Investigating Targets of miR-383-5p in Immune-Induced Regeneration

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TABLE OF CONTENTS

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ABSTRACT

Injury in the CNS often leads to permanent damage due to neurons' inability to initiate regenerative programming and the lack of support from the surrounding tissue environment. While inflammatory activity is a known deleterious force in CNS injury, there is evidence that suggests some forms of inflammation can promote outgrowth and repair. Understanding the underlying mechanisms behind inflammatory-induced regeneration (IIR) may assist in separating beneficial effects from adverse ones and uncovering novel therapeutic targets. microRNAs (miRNAs) are epigenetic inhibitory regulators known to modulate large gene networks. We previously observed the differential regulation of a miRNA, mmu-miR-383-5p, in neurons across different inflammatory contexts. miR-383 was downregulated in retinal ganglion cells (RGCs) and cortical neurons across models of IIR. Inhibition of miR-383 was sufficient to induced regeneration of RGC fibers after optic nerve crush injury. Collectively, these data suggest that miR-383 is regulated by inflammatory activity and acts to inhibit factors involved in regeneration. This thesis aimed to identify the downstream targets of miR-383 and their roles in IIR. Here, we found that miR-383 is implicated in the CNTF pathway which is known to contribute to IIR in the eye. CNTF treatment in neuron cultures led to a downregulation in miR-383 expression. Furthermore, miR-383 was confirmed to target two CNTF receptor subunits, LifR and gp130 through 3'UTR reporter assays. In RGCs, miR-383 inhibition was sufficient in upregulating LifR and gp130 protein expression. Together, the present evidence suggests that miR-383 may participate in a positive feedback loop with CNTF. This interaction allows CNTF to release the brakes on the expression of its receptor subunits and thus potentiate signaling.

RÉSUMÉ

Les blessures du système nerveux central (SNC) entrainent des lésions permanentes des neurones à cause de leurs incapacités à initier des programmes de régénération et en raison de l'absence du soutien des tissus avoisinants. Bien que l'activité inflammatoire dans les lésions du SNC soit souvent néfaste, des données suggèrent que certains processus inflammatoires peuvent promouvoir la croissance et la réparation neuronale. La compréhension des mécanismes sous-jacents à la régénération induite par l'inflammation (RII) permettrait de faire la distinction entre les effets bénéfiques et néfastes de la RII, et ainsi mener à la découverte de nouvelles cibles thérapeutiques.

Les microARNs (miARNs) sont des inhibiteurs épigéniques qui régulent un vaste réseau de gènes. Nous avons préalablement observé une régulation différentielle de l'expression neuronale d'un miARN, mmu-miR-383-5p, dans différents contextes inflammatoires. miR-383 était sous-régulé dans les cellules ganglionnaires de la rétine (CGRs) et dans les neurones corticaux, dans des modèles de RII. L'inhibition de miR-383 était suffisante pour induire la régénération des fibres nerveuses des CGRs, suite un écrasement transversal (axotomie) du nerf optique.

Mis ensemble, ces résultats suggèrent que miR-383 est régulé par l'activité inflammatoire et intervient comme inhibiteur des facteurs impliqués dans la régénération. Cette thèse a pour but d'identifier les cibles en aval de miR-383 ainsi que leurs rôles dans la RII. Nous avons trouvé que miR-383 est impliqué dans la voie de signalisation du facteur neurotrophique ciliaire (CNTF), lequel est déjà connu pour son implication dans la RII dans l'œil. Le CNTF induit une sous-régulation de l'expression du miR-383 chez des neurones en culture. De plus, miR-383 cible deux sous-unités du récepteur CNTF- LifR et gp130- testés

via un système rapporteur utilisant le 3'UTR du gène cible. Dans les CGRs, l'inhibition de miR-383 suffisait à induire une surrégulation de l'expression de LifR et gp130. Mis ensemble, ces observations suggèrent que miR-383 participe à une boucle de rétroaction positive avec la CNTF. Cette interaction permettrait au CNTF de relâcher le frein sur l'expression des sous-unités de ces récepteurs, et de ce fait, potentialiser la signalisation ci-rapportant.

PREFACE AND CONTRIBUTION OF AUTHORS

Preliminary data on miR-383 expression and outgrowth effects were completed by Camille Juźwik. Graphs and microscopy images in figures 1-2 were compiled from her experimental data and included with her permission.

All other present experiments and analyses were completed by Julia Zhang with the exception of the following. Animal procedures, including miR-383 LNA inhibitor injections and dissections of eyes, were performed by Barbara Morquette. Luciferase experiments and RT-qPCR analysis of target transcripts were completed in collaboration with Nemo Liu. The latter experiments were also assisted by Camille Juźwik. Analysis of microscopy images for target expression were performed in collaboration with Shan Shi.

LIST OF ABBREVIATIONS

3'UTR	3' untranslated region
ACM	Astrocyte conditioned media
BBB	Blood brain barrier
CM	Conditioned media
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
DRG	Dorsal root ganglion
IIR	Inflammatory-induced regeneration
IHC	Immunohistochemistry
LIF	Leukemia inhibitory factor
LNA-383	miR-383 LNA inhibitor
M383	miR-383 mimic
mRNA	Messenger ribonucleic acid
miRNA	microRNA
NC	Negative control
ONC	Optic nerve crush
PNS	Peripheral nervous system
RGC	Retinal ganglion cells
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
TBI	Traumatic brain injury
SCI	Spinal cord injury

INTRODUCTION

In the mammalian central nervous system (CNS), neurons fail to regenerate following injury(Kaplan, Ong Tone et al. 2015). While neuron stumps exhibit some regrowth after damage, these temporary measures do not contribute to meaningful repair(Benowitz and Yin 2007). This regenerative failure is what leads to the poor long-term recovery seen in spinal cord injury (SCI) patients. CNS injury can lead to permanent disability and become physically, emotionally, and economically devastating for patients and their families. As of 2016, there are approximately 27.04 million cases of SCI across 195 countries(James, Theadom et al. 2019). National spending on the initial treatment and hospitalization for SCI patients totals $1.69 billion per year in the US and $2.67 billion in Canada(Krueger, Noonan et al. 2013, Mahabaleshwarkar and Khanna 2014). These expenses do not include the long-term costs associated with CNS injury, which includes physical therapy and rehabilitation, potential rehospitalization, and lost wages due to disability(Merritt, Taylor et al. 2019). Several pharmacological treatments have been trialed, including high-dose steroid injections and anti-excitotoxic ganglioside(Eckert and Martin 2017). However, these remedies failed to show consistent regenerative effects and induced numerous harmful side effects(Eckert and Martin 2017). There is a clear and current need for effective treatments in CNS injury. A better understanding of the cellular mechanisms underlying neuron damage and repair can expand therapeutic options and address this demand.

Barriers Against Regeneration

Following injury, neurons must shift cellular programming towards repair and growth, a state reminiscent of early development(Mahar and Cavalli 2018). Growth-competent neurons, such as those in the peripheral nervous system (PNS), begin to sprout growth cones

within hours after injury(He and Jin 2016). These highly dynamic structures extend into the environment and elongate axons towards their innervation targets, leading to functional recovery(Huebner and Strittmatter 2009). In contrast, CNS neurons do not share this intrinsic regenerative ability. Fluorescent imaging studies have shown that immediately following spinal cord trauma in mice, transected axons begin to retract and die(Kerschensteiner, Schwab et al. 2005). Several hours post-injury, growth cone-like structures appear but fail to extend due to poor navigation through the extracellular environment(Kerschensteiner, Schwab et al. 2005). There are several explanations for why CNS neurons experience little growth compared to their parallels in the PNS. One potential reason is an age-related decline in regenerative ability. Following development, mature neurons switch from growth-focused processes to synapse formation and electrical signaling(Mahar and Cavalli 2018). This transition can be reverted in growth-competent neurons; however, this does not occur in the CNS(He and Jin 2016).

External factors present another barrier against successful regeneration in the CNS. Following injury, a cascade of cellular events creates an inhibitory environment against growth(Kaplan, Ong Tone et al. 2015). This was first demonstrated in grafting experiments where bridges of PNS tissue were transplanted onto rat spinal cords. While PNS axons extended only 2 mm into the spinal cord, CNS axons that crossed over onto the PNS bridge grew 15-times longer, almost the entire length of the graft(David and Aguayo 1981). These pioneering experiments demonstrated the contrasting environments between the CNS and PNS. Since then, several external inhibitory factors have been identified within the CNS milieu including myelin-associated inhibitors, chondroitin sulfate proteoglycans, and components of the glial scar(Yiu and He 2006, Kaplan, Ong Tone et al. 2015). Another large

group of external factors that affect regeneration is the invasion of inflammatory cells into the injury site. Recruited immune cells secrete inflammatory cytokines and other cytotoxic factors that further damage already injured neurons(Zhou, He et al. 2014). However, these cells can also be beneficial for injury as they clear debris and help to contain damage to the site of injury(Zhou, He et al. 2014). The roles of inflammation in CNS injury are complex and while some processes can be damaging, the potential benefits for regeneration warrant further investigation.

Inflammation and CNS Injury

In CNS injury, the inflammatory response initiates almost immediately following damage and continues for several months(Zhou, He et al. 2014). This response is traditionally thought to be detrimental towards repair due to the release of cytotoxic factors that limit regeneration. Recruited peripheral cells and activated residential glia present at the site of injury will produce inflammatory cytokines and oxidative and proteolytic enzymes(Zhou, He et al. 2014). Continuous inflammatory processes create a secondary injury site that extends past the initial wound, becoming a barrier against growth for weeks post-injury(Fitch, Doller et al. 1999, Zhou, He et al. 2014, Simon, McGeachy et al. 2017). However, the deleterious effects of post-injury inflammation are not as clear-cut as initially believed. Numerous clinical trials investigating the use of anti-inflammatory treatments in traumatic brain injury (TBI) found little benefit(Simon, McGeachy et al. 2017). Furthermore, evidence suggests that recruitment and activation of immune cells help to clear neuron debris and improve tissue repair(Kawabori and Yenari 2015). Ultimately, inflammation plays a nuanced role within CNS injury and remains a highly determinant factor for patient recovery(Zhou, He et al. 2014, Gadani, Walsh et al. 2015, Simon, McGeachy et al. 2017). A better understanding of these

intricate processes may reveal new therapies that utilize the beneficial aspects of inflammation without exacerbating injury.

Inflammatory Events Following Injury

Upon disruption of the plasma membrane, damage associated molecular patterns (DAMPs) are released into the extracellular environment(Gadani, Walsh et al. 2015). Endogenous or "self" DAMPs are referred to as alarmins. This diverse category of factors includes nucleic acids, lipids, metabolites, high mobility group protein B1, and the S100 protein class(Corps, Roth et al. 2015). Once released, alarmins are recognized by pattern recognition receptors (PRRs) which triggers a cascade of cytokines that will initiate and amplify the immune response(Gadani, Walsh et al. 2015). These early inflammatory factors, including TNF-α, IL-1β, IL-6, and IL-17, promote the expression of adhesion molecules on endothelial cells to signal for the migration of peripheral immune cells(Amulic, Cazalet et al. 2012, Ransohoff and Brown 2012).

Peripheral neutrophils are the first immune cells to cross the blood-brain barrier (BBB), becoming detectable as early as two hours after injury(Zhou, He et al. 2014). While neutrophils are well-characterized specialists against microbial pathogens in wounds, their roles in sterile injury are not fully understood(Gadani, Walsh et al. 2015). They secrete proteases, cytokines, and free oxygen radicals in injury sites that help to clear cell debris and combat microbial invasion(Amulic, Cazalet et al. 2012). However, these effectors are non-specific and ultimately lead to damage in surrounding neuronal tissue(Gadani, Walsh et al. 2015). Blocking neutrophil invasion, through knockout of CXCR2 receptors, in mice was shown to reduce neuronal cell loss and tissue damage following brain injury(Semple, Bye et al. 2010). Neutrophil numbers peak at around one to two days post-injury before undergoing

apoptosis and are phagocytosed by the next wave of invading immune cells: macrophages(Soares, Hicks et al. 1995, Elliott, Koster et al. 2017).

