Chromosome missegregation in Alzheimer's disease caused by presenilin 1

Debrah I. Boeras
University of South Florida

Follow this and additional works at: http://scholarcommons.usf.edu/etd

Part of the American Studies Commons

Scholar Commons Citation
http://scholarcommons.usf.edu/etd/2786
Chromosome Missegregation in Alzheimer’s Disease Caused by Presenilin 1

by

Debrah I. Boeras

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
Department of Biochemistry and Molecular Biology
College of Arts and Sciences
University of South Florida

Major Professor: Huntington Potter, Ph.D.
R. Ken Keller, Ph.D.
David Morgan, Ph.D.
Larry P. Solomonson, Ph.D.
Maxine Sutcliffe, Ph.D.

Date of Approval:
November 4, 2005

Keywords: aneuploidy, cell cycle, mitosis, in situ hybridization, Down Syndrome

© Copyright 2006, Debrah I. Boeras
Acknowledgments

I would like to thank Dr. Huntington Potter for his guidance and direction, and for providing me with an excellent laboratory in which to work. A special thanks to Dr. David Morgan, Dr. Maxine Sutcliffe, Dr. Larry Solomonson, and Dr. Ken Keller for their invaluable advice and support and keeping me on track. I could not have chosen a more excellent dissertation committee.

Thank you to my great friend and collaborator, Antoneta Granic, for all her hard work and expertise, and providing me with much needed moments of hope and sanity - sometimes to be found at the bottom of a good cup of coffee. Thanks to Dr. Jaya Padmanabhan for being an endless resource of information and a good friend. Her beautiful inner strength fueled me both intellectually and emotionally. Thanks to Dr. Inge Wefes for all her generous time and effort.

Thanks to my many friends and colleagues, Tanya Butler, David Chappell, Don Cameron, Melinda Miller Horne, Ainsley Davis, Friduca, and Steve Bennett, for making the life of a scientist not only normal but also fun.

Thanks to my dear friend, Elsie Gross, for allowing me to see the beauty and diversity within science and for opening the world for me. I am grateful for all the exotic places we have been and will go to. She’s right, one day when we’re old and gray we’ll still have those memories. Thanks to Susan and Eric for the
opportunity of a lifetime - the icing on my cake, for their extended patience, and their faith in me. Thanks to my Mom and Dad for their love and for supporting me in every decision that I have made in my life.

Eternal thanks to my best friend Joelle, for being there when truly needed in times of joy and despair, and everything that fell between. Thanks for all her patience and advice, agreeing with me even in my most lunatic moments, catching all my tears, and in the end, pushing me right back up again.

Thanks finally to Angie and Edu, without whom none of this would have been possible. I cannot even find words that express my gratitude and appreciation. They truly have been my oars that guided me through the storms and my rock that anchored me down when I needed rest.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Tables</td>
<td>v</td>
</tr>
<tr>
<td>List of Figures</td>
<td>vi</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>ix</td>
</tr>
<tr>
<td>Abstract</td>
<td>xi</td>
</tr>
<tr>
<td>Chapter One – Introduction</td>
<td>1</td>
</tr>
<tr>
<td>General Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Alzheimer’s Disease</td>
<td>2</td>
</tr>
<tr>
<td>Alois Alzheimer</td>
<td>2</td>
</tr>
<tr>
<td>Alzheimer’s disease pathology</td>
<td>2</td>
</tr>
<tr>
<td>Genetics and risk factors</td>
<td>5</td>
</tr>
<tr>
<td>Familial Alzheimer’s disease</td>
<td>7</td>
</tr>
<tr>
<td>Abeta</td>
<td>8</td>
</tr>
<tr>
<td>APP processing</td>
<td>9</td>
</tr>
<tr>
<td>Amyloid cascade hypothesis</td>
<td>12</td>
</tr>
<tr>
<td>Presenilins</td>
<td>16</td>
</tr>
<tr>
<td>Brief overview</td>
<td>16</td>
</tr>
<tr>
<td>Cloning and analysis of the presenilins</td>
<td>17</td>
</tr>
<tr>
<td>Localization and expression of PS-1</td>
<td>19</td>
</tr>
<tr>
<td>Gamma secretase</td>
<td>20</td>
</tr>
<tr>
<td>γ-secretase/APP interaction</td>
<td>21</td>
</tr>
<tr>
<td>Formation of the γ-secretase complex</td>
<td>23</td>
</tr>
<tr>
<td>Presenilin 1 metabolism</td>
<td>24</td>
</tr>
<tr>
<td>Functions of PS-1</td>
<td>27</td>
</tr>
<tr>
<td>Other PS substrates</td>
<td>28</td>
</tr>
<tr>
<td>Presenilin 1 FAD mutations</td>
<td>28</td>
</tr>
<tr>
<td>General phenotypes associated with PS-1 mutations</td>
<td>31</td>
</tr>
<tr>
<td>Presenilin-1 dysfunction associated with FAD mutations</td>
<td>31</td>
</tr>
<tr>
<td>Concluding remarks for PS-1</td>
<td>32</td>
</tr>
<tr>
<td>Involvement of the Cell Cycle in Alzheimer’s Disease</td>
<td>33</td>
</tr>
<tr>
<td>Brief introduction to the cell cycle, mitosis, and AD</td>
<td>33</td>
</tr>
<tr>
<td>The cell cycle and mitosis</td>
<td>34</td>
</tr>
<tr>
<td>An aberrant cell cycle and AD</td>
<td>34</td>
</tr>
</tbody>
</table>
Evidence of mitotic epitopes 36
Phosphorylation of APP during the cell cycle 37
Involvement of PS-1 in the cell cycle and mitosis 38
PS-1 and p53 40
Neurogenesis 41
  Neurogenesis in the AD brain 41
  PS-1 and neurogenesis 42
Aneuploidy in AD 42
  PS-1 and aneuploidy 44
AD and apoptosis 45
  PS, apoptosis, and neurodegeneration 46
Alzheimer’s Disease and Down Syndrome 48
  Brief introduction 48
  Chromosome 21 overexpression and gene dosage 49
  AD and DS in families 49
  PS-1 and DS 51
  Possible shared mechanism between DS and AD 51
How Aneuploidy May Lead to Alzheimer’s Disease 53
Thesis Statement 56

Chapter Two – Results and Conclusions 57
Part I: Aneuploidy in the Lymphocytes and Neurons of FAD Mutant PS-1 Transgenic Mice Detected by Metaphase Chromosome Analysis and In Situ Hybridization. 57
  Abstract 57
  Introduction 58
  Results 62
    Detection of aneuploidy in chromosome spreads of lymphocytes of FAD mutant PS-1 transgenic mice via Giemsa staining 62
    Construction of a BAC probe to detect chromosome 16 in metaphase and interphase cells 65
    Detection of trisomy 16 in lymphocytes of FAD mutant PS-1 transgenic mice via fluorescence in situ hybridization (FISH) 68
    Detection of trisomy 16 in primary neurons of FAD mutant PS-1 transgenic mice via fluorescence in situ hybridization (FISH) 71
    Detection of trisomy 16 in primary neurons of FAD mutant PS-1 knock-in mice via fluorescence in situ hybridization (FISH) 78
  Conclusions 83
    Aneuploidy in lymphocytes from FAD mutant PS-1 transgenic mice 83
    BAC probe 84
Aneuploidy in neurons from FAD mutant PS-1 transgenic mice 85
Aneuploidy in neurons from FAD mutant PS-1 knock-in mice 86

Part II: Aneuploidy Develops in PS-1 Transfected Human Cells, But Not in Cells Transfected With Dominant Negative PS-1 Genes Lacking γ-Secretase Activity. 87

Abstract 87
Introduction 88
Results 92
PS-1 transiently transfected hTERT-HME1 cells develop aneuploidy 92
FISH reveals trisomy 21 in PS-1 transiently Transfected hTERT-HME1 cells 95
Aneuploidy in hTERT-HME1 cells expressing PS-1 is not chromosome specific 103
Transiently transfected hTERT-HME1 cells exhibiting aneuploidy showed no evidence of tetrasomy, only trisomy 107
hTERT-HME1 cells transiently transfected with dominant negative PS-1 lacking γ-secretase activity do not develop aneuploidy 109

Conclusions 111
hTERT-HME1 cells transiently transfected with PS-1 expressing plasmids develop aneuploidy as detected by metaphase chromosome analysis and FISH 113
FISH reveals trisomy 21 and trisomy 12 in PS-1 Transiently transfected hTERT-HME1 cells 115

Chapter Three - Materials and Methods 118
Materials 118
Mice 118
Tissue and cell culture 119
Molecular biology and cytogenetic reagents 120
Methods 121
Primary cells 121
Cell line 122
Plasmids 123
Transient transfections 126
Metaphase chromosome analysis 127
Bacterial artificial chromosome 128
In situ hybridization 130
Image acquisition and analysis 133
Immunocytochemistry 135
List of Tables

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Proteins shown to interact with PS-1</th>
<th>29</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2</td>
<td>Aneuploidy in spleen cells of PS-1 transgenic mice related to mutation and age</td>
<td>73</td>
</tr>
<tr>
<td>Table 3</td>
<td>Transgenic and Knock In mice analyzed for chromosome missegregation</td>
<td>82</td>
</tr>
</tbody>
</table>
List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Alzheimer's disease plaques and tangles</td>
<td>4</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Alzheimer's disease pathology</td>
<td>6</td>
</tr>
<tr>
<td>Figure 3</td>
<td>APP processing by α-, β-, and γ-secretases</td>
<td>11</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Amyloid cascade hypothesis</td>
<td>13</td>
</tr>
<tr>
<td>Figure 5</td>
<td>The presenilins</td>
<td>18</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Formation of the active γ-secretase complex</td>
<td>25</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Mitosis</td>
<td>35</td>
</tr>
<tr>
<td>Figure 8</td>
<td>How the presenilins and aneuploidy can lead to AD</td>
<td>55</td>
</tr>
<tr>
<td>Figure 9</td>
<td>G-band karyotype analysis of mouse chromosomes</td>
<td>60</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Metaphase chromosome analysis of mouse lymphocytes</td>
<td>63</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Aneuploidy induced in mouse spleen cells by a FAD mutant presenilin transgene as detected by chromosome metaphase analysis</td>
<td>66</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Fluorescence in situ hybridization (FISH)</td>
<td>67</td>
</tr>
<tr>
<td>Figure 13</td>
<td>Fluorescence in situ hybridization with a BAC probe detects chromosome 16 in both interphase and metaphase cells</td>
<td>69</td>
</tr>
<tr>
<td>Figure 14</td>
<td>Mouse cells demonstrating three signals (trisomy) identified as aneuploid</td>
<td>70</td>
</tr>
<tr>
<td>Figure 15</td>
<td>Aneuploidy induced in mouse spleen cells by a FAD mutant presenilin transgene as detected by fluorescence in situ hybridization</td>
<td>72</td>
</tr>
</tbody>
</table>
Figure 16  Primary mouse neurons isolated from whole brain in preparation for in situ hybridization

Figure 17  Aneuploidy identified in PS-1 FAD transgenic mice primary neurons by FISH with a BAC probe

Figure 18  Aneuploidy induced in primary mouse brain neurons by a FAD presenilin transgene

Figure 19  Aneuploidy detected in primary mouse neurons of M146V PS-1 Knock In mice

Figure 20  Chromosome metaphase analysis of Giemsa stained hTERT-HME1 cells

Figure 21  Metaphase chromosome analysis revealed aneuploidy induced in hTERT cells transfected with normal and particularly FAD mutant PS-1

Figure 22  Transiently transfected hTERT cells hybridized with LSI 21 Spectrum Orange

Figure 23  Score sheet used for Fluorescence in situ hybridization assays

Figure 24  FISH revealed trisomy 21 induced in hTERT-HME1 cells transfected with normal and particularly FAD mutant PS-1

Figure 25  PS-1-transfected human cells develop both trisomy 12 and trisomy 21 aneuploidy

Figure 26  FISH revealed trisomy 12 induced in hTERT-HME1 cells transiently transfected with normal and particularly FAD mutant PS-1

Figure 27  hTERT-HME1 cells transiently transfected with PS-1 expressing plasmids show no evidence of tetrasomy 21

Figure 28  Dominant negative PS-1

Figure 29  γ-secretase activity is required for presenilin-induced chromosome missegregation
Figure 30  hTERT-HME1 cells transiently transfected with PS-1 expressing plasmids develop aneuploidy as detected by metaphase chromosome analysis and FISH  114

Figure 31  The pcDNA3 and pAG expression vectors  125

Figure 32  The LSI 21 SpectrumOrange Probe  131

Figure 33  The LSI TEL/AML1 ES Dual Color Translocation Probe  134

Figure 34  An inflammatory pathway leading to AD  147
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-secretase</td>
<td>alpha secretase</td>
</tr>
<tr>
<td>Aβ</td>
<td>amyloid beta</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>AICD</td>
<td>amyloid precursor protein intracellular domain</td>
</tr>
<tr>
<td>ApoE</td>
<td>apolipoprotein E</td>
</tr>
<tr>
<td>APP</td>
<td>amyloid precursor protein</td>
</tr>
<tr>
<td>APP-C83</td>
<td>amyloid precursor protein C-terminal fragment of 83 amino acids</td>
</tr>
<tr>
<td>β-secretase</td>
<td>beta secretase</td>
</tr>
<tr>
<td>BAC</td>
<td>bacterial artificial chromosome</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CTF</td>
<td>C-terminal fragment</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribose nucleic acid</td>
</tr>
<tr>
<td>DS</td>
<td>Down syndrome</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ER/IC</td>
<td>endoplasmic reticulum/intermediate compartment</td>
</tr>
<tr>
<td>FAD</td>
<td>familial Alzheimer’s disease</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescence in situ hybridization</td>
</tr>
<tr>
<td>γ-secretase</td>
<td>gamma secretase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>hTERT-HME</td>
<td>human telomerase reverse transcriptase – human mammary epithelial</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kD</td>
<td>kilodalton</td>
</tr>
<tr>
<td>KI</td>
<td>knock in</td>
</tr>
<tr>
<td>LSI</td>
<td>locus specific identifier</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NICD</td>
<td>Notch intracellular domain</td>
</tr>
<tr>
<td>PCD</td>
<td>premature centromere division</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>PS</td>
<td>presenilin</td>
</tr>
<tr>
<td>PS-1</td>
<td>presenilin-1</td>
</tr>
<tr>
<td>PS-2</td>
<td>presenilin-2</td>
</tr>
<tr>
<td>RNA</td>
<td>ribose nucleic acid</td>
</tr>
<tr>
<td>sAPPα</td>
<td>soluble amyloid precursor protein alpha</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
</tbody>
</table>
Chromosome Misseggregation in Alzheimer’s Disease Caused by Presenilin-1

Debrah I. Boeras

ABSTRACT

Mutations in the presenilin 1 gene account for most early-onset familial Alzheimer’s disease (FAD). The presenilins and AD may also be related through a common involvement in the cell cycle. Here we report that one important aspect of the cell cycle—proper chromosome segregation—is dependent on presenilin function and therefore may be involved in AD pathogenesis. Specifically we find that FAD mutations in presenilin 1 (M146L and M146V) lead to chromosome missegregation and aneuploidy in vivo and in vitro: 1) Both metaphase chromosome analysis and in situ hybridization reveal significant aneuploidy in the lymphocytes and neurons of PS-1 transgenic mice. 2) Transiently transfected human cells expressing normal and, especially, mutant PS-1 develop aneuploidy within 48 hours, including trisomy 21, while cells transfected with dominant negative PS-1 genes lacking γ-secretase activity have no effect on chromosome segregation. 3) Analysis of mitotic spindles in the transfected cells reveals abnormal microtubule arrays and lagging chromosomes. The possible mechanisms by which cell cycle defects and chromosome
missegregation induced by $\gamma$-secretase may contribute to Alzheimer’s disease will be discussed.
Alzheimer’s disease (AD) is the most common form of dementia, currently affecting 4.5 million men and women in the United States alone, with alarming estimates of soaring numbers due to increased longevity. AD is a neurodegenerative disorder that arises when neurons in the human cortex and hippocampus, both involved in memory and cognition, are damaged and ultimately killed. The characteristic psychological features of this disorder are memory loss and decline in general mental function. The pathophysiological features are extracellular amyloid deposits, intraneuronal neurofibrillary tangles and inflammation.

Since the initial reporting by the German neuropathologist and psychiatrist Alois Alzheimer in 1907, the search for tools to understand, diagnose, and treat this debilitating disease has exploded and reached into many diverse regions of science. One of the controversial issues in this research is whether the neurodegeneration is a direct consequence of the neuropathology that
characterizes the AD brain or caused independently, or both. Finding means for the prevention and cure for this disease will therefore require a clear understanding of all components of the Alzheimer pathogenic pathway that eventually leads to neuronal death.

Alzheimer’s Disease

Alois Alzheimer

In 1907, the German neuropathologist and psychiatrist Alois Alzheimer reported on his findings of “a peculiar disease of the cerebral cortex” which was later to bear his name (Alzheimer A., 1907). He was intrigued by a 46 year old woman, Augusta D., who had been admitted to the mental asylum with symptoms of memory deficits which later escalated to hallucinations and more serious loss of higher mental functions. Upon her death at age 51 he examined his patient’s brain and found distinguishing abnormalities in the cerebral cortex. Alzheimer described them as “miliary bodies” and “dense bundles of fibrils” which later became to be known as plaques and tangles.

Alzheimer’s disease pathology

The very neuropathological lesions identified by Alois Alzheimer in the early 1900’s –plaques and tangles - have become the hallmark features of the
disease that was later to carry his name. They are the classic pathological
elements that allow for a certain postmortem diagnosis of Alzheimer’s disease
(AD) and the exclusion of all other types of dementia.

The extracellular amyloid plaques are made up of a dense central core
containing primarily Aβ peptides (Allsop et al., 1983; Glenner and Wong, 1984a;
Glenner and Wong, 1984b) which are derived from a larger amyloid precursor
protein (APP) (Goldgaber et al., 1987a; Goldgaber et al., 1987b; Kang et al.,
1987; Robakis et al., 1987a; Robakis et al., 1987b). The neurofibrillary tangles
are intracellular twisted filaments containing the microtubule associated protein
tau (Ihara et al., 1986; Nukina and Ihara, 1986). (Figure 1) Additional
pathological markers of AD include diffuse plaques, which are not associated
with significant cell loss, glial cell-based inflammation, granulovacular
degeneration, loss of synapses, and decreases in cell density mostly in the
hippocampus and the cortex (Neve et al., 2000).

Although plaques contain large amounts of the insoluble beta amyloid
protein (Aβ), it is still unclear whether this protein is the direct cause or an
intermediate step in the neurodegeneration. Some scientists blame the
neurofibrillary tangles containing the protein tau as the culprit. Tau is a
microtubule associated protein that is normally involved in the formation of
microtubules, which play a critical role in intracellular transport and structure, and
in mitosis. However, because the tangles are another hallmark feature of AD,
tau has also become a suspect in causing Alzheimer’s disease. Ultimately, it
Figure 1  Alzheimer’s disease plaques and tangles.
Two hallmark features of the Alzheimer’s disease brain are plaques and tangles. The cartoon image (A) depicts a normal versus Alzheimer’s brain with AD neurons containing neurofibrillary tangles and extra cellular plaques nearby. Figure 1B shows an actual AD brain stained to identify plaques (left) and tangles (right).
may be that both beta amyloid and tau will have to share the blame. Another uncertainty is which disease feature develops first, the amyloid plaques or the tangles, and if they are even directly involved in the pathology. The true mechanism of the disease development might possibly even be quite distinct from those currently proposed.

All pathological hallmarks of Alzheimer’s disease are found to affect regions of the brain that are important for memory, language, and reasoning. Preclinical AD begins in the entorhinal cortex probably 10 – 20 years before any clinical symptoms are evident. The disease then progresses to the hippocampus, specifically the CA1 region, and thereby causes noticeable memory deficits. As the plaques and tangles become more abundant, the disease moves on to affect various other areas such as the cerebral cortex. Ultimately, at the last stages of AD, plaques and tangles are widespread and severe atrophy has affected the entire brain. (Figure 2) Interestingly, many older people develop some plaques and tangles without being demented. It must therefore be assumed that increased amount of plaques and tangles in the brains of AD patients contribute to the decisive difference in the mental capacity of AD diseased versus healthy elderly.

**Genetics and risk factors**

Alzheimer’s disease likely results from many interrelated factors, including genetic, environmental, and others that are not yet identified. Two types of AD
Figure 2  Alzheimer’s disease pathology.
The images above demonstrate some of the major changes to occur in the Alzheimer’s brain as the disease progresses. (top to bottom) Most importantly are the affects to the hippocampus, where memory is processed, and the overall brain atrophy caused by the disease.
are currently distinguished: familial AD (FAD), which is a rare autosomal dominant inherited disease, and sporadic AD, which has no obvious pattern of inheritance. AD is also described in terms of the age of the disease onset. Early onset AD refers to patients where the disease is diagnosed before the age of 65. Late-onset AD describes the disease when it is diagnosed at the age of 65 and older (Seltzer and Sherwin, 1983). Early on-set AD comprises about 5-10% of AD cases, can affect individuals as early as 35 years of age, and is almost exclusively of the familial type.

While genetics play a direct role in some early onset AD cases, the familial AD (FAD), certain other risk factors are assumed to play a dominant role in late on-set AD. Of those, the most significant risk factor that increases a person’s likelihood of developing the disease is old age. In addition, polymorphisms in the gene for apolipoprotein E (APOE) have been well established as being associated with a very high risk for late on-set Alzheimer's disease, with E4 variant causing an increase in deposition of fibrillar beta-amyloid in 40% of AD cases.

Familial Alzheimer's disease

So far our understanding of the pathogenesis of Alzheimer's disease (AD) has primarily been derived from the study of the rare inherited forms of the disease, familial AD (FAD), where specific gene mutations have been discovered to be involved in the cause of the disease. However, even though the familial
forms of AD are a specific subtype of the Alzheimer's disease and represent only a small percentage of all AD cases (5-10%), the neuropathology of the disease is the very same as found in the more common, late onset form of the disease. In familial AD, a person has inherited a mutation in one of three genes: the amyloid precursor protein (APP) on chromosome 21 (Goate et al., 1991), presenilin 1 (PS-1) on chromosome 14 (Sherrington et al., 1995), or presenilin 2 (PS-2) on chromosome 1 (Levy-Lahad et al., 1995; Li et al., 1995). To date, there are approximately 23 APP mutations, 100 PS-1 mutations, and 8 PS-2 mutations (alzforum.org). While the presenilin mutations are found throughout the PS proteins, the APP mutations occur at, or close to the sites where the protein is later cleaved. A common feature of these mutations is that they all result in an increased production of the toxic, amyloidogenic Aβ 42/43. About half of all FAD cases are linked to mutations in the PS-1 gene on chromosome 14. Even though familial AD cases have a low prevalence, the disease is transmitted as an autosomal dominant trait and is therefore 100% penetrant – i.e. even though the FAD related mutations do not occur very often, they will with certainty lead to the disease onset in people who carry those mutations.

