TOP2B DEFICIENCY AFFECTS RETINAL CELL DEVELOPMENT

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ABSTRACT OF THE THESIS

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Topoisomerase II beta (Top2b) is an enzyme that assists with DNA transcription by controlling the topological state of DNA to prevent supercoiling of the DNA and is widely expressed in postmitotic neurons. It is known to play a role in neuronal migration, cell adhesion, voltage-gated calcium channel activity, synaptic transmission, and cytoskeleton formation. In the retina, Top2b contributes to the late-stage differentiation and maturation of photoreceptors and affects the expression of key genes linked to retinopathies. Top2b deficiency has shown to lead to degeneration of the plexiform layers and outer segment of photoreceptors and reduction of cell number in the retina. However, the specific morphological differences between Top2b deficient retinal cells and normal retinal cells are not well documented. Additionally, the role of Top2b in interkinetic nuclear migration during retinal development has not been well defined. In this study, the role of Top2b in the developing retina was studied in two model systems: 1) the chick model was used to perform shRNA-based gene knockdown on cultured cells and retinal explants at embryonic day 10 (E10) to determine the function of Top2b at the embryonic stage; and 2) the gene knockout mouse model was used to examine tissue sections with Top2b deficiency at postnatal day 7 (P7), P14, and P21. *In vitro* shRNA knockdown was conducted to examine the role of Top2b in morphological development of the retina at the cellular level, while *ex vivo* and *in vivo* studies were used to examine cellular migration and morphology at the tissue level. Results show that i) after 3 days of Top2b knockdown *in vitro*, E10 retinal cells exhibit less surface area (3-fold reduction, p < 0.05) and shorter cellular processes (2-fold reduction, p < 0.05); ii) after 3 days of Top2b knockdown in the developing E10 chick retina, cells have shortened cellular processes but do not exhibit any migratory delays; iii) Top2b knockout delays interkinetic nuclear migration at P7 in the developing mouse retina, but does not inhibit migration of cells into the ONL in the long-term. This study helps to further characterize the role of Top2b in the developing retina and may provide insights into pathogenesis of various retinal disorders.

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LIST OF ABBREVIATIONS

CNS	Central nervous system
DMEM	Dulbecco's Modified Eagle
	Medium
Е	Embryonic day
FBS	Fetal bovine serum
f/f	Floxed/floxed
GAPDH	Glyceraldehyde 3-phosphate
	dehydrogenase
GCL	Ganglion cell layer
GFP	Green fluorescent protein
НН	Hamburger & Hamilton
INBL	Inner neuroblastic layer
INL	Inner nuclear layer
IPL	Inner plexiform layer
ONBL	Outer neuroblastic layer
ONL	Outer nuclear layer
OPL	Outer plexiform layer
Р	Postnatal day

PBS	Phosphate buffered saline
qPCR	Quantitative polymerase chain
	reaction
RPC	Retinal progenitor cell
Top2b	Topoisomerase II beta

Chapter 1

Significance and Background

1.1 Introduction and Significance

Topoisomerase II beta (Top2b), a protein universally expressed in postmitotic neurons, assists with DNA transcription by controlling the topological state of DNA to prevent supercoiling of the DNA (Nitiss, 2009; Wang, 2002). Studies demonstrated that Top2b is involved in the expression of extremely long genes (>200kb) that play a role in brain development and autism spectrum disorder (King et al., 2013). Top2b also plays a vital role in retinal development, neuron migration, cell adhesion, voltage-gated calcium channel activity, synaptic transmission, and cytoskeleton formation (Heng and Le, 2010). Top2b is crucial in neural/retinal development, because it is necessary for proper neurite outgrowth and axon path-finding (Nur et al., 2007). Neurite outgrowth is a key developmental process, because it allows for active migration of the neuron from its site of origin to its destination site (Khodosevich and Monyer, 2010). Neurite outgrowth also allows for the establishment of connections between the neuron and its target for proper signal transduction (Laketa et al., 2007).