Upon their arrival in neuronal tissue, blood monocytes differentiate into macrophages(Auffray, Fogg et al. 2007). These peripheral immune cells, along with residential microglia, represent a substantial population of cells present in the injury site and will reside for several months(Greenhalgh, Zarruk et al. 2018). Microglia are dynamic residential glial cells that move through CNS tissue surveying for foreign compounds(Nimmerjahn, Kirchhoff et al. 2005). Once activated, microglia retract their processes and morph into a round amoeboid state. In this form, microglia resemble macrophages both in morphology and functional behavior(Kreutzberg 1996). Together, both cell populations clear damaged cell debris and help contain the wound perimeter(David, Kroner et al. 2018, Liu, Peng et al. 2019). However, like neutrophils, through the expression of inflammatory and cytotoxic factors, their activity presents detrimental effects against neuronal survival and repair.

Both microglia and macrophages contribute to glucotoxicity(Gadani, Walsh et al. 2015). In macrophages, this occurs through the production of glutamate by the enzyme glutaminase and extracellular glutamine(Yawata, Takeuchi et al. 2008). Pharmaceutical inhibition of glutaminase was sufficient to rescue the elevated levels of neuronal cell death normally seen in cultures treated with LPS-activated macrophage conditioned media (CM)(Yawata, Takeuchi et al. 2008). Additionally, microglia work in concert with TNF-α to potentiate glutamate release. Through TNFR1 signaling, TNF-α promotes microglial release of glutamate which activates mGluR2 receptors in an autocrine fashion, signaling for more TNF-α release(Olmos and Llado 2014). This positive-feedback cycle not only promotes

excitotoxicity but also further stimulates the inflammatory response. This effect is extended into the surrounding healthy tissue when viable neurons, stressed by elevated glutamate levels, transiently express phosphatidylserine on their cell surface(Neher, Emmrich et al. 2013). This “eat-me” marker targets neurons for phagocytosis by microglia and thus increases neuronal cell loss. Inhibition of phagocytosis, through knock-out of MerTK and MFG-E8, was sufficient to reduce tissue atrophy and prevent motor deficits following ischemic injury(Neher, Emmrich et al. 2013). Microglia and macrophages are also major contributors to oxidative stress through the production of free radicals. Nitric oxide synthase becomes upregulated in recruited macrophages and microglia following nerve injury, leading to increased synthesis of nitric oxide (NO)(Shafer, Chen et al. 1998, Satake, Matsuyama et al. 2000). NO is inherently cytotoxic as a free radical and additionally acts as a signaling factor to further promote inflammation(Guzik, Korbut et al. 2003, Kobayashi 2010). It also contributes to excitotoxicity by blocking glutamate reuptake receptors on the presynaptic terminus of neighboring neurons(Yuste, Tarragon et al. 2015). Ultimately, microglia and macrophages present numerous inhibitory pathways against neuron repair.

Astrocytes are another population of residential glial cells that contribute to inflammation following injury. In the healthy brain, their main responsibilities are to maintain homeostatic levels of extracellular factors, provide metabolic support for neurons, and form part of the BBB(Colombo and Farina 2016, Karve, Taylor et al. 2016). Astrogliosis describes the increase in astrocyte reactivity following damage to CNS tissue. In this activated state, astrocytes undergo morphological changes as their cell bodies swell and processes extend(Karve, Taylor et al. 2016). This process begins about three days post-injury and may continue for up to two months(Villapol, Byrnes et al. 2014). Reactive astroglia produce

inflammatory chemokines and disruptive matrix metalloproteases that damage neurons adjacent to the injury site(Colombo and Farina 2016). The numerous homeostatic and maintenance roles of astrocytes are also disrupted upon injury. Astrocytic end feet that normally encompass endothelial cells and maintain the BBB become damaged and swollen following TBI(Vajtr, Benada et al. 2009). This damage to the BBB can lead to an increase in immune cell infiltration and exacerbates tissue damage during the secondary phase of injury(Jin, Ishii et al. 2012, Karve, Taylor et al. 2016). Homeostatic glutamate levels normally controlled by astrocytes become disrupted due to the misregulation of release and reuptake processes(Ransohoff and Brown 2012, Karve, Taylor et al. 2016). CXCL12, an inflammatory cytokine commonly released in injury sites, stimulates astrocytes via CXCR4 receptors to release glutamate and TNF-α(Bezzi, Domercq et al. 2001). Similar to its effect on microglia, TNF-α regulates the glutamate-release signaling cascade within astrocytes and creates a feed-forward system that potentiates glutamate release(Bezzi, Domercq et al. 2001). Glutamate reuptake transporters, EAAT1 and EAAT2, become downregulated in astrocytes of TBI patients, suggesting an impairment in the sequestration of glutamate(van Landeghem, Weiss et al. 2006). Together, the amplified release of glutamate and its attenuated reuptake by astrocytes contributes to excitotoxicity, a common form of secondary injury(Karve, Taylor et al. 2016).

Benefits of Inflammatory Activity

It is well understood that post-injury inflammation presents a large barrier against regeneration within the CNS. This suggests that therapies limiting inflammatory activity after CNS trauma may aid neuronal recovery. Contrarily, several clinical trials have been unable to find such benefits in anti-inflammatory therapies, with some treatments being more

deleterious for repair or even leading to increased mortality(Simon, McGeachy et al. 2017). Several of these therapies use generalized anti-inflammatory methods such as corticosteroid injections, progesterone, or hypothermia(2004, Saxena, Andrews et al. 2014, Skolnick, Maas et al. 2014). The non-specific nature of these therapies likely contributed to the lack of benefits seen in such trials(Simon, McGeachy et al. 2017). This is further supported by animal studies that found depleting populations of neutrophils or macrophages after SCI led to exacerbated pathologies and worsened behavioral outcomes post-injury(Shechter, London et al. 2009, Stirling, Liu et al. 2009). Similarly, the ablation of inflammatory signaling through knockdown of toll-like receptors 2 and 4 (TRL2 and 4) in mice led to impaired motor recovery following spinal contusion injury(Kigerl, Lai et al. 2007). These results suggest that global suppression of the inflammatory response produces little benefits for recovery and can even be detrimental in certain cases. Clearly, inflammatory activity cannot be graded on a binary scale of two extremes and instead holds a nuanced role within CNS injury and regeneration.

Inflammatory-induced Regeneration Following Optic Nerve Crush

There are several studies that have shown the potential benefits of inflammatory activity following injury. Perhaps the most well-studied model of inflammatory-induced regeneration (IIR) within nerve injury is the zymosan-treated eye(Fitch and Silver 2008, Gadani, Walsh et al. 2015). The optic nerve is composed of the axon projections of retinal ganglion cells (RGCs) residing in the ganglionic layer of the retina(Sharma, McDowell et al. 2014). These neurons are surrounded by supportive glial cells including astrocytes, microglia, and Müller cells(Vecino, Rodriguez et al. 2016). As a cranial nerve of the CNS, injury to the optic nerve results in neuronal cell death and functional damage(Levkovitch-Verbin, Harris-

Cerruti et al. 2000). Several studies have shown that activating inflammatory activity within the eye following crush injury can protect RGCs and induce regeneration(Leon, Yin et al. 2000, Yin, Cui et al. 2003). Initial investigations found that a lens injury, such as a puncture wound, led to the activation of residential Müller cells and recruitment of peripheral macrophages into the retina(Leon, Yin et al. 2000). When lens injury was performed alongside optic nerve crush (ONC), there was nearly a 100-fold increase in RGC axon growth and an 8-fold increase in cell survival compared to minimally invasive puncture wounds(Leon, Yin et al. 2000). The study pointed to inflammatory activation as the key mediator because zymosan, an inflammatory stimulant, created a similar regenerative effect(Leon, Yin et al. 2000). Zymosan, a fungal-derived glucan, is a robust TLR-2 agonist that stimulates macrophage recruitment and astrocyte activation(Yin, Cui et al. 2003, Muller, Hauk et al. 2007). Injections of this inflammatory agonist produced even greater levels of RGC regeneration than lens injury, likely due to its focused inflammatory effects(Leon, Yin et al. 2000, Yin, Cui et al. 2003, Winner and Winkler 2015). In the rat spinal cord, zymosan coupled with chondroitinase ABC treatment led to functional recovery of sensory axons following DRG crush injury(Steinmetz, Horn et al. 2005).

While the regenerative effects of zymosan are promising, its potential as a therapeutic factor is limited due to the accompanied dangers of promoting uncontrolled inflammation. In one demonstration of these hazards, microinjections of zymosan in uninjured rat spinal cords triggered demyelination and axon degeneration surrounding injection sites(Popovich, Guan et al. 2002). Another study investigated the effects of zymosan on the growth of DRG transplants in the mouse cervical spinal cord(Gensel, Nakamura et al. 2009). While zymosan treatment instigated significant outgrowth, its effects quickly became neurotoxic killing

approximately 33% of transplanted DRGs within 72 h. An attempted to titrate the effects of zymosan was ultimately unsuccessful and its growth-promoting effects could not be fully disassociated from its neurotoxic ones(Gensel, Nakamura et al. 2009). Thus, zymosan likely has an extremely narrow therapeutic window, and missing this margin can exacerbate cell loss. Understanding the mechanisms behind IIR may offer more specific and safer therapeutic options. Several studies have outlined the pathways underlying the regenerative effects of lens injury and zymosan treatment, the two major mechanisms being oncomodulin and the ciliary neutrophic factor (CNTF) pathway.

Oncomodulin is a Ca^{2+} binding protein expressed and secreted by macrophages(Yin, Henzl et al. 2006). It was initially purified from outgrowth-promoting macrophage CM (MCM) via size-exclusion chromatography and mass spectrometry(Yin, Cui et al. 2003). Depleting oncomodulin from MCM blocked this growth promotion and treating cultures directly with the protein doubled the level of growth seen compared to control-treated neurons(Yin, Henzl et al. 2006). In rats, intraocular injections of oncomodulin post-ONC increased axon regeneration up to 7-fold(Yin, Henzl et al. 2006). Oncomodulin is thought to initiate a transcriptional cascade involving calcium-dependent protein kinase II (CaMKII) eventually leading to the phosphorylation and activation of cAMP/Ca^{2+} response element binding protein (CREB), a transcriptional activator(Yin, Henzl et al. 2006). Indeed, pharmaceutical inhibition of CaMKII and transcription activity were both sufficient to block oncomodulin's regenerative effects(Yin, Henzl et al. 2006). While it is clear that oncomodulin is a key mediator of inflammatory-induced regeneration, it likely does not work alone. The regenerative effects seen in oncomodulin treatment are highly dependent on intracellular cAMP levels. Co-treatment with forskolin and cAMP is necessary for oncomodulin to exert

its effects, while MCM treatment does not require these additional factors to induce growth(Yin, Cui et al. 2003, Yin, Henzl et al. 2006, Muller, Hauk et al. 2007). Oncomodulin also does not mimic the neuroprotective effects of zymosan treatment as RGCs show poor survivability post-axotomy under oncomodulin treatment(Yin, Henzl et al. 2006). This suggests the presence of other factors contributing to IIR.

