Relative Distribution of Polyglutamylated Tubulin and Alpha Tubulin Within Mitotic Glial Cells

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Introduction

Mitosis is the visually dramatic process of eukaryotic cell division (Nigg, 2001). Microtubules (MTs) are the fibers that structure the mitotic spindle and integral components of cell division (Walczak & Heald, 2008). MTs are polymers of tubulin, a protein with a heterodimer tertiary structure consisting of one alpha-subunit and one-beta subunit (Kandel, 2013). Fascinatingly, MTs display dynamic and disparate properties within the mitotic spindle (De Forges, 2012).

Post-translational modifications of tubulin have been found to influence properties of MTs that determine their function in the cell (Hammond, Cai & Verhey, 2008). Particularly, there is evidence to support that the poly-glutamylation, or addition of multiple glutamate residues to alpha and beta tubulin (van Dijk, 2008) is prevalent in neuronal cells, centrioles, axonemes, and the mitotic spindle and could influence the stability and/or structure of MT assemblies (Hammond, Cai & Verhey, 2008). Other studies have examined distribution tyrosination and detyronsination of alpha-tubulin during mitosis using immunofluorescence and found a relative abundance of post-translationally modified tubulin in the polar regions containing the centrosome (Gundersen & Bulinski 1986). Further, a study of the distribution of glutamylated tubulin isoforms along the sea urchin sperm axoneme found gradiated distribution that correlates with nonuniform properties along the axoneme (Huitorel et al., 2002). Past research also using immunofluorescence techniques concluded that centrioles are poly-glutamylated in all cell types tested (Kahn et al., 2003). Alas, the distribution of post-translational modifications of tubulin is suggested to play a role in the structure and function of the mitotic spindle and work in sea urchin axoneme specifically displays the role of poly-glutamylation in heterogeneous behavior of MTs. However, no studies could be found that integrate these findings and specifically examine distribution of poly-glutamylation within the mitotic spindle. Accordingly, this study examines the hypothesis that there is a higher relative abundance of poly-glutamylated tubulin than alpha tubulin near the poles of a mitotic Schwann cell.

Glial cells are the most plentiful cell type in the nervous system and function to support neurons. Macroglia are categorized into three major subcategories, astrocytes, oligodendrocytes and Schwann cells (Kandel, 2013). Schwann cells function to myelinate axons of neurons and are found in the peripheral nervous system. Thus the glia in this study obtained from primary culture of 10 day-old domestic chicken (*Gallus gallus)* embryos are Schwann cells. Chick (*Gallus gallus)* embryos are used because dorsal root ganglia and sympathetic nerve chains that are fairly easy to obtain through dissection grow well in the culture method used.

Research on the intricacies of the mitotic spindle is of the utmost significance because MTs are a known target for anticancer drugs (Kavallaris, 2001;Islam&Iskander, 2004;Dumontet&Jordan, 2010; Jordan&Wilson, 2004; Rowinsky&Calvo, 2006). A recent review discussed improvements in anti-tubulin agents that exhibit enhanced tumor specificity and reduced neurotoxicity (Loong &Yeo, 2014). Thus, knowledge gained in this study could help to improve anti-tubulin agents that cause apoptosis in cancer cells by attacking the mitotic spindle. In addition, this study could be useful for research on traumatic injury to the peripheral nervous system, particularly to the sciatic nerve (Yao, L. et al., 2014) or demyelinating diseases such as Charcot-Marie-Tooth type 1A disease (Nobbio, L. et al., 2004) and Guillain-Barre Syndrome (Rubin, 2014) because of the type of glial cells used in this study. Many traumatic injurys to the peripheral nervous system and demyelinating diseases impact the body through lack of cell division so this study could provide novel information for possibly promoting mitosis.

In short, this study will first confirm the hypothesis that glial cells are mitotic. If said objective is accomplished, this study will use ratiometric analysis to test the hypothesis that there is a higher relative abundance of poly-glutymylated tubulin compared to alpha-tubulin at the poles of a mitotic Schwann cell.

