www.jnbs.org

2016 Published by Üsküdar University

JNBS

PATIENT- DERIVED STEM CELLS AS NEW FRONTIERS FOR DISEASE MODELLING WITH FOCUS ON NEURODEGENERATIVE DISEASES

NÖRODEJENERATİF HASTALIKLAR ÜZERİNE ODAKLANAN HASTALIK MODELLEMELERİ İÇİN YENİ SINIRLAR OLARAK HASTA-KAYNAKLI KÖK HÜCRELERİ

Kuldip S. Sidhu*

Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder and represents the most common form of dementia, affecting over 46.9 million people worldwide. AD is characterized by the progressive loss of specific neurons in the brain, which leads to gradual loss of bodily functions, long term memory loss and eventually death. The pathology of AD remains elusive due to the lack of appropriate animal and/or in vitro models, which recapitulate the human AD. The induced pluripotent stem (iPS) cells derived from patient's somatic cells and thus patient-specific and disease-specific iPS cells offer great potential in regenerative medicine, in drug discovery and modelling disease processes in vitro. Here we report the first generation of feeder-free iPS cells from Alzheimer's patients with an early onset of disease using a polycistronic lentiviral vector containing four pluripotent genes, Oct4, Sox2, Klf4 and cMyc. These iPS cells are pluripotent as demonstrated by both the in vitro and in vivo assays i.e. stem cell surface markers, gene expressions and teratomas formation after injecting these cells into the SCID mice. These iPS cells from patients that are predisposed to Alzheimer's disease have been analyzed by using the microarray chip and the computation of data is assisting in developing the in vitro models for this disease and for future regenerative medicine. Genome-wide microarray analysis revealed that AD-iPS cells are similar to control iPS cells and hESC lines; however, eight candidate genes differentially expressed between familial iPS cells and sporadic iPS cells. Some Alzheimer's specific genes and pathways were overrepresented in these cells hence in vitro disease modelling possible.

Keywords: Alzheimer's disease, neurodegenerative disorder

Özet

Alzheimer Hastalığı(AH) nörodejeneratif bir bozukluktur ve dünya çapında 46.9 milyonun üzerinde insanı etkileyen demans hastalığının en yaygın şeklini temsil eder. Alzheimer Hastalığı(AD), beyindeki spesifik nöronların artan kaybı sonucu uzun süreli hafıza kaybı ile başlayan, vücut fonksiyonlarının yavaş ve sürekli olarak yitirildiği, ve en sonunda ölüm ile neticelenen ciddi bir hastalıktır. Alzheimer Hastalığı'nın patolojisi, Alzheimer Hastalığı'nı yeniden özetleyen uygun hayvan ve/ya da deney ortamındaki modellerin eksikliği nedeniyle tarif edilmesi zor bir hastalık olarak kalmıştır. Hastanın somatik hücrelerinden edinilen uyarılmış pluripotent kök hücreleri(iPS) ve dolayısıyla hasta-özgü ve hastalık-özgü iPS hücreleri rejeneratif tıpta, ilaç keşfetmede ve deney ortamında hastalık modellemede büyük bir potansiyel arz eder. Bu çalışmada, 4 pluripotent geni olan Oct4, Sox2, Klf4 ve cMyc'i içeren polycistronic lentiviral vektörü kullanan erken hastalık başlangıcı olan Alzheimer hastalarından besleyici eksik İPS hücrelerinin ilk jenerasyonunu raporladık. Bu İPS hücrelerinin SCID farelerine enjekte edilmesinden sonra kök hücresi yüzey yapıcıları, gen ifadeleri ve teratoma formasyonu gibi hem deney ortamlarında hem yaşayan organizma denemelerinde gösterildiği gibi İPS hücreleri pluripotenttir. Alzheimer hastalığına yatkınlığı olan hastalardaki bu İPS hücreleri mikroçipler kullanılarak analiz edilmiştir ve data ölçümü bu hastalık ve gelecek rejeneratif tıp için deney ortamındaki modellerin gelişimine yardımcı olacaktır. Genom-kapsamlı mikroçip analizi ortaya çıkarmıştır ki; Alzheimer hastalığıiPS hücreleri İPS hücrelerinin ve hESC yollarının kontrolüne benzerdir. Ancak, 8 aday gen ailevi ve sporadik hücreler arasında farklılar ortaya koymuştur. Bazı Alzheimer'a özgü genler ve yolları, bu hücrelerde ve bunun sonucu olarak deney ortamında hastalık modellemede fazla temsil edilmiştir.

