ABSTRACT

Characterization of Tau Phosphorylation During the Differentiation of Human Embryonic Stem Cells

Alzheimer's disease is the seventh leading cause of death in the United States with an estimated cost of 100 billion dollars. The severity of this neurodegenerative disease is strongly correlated with the number of neurofibrilary tangles (NFT) composed of hyperphosphorylated tau, a microtubule associated protein. The phosphorylation of tau is known to be tightly regulated by glycogen synthase kinase 3β (GSK-3β) and cyclin-dependent kinase 5 (Cdk-5). These two kinases also play a vital role in neuronal cell development. We characterized the phosphorylation of tau and expression of tau, GSK-3β, and Cdk-5 during early embryo development using human embryonic stem cells (hESC) as our model system. We determined that GSK-3β plays an important role in the regulation of proliferation, tau phosphorylation, and neuroectodermal differentiation of hESC. However, the inhibition of Cdk-5 did not significantly change hESC proliferation and the expression of tau, GSK-3β, and Cdk-5 throughout hESC differentiation.

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May 29, 2008
Date

COVER SHEET

TITLE: _Characterization of Tau Phosphorylation During the Differentiation of Human Embryonic Stem Cells

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YEAR: 2008

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CHARACTERIZATION OF TAU PHOSPHORYLATION DURING THE DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS

by

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A Senior Honors Thesis submitted in partial fulfillment of the requirements for the degree of

Bachelor of Science

(Biochemistry)

at the

UNIVERSITY OF WISCONSIN – MADISON

2008

To my parents - тцунк

ACKNOWLEDGMENTS

First, I would like to thank my great research mentor, Dr. Craig Atwood for giving me the amazing opportunity to work in his lab, his advice, and support. A special thank you to Prashob Porayette and Miguel Gallego for teaching me everything I know about human embryonic stem cell research. I would also like to convey my appreciation for their patience, friendships, endless advice, and setting an amazing example of a great scientist. I also thank Dr. Sivan Vadakkadath Meethal, Ryan Haasl, Andrea Wilson, Tianbing Liu, Jacob Basson, Stephanie Weinecke, and Susan Bright for creating a wonderful working environment. In addition, our “friendly neighbors” in Dr. Luigi Puglielli’s laboratory have been especially helpful – thank you Mi Hee Ko, Mariana Pehar, Mary Cabell Jonas, and Lisa Hui Li. Thanks to all of my mentors throughout my undergraduate career, especially Dan Barnish and David Nelson. Finally, I would like to thank Caitlin McKeown for her kindness and understanding. It has been quite an adventure and thank you all for making it a wonderful and memorable one!

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ABSTRACT

Alzheimer's disease is the seventh leading cause of death in the United States with an estimated cost of 100 billion dollars. The severity of this neurodegenerative disease is strongly correlated with the number of neurofibrilary tangles (NFT) composed of hyperphosphorylated tau, a microtubule associated protein. The phosphorylation of tau is known to be tightly regulated by glycogen synthase kinase 3β (GSK-3β) and cyclin-dependent kinase 5 (Cdk-5). These two kinases also play a vital role in neuronal cell development. We characterized the phosphorylation of tau and expression of tau, GSK-3β, and Cdk-5 during early embryo development using human embryonic stem cells (hESC) as our model system. We determined that GSK-3β plays an important role in the regulation of proliferation, tau phosphorylation, and neuroectodermal differentiation of hESC. However, the inhibition of Cdk-5 did not significantly change hESC proliferation and the expression of tau, GSK-3β, and Cdk-5 throughout hESC differentiation.

Chapter 1: Introduction and Literature review

Background

Alzheimer's disease (AD), first described by Dr. Alois Alzheimer in 1906, is currently the 7th leading cause of death and the most common cause of dementia in the United States. The inevitable institutionalization of morbid AD patients results in an estimated annual cost of $100 billion in the United States.[1] It is evident that any gain in understanding of this detrimental disease will not only improve the quality of life of those affected, but also alleviate the cost burden on the health system.

