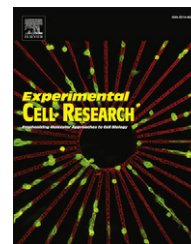


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Research Article

Modeling neurogenesis impairment in down syndrome with induced pluripotent stem cells from Trisomy 21 amniotic fluid cells

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ABSTRACT

Down syndrome (DS), or Trisomy 21 (T21) syndrome, one of the most common chromosomal abnormalities, is caused by an extra duplication of chromosome 21. In studies of neuron development, experimental models based on human cells are considered to be the most desired and accurate for basic research. The generation of diseased induced pluripotent stem (iPS) cell is a critical step in understanding the developmental stages of complex neuronal diseases. Here, we generated human DS iPS cell lines from second trimester amniotic fluid (AF) cells with T21 by co-expressing Yamanaka factors through lentiviral delivery and subsequently differentiated them into neuronal progenitor cells (NPCs) for further analyses. T21 AF-iPS cells were characterized for the expression of pluripotent markers and for their ability to differentiate into all three germ layers by forming embryoid bodies *in vitro* and teratomas *in vivo*. The T21 AF-iPS cells maintained their unique pattern of chromosomal karyotypes: three pairs of chromosome 21. The level of amyloid precursor protein was significantly increased in NPCs derived from T21 AF-iPS cells compared with NPCs from normal AF-iPS cells. The expression levels of miR-155 and miR-802 in T21 AF-iPS-NPCs were highly elevated in the presence of low expression of MeCP2. We observed that T21 iPS-NPCs generated fewer neurons compared with controls. T21 iPS-NPCs exhibit developmental defects during neurogenesis. Our findings suggest that T21 AF-iPS cells serve as a good source to further elucidate the impairment neurogenesis of DS and the onset of Alzheimer's disease.

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Introduction

Down syndrome (DS), or Trisomy 21 (T21) syndrome, one of the most common chromosomal abnormalities, is caused by an extra duplication of chromosome 21 [1]. The characteristics of DS include cognitive impairment, congenital heart defects, craniofacial abnormalities, gastrointestinal anomalies, leukemia, mental retardation, seizures and early onset Alzheimer's disease (AD) [2].

The Ts65Dn mouse used to study DS is an invaluable tool, but it provides a limited representation of human pathophysiology [3]. In this case, Ts65Dn mice are trisomic at chromosome 16, which is only homologous to approximately 55% of the human chromosome 21 [4]. This shows that Ts65Dn mouse is not always a faithful mimic of humans for human T21 syndrome. Studying human cells *in vitro* is the backbone of basic research, and numerous findings regarding both normal and pathologic cellular processes have been reported. Human cell culture provides an essential complement to research with animal models, where induced pluripotent stem (iPS) cells can serve as *in vitro* human disease models [5–7]. Disease-specific pluripotent cells capable of differentiating into various tissues; in this specific case, differentiation into neurons can provide new insights into disease pathophysiology that were not possible with murine models [8–11].

Here, we generated human DS iPS cell lines from human second trimester amniotic fluid (AF) cells with T21 by co-expressing Yamanaka factors through lentiviral delivery and subsequently differentiated them into neuronal progenitor cells (NPCs) for further analyses. We report that T21 AF-iPS cells were positive for pluripotent markers and differentiate into three germ layers, as shown by the generation of embryoid bodies (EBs) *in vitro* and teratomas *in vivo*. The T21 AF-iPS cells maintained the abnormal chromosomal karyotype signature. The human gene for amyloid precursor protein (APP) is located on chromosome 21. APP is a ubiquitously expressed transmembrane protein whose cleavage product, the β -amyloid ($A\beta$) protein, is deposited as amyloid plaques in the neurodegenerative conditions of AD and DS. We found that the expressed protein level of APP in NPCs derived from T21 AF-iPS cells was significantly overexpressed. In addition, several studies reported that developing DS cortical neurons have shorter dendrites, fewer dendritic spines and abnormal dendritic morphology [12]. New insight was also provided by a recent study that described the developing DS neurons on the basis of miRNA regulation [13]. Human chromosome 21 harbors 5 miRNAs: miR-99a, let-7c, miR-125b-2, miR-155 and miR-802 [14]. In DS neurons, over expression of miR-155 and miR-802 inhibit the expression of the target, methyl-CpG-binding protein 2 (*MeCP2*) and in turn cause aberrant expression of two downstream target genes, *CREB1* and *MEF2C*, both which play important roles in neurogenesis. Subsequent impairments in neuronal maturation also appear to be reflected in the DS brain [13]. We found that over expression of miR-155 and miR-802 result in the degradation of *MeCP2* in T21 iPS-NPCs. We observed that T21 iPS-NPCs generated fewer neurons compared with controls. T21 iPS-NPCs exhibited a developmentally regulated defect in neurogenesis. Our findings demonstrate that T21 AF-iPS cells serve as a good source to further elucidate the impairment neurogenesis of DS and the onset of Alzheimer's disease.