Materials and Methods

Primary Tissue Culture

Procedure generally followed protocol for “Primary Culture Of Chick Embryonic Peripheral Neurons 1: DISSECTION” (Morris, 2014) with these few exceptions. Ten day *Gallus gallus* embryos were utilized to extract, disassociate and plate sympathetic nerve chains and dorsal root ganglia on coverslips by Professor Robert Morris (Morris, 2014). The coverslips were treated with poly-lysine for one hour 25 minutes, laminin for one hour and trypsin for 20 minutes in 37 degrees C. The culture was incubated at 37 degrees C in F+ medium consisting of Leibovitz L-15, 0.5% methyl cellulose, 2 mM Glutamine, 0.6% glucose, 100 U, µg/mL penicillin, streptomycin, 10% fetal bovine serum, and 50 ng/mL nerve growth factor for 24 hours (Morris, 10/1/2014).

Immunofluorescence

Procedure generally followed “immunofluorescent staining of sea urchin embryos” with these following exceptions. Primary cultures were chick embryonic nervous tissue instead of sea urchin and fixed with a formaldyhyde-gluteraldehyde crosslinking fixative. Experimental coverslips were blocked with 3% Bovine Serum Albumin (BSA) in PBS-T then labeled with anti-poly-glutamylated-tubulin Ab at 1:1000 dilution, incubated for one hour at room temperature (RT), anti-mouse IgG Ab at 1:200 dilution incubated overnight at four degrees C, anti-alpha-tubulin (DM1A) at 1:100 dilution incubated for one hour at RT, and 10 µg/mL Hoechst stain for DNA incubated for five minutes at RT. All coverslips

were washed three times with PBS-T after labeling.

Imaging

Nikon Eclipse E200 fluorescent microscope, Mercury 100W fluorescence machine with a Sony DWF-X700 camera and a 1.0X camera mount was used to view mitotic glia (the cell of interest (COI), based on mitotic signatures of DNA stain) with a 40x objective lens. An iMac computer (capicorn) with SPOT software was used to collect images of the COI in the blue, green and red fluorescent channels that labeled for DNA, alpha-tubulin and poly-glutamylated tubulin respectively for experimental, positive control and negative control. All equipment was provided by Wheaton College in Norton, MA in the Imaging Center for Undergraduate Collaboration, ICUC, of the Mars Center for Science and Technology.

Data Analysis

Quantitative Analysis of Mitotic Signature

First the scale of the 40X image containing the cell of interest (COI) was calculated on ImageJ. Then DNA (designated by the blue spots) of every cell within the field of view was outlined as precisely as possible using the freehand selection tool. The area in μm^2 and mean brightness of each cell was collected. Then a ratio of mean brightness per μm^2 was calculated. Averages of the area, mean brightness and mean brightness per μm^2 was calculated for each cell then compared to the same designation for the COI with mitotic signatures.

Ratiometric Analysis of Poly-glutamylated Tubulin and alpha Tubulin

Ratiometric image analysis followed procedure outlined in Patterns of Ciliary Retraction in Echinoid Embryos (Sholi, 2014). Images of the poly-glutamylated labeled tubulin channel and the alpha tubulin labeled channel of were opened in ImageJ. The type of images was changed to 32 bit. Then from the process tab, image calculator was used to divide the image of poly-glutamylated labeled tubulin by image of the alpha tubulin labeled channel to produce a ratiometric image. This stacked image represents a mathematical ratio of two labeled tubulins. This procedure was used because a simple comparison of measured brightness of the green and red channel would not test the hypothesis of relative abundance seeing that brightness values are effected by exposure time and fluorchromes. Therefore, if a ratiometric image is created, the problem of exposure time is no longer a confounding variable because it is simply a visual representation of the mathematic ratio. The only deviation from the procedure outlined in Patterns of Ciliary Retraction in Echinoid Embryos was that the ratiometric image brightness was not multiplied by an appropriate factor because the details were already evident without multiplication.

Statistical Analysis

A one-tailed two-sample Student's t-test was calculated to determine if the brightness to area ratio was significantly different for a mitotic cell of interest (COI) than the average brightness to area ratios with in the field of view.

Results

Quantitative Analysis of Mitotic Signature

Immunofluorescence images of a glial cell found in the experimental culture did display mitotic signatures. The COI (designated with two arrows pointing to the poles in Figure 1) has a spindle shaped network of MTs surrounding condensed DNA that could be a textbook picture of mitosis. The shapes of the MTs in other cells of the image are more outspread and display the more typical triangular shape of glia indicated by the green and red fluorescence. In comparison, the COI appears more condensed and polarized with the DNA compacted in the middle.