The neuropathology of AD is mainly characterized by the intracellular accumulation of neurofibrillary tangles (NFT) and the extracellular deposition of senile plaques.[2] NFTs consist of hyperphosphorylated tau in paired helical filaments (PHF), while the senile plaques are composed mostly of fibrillar amyloid-β (Aβ).[2] However, the severity of AD dementia symptoms strongly correlates to the number of NFTs, while such a correlation is not seen with senile plaques.[3,4] Furthermore, it has been shown that the ratio of phosphorylated tau (p-tau) levels to total tau (t-tau) levels at the Ser-396/Ser404 residues is a promising marker in cerebrospinal fluid (CSF) for the severity and abundance of AD symptoms.[3] These observations suggest that tau is more directly associated with the disease mechanism leading to AD.[4] While all these pathological and biochemical changes are certainly involved in the disease process, the etiologic events that lead to the generation of these pathological hallmarks are not well understood.

Tau

Tau is a microtubule-associated protein normally expressed in neuronal and glial cytoplasm including cell bodies, neurites and axons, where they bind to and stabilize microtubules.[5,6] Research in this field suggest that tau's major cellular role involves regulation of neuronal microtubule assembly and stabilization of microtubules against severing and depolymarization in vivo.[7-9] It has been shown that disassembly of the rigid microtubule structure of neurons for neuronal division is achieved by the removal of the microtubule stabilizing protein tau via its phosphorylation. Phosphorylation of tau normally occurs during metaphase of neuronal division, and during differentiation hyperphosphorylated tau is observed in neurons of the fetal brain, suggesting that it is also developmentally regulated.[10] Goedert *et al.* experimentally showed that while Ser-202 is a normal phosphoryalation site in development, in AD patients this key site undergoes abnormal phosphorylation.[11] In addition, among the 37 serine and threonine residues that have been found to be phosphorylated in PHF-tau, the C-terminal Ser-396 and Ser-404 represent one of the major AD epitopes.[3,4]

Tau Regulation

This high and residue-specific phosphorylation of tau in mitotically active neurons is driven by cyclin-dependent kinases (Cdk's).[10] Several Cdk's are associated with phosphorylated tau in AD and *in vitro* tau is phosphorylated in a manner similar to that found in AD.[12] A number of other kinases such as glycogen synthase kinase-3β (GSK3β) also are pivotal in tau phosphorylation.[13] These two

kinases largely phosphorylate the same Serine residues in tau and both are known to play a role in neuronal cell development (***Figure 1***).[14] Since increased phosphorylation and altered

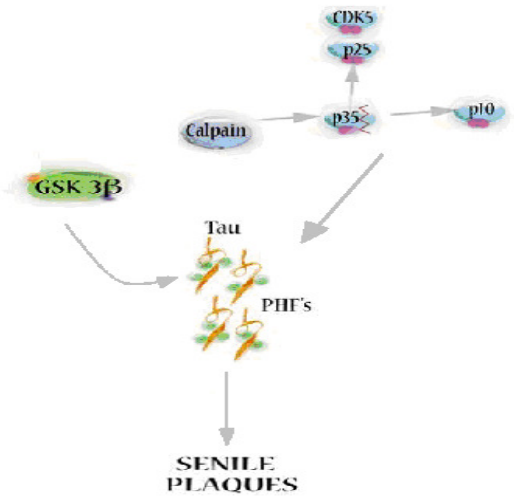

Figure 1: Interaction of GSK-3β, Cdk-5, and tau. In AD, hyperphosphorylation of tau by GSK-3β and Cdk-5 results in the formation of paired helical filaments and neurofibrillary tangles leading to senile plaques. (Edited from BioSource Internationale)

microtubule stability occur during progression through the cell cycle, it is likely not coincident that there are microtubular abnormalities associated with AD[14].