Abeta

One of the defining features of Alzheimer’s disease has been the increased presence of the 4 kD amyloid beta peptide, specifically Aβ 1-42, in the brain. The 42-43 amino acid peptide initially described as a smaller 1-28 peptide
by Allsop et al. (Allsop et al., 1983) and Glenner and Wong (Glenner and Wong, 1984a; Glenner and Wong, 1984b) is now known to be a major component of the amyloid deposition in both diffuse and neuritic plaques. The toxic characteristics associated with this “sticky” peptide are attributed to its ability to form polymerized filaments which are deposited as extracellular aggregates (Masters et al., 1985a; Masters et al., 1985b). Several lines of evidence suggest that deposition of Aβ1-42 is an important initial step in the pathogenesis of Alzheimer's disease: Aβ1-42 aggregates more rapidly and is deposited earlier in Alzheimer's disease plaques than Aβ1-40 (Iwatsubo et al., 1994). A prevailing theory in AD research, the amyloid cascade hypothesis, has revolved around the processing and deposition of this peptide and its eventual involvement in the disease process.

**APP processing.** The Aβ peptide is generated by the proteolytic processing of the amyloid precursor protein (APP) by various proteases termed secretases. APP is a ubiquitous, single transmembrane domain protein that is post-translationally modified. Its actual normal function still remains unknown. One line of evidence suggests that APP may most likely function in the brain as a cell surface signaling molecule whose normal disruption could lead to neuronal cell cycle abnormalities resulting in neurodegeneration and hence Alzheimer’s disease (Neve et al., 2000)
APP has several isoforms generated by alternative splicing of the 19 exons in the transcript. The predominant mRNAs encode proteins termed APP695, APP751, and APP770. APP695 is primarily found in neurons while APP751 is ubiquitous. In addition, APP has a short half life and is metabolized rapidly along two pathways. In the non-amyloidogenic pathway, cleavage of APP by α-secretase (Sisodia et al., 1990) releases a large soluble fragment called sAPPα and a shorter C-terminal membrane bound fragment of 83 amino (APP-C83). This membrane bound fragment can then be cleaved by γ-secretase to release an APP intracellular domain (AICD) and a p3 fragment. (Figure 3) In the AD amyloidogenic pathway, which is found to occur predominantly in neurons, a β-secretase, recently identified as BACE (Hussain et al., 1999; Sinha and Lieberburg, 1999; Vassar et al., 1999; Yan et al., 1999) cleaves APP at the N-terminus. This cleavage leads to the production of a soluble sAPPβ fragment and a C-terminal membrane bound 100 kD fragment (APP-C99). Like APP-C83, this membrane bound fragment which becomes the substrate for presenilin-dependent γ-secretase cleavage (Haass and Selkoe, 1993) that releases an APP intracellular domain (AICD) and an Aβ peptide. (Figure 3) In summary, cleavage of APP by α-secretase occurs within the Aβ sequence and thus prevents the formation of Aβ peptides. Yet, cleavage of APP by β- and γ-secretases results in the release of Aβ peptides of 40 or 42/43 amino acids.
Figure 3  APP processing by α-, β-, and γ-secretases.
APP is metabolized down two pathways. In the non-amyloidogenic path, α-secretase cleaves within the Aβ sequence thus preventing its formation. In the amyloidogenic path, cleavage by both β- and γ-secretase produces Aβ peptides. Both pathways produce a large soluble extracellular APP fragment followed by presenilin-dependent γ-secretase cleavage of the remaining C terminus membrane bound fragment. The final products in the α-secretase processing are an extracellular P3 fragment and an intracellular C-terminal fragment (CTF), also referred to as the APP intracellular domain (AICD), and both have unclear functions to date. The final products in the β-secretase processing are again an intracellular CTF/AICD and an extracellularly secreted Aβ peptide. The exact cellular localization of APP processing still remains unclear but it is generally thought to occur predominantly in intracellular membrane compartments, including both early and late secretory compartments, and to some extent directly at the cell surface plasma membrane.
**Amyloid cascade hypothesis.** The amyloid cascade hypothesis formulated by Hardy and Higgins in 1992 (Hardy and Higgins, 1992) proposes – a) that the deposition of Aβ is central to the disease process and b) that any other lesions found in the AD brain are resulting from the cascade initiated by the amyloid peptide. (Figure 4) This hypothesis was brought about by the fact that all known familial AD mutations lead to increased formation of the longer, “sticky” Aβ1-42 which aggregates into amyloid plaques in the AD brain (Borchelt et al., 1996; Kosaka et al., 1997; Mann et al., 1996; Scheuner et al., 1996; Younkin, 1998). Those mutations are found to occur directly in the gene for the amyloid precursor protein (APP) or in the genes for the presenilin proteins, which are an integral part of the γ-secretase complex.

Various studies have supported the claim that the deposition of Aβ is central to the disease process. In 1999, Mayeux demonstrated that plasma Aβ 1-42 levels are higher in AD individuals (Mayeux et al., 1999) compared to non-AD patients. Others have supported the prediction that brain levels of Aβ 1-42 increase during the disease process and are strongly correlated with cognitive decline (Younkin, 1998; Cummings and Cotman, 1995; Naslund et al., 2000). One of the strongest facts in support of the amyloid cascade hypothesis has been the that PS mutations lead directly to an increase in Aβ 1-42. Specifically, it was shown that individuals with a PS mutation have increased Aβ 1-42 plasma levels (Scheuner et al., 1996), abundant brain deposits of Aβ (Cruts et al., 1996; Lemere et al., 1996), and that fibroblasts of those patients
Figure 4  Amyloid cascade hypothesis.
The amyloid cascade hypothesis proposes a sequence of pathogenic events leading to AD starting with the familial missense mutations known to cause the disease. The premise of this hypothesis is that Aβ is central to the disease process and all other lesions found in the AD brain are resulting from the cascade initiated by the amyloid peptide.
appear to produce more Aβ 1-42 than in non-diseased individuals (Scheuner et al., 1996). In addition, other studies have shown that transgenic animals overexpressing mutant PS-1 produce more Aβ 1-42 than their WT counterparts (Borchelt et al., 1996; Duff et al., 1996; Scheuner et al., 1996) and cells transfected with mutant PS-1 produce more Aβ 1-42 than untransfected cells (Borchelt et al., 1996).

The exact mechanism by which the amyloid cascade hypothesis functions is not known except that it appears that the FAD mutations somehow alter APP processing to increase Aβ 1-42 production. Since the presenilins are an integral part of the γ-secretase complex, it is conceivable that mutated presenilins could adversely affect the complex’s ability to function normally. For this reason, these FAD mutations are commonly referred to as a toxic gain of function mutations. This topic will later be discussed in more detail. A consequence of these mutations is either an elevation of total Aβ levels or an increase in the ratio of toxic Aβ 1-42 to Aβ 1-40 (Selkoe, 2000; Selkoe, 2001).

The amyloid cascade hypothesis has been pivotal to Alzheimer’s research for over 10 years and has prevailed as the central hypothesis despite other models and theories. Due to many supporting facts, the amyloid cascade hypothesis has been on center stage for so long that challenging it is often treated like Galileo’s attempt to locate the earth off center of the solar system. Conflicting with the amyloid cascade hypothesis are, for example, the results of Schonheit et al. (Schonheit et al., 2004) that demonstrate that tangles may form
without or prior to the presence of plaques, and that therefore Aβ cannot be the causative agent for neurofibrillary lesions. In addition, various laboratories have focused their research on the involvement of the cell cycle in AD and provided strong evidence that an aberrant cell cycle in the brain leads to neurodegeneration independent of Aβ plaque formation. Finally, more recent studies have shown that while the disease process may indeed stem from the amyloid precursor protein, the causative agent might not be the secreted Aβ peptide but rather the intracellular domain (AICD) fragment which is generated simultaneously during the proteolytic cleavage. Therefore, most likely multiple pathways will contribute to the disease process. For example, while one of the strongest supports for the amyloid hypothesis is the direct effect of the PS mutations on Aβ 1-42 production, the current dissertation will present striking evidence that during the AD process, presenilins cause dysfunctions in the cell cycle which lead to chromosome missegregation. It is possible that upon further investigations, this hypothesis works in combination with or independent of Aβ production to lead to AD neurodegeneration but with such overwhelming data from various fields it would seem too simplistic to assume that a disease as complex as Alzheimer's is dependent upon one mechanism.
Presenilins

Brief overview

After the initial discovery of the abeta peptide, reverse molecular genetic studies led to the discovery of the APP gene followed thereafter by the discovery of mutations in APP. The study of familial AD cases led to the discovery of the presenilins and shortly thereafter the presenilin mutations. The identification of mutations in these three genes (i.e. APP, PS-1, PS-2) which cause increases in Aβ, particularly Aβ 1-42, provide the foundation of the amyloid cascade hypothesis. After the amyloid cascade hypothesis took hold, there was a race to discover the mechanism by which APP was cleaved to produce Aβ 1-42. The work of Schellenberg et al. in 1992 (Schellenberg et al., 1992) provided the first proper evidence that a locus towards the telomere of chromosome 14 might be involved. The locus turned out to encode on one of the presenilin genes and this discovery indeed has led to a greater understanding of the Alzheimer’s disease process, specifically the molecular mechanism behind the Aβ production. It turned out that the presenilins are an integral part of the γ-secretase complex and appear to directly promote its proteolytic activity. This fact alone already underscores the importance of understanding the full normal and pathological functions of the presenilins. Recent findings by various groups strongly suggest that the role of presenilins in the Alzheimer’s disease process may actually be not only more complex but more pivotal than originally suspected.
The first documentation of proper genetic linkage studies related to Alzheimer’s was published almost 10 years after the initial 1983 report where a series of polymorphic genetic markers linked to the early on-set familial form of AD were identified on chromosome 14 (Schellenberg et al., 1992). Shortly thereafter confirmatory reports came from St. George-Hyslop et al. (St George-Hyslop et al., 1992), Van Broeckhoven (Van Broeckhoven et al., 1992), and Mullan et al (Mullan et al., 1992) linking these genetic markers to the aggressive early on-set Alzheimer susceptibility locus (AD3). The region containing the locus was then further narrowed down (Cruts et al., 1995) and in 1995, the novel gene on chromosome 14 corresponding to the AD3 region was discovered and called presenilin 1 (Sherrington et al., 1995). Eventually presenilin 2 was discovered on chromosome 1, but this defect accounts for less than 1% of FAD cases (Rogaev et al., 1995).

The presenilin gene has been found in many different animals (i.e. mouse, rat, chimp, dog, fowl, C. elegans, etc.) including the Arabidopsis thaliana plant and the fruitfly with substantial homology. Specifically, the human and the mouse share quite high homology – approximately 90%.

Presenilin 1 and 2 are serpentine polytopic membrane proteins sharing 67% of amino acid sequence. (Figure 5) Their transmembrane domains are even more similar, with 84% identity (Levy-Lahad et al., 1995). Most models depict the presenilins as having 8 transmembrane domains (TM) with the N- and
Figure 5 The presenilins
Presenilin 1 (PS-1) and presenilin 2 (PS-1) are homologous transmembrane domain proteins. Most mutations are found on PS-1 within the transmembrane domains or within the large hydrophilic loop. PS-1 is located on chromosome 14 and PS-2 is located on chromosome 1.
C-terminal tails facing the cytoplasm or nucleoplasm (Li and Greenwald, 1998). However, other views describing the tails as part of the plasma membrane have been published as well. There is a large acidic loop domain between transmembrane domains 6 and 7 which also faces the same direction as the N- and C-terminal. The N-terminal domain and the large loop domain between TM 6 and TM 7 are hydrophilic while the C-terminal domain is hydrophobic (De Strooper et al., 1997; Doan et al., 1996; Li and Greenwald, 1996).

The presenilin 1 gene spans approximately 60 kb and contains 13 exons encoding a 467 amino acid protein. The open reading frame is encoded by 10 exons, specifically exons 3-12 (Rogaev et al., 1997). Both presenilins undergo alternative splicing whereby the inclusion of a VRSQ motif in PS-1 creates potential phosphorylation sites for casein kinase I and protein kinase C (Clark et al., 1996).

Localization and expression of PS-1. PS-1 has been found expressed ubiquitously and at comparable levels in most human and mouse tissues, including developing and adult brains (Rogaev et al., 1995; Sherrington et al., 1995). In situ hybridization showed that PS-1 expression is predominantly in neurons with highest levels in hippocampus and cerebellum (Kovacs et al., 1996). Inside the cells, overexpressed PS-1 has been localized to the smooth and rough endoplasmic reticulum and Golgi apparatus (Annaert et al., 1999) while endogenous PS-1 was shown to reside on the nuclear membrane,
interphase kinetochores, and centrosomes (Li et al., 1997) with no difference in expression between the normal wild-type and FAD mutant presenilin proteins (Cook et al., 1996). In addition to localizing the full length PS-1 to the nuclear envelope, studies have also reported its presence in other mitotic structures (Annaert et al., 1999; Honda et al., 2000a; Jeong et al., 2000). Importantly, in the AD brain, PS-1 expression has been specifically observed in hippocampal neurons and the entorhinal cortex, the same regions most affected by the disease process.

**Gamma secretase**

Rather early in the history of Alzheimer’s research, the abeta peptide was recognized to contribute to AD pathology. With this knowledge, the processing of APP by various secretases became a major focus of research. Specifically, the secretase responsible for the generation of Aβ 1-40 and 1-42, the γ-secretase, came under intense scrutiny. It was thereafter that the presenilins were found to be directly involved in this process and for this reason they are commonly referred to as the γ-secretase itself.

Initial evidence for the direct involvement of the presenilins in Aβ production came when two groups reported the loss of Aβ secretion and the accumulation of APP C- terminal fragments in neurons of PS-1 knock-out mice (De Strooper et al., 1998; Naruse et al., 1998). Around the same time coimmunoprecipitation studies revealed that the presenilins were part of a much
larger high molecular weight complex (Capell et al., 1998; Thinakaran et al., 1998). Then in 1999 Wolfe et al. (Wolfe et al., 1999) demonstrated that the presenilins contained two aspartate residues which were responsible for the γ-secretase activity. This was shown by mutating one, or both aspartate residues, and then measuring reduced Aβ production and increased C-terminal fragment of APP. These aspartyl residues are located in transmembrane domains six and seven, D257 and D385, respectively. These findings were the first indication that the presenilins might be unique di-aspartyl proteases which require both functional aspartates for normal activity and can cleave within a hydrophobic environment. Upon further investigation, Li et al. (Li et al. 2000) reported photoaffinity labeling of PS-1 by potent γ-secretase inhibitors that were designed to function as transition-state analog inhibitors directed to the active site of the aspartyl protease. Thus, this finding suggested that the presenilins contain the active site of the γ-secretase complex. While the aspartyl mutations, previously described, annihilated the proteolytic activity of the γ-secretase, they did not affect the assembly or trafficking of the γ-secretase complex (Nyabi et al., 2003).

γ-secretase/APP interaction. Since these initial studies, it has been shown that along with the presenilins ubiquitously expressed in all cells, γ-secretase activity is ubiquitously present as well (Doerfler et al., 2001; Geling et al., 2002; Hadland et al., 2001; Wong et al., 2004).
The actual site of cleavage by the γ-secretase enzyme occurs at the C-terminus of the APP protein. For example, in the case of APP751, the protein is cleaved at the C-terminus of residue 712 on APP. If cleavage occurs at residue 712-713, Aβ 1-40 results; if it is after residues 714, long Aβ (1-42/43) is generated (Seubert et al., 1992). Specifically, Annaert et al. has identified an 11 amino acid binding site within the transmembrane region of APP where PS-1 interacts and it has been suggested that this binding region is crucial for the presentation of APP to the catalytic domain of γ-secretase (Annaert et al., 2001).

There are currently many investigations to determine the exact location within the cell where the PS-1/γ-secretase complex processes APP, and it might be important to keep in mind that different phases in the cell cycle might require PS-1 to be located in different places. So far PS-1 has been localized to the ER, Golgi complex, plasma membrane, nuclear membrane, interphase kinetochores, and centrosomes (De Strooper et al., 1997; Kovacs et al., 1996; Li et al., 1997; Cook et al., 1996; Walter et al., 1996). It was previously thought that APP was synthesized in the endoplasmic reticulum (ER) and trafficked through the Golgi network to the cell surface. From the cell surface it was reinternalized via endocytosis into the endosomal/lysosomal system to be processed at various locations to generate Aβ, which was then secreted. Aβ generation was thought to occur within the endosomal-lysosomal system, the Golgi apparatus, and the endoplasmic reticulum/intermediate compartment ER/IC (Golde et al., 1992; Hartmann et al., 1997; Koo and Squazzo, 1994; Xia et al., 1998). There was
obviously some disparity between the sites where Aβ production was thought to occur and the compartments where most of the PS-1 is detected. Also, of the three locations, i.e. the endosomal/lysosomal system, the Golgi apparatus, and the endoplasmic reticulum/intermediate compartment ER/IC, most Aβ 1-42 was thought to be generated at the ER/IC but some studies showed that this Aβ was not destined for secretion (Cook et al., 1997; Hartmann et al., 1997; Wild-Bode et al., 1997).

A more recent study has identified both PS-1/γ-secretase and APP at the plasma membrane where APP is proteolytically cleaved by an active γ-secretase complex to produce Aβ. Specifically, an actively assembled γ-secretase complex is found directly associated with its APP substrates, C83 and C99 (Dewji and Singer, 1996; Chyung et al., 2005). Previous studies analyzing PS mutants have reported increased levels of C83 and C99 (Capell et al., 2000; Kim et al., 2001b; Leem et al., 2002).

*Formation of the γ-secretase complex.* The active γ-secretase is a high molecular weight multi-protein complex that consists of at least four proteins - presenilin, nicastrin (NCT) (Yu et al., 2000), APH-1, and PEN-2 (Francis et al., 2002; Lee et al., 2002; Steiner et al., 2002). The formation of the complex is highly regulated and all four subunits must be present to build a functionally active complex. Recent studies showed that Aph-1 and nicastrin form a subcomplex to bind and stabilize the presenilin, and then PEN-2 bind the
complex and facilitates endoproteolysis of presenilin (Takasugi et al., 2003). (Figure 6) It has also been demonstrated that the maturation of the active γ-secretase complex takes place rather early on in the ER and that the complex then migrates to the cell membrane (Kaether et al., 2004).

Just recently, CD147 has been identified as an additional subunit of the active γ-secretase complex with a regulatory role (Zhou et al., 2005). The presence of CD147, a transmembrane glycoprotein with two Ig-like domains, within the γ-secretase complex is shown to down-modulate the production of Abeta-peptides.

**Presenilin 1 metabolism**

PS-1 not only cleaves proteins but undergoes cleavage itself. In vivo studies have shown that the approximately 43 kD PS-1 undergoes endoproteolytic cleavage within the large hydrophilic loop, between amino acids 260 and 320, generating an approximately 27-28 kD N-terminal protein and an approximately 17-18 kD C-terminal protein (Mercken et al., 1996; Thinakaran et al., 1996). Work done in transgenic mice expressing wild-type PS-1, show that these PS-1 derivatives accumulate to saturable levels and to approximately 1:1 stoichiometry (Thinakaran et al., 1996). The endoproteolysis occurs somewhere between TM 6 and TM 7, a region encoded specifically within exon 9, by an unidentified “presenilinase”. The prospective presenilinase was thought to have similar protease activity as the γ-secretase itself. Studies showed that mutation
Figure 6 Formation of the active γ-secretase complex.
The four subunits that make up the active γ-secretase complex are assembled in an orderly manner. First, APH-1 and nicastrin (NCT) form a low molecular weight (LMW) subcomplex. Next, newly generated presenilin (PS) joins the subcomplex and any presenilin that is not taken into the complex is immediately ubiquitinated for proteasomal degradation. And lastly, PEN-2 joins to complete the active γ-secretase complex. All four subunits are required for proteolytic activity even though the presenilin contains the active sites.
of two aspartate residues residing in TM 6 and TM 7 of PS-1 not only inhibited γ-secretase activity on APP but also inhibited presenilin endoproteolysis (Wolfe et al., 1999). Later, separate studies showed that PS-1 N-terminal and C-terminal fragments were reduced when a γ-secretase inhibitor was used (Beher et al., 2001; Campbell et al., 2002). These observations caused speculation that perhaps presenilin cleavage occurred by autoproteolysis. Yet, more recent work by Campbell et al (Campbell et al., 2003) showed that the “presenilinase” has characteristics of an aspartyl protease, similar to the γ-secretase, but its activity is distinct from the γ-secretase.

One of the questions brought about by the discovery that the presenilins undergo endoproteolysis is whether the holoprotein, the endoproteolytic fragments, or both, form the functional protein responsible for γ-secretase. There are two relevant points to be made. First, the endogenous detection of the presenilins shows that the full-length holoprotein is barely detected. This implies that it is processed to generate the two fragments and that these two fragments make the functional protein (Capell et al., 1998; Thinakaran et al., 1998; Yu et al., 1998). Most recent studies show that these two fragments form a heterodimer and, along with other proteins, comprise a high molecular weight complex referred to as the γ-secretase complex. Second, even though the majority of the presenilins undergo endoproteolysis, the cleavage is not absolutely required for protein function and the disease development. The PS-1 FAD mutant which lacks exon 9 (Delta 9 mutation) no longer undergoes endoproteolytic cleavage
but is still found as part of the functional γ-secretase complex. Therefore, it appears that endoproteolytic cleavage of the presenilins is not required for the development of AD.

Functions of PS-1

It is now well known that the presenilins play a major role in the γ-secretase complex and that FAD mutations of PS-1 lead to altered APP processing. However, the exact mechanism by which these mutations affect APP processing still remains unclear. One assumption is that the PS mutations may directly influence γ-secretase cleavage of APP while complexed with the presenilins or that the PS mutations may cause a subtle alteration in the trafficking of APP such that a greater proportion enters a pathway that leads to the generation of Aβ1-42/43 (Esler and Wolfe, 2001; Hutton and Hardy, 1997). In this later mechanism, PS would function more as a chaperone than the actual γ-secretase.

Alternative view points on the role of the presenilins in the AD process focus on their direct involvement in the cell cycle. However, this specific approach will be discussed in much greater detail later in the cell cycle and AD section of this introduction.

So far, the normal function of the presenilins is still not completely understood. Based on studies on the isologues spe-4 and sel-12 proteins in C. elegans, they have been shown to play a fundamental role in the Notch signaling
pathway (Baumeister et al., 1997; Levitan et al., 1996). Gamma-secretase mediated cleavage of the receptor protein Notch is involved in cell fate during development via the release of the Notch intracellular domain (NICD). Therefore, mutations in the presenilins also affect this process (De Strooper et al., 1999). Also, the knock-out of the PS-1 gene in mice leads to death immediately before or after birth and there is evidence of drastic skeletal malformation during embryogenesis (Davis et al., 1998; Donoviel et al., 1999; Shen et al., 1997).