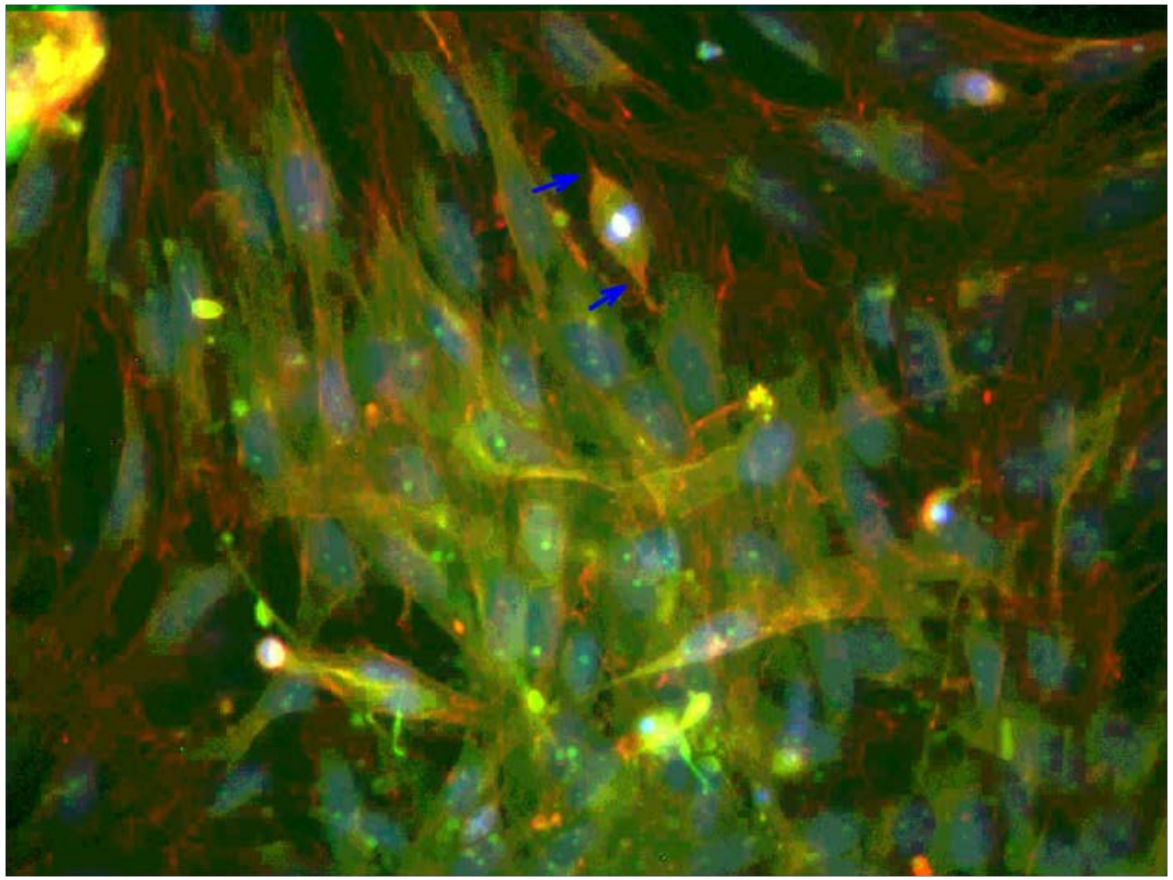

Figure 1. Overlaid image with arrows pointing to the poles of mitotic COI. This image layered blue, green and red immunofluorescence channels labeling DNA, alpha-tubulin and poly-glutamylated tubulin respectively.

Furthermore, as seen in Figure 2A, which contains only the DNA stained channel, the COI (indicated with an arrow) is much brighter and appears more condensed than other nuclei in the frame. Additionally, there are slight variations within the oblong DNA, possibly displaying individual chromosomes. Figure 2B shows an image of the negative control with no blue immunofluorescence and Figure 2C is an image of the positive control was sea urchin *(L. pictus)* with bright blue fluorescence.