Human Embryonic Stem Cells and Embryogenesis

Currently, the best human model available to study early embryonic development is human embryonic stem cells (hESC). Embryonic stem cells are pluripotent cells derived from embryos generated by *in vitro* fertilization. hESC are derived from the inner cell mass of the human blastocyst and are cultured in defined media to maintain their pluripotent nature. These cells can be differentiated to neuro-ectodermal cells akin to those that form the neural tube, and can be further differentiated into different neural lineages (***Figure 2***).

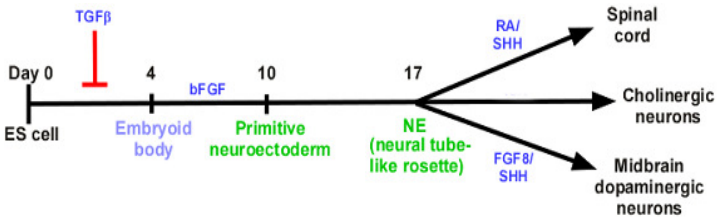

Figure 2. Neuronal subtype specification from human embryonic stem cells. hESC can be differentiated into primitive neuroectodermal cells (NE) at around day 10 which then exhibit neural tube-like rosettes with 14–17 days of differentiation in a chemically defined neural medium ([15,16]). This transition is negatively regulated by TGFβ signaling. Treatment of NE (14-17 days) with RA and SHH results in mostly spinal interneurons. Similarly, FGF8 and SHH treatment at this later stage results in differentiation of dopamine neurons expressed by forebrain cells.

Research Objectives and Thesis

Since the phosphorylation of tau occurs both during embryogenesis and neurodegeneration, the re-expression of these developmental proteins in the brain with aging and AD suggests a developmental component to this disease state. Utilizing the hESC model to understand the developmental functions of tau phosphorylation would almost certainly promote a better mechanistic understanding of the functions of this protein in AD. An understanding of the mechanisms involved in the positive actions of tau could be valuable in exploiting the regenerative and plastic potential of the brain in preventing and treating AD.

Chapter 2: Materials and Methods

Propagation of Human Embryonic Stem Cells

Undifferentiated human embryonic stem cells (hESC) cells, H9 (passage 22–46 XX karyotype) (also known as WA09, a National Institutes of Health registered line) were purchased from WiCell Research Institute (WI, USA) and maintained according to the supplier's instructions. Each well of a 6-well plate (Fisher Scientific, PA, USA) was first coated with 1ml of sterile 0.1% gelatin (Sigma-Aldrich Co., MO, USA) solution. After 24 hours, irradiated mouse embryonic fibroblast (MEF) cells (Biovintage, CA, USA) were plated as a monolayer (1.875 X 10^5 cells per well) on the gelatin-coated wells in DMEM media (Invitrogen, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen) and 1% Non-Essential Amino Acids (NEAA; Invitrogen). After 24 hours of MEF plating, H9 cells were subsequently plated on this MEF feeder layer and grown in the presence of DMEM-F12 media (Invitrogen) supplemented with 1% NEAA, 1mM L-glutamine (Invitrogen), 0.1mM 2-mercaptoethanol (Sigma-Aldrich), 4ng/ml bFGF (Invitrogen) [from a stock solution of 2µg/ml of 0.1% bovine serum albumin (Sigma-Aldrich)] and 20% Knockout Serum Replacer™ (KOSR) (Invitrogen). Continual propagation of cells required colonies to be enzymatically lifted with 1ml of a sterile solution of collagenase type IV (Invitrogen) (1mg/ml of DMEM-F12), dissected into multiple small pieces and transferred onto a fresh MEF feeder layer once or twice a week. hESC also were grown on Matrigel™ (BD Biosciences, CA, USA) in the presence of a defined culture media called mTeSR1 media developed by WiCell Research Institute[17]. 1ml of Matrigel™ (100□g/ml in DMEM-F12) was added to each well of 6-

well plate and left for 1 h at room temperature or at 4ºC overnight. These plates were either used immediately or stored at 4ºC for a maximum of 1 week. For further passaging on mTeSR1, cells were enzymatically lifted using a sterile solution of dispase (Invitrogen) (1mg/ml in DMEM-F12), dissected into multiple small pieces and transferred onto freshly plated Matrigel™ on mTeSR1 media (2.5ml per well). The culture media was replaced every day in all of these culture techniques.