Studies investigating activated astrocytes within the zymosan-treated eye model have identified the CNTF pathway as a second contributing mediator (Muller, Hauk et al. 2007, Leibinger, Muller et al. 2009, Muller, Hauk et al. 2009, Leibinger, Andreadaki et al. 2013). CNTF is a protein member of the IL-6-like cytokine family(Fischer 2017). Western blot and immunostaining analysis showed that CNTF becomes upregulated one day post-ONC and zymosan treatment(Muller, Hauk et al. 2007). This expression was specific to astrocytes and not observed in surrounding Müller cells or infiltrating macrophages. Blocking this CNTF peak with antibody treatment significantly reduced lens injury-associated outgrowth by about 30%. In contrast, anti-oncomodulin antibody treatment in the same experiment had no effect, suggesting that while oncomodulin expression is sufficient for outgrowth, it is not necessary like CNTF. Intravitreal injections of CNTF post-ONC injury notably increased RGC outgrowth and survivability, replicating a portion of the levels normally seem with IIR(Muller, Hauk et al. 2007). The effects of CNTF were later identified to be mediated by the JAK/STAT3 pathway(Leibinger, Andreadaki et al. 2013, Fischer 2017). The CNTF receptor is a tertiary complex composed of leukemia inhibitory factor receptor (LifR), glycoprotein 130 (gp130), and CNTF receptor-(CNTFr-α). Upon the binding of CNTF, Janus-kinases (JAK) 1 and 2 are recruited to the receptor complex leading to the phosphorylation of tyrosine residues. This creates docking sites for signal transducer and activator of

transcription 3 (STAT3) and SHP2, a phosphorylase. Through transference by SHP2, STAT3 becomes phosphorylated and activated. In this state, pSTAT3 forms dimers that travel to the nucleus and activate various genes containing the STAT3 response promoter element(Fischer 2017). In zymosan-treated eyes, the peak in CNTF expression is followed by a similar peak in pSTAT3 within RGCs which temporally matches with their transition into a regenerative state(Muller, Hauk et al. 2007). Furthermore, selective knockdown of STAT3 in RGCs significantly dampens both the regenerative and neuroprotective effects of inflammatory-stimulation following ONC(Leibinger, Andreadaki et al. 2013). Knocking out SOCS3, a JAK/STAT3 inhibitory regulator, increases the outgrowth levels of RGC fibers after crush injury(Smith, Sun et al. 2009). Overall, CNTF and its downstream JAK/STAT3 pathway are major contributors to IIR.

While oncomodulin and CNTF both trigger some levels of regeneration, both cannot completely capture the full effects of ocular inflammation. Oncomodulin does not protect against cell death like zymosan treatment or lens injury(Yin, Henzl et al. 2006). CNTF also cannot fully capture the level of regrowth seen in either treatment. Additionally, inhibition of CNTF does not completely ablate the benefits of inflammation in ONC models, suggesting the presence of other mediators(Muller, Hauk et al. 2007). Ultimately, the processes behind IIR is exceedingly complex and likely cannot be captured by a single pathway. Thus, it would be beneficial to investigate targets that would reveal networks of molecular mediators. Studying the regulation of microRNAs in these nerve injury models is one potential approach in addressing this goal.

microRNAs

microRNAs (miRNAs) are short, non-coding RNA sequences that function as epigenetic regulators within cells(Bartel 2004). Since their initial discovery in *Caenorhabditis elegans*, miRNAs have emerged as key cellular regulators with duties beginning early in development and continuing on throughout adulthood(Bartel 2004, Cai, Yu et al. 2009). miRNAs negatively regulate their messenger RNA (mRNA) targets through a variety of mechanisms. Because of their short structure and promiscuous targeting, a single miRNA may control up to hundreds of downstream mRNAs(Agarwal, Bell et al. 2015). This makes miRNAs attractive targets to study because understanding a small set of miRNAs can reveal a wide range of downstream mechanisms.

Biogenesis and Mechanism of Action

miRNAs' journey begins in the nucleus where they are transcribed by RNA polymerase II or III (Lee, Kim et al. 2004, Borchert, Lanier et al. 2006, Winter, Jung et al. 2009). These immature hairpin pri-miRNAs are edited by Drosha-DCR8 before they are shuttled out into the cytoplasm as pre-miRNAs(Lee, Ahn et al. 2003). Here, adolescent miRNAs undergo further editing and are prepared for loading into the RNA-induced silencing complex (RISC)(Gregory, Chendrimada et al. 2005, Winter, Jung et al. 2009). The construction of RISC begins with the union of Dicer, TRBP, and Argonaute 2 (Ago2) which altogether forms the RISC loading complex (RLC)(Gregory, Chendrimada et al. 2005). This structure accepts the incoming pre-miRNA; Dicer cleaves the hairpin sequence into a duplex structure before dissociating from the RLC with TRBP(Winter, Jung et al. 2009). What remains is a duplex miRNA sequence associated with Ago2. In its final processing stage, the double-stranded miRNA is unwound by helicases and the "template strand" is

degraded(Khvorova, Reynolds et al. 2003). The remaining strand is complementary to targets and appropriately termed the “guide strand”. This mature miRNA along with Ago2 forms the final RISC(Khvorova, Reynolds et al. 2003).

Activated RISCs inhibit mRNAs by scanning transcripts for complementation with its loaded miRNA(Bartel 2004, Huntzinger and Izaurralde 2011). Within its compact 22-nucleotide structure is the targeting seed sequence spanning from base pair positions 2-7(Agarwal, Bell et al. 2015). The seed recognizes and binds onto complementary sequences within the 3’ untranslated region (3’UTR) of mRNA targets(Bartel 2009). Upon target recognition, suppression of the mRNA occurs through two major mechanisms: transcript degradation or inhibition of translational machinery(Huntzinger and Izaurralde 2011, Ameres and Zamore 2013). Degradation of transcripts may be carried out through direct endonucleolytic cleavage by Ago2 or deadenylation of 5’-ends by the CAF1-CCR4–NOT complex followed by degradation by exonuclease XRN1(Meister, Landthaler et al. 2004, Behm-Ansmant, Rehwinkel et al. 2006, Wu, Fan et al. 2006, Eulalio, Huntzinger et al. 2009). The former occurs in cases of perfect seed-target matching, commonly seen in plants, while the latter is observed in mammals where seed-target binding is often imperfect(Huntzinger and Izaurralde 2011). miRNAs can also inhibit targets through inhibition of translational machinery. Here, transcripts are spared from degradation, and instead, the production of the final protein product is halted. This may occur in several ways, such as inhibition of elongation, concurrent degradation of protein products during translation, or premature termination(Huntzinger and Izaurralde 2011). The predominant method likely occurs at initiation where translational machinery is prevented from loading onto the 3’-cap end of transcripts(Mathonnet, Fabian et al. 2007, Huntzinger and Izaurralde 2011). The relative

prevalence of the two major miRNA silencing mechanisms remains debated(Iwakawa and Tomari 2015). Ribosomal profiling studies suggest that transcript decay is the predominant mechanism of miRNA silencing within animal cells, citing a strong correlation between changes in mRNA and protein expression(Guo, Ingolia et al. 2010, Eichhorn, Guo et al. 2014). However, the same studies also acknowledge that transcript decay cannot account for all miRNA activity and that translational inhibition may contribute up to 34% of total silencing(Guo, Ingolia et al. 2010, Eichhorn, Guo et al. 2014). Ultimately, the exact mechanism of action depends on the miRNA seed sequence, RISC components, mRNA target binding site, degree of inhibition, and cell type(Baek, Villén et al. 2008, Iwakawa and Tomari 2015).

In mammals, perfect complementation between the seed and target is not required for silencing to occur(Huntzinger and Izaurralde 2011, Agarwal, Bell et al. 2015). This inexact targeting is a key characteristic that allows single miRNAs to act as high-level epigenetic regulators, controlling up to thousands of downstream targets(Agarwal, Bell et al. 2015). Computational studies estimate that more than 60% of human protein-coding genes hold miRNA-binding sites(Friedman, Farh et al. 2008). Our lab has chosen to study miRNAs because of their high-yield nature in studying complex processes like IIR.

miR-383 in inflammatory-related outgrowth

We have previously screened various inflammatory conditions to investigate the miRNA expression in neuron regeneration. Candidate miRNAs were drawn from a past study that found significant expression changes in injured dorsal root ganglion (DRG) neurons after they were conditioned for regeneration by a sciatic nerve injury(Strickland, Richards et al. 2011). We profiled these candidates in mouse RGCs treated with zymosan. Mice were

unilaterally injected with zymosan and retinas were later collected for RGC isolation using laser capture microdissection. miRNA expression was quantified in purified samples using reverse transcription quantitative PCR (RT-qPCR). One candidate, mmu-miR-383-5p, was significantly downregulated in these regenerating neurons **(Fig. 1A-B)**. This matched data from conditioned DRG neurons, where a 1.9-fold decrease was observed in miR-383 expression compared to non-axotomized controls(Strickland, Richards et al. 2011). We also found a similar regulatory pattern across different *in vitro* inflammatory models (**Fig. 1C-F**). Embryonic mouse cortical neuron cultures treated with astrocyte conditioned media (ACM) demonstrated increased neurite outgrowth (**Fig 1C**). Similar to the zymosan model and in conditioned DRG neurons, we found that this growth-inducing treatment lead to a significant downregulation in miR-383 expression compared to vehicle-treated controls (**Fig. 1D**). Contrasting ACM-treatment, when cultures were treated with CM from peripheral blood mononuclear cell cultures (PBMC-CM), significant degeneration and inhibition of outgrowth were both observed (**Fig. 1E**)(Pool, Rambaldi et al. 2012, Morquette, Juzwik et al. 2018). Neurons in these cultures exhibited a significant upregulation of miR-383 expression (**Fig 1F**). Together, these expression data across *in vivo* and *in vitro* models point to a negative correlational relationship between miR-383 expression and neuron regeneration and outgrowth.