Figure 2. A. Immunofluorescence image at 40X of the blue channel with an arrow indicating DNA of COI in the experimental condition B. Immunofluorescence image at 40X of the blue channel negative control C. Immunofluorescence image of the blue channel in the positive control condition that used the same procedure on *L. pictus* embryos

Next the area in μm^2 and the mean brightness of each nucleus (n=47) was measured. Then the ratio of mean brightness to area was calculated by dividing the mean brightness by the area of the spot. Said quantification is displayed below in Figure 3. The ratio of brightness to area is almost nine times larger than average for the COI, derived from dually smaller area and larger mean brightness than average. The COI value was significantly different than the average values ($p < 0.0001$; $df=46$) when a students' t-test was calculated, therefore there is a less than 0.01% probability the difference occurred by chance.

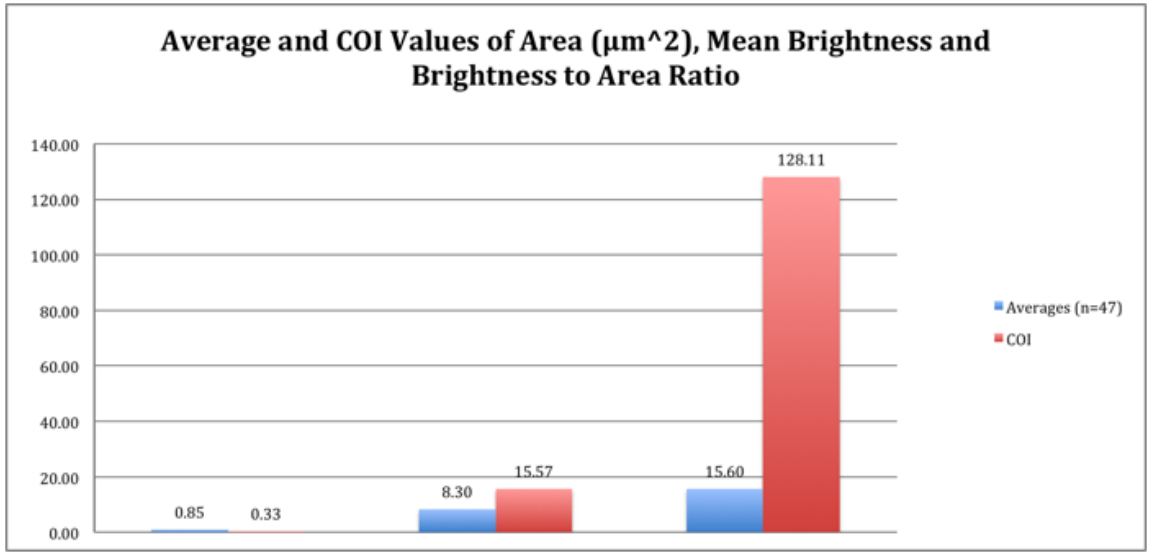

Figure 3. Comparison of the average and COI values for area, brightness and brightness per area values for all nuclei (n=47) within the field of view. The brightness per area value for the COI is larger than average by considerable statistical significance.

Ratiometric Analysis of Poly-glutamylated Tubulin and Alpha Tubulin

After mitosis in the glial cell was confirmed, the fluorescence images appeared to show an unequal distribution of the two labeled tubulins across the mitotic cell. Therefore, ratiometric analysis was used to test the hypothesis that there is a relative abundance poly-glutamylated tubulin to alpha-tubulin in the poles of the mitotic cell.

First of all, Figure 4A and 4B confirm the presence of both poly-glutamylated tubulin and alpha-tubulin respectively within the mitotic spindle. The results of the ratiometric image in Figure 4C display the mathematical ratio of the two images. Thus the brightness of the ratiometric image denotes a relative abundance or ratio of poly-glutamylated tubulin

to alpha tubulin. Where ever poly-glutamylatd tubulin is in relative abundance will be brighter because it is the numerator of the ratio. At the poles of the mitotic cell (indicated with arrows in Figure 4C), the image is brighter than the middle of the cell (indicated with an arrow in Figure 4C), which appears dimmer. Also, the ratio of polyglutamylated tubulin to alpha tubulin looks to be fairly constant across the cell denoted by the similar brightness excluding the poles.