Differentiation of hESC to Neuroectodermal Cells

The protocol described below for the differentiation of hESC into columnar neuroectodermal cells (neural precursor cells; NPC) mimics *in vivo* neuroectodermal development in terms of timing and morphology[18]. *In vitro*, hESC differentiate into columnar neuroectodermal cells that organize into neural tube-like rosettes within 12-14 days. Since hESC are equivalent to a 5-6 day embryo, development of the neuroectodermal *in vitro* takes about 18-20 days. Physiologically, this is the time window when the neural tube forms in a human embryo[19,20].

Undifferentiated hESC (H9) colonies grown on MEF in a 6-well plate were rinsed twice with 1ml of DPBS per well. They were then treated with 1ml of dispase (1mg/ml in DMEM-F12) and incubated at 37℃, 5% CO_2 till the edges of the colonies began to curl up. The plate was then swirled to detach the colonies intact but without dispersing the colonies into individual cells. The hESC colonies were grown in T25 flasks in a special ES cell growth medium (78.5% DMEM-F12, 20% KOSR, 1% NEAA, 1mM L-glutamine, 0.1 mM 2-mercaptoethanol) for 4 days with daily replacement of media to form ES cell aggregates. They were then adhered to the

culture surface where they formed monolayer colonies in a chemically defined neural induction medium [32.6% F-12 (Invitrogen), 65.2% DMEM, 1% N2 supplement (Invitrogen), 1% NEAA, 0.2% of 1mg/ml Heparin (Sigma-Aldrich), and 10ng/ml bFGF]. Under these conditions, columnar neuroectodermal cells (Rosettes) appeared in the center of each colony and organized into neural tube-like rosettes after a total of 9-10 days of differentiation culture. The neural induction media was replaced every other day. The neuroectodermal cells in the rosettes were selectively isolated through differential enzymatic treatment using dispase (0.5mg/ml in DMEM-F12) and incubated for 2 h in neural induction medium to allow the non-neural cells to differentially attach to the T25 flasks. After this, the floating cells (mostly aggregates of neural rosette cells) were transferred to new T25 flasks where they rolled up to form round clusters. Some of these clusters were collected and probed for nestin as a marker of neuroectodermal cells to confirm proper differentiation.

GSK-3β and Cdk-5 inhibition of hESC

Undifferentiated H9 hES cells were evenly plated in 6-well plates coated with Matrigel^{TM} in 2.5ml of mTeSR1 media per well. The cells in each well of one plate were trypsinized after 24 hours using 400µl of TrypLE^{TM} Express (Invitrogen) per well. TrypLE was neutralized after 5 min. by adding 1ml of MEF culture media. To confirm even plating, cells were counted using a hemocytometer after mixing equal volumes of neutralized sample and trypan blue stain (0.4%, Invitrogen). After confirmation of even plating, the remaining plates were treated with GSK-3b inhibitor, LiCl (100µM; Sigma-Aldrich Co., MO, USA), and Cdk-5 inhibitor, roscovitine

(300nM; Calbiochem, CA, USA) in mTeSR1 media. A stock solution of LiCl was prepared in ddH_2O. Corresponding concentrations of ddH_2O or NaCl (Fisher Scientific, PA, USA) dissolved in ddH_2O were added to control wells. A stock solution of roscovitine was prepared in dimethyl sulfoxide (DMSO; Fisher Scientific). A corresponding concentration of DMSO was added to control wells. After 5 days of treatment, the cells were trypsinized and cell proliferation was assessed using the trypan blue assay. Cells were also collected in DPBS and stored at -80℃ for examination of the effect of GSK-3β and Cdk-5 inhibition on the differentiation of hESC.