Anahtar Kelimeler: *Alzheimer hastalığı, nörodejeneratif bozukluk*

*Correspondign author: Centre for Healthy Brain Ageing, Faculty of Medicine, University of New South Wales, Australia. E-mail: k.sidhu@unsw.edu.au

1. Introduction

Our interest is in Alzheimer's and Parkinson's diseases they both come under a broader term, dementia. As it on 2015 now there are 46.9 million people living with dementia worldwide, 342, 800 Australian. This number will be double in 2030 and triple in 2050. These people are and will continue to populate mostly the low and middle income countries. The current cost to manage this disease is around 818 billion, soon will be 1 trillion in 2018 worldwide and about 4.9 billion in Australia. If dementia was a country, it would be the 18th largest economy of the world with budget as good as of Apple and Google combined. There are 23 million residing in Asia-Pacific. There is no cure but only the management and hence growing burden. It is a gigantic problem to sustain unless we do something about it.

There is a strong need to bring in focus the need of the Brain Initiative. This is because 1/3 of world population is inflicted with some sort of mental and psychiatric illnesses including neurological disorders like Alzheimer's, Parkinson etc.These all account for 13% of disease burden that surpasses cancer (10%) and cardiovascular (5%) diseases Worldwide funding is not in commensurate with disease burden. Majority goes to cancer and cardiovascular. This inequality is imposing a huge in balance & impact on quality of human life Medical funding is required to increase globally to offset this in balance & improve quality of life.

2. What is an Alzheimer's disease

There are over 100 billion neurons in a normal human brain and each neuron makes about 15 000 connections (synapses) with other neurons. This massive network of neurons is responsible for the cognition, reasoning, language, and storage and retrieving of information and all that is affected in AD patients

There are two major hall marks of AD – extracellular Amyloidal plaques probably caused by mutations in APP, PS1/PS2 genes and intracellular neural fibrillar tangles in the brain in addition to oxidative stress, mitochondrial dysfunction, neuroinflammation, co-morbidity with PD all appear to be contributing pathological factors.

The traditional route for drug discovery is a long and expensive journey. Each new drug discovery costs around 1.5 billion US$. A part of the reason is that it involves using preclinical animal trials and modelling. Animal models of diseases are invaluable often do not faithfully mirror human pathophysiology. We have an alternative, with the advent of patient-derived induced pluriptoent stem cells technology (Takahashi & Yamanaka, 2006) we can turn the clock back on these diseases by reproducing these diseases in the Petri dish that is discussed here. The iPS cells obtained by reprogramming from patients promise unique insights into the disease modelling, & development of customized cellular therapies (Sidhu, 2015).

The primary source of pluripotent stem cells has been the embryos, however, due to ethical issues involved, in 2006 a remarkable discovery by Yamanaka from Japan that an adult somatic cell can be reprogrammed to its pluripotent state by simply transducing with four pluripotent genes, Oct4, Sox2, Klf4, c-Myc either by viral or protein transduction has taken the debate away from human embryos. There is a paradigm shift with the birth of iPSC technology. Such pluripotent cells called as induced pluripotent stem cells though can generate different lineages all derived from three germ layers for future regenerative medicine, but has limitations because of transgenes and these as such as currently being developed for drug discovery and disease modelling. There are advances now to produce non-integrating iPSC that can be converted to relevant neurons, probably that will move towards custom-made cell therapies for such patients a whole new world of regenerative medicine.

In Alzheimer's patients, there is extensive loss of specific neurons in the brain called basal forebrain cholinergic neurons (BFCN) that leads to shrinkage of the brain. In our lab we have used iPSC derived from such patients and converted these into BFCN using our optimised protocols. The major question now is to fully understand and characterise these cells before they can used for therapy for example study their electric conductivity through synapses called electrophysiology. We have done that though in individual cells in a Petri dish, but in the real world million of these neurons in the brain talk to each other and that we want to simulate in the Petri dish, I called human brain in the Petri dish. So that we can understand their function in totality more specifically so for example n AD, how they fire together and produce response and how that is affected under disease situation. This has relevance in studying disease pathophysiology and disease modelling. As there is a latency of 10-15 years in AD before symptoms appear and this technology can recapitulate human development in the Petri dish, and hence the disease process and we can reset the clock from numbers of years to number of days by this technology.