Other PS substrates

Interestingly, besides Notch, PS-1 interacts with many other proteins which might play a role in the disease process. To date, most of these interacting proteins have not been shown to increase Aβ production when complexed with PS-1 but they may be contributing to the disease process along other pathways. Table 1 lists some of those proteins which have been shown to interact with PS-1.

Presenilin 1 FAD mutations

Mutations in the presenilin genes promote the cleavage of APP to produce the amyloidogenic, 42-43 amino acid form of Aβ, thereby fostering Aβ deposition in the brain. Studies have shown that presenilin mutations elevate extracellular Aβ levels about 1.2 – 3 fold (Borchelt et al., 1996; Citron et al., 1997; Duff et al., 1996; Scheuner et al., 1996). So far, over 100 PS-1 mutations and 8 PS-2
<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filamin</td>
<td>actin binding protein</td>
</tr>
<tr>
<td>calsenilin</td>
<td>calcium binding proteins</td>
</tr>
<tr>
<td>calmyrin</td>
<td></td>
</tr>
<tr>
<td>sorcin</td>
<td></td>
</tr>
<tr>
<td>mu-calpain</td>
<td></td>
</tr>
<tr>
<td>Bcl-XL</td>
<td>antiapoptotic molecule</td>
</tr>
<tr>
<td>Rab11</td>
<td>a small GTPase involved in regulation of vesicular transport</td>
</tr>
<tr>
<td>G0</td>
<td>brain G-protein</td>
</tr>
<tr>
<td>TPIP</td>
<td>a tetratricopeptide repeat containing protein</td>
</tr>
<tr>
<td>glycogen synthase</td>
<td></td>
</tr>
<tr>
<td>kinase-3β</td>
<td>a protein serine kinase implicated in phosphorylating Tau</td>
</tr>
<tr>
<td>Tau</td>
<td>microtubule-associated protein that is involved in microtubule assembly and stabilization</td>
</tr>
<tr>
<td>Notch 1</td>
<td>involved in cell signaling and differentiation</td>
</tr>
<tr>
<td>β-catenin</td>
<td>a multifunctional protein involved in Wnt signal transduction, cell adhesion, and tumor progression</td>
</tr>
<tr>
<td>LRP</td>
<td>LDL receptor related protein</td>
</tr>
<tr>
<td>CD44</td>
<td>a cell surface adhesion molecule</td>
</tr>
<tr>
<td>N- and E-cadherins</td>
<td>cell surface transmembrane proteins</td>
</tr>
<tr>
<td>DCC</td>
<td>deleted in colorectal carcinoma</td>
</tr>
<tr>
<td>p75</td>
<td>neurotrophin receptor</td>
</tr>
<tr>
<td>Syndecan 3</td>
<td>heparan sulfate proteoglycan involved in axon guidance</td>
</tr>
</tbody>
</table>

**Table 1 Proteins shown to interact with PS-1.**
Summary of proteins which interact with PS-1 and could potentially play a role in the Alzheimer’s disease process.
mutations have been identified. A current list is available at (http://www.molgen.ua.ac.be/ADMutations/). The majority of these mutations are missense mutations, where a single amino acid has been substituted. Other mutations have been shown to lead to deletions or insertions mutations. Two splice defect mutations have been found as well. With the exception of one PS-1 mutation (I143F) that has been identified in an English family, each PS-1 mutation was found to exhibit 100% penetrance (Rossor et al., 1996).

Most mutations are found to occur at residues which are conserved between the two proteins and most are located within the transmembrane domains or within the large hydrophilic loop between TM 6 and TM 7. Because of the large number of mutations found in PS-1, the study of this presenilin and its involvement in the Alzheimer's disease process has taken preference over the investigation of PS-2. For this reason, the report and discussion in the following chapters will focus mainly on PS-1.

Studies have shown that there are two major clusters of mutations on PS-1. One of these clusters is located on the transmembrane regions 2 (TM 2) (Crook et al., 1997). However, it is the region between TM 6 and TM 7, specifically exon 8, that contains the highest density of FAD mutations (Cruts et al., 1996; Perez-Tur et al., 1996). This high density of mutations occurs near the site where PS-1 itself is endoproteolytically cleaved and as will be discussed later, near the active site of the γ-secretase. Yet, other than the delta 9 mutation, these missense mutations do not affect PS-1 processing.
General phenotypes associated with PS-1 mutations. FAD PS-1 mutations are associated with the most severe AD phenotypes. They have an approximate frame of on-set between 35 – 65 years of age with some rare reports of occurrence as early as age 28. Specific single PS-1 mutations do not show significant differences in age of on-set of the disease. However, specific clusters of mutations can be strongly correlated to the age of the disease on-set. For example, the cluster within TM 2 has a lower mean age of on-set than the cluster around TM 6. Then again, the cluster around TM 6 has a lower mean age of on-set than the other mutations in the protein (Hutton and Hardy, 1997). While all the FAD PS-1 mutations lead to similar pathology and Aβ deposition, still a few slight differences exist. For example, with the exception of the delta 9 mutation which is associated with large, diffuse plaques termed "cotton wool" plaques, most mutations are associated with dense cored plaques. Other mutations show predominantly vascular deposition, with severe angiopathy, and only some are associated with enhanced neurofibrillary tangle formation and increased neuronal loss (Lleo et al., 2004).

Presenilin-1 dysfunction associated with FAD mutations. The general consensus has been that FAD PS-1 mutations lead to a toxic gain of function (Van Broeckhoven, 1995). Strong evidence of increased extracellular Aβ 1-42 levels in PS transfected cell lines (Borchelt et al., 1996; Xia et al., 1997; Citron et al., 1996; Tomita et al., 1997), in transgenic mice (Borchelt et al., 1996;
Citron et al., 1996; Duff et al., 1996), and in the plasma (Scheuner et al., 1996), skin fibroblast media (Scheuner et al., 1996), and brain tissue (Lemere et al., 1996) of humans carrying FAD PS mutations supported this idea. Yet, there has been some speculation that the mutation may block the normal function of the protein in a dominant negative way. More recent research has shown that the presenilin dysfunction leading to AD may actually be a partial loss of function as opposed to a toxic gain of function (Saura et al., 2004). One example along this line is some FAD PS-1 mutations allow for the accumulation of the C-terminal fragment of APP, the γ-secretase substrate (Casas et al., 2004), hence demonstrating a loss of the PS function.

Concluding remarks for PS-1

In the scope of the present dissertation, it is not be possible to provide a comprehensive review of PS-1 and the γ-secretase. In addition to the many different theories which are currently being investigated from all angles, the attempt is challenged by the fact that many current theories are opposed by equally strong counter theories. This dissertation will attempt to shed some new light on the role of the presenilins in the Alzheimer’s disease process and provide the insight that the presenilins may be involved in more ways than one.
Involvement of the Cell Cycle in Alzheimer’s Disease

Brief introduction to the cell cycle, mitosis, and AD

As previously stated, Alzheimer’s disease is a complex process which should be studied with different independent approaches. Thereby, it should be kept in mind that the understanding and eventual cure of the disease depends on understanding all pathogenic pathways that lead to neuronal death in the AD brain. One area that is currently investigated with great enthusiasm is the role of the cell cycle in AD. The fact that many cell cycle related proteins are found to be expressed in neurons in AD brains and that cell cycle defects can lead to apoptosis makes it essential to analyze the role of the cell cycle in Alzheimer’s disease. Indications that an aberrant mitosis might be a part of the disease feature came from initial findings of similar pathology between the brains of AD and Down syndrome individuals. Down syndrome individuals carry an additional copy of chromosome 21 due to a defective chromosome segregation in the oocyte. Because all Down syndrome patients eventually develop Alzheimer’s disease, it is worth investigating if and how defective mitosis and the resulting chromosome imbalance could contribute to the disease development. Interestingly, the gene for APP is encoded on chromosome 21. However, up to date, no conclusive evidence has been reported indicating that either an enhanced expression of APP due to the additional copy of chromosome 21 or specific APP mutations in Down syndrome patients are the specific cause of the
unescapable combination of Down syndrome with AD. As will be discussed in more detail below, it is therefore important to investigate the probability that the aneuploidy itself plays a major role in the disease development.

The cell cycle and mitosis

For the purpose of this dissertation, a brief overview of the cell cycle and mitosis with relevance to the Alzheimer disease process will be discussed. In brief, there are four phases to the cell cycle (G1, S, G2, M) all of which are tightly regulated by cyclins and cyclin dependent kinases. With respect to AD, the interest lies in the G2/M transition and specifically mitosis. After the DNA has duplicated during S phase, the cell cycle proceeds through to mitosis where the chromatin condenses into compact chromosomes. Mitotic spindles attach to the kinetochores at the centromere region and move the chromosomes into alignment to the metaphase plate. There the chromosomes are separated at their centromeres so that after moving in opposite directions, each sister chromatid reaches its respective spindle pole. (Figure 7)

An aberrant cell cycle and AD

There are many evolving hypotheses regarding the involvement of the cell cycle and the AD process, some of which offer possible mechanisms for the neurodegeneration observed with the disease. Most of cell cycle related hypotheses revolve around the idea that the cell cycle is inappropriately activated
Figure 7 Mitosis
The illustration above depicts different stages of mitosis. The first image shows the condensation of chromatin into chromosomes. Two centrioles move toward the opposite ends (poles) of the cell. Microtubules radiate from each centriole, forming a structure called a spindle. Next the nuclear envelope breaks down as the chromosomes align at the equatorial plane of the cell, as directed by the microtubules attached to the kinetochore (located at the centromere of each chromosome). This phase is called Metaphase. Following this arrangement, the sister chromatids separate and move apart simultaneously for the entire set of chromosomes. Microtubules attached to the kinetochore also play a critical role in this process. Now located on opposite poles of the cell, the chromosomes begin to uncoil and become less condensed. The spindle disappears while a nuclear envelope reappears. The cell elongates and eventually divides into two daughter cells.
in otherwise undividing neuronal cells. It is assumed that the reactivation of the
cell cycle also involves DNA replication and that specifically this process is an
early and important event in the development of AD.

Evidence of mitotic epitopes. Some of the most striking evidence
that the cell cycle is involved in AD has been the discovery of mitotic epitopes in
the diseased brain versus the normal brain, specifically in those regions of
neurodegeneration related to the AD process. More specifically, these mitosis
specific phospho-proteins and enzymes (such as phosphorylated cyclins and
cyclin dependent kinases) which are involved in regulating the cell cycle and are
known to be activated only during cell cycle progression have been found
present in the post-mitotic neurons of AD brains (Arendt et al., 1995; Arendt et
al., 1996; Husseman et al., 2000; Kondratick and Vandre, 1996; McShea et al.,
1997; Nagy et al., 1997a; Nagy et al., 1997b; Raina et al., 2004; Smith and
Lippa, 1995; Smith et al., 2004; Vincent et al., 1997). This might suggest that an
imprecise mitotic cascade initiates the neurodegenerative process.

Markers from every phase of the cell cycle have been found activated in
degenerating neurons even before the formation of AD lesions within the brain.
These activated phosphoproteins have been consistently found at sites of
neuronal cell death in the AD brain (Busser et al., 1998). Along with evidence of
duplicated chromosomes in degenerating neurons, which will be discussed later,
this has led to the hypothesis that vulnerable neurons re-enter the cell cycle. The cells go through S phase, but prior or during mitosis, a still unidentified mechanism prevents the cell from a normal division and thereby triggers its degeneration.

Phosphorylation of APP during the cell cycle. From the standpoint of Alzheimer’s disease, perhaps the most interesting phosphoprotein is APP, which undergoes cell cycle dependent phosphorylation. Interestingly, changes in a cell's phosphorylation/dephosphorylation balance has been shown to alter its APP processing (Gandy et al., 1988; Pope et al., 1994; Preuss and Mandelkow, 1998; Suzuki et al., 1994). More recent data support this idea that phosphorylation of APP, specifically at the thr668 site, affects Aβ production (Ando et al., 2001). Some specific kinases that are thought to phosphorylate the thr668 site include cdk5 (Iijima et al., 2000), cdc2 (Milton, 2001; Milton, 2002; Suzuki et al., 1994), and GSK-3β (Aplin et al., 1996), just to name a few.

In addition, secreted APP is found to enhance proliferation of CNS-derived neural stem cells (Hayashi et al., 1994; Ohsawa et al., 1999) and induce mitosis of Schwann cells (Alvarez et al., 1995). Both the overexpression of wild-type APP and FAD mutant APP is shown to induce DNA synthesis in primary cortical neurons (Neve et al., 2000).

Interestingly, Aβ itself plays a direct role in cell cycle events. Specifically, it is shown to induce cell cycle signaling and neuronal death (Copani et al., 1999;
Giovanni et al., 1999; Wu et al., 2000). One example of this is its relationship to an important cell cycle regulator, cdc2. It was first demonstrated that cdc2 directly binds and phosphorylates Aβ (Milton, 2001) and then, a year later, it was shown by the same author that the Aβ peptide also activates cdc2 directly and binds cyclin B1 (Milton, 2002). This evidence strongly suggests that cell cycle abnormalities may influence the plaque formation and neurodegeneration associated with elevated Aβ.

In general, the available data provide a strong correlation between at least two well known etiologic factors and the proposed involvement of the cell cycle in the disease process.

**Involvement of PS-1 in the cell cycle and mitosis**

PS-1 is another known etiologic factor for AD that shows direct involvement in the cell cycle as well. For example, overexpressed PS-1 accumulates at the microtubule organizing centers (Haass and De Strooper, 1999). Yet, perhaps the most compelling evidence for its involvement comes from its endogenous location at mitosis specific structures; the centrosomes, interphase kinetochores, and nuclear membrane (Li et al., 1997).

Briefly, in order for a cell to leave G2 and enter mitosis, the kinetochores and their associated centrosomes must be released from the surface of the inner nuclear membrane and its associated proteins such as PS-1. Two possible ways in which the putative PS-1 binding to centrosomes/kinetochores might be
regulated during this process are by phosphorylation or proteolytic cleavage. Evidence for PS-1 phosphorylation will be discussed below and it is well established that PS-1 is endoproteolytically cleaved to generate two fragments which become part of the γ-secretase complex, the enzyme involved in Aβ generation.

There are precedents for nuclear membrane proteins having a role in mitosis. Specifically, the yeast NDC-1 protein and the lamin B receptor both have seven-eight transmembrane domains, are located in the nuclear membrane, contain phosphorylatable S/TPXX motifs, and, in the case of NDC-1, cause chromosome missegregation when. In addition to the evident transmembrane domains, PS-1 carries several S/TPXX amino acid motifs which are target sites for phosphorylation by cdc2 kinase in vitro (Li et al., 1995), and casein kinase in vivo (Walter et al., 1998).

There is also evidence that PS-1 arrests the cell cycle at various phases. Data from several labs show while FAD presenilin mutations inhibit the cell cycle (Janicki and Monteiro, 1999; Janicki et al., 2000; Kim et al., 2001a), and prevent the full-length proteins from being translocated to the nuclear envelope (Honda et al., 2000a) overexpression of PS-1 can arrest cells at G1 (Janicki and Monteiro, 1999).

Recently, Louvi et al demonstrated that the loss of PS-1 affects brain development, specifically the morphogenesis and migration of neurons in the CNS, probably because PS-1 is intimately involved in cellular signaling (Louvi et
al., 2004). Similarly, PS-1 deficiency affects differentiation and cell cycle control of neuronal precursor cells (Yuasa et al., 2002).

**PS-1 and p53.** An interesting aspect of the role of PS-1 in cell cycle control is its intimate relationship with the well-studied tumor suppressor gene, p53, whose mode of action is still not completely understood. The p53 gene is the site of the most common mutation in human cancers and both the mutation of p53 or lack of p53 causes cell cycle dysregulation resulting in abnormal centrosome duplication, chromosome missegregation, and aneuploidy (Donehower et al., 1995; Fukasawa et al., 1996; Donehower et al., 1997). p53 mutant cells, animals, and humans show abnormal centrosome duplication, chromosome missegregation, and aneuploidy (Donehower et al., 1995; Fukasawa et al., 1996; Donehower et al., 1997). It is therefore tempting to presume that p53 may exert its tumor suppressor function at least partly by promoting the orderly segregation of chromosomes during mitosis, with loss of p53 leading to aneuploidy. Interestingly, Roperch et al demonstrated that PS-1 deficiency in cancer cells leads to tumor suppression, suggesting that PS-1 is required for tumor formation (Roperch et al., 1998). This not only further implicates PS-1 in the cell cycle but specifically places it in a central function for cell cycle control including correct mitosis and chromosome segregation.
Neurogenesis

The idea that neurons in the brain can undergo cell division defies a long
standing dogma within the science community. It was not until 1995 when Fred
Gage identified substantial neurogenesis occurring within specific regions of the
adult monkey and human brain that the idea of adult neurogenesis took a firm
hold (Gage et al., 1995; Gage, 1998; Gould et al., 1999a). Gage estimated that
approximately up to 1 in every 2000 neurons is replaced by neurogenesis each
day. This neurogenesis occurs primarily in the dentate gyrus which is not only
neuron rich but it is also the very area where the degeneration of those neurons
occurs that are most intimately involved in the AD process (Eriksson et al., 1998).

Presumably, the ability or capacity for neurogenesis in the normal brain is
distinct from the disease-associated reappearance of mitosis specific epitopes,
which takes place in neurons that have been post-mitotic for decades (Vincent et
al., 2003). There are obvious similarities between neurogenesis and
neurodegeneration during the AD process but the combining mechanism is still
not known.

Neurogenesis in the AD brain. It is now better understood that
neurogenesis can be stimulated further by pathological processes. For
example, there is data from adult rats demonstrating that brain injury, such as
induced ischemia, promotes increased cyclin D, cdk4, and neurogenesis
(Arvidsson et al., 2001; Jiang et al., 2001; Nakatomi et al., 2002; Osuga et al.,
2000; Zhang et al., 2004). More recent data show specific cell cycle/neurogenesis markers to be overexpressed in the AD hippocampus (Jin et al., 2004) while Aβ disrupts neurogenesis in AD mouse models (Haughey et al., 2002).

**PS-1 and neurogenesis.** In the Xenopus system, Panganelli et al. demonstrated PS-1 directly promotes neurogenesis (Panganelli et al., 2001). Furthermore, mice lacking the endogenous PS-1 genes or harboring a knock-in FAD mutant human PS-1 have reduced neurogenesis compared to normal mice (Chevallier et al., 2002; Feng et al., 2004; Wen et al., 2004) and neuronal precursors lacking PS-1 have a 50% longer cell cycle (Yuasa et al., 2002).

Importantly, these findings demonstrate that cell cycle activity (i.e. neurogenesis) not only occurs in the specific brain regions that are affected by AD but that once again PS-1 is a key component and requires further investigation.

**Aneuploidy in AD**

Aneuploidy refers to the loss or gain of one or more chromosomes which could result in serious consequences for a single cell (i.e. apoptosis) as well as the entire organism. Genetics and biochemistry have shown that many proteins
are required for proper chromosome segregation and the disruption, dysfunction, or loss of any one can lead to asymmetrical mitosis resulting in abnormalities in chromosome numbers.

Previous cytogenetic analyses have yielded mixed results with regards to aneuploidy in AD individuals (Buckton et al., 1983; Moorhead and Heyman, 1983; Nordenson et al., 1980; Ward et al., 1979; White et al., 1981). With current advancements in cytogenetics and molecular biology, various laboratories have now demonstrated definite and consistent evidence of aneuploidy in AD. Specifically, fluorescence in situ hybridization (FISH) has proven to be a powerful tool which allows for the detection of low levels of aneuploidy in cells independent of the stage of the cell cycle.

Initial studies by Potter and colleagues employed quantitative fluorescence in situ hybridization to measure aneuploidy, specifically trisomy 21 cells, in primary fibroblast cultures from AD and unaffected subjects (Potter et al., 1995). Individuals carrying FAD mutations exhibited a significant approximate twofold increase in the number of trisomy 21 cells with a suggestion of aneuploidy affecting chromosome 18 as well (Geller and Potter, 1999). Then in 2001, Yang and Herrup used FISH to identify hippocampal neurons in the AD brain that had reentered the cell cycle, underwent full or partial DNA replication, developed substantial aneuploidy, and died (Yang et al., 2001). Both tetrasomy and some trisomy 21 were identified in regions of significant cell loss accompanied by tissue atrophy. In another line of investigation, Migliore et al
found the preferential occurrence of trisomy 21 missegregation in the lymphocytes of AD patients (Migliore et al., 1999). Again using FISH, an increase in both spontaneous and chemically induced aneuploidy was detected. Specifically, there was evidence of premature centromere division (PCD) and micronuclei, both mitotic abnormalities associated with the development of aneuploidy. More recently, Migliore and colleagues confirmed the occurrence of chromosome 21 missegregation in the lymphocytes of AD patients (Trippi et al., 2001; Petrozzi et al., 2002). The presence of significantly higher spontaneous micronuclei levels in the peripheral lymphocytes and skin fibroblasts of AD patients was detected. Also, FISH was used to identify micronuclei resulting from chromosome breakage and micronuclei that derive from lagging whole chromosomes.

PS-1 and aneuploidy. With PS-1 being so intimately involved in the cell cycle, it is reasonable to assume that a mutation or overexpression of PS-1 could result in mitotic dysregulation and chromosome missegregation. In fact, this was demonstrated in a study by Geller and Potter, previously described, where fibroblast cultures from FAD individuals were analyzed using FISH (Geller and Potter, 1999). Specifically, individuals carrying FAD mutations in presenilin 1 or 2 exhibited significant levels of trisomy 21 thereby demonstrating that FAD mutations predispose to chromosome missegregation.
The findings reviewed so far demonstrate the involvement of PS-1 in the cell cycle and mitosis and the specific requirement for normal, physiological levels of PS-1 to secure accurate cell cycle progression. The present thesis analyzes the effect of FAD mutant PS-1 on the cell cycle and specifically, its affect on chromosome segregation.

**AD and apoptosis**

In addition to the discovery of cell cycle activity and mitotic epitopes in the AD brain, apoptosis and apoptosis related proteins have been reported in the AD brain as well (Engidawork et al., 2001; Su et al., 1994). Morphologically, AD neurons show an increase in DNA damage and DNA fragmentation (Anderson et al., 2000). While various caspases have been identified in the AD brain, some of the most exciting findings have come from investigations looking specifically at apoptosis related proteins, such as Par-4. Levels of Par-4 are increased in the AD brain and cultured neurons and the inhibition of Par-4 blocks apoptosis (Guo et al., 1998; Mattson et al., 1999). Recent confocal fluorescence microscopy studies have shown that Par-4 interacts with and binds to the cytoskeleton and that this association is necessary for apoptosis (Vetterkind et al., 2005a; Vetterkind et al., 2005b). Previously, Xie et al showed that Par-4 is directly involved in the apoptosis of hippocampal neurons in FAD mutant (M146V) PS-1
knock-in mice (Xie et al., 2001). This observation does not only provide strong support for the role of PS-1 in apoptosis but also offers a possible explanation about the mechanism of presenilin’s involvement in AD disease progress.

