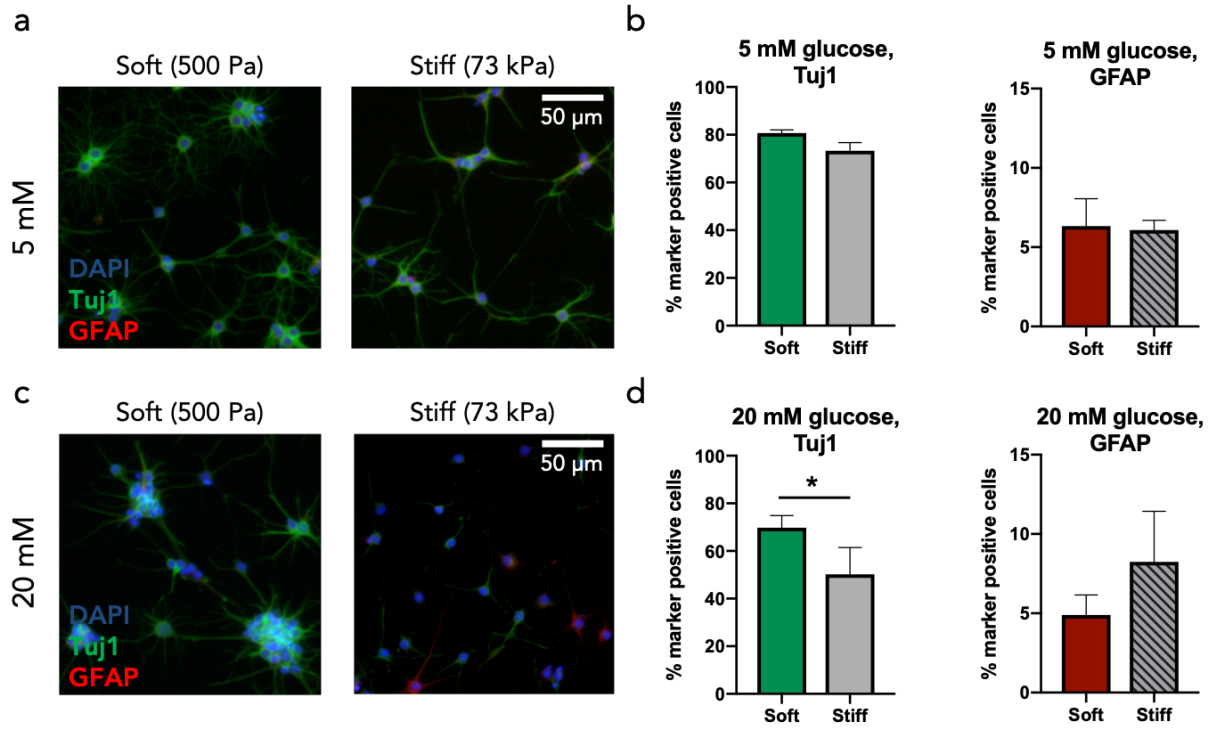

Figure 4.7 Elevated glucose is required for mechanosensitive differentiation of NSCs. NSCs were primed in 5 mM glucose for 48 hours and then differentiated in mixed differentiation conditions (1 μM retinoic acid + 1% FBS) for 6 days on soft (500 Pa) or stiff (73 kPa) substrates in either (a-b) 5 mM glucose or (c-d) 20 mM glucose. Cells were fixed in 4% PFA and stained for nuclei (blue), the neuronal marker Tuj1 (green), the astrocytic marker GFAP (red). *$p<0.05$ by unpaired t-test.

