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2012 5th Annual Maryland Stem Cell Research Symposium - Poster presentation
Maryland Stem Cell Research Fund
2011 International Proteomics Workshop – Organizer / Lecturer
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2010 7th Molecular Biology and Genetics Winter School - Poster Presentation
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2009 6th Molecular Biology and Genetics Winter School - Organizer
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- Genomic DNA & RNA extraction from a range of source materials such as animal tissue, white blood cells, cell pellets, whole blood, plants and maize containing processed food sources through the use of various manual and commercial methods.
- DNA purification using Roche High Pure PCR kit to successful and effective PCR analysis.
- Primer design.
- RT-, qRT- and nested PCR.

Molecular biology & genetics

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- RT-, qRT- and nested PCR.

Cell biology

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- Ability to generate, differentiate and culture embryoid bodies and neurons from hESCs.
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- Light and fluorescence imaging.
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ABSTRACT

Title of Thesis: Potential Role of Tazarotene-Induced Gene 3 and Transglutaminase 1 in Tauopathies

Yasin Kizilyer, Master of Science, 2013

Thesis Directed by: Richard L. Eckert, Ph.D., John F.B. Weaver Distinguished Professor and Chair of Biochemistry and Molecular Biology Department

The family of neurodegenerative diseases, called tauopathies, includes Alzheimer's disease, progressive supranuclear palsy, and Pick's disease. These diseases are associated with the formation of tau inclusions and neuronal cell death. Tau inclusions are characterized by the presence of neurofibrillary tangles (NFTs), where protein Tau, a member of microtubule-associated proteins (MAPs), is found to be, hyperphosphorylated and aggregated. The formation of these aggregates leads to microtubule instability and cellular toxicity. There are several lines of evidence that demonstrate this aggregation is due to transglutaminase mediated cross-linking of Tau protein. However, little is known about the mechanism of the hyperphosphorylation, aggregation and tau-associated toxicity. Analysis of brains with tau pathologies showed the colocalized expression of transglutaminase 1 (TG1) and its activator, tazarotene-induced gene 3 (TIG3) in NFTs, suggesting functional relevancy. Hereby, we hypothesized that TIG3 regulates TG1-catalysed cross-linking of Tau, and therefore the formation of Tau inclusions in neurons. To test this hypothesis, neurons differentiated from human embryonic stem cells used to develop an *in-vitro* tauopathy model. Endogenous expression analyses of TIG3, TG1 and

Tau were performed and induction of Tau occlusions via overexpression of TIG3 studied. Results have shown endogenous TIG3 mRNA and protein were expressed in neurons derived from embryonic stem cells. Endogenous TIG3, TG1 and TAU have shown colocalization. Cells overexpressing TIG3 have shown a higher level of colocalization and increased Tau aggregation, which confirmed our hypothesis. The results of this study provide new mechanistic insights into the formation of neurofibrillary tangles and the resulting pathology.

Potential Role of Tazarotene-Induced Gene 3 and Transglutaminase 1 in Tauopathies

by
Yasin Kizilyer

Thesis submitted to the Faculty of the Graduate School of the
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I also would like to thank my family for their constant unconditional support, and to my sister, Ayse, for being an on-call tutor for me throughout my collegiate education. Last, but not least, I also would like to thank my friends from Molecular Medicine – Master's program for making this duration an invaluable part of my life.

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LIST OF ABBREVIATIONS

Ad5-TA: adenovirus transactivor

AD: Alzheimer's disease

BDNF: brain derived neurotrophic factor

CNS: central nervous system

DMEM: Dulbecco's Modified Eagle Medium

DMSO: dimethyl sulfoxide

DTT: dithiothreitol

EB: embryoid bodies

EDTA: ethyleneglycol-bis(aminoethylether)-tetraacetic acid

ERK: extracellular signal-regulated kinase xii

EV: empty vector

FGF2: fibroblast growth factor

hESCs: human embryonic stem cells

KO: knockout serum

MBD: microtubule binding domain

MEF: mouse embryonic fibroblasts

MOI: multiplicity of infection

NEAA: non-essential amino acids

NFT: neurofibrillary tangles

NP: neural progenitors

NT3: neurotrophic factor 3

OA: okadaic acid

PBS: phosphate buffered saline

PHF: paired helical filaments

PMSF: phenylmethylsulfonyl fluoride

PP2A: protein phosphatase-2A

RA: retinoic acid

RARRES3: retinoic acid receptor responder 3

RIG1: retinoid-inducible gene 1

SCC: squamous cell carcinoma

SD: standard deviation

SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM: standard error of the mean

SHH: sonic hedgehog

TAU-*P*: phospho-“paired helical filament”-tau serine202/threonine205

TBS: tris-buffered saline

TG1: type I transglutaminase

TG2: type II transglutaminase

TG: transglutaminase

TGase: transglutaminase

TIG3: tazarotene-induced gene 3

TUJ1: neuronal class III β-tubulin

CHAPTER 1: BACKGROUND

INTRODUCTION

The family of progressive neurodegenerative diseases, called tauopathies, includes Alzheimer's disease (AD), progressive supranuclear palsy (PSP), Pick's disease (PiD) and frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) [1]. Manifestation and progression of these diseases are associated with the accumulation of neurofibrillary tangles (NFTs). NFT is a pathological term used to describe large Tau aggregates which accumulate over time and are present in the tissue after neuron death. These develop from small Tau aggregates which are formed by the hyperphosphorylation and aggregation of the microtubule associated protein, Tau or MAPT [2-4]. Tau functions by binding to tubulin monomers and stabilizing microtubule structure [5, 6], while hyperphosphorylation of the protein causes its dissociation from microtubules in the cytoplasm [7]. Microtubules are a primary component of the cytoskeletal architecture of neurons and their axons. Thus, the formation of these NFTs leads to the microtubule instability, disturbance in cytoskeletal dynamics and cellular toxicity in these cells [8, 9]. The cellular and molecular mechanisms causing hyperphosphorylation, aggregation and Tau-associated toxicity in neurons are not well understood.

Transglutaminases (TGs) are a family of enzymes that catalyzes the formation of (g-glutamyl) polyamine bonds [10], molecular cross-links and deamidation of protein substrates [11]. Several lines of evidence points that members of this family, and particularly a few studies demonstrating transglutaminase 1 (TG1) and most studies showing transglutaminase 2 (TG2) might be involved in the aggregation of protein Tau.

Several studies have been shown that TG2 has the ability to cross-link both phosphorylated and unphosphorylated Tau forms in an acellular environment [12, 13]. It has also been shown that both TG1 and TG2 colocalizes in NFTs of Alzheimer's brain [14]. Lastly, analysis of brains from patients afflicted with several different tauopathies has revealed the colocalized expression of TG1 and an activator of TG1 in the skin, tazarotene-induced gene 3 in NFTs [15].

Tazarotene-induced gene 3 (TIG3, also named as retinoid-inducible gene 1, RIG1 and retinoic acid receptor responder 3, RARRES3) is classified as a class II tumor suppressor gene and has reduced expression in tumors and skin cancer cell lines [16-22]. Its function is well-established in epithelial tissues, including skin. In these tissues, TIG3 acts as a pro-differentiation factor due to its ability to increase TG1 activity [16, 20-24]. TG1's (also known as keratinocyte transglutaminase) function and interaction with TIG3 is well-documented by our laboratory and shown to be essential in the differentiation of epidermal keratinocytes, which suggests functional relevancy to the colocalization observed in the brain samples with different tauopathies [15, 25]. Hereby, we hypothesize that TIG3 regulates TG1-catalysed cross-linking of protein Tau, to form Tau aggregates. To test this hypothesis, neurons differentiated from human embryonic stem cells (hESC) were used to develop an *in-vitro* tauopathy model. Endogenous expression analysis of TIG3, TG1 and Tau were performed and induction of Tau occlusions via overexpression of TIG3 studied. The results of this study provide new mechanistic insights into the formation of neurofibrillary tangles and the resulting pathology.

TAUOPATHIES

Tauopathies are progressive neurodegenerative diseases, which are characterized by the manifestation and disease progression correlated accumulation of NFTs. NFTs, a major hallmark of this family of diseases, are formed by the aggregation of hyperphosphorylated Tau [2-4]. Some of these diseases are shown in Table 1.

Table 1: Members of tauopathies, the family of progressive neurodegenerative diseases (*adopted from Iqbal et. al, 2005* [26])

Alzheimer's disease (AD)
Down syndrome, adult cases
Guam parkinsonism dementia complex
Dementia pugilistica
Pick's disease (PiD)
Dementia with argyrophilic grains
Frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17)
Cortico-basal degeneration
Pallido-ponto-nigral degeneration
Progressive supranuclear palsy (PSP)
Gerstmann–Sträussler–Scheinker disease with tangles

Alzheimer's disease is the most common and the best-known member of this family. Once considered as a rare disorder, AD is now recognized that over 5.1 million Americans have this disease. As the main cause of dementia in people age 65 and older, AD is the 6th leading cause of death in the US. While it is known that genetics and

environmental factors are involved, there is unfortunately no cure for this disease and average survival is only 4-8 years of after diagnosis. As such, the estimated cost of AD in 2013 in the US was $203 billion. As all tauopathies are just as debilitating there is therefore a need for a better understanding of the mechanisms contributing to these diseases, and it is greatly needed to discover new forms of treatments.

MICROTUBULE ASSOCIATED PROTEIN TAU (MAPT, TAU)

Tau belongs to microtubule associated proteins (MAPs) family and was shown in the mid-1970s to promote microtubule assembly in an acellular environment [5, 6]. It is found in eukaryotes and is highly conserved between species [27-29]. In humans, Tau is encoded by the gene MAPT, which is localized on chromosome 17q21; a gene consists of 16 exons and spans a region larger than 50kb. The gene expression is developmentally regulated [30], found in many organs and tissues [31] and produces 9 isoforms with varying sizes via posttranscriptional modifications [31-33]. The protein contains 4 domains, which include: the C-terminal domain, the microtubule binding domain, the proline-rich domain and the N-terminal domain [34]. In the central nervous system (CNS), alternative splicing of N-terminal domain and microtubule binding domain (MBD) produces 6 isoforms, with amino acid lengths varying from 352 to 441 [28, 34].

In mammals, Tau is present in ample amounts within neuronal axons [1, 35] and to a lesser extent in the somatodendritic regions of neurons and in oligodendrocytes [36]. Following its discovery in porcine brain, Caceres *et. al.* [37] showed a critical role of the Tau for the first time by demonstrating that primary neurons treated with Tau antisense oligonucleotide were unable to establish axonal outgrowth, indicating its role in axonal integrity. *In vivo* studies has shown regional axonal abnormalities and altered gene

expression in the brains of knockout animals, and when cultured, their cells exhibited delayed axonal maturation. Interestingly, no other behavioral or developmental deficits were found [38, 39]. Within these animals, microarray analysis of brains has shown that the majority of the genes with major expression alterations were not related to cytoskeleton, suggesting both, the presence of a compensatory mechanism and more diverse functions of Tau than a single involvement in microtubule stabilization [40]. The lack of toxicity and weak alterations of cytoskeletal components seen in knock-out animals might be due to the presence of multiple microtubule associated proteins (MAP) types in a given cell.

While MAPs share structural homology and in some cases overlapping functions [41-45], in other cases they exhibit distinct structural properties and functions. For example, the defects in the neuronal development exhibited by knockouts of MAP1B, which is another MAP found in axons, increased toxicity and lethality in the double knockouts of MAP1B and Tau, underlining this likely compensatory relationship in mice [46]. Nonetheless, out of all MAPs, only Tau is involved in the formation of neurofibrillary tangles.

Additionally, it is now known that alongside its function as a cytoskeletal regulator, Tau might be involved in several other pathways in neurons and other systems. For instance, when compared to wild-type, Tau knockout mice demonstrated increased muscle weakness and enhanced protection against experimentally induced seizures [47, 48]. Moreover, although Tau's prominent expression is in neurons and it is predominantly localized on axons, its expression has been shown in many other non-neuronal systems, such as kidneys, liver and muscle tissue [31, 49], as well as in tissues and cell lines of gastric, prostate and breast cancers [50-54].