*PS, apoptosis, and neurodegeneration.* Various studies have shown that the presenilins are specific modulators of apoptosis. It was shown that overexpression of wild type as well as FAD presenilin mutations in transfected cell lines make those cells more prone to apoptosis (Deng et al., 1996; Eckert et al., 2003; Guo et al., 1996; Guo et al., 1997; Janicki and Monteiro, 1997; Vito et al., 1996a; Vito et al., 1996b; Wolozin et al., 1996; Yang et al., 2004). In addition, Xiang et al have shown that cdk inhibitors block neuronal apoptosis in APP/PS transgenic mice (Xiang et al., 2002) again implicating PS-1 in the role of apoptosis in the AD brain. Also, in the previously discussed study by Roperch et al, the link between PS-1 and p53 was investigated and it was found that inhibition of PS-1 expression resulted in apoptosis (Roperch et al., 1998). These are just a few of the many examples providing direct confirmation that a cell placed under stress undergoes a form of cell suicide eventually leading to the neurodegeneration observed in the AD brain. While there are many reports that support PS-1 involvement in AD related apoptosis, other studies dispute this role. There are some conflicting studies showing that the presenilins either are not involved in apoptosis or perhaps protect cells from apoptosis (Bursztajn et al., 1998; Giannakopoulos et al., 1997).
While the debate about the specific and possibly cell type specific involvement of the presenilins is still ongoing, there seems to be no doubt that the presenilin effects have so many more possible outcomes than just APP processing. Even more so that mice expressing mutant presenilins have been found to exhibit age-related neurodegeneration even in the absence of other aspects of AD pathology such as amyloid deposition.

In a separate study, Roperch et al investigated the link between PS-1 and p53 and found that inhibition of PS-1 expression resulted in apoptosis (Roperch et al., 1998). p53 is a well-studied tumor suppressor gene whose mode of action is still not completely understood. The p53 gene is the site of the most common mutation in human cancers, and p53 mutant cells, animals, and humans show abnormal centrosome duplication, chromosome mis-segregation, and aneuploidy (Donehower et al., 1995; Donehower, 1997; Fukasawa et al., 1996). Thus p53 may exert its tumor suppressor function at least partly by promoting the orderly segregation of chromosomes during mitosis, with loss of p53 leading to aneuploidy.
Alzheimer’s Disease and Down Syndrome

Brief introduction

All individuals with Down syndrome (DS) share neuropathological changes consistent with an AD individual by the time they reach 40 years of age (Mann, 1988; Olson and Shaw, 1969; Wisniewski et al., 1995). The finding of these shared neurological changes and the fact that DS individuals are trisomic for chromosome 21 guided AD researchers to chromosome 21. This effort was rewarded by the identification of the amyloid precursor protein gene on chromosome 21 and somewhat later by the discovery of FAD mutations in APP (Glenner and Wong, 1984b; Goate et al., 1991; Masters et al., 1985b). Immediately after this genetic linkage between the two diseases was established, many questions arose revolving around the cell cycle and chromosome missegregation. Knowing that the premature ageing of DS is the result of an aberrant cell cycle made it an obvious task to see if AD individuals shared this aberrancy as well (Potter, 1991). Specifically, the basic hypothesis of the current thesis is that a cell cycle dysfunction mediated by the presenilins leads to chromosome missegregation and that over time the accumulation of aneuploid cells supports the development of the classic disease features observed in AD.
Chromosome 21 overexpression and gene dosage

One possible explanation for the link between Down syndrome and Alzheimer’s disease is that, in both disorders, a gene on chromosome 21 is overexpressed (Schweber, 1985; Schweber, 1989). The overexpression of genes located on chromosome 21, due to extra gene load, is thought to be central to the pathogenesis of DS. APP is actually overexpressed in DS individuals somewhat more than the 50% that the gene dosage alone would lead to expect (Neve et al., 1988; Oyama et al., 1994; Pallister et al., 1997; Rumble et al., 1989). However, so far no cases of Alzheimer’s disease could be solely contributed to the simple duplication of the APP gene and the overexpression of APP. It therefore seems more likely that trisomy 21, or perhaps other forms of aneuploidy, result in multiple abnormalities that lead to AD decades later.

AD and DS in families

A statistically significant association between AD and a family history of DS in first degree relatives was reported in 1991 by van Duijn et al (van Duijn et al., 1991). Similarly, Yatham et al showed significant incidence of AD amongst the first and second degree relatives of DS families (Yatham et al., 1988). Some of the earlier studies in this area showed that women in AD families in which the disease is inherited as an autosomal dominant trait give birth to a high frequency of DS children (Heston and Mastri, 1977; Heston et al., 1981; Heyman et al., 1983). On the other hand, Schupf et al showed that mothers who give birth to
DS children before the age of 35 have a fivefold greater risk of developing AD compared to other mothers (Schupf et al., 1994). Prior to this study, Ford concluded that DS parents may also demonstrate defects in mitosis as evidenced by an increased frequency of chromosomes displaced from the mitotic spindle after colchicine treatment (Ford, 1984).

Trisomy 21 mosaicism was specifically found in two women who, though not mentally retarded and not characterized as having Down syndrome, developed AD-like dementia by age 40 (Hardy et al., 1989; Rowe et al., 1989; Schapiro and Rapoport, 1989). One of the two women also had a DS child. An unusual family with an inherited aberrant chromosome 22-derived mini-chromosome was studied by Percy et al and found to have a high frequency of AD (Percy et al., 1991). The two living Alzheimer-affected members of the family carried the marker chromosome and one was also found to be mosaic for trisomy 21. A more recent case presented by Prasher et al also demonstrated trisomy 21 mosaicism in a 78 year old woman who experienced several of the common age-related conditions of DS individuals (Prasher et al., 1998). These epidemiological findings further support the hypothesis that AD is associated with trisomy 21 and that it is not necessary for every cell of an individual to be trisomy 21 for the aberrant effects of this chromosome imbalance to result in Alzheimer dementia.
More recent reports show an association between PS-1 and DS, thereby providing further evidence that the Alzheimer’s disease process could indeed originate from a defect in the cell cycle. A study from Peterson et al shows an increase in association of PS-1 intron 8 polymorphism in mothers of DS patients with meiosis error (Petersen et al., 2000) thereby suggesting that this polymorphism may be involved in chromosomal missegregation through an influence on the expression level of PS-1. Another report, by Lucarelli et al, shows an association between the PS-1 -48C/T polymorphism and DS (Lucarelli et al., 2004). Specifically, this study provides evidence that the −48C/T polymorphism in the PS-1 gene promoter, which is involved in the modulation of Aβ load in human AD brains, is associated with DS.

Possible shared mechanism between DS and AD

The observation that both DS and AD are characterized by cell cycle abnormalities together with the location of the APP gene on chromosome 21, has led to speculations regarding a common mechanism governing both these pathologies. Some of the original work presented by Heston et al in 1977 suggested that both AD and DS genetic defects may be expressed through disorganization of microtubules resulting in chromosomal aberrations (Heston and Mastri, 1977). In 1989, Matsuyama et al hypothesized that the microtubules were indeed the key in the Alzheimer’s disease process and even suggested that this may provide an excellent diagnostic tool. This hypothesis was based on
previous suggestions that microtubule dysfunction was the underlying mechanism in DS (Matsuyama and Jarvik, 1989). Then in 1991, Potter directly hypothesized that AD and DS individuals not only share a similar pathology but also a common mechanism governing chromosome missegregation and resulting in trisomy 21 (Potter, 1991). Shortly thereafter, Schupf et al demonstrated an increased risk for AD in mothers of adult DS individuals and postulated a shared genetic susceptibility which resulted in chromosome non-disjunction (Schupf et al., 1994). Individually, these findings have all revolved around the common speculation that DS and AD individuals share cell cycle abnormalities. Taken together, these findings suggest a common microtubule dysfunction which clearly results in trisomy 21 in DS individuals and common pathology in both.

Based on the fact that microtubules are a governing mechanism responsible for proper chromosome segregation, the hypothesis by Potter that AD and DS individuals share a common mechanism governing chromosome missegregation and resulting in trisomy 21 seems quite reasonable. Others found the cytogenetic abnormality termed premature centromere division (PCD), which is a correlate and potential cause of improper chromosome segregation, to be involved in the disease process. Specifically, PCD is found to be positively linked with age and increased in women with FAD (Buckton et al., 1983; Fitzgerald, 1975). Also, trisomy 21, 18, and X occurred in the lymphocytes and fibroblasts of a woman prone to PCD, who also had three trisomy 21 conceptuses (Fitzgerald, 1975).
How Aneuploidy May Lead to Alzheimer’s Disease

Based on studies presented thus far, several potential mechanisms might explain how aneuploidy could lead to Alzheimer’s disease. For example, chromosome missegregation may make cells more prone to apoptosis due to the dramatic change in gene dosage (Nicklas, 1997). Specifically in the case of trisomy 21, altered gene dosage not only results in DS but there is also some evidence that the cortical neurons from DS fetuses undergo spontaneous apoptosis in vitro (Busciglio et al., 2002). Apart from such neurodegeneration leading directly to apoptosis, it can also indirectly affect APP processing and the production of the Aβ peptide. There is direct evidence that DS fetal brains and adult sera actually contain a higher ratio of the pathogenic Aβ1-42 compared to Aβ1-40 (Teller et al., 1996). When apoptosis is induced in normal human neurons by serum starvation or other treatments, their secretion of the Aβ peptide is increased (LeBlank, 1995; Galli et al., 1998). These findings show strong relationships between AD and DS pathologies, aneuploidy, apoptosis, and Aβ levels.

A more direct connection between AD and apoptosis comes from studies showing that FAD mutant presenilin or overexpression even of normal presenilin genes results in increased sensitivity to apoptotic stimuli (Vito et al., 1996a; Vito et al., 1996b). Interestingly, studies by Potter and colleagues have shown that the presenilins are located at the nuclear membrane, interphase kinetochores,
and centrosomes thereby implicating them in mitosis and chromosome segregation (Li et al., 1997). This observation is further supported by evidence that FAD mutant PS-1 individuals carry significant levels of trisomy 21 in their fibroblasts (Potter et al., 1995; Geller and Potter, 1999). These findings allow for the possibility that a mutation of the presenilins would alter mitosis, resulting in chromosome missegregation, and these aneuploid cells would be more prone to apoptosis.

The exact mechanism by which the presenilins could cause aneuploidy is still under investigation but there are many possibilities. For example, it is highly interesting that the microtubule-associated protein CLIP 170, present in the centrosome, is required for centrosome function, and that its interaction with PS-1 is required for Aβ peptide production (Johnsingh et al., 2000; Tezapsidis et al., 2003). Similarly, there must be other cytoskeletal proteins still not identified that associate with the presenilins and are adversely affected by the presenilin mutations.

In summary, various reports have provided strong evidence that the presenilins are not only involved in Aβ generation, but have a decisive functional role in several of the processes which lead to AD, including the cell cycle. Figure 8 attempts to summarize the possible involvement of presenilin and chromosome missegregation in the development of Alzheimer’s disease.
Figure 8  How the presenilins and aneuploidy can lead to AD.  
The schematic above depicts various pathways by which PS can lead to AD via chromosome missegregation. An initial finding in the disease process was the shared pathology between DS and AD individuals. This led to the discovery of the presenilins and their direct involvement in generating the Aβ peptide. Further investigations revealed that the presenilins are located at mitotic structures. This along with other independent findings of the cell cycle involvement in AD have provided further support that the presenilins cause chromosome missegregation and that aneuploidy is an integral part of Alzheimer’s disease.
Various reports have provided strong evidence implicating the presenilins in cell cycle activities including a requirement for presenilins in normal mitotic progression. In light of all the evidence provided, this study postulates that presenilins are cell cycle regulators, and set out to test this hypothesis directly.

Specific aim one of this dissertation was to a) assay and compare levels of aneuploidy in transgenic mice expressing normal human wild-type PS-1, FAD mutant (M146L) PS-1, and FAD mutant (M146V) PS-1; and b) assay levels of aneuploidy in FAD mutant (M146V) PS-1 knock-in mice expressing normal levels of human FAD mutant PS-1. The levels of aneuploidy in lymphocytes and neurons of those animals were identified by chromosome metaphase analysis and DNA fluorescence in situ hybridization (FISH).

Specific aim two was to a) determine whether the FAD mutant PS-1 was indeed the causative agent for the aberrant chromosome segregation; and b) determine the role of γ-secretase on chromosome segregation. For the first part of this specific aim karyotypically stable mammalian cells, hTERT-HME1, were transiently transfected with WT PS-1 and FAD mutant (M146L) PS-1. Aneuploidy was assayed and compared by chromosome metaphase analysis and FISH. For the second part of this specific aim hTERT-HME1 cells were transiently transfected with dominant negative PS-1 genes lacking γ-secretase activity and aneuploidy was assayed and compared with FISH.
Chapter Two
Results and Conclusions

Part I: Aneuploidy in the Lymphocytes and Neurons of FAD Mutant PS-1
Transgenic Mice Detected by Metaphase Chromosome Analysis and In Situ Hybridization

Abstract

Aneuploidy, specifically trisomy 21, was previously shown in fibroblasts from both sporadic and familial AD patients. Remarkably, particularly those cells were effected that carried a mutant presenilin 1 or 2 gene (Geller and Potter, 1999; Potter et al., 1994; Potter, 1999). To extend these findings along with novel results, the role of the presenilins in chromosome segregation and the cell cycle was investigated. For this purpose, transgenic mice harboring and expressing either the normal wild-type human PS-1 gene or one of two FAD mutant PS-1 genes (M146V or M146L) were compared. Both metaphase chromosome analysis and DNA fluorescence in situ hybridization were used to assay chromosome missegregation and aneuploidy in lymphocytes and neurons. Those animals harboring and expressing FAD mutant PS-1 had significantly
higher levels of aneuploidy, specifically trisomy 16, the mouse homolog to human chromosome 21, than mice with wild type presenilins. Supportingly, primary neurons from FAD mutant PS-1 knock-in mice expressing wild type levels of FAD mutant PS-1 from the endogenous location of the gene were also found to have significant trisomy 16. Thus, one of the major effects of mutant PS-1 is the development of Alzheimer’s disease that might be mediated through its role in the cell cycle and chromosome segregation.

Introduction

Aging transgenic mice expressing FAD mutant genes develop many features of Alzheimer’s disease as they are found in humans and therefore provide an excellent tool for the investigation of this disease. Specifically, the effect of PS-1 on chromosome segregation and aneuploidy was first examined using transgenic mice in which the PDGF promoter induces high PS expression in CNS neurons (Duff et al., 1996). Unfortunately, neurons, which have proven to be central to AD research, cannot be analyzed by classical cytogenetics such as karyotyping because they do not divide easily in culture. Although chosen because of its ability to direct expression predominantly in neurons, the PDGF promoter is also weakly active in the spleen. Primary mouse lymphocytes harvested from mouse spleen can be easily induced to divide and provide an excellent source for analysis of metaphase chromosomes. Because transgenic mice can exhibit features that are due in part to the location of the transgene
and/or an abnormal expression of the transgene, the knock-in approach represents a powerful and proven technique for evaluating the significance of the targeted gene at endogenous levels.

Cytogenetics is the study of chromosomes and chromosome abnormalities. During most of the cell cycle, specifically interphase, the DNA is not condensed and therefore not individually visible with light microscopic techniques. However, during mitosis, the DNA become highly condensed and visible as dark distinct chromosomal bodies within the cell nuclei. As a result, individual chromosomes are most easily seen and identified at the metaphase stage of cell division. Unfortunately, while the analysis of mouse chromosomes is important to many fields of genetic research, mouse chromosomes are much harder to classify than human chromosomes. For example, a standard cytogenetic techniques used to identify specific chromosomes, such as G-band karyotyping, are very difficult because mouse chromosomes are acrocentric and of similar size and do not easily reveal banding patterns. (Figure 9) Yet, Giemsa staining and fluorescence in situ hybridization (FISH) are two excellent standard cytogenetic techniques that are quite frequently employed to determine abnormal chromosome numbers within mouse cells.
Mouse chromosomes are organized according to their size, however all of the chromosomes are acrocentric (having the centromere close to one end and thus no p-arm) and of similar size, making karyotyping more of a challenge. The normal mouse karyotype consists of 40 chromosomes.
The use of fluorescence in situ hybridization to enumerate mouse chromosomes has been traditionally limited to the use of whole chromosome paints or centromeric probes. While both techniques represent advancements in the field of mouse molecular cytogenetics, each has its limitation. The whole chromosome paints have limited use because they paint the entire chromosome and are therefore only useful as a valid assay for aneuploidy on metaphase spreads. The centromeric probe, however, is capable of detecting aberrations in the chromosome number in both interphase and metaphase spreads as it is designed to identify the centromeric region of each chromosome. Still, because it recognizes every centromere it is only as valuable an assay as Giemsa staining in that it does not identify specific chromosomes.

More recently, bacterial artificial chromosomes (BACs) are being used in the field of mouse cytogenetics to enumerate and identify chromosomes (Korenberg et al., 1999). A BAC is a vector used to clone large DNA fragments (100- to 300-kb insert size) in *Escherichia coli* cells and is based on the naturally occurring replication origin in the F-factor plasmid found in *E. coli*. Bacterial artificial chromosomes (BACs) are plasmid-based and thus the large DNA fragments are still easily isolated and manipulated which makes them especially well suited for fluorescence in situ hybridization (FISH). Large DNA fragments are critically important for FISH because the cloned DNA must be sufficiently large to produce visibly bright, well-defined signals on metaphase spreads and interphase nuclei preparations.
Results

Detection of aneuploidy in chromosome spreads of lymphocytes of FAD mutant PS-1 transgenic mice via Giemsa staining. The effect of PS-1 on chromosome segregation was first observed in primary spleen cells of transgenic mice harboring either the M146L or the M146V mutation. Primary lymphocytes readily divide in culture and thereby allow for standard cytogenetic chromosome metaphase analysis and hence detection of aneuploidy. After harvesting mouse spleens, mitogenic stimulation of T lymphocytes was induced in culture by addition of Concanavilin A. The cells were then treated with colcemid which prevents formation of the spindle and thereby halts chromosome separation at the metaphase-anaphase border. This technique is routinely used to prepare chromosome for further cytogenetic analysis such as karyotyping or fluorescence in situ hybridization. Mouse karyotyping is difficult due to the very similar banding patterns of individual chromosomes. Fortunately, Giemsa staining alone allows the reliable identification of additional chromosome copies in aneuploid cells. For this purpose, chromosome metaphase spreads were fixed onto slides with cold 3:1 methanol:acetic acid for 30 minutes and then stained with Giemsa staining solution for 2 minutes to visualize the chromosomes. With this procedure chromosomes in each cell could be counted and imbalances (aneuploidy) be identified. (Figure 10)
Figure 10  Metaphase chromosome analysis of mouse lymphocytes reveals aneuploid cells.
Mouse spleens were harvested and lymphocytes were stimulated to divide in culture with concanavalin A. The mitotic spindle was disrupted with colcemid and metaphase spreads were stained with Giemsa so that the chromosomes could be counted. Image above shows a normal (non-aneuploid) mouse cell consisting of 40 chromosomes.
To compare the chromosome number of cells from FAD mutant PS-1 transgenic mice with those from non-transgenics, the chromosome content of thousands of lymphocytes were individually counted. Basically, the spleen cells were harvested after concanavalin A and colcemid treatment, then fixed with 3:1 methanol:acetic acid, dropped onto slides, and Giemsa stained for counting (as shown in previous figure). Lymphocytes from more than 50 animals 15 – 17 months of age were blinded and used for unbiased counting. A normal non-aneuploid mouse lymphocyte contains 40 chromosomes. Therefore, any cell with more or less than 40 chromosomes was considered to be aneuploid. Only intact unbroken metaphase spreads were counted and extra precautions were taken to ensure that an aneuploid cell was indeed truly aneuploid. For this purpose, the area surrounding each aneuploid metaphase spread containing more than 40 chromosomes was analyzed for evidence of nearby broken cells that may have contributed extra chromosomes to the cell being counted. In addition, the area surrounding those metaphase spreads containing less than 40 chromosomes was analyzed for evidence of a trailing chromosome indicating that the apparent unbroken cell being counted had been damaged probably during the fixation process. After bad chromosome spreads were eliminated, chromosomes from over 3500 lymphocytes were counted and the analysis clearly showed twice the amount of aneuploidic lymphocytes from mice that were transgenic for the FAD mutant PS-1 gene (~30%) in comparison to lymphocytes from PS-1 WT (15.2%) -or non-transgenic animals (15.2%) (Figure 11).
Interestingly, the M146V mutation resulted in a slightly higher percentage of aneuploidy (33.7%) when compared to the M146L mutation (27.3%). This might indicate that this mutation may account for a more aggressive phenotype in FAD and indeed patients having the M146V mutation show an earlier age of onset than patients with no M146V mutation.

*Construction of a BAC probe to detect chromosome 16 in metaphase and interphase cells.* In contrast to Giemsa staining, fluorescence in situ DNA hybridization (FISH) allows analysis of particular chromosomes within the cell independently of the cell cycle phase. In situ hybridization is a method of localizing and detecting specific mRNA or DNA sequences in morphologically preserved tissue or cell preparations by hybridizing the complimentary strand of a nucleotide probe to the sequence of interest. (Figure 12) For this assay, a DNA hybridization probe was generated from a bacterial artificial chromosome (BAC) carrying a 300 kb fragment of mouse chromosome 16, the mouse chromosome most similar to human chromosome 21. The DNA was labeled with spectrum green by nick translation and the probe was then used to detect the complimentary sequence of mouse chromosome 16 in preserved cell preparations. The visualization of any uneven number of hybridization signals would account for aneuploidy.
Figure 11  Aneuploidy induced in mouse spleen cells by a FAD mutant presenilin transgene as detected by chromosome metaphase analysis. Metaphase chromosome analysis of lymphocytes of transgenic (WT = normal human PS-1, M146L = FAD mutant M146L PS-1, M146V = FAD mutant M146V PS-1) and non transgenic mice (NON) revealed significantly higher levels of aneuploidy in the mutant PS-1 transgenic lymphocytes. Mice were 15-17 months of age.
**Figure 12  Fluorescence in-situ Hybridization (FISH).**

In situ hybridization is a method of localizing and detecting specific mRNA or DNA sequences in morphologically preserved tissue or cell preparations by hybridizing the complimentary strand of a nucleotide probe to the sequence of interest.
Over 3000 cells were hybridized and blindly scored for the number of fluorescent spots to measure both the hybridization efficiency and the aneuploidy for chromosome 16. Any nuclei that were overlapping or even slightly touching were not counted. Depending on the cell cycle phase in which the individual cells were harvested, WT metaphase and interphase cells were expected to harbor an even number of chromosomes and thus show usually two and sometimes four signals. In Figure 13, the image on the left represents a normal non-aneuploid interphase cell and the image on the right a normal non-aneuploid metaphase cell. These two images are both from a PS-1 WT transgenic animal.