Transcriptome analysis conducted in our lab revealed that the expression of key genes in the reelin signaling pathway were downregulated in the brain of Top2b knockout mouse embryos. The extracellular matrix protein reelin can activate Rap1/Dab1-mediated cell adhesion processes to control the radial migration of cortical neurons (Franco et al., 2011). The decreased expression of genes in the reelin signaling pathway may contribute to the neuronal migration defects identified in Top2b knockout neocortex (Lyu and

Wang, 2003). Transcriptome analysis also revealed the downregulation of major cell adhesion genes, including several cadherins such as Cdh2, Cdh8, and Cdh10. Since proper cell adhesion is critical in cortical neuron migration, it is suspected that the downregulation of these genes may also contribute to the neuronal migration defects observed. Additionally, cadherins play a vital role in tissue homeostasis and morphogenesis (Maitre and Heisenberg, 2013). They are also known to stabilize neuronal contact and may play a role in the formation of correct synapses during development of the mammalian retina (De la Huerta et al., 2014).

The vertebrate retina can serve as a useful model for studying the function and development of the central nervous system (CNS) since it is the most accessible extension of the CNS. In the retina, Top2b deletion leads to shorter photoreceptor outer segments, cell loss during photoreceptor differentiation, and derangement of horizontal cells during development (Li et al., 2014). Additionally, Top2b contributes to the late-stage differentiation and maturation of photoreceptors by affecting the expression of key genes linked to retinopathies (Li et al., 2017). The Top2b zebrafish gene was shown to play a vital role in the precise targeting of retinal neurites to their synaptic laminae (Nevin et al., 2011). These findings suggest that Top2b plays a vital role in maintaining normal function of the retina. Unfortunately, the specific role of Top2b in interkinetic nuclear migration of retinal cells and its effects on cell size and morphology are still widely undocumented. Better understanding the role of Top2b in the retina can provide us knowledge about retinal development and may provide insights into pathogenesis of various retinal disorders.

We hypothesize that Top2b deficiencies will delay migration of retinal cells to their appropriate layer and reduce the length of cellular processes. This study utilized the Cre-mediated Top2b knockout system to observe the effects of Top2b on interkinetic nuclear migration. This study also utilized the chick embryo model. The chick embryo model is a practical model for research because of its rapid development and resemblance to a human embryo at the anatomical, cellular, and molecular levels (Vergara and Canto-Soler, 2012). The number of genes in the chick is very similar to the number of genes in humans (Vergara and Canto-Soler, 2012). The chick embryo's large size during development makes it an ideal research model, because it allows for easy visualization and the collection of more tissue (Vergara and Canto-Soler, 2012).

1.2 Retinal Structure and Development

The retina is a highly organized neurosensory surface lining the back of the eye. It is formed from many microcircuits with distinct patterns of synaptic connections between various types of cells (Young, 1985). These cells are interconnected to form a complex network that sends visual stimuli to the brain. The vertebrate retina contains six types of neurons and one type of glia. The layers of the retina include the ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), and outer nuclear layer (ONL) (Young, 1985). The GCL contains the cell bodies of the ganglion cells and a few amacrine cells as well. The INL contains the cell bodies of the horizontal cells, bipolar cells, and majority of the amacrine cells. The ONL contains the cell bodies of the rods and cones. The OPL contains the synaptic contacts between the photoreceptor cells and the bipolar and horizontal cells. The IPL contains the synaptic contacts between the bipolar cells and the ganglion cells (Young, 1985). During embryonic retinal development, the INL develops from the inner neuroblastic layer (INBL) and the ONL develops from the outer neuroblastic layer (ONBL).

In retinal development, the cell cycle occurs as a back-and-forth movement. Mitosis is confined to the apical surface of the retina, while the G1-, S-, and G2-phases occur at the basal surface (Baye and Link, 2008). The nuclei of both daughter cells are produced at the apical surface. They then migrate to the basal surface of the retina where the DNA is replicated. Afterwards, the cell returns to the apical surface and the nucleus divides again. After each cell division cycle, fewer and fewer cells return to the outer segment and continue to divide. Cells that do not divide further differentiate into one of the postmitotic cell types in the retina (Young, 1985). Of the cells produced postnatally in mice, 73% differentiate into rods, 20% into bipolar cells, 6% into Muller cells, and 1% into amacrine and ganglion cells (Young, 1985). In embryonic day 10 (E10) chick retina, the expression of photoreceptor marker Xap-1 has already begun in the outer segment of the ONBL (Doh et al., 2010). At this stage, the expression of another photoreceptor marker Visinin peaks throughout the entire ONBL (Doh et al., 2010). For these reasons, the postnatal mouse retina and the E10 chick retina can serve as good models for examining the effects of Top2b on the migration and morphology of photoreceptor cells.