4.4.7 Effects of Meformin and Compound C on AMPK activation and NSC mechanosensing

AMPK is a well-known intracellular sensor for the energetic stage of a cell[187]. It plays a critical role in the regulation of cellular metabolism and its activity is influenced by the concentration of glucose in the extracellular space, which we observed previously (Fig. 4.6). Additionally, cytoskeletal re-organization and tension generation as well as the differentiation process itself are energy-consuming processes[122,188] , so AMPK may play a critical role during mechanosensitive fate commitment. Previously, we found that low glucose (5 mM) conditions supported a high degree of neurogenesis, even on stiff substrates that typically suppress neurogenesis compared to softer substrates. In contrast, NSCs cultured in higher glucose (20 mM) were seemingly mechanosensitive and displayed reduced neurogenesis on stiffer substrates compared to soft substrates. Therefore, since AMPK is activated by reduced glucose concentrations, we sought to directly test if AMPK activity was a critical component of glucose-sensitive mechanosensitive differentiation in NSCs. To do this, we used metformin, a known activator of AMPK[189], and compound C (Dorsomorphin), an AMPK inhibitor[190]. Strikingly, we observed that countering AMPK inhibition in high glucose concentrations (25 mM) with metformin treatment rescued neurogenesis on stiff substrates (Fig. 4.8a-b). Conversely, inhibition of AMPK with compound C treatment reduced neurogenesis in both low and high glucose concentrations. In addition to affecting fate commitment, we observed striking morphological differences upon metformin or compound C treatment. Metformin treated cells displayed reduced neurite branching and neurite length during differentiation, while compound C treated cells had extensive neurite branching, were flattened, and had larger nuclei (Fig. 4.8a).

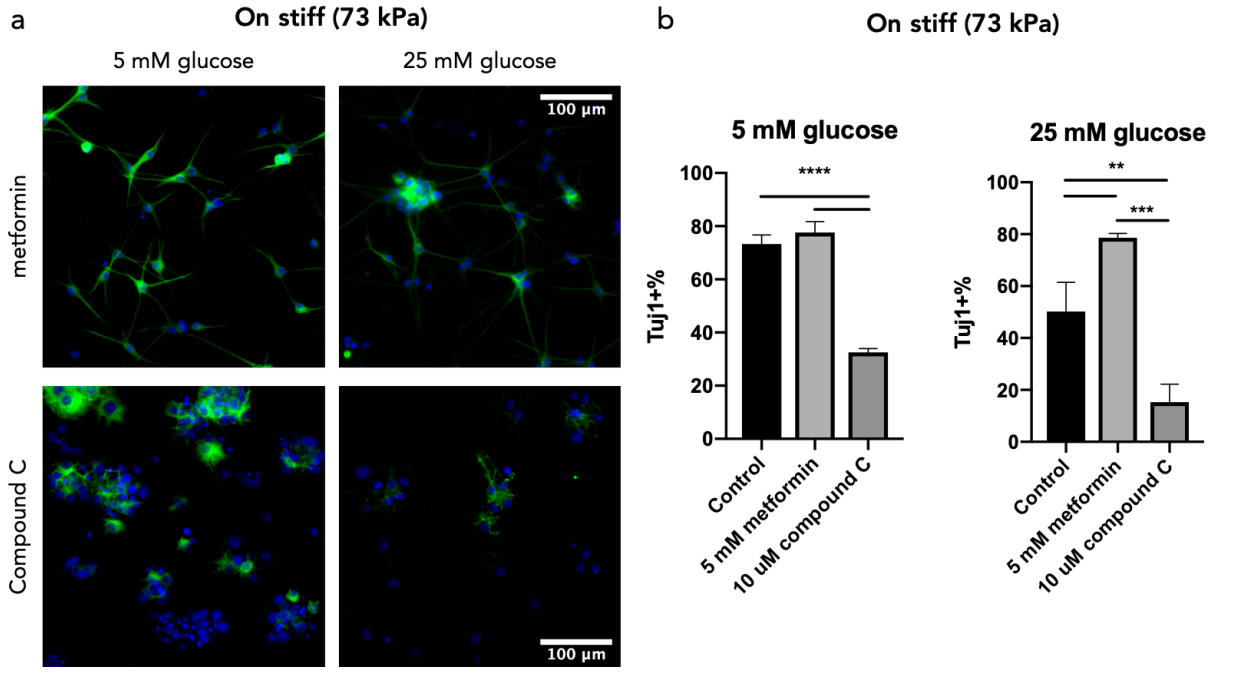

Figure 4.8 AMPK activity modulation with metformin and compound C affect mechanosensitive differentiation of NSCs.
(a) Representative images of NSCs that were differentiated in mixed differentiation conditions (1 μM retinoic acid + 1% FBS) in either low (5 mM) or high (25 mM) glucose concentrations and either 5 mM metformin or 10 μM compound C for 6 days. Cells were then fixed in 4% PFA and stained for nuclei (blue) or the neuronal marker Tuj1 (green). (b) Quantification of Tuj1+ cells in the aforementioned conditions. **p<0.005, ***p<0.001, ****p<0.0001 by one-way ANOVA followed by Tukey's post-hoc test.