GSK-3β and Cdk-5 inhibition of Neuroectodermal Cells

Undifferentiated H9 hESC colonies grown on MEF were treated with hESC culture media for 4-5 days. hESC were then treated as described above to induce the formation of NPC in the presence or absence (controls) of LiCl (5mM), roscovitine (300nM), or GSK-3β inhibitor VIII (200nM, Calbiochem) in their respective neural induction medias. Corresponding concentrations of ddH_2O, NaCl, or DMSO were added to the control flasks. Fully formed NPCs were then collected in DPBS after 14 days and stored at -80℃ for immunoblot analyses.

Immunoblotting

Cells were collected in DPBS and homogenized in lysis buffer (20mmol/L Tris–HCl, pH 7.6, 150mmol/L NaCl, 1mmol/L EDTA, 1mmol/L EGTA, 1% sodium dodecyl sulfate (SDS), and protease inhibitors (10μg/mL aprotinin, 10μg/mL

leupeptin, 1μg/mL pepstatin A, 1mmol/L phenylmethylsulfonyl fluoride; Roche Diagnostics, IN, USA) and then sonicated indirectly for 30 s. Following protein assay (Bicinchoninic Acid Protein Assay Kit; Pierce), equal amounts of protein were loaded onto 10–20% tricine gels (Invitrogen) for SDS–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, CA, USA), fixed with 4% glutaraldehyde in TBS Tween-20 (TBST), blocked with milk (10%, w/v) for 4 h and then probed with primary antibody overnight at 4℃. The blot was then incubated with the corresponding horseradish peroxidase conjugated secondary antibody (Santa Cruz Biotechnology, CA, USA) for 1 h at room temperature, washed again and developed with ECL plus reagent (Amersham ECL™ Advance Western Blotting Detection Kit, GE Healthcare, UK) as per the manufacturer's instructions. The chemiluminescent signal was captured on autoradiograph (Eastman Kodak, NY, USA). The membrane was then stripped using a stripping buffer (2% SDS, 60mM Tris, pH 6.8, 1% 2-mercaptoethanol) and probed with the next primary antibody of interest.

The following antibodies (with dilution ratio) were used throughout this study: Anti-human nestin monoclonal antibody (Chemicon International, CA, USA; 1:4000), Anti-human tau-1 monoclonal antibody (Chemicon; 1:1000), Anti-human PHF-tau monoclonal antibody (clone AT8) (Pierce; 1:400), Anti-human PHF-tau monoclonal antibody (kindly gifted by Dr. Peter Davies, NY, USA; 1:50), Anti-human GSK-3β polyclonal antibody (Biolegend, CA, USA; 1:1000), Anti-human Cdk-5 polyclonal antibody (Stressgen Bioreagent Corp., Victoria, BC Canada, 1:1000), Anti-human

Oct-3/4 monoclonal antibody (Santa Cruz Biotechnologies, CA, USA; 1:1000), Anti-human actin (C-11) (Santa Cruz; 1:1000), Anti-human GAPDH (V-18) (Santa Cruz; 1:1000), Horseradish peroxidase-linked goat anti-mouse (Santa Cruz; 1:6000), goat anti-rabbit (Santa Cruz; 1:15,000), and donkey anti-goat IgG (Santa Cruz; 1:6000)

Statistical Analysis

Changes between treatments were analyzed with Student's t-test and significance was set using a Type I error rate of or equal to or less than 0.05.