Materials and methods

Cell culture and iPS cell generation

T21 and normal AF samples were obtained with written informed consents from pregnant donors. The protocols of this study were approved under the Institutional Review Board (IRB) of Cathay General Hospital, Taipei, Taiwan. AF cells were isolated from second-trimester amniocenteses according to our previous report [15]. T21 AF cells were cultured and passaged routinely at 80–90% confluence with alpha-modified minimum essential medium (α -MEM, Hyclone, Logan, UT) containing 20% fetal bovine serum (FBS, Hyclone), 4 ng/mL basic fibroblast growth factor (bFGF, Peprotech, London, UK), 50 U/mL penicillin and 50 μ g/mL streptomycin (Sigma, St. Louis, MO) at 37 °C and 5% CO₂ in a humidified incubator. Briefly, pre-prepared lentivirus packaged with Yamanaka factors (OCT4, SOX2, KLF4 and c-MYC) was purchased from Vectorite Biomedica Inc. (VBI, Taipei, Taiwan). The virus titer used for transduction was 1×10^9 with a multiplicity of infection (MOI) of 20. For viral transduction, 1×10^5 T21 AF cells were seeded per well in 12-well culture plates. The viral mixture was then added into the culture medium with 2 μ g/mL polybrene (Sigma) and incubated for two days. On day 2, the cells were passaged and plated onto 100 mm tissue culture dishes coated with 1×10^6 irradiated mouse embryonic fibroblasts (MEFs). The next day, the culture medium was exchanged with human iPS cell culture medium composed of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, Invitrogen, Carlsbad, CA) medium supplemented with 20% KnockOut serum replacement (KOSR, Invitrogen), 0.1 mM nonessential amino acids (NEAA, Invitrogen), 1 mM GlutaMax-1 (Invitrogen), 0.1 mM 2-mercaptoethanol (Invitrogen), 10 ng/mL bFGF, 50 U/mL penicillin and 50 μ g/mL streptomycin. On day 10 post-transduction, embryonic stem cell(ES)-like colonies were picked based on morphology and cultured on irradiated MEF feeder layers with iPS culture medium. On day 17, T21 AF-iPS colonies were transferred for expansion and further characterization. The workflow chart is shown in Fig. 1A.

Alkaline phosphatase and immunofluorescence staining

The alkaline phosphatase (AP) activity detection kit used in this study was obtained from Millipore (Millipore, Billerica, MA) and used following the manufacturer's instructions. For immunofluorescence staining, cells were fixed with 4% paraformaldehyde (Sigma), permeabilized with 0.05% Triton X-100 (Sigma) if necessary and blocked with 10% donkey serum. For the identification of ES markers in T21 AF-iPS cells, colonies were stained with primary antibodies against OCT4 (1:400, Abcam), SOX2 (1:200, Millipore), NANOG (1:500, Abcam, Cambridge, MA), SSEA-1 (1:200, Millipore), SSEA-3 (1:100, Abcam), SSEA-4 (1:100, Abcam), TRA-1-60 (1:200, Abcam) and TRA-1-81 (1:200, Abcam). For the detection of lineage markers in differentiated T21 AF-iPS cells *in vitro*, cells were stained with primary antibodies against TUJ1 (1:400, Sigma), NESTIN (1:100, R&D Systems, Minneapolis, MN), α -SMA (1:100, Abcam), TROPONIN (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) and ALBUMIN (1:200, Abcam). For the detection of lineage markers in NPCs