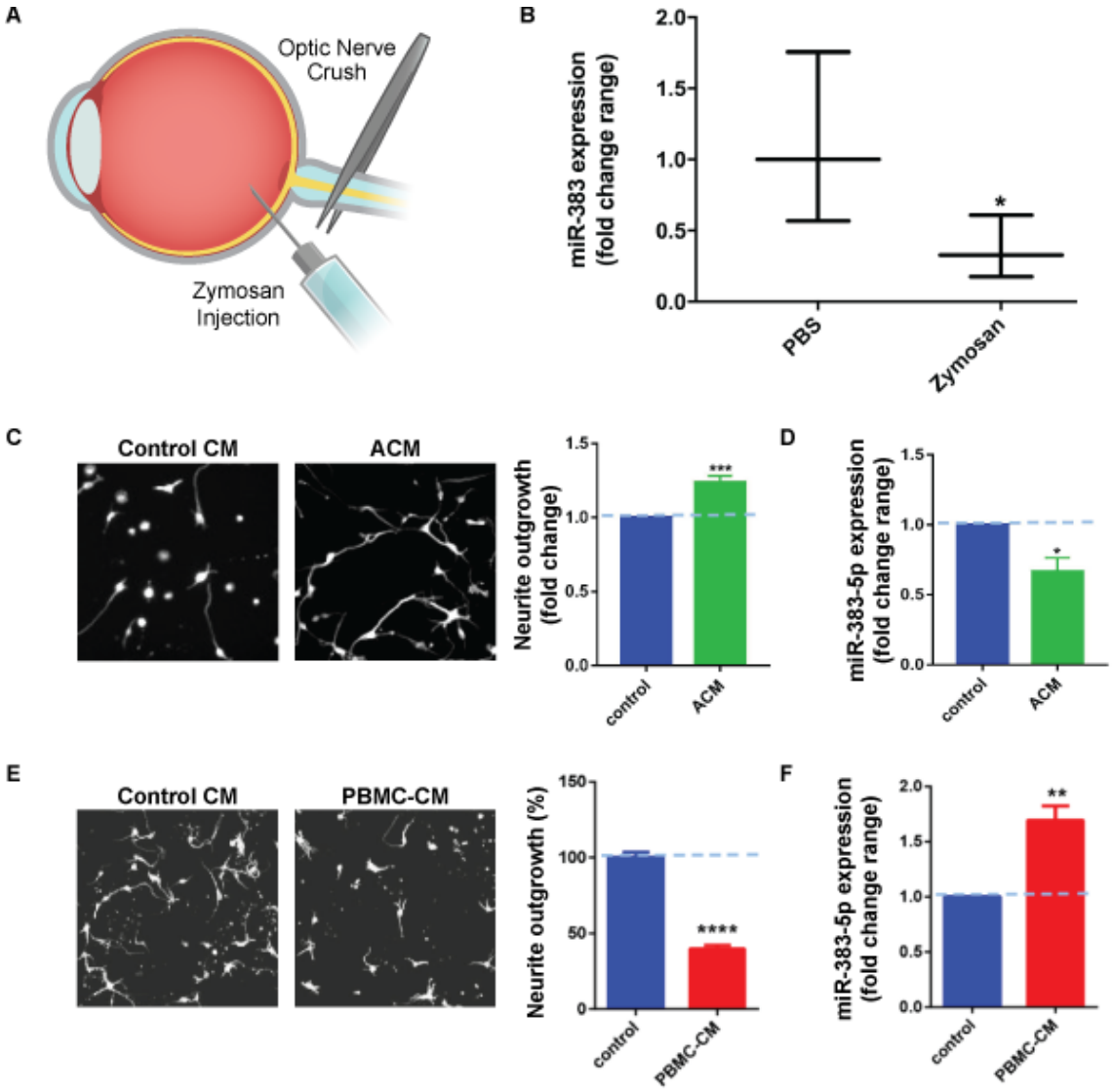

Figure 1. miR-383 is inversely regulated in growth promoting and inhibiting contexts. (A) A schematic of the zymosan-treated eye model. **(B)** RT-qPCR quantification of miR-383 expression in the retina showed significant downregulation following zymosan-treatment compared to PBS control. **(C)** βIII-tubulin staining of dissociated cortical neurons treated with control conditioned media (CM) or astrocyte conditioned media (ACM). **(D)** miR-383 expression in ACM-treated neurons, quantified by RT-qPCR. ACM-associated outgrowth matched a simultaneous downregulation in miR-383 expression. **(E)** βIII-tubulin staining of cortical neurons treated with control or peripheral blood mononuclear cell CM (PBMC-CM). **(F)** miR-383 expression within PBMC-CM treated neurons. The significant decrease in outgrowth seen with PBMC-CM treated neurons coincided with increases in miR-383 expression. Student's T-test. *p<0.05. **p<0.01. ***p<0.001. ****p<0.0001.

In support of this, follow-up experiments found that manipulating miR-383 levels in cortical neuron cultures led to changes in outgrowth levels and response to growth stimulants (**Fig. 2**). Upregulation with a synthetic miR-383 mimic ablated the increased neurite

outgrowth normally seen with ACM treatment (**Fig. 2A**). This suggests that the downregulation of miR-383 in neurons is not only consequential of ACM-treatment but also necessary for its growth-promoting effects. Furthermore, downregulation of miR-383 in cortical neuron cultures by locked-nucleic acid (LNA) inhibitors led to an increase in baseline outgrowth compared to scrambled controls (**Fig. 2B**). Animal experiments showed that miR-383 knockdown with LNA inhibitors can stimulate regeneration of RGC axons (**Fig. 2C**). Together, these lines of evidence strongly implicate miR-383 as a negative regulator of outgrowth and regeneration.

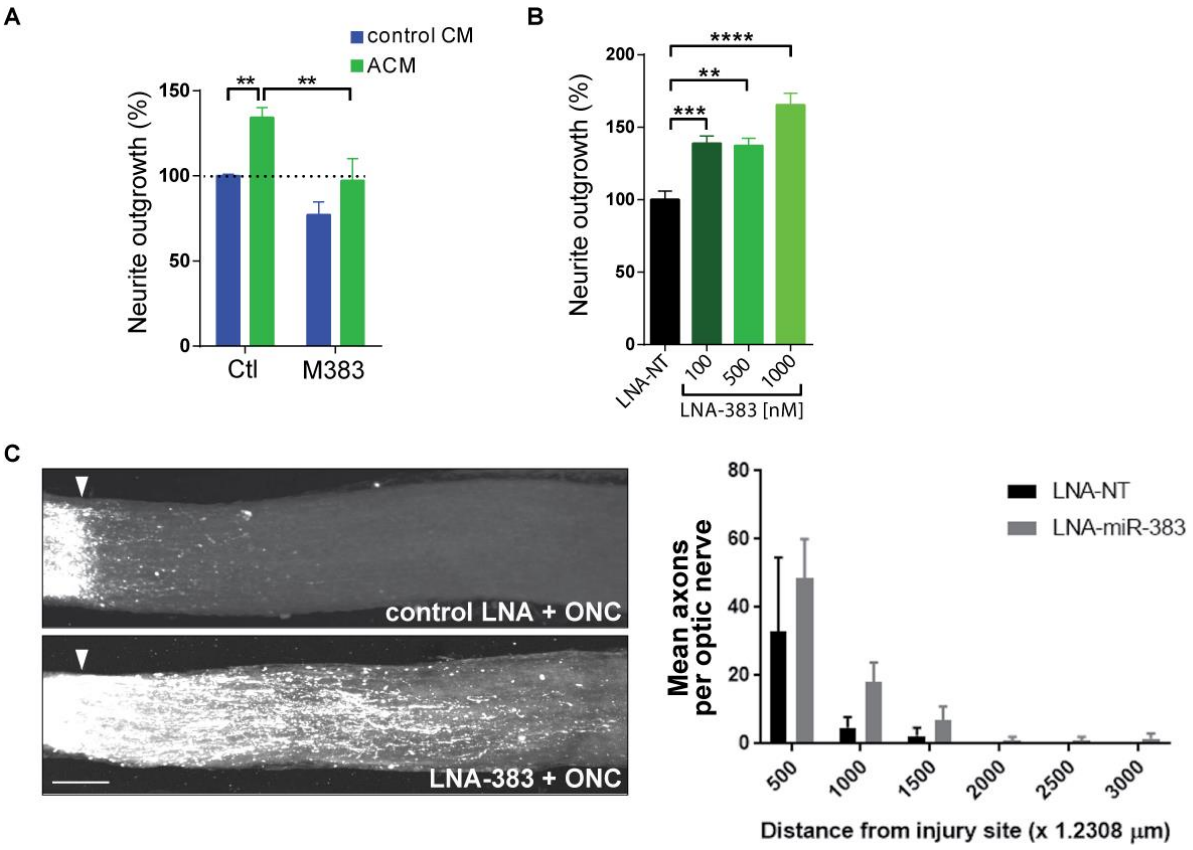

Figure 2. miR-383 inhibits baseline and ACM dependent outgrowth of cortical neurons. (A) Neurite outgrowth levels of neurons treated with ACM, with or without miR-383 mimic (M383) transfection. Forced overexpression of miR-383 in these cultures ablated the outgrowth-promoting effects of ACM. Two-way ANOVA, Dunnet's post-hoc. **(B)** Quantified outgrowth of neurons treated with varying concentrations of miR-383 LNA-inhibitor (LNA-383). Inhibition of miR-383 function promoted significant baseline neurite outgrowth compared to non-targeting controls (LNA-NT). One-way ANOVA, Dunnet's

post-hoc. (**C).** Cholera-red staining of regenerating RGC axons after ONC and LNA-383 or control intraocular injections. Axons of eyes injected with LNA-383 trended towards greater growth past crush injury site compared to controls. Arrow indicates crush site. Scale bar measures 100 μm. **p<0.01. ***p<0.001. ****p<0.000.

In the literature, miR-383 is outlined as a regulator within the reproductive system, controlling spermatogenesis and follicular development(Lian, Tian et al. 2010, Yin, Lu et al. 2012, Tian, Cao et al. 2013). In cancer research, miR-383 has been described as a suppressor against growth and metastasis of numerous cancer including medulloblastoma, prostate, hepatocellular, colorectal, and gastric cancers(Li, Pang et al. 2013, Bucay, Sekhon et al. 2017, Fang, He et al. 2017, Li, Smith et al. 2018, Wei and Gao 2019, Zhou, Zhang et al. 2019). Currently, there is little known about miR-383 targets in nerve injury or inflammation. Our experimental data suggests that miR-383 is regulated by inflammatory activity and may negatively regulate regenerative pathways. Understanding the downstream targets of miR-383 can offer insight into the regulation and mechanisms behind IIR. The present thesis aims to identify such downstream pathways and characterize their roles in IIR.

MATERIALS AND METHODS

Mouse cortical neuron cultures

Mouse cortical neuron cultures were obtained from cortical dissections of E16 mouse as previously described(Juzwik, Drake et al. 2018). Briefly, cortical tissues were dissected from embryonic day 15-17 (E15-17) C57BL/6 mice and dissociated using 0.25% trypsin-EDTA (Life Technologies) for 15-20 min at 37°C, 5% CO_2. Neurons were seeded onto 0.001% poly-L-lysine (PLL) coated 6-well plates and incubated in 10% FBS, DMEM (Life Technologies) at 37°C, 5% CO_2. Media was replaced with neurobasal (Life Technologies) supplemented with 1% N2, 2% B27 nutritional additives (Life Technologies), 1% glutamine, and 1% penicillin-streptomycin 1 h after cell seeding. Cultures were kept at 37°C, 5% CO_2 for the duration of experiments.

For miR-383 overexpression and inhibition assays, or biotinylated-miRNA pulldowns, cortical neurons were transfected with synthetic mimics (ThermoFisher), LNA-inhibitors (QIAGEN), or 3’ biotin-tagged mimics (QIAGEN) using lipofectamine RNAiMAX (Invitrogen) according to instructions. For both mimics and LNA-inhibitors, scrambled sequences were provided as negative controls (NCs) by manufacturers. In the case of biotinylated mimics, a *C. elegans* miRNA, a tagged cel-miR-63-3p was used. The final transfection concentrations were 20nM/well for mimic and biotinylated mimics, 15 nM/well for inhibitors. Cultures were transfected at 1-2 DIV, depending on their maturity.

For CNTF treatment, dilutions of rat CNTF (PeproTech) were added to culture media upon initial seeding. The diluted concentrations used ranged from 0.1 ng/μL to 10 ng/μL. Every 2-3 days, 50% of media was discarded and replenished with fresh media. Neuron

cultures were grown for a total of 8 days. Lysates were collected using QIAzol lysis reagent (QIAGEN) for subsequent miRNA purification.

Biotinylated miRNA pulldown and validation

Biotinylated miRNA pulldowns were performed was previously described(Wani and Cloonan 2014). Cultures were first washed with cold PBS to remove excess mimic present in the media. Lysates were then collected from cultures transfected with biotinylated mmu-miR-383-5p or cel-miR-63-3p negative control mimics. Protein concentrations of lysates were quantified using the Pierce BCA-RAC Protein Assay Kit (ThermoFisher). 10% of cell lysate by protein was aliquoted as an input control. The remaining lysate was incubated with Dynabeads MyOne Streptavidin C1 (Invitrogen) magnetic beads. Beads were previously prepped and blocked with 5% BSA and yeast tRNA (Invitrogen) overnight. After a 12-hour incubation, the beads were washed and eluted by boiling with loading buffer.

Eluants and input samples were assessed through SDS-PAGE and western blot analysis as previously described(Girouard, Simas et al. 2020). Separated gels were transferred onto PVDF membrane, blocked with 5% BSA, and incubated with anti-panAgo (Sigma Aldrich), diluted at 1:200, at 4°C overnight. Blots were washed and finally probed with HRP-conjugated antibodies, 1:10000, for 1 h. Chemiluminescence was detected using Western Lightning Plus-ECL (PerkinElmer).