Expression and Post Translational Modifications of Tau

Expression and translation of Tau is highly regulated depending on the developmental stage and the tissue type [30]. In the CNS and peripheral nervous system, of the 16 exons of the MAPT gene, exons 2, 3 and 10 are found to be alternatively spliced, resulting with non-translating exons 6 and 8. In the peripheral nervous system, specific expression splicing of exon 4A also occurs [55-57]. In the adult brain, Tau alternative splicing produces 6 isoforms in neurons, with amino acid lengths of 352 to 441 amino acids and 60 – 74 kDa molecular weights [55, 56, 58] (Figure 1). The proteins produced contain 4 domains: The acidic N-terminal domain encoded from exons 1 – 5; the proline rich region encoded from exon 7 and partially from exon 9; MBD, which contains 3-4 binding repeat sequences termed as R_1, R_2, R_3 and R_4, from exons 9 – 12 and lastly, the C-terminal domain from exon 13 [34, 59, 60]. Although exon 14 is transcribed as well, it contains a premature stop codon that prevents translation [56]. Alternative splicing of exon 10 results with products carrying different amounts of R sequences, and are named as 3R (3 repeat sequence containing, the exon 10 absent product) or 4R (4 repeat sequence containing, the exon 10 present product). Additionally, alternative splicing of exons 2 and 3 results with products carrying the different amount of N-terminal inserts, which are named as 0N, 1N or 2N, which together forming the six isoforms of CNS; 3R/0N, 3R/1N, 3R/2N, 4R/0N, 4R/1N, 4R/2N.

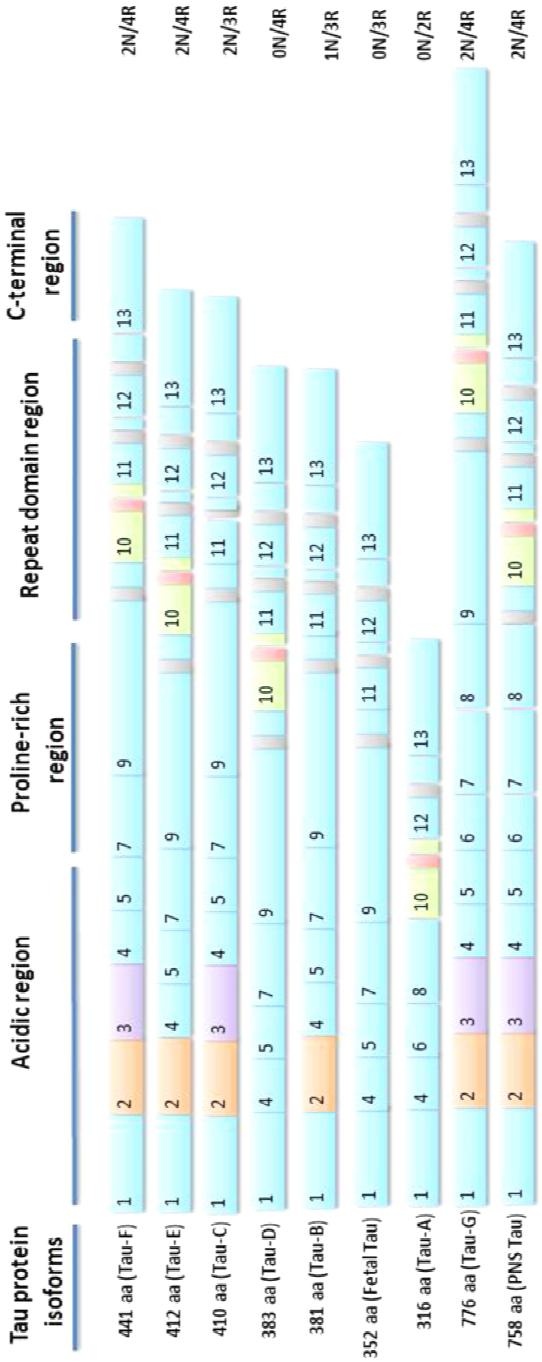

Figure 1: Tau isoforms in human.

Developmentally regulated expression of the MAPT gene and post-translational modifications of its product results with the production of 9 Tau isoforms. The first six isoforms, including fetal Tau, are expressed in adult brain.

The majority of our knowledge related to the Tau posttranslational modifications comes from studies related to the pathological conditions, and from AD in particular. To date, 131 different Tau post-translational modification sites and 11 different modification types are identified in brains from AD patients. Some of these modifications are phosphorylation (on 85 different sites), glycation (on 12 different sites), glycosylation (on 11 different sites), polyamination (on 8 different sites), and ubiquination (on 3 different sites). Phosphorylation, the most common modification found in AD brains, is found at 85 of 131 potential sites in the protein [61].

In our study, we focused on the phosphorylation and polyamination of the Tau. Briefly, Tau phosphorylation is known to cause Tau to lose its affinity for tubulin, leading to its dissociation and microtubule destabilization [62-64]. Due to this, the balance between two forms is hypothesized to be important for the hyperdynamic functioning of the microtubules [57]. In glial cells and neurons of a family of diseases, called tauopathies, protein Tau is found to be hyperphosphorylated and aggregated [4], causing problems that are not limited to: destabilization of the microtubule and axonal structure, disruption of cellular and axonal transport and disruption of axonal growth [61, 65, 66]. Researchers have shown that abnormal Tau phosphorylation precedes the formation of NFTs, an important hallmark of tauopathies [67, 68]. Moreover, mutant Tau studies indicate that Tau phosphorylation on certain domains can induce conformational changes, and promote self-aggregation [69-72], which shows the critical role of phosphorylation in this process. Levels of Tau phosphorylation is controlled by various kinases and phosphatases [30, 73, 74], whereby increases in kinases or downregulation in phosphatases results in its dysregulation. For instance, many studies have shown that a Tau-kinase, glycogen

synthase kinase 3 is over-expressed and/or upregulated in Alzheimer's patients [75-78]. Moreover, protein phosphatase-2A (PP2A) a protein responsible for higher than 70% of cellular phosphatase activity in cells [79], is hypothesized to carry out the majority of Tau dephosphorylation activity in neurons [80-83]. Specifically, in the brains of AD, PP2A activity was demonstrated to be downregulated by 50% [79, 80, 84].

After Tau becomes phosphorylated, it falls off of microtubules and gets further modified in tauopathies. One primary modification is polyamination of the Tau protein. In epithelial and neural cells, Tau polyamination gets carried out by transglutaminases. This reaction occurs between a lysine as an acyl acceptor on one Tau molecule and a glutamine as an acyl donor on another Tau molecule. The reaction results in cross-linking of Tau proteins via the formation of γ-glutaminyl-ε-lysine isopeptide bonds [85]. Although the role of this Tau cross-linking under physiological conditions is currently unknown, transglutaminase activity and expression is found to be upregulated in the AD brain. In addition, the results show that polyamination of Tau precedes NFT formation and transglutaminases colocalizes with Tau aggregates in tauopathies. Together, these suggest that polyamination and transglutaminases are possibly involved in the Tau aggregation and the development of NFTs.

Neurofibrillary Tangle Formation and Tauopathies

Manifestation and progression of tauopathies are characterized with the presence and accumulation of neurofibrillary tangles. NFTs, a major hallmark of this family of diseases, are formed by the aggregation of hyperphosphorylated Tau [2-4]. Today, whether or not these Tau aggregates are factitive or secondary characteristics of the tauopathies remains unknown [61, 86], as well as are the mechanisms underlying their

formation [15]. When combined, the current evidence suggests a multistep process for NFT formation, where the impairment of Tau binding to tubulin is the initiating event, resulting in soluble Tau monomers. These monomers are then modified through post-translation modifications, altering the conformation of Tau and promoting its anti-parallel self-dimerization. Further aggregation then causes the formation of oligomers which leads to the development of longer Tau filaments called protomers. Similar to the self-dimerization process, these protomers form paired helical filaments (PHFs) which are the main subunits of neurofibrillary tangles. Lastly, PHFs assemble to form the final, insoluble, covalently bonded neurofibrillary tangles which are detected pathologically at sites of tauopathy lesions and neuronal deaths [69]. Several lines of evidence have shown that the covalent bonding of Tau is carried out by the enzymes called transglutaminases (TGs), and especially by TG1 and TG2. Experiments have shown that TG2 has the ability to cross-link both phosphorylated and unphosphorylated Tau forms in an acellular environment [12, 13], and both TG1 and TG2 colocalize in NFTs of Alzheimer's brains [14]. More recently, analysis of brains with various Tau pathologies showed the colocalized expression of transglutaminase 1 (TG1), but not TG2 in aggregates. Additionally, it was also shown that the activator of TG1, tazarotene-induced gene 3 was also colocalized with TG1 within these NFTs [15]. Together, these data suggest that cross-linking of Tau, an important step in NFT formation, might be carried out via TG1 through its modulation by TIG3.

TRANSGLUTAMINASES

Transglutaminases (TGases, TGs) are a family of which catalyze the formation of (γ-glutamyl)polyamine bonds [10] and molecular cross-links as well as deamidation of

protein substrates [11]. The family consists of 9 members, some of which are well-characterized (Table 2).

Table 2: The transglutaminase (TGase) enzyme family and their known functions (*adopted from Eckert et. al., 2005* [25]).

TGase	Synonyms	Chromosome location	Gene	Function	Ref.
Factor XIII	Fibrin stabilizing factor	6p24–25	F13A1	Blood clotting and wound healing	[11, 87, 88]
Band 4.2	Erythrocyte membrane protein	15q15.2	EPB42	Structural protein in erythrocytes—no activity	[89]
TGase 1	Keratinocyte TGase	14q11.2	TGM1	Cornified envelope assembly in surface epithelia	[90-92]
TGase 2	Tissue TGase, tTG, cTG	20q11–12	TGM2	Apoptosis, cellular differentiation and adhesion, matrix assembly	[93-96]
TGase 3	Epidermal TGase, eTG	20q11–12	TGM3	Cornified envelope assembly in surface epithelia	[97, 98]
TGase 4	Prostate TGase	3q21–22	TGM4	Coagulation and immunogenicity suppression of semen	[99, 100]
TGase 5	TGase X	15q15.2	TGM5	Epidermal differentiation	[101-104]
TGase 6	TGase Y	20q11	TGM6	Unknown	[101-104]
TGase 7	TGase Z	15q15.2	TGM7	Unknown	[101-104]

The enzymes carry out their activities in a two-step process. Binding of Ca^{2+} activates the enzyme by exposing its active-site cysteine residue. There are two steps following its activation; step one is the formation of thioacyl-enzyme intermediate and ammonia through the interaction of the active-site cysteine residue and the substrate protein/peptide's glutaminyl γ-carboxamide residue; and step two is the reaction of the thioacyl-enzyme intermediate with a nucleophilic primary amine substrate, leading to the active-site cysteine residue regeneration and the formation of a covalent attachment in between the substrate glutaminyl acceptor and the amine-containing donor [105].

Transglutaminase expression is modulated by complex transcriptional and translational mechanisms, leading to the precise distribution patterns seen in many cells and tissues [106]. This fine and meticulous balance of the expression patterns reflect the intracellular and extracellular physiological roles of these enzymes. Different TGs are expressed in different cellular or tissue compartments at a given time and a single cell can carry several splice variants of a given TGase [107, 108]. Although these complex regulations led researchers to believe that TGases would be an essential components for the cells, knockouts in mice were not entirely consistent with this theory. For example, even though TG2 is ubiquitously expressed and has complicated roles, knockouts of this protein were phenotypically normal at birth [109]. The reason for mice's survival was shown to be due to the expression of multiple TGases and their ability to substitute for the missing isoforms [110]. This is not surprising given that bioinformatics show that TGs share sequence homologies in their active site and the calcium binding regions. This homology is shown to be stronger and highly preserved in these regions of TG2, TG3 and FXIIIa secondary structures, suggesting that similar relationships for other TGs also might be possible [11, 111]. This section will focus on TG1 for which very little is known about its expression and function in neurons, and on TG2, but to a lesser extent.