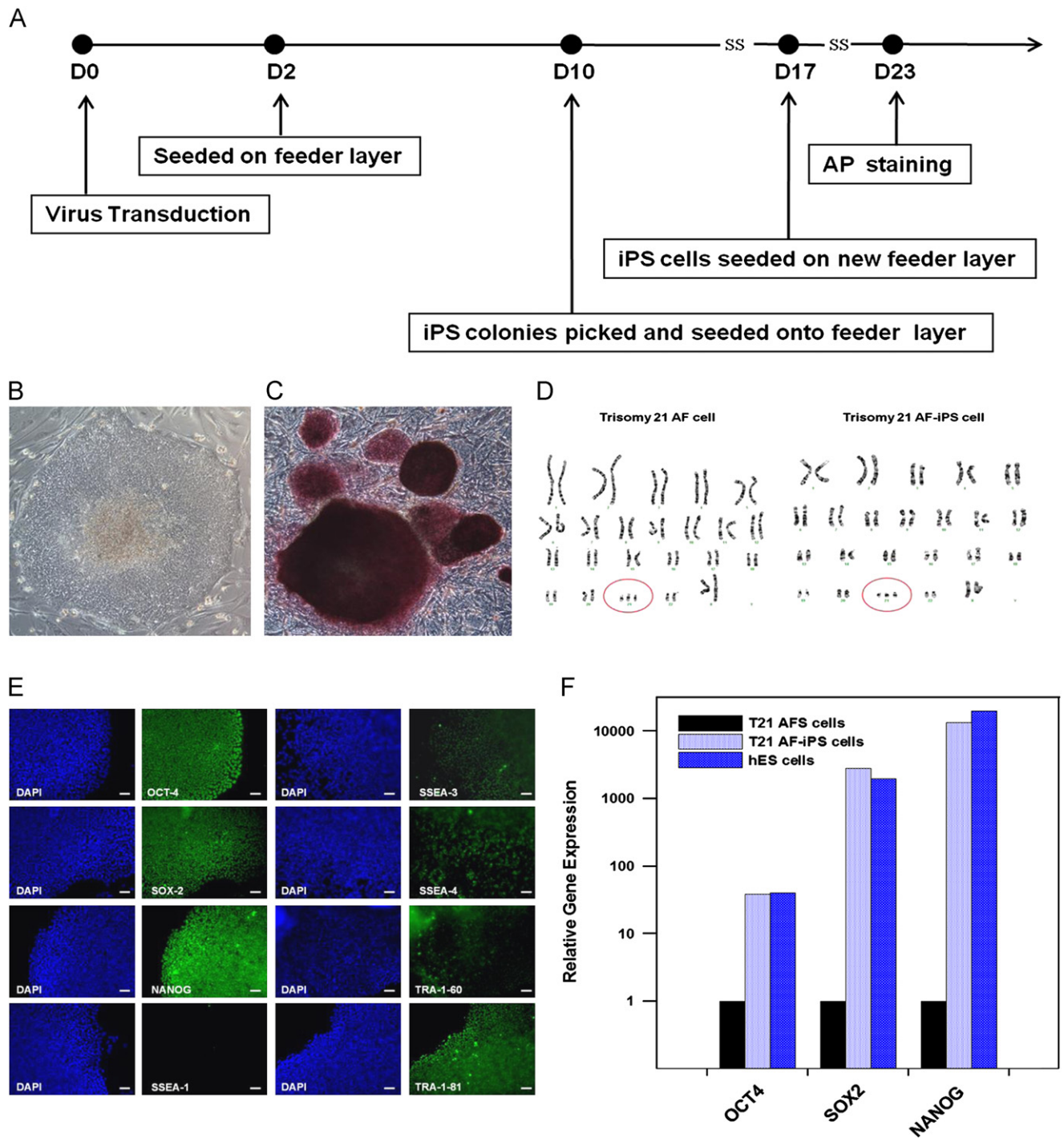


Fig. 1 – Generation of T21 AF-iPS cells. (A) Schematic representation of the protocol used for T21 AF-iPS cell generation. T21 AF cells were transduced using the same protocol as previously described. We observed colonies with ES-like morphology appearing on day 7 and manually picked select colonies for further growth and characterization on day 10 post-transduction. (B) Phase contrast captures of T21 AF-iPS cells grown on feeder layers. (C) T21 AF-iPS cells have alkaline phosphatase activity. (D) T21 AF-iPS cells maintained the abnormal and typical trisomy karyotypes. (E) Immunofluorescence staining of pluripotent markers in T21 AF-iPS colonies, including nuclear staining for OCT4, SOX2, NANOG and cell surface staining for SSEA-1, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81. Scale bar, 50 μ m. (F) Gene expression of *OCT4*, *SOX2* and *NANOG* in T21 AF-iPS cells by qRT-PCR analysis.

in vitro, cells were stained with primary antibodies against ABCG2 (1:20, R&D Systems) and MUSASHI (1:250, Abcam) followed by staining with secondary AlexaFluor-conjugated antibodies (Invitrogen) and observation under a fluorescent inverted microscope (Axio Observer, Zeiss, Germany).

Karyotype analysis

Karyotype analyses of T21 AF-iPS cells were performed at passage 8 in the laboratory of Prenatal Diagnosis, Cathay General Hospital, Taipei, Taiwan using standard protocols for G-banding

of chromosomes. The karyotype description follows the International System for Human Cytogenetic Nomenclature (<http://atlasgeneticsoncology.org/ISCN09/ISCN09.html>).

RT-PCR and qRT-PCR

Total RNA was extracted from each sample using TRIzol reagent (Invitrogen) and reverse transcription was performed using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, MD). Specific cDNA was amplified by PCR using DreamTaq PCR Master Mix (Fermentas) and subsequently analyzed by gel electrophoresis. The primers and conditions used were reported previously [16,17]. For qRT-PCR, we detected the expression levels of *APP* and *MeCP2* in T21 and normal AF-iPS-NPCs and the expression levels of *OCT4*, *SOX2* and *NANOG* in T21 AF-iPS, T21 AF and human ES cells, H9. For evaluating the miRNA profile, single-stranded cDNA was synthesized from total RNA samples using the TaqMan® MicroRNA Reverse Transcription Kit. We detected the expression levels of the miRNAs located on chromosome 21 (Hsa21-derived miRNA), including miR-99a, miR-125b-2, let-7c, miR-155 and miR-802. Amplification data were collected using the ABI sequence detection system 7700.