***in silico* target analysis**

Targets were compiled from seven different prediction tools and filtered, similar to a previously described method(Juzwik, Drake et al. 2018). The programs used were: TargetScan(Agarwal, Bell et al. 2015), DIANA microT-CDS(Paraskevopoulou, Georgakilas et al. 2013), miRanda(Betel, Wilson et al. 2008), miRDB(Wong and Wang 2015),

miRTarBase(Chou, Shrestha et al. 2018), rna22(Miranda, Huynh et al. 2006), TarBase(Vergoulis, Vlachos et al. 2012). A flowchart of the complete mining and analysis process is depicted in **Fig. 4A**. TargetScan, DIANA microT-CDS, miRANDA, miRDB, and rna22 are algorithm-based prediction programs, while miRTarBase and TarBase are databases of experimentally validated targets. The input entry for all seven databases was "mmu-miR-383-5p," with targets searched within the *Mus musculus* genome. In TargetScan, two registered seed sequences for miR-383, "mmu-miR-383-5p.1" and "mmu-miR-383-5p.2", were used. For these entries, conserved and non-conserved target lists were also taken. The total compiled list was filtered for targets that were predicted by at least two programs. This list was compared against CNTF and oncomodulin pathway components obtained from the STRING interaction network database(Szklarczyk, Gable et al. 2019).

Reverse transcription and RT-PCR

Total RNA was purified from QIAzol cell lysates using the miRNEasy Mini Kit (QIAGEN). cDNA was generated using the TaqMan microRNA Reverse Transcription Kit (Applied Biosystems) for miRNA quantification or SuperScript VILO cDNA synthesis kit (Invitrogen) for mRNA transcript quantification. RT-qPCR was then performed using FAM-tagged mmu-miR-383-5p, LifR or gp130 probes (Applied Biosystems) with TaqMan Fast Advanced Master Mix (Applied Biosystems). FAM-tagged snoRNA202 or VIC-tagged GADPH probes (Applied Biosystems) were used as endogenous controls. Expression levels of miR-383 or targets were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to endogenous controls(Livak and Schmittgen 2001). For experiments that calculated absolute miRNA concentrations, levels were obtained using a known standard-curve of human β-actin.

Dual-luciferase reporter assay

HEK293T cells were first seeded in 24-well plates in 10% FBS, DMEM. 24 h later, cultures were transfected with mmu-miR-383-5p or negative control mimics (Ambion) and pEZX-MT06 dual-luciferase constructs (GeneCopoeia). Experimental vectors contained 3'UTR of LifR or gp130 cloned directly downstream of a firefly luciferase gene. Negative control constructs had no additional regulatory constructs. All vectors also contained a renilla luciferase gene under no experimental regulation, used as a normalizing control. 50 ng of vector and 10 nM of mimic were transfected with lipofectamine 2000 (Invitrogen) in Opti-MEM media (Gibco) using provided protocols. Cell lysates were collected 24 h later for analysis using the Luc-Pair Duo-Luciferase HS assay kit (GeneCopoeia) following manufacturer's instructions. Firefly and renilla activity were detected using the Infinite 100 microplate reader (Tecan). Measures of firefly luminescence were normalized to background renilla activity.

***in vivo* miR-383 inhibition and analysis of target expression**

Adult C57BL/6 mice were anesthetized with isofluorane and intravitreally injected with 2 μL of mmu-miR-383-3p LNA-inhibitor or negative control. Animals were sacrificed at 3 days post injection (DPI). The eye cups were isolated and cryoprotected in 30% sucrose at 4°C overnight. The LNA-injected retina and the non-injected contralateral retina were embedded together with Tissue-Tek OCT compound (Sakura) and stored at -80°C. Cross-sections of mounted retinas were obtained using a Leica CM3050 S cryostat. The edges of eye cups were first trimmed until a clear round cup was visible.12 μm thick sections were then obtained and were positioned in a non-consecutive manner onto slides. Samples were left to

dry at room temperature for an hour and then stored at -80°C before immunohistochemistry (IHC) was performed.

For staining, slides were blocked and permeabilized with a 3% BSA and 0.3% Triton X-100/PBS solution for 1 h. Samples were then incubated with anti-Brn3a (Santa Cruz) and either anti-LifR (abcam) or anti-gp130 (abcam) overnight at 4°C. Antibodies were diluted in 1% BSA 0.3% Triton X-100/PBS at 1:300, 1:250, and 1:100 respectively. Slides were then washed in preparation for secondary staining with Hoescht blue, Alexa Fluor 488 (ThermoFisher) for targets and Alexa Fluor 555 (ThermoFisher) for Brn3a. All secondary antibodies were diluted in 1% BSA 0.3% Triton X-100/PBS at 1:1000. Secondary staining was completed for 1 h at room temperature. Final slides were mounted with Fluoromount-G (SouthernBiotech) and cover glass and left to cure overnight at room temperature. All slides were stored at 4°C until imaging.

Fluorescent imaging and analysis

Retina samples were imaged using an Axiovert A1 inverted microscope (Zeiss) at 20x objective and ZEN software. Retinas were captured in their entirety through individual photos taken through the automated tile function and stitched together in ZEN. Final stitched images were exported as multi-channel TIFF files for analysis in ImageJ. Fluorescent intensity of protein targets was assessed using corrected total cell fluorescence (CTCF). RGCs were traced based on Brn3a signals and this mask was used to measure integrated density, area, and mean grey value of fluorescence in the target channel. The same mask was used to obtain a background reading. This was repeated for every visible RGC along the retina. CTCF values for individual RGCs were calculated using the following formula:

$$\boldsymbol{CTCF} = Integrated\ density - (Area \times Average\ grey\ value\ of\ background\ readings)$$

CTCF values for LNA-treated RGCs were normalized to their respective contralateral controls.

Statistical Analysis

Measures were statistically measured for significant using GraphPad Prism, as previously described(Morquette, Juzwik et al. 2018). Unless stated otherwise, triplicate trials were performed for each experiment. For *in vitro* culture experiments, a single trial or n was defined as a neuron culture derived from the E16 pups of a single pregnancy, or a single split cycle of HEK293T cells. For the *in vivo* animal experiments, an n indicated a retina set (injected and non-injected contralateral) from a single animal. All graphical data are presented with means ±SEM. Specific statistical tests and significance markers are described at the end of each figure caption.

RESULTS

Biotin-tagged miR-383 does not reliably associate with Ago-2

To identify bona fide mRNA targets of miR-383 we first attempted a biochemical approach to identify mRNAs that bind to miR-383. Biotinylated miRNAs offer a relatively unbiased approach towards identifying mRNA targets(Orom and Lund 2007). Synthetic miRNA mimics tagged with a biotin marker on the 3' end can be transfected into cells where they associate with mRNA targets. These targets can be isolated using streptavidin-coated beads and then sequenced (**Fig. 3A**)(Orom and Lund 2007, Wani and Cloonan 2014). In the literature, biotinylated miRNAs have been used to successfully identify targets for numerous miRNAs across different *in vitro* models(Orom, Nielsen et al. 2008, Lal, Thomas et al. 2011, Tan, Kirchner et al. 2014). However, reports have suggested that biotinylated miRNAs cannot functionally incorporate into RISC. Through Ago co-immunoprecipitation and Northern blot analysis, a study showed that biotinylated miR-27 associated with Ago at much lower the levels than its non-modified counterpart(Guo and Steitz 2014). It was argued that the biotin modification inhibits proper association with Ago due to its added bulk. Without incorporation into RISC, these mimics could not function like native miRNAs and as a result, isolated mRNA targets are possibly invalid(Guo and Steitz 2014). In direct contrast, other labs have successfully shown normal Ago-association in biotinylated miRNAs compared to non-modified controls(Lal, Thomas et al. 2011, Tan, Kirchner et al. 2014). Ultimately, these studies suggest that the effect of biotin modification on RISC association varies from miRNA to miRNA. In accordance with these studies, biotinylated miR-383 (bi-383) was first validated for functional association with Ago.

Modified miR-383 and control mimics were transfected into HEK293T cells and a pulldown was performed using streptavidin magnetic beads. RT-qPCR showed significant levels of miR-383 in the eluate, confirming the successful pulldown of bi-383 (**Fig. 3B**). The presence of Ago1-4 was assessed using western blot analysis. Out of three trials, panAgo signals were detected in only one sample set (**Fig. 3C**). This is in contrast to respective input fractions which showed the clear presence of Ago proteins (**Fig. 3D**). These results suggest that the biotin tag interferes with bi-383's ability to associate with Ago proteins. By extension, the modified mimic may not functionally incorporate into RISC. Accordingly, this method was discarded for an alternative target screening approach using predictive bioinformatic tools.

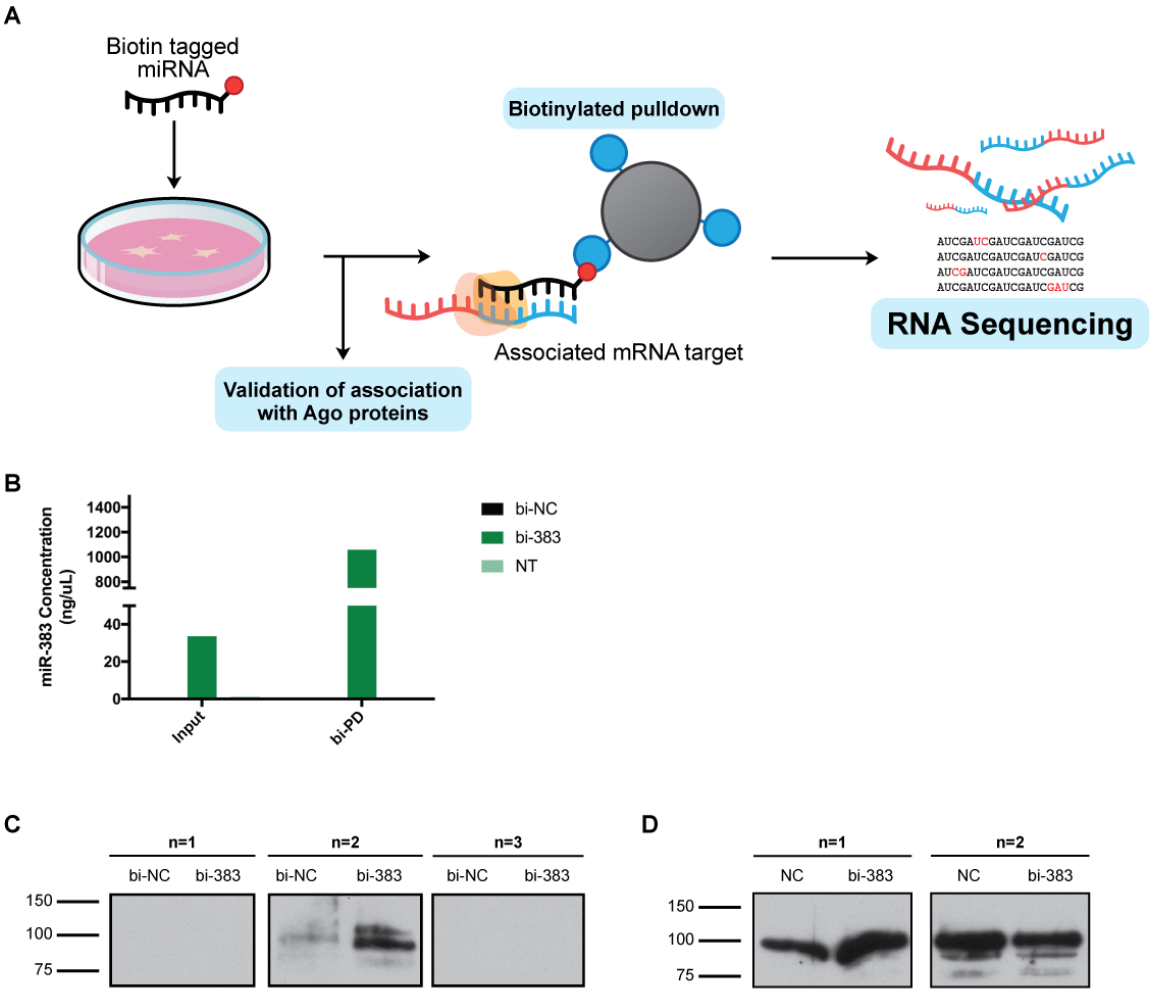

Figure 3. Ago proteins do not consistently associate with 3'-biotin tagged miR-383. (A) A workflow schematic of a biotinylated-miRNA pulldown and target sequencing. **(B)** Quantification of miR-383 concentrations in eluant fractions with RT-qPCR confirmed successful pulldown. **(C)** Eluant fractions were probed for panAgo proteins (Ago1-4). Only a single trial showed the presence of Ago proteins. **(D)** Western blots of respective input fractions confirm the presence of panAgo in lysates prior to pulldown.