In humans, transglutaminase 1 (keratinocyte TGase, TGase 1, TG1) is encoded by the gene TGM1, which is localized on chromosome 14q11.2, spanning 14.3 kb and consisting of 15 exons [92]. Its expression is shown in various epidermal and neuronal cell lineages [112, 113], while its function is best characterized in keratinocytes (hence the name keratinocyte TGase), showing an essential differentiation-dependent expression [25, 114]. The expression of the gene results in a product 817 amino acid long (90 kDa)

that is modified by co-translational myristoylation and post-translational palmitoylation of the protein at its N-terminus [115-117]. The majority of TG1 is found to be anchored through these myristate and palmitate residues to the cellular membrane in keratinocytes. A fraction of this membrane bound form undergoes proteolytic cleavage during maturation, yielding 3 fragments [117, 118]. This in turn increases the activity of the TG1's portion representing its pro-peptide form over 100 fold in comparison to the full length active protein [97, 119]. The computerized 3D model of TG1 which was created based on the crystal structure of factor XIIIa revealed that the protein carries four discernible domains: the N-terminal β-sandwich domain; the catalytic core, a transamidation domain (domain 2); and two carboxy-terminal β-barrel domains. The same 3D modeling also showed that TG1 would function as dimers [111].

Transglutaminase 2 (tissue transglutaminase, tTG, TGase 2, TG2) is encoded by TGM2 gene, which is located on human chromosome 20q11–12, spans 37 kb and consists of 13 exons [98]. The expression of the gene results in a product 686 amino acid long (78 kDa) [120]. TG2 is ubiquitously expressed [106], located predominantly in cytosol and to a lesser extent in the nucleus and extracellularly [120]. It is implicated in diverse mechanisms, including but not limited to cellular differentiation, adhesion, apoptosis, and matrix assembly [93, 95, 96, 106, 121, 122]. In addition to its classical transglutaminase activities, TG2 has been shown to modulate cell-matrix interactions [123-125] and performs protein kinase [126] and protein disulfide isomerase activities [127]. Surprisingly, despite these diverse roles, TG2 knockout mice generated by different groups are viable and without apparent physical abnormalities [109, 128]. Like TG1

knockout mice, this is attributed to the compensation by other members of the family [110].

An ever-growing body of evidence points out that members of this family, TG1 and TG2 in particular, might be involved in the cross-linking of Tau and therefore the formation of neurofibrillary tangles. Following the demonstration of likely transglutaminase involvement in the cross-linking of brain proteins and formation of insoluble polymers in Alzheimer's disease (AD) [129], TG2 is shown to be able to cross-link both phosphorylated and unphosphorylated Tau forms in an acellular environment [12, 13]. Additionally, the overall TG activity, as well as TG1 and TG2 expression are increased in Alzheimer's disease [107, 113] and both TG1 [15] and TG2 [14, 130] are colocalized in NFTs of Alzheimer's brain, indicating that both TG1 and TG2 are likely to be involved in the formation of NFTs. Lastly, a recent immunological study which was done in the human brain sections of PiD, PSP, and FTDP-17T has shown the colocalized expression of TG1 as well as its activator, tazarotene-induced gene 3 in NTFs, while TG2 had very little or no expression [15]. This data highlights a potential role for TG1 in the development of NFTs and provides a potential mechanistic cue from a known activation of TG1, by TIG3 in neurons and tauopathies.

TAZAROTENE-INDUCED GENE 3

Tazarotene-induced gene 3 (TIG3; retinoid-inducible gene 1, RIG1; retinoic acid receptor responder 3, RARRES3) is a member of the NlpC/P60 superfamily [131, 132], an H-rev family and classified as a class II tumor suppressor gene; located on chromosome 11q12.3, spanning ~9.5 kb and consisting of 4 exons. The expression of the gene results in a product 164 amino acid long (18 kDa) [16, 133] that consists of 2 main domains; the

hydrophilic N-terminal region (amino acids 1 to 134) and the hydrophobic C-terminal domain (amino acids 135 to 164) (Figure 2).

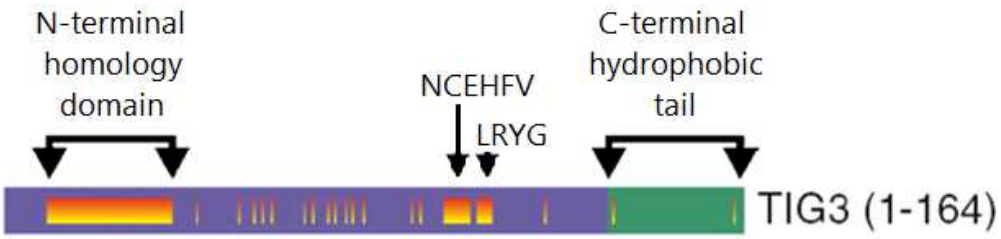

Figure 2: Structure of TIG3.

The protein is composed of 164 amino acids, with purple region representing a 134 amino acid long N-terminal domain, and green region representing its 30 amino acid long hydrophobic, membrane anchorage domain. Areas depicted in orange represent conserved regions and are present in both hydrophilic and hydrophobic domains. The notable conserved regions are the NCEHFV and LRYG motifs of N-terminal domain, which are shared within the lecithin retinol acyltransferase (LRAT) subfamily of the NlpC/P60 super family [131, 134-137] (*adopted from Jans et. al., 2008* [138]).

Following its discovery in psoriatic keratinocytes treated with the synthetic retinoid drug tazarotene [16], TIG3 has been identified in other cells and tissues as well, including liver, heart, brain, lung, skeletal muscle and kidney [18, 139-142]. Today, the function of TIG3 is best-established in epithelial cells. It is shown to induce differentiation through modulating the TG1 activity [23, 24], which is a key enzyme in cornified layer formation [90, 116]. Further studies pointed out that TIG3 was modulating TG1 activity at a posttranslational level, by functioning as its substrate [24]. Additionally, it has also been shown to cause cessation of the cell cycle progression at G1/S phase and apoptosis induction in skin cancer cell lines and normal keratinocytes. The protein is found to carry out these functions by associating with and altering the function of centrosomes, where it

reorganizes microtubule network, as well as reduces cell division and proliferation [20, 143]. In several hyperproliferative diseases and skin cancers TIG3 expression is found to be reduced, and reconstitution of its levels resulted in decreased cell proliferation [16], and/or tumor size reduction [19, 144]. Together, these results suggests that TIG3 and its interaction with TG1 might be important in complete – terminal differentiation of skin cells, and loss of TIG3 could be necessary in the formation of these diseases and carcinogenesis [16, 19, 22, 138, 145, 146].

Evidence suggests that transglutaminases and TG1 and TG2 in particular, might be involved in the formation of neurofibrillary tangles, which is a major hallmark of tauopathies. Alongside with the observation of likely transglutaminase involvement in the cross-linking of brain proteins and formation of insoluble polymers in Alzheimer's disease [129], TG2's ability to cross-link phosphorylated and unphosphorylated forms of Tau in an acellular environment [12, 13], both TG1 [15] and TG2 [14, 130] are shown to be colocalized in the NFTs of AD. Moreover, the overall TGase activity, and TG1 and TG2 expression are found to be increased in Alzheimer's disease [107, 113], indicating that both TG1 and TG2 are likely to be involved in NTF formation. A recent study where human brain sections of PiD, PSP, and FTDP-17T were analyzed by immunological methods demonstrated strong colocalization of TG1 in NFTs, while TG2 showed very little or no immunoreactivity [15]. These results suggest a potential involvement of TG1 in the formation NFTs. Additionally, the same study showed colocalized expression of TIG3 and TG1 in NFTs, attributing a functional relevancy to TIG3 similar to the one observed in the cells of epithelial background [15]. Therefore, we hypothesize that TIG3 regulates TG1-catalysed cross-linking of protein Tau, to form Tau aggregates in neurons.

To test this hypothesis, neurons differentiated from human embryonic stem cells were used as an *in vitro* tauopathy model to study the potential contribution of TIG3 and TG1 in forming Tau aggregates. For this purpose, endogenous expression analysis of TIG3, TG1 and Tau was performed and induction of Tau aggregation via overexpression of TIG3 was also examined. The results of this study might provide new mechanistic insights into the formation of neurofibrillary tangles and the resulting pathology.

HYPOTHESIS

We hypothesized that the TIG3, TG1 and NFT colocalization that was shown by Wilhelmus *et. al*. [15] points out a functional relevancy, similar to the role of TIG3 and TG1 in epidermal keratinocytes. In tauopathies, we believe that TIG3 is the inducer of TG1 mediated cross-linking of the protein Tau. Studies herein have tested this hypothesis while future studies are needed to address how TIG3 performs this function. To date, no study other than Wilhelmus *et. al.* [15] has described the presence of TIG3 in neurons. Therefore, this is the first confirmative report to this study using multiple approaches to prove TIG3 expression is found in neurons and that TIG3 and TG1 are present when Tau aggregation occurs.

SIGNIFICANCE

Tauopathies are progressive neurodegenerative diseases, without any available treatment options that can cure the disease. The results from this study might provide new mechanistic insights into the events leading to pathological conditions, as it addresses the key hallmark of the diseases. Additionally, it tries to elucidate the presence of a known pathway in keratinocytes, TIG3-TG1, in a new system such as neurons.

CHAPTER II: MATERIALS AND METHODS

Reagents And Antibodies

Reagents DMEM (11965-092), DMEM-F12 (11330-057), neurobasal media (21103-049) 0.25% trypsin-EDTA (25200-056), GlutaMAX (35050), N-2 supplement (17502-048), non-essential amino acids (NEAA, 11140-050) sodium pyruvate (11360-070), collagenase (type IV, 17104-019) and knock serum (KO, 10828-028) were purchased from Gibco (Grand Island, NY). Fetal bovine serum (FBS, SH300703) was obtained from Hyclone (Thermo Scientific, Logan, UT, USA). Albumin from bovine serum (BSA; A4503), gentamycin (G1397), okadaic acid (OA, O8010), polybrene (H9268), TritonX-100 (T8787) and dimethyl sulfoxide (DMSO, D2650) were obtained from Sigma (St. Louis, MO). Fibroblast growth factor (FGF2, 233-FB), brain derived neurotrophic factor (BDNF, 248-BD), sonic hedgehog (SHH, 1845-SH) and neurotrophin-3 (NT3, 267-N3) were obtained from R&D Systems (Minneapolis, MN). Mitomycin C (10107409001) was from Roche (Indianapolis, IN). "Cell lysis buffer" (#9803) and "protease inhibitor cocktail" (#5871) were purchased from Cell Signaling (Danvers, MA). Matrigel (354277) was obtained from BD Biosciences (Bedford, MA).

Antibodies Monoclonal anti-mouse β-Actin IgG1 (A5441; Sigma; St. Louis, MO) at 1:5000, monoclonal mouse anti-human TG1 IgG2a (sc-166467; Santa Cruz Biotechnology; Santa Cruz, CA) at 1:1000, monoclonal mouse anti-bovine Tau IgG1 (ab80579; Abcam; Cambridge, MA) at 1:1000 and monoclonal mouse anti-human anti-Phospho-PHF-tau pSer202/Thr205 IgG1 (Tau-*P*, MN1020; Thermo; Rockford, IL) antibody is used at 1:1000 dilution. Rabbit anti-TIG3 serum was produced in our laboratory and used at a 1:1000 dilution. Horseradish peroxidase-conjugated secondary

antibodies, sheep anti-mouse IgG (NXA931) and donkey anti-rabbit IgG (NA934) were obtained from GE Healthcare (Buckinghamshire, UK) and used at a 1:5000 dilution. Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200), Alexa Fluor 594-conjugated goat anti-mouse IgGs (1:200), Alexa Fluor 594-conjugated donkey anti-goat IgGs (1:200) and Alex Flour 488-conjugated goat anti-mouse IgM. All Alexa Fluor conjugated secondary antibodies were purchased from Molecular Probes (Eugene, OR).