In vitro differentiation

For the generation of EBs, T21 AF-iPS cells were treated with 200 U/mL collagenase IV (Biochrom, Cambridge, UK) in DMEM/F12 basal medium and suspended in ultralow attachment 6-well plates (Corning, Lowell, MA) with DMEM/F12 supplemented with 20% FBS, 0.1 mM NEAA, 1 mM GlutaMax-1, 0.1 mM 2-mercaptoethanol, 50 U/mL penicillin and 50 µg/mL streptomycin for 7 days. EBs were then transferred onto gelatin-coated plates and cultured for another 10 days. The spontaneously differentiating T21 AF-iPS cells were analyzed for ectodermal (*NESTIN* and *TUJ1*), mesodermal (*TROPONIN* and α -*SMA*), and endodermal (*ALBUMIN*) markers by immunofluorescence staining as described above.

In vivo differentiation

The animal study conducted conformed to the Animal Protection Law (2010.01.10 Amended) published by the Council of Agriculture, Taiwan and approved by the Animal Care and Use Committee of the Food Industry Research and Development Institute, Hsinchu, Taiwan. For teratoma formation, 2×10^6 T21 AF-iPS cells were injected intramuscularly into non-obese/severe combined immunodeficiency (NOD-SCID) mice (Biolasco, Taipei, Taiwan). Six to eight weeks post-injection, teratomas were harvested and fixed with 10% formaldehyde (Sigma). Tissue sections were embedded in paraffin and stained with hematoxylin and eosin. Histopathological analysis was performed by the Taipei Institute of Pathology (Taipei, Taiwan).

Neuronal progenitor cell and neuronal differentiation

For the generation of NPCs, normal AF-iPS cells and T21 AF-iPS cells were differentiated into EBs for 4 days as described above. EBs were plated onto poly-ornithine/laminin-coated (Sigma) dishes in DMEM/F12 supplemented with 0.5X N2. After 7 days, rosette-like cells formed and were dissociated with accutase (Chemicon) and then seeded onto coated dishes with NPC culture media (DMEM/F12,

0.5X N2, 0.5X B27, 10 µM SB431542 and 10 ng/mL bFGF). To induce neuronal differentiation, NPCs were plated onto poly-ornithine/laminin-coated dishes and cultured with NPC culture media without bFGF for 15 days, and further analyses were subsequently performed.

Western blot analysis

The cells were harvested and resuspended in lysis buffer (20 mM HEPES at pH 7.6 containing 7.5 mM NaCl, 2.5 mM MgCl₂, 0.1 mM EDTA, 0.1% Triton X-100 and protease inhibitor cocktail). The cell lysates were centrifuged at 10,000 g for 5 min, and the supernatant was retained for further analysis. The supernatant was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Expedeon, Cambridge, UK) and transferred to polyvinylidene difluoride (PVDF) transfer membranes (Millipore). The membrane was probed with anti-beta-ACTIN (1:7000, Abcam), anti-APP (1:400, Millipore) and anti-MeCP2 (1:200, Novus). The secondary antibodies utilized were goat anti-mouse IgG-HRP (1:10,000, R&D) or goat anti-rabbit IgG-HRP (1:10,000, Millipore).

Results

Generation of iPS cells with trisomy 21

T21 AF cells were transduced and processed as shown in the flowchart in Fig. 1A. We observed colonies with ES-like morphology appearing on day 7 and manually picked select colonies for further growth and characterization on day 10 post-transduction. The T21 AF-iPS colonies maintained their ES-like morphology (Fig. 1B) and the chromosome 21 trisomy karyotype (Fig. 1D) on feeder cells. T21 AF-iPS cells were further characterized for AP activity (Fig. 1C) and the expression of human ES cell markers by immunofluorescence staining analyses for *OCT4*, *SOX2*, *NANOG*, *SSEA-3*, *SSEA-4*, *TRA-1-60* and *TRA-1-81* (a typical example is shown in Fig. 1E). Gene expression levels of *OCT4*, *SOX2* and *NANOG* in T21 AF-iPS cells were also confirmed as being similar to the levels in hES cells by qRT-PCR analyses (Fig. 1F).

Differentiation potential of T21 AF-iPS colonies

To determine the pluripotency of the T21 AF-iPS cells, their ability to differentiate into the three germ layers was assayed *in vitro* and *in vivo*. EBs were formed in suspension on ultralow attachment plates for one week and then seeded on gelatin-coated plates for adherence and further differentiation *in vitro*. The resulting cell populations stained positively for lineage markers *NESTIN* and *TUJ1* (ectoderm), α -*SMA* and *TROPONIN* (mesoderm) and *ALBUMIN* (endoderm), indicating the presence of all three germ layers (Fig. 2A). The gene expression levels of genes specific to the three germ layers were also confirmed by an RT-PCR analysis for the expression of *RUNX1* and *COLII* (mesoderm), *GATA4* (endoderm), *NESTIN* and *SOX1* (ectoderm) (Fig. 2B). To investigate the teratoma-forming ability of T21 AF-iPS cells *in vivo*, we injected 2×10^6 cells intramuscularly into NOD/SCID mice. Typical teratoma-like masses were observed and harvested for histopathological analysis six to eight weeks post-transplantation. We observed that the teratomas comprised of tissues from all three germ layers, such as the respiratory epithelium (endoderm), cartilage (mesoderm), muscle (mesoderm), and neural tube (ectoderm) (Fig. 2C).