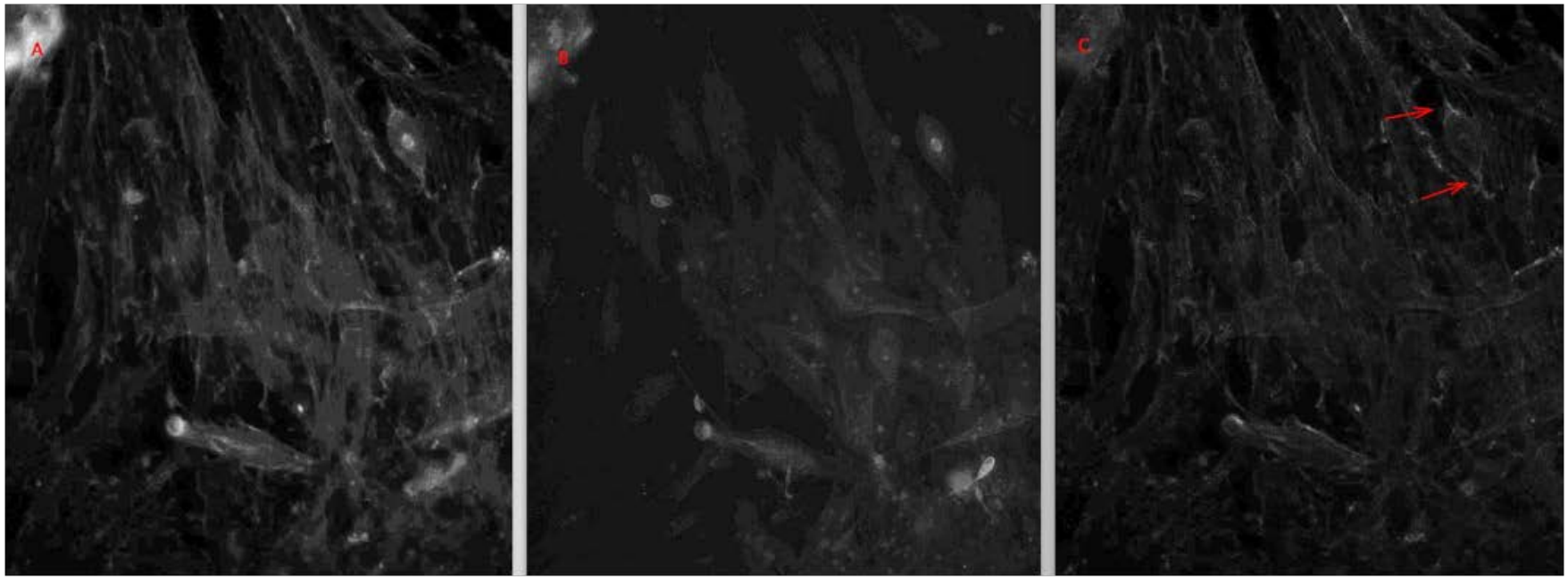

Figure 4. Displays 32 bit image of A. Image of polyglutamylated-tubulin channel at 40X B. Alpha-tubulin channel at 40X and C. Ratiometric image (created by dividing A by B) suggesting that poly-glutamylated tubulin is relatively more abundant at the poles of the mitotic cell by the brightness in this region.

Discussion and Conclusions

Quantitative Analysis of Mitotic Signature

These qualitative and quantitative results support the hypothesis that glial cells are mitotic in primary culture. The qualitative data in Figure 1 displays the condensed bright DNA surrounded by a network of microtubules; clear mitotic signatures (Nigg, 2001). The image of the negative control in Figure 2B measured the validity of the Figure 1A and we were therefore able to conclude the extreme brightness of the DNA in the COI was not due to autofluorescence. Furthermore, we confirmed the Hoechst stain did in fact label DNA from the positive control in Figure 2C. Thus this study developed a method for quantifying mitotic DNA through relative brightness per area ratio after proper mitotic signatures have been found. These findings make sense because glial cells proliferate in culture therefore should be mitotic. These results are in concordance with other findings by Ratner (1986), Conti (2002) and Nobbio (2004) that found glia from different organisms are mitotic in culture but this study confirms that glia extracted specifically from domestic chick embryos are indeed mitotic.