3. Disease Modelling

3. 1. Production of iPS cells & Characterisation

We used Yamanaka cocktail consisting of four pluripotent markers, Oct2, Sox2, Klf4 and c-Myc using a stem cell lentiviral cassette with a constitutive promoter EF-1alpha. With experience on iPSC technology, we have now produced patient-specific iPSC from AD (Chung et al., 2015). We recruited a cohort of 12 patients both sporadic and familial cases of AD including age and sex-matched control and have produced > 100 iPSC clones that are a various stages of characterisation

These clones express pluripotent surface markers i.e Oct4, Nanog, SSEA4, TRA-160. These cells do undergo spontaneous differentiation to three germ layers i.e. ecto- endo-, meso- with specific immunostaining. In vitro pluripotency is also matched with that seen in in vivo after injecting these cells into SCID mice and formation of solid teratomas (Figure)
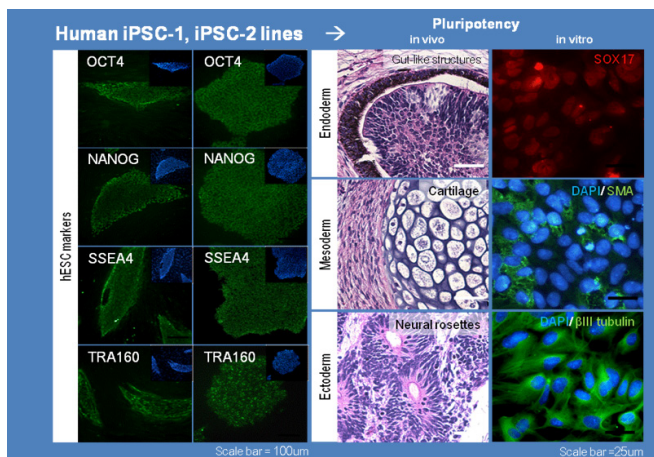
Figure 1: In vitro and in vivo analysis of pluripotency of iPSCs. Surface markers for pluripotency, Oct4, Nanog, SSEA4 and TRA160 in iPSC 1&2 lines. In vivo teratoma formation assays were performed by intratesticular injection of AD-iPSCs into SCID mice. In vitro Immunofluorescence staining of early lineage markers after in vitro differentiation.
Representative tissues of the 3 embryonic lineages were observed.
(From Chung et al. 2011)

Microarray analyses and annotation of differentially expressed genes indicated that cell cycle and DNA replication that are indicated of pluripotency are over expressed in ff-iPSCs (figure2).
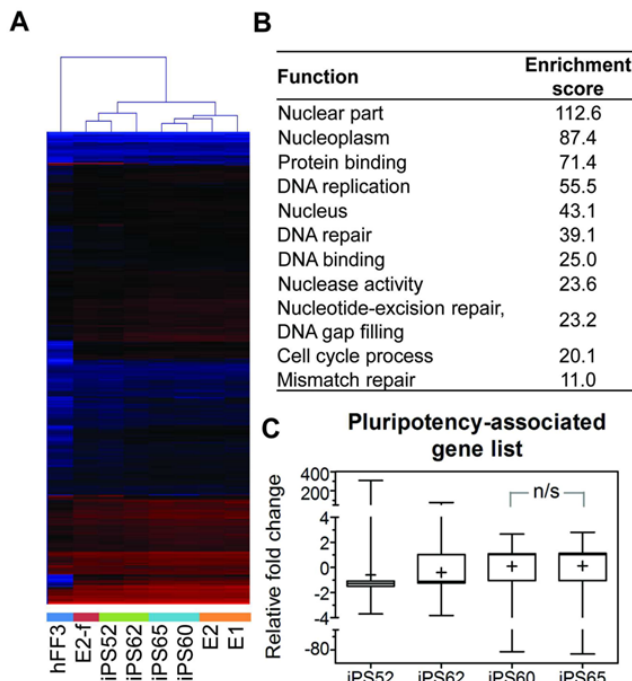

Figure 2: A. Heatmap of 951 pluripotency-associated genes across all cell lines. ff-iPSC lines (iPS60 and iPS65) were tightly clustered to hESCs (E1, E2), whereas f-iPSCs (iPS52 and iPS62) clustered separately. B, GO enrichment analysis of these 951 genes reveals they are highly related to DNA metabolic processes and mostly occur within the nucleus. C, Quantitative fold changes of these genes in all iPSC lines relatively to hESCs. Only ff-iPSCs were not significantly (n/s) different (from Chung et al. 2011).

3. 2. Production of Basal Forebrain Cortical Neurons (BFCN)

In AD there is a substantial loss of BFCN in addition to other clinical symptoms. Therefore, following ontological approach we produced BFCN from pluripotent stem cells using BMP9 signalling. BFCN is 36 days protocol consisting of first generating NPC by neural rosettes assay for 15 days followed by specific BFCN induction using BMP9 signalling after traditional neural patterning with FGF8/SHH for 21 days followed by maturation.