Differentiation of T21-iPS into neuronal progenitor cells

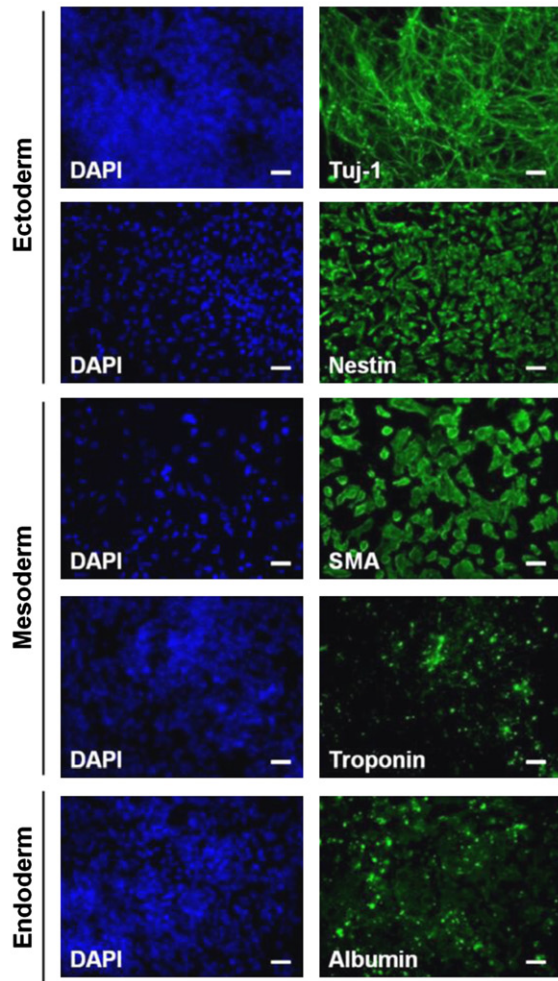
T21 AF-iPS cells and AF-iPS cells were differentiated into NPCs as described in the Materials and Methods. NPCs were cultured on poly-ornithine/laminin-coated plates and expressed NPC markers, including ABCG2, NESTIN, SOX2 and MUSASHI (Fig. 2D).

Homogeneous populations of NPCs could be generated after 1–2 passages for further analyses.

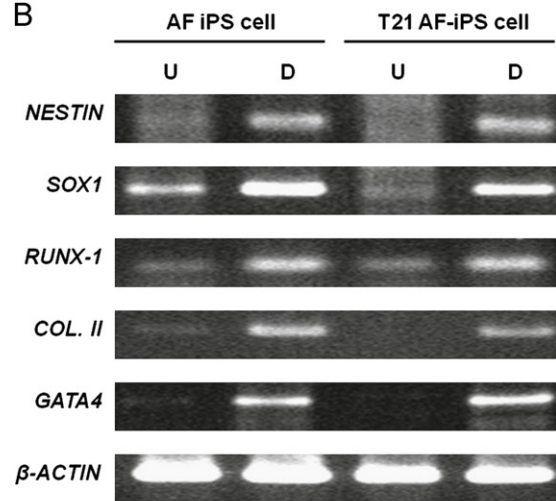
Hsa21-derived miRNA, gene and protein expression levels

Quantitative analyses of miRNAs were performed using primer sets specific for the 5 Hsa21-derived miRNAs. The results demonstrated