To assure the accuracy of the data and to avoid any wrongful counting due to incomplete hybridization, only triple signals (trisomy) were scored as aneuploid. Cells with only one signal were not considered aneuploid but were scored as incomplete hybridizations (Figure 14). A comparison of the number of cells with one and two hybridization spots showed that the labeled 300 kb BAC hybridized with greater than 90% efficiency.

*Detection of trisomy 16 in lymphocytes of FAD mutant PS-1 transgenic mice via fluorescence in situ hybridization (FISH).* When trisomy 16 cells were scored, lymphocytes from nontransgenic and PS-1 WT transgenic mice showed 6.4% and 7.7% aneuploidy respectively. In contrast, the FAD mutant PS-1 (M146L) transgenic mice had 15% aneuploidy and the PS-1 (M146V) mice 19% aneuploidy, 19% aneuploidy exceeds twice the amount of aneuploidy that had
Figure 13  Fluorescence in situ hybridization with a BAC probe detects chromosome 16 in both interphase and metaphase cells.

Fluorescence in situ hybridization with a BAC probe (labeled with spectrum green) was used to score the numbers of chromosome 16 in both interphase and metaphase spleen cells from mice transgenic for human PS-1 compared to nontransgenic mice. DAPI was used as a counterstain. Diploid chromosome 16 is seen as two signals in the interphase cell on the left and seen as 4 signals or 2 pairs of signals in the metaphase cell on the right.
Figure 14 Mouse cells demonstrating three signals (trisomy) identified as aneuploid.
Fluorescence in situ hybridization generated cells with one to four signals. To avoid scoring incomplete hybridizations as aneuploid, cells with one signal were not considered aneuploid but were used as a measure of incomplete hybridization and only cells with three signals were scored as aneuploid.
been scored in mice expressing the normal WT human PS-1 gene (Figure 15). Also, consistent with the chromosome analysis by Giemsa staining, FISH revealed an increased level of aneuploidy in cells carrying the M146V mutation compared to cells carrying the M146L mutation.

So far, using two independent techniques for assaying aneuploidy, it could be demonstrated that FAD mutant PS-1 genes induce aneuploidy in lymphocytes of transgenic mice. In addition, there was a clear correlation between the level of aneuploidy and the age of the mice and the level of aneuploidy in relation to the more aggressive M146V mutation. (Table 2)

Detection of trisomy 16 in primary neurons of FAD mutant PS-1 transgenic mice via fluorescence in situ hybridization (FISH). Because FISH works independently of a specific cell cycle phase, it can be used to measure aneuploidy in dividing as well as non-dividing cells. This becomes extremely important in assessing levels of aneuploidy in the degenerating neurons of an AD mouse model because with the exception of a small percentage of neurons residing in the hippocampus, neurons are considered to be non-dividing cells (Eriksson et al., 1998; Gage et al., 1995; Gould et al., 1999a; Gould et al., 1999b; Kempermann et al., 2003; Kuhn et al., 1996; Rakic, 1985; Rakic, 2002; Taupin and Gage, 2002). Therefore, FISH is an excellent tool to detect and analyze aneuploidy in neurons. The key steps to this procedure were to ensure that only
Quantitative in situ hybridization of spleen cells from transgenic (WT = normal human PS-1, M146L = FAD mutant M146L PS-1, M146V = FAD mutant M146V PS-1) and non transgenic mice (NON) revealed significantly higher levels of trisomy 16 in the mutant PS-1 spleen cells. Mice were 14-19 months of age.

Figure 15  Aneuploidy induced in mouse spleen cells by a FAD mutant presenilin transgene as detected by fluorescence in situ hybridization.
### Aneuploidy in Spleen Cells from PS-1 Mutant Mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Age (mo)</th>
<th>% Aneuploidy</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS1 6.2 M146L</td>
<td>8</td>
<td>1.6</td>
</tr>
<tr>
<td>PS1 6.2 M146L</td>
<td>13</td>
<td>3.2</td>
</tr>
<tr>
<td>PS1 6.2 M146L</td>
<td>17</td>
<td>25.9</td>
</tr>
<tr>
<td>PS1 6.2 M146L</td>
<td>20</td>
<td>19.2</td>
</tr>
<tr>
<td>PS1 6.2 M146L</td>
<td>&gt;24</td>
<td>21.4</td>
</tr>
<tr>
<td>PS1 5.1 M146L</td>
<td>20</td>
<td>28.6</td>
</tr>
<tr>
<td>PS1 8.9 M146V</td>
<td>17</td>
<td>31.8</td>
</tr>
<tr>
<td>PS1 8.9 M146V</td>
<td>20</td>
<td>41.5</td>
</tr>
<tr>
<td>PS1 wt 4.8</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>PS1 wt 4.8</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>PS1 wt 4.8</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>PS1 wt 10</td>
<td>17</td>
<td>12.7</td>
</tr>
<tr>
<td>Non Tg</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Non Tg</td>
<td>17</td>
<td>12.7</td>
</tr>
<tr>
<td>Non Tg</td>
<td>&gt;24</td>
<td>21</td>
</tr>
</tbody>
</table>

**Table 2** Aneuploidy in spleen cells of PS-1 transgenic mice related to mutation and age. The table above shows a correlation between the level of aneuploidy and the age of the mice and the level of aneuploidy in relation to the more aggressive M146V mutation.
neurons were isolated and that they were assayed as quickly as possible in order to maintain their in vivo conditions. Whole mouse brains from the PS-1 transgenic and non-transgenic mice were rapidly processed as described in Materials and Methods to yield primary neuronal cultures. Basically, after harvesting the whole brain, the meninges was removed and the tissue was then triturated in serum-free media. The cell suspension was plated on single well chamber slides and allowed to incubate at 37°C for one hour. The medium was removed, thereby eliminating debris and non-neuronal cells and the adhered neurons were incubated in fresh medium overnight. The next day the cells were fixed with 3:1 methanol:acetic acid and stained with the neuronal marker, NeuN. Cells prepared with this procedure were over 90% positive for NeuN (Figure 16). Using both phase contrast and DAPI counterstain, the total number of cells per viewing area was determined.

The isolated neurons were then hybridized with the mouse chromosome 16 BAC probe. The hybridization efficiency was determined to be approximately 80%. As expected, most neurons were disomic, i.e. exhibiting two signals (Figure 17, A). However, there was approximately 3% aneuploidy (i.e. trisomy 16; Figure 17, B) in neurons from the FAD mutant PS-1 (M146L) transgenic mice and 4% trisomy 16 in neurons from the FAD mutant PS-1 (M146V) transgenic mice (Figure 18). Again, the M146V mutation did result in a slightly higher percentage aneuploidy in comparison to the M146L mutation but the difference was not statistically significant. Neurons from the nontransgenic mice showed
**Figure 16** Primary mouse neurons isolated from whole brain in preparation for in situ hybridization. Primary neurons were isolated from transgenic and non transgenic mice and labeled with mouse anti-neuronal nuclei (NeuN) monoclonal antibody (1:100 dilution). Visualization of neurons was performed with Alexa 568-conjugated anti-mouse antibody (1:1000 dilution).
Figure 17  Aneuploidy identified in PS-1 FAD transgenic mice primary neurons by FISH with a BAC probe. Fluorescence in situ hybridization with a BAC probe (labeled with spectrum green) was used to score chromosome 16 in cultured neurons from PS-1 transgenic and non transgenic mice. DAPI used as a counterstain. Chromosome 16 trisomy (B) reveals aneuploidy in neurons of PS-1 transgenic mice. Only 2 signals (disomy) were usually observed in neurons from nontransgenic mice (A).
Figure 18 Aneuploidy induced in primary mouse brain neurons by a FAD presenilin transgene.
Quantitative in situ hybridization of neurons from transgenic (WT = normal human PS-1, M146L = FAD mutant M146L PS-1, M146V = FAD mutant M146V PS-1) and non transgenic mice (NON) revealed significantly higher levels of trisomy 16 in the neurons from mice transgenic for FAD mutant PS-1. All mice were 14-19 months of age.
only 0.2% trisomy 16 aneuploidy, and the PS-1 WT neurons 0.6%. Approximately
1500 neurons from more than 15 animals 14 – 19 months of age were counted in
blinded studies.

Detection of trisomy 16 in primary neurons of FAD mutant PS-1 knock-in
mice via fluorescence in situ hybridization (FISH). Transgenic mice can
exhibit features that are partly due to the location of the transgene and/or an
abnormal level of the transgene expression. To assure that the significant
increases in levels of aneuploidy that were found in transgenic mice harboring
the FAD mutant PS-1 gene are due to the mutation and not the overexpression
of the FAD mutant PS-1 gene, primary neurons of PS-1 knock-in mice were
analyzed. In these mice, a mutant human PS-1 (M146V) gene replaces the
mouse PS-1 gene at its endogenous location and is expressed under the control
of the mouse PS-1 promoter. Therefore, these knock-in mice lack mouse wild-
type PS-1 and express mutant human PS-1 at endogenous levels. One feature
of these PS-1 knock-in mice that has been reported so far is an increased
amount of Aβ 1-42 in the brain (Guo et al., 1999; Guo et al., 1999; Mattson et al.,
2000).

To investigate chromosome aneuploidy in the neurons of the PS-1 knock-
in mice, primary neuronal cultures were prepared for in situ hybridization with the
BAC 16 probe as previously described. As shown in Figure 19, there was almost
4% trisomy 16 in neurons from the FAD mutant PS-1 (M146V) knock-in mice while nearly no trisomy 16 aneuploidy was found in neurons from nontransgenic mice. Interestingly, the score of trisomy 16 aneuploid in neurons of the PS-1 (M146V) knock-in mice was almost identical to the aneuploidy detected in neurons from the PS-1 (M146V) transgenic mice. This result strongly indicates that the effect of the human PS-1 transgenes reported earlier for mouse neurons is not due to an increased expression level of the PS-1 mutant but to an inherent capacity of the mutant itself.

Transgenic and knock-in mice used for this study have been summarized in Table 3 listing strain, age, tissue, and technique analysis.
Figure 19  Aneuploidy detected in primary mouse neurons of M146V PS-1 KI mice. Quantitative in situ hybridization of cultured neurons from mutant (M146V) PS-1 Knock In (PS KI) and non transgenic mice revealed significantly higher levels of trisomy 16 in the (M146V) PS-1 KI neurons. PS KI mice were 10-15 months of age and the non transgenics were 17 months of age.
<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Geno</th>
<th>Age (mo)</th>
<th>Tissue</th>
<th>Assay</th>
<th>Aneuploidy %</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT2-2</td>
<td>Non</td>
<td>24.5</td>
<td>spleen</td>
<td>karyo</td>
<td>25.5</td>
</tr>
<tr>
<td>T73-6</td>
<td>Non</td>
<td>23</td>
<td>spleen</td>
<td>karyo</td>
<td>23</td>
</tr>
<tr>
<td>PA61-1</td>
<td>Non</td>
<td>27</td>
<td>spleen</td>
<td>karyo</td>
<td>31</td>
</tr>
<tr>
<td>WT5-7</td>
<td>Non</td>
<td>17</td>
<td>spleen</td>
<td>karyo</td>
<td>12.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>BAC</td>
<td>8.5</td>
</tr>
<tr>
<td>WT5-1</td>
<td>Non</td>
<td>17</td>
<td>spleen</td>
<td>karyo</td>
<td>14.8</td>
</tr>
<tr>
<td>WT5-3</td>
<td>Non</td>
<td>17</td>
<td>spleen</td>
<td>karyo</td>
<td>18.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17 neurons</td>
<td>0</td>
</tr>
<tr>
<td>WT5-8</td>
<td>Non</td>
<td>17</td>
<td>spleen</td>
<td>karyo</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>BAC</td>
<td>5.5</td>
</tr>
<tr>
<td>WT6-5</td>
<td>Non</td>
<td>17.5</td>
<td>spleen</td>
<td>karyo</td>
<td>27.5</td>
</tr>
<tr>
<td>WT6-2</td>
<td>Non</td>
<td>17.5</td>
<td>spleen</td>
<td>karyo</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17 neurons</td>
<td>0</td>
</tr>
<tr>
<td>WT2a-8</td>
<td>Non</td>
<td>26</td>
<td>spleen</td>
<td>karyo</td>
<td>15</td>
</tr>
<tr>
<td>WT5-3</td>
<td>Non</td>
<td>17</td>
<td>spleen</td>
<td>BAC</td>
<td>5.3</td>
</tr>
<tr>
<td>WT8-3</td>
<td>Non</td>
<td>17</td>
<td>neurons</td>
<td>BAC</td>
<td>0.8</td>
</tr>
<tr>
<td>WT5a-1 or WT8a-1</td>
<td>Non</td>
<td>17.5</td>
<td>neurons</td>
<td>BAC</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Non</td>
<td>17</td>
<td>spleen</td>
<td>karyo</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Non</td>
<td>&gt;24</td>
<td>spleen</td>
<td>karyo</td>
<td>21</td>
</tr>
<tr>
<td>WT2a-6</td>
<td>WT</td>
<td>24</td>
<td>spleen</td>
<td>karyo</td>
<td>12.7</td>
</tr>
<tr>
<td>WT5-9</td>
<td>WT</td>
<td>17</td>
<td>spleen</td>
<td>karyo</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>BAC</td>
<td>10.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>neurons</td>
<td>0</td>
</tr>
<tr>
<td>WT6-1</td>
<td>WT</td>
<td>17.5</td>
<td>spleen</td>
<td>karyo</td>
<td>19.5</td>
</tr>
<tr>
<td>WT6-5</td>
<td>WT</td>
<td>17</td>
<td>spleen</td>
<td>BAC</td>
<td>4.9</td>
</tr>
<tr>
<td>WT6-6</td>
<td>WT</td>
<td>17.5</td>
<td>spleen</td>
<td>karyo</td>
<td>20</td>
</tr>
<tr>
<td>WT8-1</td>
<td>WT</td>
<td>17</td>
<td>spleen</td>
<td>BAC</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>neurons</td>
<td>1.8</td>
</tr>
<tr>
<td>WT5-2</td>
<td>WT</td>
<td>17</td>
<td>spleen</td>
<td>karyo</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>neurons</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>13</td>
<td>spleen</td>
<td>karyo</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>16</td>
<td>spleen</td>
<td>karyo</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>20</td>
<td>spleen</td>
<td>karyo</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>17</td>
<td>spleen</td>
<td>karyo</td>
<td>12.7</td>
</tr>
<tr>
<td>M146L</td>
<td>&gt;24</td>
<td>spleen</td>
<td>BAC</td>
<td></td>
<td>28.3</td>
</tr>
<tr>
<td>GV53-2</td>
<td>M146L</td>
<td>15</td>
<td>spleen</td>
<td>karyo</td>
<td>31.6</td>
</tr>
<tr>
<td>NX9-1 or NX3-1</td>
<td>M146L</td>
<td>17</td>
<td>spleen</td>
<td>karyo</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>neurons</td>
<td>2.9</td>
</tr>
<tr>
<td>HN3-4</td>
<td>M146L</td>
<td>17</td>
<td>spleen</td>
<td>BAC</td>
<td>11.5</td>
</tr>
<tr>
<td>GV58-7</td>
<td>M146L</td>
<td>15.5</td>
<td>spleen</td>
<td>karyo</td>
<td>12.7</td>
</tr>
<tr>
<td>NX9-3</td>
<td>M146L</td>
<td>18</td>
<td>spleen</td>
<td>karyo</td>
<td>20.4</td>
</tr>
<tr>
<td>NX9-4</td>
<td>M146L</td>
<td>18</td>
<td>neurons</td>
<td>BAC</td>
<td>2.9</td>
</tr>
<tr>
<td>GV53-8</td>
<td>M146L</td>
<td>16</td>
<td>neurons</td>
<td>BAC</td>
<td>6.2</td>
</tr>
<tr>
<td>Transgenic/Knock In</td>
<td>Age</td>
<td>Tissue</td>
<td>Method</td>
<td>Aneuploidy</td>
<td></td>
</tr>
<tr>
<td>--------------------</td>
<td>-----</td>
<td>--------</td>
<td>--------</td>
<td>------------</td>
<td></td>
</tr>
<tr>
<td>GV58-5</td>
<td>15.5</td>
<td>spleen</td>
<td>karyo</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>M146L</td>
<td>20</td>
<td>spleen</td>
<td>karyo</td>
<td>28.6</td>
<td></td>
</tr>
<tr>
<td>51A33a.2</td>
<td>14</td>
<td>spleen</td>
<td>karyo</td>
<td>12.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>BAC 14.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>neurons BAC 2.7</td>
<td></td>
</tr>
<tr>
<td>51A33a-3</td>
<td>14</td>
<td>neurons</td>
<td>BAC</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>51A33a-8</td>
<td>14</td>
<td>spleen</td>
<td>BAC</td>
<td>19.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>neurons BAC 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>spleen</td>
<td>karyo</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>spleen</td>
<td>karyo</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>spleen</td>
<td>karyo</td>
<td>25.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>spleen</td>
<td>karyo</td>
<td>19.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>spleen</td>
<td>karyo</td>
<td>13.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;24</td>
<td>spleen</td>
<td>karyo</td>
<td>21.4</td>
<td></td>
</tr>
<tr>
<td>PA91.6</td>
<td>20.5</td>
<td>spleen</td>
<td>karyo</td>
<td>8.4</td>
<td></td>
</tr>
<tr>
<td>PA91.7</td>
<td>20.5</td>
<td>spleen</td>
<td>karyo</td>
<td>41.5</td>
<td></td>
</tr>
<tr>
<td>PA213-7</td>
<td>17</td>
<td>spleen</td>
<td>karyo</td>
<td>41.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>BAC 15.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>spleen</td>
<td>karyo</td>
<td>26.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>BAC 24.8</td>
<td></td>
</tr>
<tr>
<td>HPS 188-7</td>
<td>19</td>
<td>spleen</td>
<td>karyo</td>
<td>20.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>BAC 16.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>neurons BAC 5.1</td>
<td></td>
</tr>
<tr>
<td>PA194b-5 or 8H2-5</td>
<td>18.5</td>
<td>neurons</td>
<td>BAC</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>PA208-3</td>
<td>18</td>
<td>neurons</td>
<td>BAC</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>spleen</td>
<td>karyo</td>
<td>31.8</td>
<td></td>
</tr>
<tr>
<td>1257 WT</td>
<td>6</td>
<td>neurons</td>
<td>BAC</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>1258 WT</td>
<td>6</td>
<td>neurons</td>
<td>BAC</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>1259 WT</td>
<td>6</td>
<td>neurons</td>
<td>BAC</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>797 KI</td>
<td>15</td>
<td>neurons</td>
<td>BAC</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>834 KI</td>
<td>11</td>
<td>neurons</td>
<td>BAC</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>1003 KI</td>
<td>10</td>
<td>neurons</td>
<td>BAC</td>
<td>2.9</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3** Transgenic and Knock In mice analyzed for chromosome missegregation. Transgenic mice used to generate all graphs are listed in the table above along with age, tissue sample assayed, method of analysis, and percentage of aneuploidy.
Conclusions

The current study has examined whether transgenic mice harboring and expressing either the normal WT human PS-1 gene or one of two FAD mutant PS-1 genes (M146L or M146V) developed chromosome aneuploidy, specifically in their lymphocytes or neurons. While previous studies implicated the involvement of PS-1 in the cell cycle and in chromosome segregation, the data presented here provide the first definitive in vivo evidence that PS-1 FAD mutations cause aneuploidy. Additional results in this study using FAD PS-1 knock-in mice that express the mutant human PS-1 at an endogenous level demonstrate that the aneuploidy is specifically due to the PS-1 mutation and not to overexpressed levels of protein.

Aneuploidy in lymphocytes from FAD mutant PS-1 transgenic mice. Significant levels of aneuploidy were detected in lymphocytes from transgenic mice harboring and expressing FAD mutant PS-1 genes in comparison to the normal human PS-1 gene or the non-transgenic mice. Two FAD mutations that were assayed were the M146L and the M146V. As previously stated, the M146 locus is where most of the aggressive mutations associated with a stronger phenotype occur. In accordance with this observation, the current results showed that the M146V mutation was associated with significantly higher levels of chromosome missegregation.
Additional work to confirm PS-1 mutations as cause of chromosome aneuploidy was carried out in mouse lymphocytes using chromosome metaphase analysis and fluorescence in situ hybridization. Lymphocytes are excellent for chromosome metaphase analysis because they easily divide in mitogen stimulated culture. While the chromosome metaphase analysis allowed for the detection of all chromosomes it was also quite tedious and time consuming. Therefore, fluorescence in situ hybridization provided a much more efficient technique for this study and eventually also proved to be more powerful than chromosome metaphase analysis.

BAC probe. Of particular importance to this study is the fact that in situ hybridization allows scoring of chromosomes independently of the specific cell cycle phase. For the FISH assay, a BAC probe that detects chromosome 16 was used because of its homology to human chromosome 21. Based on a similar pathology between Down syndrome individuals and AD patients, Potter and colleagues (Potter et al., 1995; Potter, 1999) had started preliminary analysis of chromosome missegregation with chromosome 21. As a first follow up to these findings, initially mouse chromosome 16 was chosen for the current investigations. However, later work demonstrated that chromosome missegregation is not unique to one specific chromosome.
In summary, chromosome metaphase analysis via Giemsa staining revealed that the FAD mutant PS-1 mice carry approximately 27-33% detectable aneuploid lymphocytes compared to 15% in the WT PS-1 and non-transgenic mice. FISH analysis detected 15-19% aneuploidy in the FAD mutant PS-1 mice and approximately 7% in the WT PS-1 and non-transgenic mice. When comparing the counts obtained from metaphase chromosome analysis with those from in situ hybridization, it was noticed that indeed when counting every chromosome via chromosome metaphase analysis you have an overall higher percentage of aneuploidy. Most importantly, in both metaphase chromosome analysis and FISH, the FAD mutant PS-1 is significantly higher.

**Aneuploidy in neurons from FAD mutant PS-1 transgenic mice.** Much of the work performed for the analysis of aneuploidy in neurons was similar to the work done on lymphocytes. An obvious difference was that neurons are mostly at interphase, which allowed analysis only by FISH. The relative percentage of aneuploidy in neurons was comparable to the results in lymphocytes. However, possibly due to the low levels of cell division in neurons, lower levels of aneuploidy were detected. Still, the neurons of FAD mutant PS-1 mice showed 3-4% aneuploidy compared to less than 1% seen in the WT PS-1 and non-transgenic mice. While it is important to show FAD mutant PS triggers aneuploidy also in neurons because Alzheimer’s pathology is primarily seen in
the brain, the current study is driven by the hypothesis that chromosome missegregation is a ubiquitous event in all cells expressing an FAD mutant presenilin.

_Aneuploidy in neurons from FAD mutant PS-1 knock-in mice._ The FAD PS-1 knock-in mice expressing normal levels of FAD PS-1 proved to be a powerful experimental tool for this study. Significant levels of aneuploidy in the FAD PS-1 knock-in mice demonstrate that the defective chromosome segregation is due to the FAD PS mutation and not the protein expression levels. In summary, the neurons of FAD PS-1 transgenic mouse showed 4% aneuploidy compared to almost 0% aneuploidy in non-transgenic mice. Aneuploid neurons were detected in equal frequency in the neurons of the PS-1 (M146V) knock-in mice and in neurons from the PS-1 (M146V) transgenic mice. This similarity again demonstrates that the aneuploidy observed in the FAD mutant PS-1 transgenic mice is a result of the PS-1 mutation and not the PS-1 expression level.