Chapter 3: Results

Tau Protein is expressed and phosphorylated during neuroectodermal differentiation of hESC

To determine when tau is first expressed during early embryonic development we probed immunoblots of hESC and NPC with the anti-tau-1 monoclonal antibody. Tau was expressed in NPC (53 kDa), but not in the pluripotent hESC ***(Figure 3).*** This observation indicates that tau expression is functionally important during the

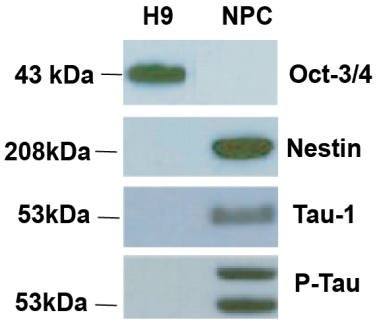

Figure 3. **Tau is expressed and phosphorylated during neuroectodermal differentiation of hESC.** Undifferentiated hESC (H9) and NPC were cultured, collected and then analyzed by immunoblot with anti-Oct3/4 (C-10) (against C-terminal 10 amino acids) monoclonal antibody**,** anti-nestin monoclonal antibody (clone 10C2), anti-tau-1 (clone PC1C6) monoclonal antibody, and anti-P-tau (AT8) monoclonal antibody. Molecular weight markers are shown on the left hand side.

development of the neural tube. Next, to examine whether the tau protein expressed in NPC is phosphorylated we probed the immunoblot with anti-human PHF-tau monoclonal antibody AT8, which recognizes tau phosphorylated at both serine 202 and threonine 205[21]. As shown in ***Figure 3,*** two isoforms of phosphorylated tau were detected in NPC at 53kDa and 61kDa. This observation indicates that the destabilization of microtubules via phosphorylation of tau is important in the *in vitro* formation of neuronal precursor cells.

Expression of GSK-3β and Cdk-5 in hESC and NPC

As previously discussed, the two main kinases known to phosphorylate tau *in vivo* are GSK-3β and Cdk-5[22,23]. The expression of these kinases during hESC

differentiation into NPC was examined by probing the previously mentioned immunoblot with the anti-GSK-3β and anti-Cdk-5 polyclonal antibodies *(**Figure 4**).*

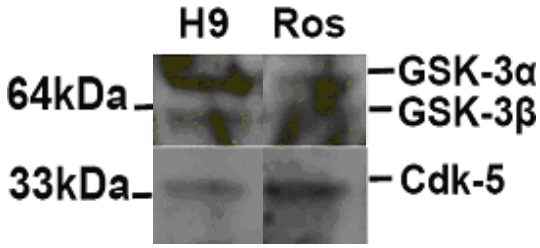

***Figure 4*. GSK-3 and Cdk-5 phosphorylate tau in neuroectodermal cells.** Undifferentiated hESC (H9) and NPC were cultured, collected and then analyzed by immunoblot with anti-GSK-3β (against C-terminal) and anti-Cdk-5 (against 268-283 residues) polyclonal antibodies. Molecular weight markers are shown on the left hand side.

GSK-3β (64-kDa) expression was detected in hESC and NPC. The GSK-3β antibody also recognized GSK-3α (~70kDa). In addition, Cdk-5 expression was detected in the hESC and the NPC. This suggests that tau phosphorylation might play a key role in the proliferation and differentiation of hESC during early human embryonic development.

Inhibition of GSK-3β results in a small but significant decrease in hESC Proliferation

We investigated the effects of GSK-3β inhibition on hESC proliferation by treating the cells with LiCl, a well-known GSK-3β inhibitor. The hESC were treated with 100μM LiCl in mTeSR1 media for 5 d prior to cell counting using the trypan blue assay. ddH_2O was added to the controls. There was a significant decrease in the proliferation of LiCl-treated hESC compared to controls (***Figure 5***), indicating that GSK-3β plays a role in hESC proliferation.