miR-383 is predicted to target components of the CNTF pathway

In order to reliably identify candidate targets, a data mining approach was taken with target prediction algorithms and databases. Some of these programs are databases of experimentally-validated targets, such as miRTarBase and TarBase(Vergoulis, Vlachos et al. 2012, Chou, Shrestha et al. 2018). The remaining majority are rule-based algorithm programs that scan genomic sequences for complementation between a miRNA seed sequence and

3’UTRs. Aside from seed-matching, contextualizing parameters, or “rules”, are used to score and refine predictions(Yue, Liu et al. 2009). These rules include sequence conservation, thermodynamic considerations, and flanking regions that stabilize and/or enhance silencing(Bartel 2009, Yue, Liu et al. 2009, Agarwal, Bell et al. 2015). The ordering of these rules greatly affects the final candidate list, thus very different results can be produced for the same miRNA between different prediction programs(Yue, Liu et al. 2009). To account for these differences, targets were compiled across seven different target tools including TargetScan(Agarwal, Bell et al. 2015), DIANA microT-CDS(Paraskevopoulou, Georgakilas et al. 2013), miRANDA(Betel, Wilson et al. 2008), miRDB(Wong and Wang 2015), miRTarBase(Chou, Shrestha et al. 2018), rna22(Miranda, Huynh et al. 2006), and TarBase(Vergoulis, Vlachos et al. 2012). This produced a list of 17,752 predicted targets (**Fig. 4A**). By far the largest contributor was the rna22 with 9,470 targets, many of which were unidentified genes. To remove irrelevant entries, only targets predicted by two or more databases were considered. This narrowed down candidates to a refined list of 7,018 genes.

Previously, our lab showed that miR-383 was downregulated in RGCs within the zymosan-treated eye model and in cortical neuron cultures treated with ACM. This regulation within these inflammatory contexts suggests that miR-383 may be involved with pathways related to IIR. To investigate this potential role, the list of predicted targets was compared against components of the CNTF and oncomodulin pathway, two known mediators of IIR in the zymosan-treated eye(Yin, Henzl et al. 2006, Muller, Hauk et al. 2007). Both pathways were compiled using the STRING interaction network database, producing a list of 11 genes in the CNTF pathway and 13 in the oncomodulin pathway(Szklarczyk, Gable et al. 2019). When cross-referenced with the candidate target list, 6 CNTF-associated genes and 1

oncomodulin gene were predicted to be regulated by miR-383 (**Fig. 4B)**. Within the CNTF pathway, miR-383 was predicted to target subunits of the CNTF receptor including LIFR and IL6ST (protein name gp130), as well as accessory receptors LEPR and EGFR. Since miR-383 appeared to have a stronger association with the CNTF network, this pathway was further explored.

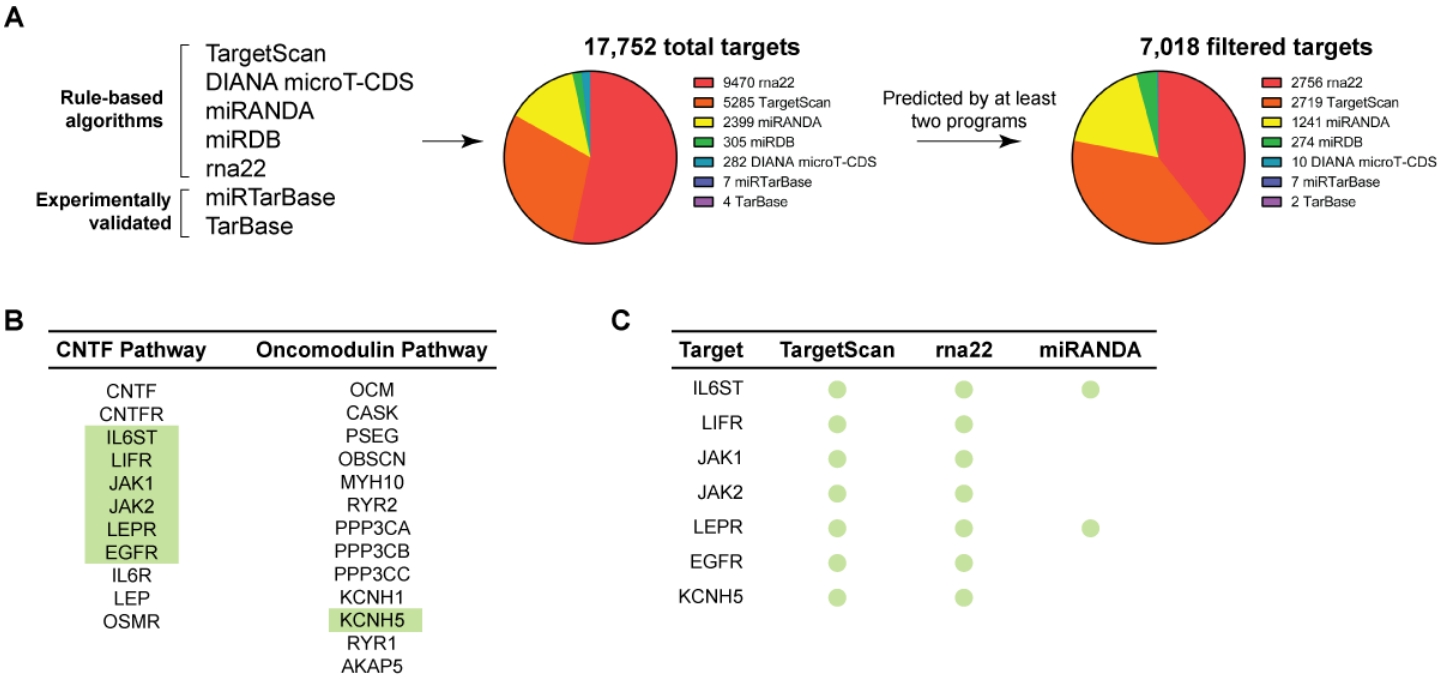

Figure 4. *in* silico screening for candidate miR-383 targets. (A) A workflow schematic of target mining and analysis. Targets were generated from seven different databases or prediction programs and filtered to remove duplicates, producing 7,018 unique targets. **(B)** Components of the CNTF and oncomodulin pathway compiled using the STRING interaction network tool. Highlighted in green are the factors predicted to be targeted by miR-383. **(C)** Table diagram of prediction programs that generated CNTF or oncomodulin pathway components.

CNTF treatment downregulates miR-383 expression in mouse cortical neurons

To better understand the direct relationship between CNTF and miR-383, we investigated the influence of CNTF treatment on miR-383 expression in neurons. CNTF is released by activated astrocytes in the retina following inflammatory stimulation(Muller, Hauk et al. 2007). We previously found that miR-383 levels are negatively regulated by

ACM. Due to its implication as an astrocyte-secreted factor, the relationship between CNTF and miR-383 was investigated here.

miR-383 expression was quantified, using RT-qPCR, from cortical neuron cultures treated with varying dilutions of CNTF. Treatment concentrations were based on a previous study that found CNTF exerted maximal outgrowth effects on rat hippocampal neurons at 10 ng/mL(Ip, Li et al. 1991). Here, we found that miR-383 expression began to significantly decrease at 0.5 ng/mL ($p < 0.05$) (**Fig. 5A**). At higher concentrations (1 ng/mL and 10 ng/mL), miR-383 expression remains significantly reduced compared to non-treated neurons ($p < 0.05$). This effect is similar to the downregulation previously seen with ACM treatment and in the zymosan-treated eye **(Fig 2B, D**). The present data suggest that the miR-383 expression pattern seen across these inflammatory contexts may be in part due to CNTF released by astrocytes.

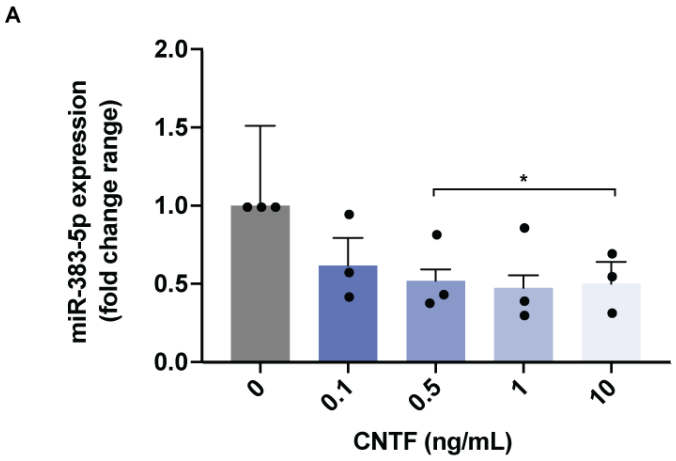

Figure 5. CNTF treatment downregulated expression of miR-383 in culture. (A) RT-qPCR quantification of mouse cortical neurons treated with various dilutions of CNTF showed significant decreases in miR-383 express. Values were normalized to the 0 ng/mL treatment condition. One-way ANOVA, Dunnet's post hoc. *p<0.05.

miR-383 inhibits LifR and gp130 through their 3'UTR

To further investigate the involvement of miR-383 in the CNTF pathway, we evaluated the targeting of two CNTF receptor subunits, LifR and gp130. Both are candidate targets of miR-383 identified by at least two predictive programs (**Fig. 4C**). Furthermore, they are expressed throughout the mouse retina and brain cortices, making them well-suited targets for our experimental models(Suzuki, Yamashita et al. 2005, Burgi, Samardzija et al. 2009, Coorey, Shen et al. 2015). In order to assess the regulation of LifR and gp130 by miR-383, a luciferase reporter assay was used. This observes the activity level of a luciferase reporter gene under the regulation of a candidate 3'UTR (**Fig. 6A**). Successful recognition and inhibition of the 3'UTR by a miRNA should lead to a decrease in detected luminescence(Jin, Chen et al. 2013). The 3'UTRs of LifR or gp130 were cloned immediately downstream of a firefly luciferase gene in pEZX-MT06 vectors. These constructs were transfected into HEK293T cells along with miR-383 or NC mimics. Vector sequences provided by the manufacturer were confirmed to contain recognition sites complementary to miR-383 seed sequence (**Fig. 6B**). Since the LifR 3'UTR was too large to be cloned in its entirety, the sequence was separated and cloned into individual vectors. This produced a non-seed, and a seed-containing vector (referred to as the LifR 3'UTR). In the presence of miR-383 mimics, the non-seed vector showed similar luminescence levels to empty vector controls (**Fig. 6C**). In contrast, cells transfected with LifR and gp130 3'UTR vectors showed a significant decrease in reporter signaling ($p < 0.05$). This strongly suggests that miR-383 negatively regulates the expression of LifR and gp130 through interaction with their 3'UTR.