4.5 Discussion

Diabetes is a prevalent and growing health issue around the world. An estimated 422 million people were living with diabetes in 2014, up from 108 million in 1980 (World Health Organization). Furthermore, in 2016, an estimated 1.6 million deaths could be directly attributed to diabetes, while in 2012, 2.2 million could be attributed to complications caused by high blood glucose[156]. Therefore, understanding how to properly diagnose, manage, and treat this disease is of critical concern in the global health community.

Over the last several decades, health professionals and researchers have discovered that the effects of diabetes and its associated hyperglycemia can extend beyond the tissues and organs that are typically impacted. In a prominent example, both large epidemiological and directed laboratory studies have begun to link diabetes to negative effects in the brain and to cognitive dysfunction. Interestingly, many of the cognitive deficits that have been associated to diabetes are related to functions of the hippocampus, a site of learning and memory formation throughout mammalian adulthood. Since the neural stem cells (NSCs) that reside within this niche are known to be responsible for the cell-level plasticity that enables many of these processes, studying how their behavior is impacted by hyperglycemia is an important step towards a more holistic understanding of how diabetes can impact individuals throughout life.

Through our studies described here, we have begun to uncover some of the mechanisms by which hyperglycemia can negatively impact NSCs. Consistent with other reports, we found that hyperglycemia stunts NSC growth and their ability to differentiate in culture. Further, our studies using cell cycle tracking suggest that hyperglycemia may influence cell cycle kinetics or progression. Other groups have also reported that hyperglycemia can lead to apoptosis[191,192], which we did not test directly in this study but could be another mechanism of how hyperglycemia can negatively affect NSC function. Beyond impacting general NSC viability and proliferation, our findings suggest that hyperglycemia can also have a direct influence on how NSCs sense and respond to their physical microenvironment. For example, we observed that hyperglycemia induces cellular hypertrophy and YAP nuclear localization in NSCs.

Like other cell types, NSCs must maintain an intracellular energy homeostasis to ensure proper function in changing conditions. To accomplish this, intracellular AMPK is an exquisite sensor that inhibits energy-consuming and activates energy-generating or -conserving processes when it is activated by low energy states. Interestingly, we found that lowering the glucose

concentration, which activates AMPK, promoted robust neurogenesis, even on stiff substrates that typically suppress neurogenic differentiation. This suggested that mechanosensitive differentiation may be an energy-consuming process that is inhibited at lower available glucose concentrations. Consistent with this hypothesis, inhibition of AMPK either by elevated glucose or pharmacological means re-suppressed neurogenesis on stiff substrates. Therefore, hyperglycemia may sensitize NSCs to physical cues from their microenvironment that is deleterious to their neurogenic capacity. Furthermore, maintaining NSCs in lower "normo-glycemic" concentrations may support consistently robust neurogenesis that is protected to some degree from the influence of biophysical cues and cytoskeletal activation.

4.6 Acknowledgements

We thank Pei Wen Xiao (Oakland High School, Oakland, CA) for her experimental assistance as a part of the Children's Hospital Oakland Research Institute (CHORI) Summer Student Research Program (SSRP) 2018. P.H.K, S.K., and D.V.S. gratefully acknowledge grant support from the National Institutes of Health (grant #5R01NS074831: Mechanisms of Neural Stem Cell Mechanoregulation), awarded to S. K. and D.V. S. and from the Siebel Scholarship, awarded to P.H.K..

Chapter 5 : Conclusions

In this dissertation, we've described our efforts to further investigate the precise mechanisms that orchestrate neural stem cell (NSC) mechanosensitive behavior. In Chapter 1, we described the discovery and significance of adult mammalian neurogenesis and the role that NSCs play in important cognitive processes as well as disease pathology. Subsequent studies have uncovered important new insights to how NSCs are regulated, which inform both our understanding of adult neurobiology as well as next-generation approaches for stem cell-based regenerative medicine in the central nervous system (CNS). We further discussed exciting new research directions undertaken in the past couple decades by groups including our own that have revealed the remarkable influence that biophysical cues, such as substrate stiffness, can have on stem cell behavior.