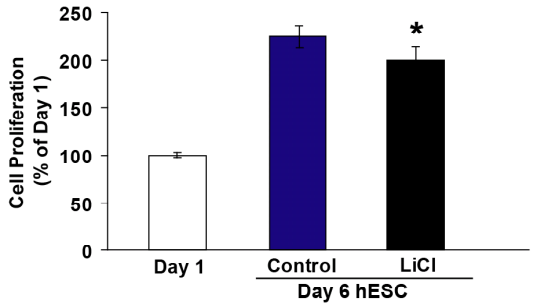

***Figure 5*. LiCl decreases hESC proliferation.** Evenly plated hESC were treated daily for 5 d with LiCl in mTeSR1 media prior to measurement of cell proliferation using the trypan blue assay. LiCl significantly decreased the rate of cell proliferation compared to untreated cells. Results are expressed as mean ± SEM, n = 5(*p<0.05 compared to 5 d Control).

Inhibition of GSK-3β results in decreased neuroectodermal differentiation of hESC

After observing that GSK-3β inhibition results in a decrease of hESC proliferation we wanted to see what kind of effect this inhibition would have on the differentiation of hESC to NPC. Therefore, we cultured pluripotent hESC and differentiated them into NPC with 5mM LiCl in neural induction media for 10 d. A decrease in the total number of NPC was observed (***Figure 6***).

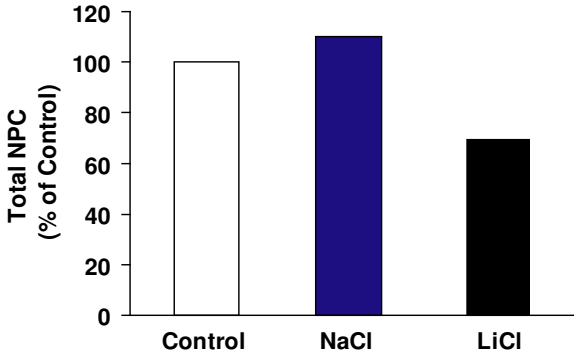

***Figure 6.* LiCl decreases formation of total NPC.** Pluripotent hESC were cultured and differentiated into NPC with 5mM neural induction media for 10 days prior to counting (n=1).

In addition, cell lysates of these LiCl-treated NPC were analyzed by immunoblotting with anti-nestin antibody, anti-P-tau (AT8) antibody, anti-tau-1 antibody, and anti-actin antibody. A decrease in expression of nestin, tau and phosphorylated tau was observed with treatment (***Figure 7***).

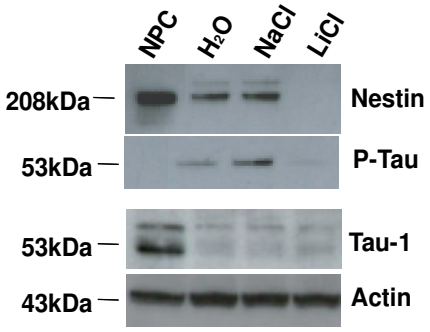

Figure 7. **LiCl decreases neuroectodermal differentiation, tau and phosphorylated tau expression.** NPC were cultured with 5mM LiCl, collected and then analyzed by immunoblot with anti-nestin antibody, anti-P-tau (AT8) antibody, anti-tau-1 antibody, and anti-actin antibody. A decrease in expression of nestin, tau and phosphorylated tau was observed. Molecular weight markers are shown on the left hand side.

These results indicated that GSK-3β plays a crucial role in the neuroectodermal differentiation of hESC.

Cdk-5 inhibition does not affect proliferation, tau expression and phosphorylation, and neuroectodermal differentiation of hESC

A similar set of experiments was conducted on Cdk-5, another main kinase linked to AD and known to phosphorylate tau. To investigate the effect of Cdk-5 inhibition on proliferation, undifferentiated hESC were treated with 300nM roscovitine, a Cdk-5 inhibitor, in mTeSR1 media for 5 days prior to cell counting using the trypan blue assay. There was no significant difference in cell proliferation between the control and the treatment (***Figure 8***).