Cell Culture

CF-1 mouse embryonic fibroblasts (MEFs) were obtained from GlobalStem (GSC-6001, Rockville, MD) and maintained in DMEM supplemented with 15% FBS, 2mM GlutaMAX, 1mM sodium pyruvate and 1% NEAA. H9/WA09 human embryonic stem cells (hESCs) were purchased from WiCell (Madison, WI) and were grown in DMEM-F12 (Invitrogen) supplemented with 20% Knockout Serum Replacement, 2mM GlutaMAX, 1% NEAA and 8 ng/ml FGF2 (complete "hESC culture medium" formulation) and cultured on MEF feeder cells that are treated with mitomycin C. The culture medium was changed daily and the cells were split to 1:5 every 5 to 6 days using 1 mg/ml collagenase.

For neuronal induction, hESCs were scraped, transferred and maintained in ultra-low attachment plates (3471, Corning Incorporated., Lowell, MA) and in the hESC culture medium for 10 - 15 days, to obtain embryoid bodies (EBs). Half of the culture replaced with fresh media every 3-4 days. EBs were then transferred into matrigel coated 100 mm cell culture dishes and maintained in the Neurobasal culture medium that was supplemented with 0.5% N-2 supplement, 500 µg/ml BSA, 25 µg/ml gentamycin and 20 ng/ml FGF2 (complete "NT2 culture medium" formulation). These EBs were attached

and started to form individual colonies within 3 days, and formed neural tube-like rosettes approximately within 12 days. These cells are considered as neuronal progenitors and kept under these conditions until neuronal induction. The culture medium was changed every other day and subcloning was carried out to new matrigel coated plates by scraping. In order to induce further neuronal differentiation and to obtain mature neurons, neuronal progenitors were then cultured on matrigel coated plates and in NT2 media supplemented with 1 µM retinoic acid, 50 ng/ml sonic hedgehog (SHH), 10 ng/ml neurotrophin-3 (NT3) and 10 ng/ml brain-derived neurotrophic factor (BDNF) (complete "neuronal differentiation media" formulation) for two to three weeks. The culture medium was changed every third day and cells were subcultured via scraping. Successful differentiations to neuronal progenitors and neurons were confirmed by immunocytochemistry, using the neural progenitor marker "nestin" and mature neuron markers "neuronal class III β-tubulin (TUJ1) and neurofilament (NeuF).

Adenovirus Infection

Adenoviruses were prepared as previously described [23]. The tetracycline-inducible virus, tAd5- TIG3 (1–164) carries the full length TIG3 gene as well as an enhancer element that is tetracycline-responsive [23, 24]. The empty tAd5-EV virus which carries the tetracycline-inducible element, but not TIG3, was used as the control virus. This system requires the delivery of a tetracycline transactivator co-transfection, which was carried by Ad5-TA (helper) virus. For infection, the cells were incubated with "neuronal differentiation medium" without any BSA, but carrying 10 multiplicity of infection (MOI) tAd5- TIG3 or tAd5-EV adenoviruses as well as 5 MOI Ad5-TA and 6 µg polybrene/ml for 5 hours. Following the 5-hour incubation, culture medium BSA levels

were reconstituted and incubation carried for an additional 67 hours. After 72 hours, DNA and RNA were collected, and the cells were fixed for qRT-PCR analysis, immunoblotting and immunocytochemistry.

qRT-PCR analysis

Following the manufacturer's instructions, RNA was isolated using illustra RNAspin Mini Isolation Kit (GE Healthcare, 25-0500-71) and cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen, 18080-044) qRT-PCR analysis is carried out using LightCycler 480 SYBR Green I Master (Roche, 04707516001). The primer sequences were as following: TIG3 (5′-CAGTATTGTGAGCAGGAACTGTGA, 5′-TTGGCCTTTTCCACCTGTTTAC), human cyclophilin (5′-CATCTGCACTGCCAAGACTGA, 5′-TTCATGCCTTCTTTCACTTTGC), human TG1 (5′- GCA CAC TCA TTG TCA CAA AGG CCA, 5′- ATA CAC ATT GGG TTT GCT GCC GTG) and human Tau (5′- CTG AAG CAC CAG CCA GGA GG, 5′- GGA TGT TGC CTA ATG AGC CAC).

Immunoblotting

The cellular extracts were prepared in “cell lysis buffer” (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM disodium ethylenediamine tetraacetate, 1 mM ethylene glycol tetraacetic acid, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM sodium vanadate, 1 µg/ml leupeptin) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM dithiothreitol (DTT) and 0.5X of protease inhibitor cocktail, kept on ice and then sonicated for 2 x 5 seconds. Protein concentration is calculated using the Bradford Bio-Rad protein assay (Bio-Rad, 500-0006). From each sample, 30 µg of protein was electrophorated on 12% SDS-PAGE gels

and transferred to nitrocellulose membranes. Membranes are blocked using 5% milk and proteins are detected using appropriate primary antibody overnight and horseradish peroxidase-conjugated secondary antibodies for 1 hour. The visualization of the proteins carried out by using Amersham ECL Prime (RPN2232) from GE Healthcare (Buckinghamshire, UK).

Immunocytochemistry

For immunocytochemistry analysis, cells are grown on matrigel coated 24-well cell culture plates, fixed in 4% paraformaldehyde for 10 minutes and permeabilized using 0.2% TritonX-100. The cells are blocked using 15% FBS, incubated with desired primary antibodies overnight and appropriate Alexa Fluor 488-conjugated secondary antibodies for 1 hour and then incubated with DAPI (1:2000) for 10 minutes. Visualization is carried out using Olympus IX81 spinning disk confocal microscope and MetaMorph image analysis software.

CHAPTER III: IN VITRO TAUOPATHY MODELING USING HUMAN EMBRYONIC STEM CELL-DERIVED NEURONS

BACKGROUND

NFTs are an important hallmark of tauopathies and consist of hyperphosphorylated Tau protein. It is known that abnormal Tau phosphorylation precedes the formation of these structures [67, 68]. Similarly, mutant Tau studies indicate that Tau phosphorylation can induce conformational changes, and promote self-aggregation [69-72], underlining the importance of these modifications. Levels of Tau phosphorylation is controlled by various kinases and phosphatases [30, 73, 74], whereby increases in kinases or downregulation in phosphatases results in its dysregulation. Protein phosphatase-2A (PP2A) is a phosphatase that is responsible for higher than 70% of cellular phosphatase activity in cells [79], and is hypothesized to carry out the majority of Tau dephosphorylation activity in neurons [80-83]. Specifically, in the brains of AD, PP2A activity was demonstrated to be downregulated by 50% [79, 80, 84]. Additionally, it is shown that PP2A treatment of NFTs leads to the dephosphorylation of Tau, as well as restoring its ability to bind tubulin monomers [147].

Okadaic acid (OA), a specific inhibitor of PP2A is widely used by many groups for *in vivo* and *in vitro* tauopathy modeling. The agent is shown to be able to induce neurodegeneration through Tau hyperphosphorylation, GSK3β activation, oxidative stress, and cause neurotoxicity and cognitive deficiency; all are the major characteristics of tauopathies [148, 149]. In this section, we aim to utilize this agent to investigate the interplay between TIG3, TG1 and Tau.

In order to accomplish this, first, we differentiated neurons from human embryonic stem cells (ESCs). Today, most studies utilize SH-SY5Y neuroblastoma cell line to study tauopathies *in vitro*. However, the use of this cell line to study a condition of non-carcinogenicity such as tauopathies might not be desirable due to their tumorigenic properties and abnormal karyotype. Alternative and possibly more relative models are the neurons derived from hESCs. These neurons are shown to be able to recapitulate Tau expression profile and physiology that is seen in a healthy adult brain [150], as well as its pathology-associated cellular alterations [151]. Additionally, this approach also allows researchers to grow and expand neurons, easily and reproducibly. Therefore, we aimed to take advantage of this approach to study the effects of OA treatment, a widely used approach for tauopathy modeling in otherwise normal cells. Following their production, these cells treated with OA and an interaction between TIG3, TG1 and Tau is evaluated. Changes in RNA and protein expression of these molecules were studied by qRT-PCR, immunoblotting and immunofluorescence.

HYPOTHESIS

We hypothesized that OA treated hESCs-derived neurons would be an appropriate model for okadaic acid-induced tauopathy and OA treatment would result in an increased Tau aggregation as well as TIG3, TG1 and Tau colocalization.

SPECIFIC AIMS

Thus, the specific aim of this study was to determine whether hESC-derived neurons provide a suitable model to study tauopathies. The second aim was to determine whether OA induced tauopathy leads to the increased colocalization of TIG3, TG1, and Tau.

RESULTS

Due to the limited availability and growth potential of primary neurons, as well as undesirable properties of cancerous cell lines, we aimed to generate embryonic stem cell derived neurons. These cells have been shown to be capable of recapitulating physiological and pathological characteristics associated with Tau [150, 151], and can be grown easily and reproducibly. In order to accomplish this, we used a chemical induction method to differentiate the hESC cell line, H9 (Figure 3A). First, embryoid bodies (EB) (Figure 3B) were generated under the conditions that are defined in Chapter 2. After several weeks, EBs were plated onto tissue culture plates in defined media to obtain neuronal progenitors (NP) (Figure 3C). Figure 3C left panel shows structures called "neural rosettes", which are the developmental signatures of neuronal progenitor cells in cell culture conditions [152]. Figure 3C middle and right panels show cells that are further differentiated and their neurites. In addition to morphological confirmations, immunocytochemistry analysis against Nestin, a marker of early neuronal differentiation was carried out. Results have shown strong Nestin expression in all cells (Figure 3D), indicating successful differentiation. Further differentiation up to 2-3 weeks resulted with elongated axonal-like structures as well as a more organized cellular network representative of mature neurons (Figure 3E). Therefore, these cells are considered as mature neurons and success of this differentiation is confirmed via immunocytochemistry analysis against markers of mature neurons; class III β-tubulin (TUJ1) and neurofilament (NeuF). Although the majority of the cells were TUJ1 positive, cells with neuronal morphology exhibited stronger signals (Figure 3F, left panels); an indication of better or complete differentiation. Similarly, the majority of the cells were NeuF positive,

however, with a more uniform staining (Figure 3F, right panels). Together, these indicate the successful generation of mature neurons. Although functional analyses were out of the scope of this project, others have shown that cells derived under similar conditions and expresses same markers are capable of promoting action potentials via patch clamp experiments [153-155].