1.3 shRNA Gene Silencing

shRNAs are short hairpin RNA that contain a sense, loop, anti-sense, and termination sequence. They are used to reduce target gene expression and offer possibilities of prolonged gene silencing. Since the shRNA gene knockdown method does not require the use of a knockout animal model, it allows for rapid and convenient experimentation. This type of knockdown is typically accomplished by delivery of plasmids. For shRNA mediated RNA interference, shRNA is first introduced into the nucleus where it is processed by Drosha and exported into the cytoplasm by Exportin-5 (Lingor, 2010). In the cytoplasm it associates with Dicer. This association results in the removal of the loop sequence. One strand of RNA is then removed to allow for association with RISC. This complex targets mRNA possessing a complementary sequence resulting in the degradation of the target mRNA and knockdown of protein production (Lingor, 2010).

1.4 Cre-Mediated Gene Knockout

The most common method of conditionally modifying gene expression is through the use of the Cre-loxP system (Kwan, 2002). Cre is an enzyme from a P1 bacteriophage that can recognize a *loxP* site and serve as a site-specific recombinase (Cox et al., 2012). A *loxP* site is a DNA sequence of 32 base pairs that is not present in the typical mouse genome (Cox et al., 2012). In Top2b^{floxed/floxed} (Top2b^{f/f}) animals, the Top2b gene is flanked by *loxP* sites that are on the same strand of DNA and are in the same orientation. The Cre protein has the ability to recognize the two *loxP* sites and their direction. Therefore, it excises the flanked DNA sequences, which, in this case, is the Top2b gene. Transfection of the pCAG-Cre:GFP plasmid allows for the production of the Cre protein in a temporally and spatially controlled manner. Cells transfected with this plasmid will have the Top2b gene deleted and will fluoresce green because of the presence of the green fluorescent protein (GFP) reporter.

Chapter 2

Materials and Methods

2.1 Analysis of Cultured Top2b Knockdown Cells

2.1.1 shRNA Targeting Top2b

For RNA interference, knockdown of Top2b expression was performed by transfecting embryonic chick retinal cells and explants with shRNA specific to the Top2b gene (shTop2b_A and shTop2b_B) or a scrambled non-targeting control shRNA. Each shRNA was constructed within the pRFP-C-RS plasmid vector. The Top2b-targeting sequences specific to *Gallus domesticus* were obtained through the Cold Spring Harbor Lab's shRNA design tool, and the shRNA vector was constructed by Origene Technologies. The Top2b-targeting sequences and the scrambled shRNA sequence are listed below.

shRNA	Sequence
shTop2b_A	5'- AAGTCTGAAAGTGATTTAGAAGAGAGTGA -3'
shTop2b_B	5'- TGCACTCAAAGTTATTCATGAAGTCGTTA - 3'
Scrambled shRNA	5'- GCACTACCAGAGCTAACTCAGATAGTACT - 3'

Table 2.1.1 Design of chick specific Top2b shRNA and scrambled shRNA

2.1.2 Chick Embryos

Fertilized, pathogen-free, white chicken (*Gallus domesticus*) eggs were acquired from Charles River (North Franklin, CT). Upon receipt, the eggs were kept at room temperature for 2 hours. They were then incubated at 37.5°C and 60% humidity for approximately 10 days to obtain embryos at Hamburger and Hamilton stage 36 (HH36) (Hamburger and Hamilton, 1951).

2.1.3 Cell Culture and Transfection

Chick embryos were euthanized by decapitation at E10 (HH36). The tissue was placed in a petri dish containing cold 1xPBS (Phosphate buffered saline, Fischer Scientific). The retinas were dissected by removing the retinal cornea, pigmented epithelium, and lens with fine forceps. Immediately afterwards, the retinal progenitor cells (RPCs) were dissociated and cultured in media containing 86% DMEM (Dulbecco's Modified Eagle's Medium, Gibco), 10% FBS (Fetal bovine serum, Gibco), 2% chick serum (Gibco), 1% L-glutamine (Gibco), and 1% penicillin-streptomycin (Gibco). After the cells properly adhered to a 6-well culture plate (Corning), they were transfected with shTop2b_A, shTop2b_B, or the scrambled shRNA using the Lipofectamine 3000 Transfection Kit (Invitrogen) as per the manufacturer's instructions. Approximately 1.5 million cells were transfected for each shRNA condition. This experiment was repeated 3 times, each with a different biological replicate.