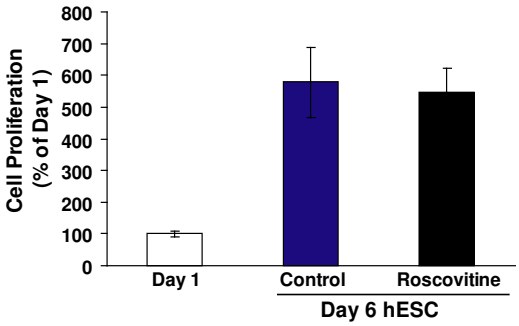

***Figure 8*. Roscovitine does not affect hESC proliferation.** Evenly plated hESC were treated daily for 5 d with 300nM roscovitine in mTeSR1 media prior to measurement of cell proliferation using the trypan blue assay. Roscovitine did not significantly change the rate of cell proliferation compared to untreated cells. Results are expressed as mean ± SEM, n = 5.

In addition, pluripotent hESC were cultured and differentiated into NPC with 300 nM roscovitine in neural induction media for 10 days. Subsequently, immunoblot analysis was conducted on the neuroectodermal cell lysates with anti-Cdk-5 antibody, anti-PHF-1 antibody, and anti-tau-1 antibody. We observed no change in the expression of tau or its phosphorylated form (***Figure 9***).

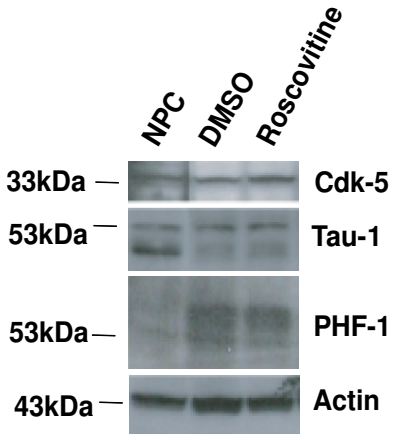

***Figure 9.* Roscovitine does not affect tau and phosphorylated tau expression.** NPC were cultured with 300nM roscovitine, collected and then analyzed by immunoblot with anti-Cdk-5 antibody, anti-tau-1 antibody, anti-PHF-1 antibody, and anti-actin antibody. No change was observed in the expression of tau and phosphorylated tau. Molecular weight markers are shown on the left hand side.

Overall, these observations suggest that Cdk-5 might not play as central a role in hESC proliferation and neuroectodermal differentiation as GSK-3β.

Chapter 4: Conclusions and Discussion

Before investigating the developmental changes as an etiology of neurodegeneration/neurogenesis in AD, it is necessary to confirm the presence and importance of tau during embryogenesis. We achieved this by using hESC as a model of neural development to study the cell-state-dependent expression of tau and the two main kinases known to phosphorylate tau. From the observed results there are several main conclusions that can be drawn. First, tau and its phosphorylated form are expressed in NPC, but not in undifferentiated hESC (***Figure 3***). In addition, the inhibition of GSK-3β decreases hESC proliferation (***Figure 5***), tau phosphorylation, and neuroectodermal differentiation of hESC (***Figure 7***). However, inhibition of Cdk-5 did not affect proliferation (***Figure 8***) and neuroectodermal differentiation (***Figure 9***) of hESC. Based on these findings, it can be concluded that GSK-3β regulates proliferation, tau phosphorylation, and neuroectodermal differentiation of hESC.

Our laboratory has demonstrated for the first time the expression of tau during the neuroectodermal differentiation of hESC suggesting it plays a crucial role in neurogenesis (***Figure 3***). We have also shown for the first time that tau is phosphorylated with neuroectodermal differentiation of hESC indicating an important role of this microtubule-associated protein in neurogenesis (***Figure 3***). Finally, our results indicate that hESC is a useful model for understanding tau metabolism.

Furthermore, our and other data illustrate the parallels that exist between embryogenesis and neurodegeneration regarding the expression and

phosphorylation of tau. Intriguingly, this suggests that a developmental component is integral to the neurodegeneration observed in the AD brain.

Overall, the physiology seen during the human embryogenesis especially during neurogenesis is strikingly similar to the pathology seen in the AD brain.

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