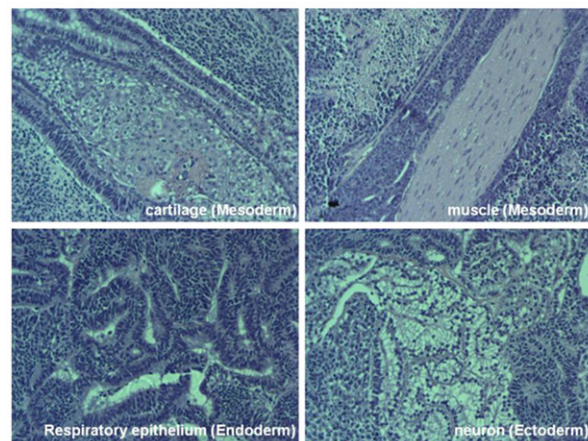
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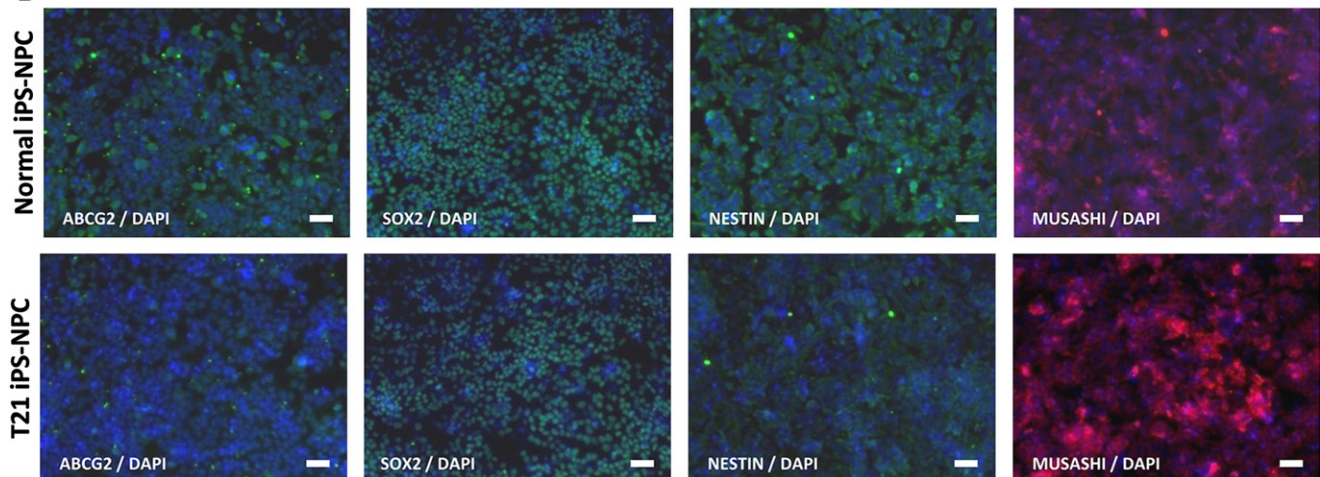
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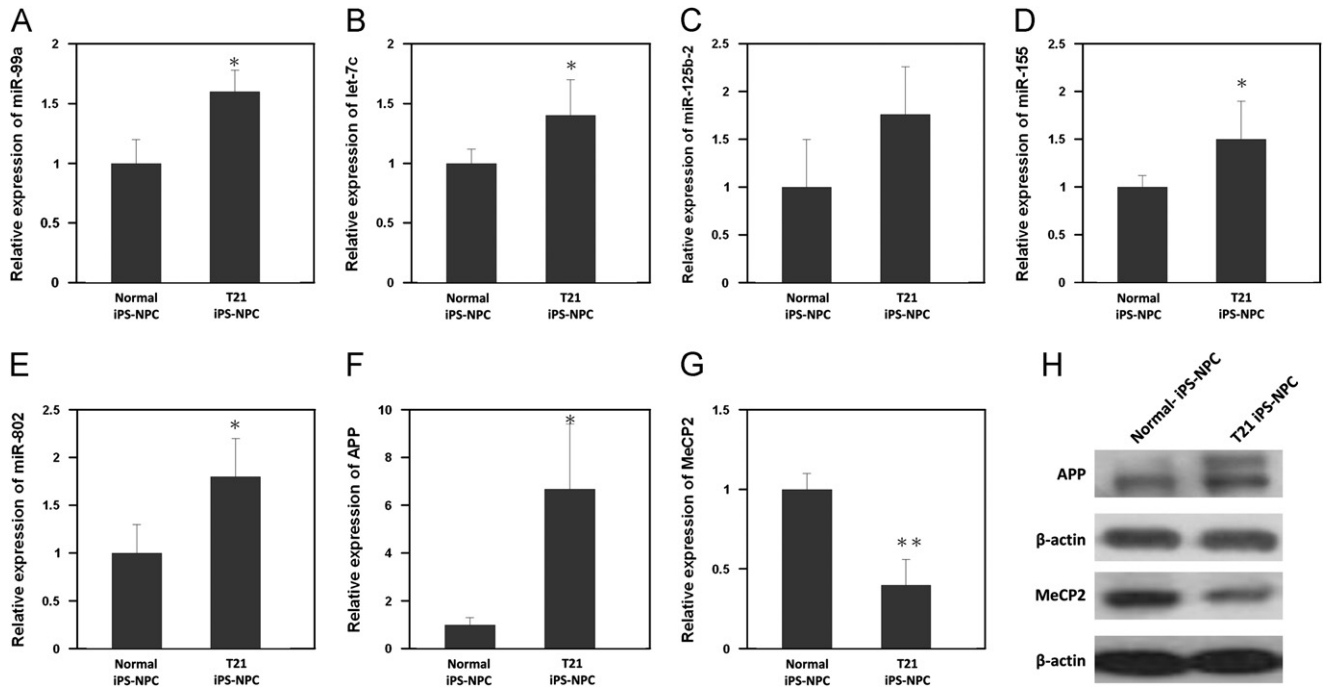


Fig. 3 – Hsa21-derived miRNA, gene and protein expression levels in T21 iPS-NPCs. Expression levels of Hsa21-derived miRNAs and mRNAs are elevated in T21 iPS-NPCs relative to normal iPS-NPCs based on qRT-PCR results (from three clones per iPS line). The relative expression levels of (A) miR-99a, (B) let-7c, (C) miR-125b-2, (D) miR-155, (E) miR-802, (F) *APP*, and (G) *MeCP2*. *: $p < 0.05$, **: $p < 0.01$ between each group. (H) Protein expression of *APP* and *MeCP2* in T21 iPS-NPCs compared with normal iPS-NPCs by Western blot analysis.