2.1.4 Imaging

Imaging was performed using an inverted motorized microscope (Zeiss Axio Observer Z1) and a digital camera, AxioCam MRc (Zeiss, Germany). For each of the 3 trials, approximately 25 images of transfected cells were taken at random for each transfection condition. Images of RFP-expressing cells were taken using the DsRed (543nm) filter.

2.1.5 Image Processing and Data Quantification

Images were binarized using Otsu thresholding in MATLAB. The MATLAB Morphology Toolbox was used to process the images. The "imclose" function was performed to close any disconnected cellular structures that were less than 5 pixels apart. The "erode" and "bwareafilt" functions were performed to convert the white pixels that were not part of the cellular structure to black pixels. The MATLAB Morphology Toolbox was also used to calculate the area and major axis of each cellular structure.

2.2 Top2b shRNA Knockdown on Chick Retinal Explants

2.2.1 Retinal Explant Culture and ex vivo Electroporation

Chick embryos were euthanized by decapitation at E10 (HH36). The retinas were dissected and cultured in explant media containing 86% DMEM, 10% FBS, 2% chick serum, 1% L-glutamine, and 1% penicillin-streptomycin. Electroporation of the cultured retinas was conducted on the same day as the dissection. For each experimental trial, a total of 6 retinas were transfected independently. The 2 retinas from the same embryo were electroporated with either shTop2b_A, shTop2b_B, or a scrambled shRNA using a pulse generator ECM 830 (BTX Harvard Apparatus). A total of 3 trials were conducted for qPCR analysis and 3 trials were conducted for histological analysis. The retinas were electroporated with 10 square pulses of 35V for 50ms with 950ms intervals. 3µl of 3µg/ml concentration of shRNA was used for each electroporation. Successful transfection was verified by examining the tissue under the fluorescent dissection microscope, Leica MZ16FA (Leica Microsystems, Germany), 24 hours after transfected region of the retina.



Figure 2.2.1 Expression of RFP in Retinal Explant

2.2.2 Verification of shTop2b_A and shTop2b_B Knockdown Efficiency using qPCR

A primer to analyze the expression of Top2b by quantitative polymerase chain reaction (qPCR) was designed using the NCBI's Primer-BLAST software. The FASTA sequence of the *Gallus domesticus* Top2b mRNA was obtained from the NCBI nucleotide database. The Primer-BLAST software was executed for species specific parameters with exon-exon junction span selection. Forward and reverse primer pairs were analyzed using IDT's OligoAnalyzer Tool to promote specificity and reliability. The ΔG values for hairpin loop and self-dimer formation and the GC content for the primer pairs were determined. The following primer pair was chosen and purchased from ThermoFisher Scientific.

Gene Name	Primer Sequence
Top2b	Forward: ACGAAACCCCTGGTCAGATG
	Reverse: TCTACTTTTGCATCATCTGTCTTC

Table 2.2.2 Design of chick specific Top2b primer for qPCR

The transfected regions of the two retinas from the same embryo were excised for each transfection condition (shTop2b_A, shTop2b_B, and scrambled shRNA) after 3 days of culture. RNA from the transfected regions was isolated by placing the transfected regions from both retinas into 400µl of TRIzol reagent (Invitrogen) and following the manufacturer's instructions for RNA isolation. After RNA isolation, cDNA was constructed using qScript cDNA Supermix (Quanta Biosciences). qPCR was performed on the Roche 480 LightCycler with SYBR Green FastMix (Applied Biosystems). The manufacturer's instructions were used to make the mixture of cDNA, primer, and SYBR Green FastMix. qPCR results were reported as relative threshold cycles (Δ Ct) and Top2b mRNA concentration was determined by normalizing the threshold cycles based on the GAPDH reference gene expression. For each shRNA condition, 3 technical replicates and 3 biological replicates were conducted.

2.2.3 Tissue Processing and Sectioning

After 3 days of culture, transfected retinas used for histological analysis were fixed in 4% paraformaldehyde (in 1xPBS) for up to 4 hours and washed in 1xPBS 3 times for 10 minutes at 4° C. Retinal tissue was then infiltrated overnight in 30% sucrose (in 1xPBS) and embedded in OCT (Electron Microscopy Sciences, Hartfield, PA). Embedded samples were stored at -80°C prior to sectioning. Samples were sectioned at 12µm using a cryostat (Thermo 0620E), mounted on Superfrost slides (ThermoFisher Scientific), and placed flat to air-dry. Nuclear staining with DAPI was performed afterwards using the Shannon Slide Rack (ThermoFisher Scientific).