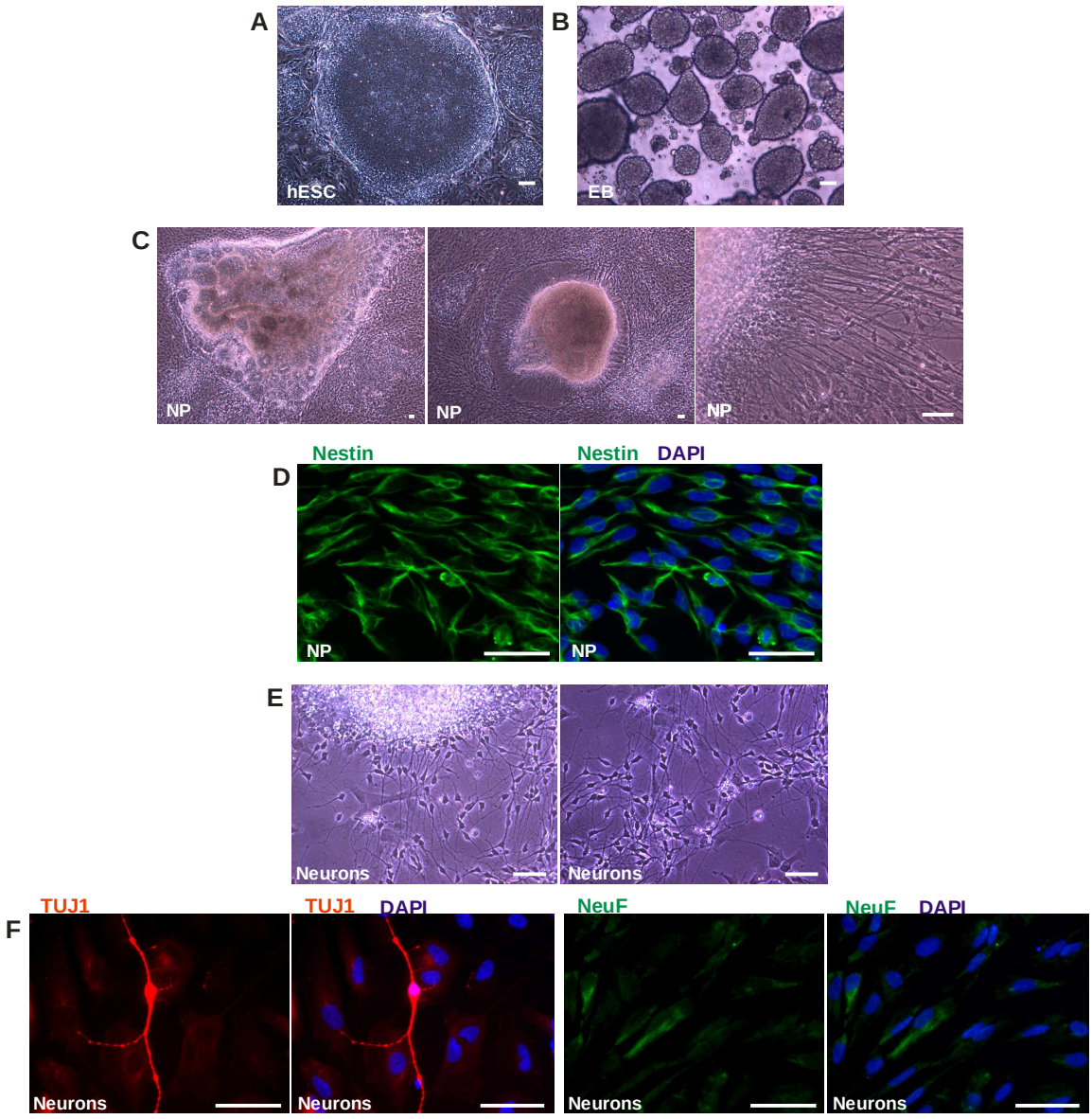

Figure 3: Generation of hESC-derived neurons.

The morphological and immunocytochemical characterization of neurons derived from hESCs. Undifferentiated hESCs (**A**) were cultured in ultra-low attachment plates for 10 - 15 days, to obtain embryoid bodies (EBs) (**B**). These EBs were cultured on matrigel coated plates, and in "NT2 culture medium" for ~12 days to obtain neuronal progenitor (NP) cells (**C**). Differentiation to NPs tested via immunocytochemistry, using a marker for neuronal progenitors, Nestin (**D**). These NPs were then cultured in "neuronal differentiation media" for further differentiation (**E**). Analysis of successful neuronal differentiation carried out using markers of mature neurons; class III β-tubulin (TUJ1) and neurofilament (NeuF) (**F**), and via immunocytochemistry. A, B, C and E. Bar = 10 µm, D and F. Bar = 5 µm.

Next, we wanted to test whether exposure to OA would alter the expression and/or interaction of TIG3, TG1 and Tau in these hESC-derived neurons. 5 nM OA concentration was selected for further analyses due to its low toxicity and optimal effect size over TIG3, TG1 and Tau proteins (data not shown). qRT-PCR analyses of hESC-derived neurons that are differentiated for 18 days has shown that mRNA expressions of all three genes were increased in neurons after 24 hours of exposure to 5nM OA (Figure 4). Similar results are observed in all three independent cell culture experiments.

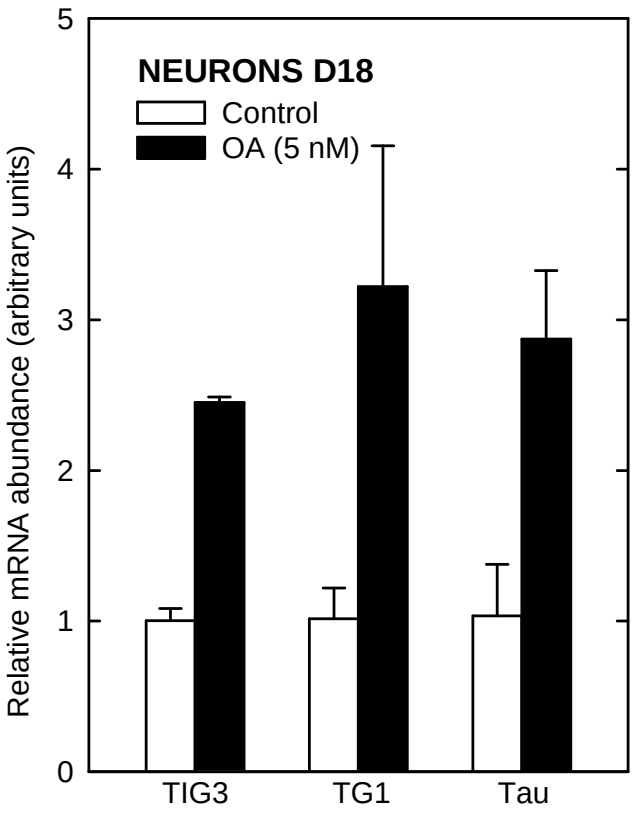

Figure 4: OA induces the transcription of Tig3, Tg1 and Tau.

Neurons differentiated from hESCs in neuronal differentiation media for 17 days were treated with culture medium containing 5 nM OA or equal amount of its carrier DMSO, for 24 hours. RNA was collected and qRT-PCR was carried out. The values are mean ± SD for 3 wells per group. Similar results were observed in each of three experiments.

Finally, we wanted to see the effect of OA treatment over TIG3, TG1 and Tau protein levels as well as localization. For this purpose, immunocytochemistry analyses of these proteins carried out within the same group of hESC-derived neuronal differentiation day 18 cells. Consistent with mRNA expression, immunocytochemistry showed increased protein expression in neurons following the 24-hours long 5 nM OA treatment (Figure 5). Cells had shown increased punctuated staining for TIG3 (Figure 5A, arrows), while both TG1 and Tau showed more diffused but stronger cytoplasmic staining (Figure 5A and B). Figure 5C shows the control staining with secondary antibodies only, and had very little or no staining. In addition to the increase in protein staining of TIG3, Tau and TG1, co localization within the cells could be seen. TIG3 and Tau (Figure 5A, arrows) as well as TG1 and Tau (Figure 5B, arrowheads) had high colocalization.

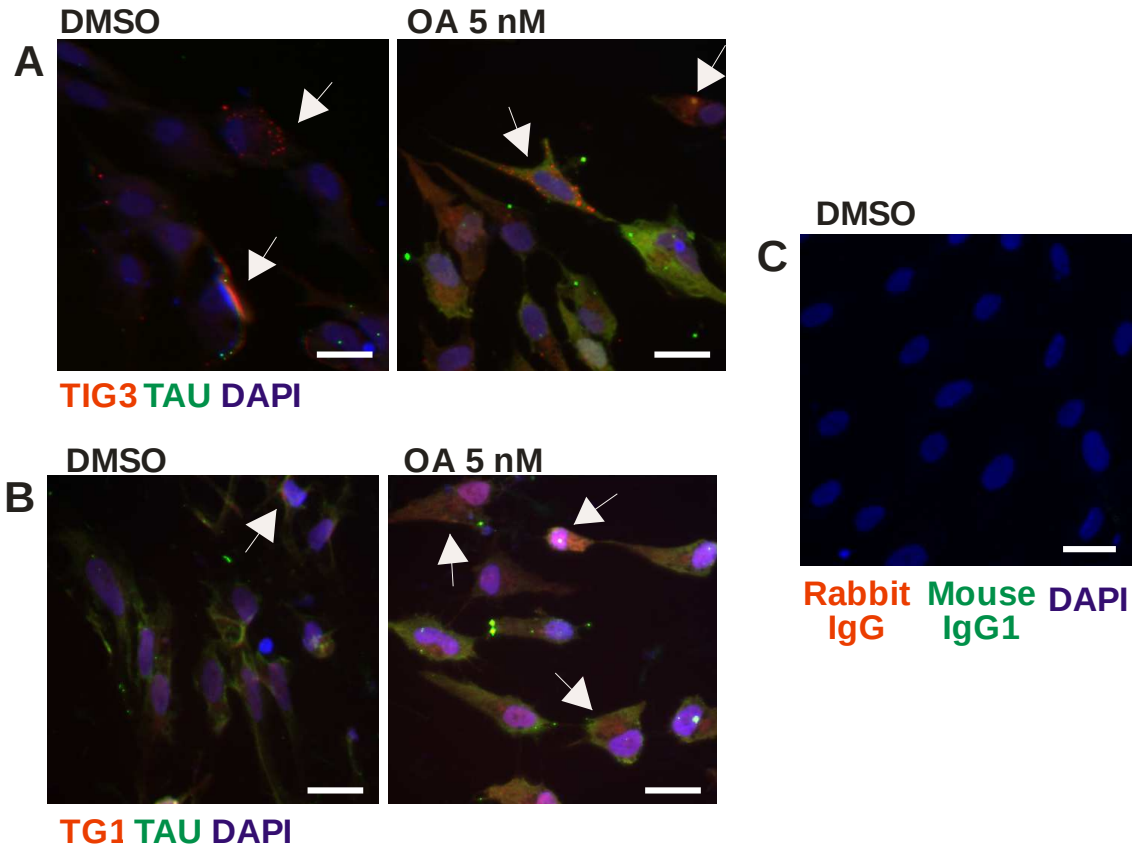

Figure 5: Endogenous TIG3, TG1 and TAU show colocalization, and OA treatment induces their expression.

Neurons differentiated from hESCs in neuronal differentiation media for 17 days were treated with culture medium containing 5 nM OA or DMSO for 24 hours. The cells were then fixed at day 18 and stained with anti-TIG3, anti-TG1 or anti-Tau, and DAPI. Arrows show areas of colocalized staining of markers. Bar = 5 µm.

CONCLUSION AND DISCUSSION

The data presented here suggested that hESC-derived neurons are a suitable cell line to study tauopathies and that TIG3 and TG1 may be key players in their underlining pathology. When the hESC-derived neurons were exposed to OA, an inducer of tauopathies *in vitro*, expressions of TIG3, TG1 and Tau mRNA and protein levels were increased, as well as their colocalization in the cytoplasm. Exposure to OA also appeared to generate more Tau aggregates with TIG3 and TG1. However, more thorough quantitation or counting of these aggregates will be required to confirm this difference. Thus, the data as presented is consistent with previous publications defining the OA as a suitable inducer of the primary trait of tauopathy, i.e. NFT formation increases along with phosphorylated Tau [81, 148, 149]. This data also showed TIG3 expression in a tauopathy model, consistent with the previous publication on human pathology samples [15]. In fact, this is the first study showing both mRNA as well as protein expression of Tig3 in human neuronal cells. TIG3 mRNA and protein expression was also confirmed in a neuroblastoma cell line SH-SY5Y and in human fetal brain tissue (data not shown).

CHAPTER IV: EFFECTS OF TIG3 OVER-EXPRESSION IN hESC-DERIVED NEURONS

BACKGROUND

Following its discovery in psoriatic keratinocytes treated with the synthetic retinoid drug tazarotene [16], TIG3 has been identified in other cells and tissues as well, including liver, heart, brain, lung, skeletal muscle and kidney [18, 139-142]. Today, the function of TIG3 is best-established in epithelial cells. It is shown to induce differentiation through the modulation of TG1 activity [23, 24], which is a key enzyme in cornified envelope formation in skin [90, 116]. Further studies pointed out that TIG3 was modulating TG1 activity at a posttranslational level, by functioning as its substrate [24]. Additionally, it has also been shown to cause cessation of the cell cycle progression at G1/S phase and apoptosis induction in skin cancer cell lines and normal keratinocytes.