The data presented so far strongly suggests that FAD mutant PS-1 causes chromosome missegregation resulting in aneuploidy. This is clearly demonstrated with both the FAD mutant PS-1 transgenic mice and the PS-1 knock-in mice which have proved to be excellent models for AD research. In addition, two well-established cytogenetic techniques were employed and the results obtained from each complemented the other.
Part II: Aneuploidy Develops in PS-1 Transfected Human Cells, But Not in Cells Transfected With Dominant Negative PS-1 Genes Lacking γ-Secretase Activity.

Abstract

Previous experiments in this study have shown that transgenic and knock-in mice harboring the FAD mutant PS-1 gene carry more than twice the amount of aneuploid lymphocytes and neurons than their WT counter parts. To further prove that the FAD mutant PS-1 as the causative agent for the aberrant chromosome segregation, WT PS-1 and FAD mutant (M146L) PS-1 were transiently expressed in hTERT-HME1 cells and aneuploidy was assayed. A preliminary obstacle to this analysis was the karyotype instability that is characteristic of oncogene-immortalized cell lines, which makes the aneugenic effect of the presenilins possible but difficult to measure (Potter, 2003). To bypass this problem, the hTERT-HME1 cell line, a primary human mammary epithelial cell line that expresses the telomerase reverse transcriptase from a permanently-transfected hTERT plasmid and is thus both immortal and karyotypically stable was used. The chromosome content was assayed by both metaphase chromosome analysis and DNA fluorescence in situ hybridization. In addition, the role of gamma secretase and its effect on chromosome segregation were investigated by transiently transfecting hTERT-HME1 cells with dominant negative PS-1 genes lacking γ-secretase activity.
Introduction

In the previous set of experiments, both transgenic and knock-in mice were used as experimental systems to assess the effects of PS-1 on chromosome segregation and thus aneuploidy. Transgenic mice harboring and expressing either the normal human PS-1 gene or one of two FAD mutant PS-1 genes (M146V or M146L) along with knock-in mice expressing endogenous levels of FAD mutant PS-1 were assayed for aneuploidogenic cells. In general, animal models are a useful tool to study the effects of genetic abnormalities. However, due to many components that contribute to the phenotype of a given organism, it can be difficult to attribute with certainty, specific effects to a single protein or its mutated form. Therefore, to prove a specific mutated protein as the predominant cause of an effect, such as chromosome missegregation being caused by FAD mutant PS-1, other experimental systems such as immortalized cell lines are valuable additional means to define and confirm the cause of specific cellular disturbances.

Immortalized mammalian cell lines have provided an invaluable research tool with which to manipulate and study a gene of interest. Specifically, this system allows for long term cell culture and hence continual expression of the transferred gene. Normal cells have a limited life span and enter senescence after a definite number of cell divisions, thereby limiting their use for experimental purposes (Hayflick and Moorhead, 1961; Hayflick, 1965; Martin et al., 1970). An even greater advantage of immortalized cells is that, due to their ability to escape
programmed cell death, they harbor many defects, such as an abnormal karyotype. The latter fact is especially critical for this study. Therefore, the effect of an overexpressed transgene, such as PS-1 on chromosome stability can not be analyzed in most immortalized cell lines.

Recently a method has been developed that generates immortalized cell lines with normal physiology and chromosome complement. For this purpose, a primary human mammary epithelial cell line permanently expressing the telomerase reverse transcriptase was chosen. It is known that a major contributing factor to the senescence of cells is the shortening of their telomeres each time the cell divides. Thus the constitutive expression of the telomerase reverse transcriptase enzyme maintains telomere length and, at the same time, allows cells to divide indefinitely while retaining normal function, phenotype, and karyotype (Jiang et al., 1999; Morales et al., 1999). The enzyme telomerase is composed of at least two subunits; an RNA that serves as a template for the synthesis of telomeric repeats (hTR) and a protein (hTERT) that has reverse transcriptase activity (Feng et al., 1995; Harrington et al., 1997). The RNA component (hTR) is ubiquitous in human cells, but the presence of the mRNA encoding hTERT is not. However, it has been shown that the forced expression of exogenous hTERT in normal human cells is sufficient to produce telomerase activity and thus to prevent the shortening of telomeres and circumvent the induction of senescence while maintaining genomic stability (Jiang et al., 1999; Morales et al., 1999).
Along with a karyotypically stable immortalized cell line (hTERT-HME1 cells), both standard molecular and cytogenetic techniques were implemented to study the direct involvement of the PS-1 gene in chromosome segregation. Transfecting the target gene into a mammalian cell system allows for the direct manipulation and analysis of this gene. Specifically, transfecting WT PS-1 and FAD mutant PS-1(M146L) into hTERT cells allows for a direct assay of aneuploidy that might have been caused by the expression of these genes.

There are various strategies available to transfer a gene of interest into mammalian cells and of these a non-liposomal reagent for transient transfections, such as Fugene6, appears to be an excellent choice for this study. It is easy to use and does not by itself cause aberrancies to the stable karyotype. A total of 10 transfections of hTERT-HME1 cells with FAD mutant PS-1 or dominant negative PS-1 were investigated.

Both metaphase chromosome analysis by Giemsa staining and fluorescence in situ hybridization (FISH) have proven to be useful cytogenetic techniques for the analysis of aneuploidy. As previously described in Results Part I of this dissertation, metaphase chromosome analysis in this study was employed only to identify aneuploidy in metaphase cells while FISH was used to identify individual chromosomes.

One advantage of working with human chromosomes versus mouse chromosomes is the availability of various, commercial ready-to-use, FISH probes for diagnostics. Vysis Inc., in particular, provides multiple DNA probes for
every human chromosome with many specific advantages for the present chromosome segregation study. For example, their Locus Specific Identifier (LSI) DNA probes hybridize to specific sequences on individual chromosomes that are gene or locus specific and thereby label the chromosome of interest. The company also supplies dual labeled probes which allow for the identification of more than one chromosome in the same nucleus. These dual labeled probes may serve more than one purpose. Either both identified chromosomes can be scored or one chromosome can be used as a control for hybridization efficiency while the other chromosome is counted. Finally, because these probes are directly labeled with fluorophores, many of the steps required to visualize the target sequence are eliminated.

For the analysis of aneuploidy in hTERT-HME1 cells, two DNA probes from Vysis – the LSI 21 SpectrumOrange Probe and the LSI TEL/AML1 ES Dual Color Translocation Probe were used. Basically, the LSI 21 SpectrumOrange Probe detects the complementary sequences on chromosome 21 while the LSI TEL/AML1 ES Dual Color Translocation Probe is a mixture of the LSI TEL probe labeled with SpectrumGreen and the LSI AML1 probe labeled with SpectrumOrange and is designed to detect both chromosome 12 and 21. Specifically, four transfections (1, 5, 6, and 7) were probed with the LSI 21 SpectrumOrange probe alone and three transfections (2, 3, and 4) were probed with the LSI TEL/AML1 ES Dual Color Translocation Probe only.
Chromosome 21 is of particular interest to this study because of the direct involvement of chromosome 21 and PS-1 in Alzheimer’s disease. Briefly, the amyloid precursor protein (APP), which is cleaved by gamma-secretase/PS-1 to release Aβ40-42, is located on chromosome 21. In addition, all Down syndrome/trisomy 21 patients who live beyond the age of 30 or 40 develop AD. Also, the first studies of aneuploidy in AD related to FAD mutant PS-1 showed significant levels of trisomy 21 in early onset AD patients. These individuals were also identified to harbor FAD mutant PS-1 genes (Geller and Potter, 1999). Taking this supportive evidence into account, it is reasonable to expect that chromosome 21 would be an ideal marker of missegregation due to PS-1 mutations. In addition to scoring for chromosome 21 aneuploidy with the LSI 21 probe, the dual labeled probe was also used to score for aneuploidy of chromosome 12.

Results

**PS-1 transiently transfected hTERT-HME1 cells develop aneuploidy.**

A primary human mammary epithelial cell line (HME1) that stably expresses the telomerase reverse transcriptase (hTERT) was used as a karyotypically stable immortalized cell line. hTERT-HME1 cells were transiently transfected with WT PS-1, mutant PS-1 (M146L), and the empty vector DNA (pcDNA3). Prior to transfections, preliminary investigations were performed on the hTERT cell line to assure a stable karyotype background. It was found that relatively low passages
(< P9) in culture showed almost no aneuploidy. Cells were maintained as described in Materials and Methods and were harvested accordingly for metaphase chromosome analysis. Basically, the cells were treated with colcemid 10 - 12 hours prior to harvesting to prevent formation of the spindle assembly and thereby collect cells at the metaphase-anaphase border.

Preliminary data analyzing time points beyond 48 hours had demonstrated that there was no change to the phenotype of vector transfected cells including levels of aneuploidy. Hence, at 48 hours post-transfection cells had sufficient time to complete one round of cell division - (BD Bioscience) and were collected. Collected cells were resuspended in 0.075 M KCl hypotonic buffer for 20 minutes to improve the spread of chromosomes and allow for better visualization and were then fixed with cold 3:1 methanol:acetic acid for 30 minutes in preparation for dropping and Giemsa staining as described in the Results section Part I. The stained chromosomes were counted and aneuploid cells scored.

To determine aneuploidy, each individual metaphase spread was counted adhering once again to all extra precautions previously described for the mouse metaphase chromosome analysis. More than 6,000 intact unbroken metaphase spreads were counted and any cell revealing more or less than the normal karyotype (46) was considered aneuploid. (Figure 20 A-C)
Figure 20 Chromosome metaphase analysis of Giemsa stained hTERT-HME1 cells. Visual counting of transiently transfected hTERT-HME1 cells revealed levels of aneuploidy. Anything less than or greater than 46 was considered aneuploid. The chromosome metaphase spread seen in Figure A contains 45 chromosomes and Figure C contains 47 chromosomes. These were scored as aneuploid cells. Figure B contains 46 chromosomes and is scored as a normal karyotype.
Within 48 hours, 29% of cells transiently transfected with the FAD mutant, (M146L) PS-1 became aneuploid by either gaining or losing chromosomes during mitosis. Cells transiently transfected with WT PS-1 expressing plasmid also showed significant yet smaller levels of aneuploidy (18%) compared to the FAD mutant (M146L) PS-1 (29%) and cells transiently transfected with empty vector pcDNA3 showed 10.7% aneuploidy. (Figure 21 A,B). Parallel transfections with Fugene6 only were performed as a control for any aneuploidy which may have resulted from the transfection reagent itself. As an additional control for metaphase chromosome analysis, unmodified hTERT-HME1 cells were cultured in parallel to the transfections. The hTERT-HME1 and Fugene6 results showed minimal if any aneuploidy. (data not shown)

FISH reveals trisomy 21 in PS-1 transiently transfected hTERT-HME1 cells. As previously mentioned, any missegregation of chromosome 21 due to PS-1 is of particular interest to this study because the amyloid precursor protein, which is processed by gamma-secretase/PS-1, is located on chromosome 21. The location of the APP gene on chromosome 21, in conjunction with the fact that all Down syndrome individuals ultimately develop AD as well, suggests some implication of the presenilins in the missegregation of chromosome 21.
Figure 21 Metaphase chromosome analysis revealed aneuploidy induced in hTERT cells transfected with normal and particularly FAD mutant PS-1. hTERT cells were transiently transfected with WT PS-1, mutant (M146L) PS-1, and empty vector (pcDNA3). (A) 6 separate transfections were performed and aneuploidy assayed by metaphase chromosome analysis. (B) Overall results revealed significantly higher aneuploidy in both WT and mutant (M146L) PS-1 when compared to cells transiently transfected with the empty vector (pcDNA3). M146L transfected cells became significantly more aneuploid than cells transfected with normal human PS-1.
To detect chromosome 21 in both interphase and metaphase cells, fluorescence in situ hybridization with a spectrum orange labeled probe specific for chromosome 21 (LSI 21 SpectrumOrange probe or LSI TEL/AML1 ES Dual Color Translocation Probe) was used. The LSI 21 SpectrumOrange Probe comes ready to use and is actually a combination of three probes in the same adjacent region in order to make the overall size of the probe carrying the fluorescent stain large enough to become easily detectable with the microscope. While the LSI TEL/AML1 ES Dual Color Translocation Probe is capable of detecting two chromosomes simultaneously, 12 and 21, in this section of the study it was only used to detect chromosome 21. Therefore, the pertinent portion of the probe is AML1 which spans a 500kb region on chromosome 21 and is labeled with spectrum orange.

Parallel cultures of hTERT-HME1 cells were transiently transfected with WT PS-1, with FAD mutant (M146L) PS-1, and with the vector DNA (pcDNA3), as previously described for metaphase chromosome analysis. The parallel transfections were carried out to assure that all conditions were as similar as possible for both assays. However, one deviation has to be noted: The hTERT-HME1 cells used for FISH did not receive colcemid or hypotonic treatment. Besides this assay specific treatment difference, all other steps were executed exactly the same and in parallel to the other transfections.
After fixed cells were dropped onto slides either the LSI 21 SpectrumOrange probe or the LSI TEL/AML1 ES Dual Color Translocation Probe was applied and allowed to hybridize to the target DNA for detection of chromosome 21. Basically, the cells were permeabilized with 2XSSC, then dehydrated in serial ethanol washes, after which the probe mixture (7 µL LSI/WCP hybridization buffer, 1 µL LSI or LSI TEL/AML1 ES probe, 2 µL purified water) was added to the target area. A HYBrite hybridization chamber was used to co-denature the probe and the target area at 75°C for 4 minutes and then hybridized at 37°C for 16 hours. After hybridization, the slides were washed in 0.4XSSC and 2XSSC/0.1% NP-40 to remove non-specifically adhering probe and then DAPI counterstained to visualize the cells.

Approximately 2500 cells for each transfection were efficiently hybridized and blindly scored for the number of fluorescent orange spots to measure aneuploidy for chromosome 21 in transiently transfected hTERT-HME1 cells. Again, any nuclei that were overlapping or even slightly touching were not counted. Similar to the mouse lymphocytes (see Results Part I), depending on the cell cycle phase in which the individual cells were harvested, WT metaphase and interphase cells were expected to harbor an even number of chromosomes and thus show usually two and sometimes four signals. It should be noted that the appearance of four signals occurred at the same frequency in the hTERT-HME1 cells as compared to the mouse lymphocytes.
According to Vysis Enumeration Guidelines, ideally signals should appear as bright and compact oval shapes but sometimes they are split into two smaller signals in very close proximity or appear as a stringy diffuse shape. (Figure 22A) For this study, signals that looked too diffuse were not scored to assure they were not a sign of incomplete hybridization. Split signals were taken into calculation when the distance between the two signals was smaller than the diameter of the smallest signal. Once again, only triple signals were scored as aneuploid as shown in Figure 22B. Rigorous adherence to these guidelines assured that rather some true aneuploid cells could be missed but false ones would not be counted and most likely resulted in conservative levels of aneuploidy to be reported.

The same conservative scoring process was applied to each sample analyzed for aneuploidy. An example of a working score sheet guideline has been included. (Figure 23)

Cells transiently transfected with a FAD mutant (M146L) PS-1 expressing plasmid showed 3.8% trisomy 21 within 48 hours. Cells transiently transfected with WT PS-1 expressing plasmid showed 2.6% trisomy 21 and cells transiently transfected with empty vector pcDNA3 showed 1.4% trisomy 21. (Figure 24 A,B) Again, parallel transfections and chromosome analysis was carried out with Fugene6 treated and unmodified hTERT-HME1 cells as a control for any aneuploidy which may have resulted from the transfection reagent itself.
Figure 22  Transiently transfected hTERT cells hybridized with LSI 21 Spectrum Orange. Cells were evaluated according to strict guidelines to ensure that only clear, compact signals were enumerated (B). Any cells containing split or diffuse signals (A) were not scored. Figure B was scored as a trisomic cell containing three copies of chromosome 21.
Figure 23  Score sheet used for Fluorescence in situ hybridization assays.
To assure consistent counting, a score sheet similar to the schematic depicted above was used. This was a modification of one provided by Vysis incorporating all strict guidelines suggested by the company as well. The use of this sheet ensured that all cells were scored with the same consistency.
Figure 24  FISH revealed trisomy 21 induced in hTERT-HME1 cells transfected with normal and particularly FAD mutant PS-1.  hTERT cells were transiently transfected with WT PS-1, mutant (M146L) PS-1, and empty vector (pcDNA3).  (A) 7 separate transfections were performed and aneuploidy assayed by fluorescence in situ hybridization.  (B) Overall results revealed significantly higher aneuploidy in both WT and mutant (M146L) PS-1 when compared to cells transiently transfected with the empty vector (pcDNA3).  M146L transfected cells became significantly more aneuploid than cells transfected with normal human PS-1.
The hTERT-HME1 and Fugene6 results showed minimal if any aneuploidy. (data not shown) In sum, hTERT-HME1 cells transiently transfected with either WT PS-1 or mutant PS-1 (M146L) expressing plasmid showed significant increased levels of trisomy 21.

Aneuploidy in hTERT-HME1 cells expressing PS-1 is not chromosome specific. While missegregation of chromosome 21 due to PS-1 is of particular interest to this study, the basic hypothesis of the current study, the intimate involvement of the presenilins in the cell cycle and chromosome segregation, predicts that presenilin induces cell cycle defects that affect ALL chromosomes in a given genome. Theoretically all chromosomes are capable of missegregating with the same frequency but the actual result is that cells are only viable with trisomy or monosomy of smaller chromosomes. Also small chromosomes missegregate more early. To test if PS-1 induced aneuploidy was specific only to chromosome 21, another chromosome was investigated with the same approach.

The LSI TEL/AML1 ES Dual Color Translocation Probe is a mixture of the LSI TEL probe labeled with SpectrumGreen and the LSI AML1 probe labeled with SpectrumOrange and is designed to detect both chromosome 12 and 21 respectively. For this part of the study, it was used to detected chromosome 12 in interphase and metaphase cells. Therefore, the pertinent portion is the LSI
TEL probe which begins between exons 3-5 of the TEL gene and extends approximately 350 kb toward the telomere on chromosome 12. (Figure 25 A,B)

The transiently transfected hTERT-HME1 samples used for this analysis have already been described in the previous section. A total of 7 transient transfections were analyzed for trisomy 21 in the previous section. Of those 7, transfections 2, 3, and 4 were probed with the LSI TEL/AML1 ES Dual Color Translocation Probe which allowed for detection of both trisomy 12 and trisomy 21.

Approximately 7500 cells were efficiently hybridized and then blindly scored for the number of fluorescent green spots to measure aneuploidy for chromosome 12 in transiently transfected hTERT-HME1 cells. The same strict guidelines previously described for signal counting were used to detect trisomy 12 in this set of experiments. Once again, these strict guidelines most likely resulted in conservative levels of aneuploidy to be reported.

The end results showed that hTERT-HME1 cells transiently transfected with PS-1 (M146L) and PS-1 WT showed a significant 3.2% and 1.8% trisomy 12 respectively in comparison to pcDNA3 which showed 0.3%. (Figure 26 A,B)
Figure 25 PS-1-transfected human cells develop both trisomy 12 and trisomy 21 aneuploidy. FISH with the LSI TEL/AML1 ES dual color probe was used to detect both chromosome 12 (Spectrum Green) and 21 (Spectrum Orange) in PS-1 transfected hTERT cells. DAPI was used as a counterstain. Chromosome 12 and chromosome 21 trisomy (B) reveals aneuploidy in a PS-1 transfected hTERT cell. A typical normal hTERT cell (A) reveals normal (disomy) karyotype as evidenced by 2 red signals and 2 green signals.
Fluorescence in situ hybridization was performed on WT PS-1, mutant (M146L) PS-1, and empty vector (pcDNA3) transiently transfected hTERT cells. (A) 3 separate transfections were probed with the LSI TEL/AML1 ES Dual Color Translocation Probe. (B) Overall results revealed significantly higher aneuploidy in both WT and mutant (M146L) PS-1 when compared to cells transiently transfected with the empty vector (pcDNA3). M146L transfected cells became significantly more aneuploid than cells transfected with normal human PS-1.
Transiently transfected hTERT-HME1 cells exhibiting aneuploidy showed no evidence of tetrasomy, only trisomy. Previous studies had reported the presence of combined trisomic and tetrasomic cells in certain regions of the human AD brain (Husseman et al., 2000; Obrenovich et al., 2003; Yang et al., 2001; Yang et al., 2003). The reports attributed the tetrasomy to possible cell cycle abnormalities which cause chromosome duplication in neurons without cell division and poor hybridization as the cause of cells showing apparent ‘trisomies’. The current study does not confirm the presence of tetrasomic cells.

Specifically, transiently transfected hTERT-HME1 cells were scored for tetrasomy of chromosome 21 with either the LSI 21 SpectrumOrange Probe or the LSI TEL/AML1 ES Dual Color Translocation Probe. All samples showed approximately the same values. No tetrasomy higher than the baseline in the control cells could be found. (Figure 27) Chromosome 12 showed similar results, as expected. (data not shown)
Figure 27 hTERT-HME1 cells transiently transfected with PS-1 expressing plasmids show no evidence of tetrasomy 21. hTERT-HME1 cells expressing PS-1 (WT PS-1 and FAD mutant PS-1) showed no increased levels of tetrasomy 21 when compared to hTERT-HME1 cells alone or hTERT-HME1 cells transiently transfected with the empty vector pcDNA3. Either the LSI 21 SpectrumOrange Probe or the LSI TEL/AML1 ES Dual Color Translocation Probe was used to detect chromosome 21.
hTERT-HME1 cells transiently transfected with dominant negative PS-1 lacking γ-secretase activity do not develop aneuploidy. The next goal was to investigate the mechanism by which presenilin overexpression or mutation leads to chromosome missegregation and aneuploidy. The only known function of the presenilin protein is to serve as an essential part of a proteolytic enzyme complex termed γ-secretase (Haass and De Strooper, 1999; Hardy and Selkoe, 2002). This proteolytic activity cleaves a number of Type 1 transmembrane proteins including Notch and the Alzheimer Amyloid Precursor Protein (APP). In each case, the cleavage occurs in the transmembrane domain of the target protein and releases an intracellular domain that then causes changes in gene expression. The mechanism of the proteolytic reaction occurs through an aspartate protease, and two aspartic acids within transmembrane domains 6 and 7 have been identified as essential for this process (Wolfe, 2003).