2.2.4 Imaging

Imaging of stained sections was performed using a fluorescence microscope (Zeiss Axio Imager A1) and a monochrome digital camera, Axiocam MRM (Zeiss, Germany). Images of the shRNA transfected cells were taken using the DsRed (543nm) filter. The DAPI (461nm) filter was used to visualize the nuclear staining. For each trial, approximately 5-10 randomly selected sections with transfected cells were imaged for each condition.

2.2.5 Data Quantification

For each condition (shTop2b_A, shTop2b_B, and scrambled shRNA), approximately 200 transfected cells per trial were used for the data quantification. The number of red cells in the INL and the ONL were quantified and the percentage of red cells in the INL vs. the ONL was determined for each condition. The percentage of red cells longer than 50µm vs. red cells shorter than 50µm was also determined for each condition.

2.3 Analysis of Top2b Knockout Mouse Retinal Cell Positioning

In this experiment, injections, retina harvesting, tissue processing, and staining was conducted by Dr. Ying Li. My major contribution to this study was the imaging, data quantification, and analysis.

2.3.1 Injections

pCAG-Cre:GFP (Addgene plasmid #13776) and RFP plasmid were mixed with 0.025% FastGreen and approximately 0.5 µl of the plasmid mixture was injected into the subretinal space of the right eye between the retina and the choroid of Top2b^{f/f} mice at postnatal day 0 (P0) (Li et al., 2017; Lyu and Wang, 2003). Electroporation was conducted following a published protocol (Li et al., 2017; Matsuda and Cepko, 2004). Electrodes (model 520; 7-mm diameter, BTX, San Diego, CA) were soaked in 1xPBS and were placed between the heads of pups. Five 80V square pulses were applied for 50ms each with intervals of 950ms using a pulse generator ECM830 (BTX, San Diego, CA). Cells transfected with pCAG-Cre:GFP will have the Top2b gene excised. These cells are called Top2b knockout cells and will fluoresce green. The RFP transfected cells are control cells, which still contain the Top2b gene. These cells will fluoresce red.

2.3.2 Retina Harvesting and Processing

Three to five mice were euthanized 7, 14, and 21 days post-injection. The tissue was placed in a petri dish containing cold 1xPBS. The retinas were dissected by removing the retinal cornea, pigmented epithelium, lens, and vitreous humor with fine forceps. Successful transfection of GFP and RFP was verified by examining the tissue under the fluorescent dissection microscope, Leica MZ16FA, immediately after harvesting. Transfected retinas were fixed in 4% paraformaldehyde (in 1xPBS) for up to

4 hours and washed in 1xPBS 3 times for 10 minutes at 4° C. Retinal tissue was then infiltrated overnight in 30% sucrose (in 1xPBS) and embedded in OCT. Embedded samples were stored at -80°C prior to sectioning. Samples were sectioned at 12µm using the Thermo 0620E cryostat, mounted on Superfrost slides, and placed flat to air-dry. Nuclear staining with DAPI was performed afterwards using the Shannon Slide Rack.

2.3.3 Imaging

Imaging of stained sections was performed using the Zeiss Axio Imager A1 fluorescent microscope and the Axiocam MRM monochrome digital camera. In total, 5-10 sections were randomly imaged for each time point. Images of GFP-expressing cells (knockout cells) and RFP-expressing cells (control cells) were taken using the FITC (488nm) and DsRed (543nm) filters, respectively. The DAPI (461nm) filter was used to visualize the nuclear staining.

2.3.4 Data Quantification

The number of red and green cells in the INL and the ONL were quantified for each harvest stage (P7, P14, and P21). Three to five independently electroporated retinas were examined and approximately 1,000 cells were imaged and counted for each stage. The percentage of red cells in the INL vs. the ONL was determined. Similar calculations were performed to determine the percentage of green cells in the INL vs. ONL.