Evidence suggests that transglutaminases and TG1 and TG2 in particular, might be involved in the formation of neurofibrillary tangles, which is a major hallmark of tauopathies. Alongside with the observation of likely TGase involvement in the cross-linking of brain proteins and formation of insoluble polymers in Alzheimer's disease [129] as well as TG2's ability to cross-link phosphorylated and unphosphorylated forms of Tau in an acellular environment [12, 13], both TG1 [15] and TG2 [14, 130] were shown to be colocalized in the NFTs of AD. Moreover, the overall TGase activity, and TG1 and TG2 expressions are found to be increased in Alzheimer's disease [107, 113], indicating that both TG1 and TG2 are likely to be involved in NTF formation.

Lastly, a recent immunological study where human brain sections of PiD, PSP, and FTDP-17T were analyzed had shown the colocalization of TG1 as well as its activator tazarotene-induced gene 3 in NTFs, while TG2 had very little or no immunoreactivity [15]. This data highlighted a potential role for TG1 in the development of NFTs and provided a potential mechanistic cue for TG1 activation in neurons and tauopathies. Therefore, we hypothesized that TIG3 modulates TG1-catalysed cross-linking of protein Tau, to form Tau aggregates in neurons. To test this hypothesis, TIG3 overexpressing neurons were produced from hESC-derived neurons and changes in Tau aggregation as well as TIG3-TG1 and TAU interactions following the likely TIG3 mediated TG1 activity induction was evaluated using qRT-PCR, immunoblotting and immunofluorescence.

HYPOTHESIS

We hypothesized that colocalization shown by Wilhelmus *et. al*. [15] points out a functional relevancy similar to the role of TIG3 and TG1 in epidermal keratinocytes [15]. In tauopathies, we believe that TIG3 is the inducer of TG1 mediated cross-linking of the protein Tau. Studies herein tested this hypothesis, while future studies are needed to address how TIG3 performs this function.

SPECIFIC AIMS

The specific aim of this study was to determine whether overexpression of TIG3 in neurons would increase TG1 expression and/or promote Tau phosphorylation and aggregation in hESC-derived neurons.

RESULTS

Overexpression of TIG3 using adenovirus infections showed a dramatic change in neuron morphology (Figure 6). Figure 6 shows 2 representative fields for each treatment. As shown, the majority of the cells with forced TIG3 over expression became flattened and lost their neuronal morphology. Extensive blebbing on the surface (arrows) of tAD5-TIG3 cells compared to tAD5-EV control cells could also be seen, suggesting cell death. This was similar to that seen in keratinocytes where TIG3 is known to induce cell cycle arrest and apoptosis. Transduction experiments were replicated three times and similar morphological changes were observed.

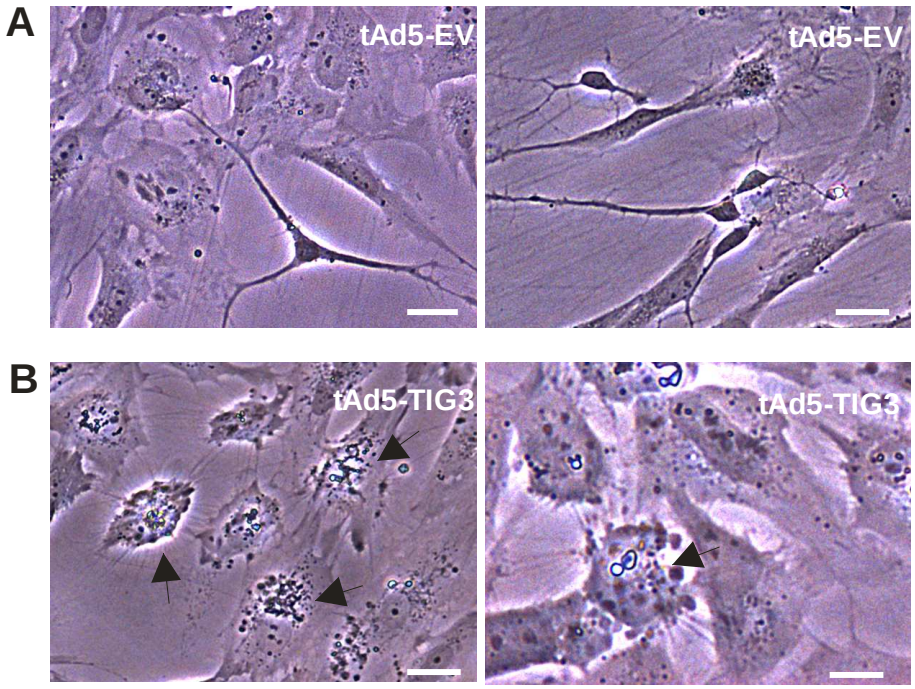

Figure 6: TIG3 overexpression alters neuronal morphology.

hESC-derived neurons that are kept in neuronal differentiation media for 22 days are infected with 10 MOI tAd5-EV (A) or tAd5-TIG3 (1-164) (B) and cultured for an additional 72 hours. Images of phase contrast microscopy were taken on day 25, prior to the sample collection. Arrows indicate the cells with high amount of blebbing, which is likely due to the cellular death. Bar = 5 µm.

Figure 7 confirms increased TIG3 expression in tAD5-TIG3 cells compared to control cells. qRT-PCR and western blot analyses also showed increased expression of TG1. Interestingly, while Tau expression remained unaltered by TIG3 overexpression, phosphorylated-Tau was reduced. The reduction in band intensity in immunoblots was primarily of soluble, non-cross-linked phosphorylated Tau. The reason for this decrease was unknown, but we had speculated that it may have been a result of an increase in the cross-linking and therefore the clearance of phosphorylated-Tau. However, evidence of cross-linked Tau proteins in the upper portions of the gel was not detected. qRT-PCR and immunoblotting analyses were performed on three independent cell culture experiments, showing similar results as those shown in Figure 7.

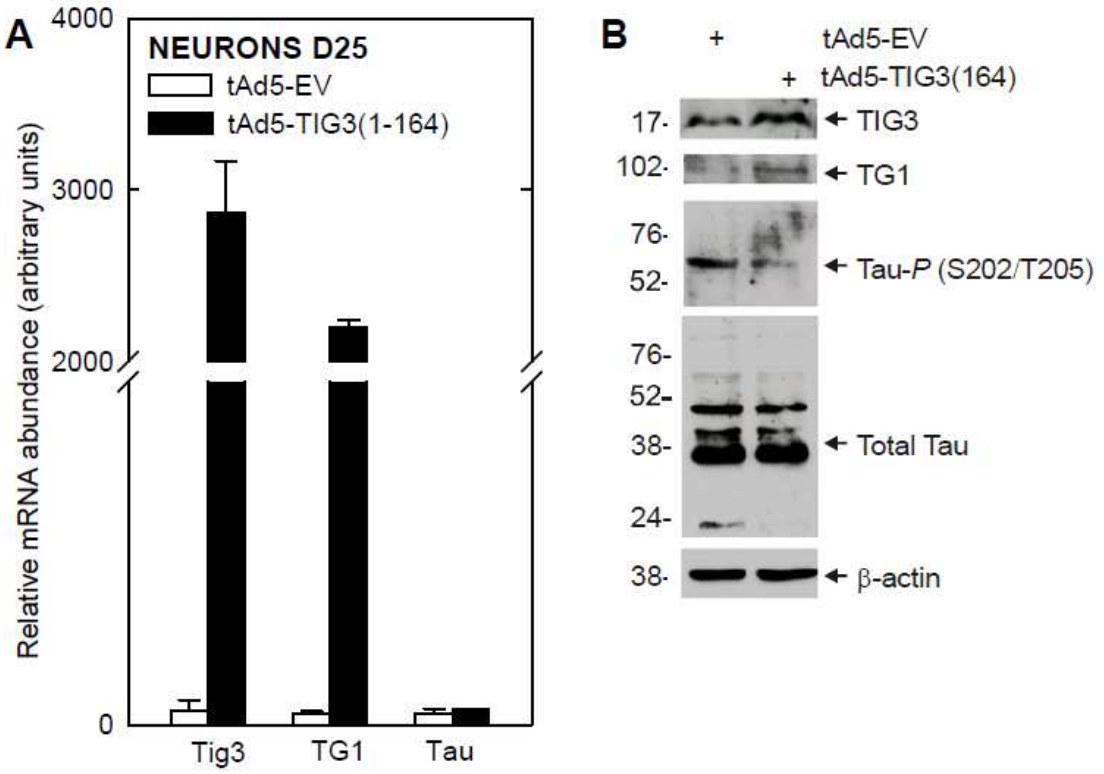

Figure 7: Overexpression of TIG3 impacts the expression of neurofibrillary tangle components.

qRT-PCR and immunoblotting analysis of neurons infected with TIG3 adenovirus. hESC-derived neurons that are kept in neuronal differentiation media for 22 days were infected with 10 MOI empty tAd5-EV or tAd5-TIG3 (1-164) and cultured for an additional 72 hours. qRT-PCR (A) and immunoblotting analyses were then carried out (B). The bars shown in qRT-PCR data represent mean ± SD for 3 wells per group.

Next we wanted to see whether TIG3 was localized with TG1 or Tau proteins in these cells. As controls, cells were stained with secondary antibodies only to determine the background level of staining. These stainings, as shown in Figure 8, were either negative or very low.

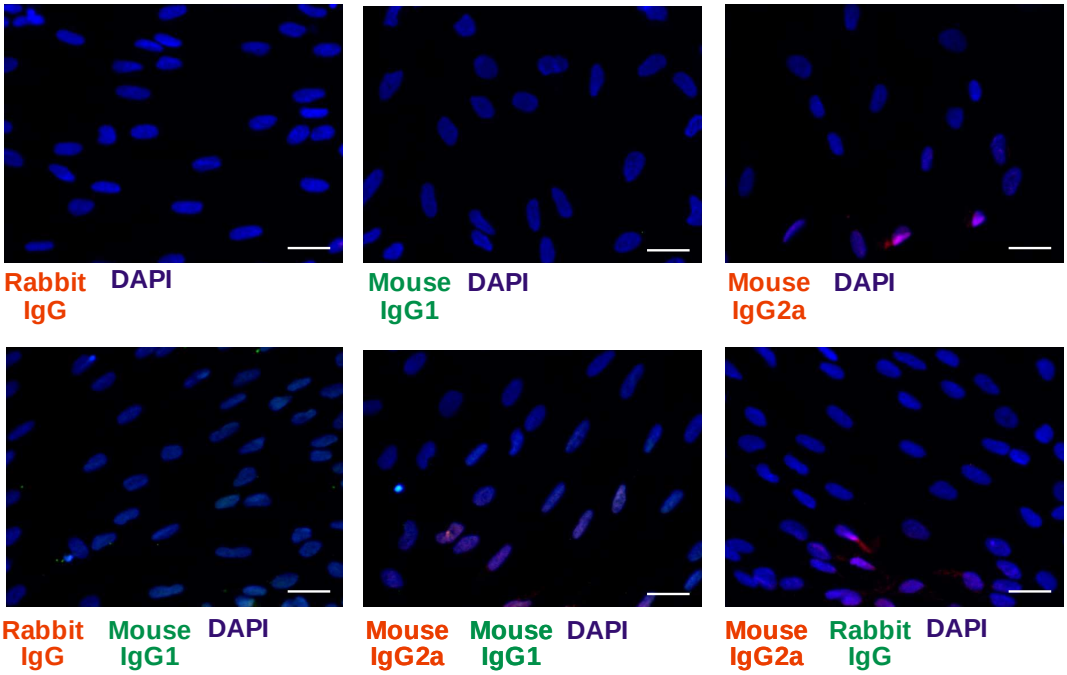

Figure 8: Immunocytochemistry analysis with secondary antibodies show negative staining.

hESC-derived neurons that are kept in neuronal differentiation media for 22 days and then infected with 10 MOI tAd5-EV or tAd5-TIG3 (1-164). These cells were cultured for an additional 72 hours and then fixed on day 25. Following the treatments with shown secondary antibodies, they were used controls for other stainings. Bar = 5 µm.