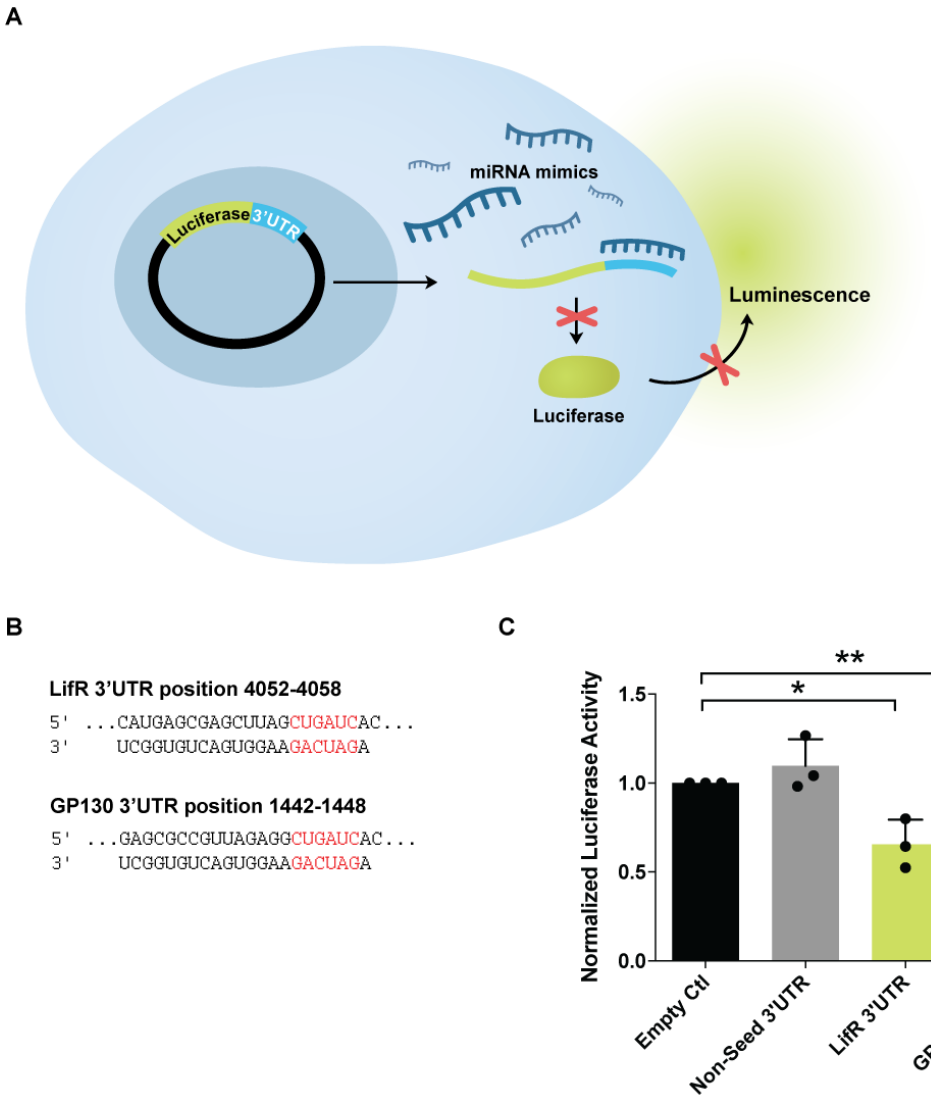

Figure 6. 3'UTRs of LifR and gp130 are negatively targeted by miR-383. (A) A molecular diagram of luciferase-3'UTR reporter assay. **(B)** Selected portions of LifR and gp130 3'UTR sequences with miR-383 recognition sites highlighted in red. Upper sequence represents target 3'UTR and the lower sequence is miR-383-5p. **(C)** Luminescence levels of HEK293T cells transfected with control or target 3'UTR dual-luciferase vectors. A significant reduction of luminescence signals was observed in LifR and gp130 3'UTR constructs while non-seed 3'UTR and control constructs exhibited no change. Measures were normalized to renilla background luminescence and then to the empty control vector condition. One-way ANOVA, Dunnet's post hoc. *$p<0.05$ **$p<0.01$.

There are multiple mechanisms in which miRNAs silence target expression. Past widescale transcriptome analyses have suggested that transcript degradation is the most common means of silencing(Huntzinger and Izaurralde 2011). However, alternative mechanisms also exist where transcripts are spared and instead, translational processes are

either inhibited or prematurely terminated(Huntzinger and Izaurralde 2011). To better understand the mechanism of regulation between miR-383 its targets, the transcript levels of LifR and gp130 in the presence of miR-383 overexpression or inhibition were quantified using RT-qPCR. In both mimic overexpression and LNA inhibition, LifR and gp130 transcript levels did not significantly deviate from negative controls (**Fig 7A-B**). These findings suggest that miR-383 does not inhibit LifR or gp130 through transcript decay. Rather, its regulation is likely exerted through the inhibition of translational machinery. This can be further evaluated by observing the influence of miR-383 on the expression levels of target protein products.

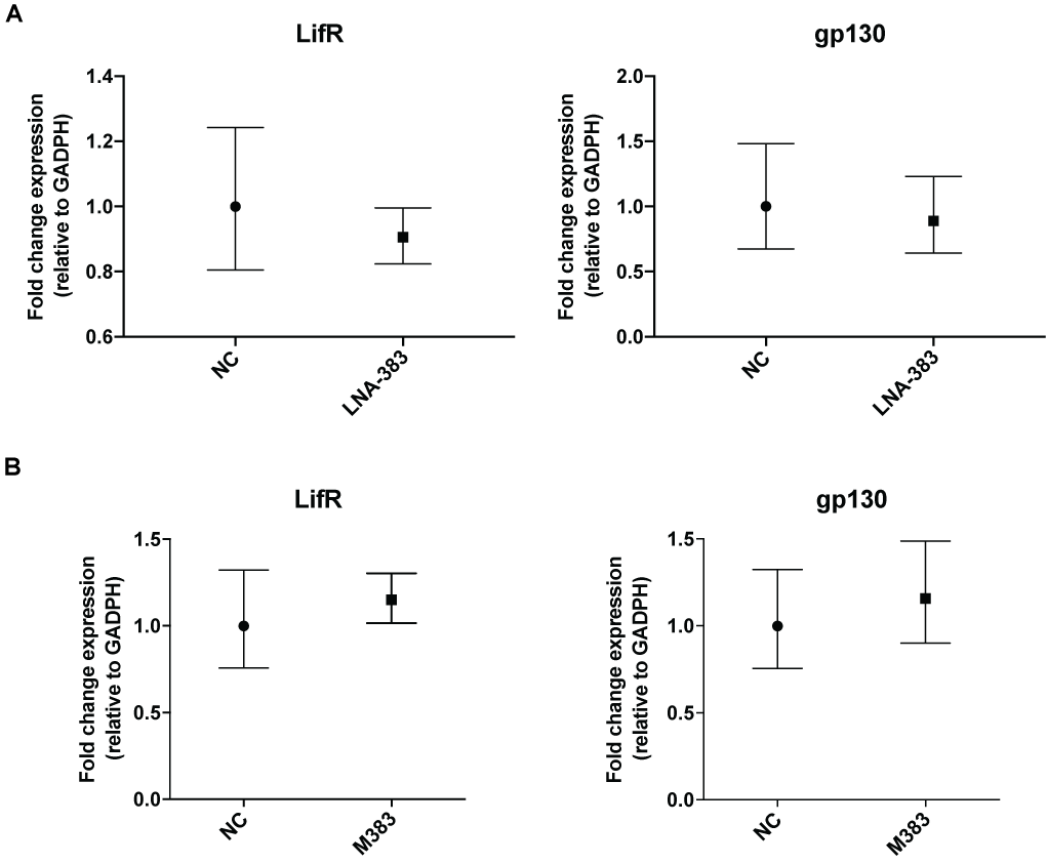

Figure 7. Variation in miR-383 expression did not affect target transcript levels. **(A-B)** Transcript levels, analysed through RT-qPCR, of LifR or gp130 in cortical neuron cultures treated with LNA inhibitors (LNA-383) or miR-383 mimics (M383). Inhibition of miR-383 did not significantly change target transcript levels compared to negative control inhibitors (NC). Likewise, no significant change was detected with mimic overexpression (M383). Values were normalized to negative control mimics (NC). Student's T-test.

miR-383 inhibition upregulates LifR and gp130 expression in RGCs *in vivo*

To evaluate the regulation of target products in RGCs, LNA inhibitors were used to downregulate miR-383 activity and target expression was evaluated using fluorescent IHC. Mice were unilaterally injected with LNA-383 or LNA-NC and sacrificed at 3 DPI. Retinal sections were prepared for immunostaining with LifR or gp130 antibodies. Both LifR and gp130 are expressed by a variety of cells throughout the retina; to selectively measure the fluorescent intensity within RGCs, retinas were co-stained for Brn3a, an RGC specific marker(Nadal-Nicolas, Jimenez-Lopez et al. 2009). Only signals that co-localized with Brn3a were analyzed. All measurements were corrected for background signaling and normalized to their respective non-injected contralateral eye. Compared to LNA-NC, LNA-383 treated retinas showed a significant upregulation in fluorescence intensity for both targets ($p > 0.05$) (**Fig. 8A-D**). Brn3a-positive cells showed a 29.9% increase in LifR expression and 32.5% in gp130. This evidence further demonstrates the negative regulation of both receptor subunits by miR-383. Given previous data showing that transcript levels remain unchanged, this current data supports the potential that miR-383 inhibits translational machinery to downregulate LifR and gp130 expression. Given that inhibition of miR-383 has previously been shown to stimulate the regeneration of RGCs post-ONC, it is possible that this regenerative effect was partially due to the upregulation of LifR and gp130 expression, which would translate to increased CNTF signaling.

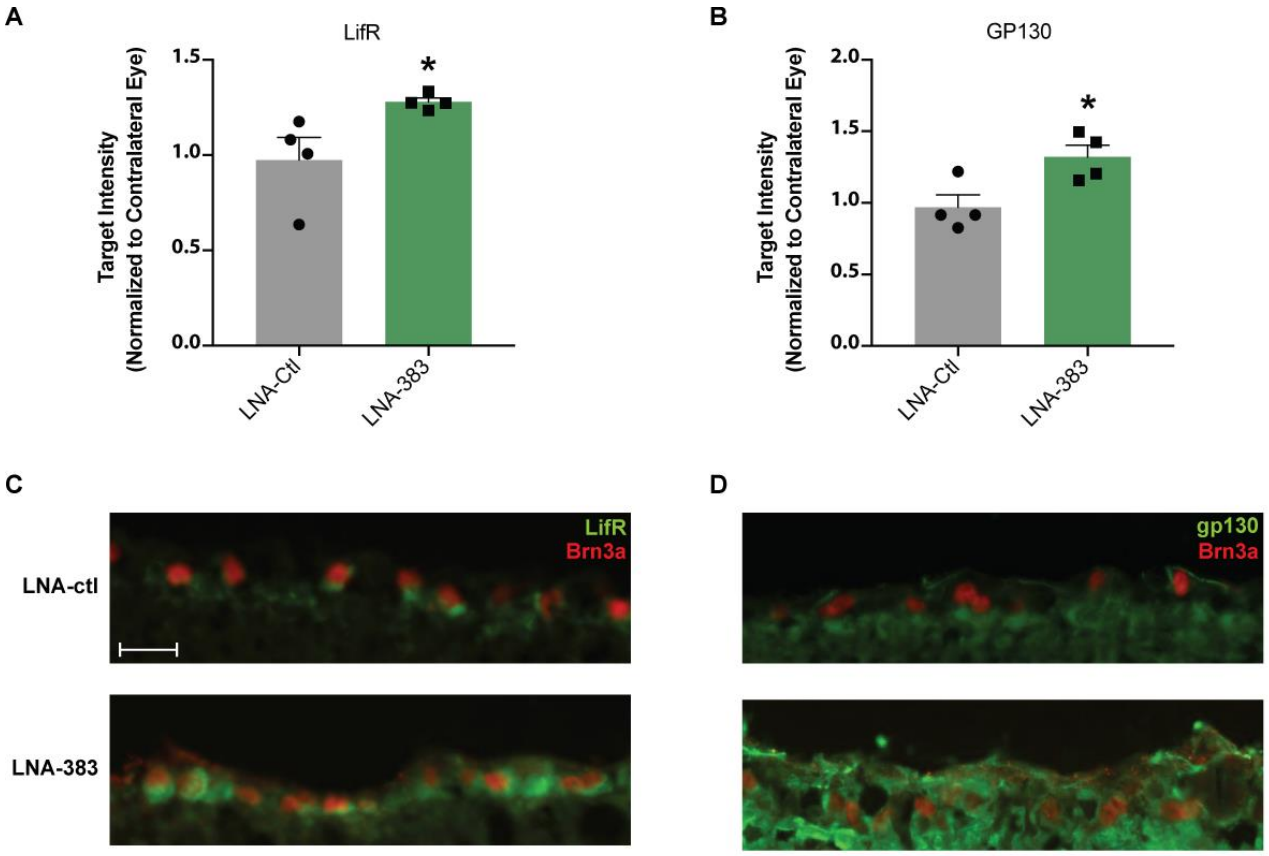

Figure 8. Inhibition of miR-383 upregulated protein expression of LifR and gp130 in RGCs. (A-B) Quantification of target fluorescent intensity in RGCs treated with miR-383 inhibitor (LNA-383) or control (LNA-ctl). LifR and gp130 expressions were both significantly increased in RGCs when miR-383 activity was dampened by inhibitors. Student's T-test. *$p < 0.05$ **(C-D)** Images of mouse retinas stained for LifR or gp130 (green), and Brn3a (red), an RGC-specific marker. Retinas injected with miR-383 inhibitors demonstrated a visible increase in fluorescent intensity. Scale bar measures 50 μm.