Chapter 3

Results

3.1 Top2b knockdown reduces cell surface area and the length of processes in cell culture

Chick RPCs were dissociated and cultured at E10. Cells were transfected with either shTop2b_A, shTop2b_B, or a scrambled shRNA. Transfected cells were randomly and blindly selected and imaged at a magnification of 10x. The areas of the transfected cells were determined by binarizing the image in MATLAB and using the MATLAB Morphology Toolbox to determine the number of white pixels that made up the entire cell. The length of the cellular process was determined by taking the binarized image and using the MATLAB Morphology Toolbox to calculate the major axis of the cell.

Cells transfected with shTop2b_A were, on average, 2.52 times smaller in area than cells transfected with the scrambled shRNA. Cells transfected with shTop2b_B were, on average, 3.65 times smaller in area than cells transfected with the scrambled shRNA. Processes of cells transfected with shTop2b_A were, on average, 2.17 times shorter than those transfected with the scrambled shRNA. Processes of cells transfected with the scrambled shRNA.





Figure 3.1.1 Top2b knockdown reduces cell surface area and the length of processes in cell culture

A: Chick RPC from stage E10 transfected with Top2b_A. B: Chick RPC from stage E10 transfected with Top2b_B. C: Chick RPC from stage E10 transfected with scrambled shRNA. Arrows indicate the ends of the cellular processes. Cells transfected with shTop2b_A or shTop2b_B are visually smaller and have shorter processes than those transfected with the scrambled shRNA. Scale bar = $20\mu m. n \ge 3$.



Figure 3.1.2 Top2b knockdown reduces cell surface area in dissociated E10 chick RPCs

The surface areas of embryonic chick retinal cells harvested at E10 and transfected with $shTop2b_A$, $shTop2b_B$, or a scrambled shRNA were determined by binarizing images of transfected cells and calculating the number of white pixels in the image. Cells transfected with the scrambled shRNA were significantly larger than those transfected with $shTop2b_A$ or $shTop2b_B$. * p-value < 0.05, ** p-value < 0.01 (t-test), $n \ge 3$.



Figure 3.1.3 Top2b knockdown reduces length of cellular processes in dissociated E10 chick RPCs

The lengths of cellular processes of embryonic chick retinal cells harvested at E10 and transfected with shTop2b_A, shTop2b_B, or a scrambled shRNA were determined by binarizing images of transfected cells and calculating the major axis of the cellular structures. Cells transfected with the scrambled shRNA had significantly longer processes than those transfected with shTop2b_A or shTop2b_B. * p-value < 0.05, ** p-value < 0.01 (t-test), $n \ge 3$.

3.2 RNAi-mediated gene knockdown reduces expression of Top2b in E10 chick retina

E10 chick retinas were explanted and transfected with shTop2b_A, shTop2b_B, or a scrambled shRNA. Levels of Top2b mRNA in retinas transfected with shTop2b_A and shTop2b_B were quantitatively determined and compared to the levels of Top2b mRNA in retinas transfected with a scrambled shRNA using qPCR. Tissue samples for qPCR analysis had a transfection efficiency of about 75%. The results from qPCR showed that samples transfected with shTop2b_A had a 17.4-fold reduction in Top2b levels compared to the samples transfected with scrambled shRNA. Samples transfected with shTop2b_B had a 3.4-fold reduction in Top2b levels compared to the samples transfected with scrambled shRNA. Samples transfected with scrambled shRNA. Samples transfected with shTop2b_B had a 3.4-fold reduction in Top2b levels compared to the samples transfected with scrambled shRNA. Samples transfected with scrambled shRNA. This suggests that both shRNAs can effectively knockdown Top2b.





Figure 3.2.1 RNAi-mediated gene knockdown reduces expression of Top2b in E10 chick retina

Fold change of Top2b mRNA levels in E10 retinas explanted and transfected with $shTop2b_A$ and $shTop2b_B$ compared to Top2b mRNA levels in E10 retinas transfected with control shRNA. Top2b levels were determined by qPCR and were normalized against levels of reference GAPDH gene. Top2b levels can be reduced using both $shTop2b_A$ and $shTop2b_B$. * p-value < 0.05, ** p-value < 0.01, $n \ge 4$, mean \pm SEM.

3.3 Top2b knockdown in E10 chick retina does not show delayed cellular migration

Chick retinas were harvested and cultured at E10. Electroporation was conducted to transfect the retinas with shTop2b_A, shTop2b_B, or control shRNA. After 3 days of culture, retinas were fixed, sectioned, stained with DAPI, and imaged. Minimal differences between the three conditions were observed in the location of the transfected cells. Results showed that for all three conditions, majority of the transfected cells were located in the INL.