Although we now appreciate cytoskeletal remodeling and stiffness-sensitive transcriptional regulators as critical components of the mechanosensitive machinery in stem cells, how these two signaling nodes are connected remains elusive in NSCs and many other cellular contexts. In Chapters 2 and 3, we described our efforts to address this gap in the field by investigating the protein angiomotin (AMOT) as an important mechanotransductive pathway component. We found that AMOT can drive neurogenesis on soft substrates by inhibiting yes-associated protein (YAP) and promoting β-catenin signaling, an effect that was strongly regulated by the RhoA signaling pathway and AMOT phosphorylation by large tumor suppressor kinase (LATS). These findings significantly contribute to our understanding of NSC mechanobiology and how these cells may respond to cues presented by their niche *in vivo* as well as engineered extracellular matrix (ECM) biomaterial platforms.

In Chapter 4, we discussed our work to further investigate how NSCs may contribute to hippocampal dysfunction in diabetes by examining NSC behavior in hyperglycemic culture conditions. Importantly, we developed and validated a low glucose priming platform that allowed us to investigate physiologically relevant ranges of glucose concentration. This is an improvement from most other *in vitro* studies in the field, which employ culture media that already contain superphysiological concentrations of glucose. Using this approach, we both validated previous reports of the negative effects of hyperglycemia on NSC differentiation and proliferation while spearheading new insights into how glucose availability can impact NSC mechanosensing. Specifically, we uncovered that neurogenic suppression observed on stiff substrates only occurs in elevated glucose conditions in an AMPK-dependent manner. In contrast, lower glucose concentrations that more closely mimic non-diabetic conditions supported high neurogenesis regardless of substrate stiffness.

In our efforts as a discipline to study and describe cellular behavior, mechanistic studies such as those described in this dissertation are a powerful approach that help illuminate the wonderfully complex signaling pathways that dictate how stem cells respond to a diverse array of cues. Historically, this kind of investigative research has helped us better understand biology on a cellular, tissue, and organism level during development, health, and disease. However, the promise of stem cells for applications in tissue engineering and regenerative medicine has added a new layer of significance to mechanistic insights as they uncover new molecular "knobs" that can be tuned for targeted therapeutic application. Already, these discoveries are aiding in the

rational design of new biomaterial-based approaches for stem cell therapy, which we have discussed previously[15]. However, there is still much left to unravel in our understanding of how NSCs sense, integrate, and respond to biophysical cues that may be presented in their hippocampal niche or by engineered biomaterials. For example, our studies and those of other groups have largely focused on the influence of two-dimensional substrate stiffness. While there have been efforts to explore other types of biophysical cues such as topography, porosity, ECM ligand density and composition, viscoelasticity, stretch, shear stress, and confinement, many aspects are still understudied. For example, studying the effect of combinatorial presentation of these cues, as they undoubtedly are *in vivo*, in a high-throughput manner is a worthwhile challenge. Additionally, the significance of the timing of these cues, which we have explored[17], and their relevance in three-dimensional contexts still warrant additional study. Finally, further study using global approaches such as RNA-seq and genome-spanning CRISPR/Cas9-mediated screens and the application of these tools with single-cell resolution has the potential to reveal a staggering number of novel mechanistic insights.

NSCs have excited the fields of neurobiology and tissue engineering since their discovery in adult mammals and continue to motivate substantial study. In this dissertation, we have described our findings regarding the role of angiomotin as a novel component of NSC mechanosensing that can link RhoA, Hippo, and Wnt pathway elements to influence differentiation. Furthermore, we found that hyperglycemia can significantly alter how NSCs respond to stiffness inputs, which may be illustrative of hippocampal dysfunction in diabetes and the relationship between diabetes and age-related cognitive decline. With continued elucidation of the mechanisms that regulate NSC behavior, we could further understand how NSCs contribute to brain pathophysiology and further advance our efforts utilize NSCs for promising regenerative therapies.

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