Ratiometric Analysis of Poly-glutamylated Tubulin and Alpha Tubulin

Through a ratiometric analysis of Figure 4C, the hypothesis that there is a higher relative abundance of poly-glutymylated tubulin to alpha-tubulin at the poles of a mitotic Schwann cell is supported. The poles of the cells are brighter in the ratiometric image allowing us to conclude the relative abundance of poly-glutamylated tubulin than alpha tubulin in polar regions opposed to the middle of the cell. Research suggesting post-translational modifications may increase stability (Hammond et al., 2008) could explain the unequal distribution of poly-glutamylation in the mitotic spindle found by this study. As previously mentioned, the mitotic spindle is highly dynamic (Walczak & Heald, 2008). Individual fibers depolymerize quickly after motor proteins translocate the chromosomes along the microtubules toward the poles (Walczak & Heald, 2008) but the poles need to remain stable for this process to occur. Therefore this study could speculatively suggest a mechanism that stabilizes the poles of the mitotic spindle could be poly-glutamylation of the tubulin if there were enough of the same result for statistical significance.

This work follows suggestions for future research by Adam Sholi and transitively confirms “there is a higher abundance of poly-glutamylated tubulin than acetylated tubulin in the spindle” (Sholi, 2014). Notably, a current study detected levels of post-translational modification of α-tubulin in spindle microtubules by immunoelectron microscopy in CHO-K1 and found varied organization in the spindle at different stages of mitosis (Balashova, 2008). The study saw tyrosinated tubulin was not uniformly distributed across the cell yet no difference in the distribution of acetylated tubulin, a finding in concordance with previous research (Gundersen & Bulinski, 1986; Sholi, 2014). Another experiment found increased microtubule stability via posttranslational modifications to beta-III tubulin in differentiating P19 neurons (Laferriere, 1996). Thus, the present study is concurrent with some of the

literature that suggests the presence of both unequal distribution of post translational modifications potential and respective increased microtubule stability. Yet the current study was the first to our knowledge to present data on the relative distribution of poly-glutamylated tubulin to alpha tubulin in the mitotic spindle.

The matter of how poly-glutamylation of the tubulin fibers could stabilize the mitotic spindle is still under much speculation. Potentially, the mechanism by which poly-glutamylated tubulin fibers are possibly able to stabilize the mitotic spindle could imaginably be caused though a role in initiation of formation of focal adhesions. Focal adhesions are molecular assemblies that act as mechanosensors (sensors of molecular force) and signal transducers between the extracellular matrix and the cytoskeleton (Riveline, 2011). There is a great deal of mechanical force at the poles of the cell during mitosis as well as rapid cellular signaling; two hallmark characteristics of focal adhesions. The interplay between post-translational modifications of tubulin and occurrence of focal adhesions still requires further research but their similar traits displayed by immunofluorescence images could be promising for future research.

There are many limitations to the current study design. The small n value for mitotic glia is of great concern and the use of the hand selection tool could account for variable results in the future. Although the statistics calculated appear definitive ($p < 0.0001$; $df = 46$), they are somewhat deceptive because there was only one mitotic glial cell analyzed. If the study were to be repeated, finding a way to standardize the measurement tool so not to use free hand may be beneficial and of course analyzing a higher n value of mitotic glia. Specifically, analyzing enough mitotic glia so the relative distribution of mitotic glia at different phases of mitosis would be of utmost importance. Also, decreasing the cell density on the coverslips for better imaging is recommended because we may have seen other mitotic glia but still, low light fluorescence microscopy has some inherent limitations. Furthermore, future studies could analyze all known post-translational modifications for tubulin in the mitotic spindle using more specific antibodies (Magiera, 2013) or other possible stabilizing mechanisms in the mitotic spindle that may not occur from post-translational modifications to tubulin. Any intellectual gains on the structure and function of the mitotic spindle could be useful for anti-tubulin agents as cancer treatments because abnormal mitosis is a hallmark of the disease (Mahindroo, 2006). Perhaps, if the post-translational modifications distribution is in fact responsible for stability of the mitotic spindle through focal adhesions, then developing an anti-post-translationally modified tubulin drug could more specifically target metastatic cells.

Overall this study confirmed that glial cells are mitotic in primary culture from domestic chick embryos and added brief yet novel insight to the relative distribution of post-translational modifications to tubulin.

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