To determine whether the effect of PS-1 on chromosome segregation is due to its γ-secretase activity, hTERT-HME1 cells were transformed with genes encoding either wild type PS-1 or a dominant negative form of PS-1. The dominant negative forms of PS-1 lack γ-secretase activity either by a mutation of one of the key aspartates (D385E) or by deletion in exons 1 and 2 (ΔTM1-2). (Murphy et al., 2000). (Figure 28) Specifically, a new set of plasmids with a different vector (PAG3) were transfected into the hTERT-HME1 cells, and trisomy 21 assayed by FISH. Figure 29 demonstrates that expression of a dominant negative mutation (D385E) of PS-1 results in 0.6% aneuploidy and
**Figure 28 Dominant negative PS-1.** Dominant negative forms of PS-1 lack γ-secretase activity either by a mutation of one of the key aspartates (D385E) or by deletion in exons 1 and 2 (ΔTM1-2).
expression of a dominant negative deletion (ΔTM1-2) of PS-1 results in 0.2% aneuploidy—both significantly less than the aneuploidy found in WT PS-1 (2.0%) transfected cells and rather comparable to the background aneuploidy in vector-transfected cells.

Conclusions

The current study shows that a karyotypically stable immortalized cell line transiently transfected with WT PS-1 and FAD mutant PS-1(M146L) developed aneuploidy after 48 hours. In addition, the use of dominant negative mutants of PS-1 determined that specifically the γ-secretase activity of PS-1 rules the underlying mechanism that causes aneuploidy. The experiments with the dominant negative mutants of PS-1 now are an important follow up to the earlier study (Results Part I) which demonstrated that transgenic mice harboring and expressing FAD PS-1 mutations (M146L and M146V) also developed aneuploidy. These results can now be attributed to mechanistic errors in the γ-secretase activity of the presenilin mutants.

One particular obstacle in carrying out these experiments, the natural chromosome instability of immortalized cell lines, was overcome by the use of the hTERT-HME1 cell line. Metaphase chromosome analysis and in situ
Figure 29 γ-secretase activity is required for presenilin-induced chromosome missegregation. hTERT cells were transiently transfected for 48 hours with either WT PS-1 (normal human PS-1), dominant negative forms of PS-1 lacking γ-secretase activity either by mutation (D385E) or deletion (ΔTM1-2), or the empty vector control (PAG3). Quantitative FISH for chromosome 21 revealed significant decreases in chromosome missegregation in cells expressing the dominant negative forms of PS-1 compared to WT PS-1.
hybridization was used to assess levels of aneuploidy and both methods revealed comparable results – overexpressed WT PS-1 and FAD mutant (M146L) PS-1 induced significant levels of aneuploidy with WT PS-1 inducing less than FAD PS-1 (M146L).

\textit{hTERT-HME1 cells transiently transfected with PS-1 expressing plasmids develop aneuploidy as detected by metaphase chromosome analysis and FISH.}

In this study, both metaphase chromosome analysis and fluorescence in situ hybridization (FISH) revealed increased aneuploidy in cells transiently expressing a presenilin gene, with significantly higher levels of aneuploidy in cells expressing FAD mutant PS-1 (M146L). For this study, we used one of the same mutations that The hTERT-HME1 cell line proved to be an invaluable tool for the examination of PS-1 induced aneuploidy alongside two well established cytogenetic techniques.

As seen in Figure 30, both chromosome metaphase analysis and FISH revealed similar trends in increased levels of aneuploidy. The use of two separate scoring techniques supports the data demonstrating the effects of PS-1 on chromosome missegregation. The absolute values shown for each technique are not expected to be exactly comparable because one method measured total metaphase levels of aneuploidy while the other measured only one chromosome, similar to the results shown in lymphocytes of the FAD mutant PS-1 transgenic mice.
Figure 30  hTERT-HME1 cells transiently transfected with PS-1 expressing plasmids develop aneuploidy as detected by metaphase chromosome analysis and FISH. Parallel cultures of hTERT-HME1 were transiently transfected with WT PS-1, FAD mutant PS-1 (M146L), and empty vector pcDNA3 as control. Levels of aneuploidy were detected using both metaphase chromosome analysis and FISH. Both techniques revealed similar results.
In summary, hTERT-HME1 cells were transiently transfected with WT PS-1, FAD mutant PS-1 (M146L), and empty vector pcDNA3 as control. After 48 hours, cells expressing FAD mutant PS-1 (M146L) showed significantly higher levels of aneuploidy compared to WT PS-1 and WT PS-1 showed significantly higher levels of aneuploidy compared to the empty vector pcDNA3.

*FISH reveals trisomy 21 and trisomy 12 in PS-1 transiently transfected hTERT-HME1 cells.* There are various cytogenetic techniques available to determine aneuploidy within cells. This study has employed metaphase chromosomes analysis and fluorescence in situ hybridization for this purpose. As previously stated, both techniques gave comparable end results when reporting levels of aneuploidy due to the overexpression or mutation of PS-1. But in the course of this study, it was discovered that FISH held many advantages that will be utilized in future investigations.

First, FISH can detect aneuploidy independent of the cell cycle. Therefore both interphase and metaphase cells are scored and the investigation is not restricted to only analyzing metaphase cells. This is particularly important in a study which wishes to look at the direct effects PS-1 has on the cell cycle. Secondly, the commercially available probes offered by Vysis allow for rapid and efficient analysis of many more cells than is possible with chromosome metaphase analysis. But for this study, probably the most important feature of
FISH was being able to identify and analyze individual chromosomes, particularly chromosome 21.

In this section of the study, FISH revealed increased levels of aneuploidy in transiently transfected hTERT-HME1 cells expressing WT PS-1 and FAD mutant PS-1 (M146L). Specifically, there was almost a 3 fold increase in trisomy 21 in cells expressing FAD mutant PS-1 (M146L). The empty vector pcDNA3 was used as a control for any increases in aneuploidy which would have resulted from the vector. Significantly, there was a 1.5 fold increase in trisomy 21 in FAD mutant PS-1 (M146L) compared to WT PS-1 thus demonstrating that the PS-1 mutation was responsible for the greatest amounts of trisomy 21 observed. Not so unexpectedly, overexpression of even WT PS-1 induced significant levels of trisomy 21. These results and observations are just being noted but will be discussed further in the Conclusions Chapter.

The use of a dual labeled probe allowed for the assessment of more than one chromosome. Specifically, chromosome 12 showed increased levels of trisomy in both WT PS-1 and FAD mutant PS-1 (M146L) transiently transfected hTERT-HME1 cells. Again, highest levels of trisomy were observed with FAD mutant PS-1 (M146L) and less, yet significant levels, with WT PS-1. These results are not so surprising in that one hypothesis of this study is that aneuploidy is not specific to one chromosome. Presumably the same mechanisms that govern missegregation of chromosome 21 would affect all other chromosomes as well. Whether missegregation of one chromosome or another, or specifically
chromosome 21, would have any special implications still remains to be
determined in future investigations. Interestingly, various studies have reported
the occurrence of aneuploidy in AD individuals with no special preference to a
chromosome (Geller and Potter, 1999; Migliore et al., 1999; Yang et al., 2001).
In summary, these results and those from counting metaphase chromosomes
show that the aneugenic activity of PS-1 expression likely affects all
chromosomes and therefore probably alters some aspect of abnormal mitosis.
Chapter Three

Materials and Methods

Materials

Mice

Transgenic mice expressing the human FAD mutant presenilins M146L or M146V, or a human wild-type presenilin under control of the PDGF promoter, and their non-transgenic littermates were generous gifts of Dr. Karen Duff, Nathan Klein Institute and New York University School of Medicine. Transgenic mice and non-transgenic littermates between 14 and 19 months of age were examined. Homozygous knock-in mice carrying a human mutant PS-1 (M146V) coding region in place of the mouse sequence and their non-transgenic littermates, all 12-15 months of age were a gift of Dr. Mark Mattson and Dr. Steven Chan of the National Institute on Aging. Mice were housed at USF, Division of Comparative Medicine and CO₂ was used for appropriate euthanasia adhering to IACUC protocols.
Tissue and cell culture

hTERT-HME1 cell line was purchased from Clontech (Palo Alto, CA).

The following medias were used and any modifications are noted in the methods: RPMI 1640 medium supplemented with 2 mM L-GLutamine and 50µM β-mercaptoethanol; Dulbecco’s Modification of Eagle’s Medium 1X (MEM) supplemented with 4.5 g/L Glucose, L-GLutamine and Sodium Pyruvate; Neural Basal Medium (NBM); Clonetics MEBM medium supplemented with Clonetics MEGM SingleQuots (52 µg/ml BPE, 0.5 µg/mL hydrocortisone, 10 ng/ml hEGF, 5 µg/ml insulin, 50 µg/ml gentamicin) and 50 ng/ml amphotericin-B.

RPMI 1640, MEM, 0.05% Trypsin-EDTA solution, FBS, PSA (100X Penicillin G-Streptomycin-Amphotericin B Antiobiotic-Antimycotic Solution), and 1XPBS were purchased from Cellgro by Mediatech, Inc. (Herndon, VA). NBM, OptiMEM, and Karyo Max Colcemid were purchased from Gibco Invitrogen Cell Culture (Carlsbad, CA). Clonetics MEBM and Clonetics MEGM SingleQuots were purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD). β-mercaptoethanol was purchased from Sigma-Aldrich (St. Louis, MO).

Plastic and glassware were purchased from Fisher Scientific (Pittsburg, PA) and VWR International (Suwannee, GA).
**Molecular biology and cytogenetic reagents**

Ethidium Bromide, Ampicillin, Triton X-100 and KCl were purchased from Sigma-Aldrich (St. Louis, MO). Bacto Agar was purchased from BD Biosciences (Sparks, MD). Agarose, low-water methanol, and acetic acid were purchased from Fisher Scientific (Pittsburg, PA). Kits for plasmid preparation were purchased from Qiagen Inc. (Valencia, CA) and BD Biosciences (Sparks, MD). FuGENE 6 and Nick Translation Kit were purchased from Roche Applied Science (Indianapolis, IN).

Giemsa Stain and Gurr tablets were purchased from Gibco (Grand Island, NJ). Vectashield mounting medium containing DAPI (4,6-dimidino-2-phenyliodone) purchased from Vector Laboratories, Inc. (Burlingame, CA). Cytoseal XYL Mounting Medium. Spectrum green dUTP, LSI 21 SpectrumOrange probe, and LSI TEL/AML1 ES Dual Color Translocation Probe were purchased from Vysis Inc. (Downers Grove, IL). Hybrisol I Hybridization Solution and Mouse COT-1 DNA were purchased from Invitrogen Life Technologies (Carlsbad, California).

Mouse anti-neuronal nuclei (NeuN) monoclonal antibody was purchased from Chemicon International (Temecula, CA). AlexaFluor 568-conjugated goat anti-mouse secondary antibody was purchased from Molecular Probes Invitrogen Detection Technologies (Eugene, OR).
**Methods**

**Primary cells**

Mouse spleens were harvested from 14-19 month old mice and 1/3 of the spleen was immediately triturated in a round 100 mm treated polystyrene culture dish containing approximately 1-2 mL of RPMI 1640 medium supplemented with 10% FBS and 5% PSA, using the ground glass ends of two Superfrost Plus slides. The cell suspension was centrifuged 5 and supernatant was removed. Pellet was resuspended in 10 mL fresh RPMI medium containing 5 µg/mL Concanavalin-A (Sigma, St. Louis, MO) and cultured in two 75 cm² tissue culture treated flasks for 42-44 hours at 37°C in a 5% CO₂ humidified atmosphere. Prior to harvesting, the cells were treated with 100 ng/mL of colcemid (Sigma, St. Louis, MO) for 40-45 minutes. To harvest cells, media and cells were centrifuged 5 minutes, 1500 rpm, at 4°C, media removed, and pellet washed three times with 3 mL 1XPBS. After harvesting, cells were prepared for metaphase chromosome analysis and in situ hybridization.

Primary mouse neuronal cultures were prepared from brains of 14–19 month old mice by a modification of the method of Liesi et al., (Liesi et al., 2001). The brains were removed under sterile conditions and immediately placed in a 55cm² treated polystyrene culture dish containing approximately 10 mL cold 1XPBS. After removal of the meninges using forceps and a stereoscope, each brain was transferred to a 50 mL conical tube and triturated in 16 mL serum-free Modified Eagle Medium (MEM) by pipetting the supernatant up and down.
approximately 20 times with a 10 mL pipet. The solid tissue was allowed to settle for 2 minutes and thereby separate from the partially dissociated cells in the supernatant. 2 mL of cell suspension in MEM were plated on single well chamber slides (Lab-Tek) previously coated with 50 µg/mL poly-d-lysine (Sigma) and incubated at 37°C in a 5% CO₂ humidified atmosphere for one hour. To coat the single well chamber slides, 2 mL of 50 µg/mL poly-d-lysine was applied to the well, allowed to sit for 30 minutes, and then rinsed with 1XPBS. After one hour, the medium together with the unattached glia was removed and replaced with fresh Neural Basal Medium (NBM) with serum-free B27 supplement (Gibco) and the cultures were further incubated at 37°C for 12 – 16 hours and then prepared for in situ hybridization and immunocytochemistry.

Cell line

The hTERT-HME1 cell line was maintained in the supplemented Mammary Epithelium Basal Medium (MEBM) medium described above. The hTERT-HME1 is a primary human mammary epithelial cell line that expresses the telomerase reverse transcriptase from a permanently-transfected hTERT plasmid. The constitutive expression of this enzyme maintains telomere length and allows the cells to divide indefinitely while retaining normal function, phenotype, and karyotype (Jiang et al., 1999; Morales et al., 1999). The cell line was passaged every 2-3 days and maintained for no more than 8 population doublings (PD) from the PD\textsubscript{initial} except where indicated. To passage cells, media
was removed, cells washed with 3 mL 1XPBS, and 1.5 mL of 0.5% Trypsin-EDTA solution added to each 75 cm$^2$ tissue culture flask. Cells were incubated in 0.5% Trypsin-EDTA solution at room temperature for 2 minutes and then further incubated at 37°C for 5-10 minutes to efficiently detach cells from flask. Cells to be used for metaphase chromosome analysis were treated with 33 ng/mL colcemid (Gibco) for 10-12 hours prior to harvesting. To harvest cells both treated with and without colcemid, media was removed, cells washed with 3 mL 1XPBS, and 1.5 mL of 0.5% Trypsin-EDTA solution added to each 75 cm$^2$ tissue culture flask. Cells were incubated in 0.5% Trypsin-EDTA solution at room temperature for 2 minutes and then further incubated at 37°C for 5-10 minutes until cells completely detached from the flask and then the cell suspension was centrifuged 5 minutes, 1500 rpm, at 4°C and supernatant containing the 0.5% Trypsin-EDTA solution was removed. After harvesting, cells were prepared for metaphase chromosome analysis and in situ hybridization.

**Plasmids**

The WT and mutant PS plasmids were constructed by inserting wild-type (WT) PS-1, FAD mutant PS-1(M146L), and FAD mutant PS-1(M146V) cDNA into the pcDNA3 expression vector. (Figure 31A) Plasmid carrying FAD mutant PS-1(M146L) was a gift from Dr. Todd Golde, Mayo Clinic, Jacksonville.

Plasmids that carried the dominant negative forms of PS-1 were also gifts from Dr. Todd Golde. Both D385E, lacking the key aspartate by mutation, and
ΔTM1-2, which contains a deletion from amino acids 81-154, were inserted into the pAG3hyg vector. (Figure 31B)

All plasmids were transformed into NovaBlue competent cells (Novagen, Madison, WI) as initial cloning host according to the manufacturer’s protocol. Singles kits were used which contained 11 x 50 µl competent cells. Briefly, 200-250 µg/µl of DNA was added to cells and placed on ice for 5 minutes, heat shocked for exactly 30 seconds in a 42°C water bath and placed on ice again for 2 minutes followed by the addition of 250 µl room temperature SOC medium. Transformants were selected by plating 10-25 µL of transformed cell suspension on LB-agar plates containing 125 µM ampicillin. Following overnight incubation at 37°C, colonies were subcultured in liquid LB broth containing 125 µM ampicillin. Upon reaching high cell density, aliquots were frozen in 40% glycerol/LB media solution (50% glycerol/50% LB media) at -80°C for future use. Plasmid DNA was isolated from the remaining culture volume using either the Qiagen Qiaprep Spin Miniprep Kit or NucleoBond Plasmid Mega Kit (BD Biosciences, Sparks, MD), according to the manufacturer’s protocol and then identified by 1% agarose gel electrophoresis in Tris-acetate buffer (1X TAE) at 80 volts for 30 minutes, visualized by Ethidium Bromide staining (0.3 µg/µL) with long wavelength ultra-violet excitation. Clones were also verified by nucleotide sequencing in both directions by the H. Lee Moffitt Cancer Center Molecular
Figure 31 The pcDNA3 and pAG expression vectors.
The WT and mutant PS plasmids were constructed by inserting wild-type (WT) PS-1, FAD mutant PS-1(M146L), and FAD mutant PS-1(M146V) cDNA into the pcDNA3 expression vector (A). Plasmids that carried the dominant negative forms of PS-1 were inserted into the pAG3hyg vector (B).
Biology Core Facility. The pcDNA3/WT PS-1 clone was verified using the T7 and SP6 primer sequences. Three separate primer sequences were used to detect pcDNA3/M146L. T7, SP6, and ATCTCCGGAGGGATATCT primer was used to sequence the middle of the clone where the mutation is found.

**Transient transfections**

For transient transfections, FuGENE 6 transfection reagent was used. FuGENE 6 transfection reagent is a multi-component lipid-based transfection reagent that complexes with and transports DNA into the cell during transfection. One to two days prior to transfection, hTERT cells (1-3 x 10^5 cells/2mL) were plated either in a 6-well plate (9.4 cm²/well) or a round 100 mm dish (55 cm²/well) with supplemented Mammary Epithelium Basal Medium (MEBM) and allowed to reach 80% confluency for day of transfection. Day of transfection fresh MEBM medium was added to the cells. 2 mL for each well in the 6-well plate and 10 mL for each round 100 mm dish. The FuGENE 6-DNA complex was prepared according to the manufacturer’s protocol using a ratio of 3 µL FuGENE 6:0.5 µg-1µg DNA for the 6-well plate and 18 µL FuGENE 6:6 µg DNA for the 100 mm dish. Briefly, the complex was prepared in a small sterile tube by first adding sufficient serum-free OptiMEM medium as a diluent for FuGENE 6 to bring the total volume to 100 µl for transfections done in 6-well plates and 300 µL for the round 100 mm dish. Next, the FuGENE 6 reagent was added and then the DNA solution was added last. After mixing the contents, the complex was allowed to
incubate for 15 minutes at room temperature and then applied dropwise to the
cells while swirling the plate or dish. Twenty four hours after transfection, the
cells were harvested and transferred to 100mm dishes or single well chamber
slides. At 48 hours post-transfection cells were either harvested immediately for
in situ hybridization or treated with 33 ng/ml colcemid for 10 hours, harvested and
scored for aneuploidy by metaphase chromosome analysis or in situ
hybridization.

Metaphase chromosome analysis

Following colcemid treatment, cells were harvested as described above
and prepared for metaphase chromosome analysis according to standard
cytogenetic procedures. After harvesting, cells were resuspended in 5-10 mL
0.075 M KCl hypotonic buffer for 20 minutes in a 37°C water bath. 0.5 mL of cold
3:1 methanol:acetic acid fixative was added dropwise to the cell suspension and
allowed to sit for 1 minutes then centrifuged 5 minutes, 1500 rpm, at 4°C. Cells
were resuspended in 10 mL cold 3:1 methanol:acetic acid fixative, placed on ice
for a minimum of 30 minutes, centrifuged again 5 minutes, 1500 rpm, at 4°C and.
Fixed cells were stored at -20°C. Prior to slide preparation, cells were
centrifuged again 5 minutes, 1500 rpm, at 4°C and resuspended in 0.5 µL - 2 mL
freshly made fixative. Cells were dropped (4-5 drops) onto Fisherbrand Frosted
Microscope Glass Slides and examined under phase with a 10X objective for
optimal metaphase index then allowed to age one day. Metaphase spreads were
stained with Giemsa to visualize and count the chromosomes. The Giemsa staining solution was prepared by adding 2 mL of Giemsa stain to 30 mL Gurr buffer. Gurr buffer was prepared by dissolving one Gurr tablet in 1 L deionized distilled water. After chromosomes were aged, slides were incubated for 2 minutes in Giemsa staining solution, rinsed with water and allowed to dry. Coverslips were mounted onto slides with CytoSeal XYL Mounting Medium and baked in a 60°C oven for 1 hour to prevent CytoSeal from leaking.