that the 5 Hsa21-derived miRNAs were elevated by at least 1.5 fold in T21 iPS-NPCs when compared with normal iPS-NPCs (Fig. 3A–E). qRT-PCR experiments demonstrated that the *APP* mRNA levels in the T21 iPS-NPCs were elevated by 6.7 fold compared with normal iPS-NPCs (Fig. 3F). *MeCP2* mRNA levels were significantly low expressed in T21 iPS-NPCs relative to normal iPS-NPCs (Fig. 3G). To support the mRNA qRT-PCR data, Western blot analyses of the same samples showed that differences in *APP* and *MeCP2* protein levels were consistent with the mRNA results in the T21 iPS-NPCs relative to controls (Fig. 3H).

Differentiation of T21 iPS-NPCs into neuronal cells

To compare neuronal differentiation abilities, normal iPS-NPCs and T21 iPS-NPCs were further differentiated in bFGF-free medium. Although both normal and T21 iPS-NPCs differentiated

into neurons, determined by positive staining of TUJ1 and *APP* on day 15 (Fig. 4A and B), we observed that T21 iPS-NPCs generated fewer neurons compared with controls. We quantified the efficiency in neuronal differentiation of normal iPS-NPCs and T21 iPS-NPCs by counting the TUJ1⁺/total cells ($n = 3$). The results demonstrated that the T21 iPS-NPCs revealed significantly lower efficiency in neuronal differentiation when compared with normal iPS-NPCs (Fig. 4C). This result showed that T21 iPS-NPCs exhibited a defect in the neuron development.

Discussion

The generation of iPS cells has a unique value for the development of *in vitro* human genetic disease models. This technology holds the promise of increased understanding for the development of complex diseases. Currently, there are several established

Fig. 2 – Differentiation potentials of T21 AF-iPS colonies *in vitro* and *in vivo* and neuronal progenitor cell differentiation. (A) Immunofluorescence staining of EB differentiation. EBs were formed on ultralow attachment plates for 7 days and then seeded on gelatin-coated plates for 10 days of growth *in vitro*. The resulting cell populations were stained for markers of the three germ layers, including α -SMA and TROPONIN (mesoderm), NESTIN and TUJ1 (ectoderm) and ALBUMIN (endoderm). Scale bar, 50 μ m. (B) Gene expression of the markers for the three germ layers in T21 AF and AF-iPS EB-differentiated cells by RT-PCR analysis, including *NESTIN* and *SOX1* (both for ectoderm), *RUNX1* and *COLII* (both for mesoderm) and *GATA4* (endoderm). (C) Teratoma formation of T21 AF-iPS cells in NOD/SCID mice *in vivo*. Mice were intramuscularly injected with 2×10^6 T21 AF iPS cells. Typical teratoma-like masses were harvested for histopathological analyses 6 to 8 weeks post-transplantation. The tissues of all three germ layers were present. Hematoxylin and eosin staining of teratoma sections. (D) T21 AF-iPS cells and AF-iPS cells were formed in suspension on ultra-low attachment plates for one week and then seeded on gelatin-coated plates for adherence and further differentiation, followed by immunofluorescence staining for NPC markers. The resulting cell populations stained positively for ABCG2, NESTIN, SOX2, and MUSASHI. Scale bar, 50 μ m.