We followed through the rigour of these protocols and shown by gene and protein expression that the relevant cells can be produced. There is down regulation of NPC markers i.e. PAX6 and SOX1 with concomitant up regulation of BFCN markers i.e. CHAT and VCHAT. There are significant differences between control and AD patients in terms of expression of BFCN markers (Figure 3).
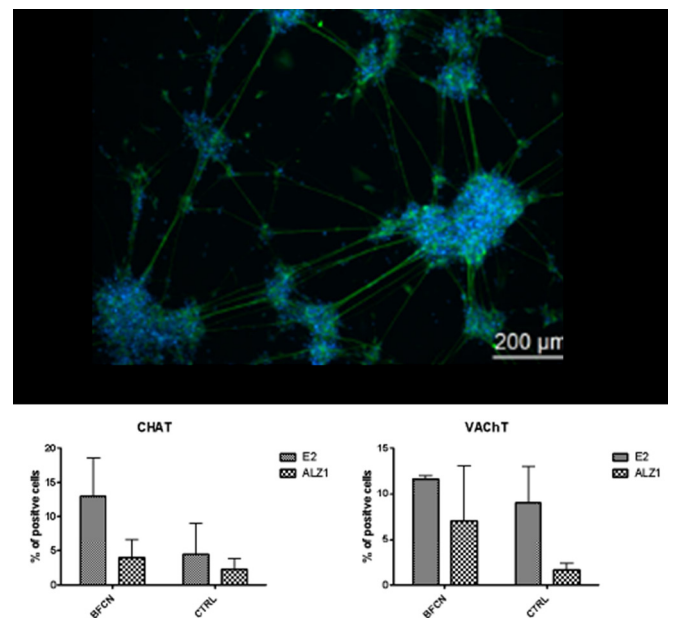

Figure 3: Production of Chat and VChat positive cells from AD-iPSC. Immunolocalisation and specific gene expression analysis.

3. 3. Characterisation of iPSC from AD Patients

We have produced iPSC from a cohort of AD patients both sporadic and familial with age-sex matched controls. We have carried out a comprehensive transcriptomics on these clones by microarray analyses. The principal component analyses clearly separated out AD clones both familial and sporadic from controls groups.

In particular our interest is in sporadic patients who far more in numbers and our Heat map analyses (B) indicated that 293 genes were differentially expressed significantly among control (CO-iPS) and sporadic cases (SAD-iPS). Gene ontology studies demonstrated the following keg pathways were significantly affected in these groups (figure 4).
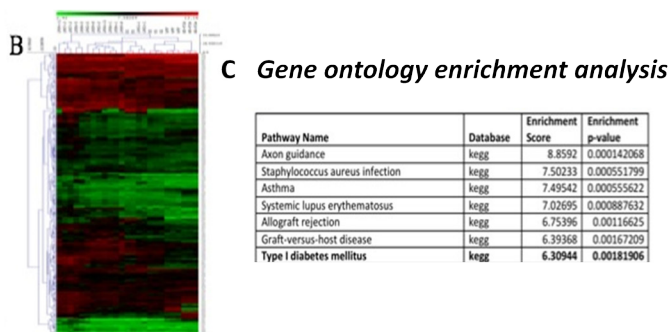
C *Gene ontology enrichment analysis*

Pathway Name	Database	Enrichment Score	Enrichment p-value
Axon guidance	kegg	8.8592	0.000142068
Staphylococcus aureus infection	kegg	7.50233	0.000551799
Asthma	kegg	7.49542	0.000555622
Systemic lupus erythematosus	kegg	7.02695	0.000887632
Allograft rejection	kegg	6.75396	0.00116625
Graft-versus-host disease	kegg	6.39368	0.00167209
Type I diabetes mellitus	**kegg**	**6.30944**	**0.00181906**

Figure 4: Trasncriptomic analysis of iPS cell lines from sporadic AD cases and controls. B. Heat map showing the 293 genes differentially expressed between sporadic AD and control lines; green = downregulated and red = upregulated. C. Summarisation of these differentially expressed genes shows that the KEGG Type 1 diabetes is among the most significantly different pathways.