Next, we tested to confirm TIG3 overexpression. Immunocytochemical staining against TIG3 confirmed the increase in TIG3 expression in tAd5-TIG3 treatment group compared to control empty vector treatment (Figure 9). Additionally, cells overexpressing TIG3 showed mostly perinuclear staining of TIG3, in contrast to the endogenous cytoplasmic staining pattern. Additionally, Figure 9 also shows that, similar to the hESC-derived neurons treated with OA, there was more aggregated TIG3 staining (arrow) in tAd5-TIG3 treatment group.

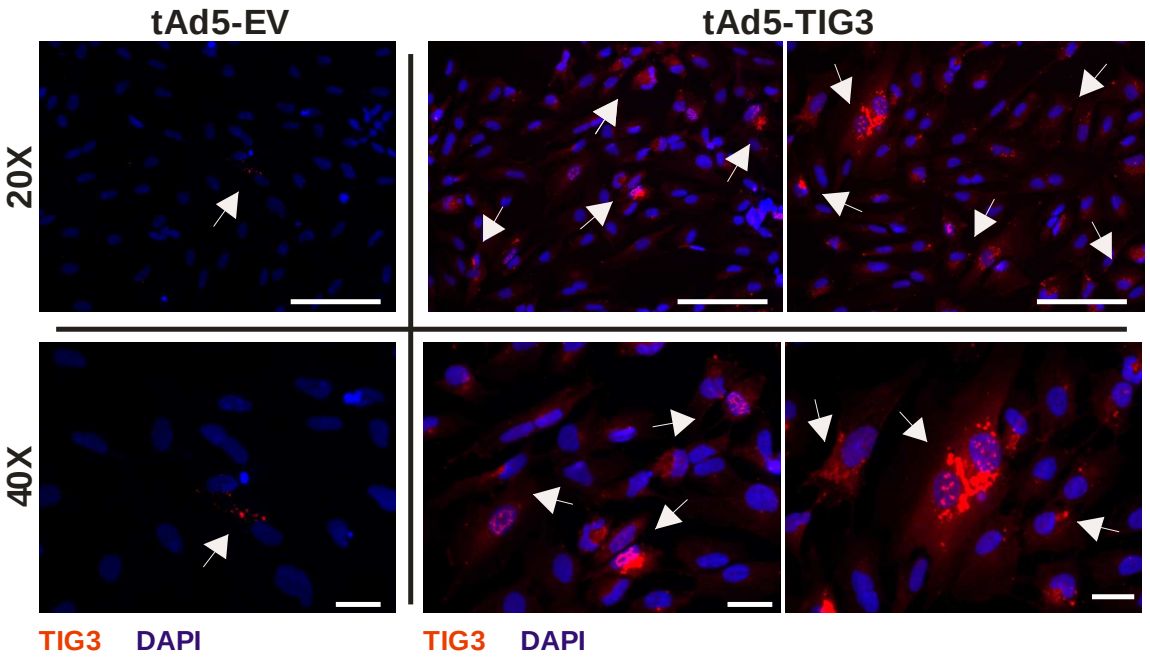

Figure 9: TIG3 overexpression was successful and resulted with accumulated TIG3.

hESC-derived neurons that are kept in neuronal differentiation media for 22 days were infected with 10 MOI tAd5-EV or tAd5-TIG3 (1-164). These cells were then cultured for an additional 72 hours and fixed on day 25. Immunocytochemistry analysis was carried out for shown markers. Arrows show the cells with high TIG3 aggregates. For 20X images Bar = 20 µm while for 40X images Bar = 5 µm.

Like TIG3, TG1 expression was also appeared to be increased in TIG3 overexpressing cells (Figure 10). TG1 and TIG3 colocalization in these cells was also seen. This colocalization was more evident in the TIG3 overexpressing neurons compared to controls. TIG3 staining was also colocalized with Tau (Figure 11), suggesting that both TIG3 and TG1 are present along with Tau. This may occur in Tau aggregates such as those shown by TIG3 and TG1 staining in Figure 11. Although Tau expression did not appear to change in tAD5-TIG3 neurons by western blot, a slight increase could be seen by immunocytochemistry. One possible explanation for this difference may be that if Tau becomes cross-linked and became insoluble, the protein may have been lost during cell

extraction or gel electrophoresis whereas the protein would be readily detectable in cell staining procedure. To answer this question further experimentation with cell extraction procedures would be necessary. Additionally, similar to the observations seen in immunoblottings, Figure 11 shows that phosphorylated-Tau was decreased with TIG3 overexpression. Importantly, the majority of phosphorylated Tau staining appeared to be localized in aggregates which were also stained for TIG3 (Figure 11).

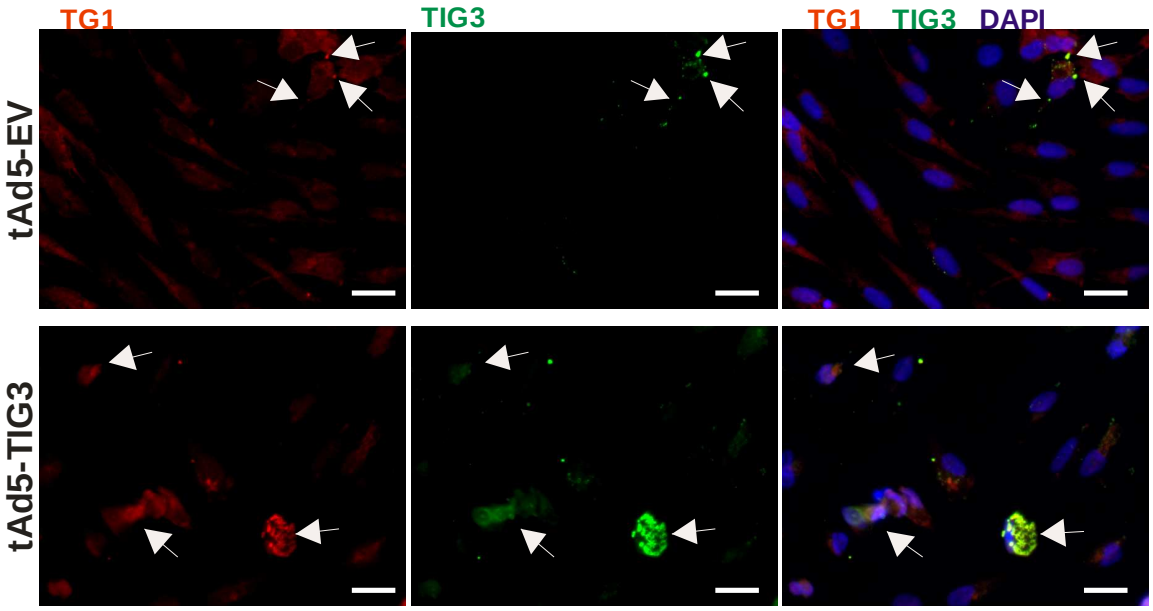

Figure 10: TIG3 colocalizes with TG1.

hESC-derived neurons that are kept in neuronal differentiation media for 22 days were infected with 10 MOI tAd5-EV or tAd5-TIG3 (1-164).These cells were cultured for an additional 72 hours and fixed on day 25. Immunocytochemistry analysis is then carried out for shown markers. Arrows show colocalization of both markers. Bar = 5 µm.

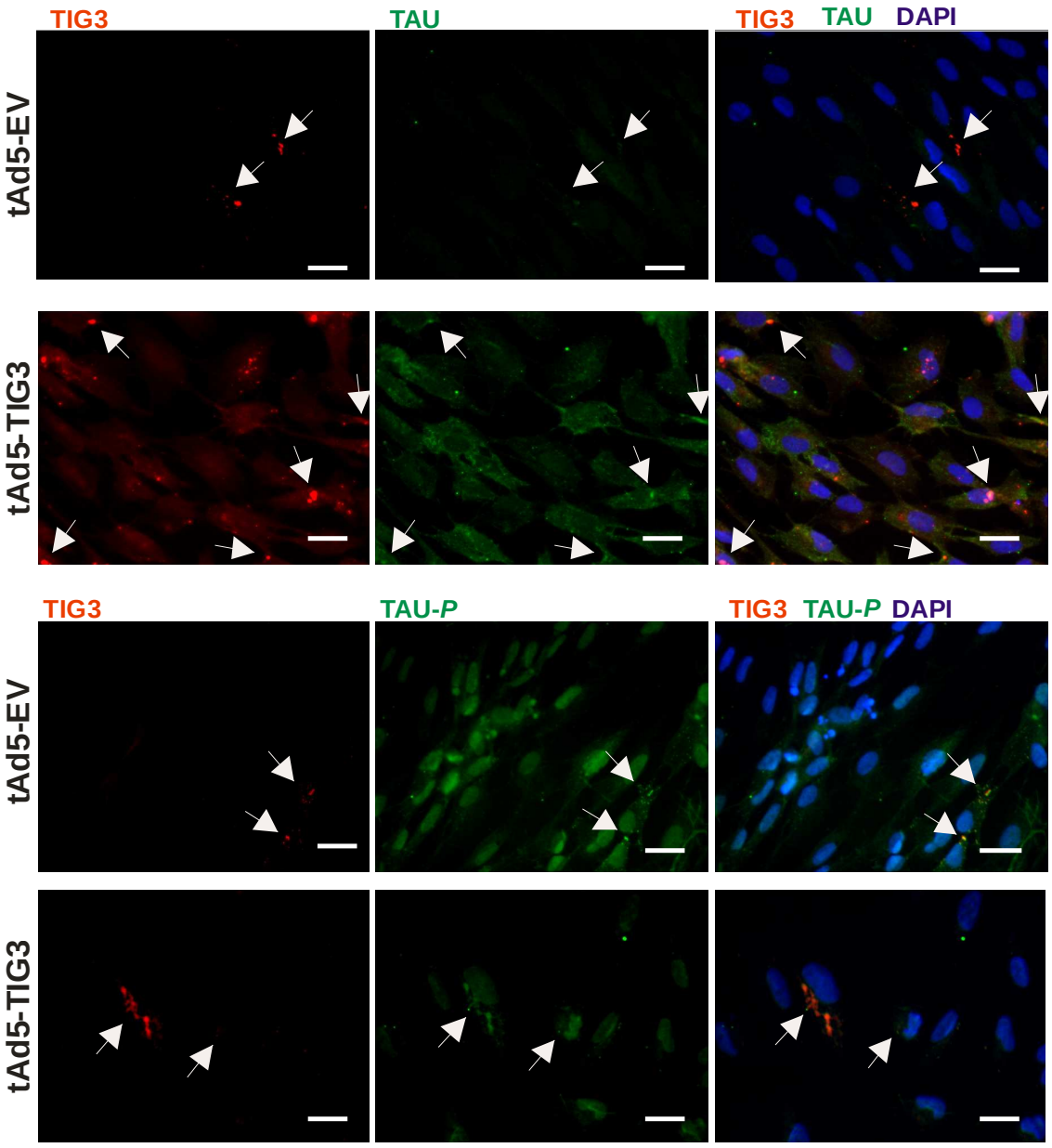

Figure 11: TIG3 colocalizes with native and phosphorylated Tau.

hESC-derived neurons that are kept in neuronal differentiation media for 22 days were infected with 10 MOI tAd5-EV or tAd5-TIG3 (1-164). These cells were cultured for an additional 72 hours and fixed on day 25. Immunocytochemistry analysis is then carried out for shown markers. Arrows show colocalization of both markers. Bar = 5 µm.

Lastly, we wanted to see if TG1 was colocalized in Tau aggregates. Like TIG3, TG1 also appeared to be colocalized with Tau and phospho-Tau staining (Figure 12). The

colocalization was most evident in the punctated staining seen in the cytoplasm of cells. TIG3 overexpressing cells also showed more punctated staining than controls. We believe that these punctates are Tau aggregates which can eventually form into NFTs. However, quantitation of these aggregates is required to confirm this apparent difference between groups. Additionally, immunocytochemistry showed an increase in TG1 and Tau expression in tAD5-TIG3 cells.