**Bacterial artificial chromosome**

A bacterial artificial chromosome (BAC) containing a mouse chromosome 16-specific sequence was generously provided by Dr. Bruce Lamb at Case Western Reserve University (Kulnane et al., 2002). BACs were transformed into NovaBlue competent cells (Novagen, Madison, WI) as previously described and transformants were selected by plating on LB-agar plates containing 125 µM chloramphenicol. Following overnight incubation at 37°C, colonies were subcultured in liquid LB broth containing 125 µM chloramphenicol. Upon reaching high cell density, aliquots were frozen in 40% glycerol/LB media solution at -80°C for future use. BAC DNA was isolated from the remaining culture volume using the Qiagen Plasmid Maxi Kit according to the manufacturer's protocol with some modification. Specifically, the supernatant was poured through Whatman 802 fluted filter paper into the equilibrated Qiagen tip and allowed to enter resin by gravity. For BAC probe labeling, 1 µg of
extracted DNA was labeled by nick translation (Nick Translation Kit, Roche Applied Science) with spectrum green dUTP according to manufacturer’s protocol. 0.5 µL of 1 mM Spectrum Green dUTP, 5 µl of 0.1 mM dTTP, 10 µL of dNTP mix (containing equal amounts of 0.3 mM dATP, 0.3 mM dCTP, 0.3 mM dGTP) 5 µL of the 10X Nick translation buffer, and 10 µL of the Nick translation enzyme were added to the 1 ug BAC DNA and total volume brought up to 50 uL with water. The reaction was incubated at 15°C at varying time points between 8 and 16 hours and then stopped in a 70°C water bath for 10 minutes. Labeled BAC probe was identified on 2% agarose gel electrophoresis in Tris-acetate buffer (1X TAE) at 80 volts for 30 minutes. For each assay, 60ng of the labeled BAC probe was precipitated by adding 0.16 mg/mL Mouse COT-1 DNA (repetitive DNA sequences used to suppress cross-hybridization), 1/10th volume of 3 M Sodium Acetate, and 2.5 volumes cold 100% Ethanol then placed in -80°C freezer for 20 minutes. After centrifuging for 12 minutes, 12,000 rpm, at 4°C the pellet was resuspend with 500 µL 70% cold ethanol and centrifuged again for 5 minutes, 12,000 rpm, at 4°C. The precipitated BAC probe was re-suspended in 10 µL Hybrisol I and incubated in a 37°C water bath overnight ready to be used for in situ hybridization the next day. All incubation time points were labeled efficiently but after they were used for in situ hybridization it was evident that the 13 hour labeled BAC probe gave best results.
**In situ hybridization**

The pre-incubated BAC probe was used for fluorescence in situ hybridization (FISH) of mouse primary cultures. Interphase and metaphase FISH of hTERT-HME1 cells was performed using either the LSI 21 SpectrumOrange Probe or the LSI TEL/AML1 ES Dual Color Translocation Probe. LSI 21 contains unique DNA sequences complementary to the loci D21S259, D21S341 and D21S342 mapping to 21q22.13-q22.2. LSI stands for locus specific identifier and is a trade name used specifically by Vysis. The letter D refers to the probe being DNA specific, 21 denotes chromosome 21, S means specific compared to a repeat region and then the number is the next consecutive number of discovery. Therefore, when you see a probe labeled D21S341 it means a specific region (locus) DNA probe on chromosome 21 and was the 341st to be assigned. For the LSI 21 probe Vysis combined three probes in the same adjacent region in order to make the overall size of the probe carrying the fluorescent stain large enough to be easily detectable under the microscope. The probe spans 200kb within the chromosome region 21q22.13 to q22.2. (Figure 32 A,B)
Figure 32 The LSI 21 SpectrumOrange Probe.
(A) The LSI 21 probe carries a spectrum orange fluorescent stain and maps to the regions 21q22.13-q22.2 of chromosome 21. (B) LSI 21 contains unique DNA sequences complementary to the loci D21S259, D21S341 and D21S342 and spans 200kb within the chromosome region 21q22.13 to q22.2.
The LSI 21 SpectrumOrange Probe detects the complementary DNA sequence only on chromosome 21 while the LSI TEL/AML1 ES Dual Color Translocation Probe is a mixture of the LSI TEL probe labeled with SpectrumGreen and the LSI AML1 probe labeled with SpectrumOrange and is designed to detect both chromosome 12 and 21. (Figure 33A) The LSI TEL probe begins between exons 3-5 of the TEL gene, highly specific for cancer, and extends approximately 350 kb toward the telomere on chromosome 12. The AML1 probe spans the entire AML1 gene, another specific cancer gene, which spans a 500kb region on chromosome 21. (Figure 33B)

All in situ hybridization procedures were done using the Hybrite hybridization chamber (Vysis Inc., Downers Grove, IL) according to manufacturer’s instructions. Cells were harvested and prepared at least one day in advance of the situ hybridization. Mouse spleen cells were harvested and prepared for in situ hybridization as described above for metaphase chromosome analysis. hTERT cells were prepared the same as mouse spleen cells except there was no hypotonic treatment. All cells were dropped onto Fisherbrand Frosted Microscope Glass Slides and examined under phase with a 10X as described above. Plated neuronal cultures were washed three times with 2 mL 1XPBS and then fixed with 2 mL cold 3:1 methanol: acetic acid in a - 20°C freezer for 30 minutes. Prior to hybridization, slides were placed in 37°C 2XSSC for 45 minutes followed by serial ethanol dehydrations (70%, 80%, 90% 2 minutes each). The slides were air dried and the probe mixture (either the pre-incubated BAC probe
in Hybrisol described above or 7 µL LSI/WCP hybridization buffer, 1 µL LSI probe, 2 µL purified water) was added. After application of the probe, the slides were coverslipped and sealed with rubber cement. The slides were placed in the HYBrite hybridization chamber, and the target and probe were co-denatured at 75°C for 4 minutes and then hybridized at 37°C for 16 hours. Posthybridization, the coverslips were removed and the slides were washed in 0.4XSSC for 2 minutes at 74°C followed by 2XSSC/0.1% NP-40 for 1 minute at room temperature to remove non-specifically adhering probe. The slides were then counterstained with 18 µL Vectashield Mounting Medium with DAPI (4,6-dimidino-2-phenylidone), immediately coverslipped, sealed, and stored at –20°C until image acquisition and analysis.

**Image acquisition and analysis**

To assure that only optimal slides were used for FISH, all slides were evaluated by phase-contrast microscopy prior to hybridization. Hybridization signals were scored and enumerated according to Vysis guidelines using a Nikon Eclipse E1000 microscope with a 4912 CCIR high performance COHU CCD Camera. Genus 2.81 software was used to process images (Applied Imaging). Only single non-overlapped cells that contained no visible cytoplasm surrounding the interphase nucleus were scored. Interphase spot counting was performed separately for each probe and only bright compact signals were counted.
Figure 33 The LSI TEL/AML1 ES Dual Color Translocation Probe.

(A) The LSI TEL/AML1 ES Dual Color Translocation Probe is a mixture of the LSI TEL probe labeled with SpectrumGreen and the LSI AML1 probe labeled with SpectrumOrange and is designed to detect both chromosome 12 and 21. (B) The LSI TEL probe begins between exons 3-5 of the TEL gene and extends approximately 350 kb toward the telomere on chromosome 12. The AML1 probe spans the entire AML1 gene which spans a 500kb region on chromosome 21.
The following Epi fluorescence filter combinations from Applied Imaging were each housed in a filter cube and included an excitation filter, dichroic mirror, and a barrier filter. The spectra for each Nikon filter cube and the applied chromophores are listed below.


**Immunocytochemistry**

Plated primary neuronal cultures were washed three times with 1XPBS, fixed with cold 3:1 methanol: acetic acid in a - 20°C freezer for 30 minutes, then permeabilized in 4XSSC at 37°C for 45 minutes. Cells were blocked with 0.1% Triton X-100/ 1% BSA in 2XSSC at room temperature for 1 hour. Neurons were stained with NeuN antibody diluted in blocking solution, 1:100, at 4°C overnight followed by AlexaFluor 568-conjugated anti-mouse secondary antibody, 1:1000, at room temperature for 1 hour. After three final washes with 1XPBS, coverslips were mounted onto slides with Vectashield mounting medium containing DAPI, sealed and stored at -20°C until image acquisition and analysis.
Calculations and statistical analysis

Percentages of aneuploidy were calculated as

\[
\frac{\text{# aneuploid cells}}{\text{# aneuploid cells} + \text{normal cells}}
\]

Percentages of trisomy were calculated as

\[
\frac{\text{# trisomic cells}}{\text{# trisomic cells} + \text{normal cells}}
\]

Percentages of tetraploidy were calculated as

\[
\frac{\text{# tetraploid cells}}{\text{# tetraploidy cells} + \text{normal cells}}
\]

The statistical significance of the differences in aneuploidy was determined using Microsoft Excel Student’s T test. A one tail paired T test was used to compare the results of transfecting different plasmids into the hTERT cells. A one tail two sample equal variance T test was used to compare the results from the primary cells. Between five and ten mice of each group and between three and seven transfections of each plasmid were analyzed.
Chapter Four
General Discussion

The studies presented in this dissertation aim to test the hypothesis that the presenilins are involved in the cell cycle, specifically in a mechanism that leads to chromosome missegregation and that the percentage of aneuploidy caused by this mechanism is sufficient to make it accountable as one of the major culprits in Alzheimer’s disease development. Although some aspects of the cell cycle involvement of the presenilins had been previously investigated, the specificity of the current hypothesis and corresponding results are unique and novel. In the process of these investigations, a new cytogenic approach was successfully applied to the analysis of murine interphase neurons. Hereby, the use of a mouse BAC probe specific for chromosome16, allowed for the first time the detection of aneuploidy in FAD mutant PS-1 mice, a mouse model of Alzheimer’s disease. The discussion of the results of this investigation, their implications for the disease process as well as possible future directions that could be taken to eventually develop means for AD diagnostics or cure, are presented in this chapter.
It is well known that individuals with trisomy 21 (Down syndrome) exhibit Alzheimer neuropathology by the time they are 30-40 years old. Somewhat later, these people also develop dementia and eventually die of AD if they survive other complications. Because the gene for the Alzheimer amyloid precursor protein (APP) resides on chromosome 21, its consequent overexpression in trisomy 21 cells is thought to contribute in part to the development of AD in Down syndrome individuals by increasing the production of the Aβ peptide (Holtzman and Epstein, 1992; Potter, 1991; Rumble et al., 1989).

The potential involvement of chromosome missegregation in classical AD was suggested by the finding of increased numbers of trisomy 21 cells among fibroblasts and lymphocytes from both sporadic and familial AD (FAD) patients (Geller and Potter, 1999; Migliore et al., 1999; Potter et al., 1995; Potter, 1999; Yang et al., 2001; Yang et al., 2003). Connecting the presenilins with this aberrant cell cycle event was suggested by the research that located the endogenous presenilin protein in structures related to mitosis, i.e. centrosomes, kinetochores, and the nuclear envelope (Honda et al., 2000b; Kimura et al., 2001; Li et al., 1997). Significantly, the microtubule-associated protein CLIP 170, which is also present in the centrosome and is required for centrosome function, binds to presenilin and is essential for the γ-secretase processing of APP to produce the Aβ peptide (Johnsingh et al., 2000; Tezapsidis et al., 2003). This is a very interesting finding which, when looked at in combination with the findings presented in this study, may possibly begin to answer perhaps the most
important question to be asked in the future. What is the mechanism(s) by which the presenilins are causing chromosome missegregation? Any microtubule or kinetochore associated protein which also associates directly with presenilin would be key in beginning to answer this question.

Other evidence connecting the presenilin proteins, chromosome segregation, and Alzheimer’s disease has been provided by the findings that a polymorphism in the presenilin 1 gene is associated with both an increased risk of developing AD (Higuchi et al., 1996; Wragg et al., 1996), and also with an increased risk of having a Down Syndrome child via a meiosis II defect (Lucarelli et al., 2004; Petersen et al., 2000). Furthermore, mothers who have a Down syndrome child before age 35 have a 5-fold increased risk of developing AD later in life as though they suffered a novel form of “accelerated aging” (Schupf et al., 1994). However, we might interpret this result, instead, as indicating that many of the young Down syndrome mothers were mosaic for trisomy 21 (as if they had a predisposition to chromosome missegregation during mitosis) and that this mosaicism was reflected in their trisomy 21 offspring and their own increased risk of developing AD. Together, these results support the hypothesis that trisomy 21 cells in AD patients contribute to their disease.

Less direct support for the chromosome missegregation hypothesis of AD has been provided by the finding that overexpressed or mutant presenilins inhibit the cell cycle (Janicki and Monteiro, 1999; Janicki et al., 2000), increase sensitivity to apoptosis (Eckert et al., 2003; Mattson and Guo, 1997; Vito et al.,
1996a; Vito et al., 1996b; Wolozin et al., 1996), and prevent their own full-length presenilin proteins from being translocated to the nuclear envelope (Honda et al., 2000a). Furthermore, mice lacking the endogenous mouse PS-1 gene or harboring a knock-in FAD mutant human PS-1 have reduced neurogenesis compared to normal mice (Feng et al., 2004; Wang et al., 2004; Yuasa et al., 2002), and mice expressing mutant presenilins exhibit age-related neurodegeneration (Chui et al., 1999; Saura et al., 2004). Finally, it is worth noting that the microtubule-associated protein CLIP 170, which is present in the centrosome and is required for centrosome function, also binds to presenilin and is essential for the presenilin-dependent processing of APP to produce the Aβ peptide (Johnsingh et al., 2000; Tezapsidis et al., 2003).

The connection between chromosome missegregation, PS-1, and AD is now further supported by the data presented here. Specifically, this study shows that PS-1 is intimately involved in the cell cycle and critically regulates the equal distribution of chromosomes to the daughter cells during mitosis. The results show that over-expression of normal presenilin and especially the expression of FAD mutant presenilin leads to missegregation of chromosomes upon completion of mitosis and the consequent development of aneuploid cells. In human cells, the presenilin-induced aneuploidy includes trisomy 12 and 21 and in mice this includes trisomy 16, the mouse homolog to human chromosome 21. In contrast to other reports (Yang et al., 2001; Yang et al., 2003), there is no evidence that the presenilin mutations caused the mouse spleen cells, the mouse
neurons, or the transfected human cells to duplicate their chromosomes and become tetrasomic without dividing. Tetrasomic cells could also not be found by other researchers who investigated primary skin fibroblast cultures or lymphocytes from AD patients—either sporadic or familial, including those carrying a PS mutation (Geller and Potter, 1999; Migliore et al., 1999; Potter et al., 1995; Potter, 1999). Still, it remains possible that some tetrasomic cells may develop in certain regions of the brain (Husseman et al., 2000; Obrenovich et al., 2003; Yang et al., 2001; Yang et al., 2003).

While the survival of aneuploid cells is dependent on the size of the multiplied chromosome, it is important to note that PS induced aneuploidy is not restricted to one specific chromosome but was successfully demonstrated for chromosome 12 and chromosome 21 as evidenced by both trisomy 12 and trisomy 21 in human cells. It is therefore assumed that the cell will tolerate aneuploidy of other small chromosomes and this is something to be looked at. Which chromosomes are capable of missegregating and leaving the cell viable? Do they all segregate at the same frequency? Interestingly, the two human chromosomes that have been chosen for this investigation do not show the same levels of missegregation. While in all cases those cells expressing FAD mutant PS-1 have increased levels of aneuploidy compared to the WT PS-1 and WT PS-1 more so than the non-transfected cells, the aneuploid levels of chromosome 12 were shown to be lower than those of chromosome 21. Future investigations will have to determine if this difference is due to a reduced frequency of chromosome
12 missegregation or if the viability of the cells with chromosome 12 trisomy is dramatically compromised. The later scenario would bear some interesting links to the Alzheimer’s related neurodegeneration.

Together, these results strongly suggest that mutations in the presenilin genes may lead to AD not only by changing the structure and function of the γ-secretase enzyme and increasing the production of the Aβ1-42 peptide, but also by inducing chromosome missegregation and the development of at least trisomy 12 and trisomy 21 mosaicism. Previous work has demonstrated that presenilin mutations are dominant and lead to a gain of function in that they cause both an increase in Aβ1-42 relative to Aβ1-40 and an increase in Notch cleavage. These changes suggest that FAD mutations increase the function of the presenilin-dependent γ-secretase (Duff et al., 1996; Haass and De Strooper, 1999; Hardy and Selkoe, 2002).

Based on the complexity of the cell cycle and the γ-secretase complex, there is no doubt the possibility that the link between presenilin and Aβ1-42 may be more complex than originally assumed. Therefore, the potential connection between PS mutation-linked changes in chromosome segregation and γ-secretase activity should be investigated more directly. One of the central questions along this line is whether the PS-1 aspartate mutations that extinguish the γ-secretase activity also maintain the genome stability or if the PS-1 induced aneuploidy is unrelated to the enzymatic activity of this protein. The current study clearly demonstrated that modifications of the γ-secretase complex do not
only lead to an increased production of Aβ1-42 but also lead to chromosomal instability.

In as much as FAD mutations in the presenilins affect both γ-secretase activity and chromosome segregation, it will be of interest to discover how these processes are related. One of the questions to be addressed is which specific product of the presenilin γ-secretase cleavage (i.e. the APP intracellular domain, the Notch intracellular domain, or Aβ, etc) could be interfering with chromosome stability. In addition, it is important to find out which specific component of the cell cycle machinery, for example the microtubule and/or centrosome, is interacting with the γ-secretase cleavage product and thereby leads to the observed chromosome missegregation. Additional questions to be answered address the connection of the described chromosome instability and the cognitive decline that characterizes Alzheimer’s disease. Several possibilities can be envisioned and need further investigating. For example, the process of mitotic spindle malformation/disruption (due to a mutated presenilin) and the consequent chromosome missegregation in neural stem cells could trigger apoptosis and/or reduced neurogenesis. Along this line, the aneuploid cells that were analyzed in the current study would represent only the very portion of the defective cells that escaped the apoptotic cleansing process. Alternatively, the observed gain-of-function mutations in presenilin could affect APP processing and the production of the Aβ peptide. It would have to be speculated that the increased Aβ production stimulates chromosome missegregation which then
might trigger apoptosis. Another possibility is that presenilin stimulated chromosome missegregation which might stimulate apoptosis, triggers the increased production of Aβ in neighboring cells. Support for this latter hypothesis is provided by the findings that inducing apoptosis in normal human neurons by serum starvation or other treatments increases their secretion of the Aβ peptide.

It is also possible that the product of chromosome missegregation—aneuploid cells, especially trisomy 21 neurons and glia in the brain—trigger a cascade of events that leads to neurodegeneration and AD in a manner similar to what occurs in Down Syndrome, but more slowly due to the compensating effect of the majority population of normal diploid cells in the same organ. This case points to the question which specific cells, when aneuploid for chromosome 21, contribute most to Alzheimer neuropathology? The answer is not obvious. Neurons are the cells whose loss leads to the clinical symptoms of AD, and neurons produce large amounts of APP and Aβ. During development, improper chromosome segregation during neurogenesis would lead to the development of aneuploid neurons, of which the trisomy 21 cells might be more prone to accumulate as they evidently are not detrimental to survival. Furthermore, neurogenesis occurs in regions of the brain, such as the dentate gyrus of the hippocampus, that are implicated in AD (Gage, 2002). Specifically, the rate of new neuron formation in the dentate gyrus of the hippocampus is reported to be on the order of one to two thousand cells per day—more than enough to cause significant numbers of trisomy 21 or other aneuploid cells to accumulate over the
course of 50 years under the influence of a presenilin mutation. This effect is further corroborated by the aging process itself because age, which is the greatest risk factor for developing AD, leads to an increase in aneuploidy in mature neurons derived from mitotic defects in neural precursor cells (Rehen et al., 2001; Yang et al., 2003).

To a greater extent than neurons, glial cells continue to divide in the adult primate brain (Rakic, 1985) and therefore could accumulate aneuploidy throughout life. The involvement of glial cells in the neuropathology of AD is indicated by increasing evidence that these cells mount an inflammatory reaction and an acute phase response that make an important contribution to the characteristic pathology and neuronal cell death of the AD brain (Potter et al., 2001). (Figure 34) Of particular interest is the fact that an abnormally high number of microglia overexpress the astrocyte-stimulating lymphokine IL-1 in the areas of AD and Down syndrome brain that exhibit, or will exhibit, AD neuropathology (Sheng et al., 1995). IL-1, in turn, induces astrocytes to express the amyloid-associated protein α1-antichymotrypsin (ACT) (Das and Potter, 1995; Nilsson et al., 2001) and to increase the translation of APP (Rogers et al., 1999). Either ACT or another amyloid-associated protein elicited by inflammation, apolipoprotein E, are required in vitro and in vivo to catalyze the polymerization of Aβ peptide into neurotoxic amyloid filaments (Nilsson et al., 2004; Potter et al., 2001). ApoE4 is the strongest promoter of amyloid formation and is the greatest risk factor for developing AD, besides age. Interestingly,
mothers carrying the ApoE4 encoding gene have also been reported having an increased chance of giving birth to a Down syndrome child (Avramopoulos et al., 1996). Together, these data indicate that a glial cell-led inflammatory cascade appears to play an essential role in Alzheimer amyloid formation, starting with the release of IL-1 from microglia. The constitutive activation of microglia to produce IL1 in a Down syndrome brain, even before birth, suggests the possibility that an additional copy of chromosome 21 in the microglia cells of a thus far normal individual might similarly lead to an increased release of IL-1 and thereby initiate the inflammatory cascade that results in the neuropathology of Alzheimer’s disease.

In summary, the results presented in this dissertation firmly establish that PS-1 plays an essential role in mitosis and chromosome segregation. These results will open new directions for dissecting the biochemical mechanisms that govern the cell cycle. Indeed, other mitotic factors (CLIP-170, Bub, aurora kinases, etc.) play essential roles in shaping and remodeling the mitotic spindle. Based on the data presented, it is proposed that the presenilins are an important component of cell cycle activities that contribute to Alzheimer’s disease pathology. Increased levels of aneuploid cells due to the mutation or overexpression of PS-1 has been demonstrated in a human cell line, mouse lymphocytes, and finally mouse neurons.
Neurons and other CNS cells release of Aβ peptide from APP, microglia activated by diffuse Aβ extracellular diffuse amyloid plaques release of cytokinesynthesis of pathological chaperones (ApoE and ACT) acceleration of amyloid plaque formation mature amyloid plaques

**Figure 34** An inflammatory pathway leading to AD.

One of the proposed paths leading to AD pathology begins with the Aβ peptide stimulating an inflammatory response from microglial cells. The activated microglia release cytokines, such as IL-1, which directly induces expression of ACT. ApoE, another pathological chaperone, is also elicited by inflammation. Both ACT and ApoE are involved in the accelerated amyloid plaque formation. This glial cell-led inflammatory cascade is observed in the DS brain as well, even before birth.
Various molecular and cytogenetic techniques were applied. Specifically, a rather novel approach employing fluorescence in situ hybridization with a BAC probe was used to detect low aneuploid levels in mouse neurons. Further investigation into PS-1 and cell cycle defects in AD will provide a more complete understanding of the mechanism by which PS-1 regulates the cell cycle and may lead to novel approaches in diagnosis and therapy.


Janicki, S. and Monteiro, M.J. (1997) Increased apoptosis arising from increased


Kim, J.W., Chang, T.S., Lee, J.E., Huh, S.H., Yeon, S.W., Yang, W.S., Joe, C.O., Mook-


human brain and spinal cord express multiple and distinct isoforms of laminin. 

and biochemical spectrum of Alzheimer disease associated with PS-1 mutations. 


Lucarelli,P., Piciullo,A., Palmarino,M., Verdecchia,M., Saccucci,P., Arpino,C. and 

125-137.

Mann,D.M., Iwatsubo,T., Ihara,Y., Cairns,N.J., Lantos,P.L., Bogdanovic,N., 
deposition of amyloid-beta 42(43) in plaques in cases of Alzheimer's disease and 
hereditary cerebral hemorrhage associated with mutations in the amyloid precursor 


Masters,C.L., Multhaup,G., Simms,G., Pottgiesser,J., Martins,R.N. and 
Beyreuther,K. (1985a) Neuronal origin of a cerebral amyloid: neurofibrillary tangles 
of Alzheimer's disease contain the same protein as the amyloid of plaque cores and 

Masters,C.L., Simms,G., Weinman,N.A., Multhaup,G., McDonald,B.L. and 
Beyreuther,K. (1985b) Amyloid plaque core protein in Alzheimer disease and Down 


pivotal player in neuronal apoptosis and neurodegenerative disorders. 

role for the endoplasmic reticulum in the pathogenesis of Alzheimer's disease? 

162


glycosylation but remain catalytically inactive in the absence of wild type Presenilin. *J.Biol.Chem.*, 278, 43430-43436.


Rowe, I.F., Ridler, M.A. and Gibberd, F.B. (1989) Presenile dementia associated with


About the Author

Debrah Isabel Boeras received her B.A. in Chemistry at the University of South Florida. In 2000 she joined Dr. Huntington Potter’s laboratory at the University of South Florida, College of Medicine, Department of Biochemistry and Molecular Biology to study Alzheimer’s disease.

Debi will pursue her postdoctoral training in HIV research whereby she will combine applied bench science along with related fieldwork in Rwanda and Zambia under the guidance of Dr. Eric Hunter and Dr. Susan Allen at Emory University. Here Debi will characterize the virus heterosexually transmitted via genital fluids from donor to recipient in hopes to better understand the selective process involved in HIV-1 infection and facilitate the development of an effective vaccine. Debi plans to continue advancing and broadening her work in the field of HIV in hopes to pursue continued bench and fieldwork in HIV vaccine development.