Figure 3.3.1 Top2b knockdown in E10 chick retina does not show delayed cellular migration

Location of embryonic chick retinal cells harvested at E10 and transfected with h^{0} shTop2b_A, shTop2b_B, or a scrambled shRNA. Minimal differences between the three conditions were observed in the location of the transfected cells. NS p-value > 0.05 (t-test), $n \ge 3$, mean \pm SEM.

3.4 Top2b knockdown in E10 chick retina reduces the length of cellular processes

Chick retinas were harvested and cultured at E10. Electroporation was conducted to transfect the retinas with shTop2b_A, shTop2b_B, or control shRNA. After 3 days of culture, retinas were fixed, sectioned, stained with DAPI, and imaged. Results showed that approximately 75% of cells transfected with shTop2b_A and 95% of cells transfected with shTop2b_B had processes shorter than 50µm. 75% of control cells had processes longer than 50µm.





Figure 3.4.1 Top2b knockdown in E10 chick retina reduces length of cellular processes

A: Section from chick retinal explant transfected with $shTop2b_A$ at E10 and fixed 3 days later. B: Section from chick retinal explant transfected with $shTop2b_B$ at E10 and fixed 3 days later. C: Section from chick retinal explant transfected with a scrambled shRNA at E10 and fixed 3 days later. Cells transfected with $shTop2b_A$ or $shTop2b_B$ have visually shorter processes than those transfected with the scrambled shRNA. Scale $bar = 20\mu m$.



Figure 3.4.2 Top2b knockdown in E10 chick retina reduces length of cellular

processes

Percentage of cells transfected with shTop2b_A, shTop2b_B, or control shRNA that have processes shorter than 50 μ m and percentage of these cells that have processes longer than 50 μ m. Majority of Top2b knockdown cells have short processes, while the majority of cells transfected with a control shRNA have long processes. ** p-value < 0.01 (t-test), $n \ge 3$, mean \pm SEM.

3.5 Top2b knockout in mouse retinal cells affects cell migration

Mouse retinas transfected with pCAG-Cre:GFP and DsRed at P0 were harvested at P7, P14, and P21. At P7, 99% of the Top2b knockout cells were present in the INL, while 1% was present in the ONL. Additionally at P7, the control cells present in the ONL were observed to be more elongated compared to knockout cells in the INL. At P14, 64% of the Top2b knockout cells were present in the ONL, while 36% were present in the INL. At P21, 86% of the Top2b knockout cells were present in the ONL while 14% were present in the INL. At all three time points, there was a statistical significance between the number of Top2b knockout cells in the ONL vs. INL, while there was no statistical difference between the number of control cells in the ONL vs. the INL. Top2b knockout in the postnatal mouse retina seems to stall the migration of cells into the ONL only at P7.





Figure 3.5.1 Top2b affects cell migration at P7

A: Retina section from sample harvested 7 days after injection. DAPI staining is in blue. Scale bar = $20\mu m$. B: Distribution of transfected cells in retinas harvested 7 days after injection. 7 days after injection, 99% of the Top2b knockout cells (green cells) are present in the INL, while there is no significant difference between the number of control cells (red cells) in the ONL vs. the INL. NS p-value > 0.05, *** p-value < 0.001 (t-test), $n \ge 3$, mean \pm SEM.





Figure 3.5.2 Distribution of Top2b knockout cells and control cells at P14

A: Retina section from sample harvested 14 days after injection. DAPI staining is in blue. Scale bar = $20\mu m$ B: Distribution of transfected cells in retinas harvested 14 days after injection. 14 days after injection, 64% of Top2b knockout cells (green cells) are present in the ONL, while there is no significant difference between the number of control cells (red cells) in the ONL vs. the INL. NS p-value > 0.05, ** p-value < 0.01 (t-test), n \ge 3, mean \pm SEM.





Figure 3.5.3 Distribution of Top2b knockout cells and control cells at P21

A: Retina section from sample harvested 21 days after injection. DAPI staining is in blue. Scale bar = 20 μ m B: Distribution of transfected cells in retinas harvested 21 days after injection. 21 days after injection, 86% of Top2b knockout cells (green cells) are present in the ONL. There is no significant difference between the number of control cells (red cells) in the ONL vs. the INL. NS p-value > 0.05, *** p-value < 0.001 (t-test), n ≥ 3, mean ± SEM.