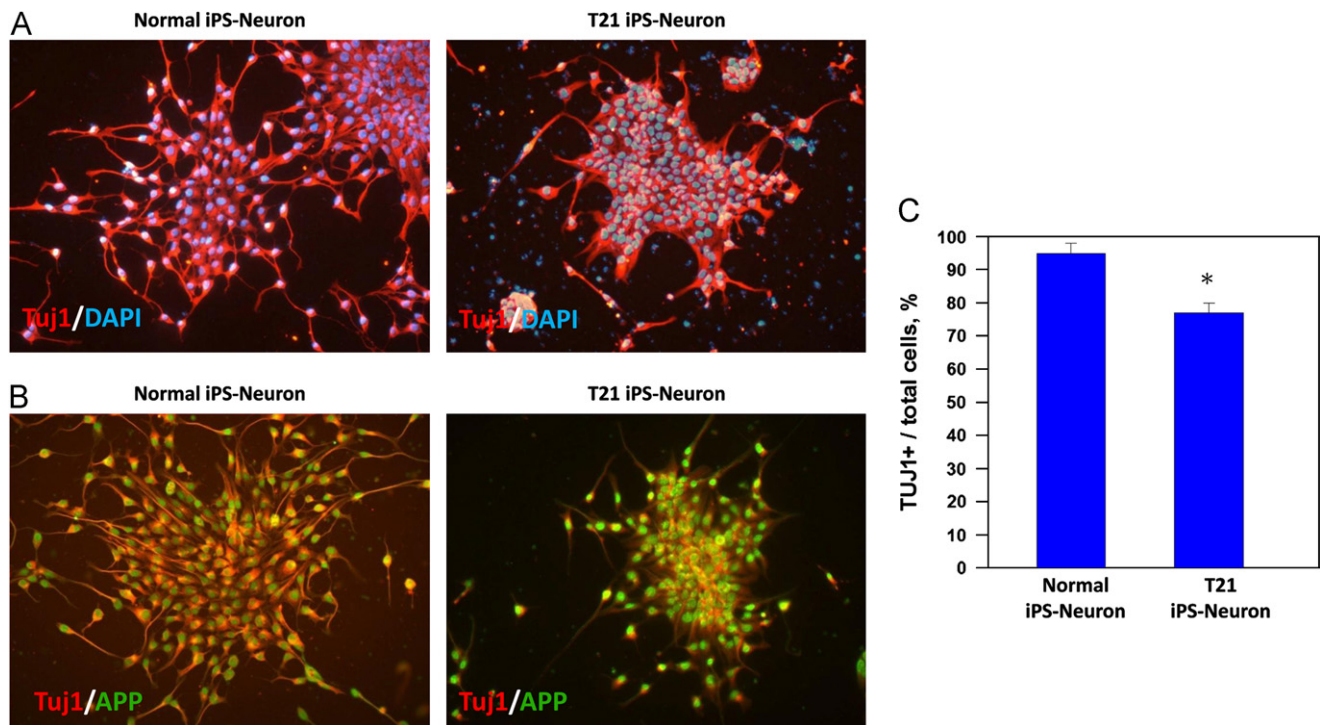


Fig. 4 – T21 iPSC-NPCs exhibit a developmentally regulated defect in neurogenesis. T21 iPSC-NPCs and normal iPSC-NPCs were differentiated into neuronal cells and subsequently processed for immunofluorescence staining. (A) Neurons stained for TUJ1. (B) Neurons double-stained for TUJ1 and APP. (C) Quantification of the efficiency in neuronal differentiation of normal iPSC-NPCs and T21 iPSC-NPCs.

patient-specific iPSC cells that pertain to genetic disorders [8,11]. Our study represents the first report of iPSC cells from second trimester AF cells with T21. In practice, amniotic fluid is routinely collected from second trimester amniocenteses for karyotype analysis. The AF cells can be easily isolated and cultured without interrupting prenatal diagnostic procedures and thus do not present significant ethical concerns [18,19]. AF cells have been recently tested for reprogramming to a pluripotent state and have exhibited higher efficiency of conversion than fibroblasts [20]. It has been reported that key senescence-related genes in fibroblasts from older donors are hypermethylated, which in turn has been shown to be a major barrier in iPSC cell generation, suggesting that donor age may affect the efficiency and quality of iPSC cells [21,22]. Prenatal AF cells, which can be easily isolated, stored and reprogrammed suggests that they may be the applicable source for iPSC cells and future patient-specific therapies, especially for studying some lethal fetal diseases [20,23].

In this study, we generated human DS iPSC cell lines from human second trimester T21 AF cells by lentiviral delivery of 4 Yamanaka factors. We report that T21 AF-iPSC cells expressed pluripotent markers and had the ability to differentiate into the three germ layers by forming EBs *in vitro* and teratomas *in vivo*. The T21 AF-iPSC cells maintained their unique pattern of chromosomal karyotype, trisomy 21. The miRNAs, especially miR-155 and miR-802 in NPCs derived from T21 AF-iPSC cells were overexpressed, contributing to the decreased expression of their specific target, *MeCP2*. These results are consistent with previous studies demonstrating that miR-155 and miR-802 overexpressed result in subsequent impairments in neuronal differentiation [13].

Porayette et al. reported that progesterone directs APP processing towards the non-amyloidogenic pathway, and then promotes hESC differentiation into NPCs [25]. In our study, we observed that the T21 iPSC-NPCs revealed significantly lower efficiency in neuronal differentiation when compared with normal iPSC-NPCs. This result demonstrated that T21 iPSC-NPCs exhibited a defect in the neuron development accompany with the overexpressed APP level. That suggests that overexpressed APP was a factor to impairments during neurogenesis.

DS is also considered to be linked with the early onset of AD, due to constitutively overexpressed *APP* gene on chromosome 21 that encodes APP protein. Shi et al. reported AD pathology could be developed in cortical neurons differentiated from iPSC cells derived from DS patient fibroblasts *in vitro*, including secretion of the pathogenic peptide fragment A β 42 and tau protein production, suggesting strong correlations between DS and AD development [24]. In our study, we demonstrated that APP levels in T21 iPSC-NPCs were significantly overexpressed in comparison with NPCs derived from normal AF-iPSC cells. It would be interesting to carry out for the future direction, especially the maturation of cortical neurons derived from T21 AF-iPSC cells.

Conclusion

In conclusion, we report that T21 AF-iPSC cells may provide the consistent quality and quantity of cells needed for the basic study of underlying molecular processes and study of various genetic disorders. The NPCs derived from T21 AF-iPSC cells serve

as a valuable disease model to further understand the role of neuronal development in individuals with DS.

Conflicts of interest

The authors indicate no potential conflicts of interest.

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