DISCUSSION AND FUTURE DIRECTIONS

Summary

CNS injury leads to permanent damage and debilitating long-term consequences due to tissues' inability to promote repair. Inflammatory activity is initiated following injury. These processes are known to block neuroregeneration and contribute to further damage. However, there is also evidence that suggests some forms of inflammation may promote regenerative programming in neurons. A better understanding behind IIR can help separate its positive effects from harmful ones and may reveal novel therapeutic targets. miRNAs are epigenetic regulators that are especially useful for studying complex processes like IIR due to their ability to regulate networks of targets. This current thesis aimed to investigate the downstream targets of a miRNA, miR-383, and its involvement in IIR-related pathways.

Previously, we have investigated the roles of miR-383 in various models of neurodegeneration and regeneration. Expression of miR-383 was found to be regulated in various inflammatory environments and negatively correlated with outgrowth activity. In the growth-promoting environments of ACM and the zymosan-treated eye, miR-383 was downregulated in neurons. Furthermore, forced overexpression of miR-383 in ACM-treated neurons dampened ACM's outgrowth-promoting effects. This suggested that miR-383 downregulation is necessary for astrocyte-induced outgrowth. In support of this, inhibition of miR-383 alone in neuron cultures was sufficient to promote baseline neurite outgrowth. Conversely, in the degenerative environment of PBCM-CM, cortical cultures demonstrated an upregulation in miR-383 expression. In the eye, miR-383 inhibition following ONC promoted regeneration of RGC fibers. Together, the past data suggests that miR-383 is a negative

regulator of outgrowth and regeneration, and its expression is likely under the regulation of inflammatory-associated pathways.

In the present study, we initially identified several thousand candidate mRNA targets through the mining of predictive algorithms and target databases. Analysis of this list revealed that miR-383 was predicted to regulate several components of the CNTF pathway, a known pro-regenerative pathway within the zymosan-treated eye. In cortical neurons, we showed that CNTF treatment led to a decrease in miR-383 expression. Additionally, the targeting of CNTF receptor subunits, LifR and gp130, by miR-383 was investigated. Potential targeting of the two factors was supported by dual-luciferase reporter assays. In the presence of miR-383 overexpression, reporter activity is significantly diminished in constructs containing LifR 3'UTR or gp130, but not in control or non-seed containing 3'UTR constructs. While the transcript levels of either target were not affected by variations in miR-383 activity, protein levels were upregulated in RGCs treated with miR-383 inhibitors. Overall, the present evidence strongly suggests a reciprocal regulatory relationship between miR-383 and the CNTF pathway. By extension, it is likely that a portion of the regenerative effects seen in miR-383 inhibition can be attributed to the downstream activation of the CNTF pathway.

Interpretation of Results and Future Directions

miR-383 and the CNTF pathway

The evidence presented here implicates miR-383 as a novel downstream effector of the CNTF network. The negative regulation of miR-383 by CNTF matches our previous data showing that ACM and zymosan-injections downregulate miR-383 expression in cortical neurons and RGCs, respectively. Since CNTF is an astrocyte-released factor, the dampened miR-383 expression observed may be attributed to the presence of CNTF in both models.

miR-383 is further implicated in the pathway through its targeting of two receptor subunits, LifR and gp130. We showed that miR-383 targets the 3'UTR of both transcripts and reducing miR-383 activity will upregulate the expression of protein products in RGCs. Through its interactions with miR-383, CNTF may be indirectly upregulating its receptors, creating a positive feedback loop and potentiating its own signaling (**Fig. 9**). This also implies that miR-383 inhibition alone may sensitize neurons to regenerative signaling. At baseline, CNTF receptors subunits are not highly expressed within mature RGCs(Fischer 2017). Although the administration of CNTF can promote fiber regeneration, this alone is not optimal in providing sufficient repair(Fischer 2017). Activation of factors adjacent to the CNTF pathway has been suggested to address this problem and amplifying regenerative effects. These alternatives include upregulating downstream JAK/STAT3 signaling through SOCS3 knockout or using synthetic hyper-IL-6 cytokines that boost activation of CNTF receptors(Fischer 2017). Our present data suggest that miR-383 inhibition may be another effective method in amplifying CNTF signaling. Administration of miR-383 inhibitors can lift the brake on CNTF receptor expression and thus sensitize neurons to IIR-related signaling.

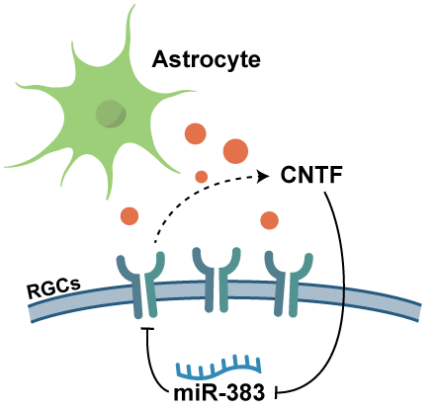

Figure 9. A visual model of the positive-feedback loop between CNTF and miR-383. Upon inflammatory stimulation in the eye, CNTF is released by astrocytes. This acts to depress miR-383 expression in RGCs which lifts inhibition of CNTF receptor subunits, LifR and gp130. Through these interactions, CNTF may increase its own signaling and promote further regeneration.

LifR and gp130 in the IL-6 cytokine family

LifR and gp130 act as signal-transducing receptor subunits for the IL-6 cytokine family(Heinrich, Behrmann et al. 2003). In addition to the CNTF pathway, both are crucial for the downstream signaling of IL-6, leukemia inhibitory factor (LIF), and cardiotrophin-1 (CT-1)(Heinrich, Behrmann et al. 2003, Davis and Pennypacker 2018). LIF and CNTF are two key IL-6-like factors expressed in the nervous system with important roles in the development of neurons, astrocytes, and oligodendrocytes(Yoshida, Satoh et al. 1993, Mayer, Bhakoo et al. 1994, Galli, Pagano et al. 2000). LIF has similar regenerative effects to CNTF. In neuron culture, the administration of LIF promotes neurite outgrowth(Gillespie, Clark et al. 2001, Leibinger, Muller et al. 2009). Furthermore, LIF likely contributes to IIR along with CNTF in the zymosan-treated eye. Researchers demonstrated that knockdown of CNTF partially reduced RGC regeneration normally seen with inflammatory-stimulation, while double CNTF and LIF KO animals showed complete ablation of all regenerative effects(Leibinger, Muller et al. 2009). This finding fills the gap in IIR as several past studies have shown that CNTF treatment alone cannot replicate the full effects of IIR.

An important differentiation between LIF and CNTF signaling is that CNTF requires the addition of an α receptor subunit, CNTFr-α, to complete its tripartite receptor complex(Ip, McClain et al. 1993). In comparison, LIF only requires dimers of LifR and gp130 to transduce downstream signaling. CNTFr-α is not highly expressed within mature neurons and is not predicted to be targeted by miR-383(Fischer 2017). Thus, its limited expression would not be promoted through the CNTF-induced downregulation of miR-383. It is possible that CNTF regulation of miR-383 may better sensitize neurons to LIF rather than CNTF itself, as all components of the LIF receptor complex are upregulated with miR-383 inhibition. LIF and

CNTF both converge onto the phosphorylation of STAT3 which is key in initiative regenerative programming(Stahl, Boulton et al. 1994). Thus, the downstream effects of LIF and CNTF are very similar, but miR-383 inhibition may ultimately promote a stronger response to LIF than CNTF. The interplay between LIF and miR-383 in neuron outgrowth and regeneration would be worthwhile examining in the future. This proposed conjecture can be investigated through simple outgrowth experiments comparing the effects of LIF versus CNTF treatment in the presence of miR-383 inhibition.

Alternative miR-383 targets

Our past *in vivo* experiments have shown that the administration of miR-383 inhibitors alone is sufficient to promote regeneration in RGC fibers. This effect cannot be completely explained by the upregulation of LifR and gp130. Without the presence of CNTF, the receptor subunits cannot transduce downstream signaling to activate STAT3, a key step in initiating regenerative programming(Tenhumberg, Schuster et al. 2006, Leibinger, Andreadaki et al. 2013). One possibility is that miR-383 inhibition bypasses ligand-signaling to promote the direct upregulation of downstream effectors. Our *in silico* search found that miR-383 was predicted to target JAK1 and 2, two key kinases downstream of CNTF that activate STAT3. Exploration of these candidates would expand understanding of miR-383's roles in regeneration and outgrowth.

It is also possible that there are alternative targets outside of the IL-6 cytokine family that may contribute to promote regeneration. Here, we compiled a list of almost 10 000 candidates. Bioinformatic tools like the PANTHER gene analysis may reveal enrichment of cellular pathways in this candidate list(Mi, Muruganujan et al. 2019). Our current investigations were based on well-studied pathways known to contribute to IIR, and on our

previous data implicating miR-383 in inflammatory-related outgrowth. It may be beneficial to adopt a non-biased approach to target identification. Here, we attempted to use a biotinylated-miRNA pulldown to carry out a widescale target search and but was ultimately unsuccessful. However other alternative methods such as miRNA knockdown followed by transcriptome or microarray analysis may be used. These further investigations may reveal novel factors that contribute to regeneration.

CONCLUDING REMARKS

Together, the line of evidence presented in this thesis demonstrates a novel interaction between miR-383 and the CNTF pathway. The regulatory relationship between miR-383, CNTF, and its receptor subunits LifR and gp130 the existence of a positive-feedback loop resulting in the potentiation of CNTF signaling. This finding is especially relevant for translating aspects of IIR into effective therapies. Inflammatory activity can promote regeneration of damaged neurons, however, a small tip of the scale may turn these beneficial effects into deleterious ones. The CNTF-miR-383 loop may present a solution to this dilemma since inhibition of miR-383 can potentially sensitize neurons to IIR-related factors. Thus, it is a possible that with miR-383 inhibitors, a lesser degree of inflammatory stimulation would be needed to trigger the same regenerative effect. Further investigations on the interaction between miR-383 and other IL-6 cytokine family members, such as LIF, as well as other downstream targets may reveal stronger effects. Ultimately, such work can expand our understanding of the molecular mechanisms behind IIR and lead the way towards new therapeutic options for CNS injury.

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