Figure 3.5.4 Distribution of Top2b knockout cells and control cells at P7, P14, and P21

Distribution of transfected cells in retinas harvested 7, 14, and 21 days after injection. Top2b knockout in the postnatal mouse retina seems to stall the migration of cells into the ONL only at P7.

Chapter 4

Discussion

In vitro experiments in this study have shown that Top2b reduces cell surface area and the length of cellular processes, confirming that Top2b plays a vital role in neurite outgrowth. Additionally, this study illustrated that even reducing the expression of Top2b, rather than eliminating its expression, can greatly affect cellular morphology. Knockdown of Top2b was shown to reduce cellular surface area 3-fold and length of cellular processes 2-fold. However, it is uncertain if Top2b reduces cell size by playing a role in cell growth or if Top2b affects cell spreading by preventing the cell from making proper connections with the culture dish and sending out projections. To answer this question, the volume of cells could be measured using transmission-through-dye microscopy (Gregg et al., 2010).

The majority of cells in E10 chick retinal culture are photoreceptor cells. However, according to ThermoFisher Scientific, different cell types can have variable transfection efficiencies. Each retinal cell type is likely to respond differently to the transfection reagents and method; therefore, a bias in the cell type studied may exist. In future studies, the biological properties of each retinal cell type must be considered to ensure that the particular transfection technology is ideal for the cell type of interest. Antibody labelling on fixed cultured cells can also be performed to mark cell types and ensure that the cell type of interest is indeed being transfected efficiently.

The explant retina culture model was used to mimic *in vivo* retinal development and provide ease during experimental manipulation. Although many key events during *in* *vivo* retinal development occur in the retinal explant model, a few differences do exist. In explant cultures, the seven major cell types are produced in the same order and ratio (Donovan and Dyer, 2006). However, photoreceptor outer segments do not fully mature in this system and shorter inner and outer segments have also been observed (Donovan and Dyer, 2006). The explants may also lack the astrocyte component (Donovan and Dyer, 2006).

The explant culture experiments were conducted utilizing the electroporation transfection method. With electroporation, a high percentage of cells, approximately 75%, are transfected without decreased viability. These experiments utilizing the chick retina model showed that Top2b knockdown cells have shorter processes than cells transfected with control shRNA, further confirming Top2b's role in neurite outgrowth. The chick explant model was unable to effectively demonstrate the role of Top2b in retinal cell migration. It is hypothesized that culturing the explant tissue for only 3 days following knockdown of Top2b may not be sufficient to observe significant differences in migration between control cells and knockdown cells. To test this hypothesis, further work needs to be conducted to develop a model that allows the tissue to be cultured for a longer period of time prior to degradation of the tissue. The use of a rotatory shaker during culture could allow the tissue to rotate continuously in an orbital manner, preventing the retina from adhering to a surface and preserving the 3D architecture of the tissue (Thangaraj et al., 2011).

The role of Top2b in retinal cell interkinetic nuclear migration has also been studied using the Top2b^{f/f} Cre-knockout mouse model. It was found that at P7, 7 days post-injection, the majority of Top2b knockout cells remained in the INL, while about

half of the control cells migrated into the ONL. This suggests that Top2b knockout in retina cells may delay the migration of photoreceptor cells into the ONL at P7. At P21, 21 days post-injection, both control cells and Top2b knockout cells had migrated into the ONL, illustrating that Top2b knockout does not prevent the migration of the retinal cells into the ONL. This may also suggest that Top2b function is required at a specific time during development in the mouse retina. It is suspected that the role of Top2b in the transcriptional control of the reelin signaling pathway contributes to the migratory delays observed in this experiment; however, the exact mechanism through which Top2b affects the migration of retinal cells is still not well understood. It is hypothesized that after about a week, other proteins in the cell may be able to replace the function of Top2b in its absence. Further research, including performing qPCR analysis on other topoisomerases and neuronal proteins, would need to be conducted to test this hypothesis and determine if an upregulation of any other key protein is present when Top2b is knocked out.

In summary, these studies demonstrate that Top2b plays a key role in regulating early-stage interkinetic nuclear migration. Top2b also functions to control cellular morphology and neurite outgrowth.

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