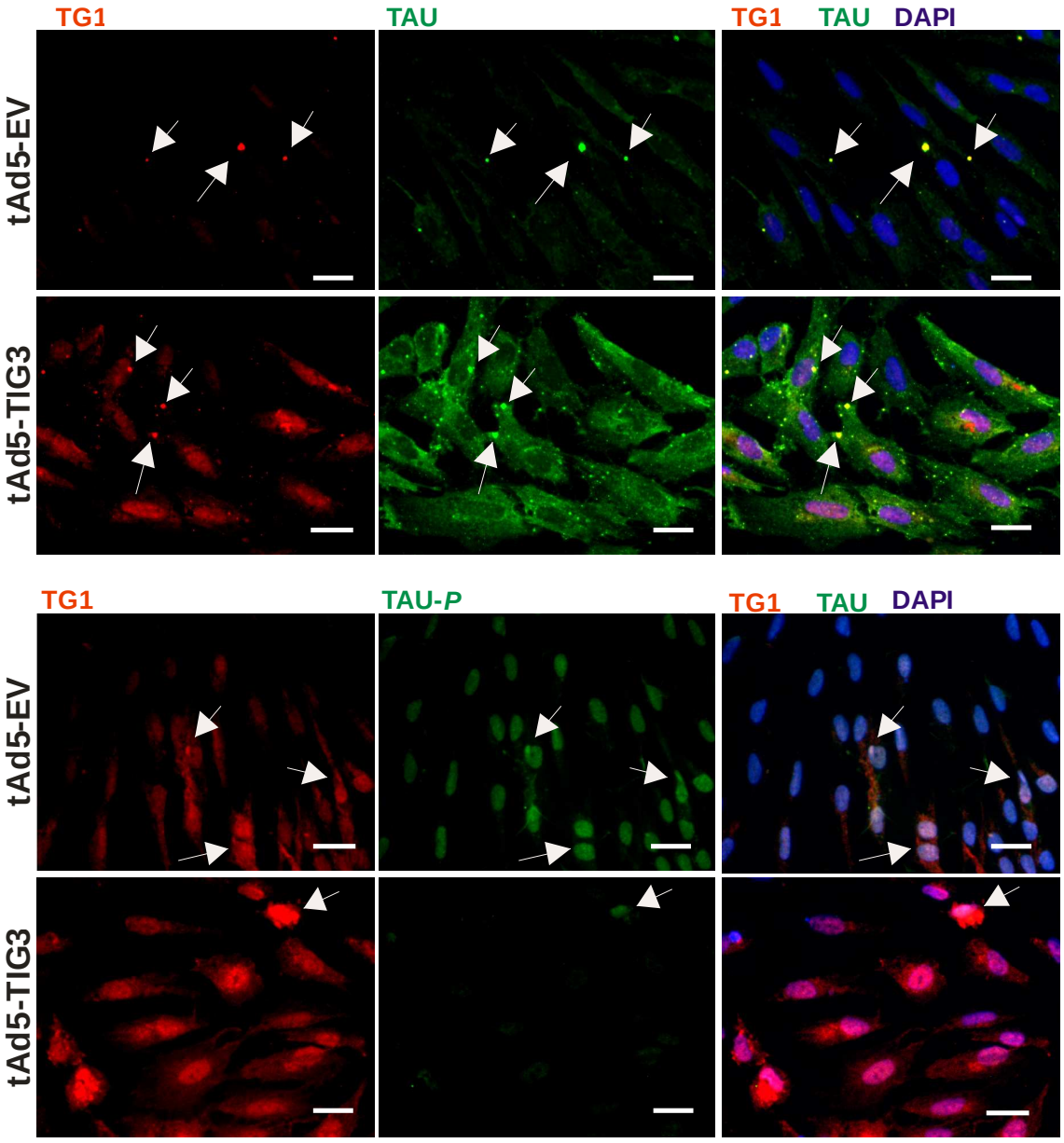

Figure 12: TG1 colocalizes with native and phosphorylated Tau.

hESC-derived neurons that are kept in neuronal differentiation media for 22 days were infected with 10 MOI tAd5-EV or tAd5-TIG3 (1-164). These cells cultured for an additional 72 hours and are fixed on day 25. Immunocytochemistry analysis is then carried out for shown markers. Arrows show colocalization of both markers. Bar = 5 µm.

CONCLUSION AND DISCUSSION

As in the previous chapter, the data presented here suggest that TIG3 is overexpressed in Tau aggregates when it was either induced using OA, like shown in the previous chapter, or forced with adenovirus infection, like in this chapter. Specifically, data presented in this chapter showed that TIG3 overexpression resulted with induction of TG1 expression. TIG3 overexpressing cells also demonstrated increased expression of Tau with immunocytochemistry, though PCR and western blot analyses were unable to detect a change in Tau levels. This discrepancy in results between methods is discussed in the next section entitled Summary and Future Directions. In contrast, phosphorylated Tau decreased with increasing TIG3 expression. Additionally, it has been shown that TIG3, TG1, native TAU and phosphorylated-Tau were colocalized in empty adenovirus treated control cells or full size TIG3-carrying adenoviruses. This colocalization was easier to see for TIG3, TG1 and TAU in TIG3 overexpressing cells because of the increased expression of these proteins as well as the generation of bigger and more aggregates.

CHAPTER V: SUMMARY AND FUTURE DIRECTIONS

In summary, Tau accumulation appears to occur in cells either exposed to OA or overexpressing TIG3. In both cases, it appears that TIG3 and TG1 expression is colocalized along with phosphorylated Tau in the cytoplasm, and in aggregates. It is unclear whether OA increased TIG3, TG1 and Tau expression, which was shown in Figure 4, was due to increased phosphorylated Tau or indirectly through other mechanisms. Indeed, both OA and PP2A are known to affect multiple pathways. From this data, I propose a model for a role of TIG3 in neural tauopathy shown in Figure 13. Here, TIG3 increases TG1 activity which results in Tau cross-linking, a required step for NFT formation. Our data show that TIG3 increases TG1 activity in part by increasing TG1 gene expression levels. TG1 in turn promotes phosphorylated Tau cross-linking, resulting in the loss of soluble phosphorylated Tau. This would explain the loss of soluble protein band that was shown in Figure 7 western blot, as well as the increased Tau staining levels which was shown in immunocytochemistry stainings.

In conclusion, this data reaffirms that neurons express TIG3 and TG1. Presently, only one study has shown the presence of TIG3 in human brain [15]. More importantly, this data suggests that these proteins may serve a vital role in the pathology of tauopathies. It also provides a foundation for future studies in determining how TIG3 plays a role in neuron pathology and suggests that TG1 plays an important role in this process.

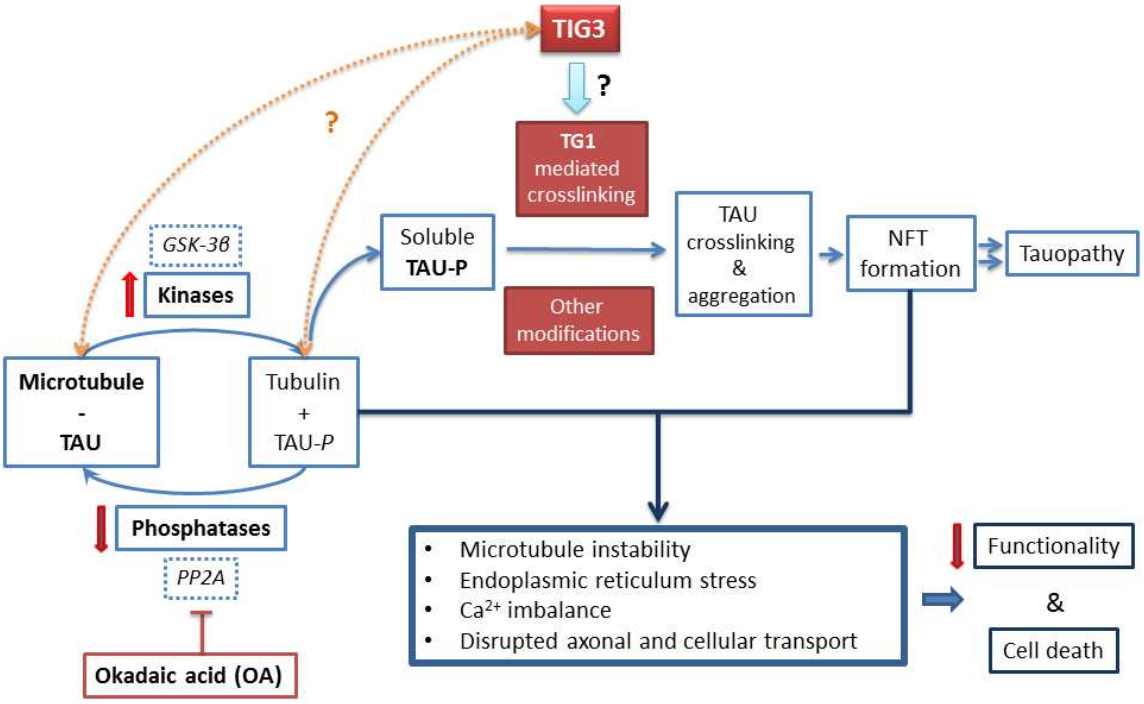

Figure 13: Theoretical model of Tau aggregation process with TIG3 and TG1 involvement.

Based on these results, I would suggest that future studies should be designed to determine how TIG3 and TG1 may regulate the Tau aggregation in neurons using this model system. Specifically, this would involve measuring TG1 activity in response to TIG3 overexpression and determining whether TIG3 modulates TG1-activated Tau aggregation. Our model suggests that TIG3 is involved in NFT formation through activity and abundance of TG1. Although TIG3 significantly increases the TG1 activity in skin, the TG1 has also been shown to function independent from TIG3 as well. For example, the diffused cytoplasmic staining of TG1 as opposed to the aggregated perinuclear staining pattern of TIG3 has been shown in keratinocytes [24, 25, 108, 146] and was also seen in the neuron shown in this study. The differences in their localization patterns

therefore suggest that TG1 may also perform functions independent of its role with TIG3 in neurons as well. Thus, since our data only shows colocalization, it would be essential to show by co-immunoprecipitation that TIG3, TG1 and Tau are, in fact, at the same protein complex. Additionally, it is important to measure the levels of TIG3-dependent TG1 activity in the formation of NFTs. In order to accomplish this, TIG3 knock in as well as knockout hESC-derived neurons would be generated and differences in TG1 activity levels quantitated. While previous techniques could not distinguish between the activity levels of TG proteins such as TG1 and TG2, a new method has been developed that uses “fluorescence-conjugated K5 peptides” [156, 157], which is a substrate that is specifically being targeted by TG1. Combining this information with corresponding changes of Tau aggregation and phosphorylated-Tau abundance levels would provide the TIG3-dependent TG1 activity information in neurons. For example, we would test whether TIG3 knockdown results in reduced TG1 activity along with decreased Tau cross-linking by measuring NFT formation, as well as possibly increased P-Tau abundance.

Next, since it is known that TG2 knockout animals were viable without any apparent defects, and TGase activity shown to be substituted by other TGs [110], it might be important to analyze the dependence of Tau-cross-linking to TG1 activity. In order to accomplish this, the formation of NFTs can be induced via the use of OA and/or Tau mutants in neuronal cells that do not carry TG1. This approach would allow us to see the importance of TG1-activity induced Tau-cross-linking. Together, from the information of TIG3-dependent TG1 activity levels and phosphorylation-Tau cross-linking, the molecular relevancy and importance of this pathway in the progression of these diseases

can be revealed. Following these, follow-up studies can be designed and performed to determine whether these proteins could be used as identification factors to either diagnose tauopathies or as potential treatment options by developing drugs that would inhibit this process.

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