In particular the following genes came out interesting out of these data, **MAPK10** -mitogen-activated protein kinase **(2.5 fold change)** - We predict that changes in the level of this protein would have implications in the regulation of the beta-amyloid precursor protein/APP signalling during neuronal differentiation by phosphorylating APP formation. **PIK3R1** - phosphoinositide-3-kinase, regulatory subunit 1 (alpha) **(1.6 folds change)** may be involved in insulin resistance. **GPX2** -glutathione peroxidase 2 **(-1.7 fold change)**- could possibly explain the increased susceptibility to oxidative stress (Chung et al. 2015).

These data conform with our in vitro phenotyping of these clones where we have shown that AD-specific iPSC clones were more susceptible to oxidated stress (Figure 5).
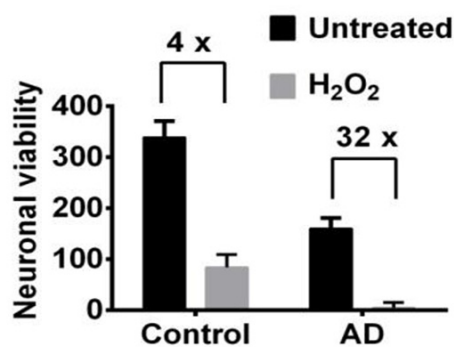

Figure 5: AD neurons showed increased susceptibility to oxidative stress. Neurons were generated from iPSCs from a familial AD patient carrying a PSEN1 P117R mutation or an age-matched control. Data shown are mean ± standard error of the mean from 3 independent experiments. Neurons were treated ± 100 μM H2O2 for 24 hours and viability of AD or control neurons was measured (from ooi & Sidhu unpublished).

Another important genotype relevant to sporadic forms of AD is the APOE. Individuals with APOE 4/4 are more susceptible than APOE3/3. The data from one of an another collaborator, Prof Brett's lab indicated recently

that a protective form of protein APOE-25 is significantly expressed more in APOE3/3 than in APOE4/4 in the brain (Hippocampus extracts from post mortem patients). We carried out similar analyses in our iPSC clones derived from these patients. Although there was no significant difference between APOE-25 in the conditioned media from iPSC-derivd neurons, however, neuronal extract brought out significant differences in APOE3/3 and 4/4 iPSC clones, the former produced more and thus may be providing protection. This is a very important validation of these in vitro model based on patient-derived iPSC technology.

3.4. Mechanism of AD

To understand the mechanisms of these differences between groups, we also carried out meta-analyses of transcriptomics from other studies. A number of go terms related to various biological phenomenons like amyloid precursor protein metabolic, apolipoprotein binding, sterol and cholesterol homomeostasis, MAPK cascade, and ageing emerged all relevant to AD disease. MAPK cascade was interesting as it related to cell cycle regulation that also emerged in our transcriptomics data. Briefly this cartoon (Figure 6) explains the role of MAPK cascade in regulating cell cycle and its implication in AD. Under normal circumstances with optimal growth factors and adhesion molecules (extracellular matrix), Raf, MEK, ERK pathways are activated and that brings about transition in cell cycle from gap phase to synthesis and mitosis in cells by derepressing transcription factor, E2F through phosphorylating various cyclin proteins (A, D, E). However, in the presence of stress factors like in AD caused by MAPK, and oxidative stress by Akt (As shown by our microarray and phenotype data) that derepression of E2F is blocked and that leads to cell cycle arrest and apoptosis in cells. However, there are other factors that may impact cell cycle directly.
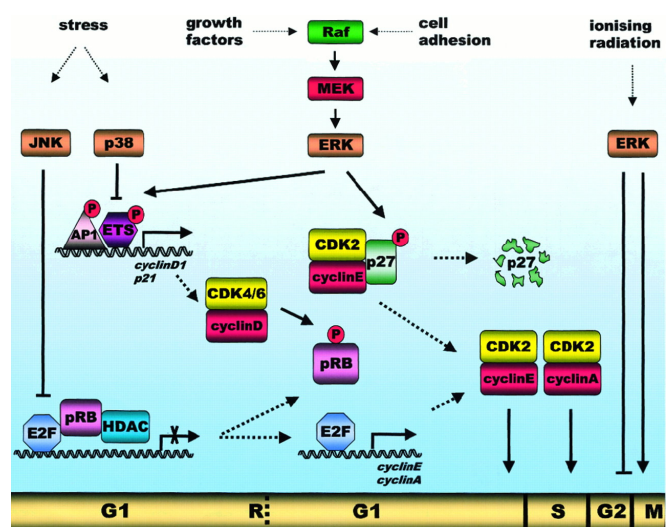

Figure 6: Mechanism of Alzheimer's disease (see text for explanation – figure taken from website)

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