GENERATION OF HUMAN INDUCED PLURIPOTENT STEM CELLS AS ISOGENIC CELLULAR MODEL FOR MYOTONIC DYSTROPHY TYPE 1

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

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To my father Enzhen Xia, who has taught me not to be just a passenger in this wonderful world but to leave something valuable behind

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Dystrophia Myotonica type 1 (DM1) is an autosomal dominant multisystem disorder with no currently effective therapy. We hypothesized that disease-specific induced pluripotent stem (iPS) cell lines would provide a translational platform for DM1 studies

The study was approved by the University of Florida Institutional Review Board. Dermal fibroblasts (passage 3) derived from skin punch biopsies obtained from 2 DM1 patients with different CTG repeats and a 51-year-old healthy male were used for reprogramming. Traditional Yamanaka factors (hOct4, hSox2, hKlf4, hc-Myc) were transduced by retroviral infection. The iPS cell lines were characterized by morphology, reverse transcriptase PCR (RT-PCR) and immunofluorescence assay of stem cell markers. Pluripotency was assessed by *in vitro* embryoid body-mediated differentiation. Generation of neural stem cells (NSC) and *in vitro* neural differentiation were assessed using morphological analyses and immunofluorescence stains of a specific NSC marker (Nestin), neuronal markers (neurofilament H, beta-tubulin III) and an astrocytic marker

(GFAP). Intranuclear foci were detected by RNA fluorescence *in situ* hybridization (FISH).

We generated two DM1 iPS cell lines and a normal control iPS cell line. DM1 iPS and control iPS cell clones showed typical stem cell growth patterns in culture with high nuclear/cytoplasm ratio with normal karyotype. The iPS colonies maintained the same growth pattern through subsequent passages. All iPS cell lines expressed stem cell markers (Oct4, Nanog, Sox2, SSEA4) by RT-PCR and immunocytofluorescence and differentiated into three embryonic layer cells *in vitro* through embryoid body formation. Upon *in vitro* neural differentiation, control iPS cells underwent normal differentiation from neurospheres, neural rosettes, and neural stem cells to neural cells. The pathognomonic features of intranuclear foci were detected in DM1 iPS cells and neural stem cells, and terminally differentiated all three embryonic layer cells.

In conclusion, we have established disease-specific human DM1 iPS cell lines. These mutant iPS cells have hESC features and the potential for *in vitro* differentiation. Pathognomonic intranuclear foci were detected in iPS cells, neural stem cell and terminally differentiated all three embryonic layer cells. The DM1 disease-specific iPS cells can provide unlimited cell resources for mechanistic studies and can function as a translational platform for future therapeutic drug development and cell replacement therapy.

CHAPTER 1
INTRODUCTION

Dystrophia Myotonica type 1 (DM1) is a dominantly inherited genetic disorder that is the most common cause of muscular dystrophy in adults, affecting one in 8500 individuals worldwide.[1] Theoretically, the incidence should decrease with the prevalence of genetic testing and genetic counseling. However, the general pool of the population who carry the pre-mutated allele won't change. Thus, we do not expect a significant change in DM1 incidence as the descendents of this population will be eventually affected due to the common phenomenon of anticipation, meaning the expansion will further expand and cause early onset and severe type. In fact, most DM1 are identified without a family history. Simply providing genetic counseling for the affected families won't eliminate the mutant gene.

The disease is caused by an unstable CTG nucleotide repeat expansion within the dystrophia myotonica protein kinase(DMPK) 3'-untranslated region on chromosome 19q13.3.[2] DM1 is a multisystemic disorder. In classic form, the major features include impaired muscle relaxation (myotonia), muscle wasting, cardiac conduction defects, cardiomyopathy, insulin resistance, frontal balding and early onset cataracts. Much has been known about these features through transgenic, knockout mice and *in vitro* fibroblast cell and myoblast cells cultures (summarized in recent reviews).[3-7] One other feature of DM1 is central nervous system (CNS) involvement. The CNS involvement in DM1 is presented by low intellectual and cognitive abilities manifested by memory impairment, poor executive function, and psychiatric disorders including personality abnormalities in the adult and mental retardation, autism spectrum disorder, attention deficit hyperactivity disorder (ADHD) in the congenital form.[8-15] The impairment is

generally correlated to CTG expansion size.[8, 14] However, the further study of this disorder is limited by difficulty in getting viable CNS cells.

DM1 is considered a disease of splicenopathy. Two antagonistic splicing factor families, muscleblind-like 1(MBNL) and CUG-binding protein 1(CUGBP1)/Elav-like family member (CELF1) proteins, are particularly affected.[16-25] In normal embryonic stage, MBNL1 nuclear levels are low and CUGBP1 levels are high. During development, MBNL1 nuclear levels increase while CUGBP1 levels decrease, inducing an embryonic-to-adult transition of downstream splice targets. In DM1, MBNL1 is sequestered by CUG repeats in intranuclear foci, resulting in a decrease of functional MBNL1, while CELF1 function is upregulated due to hyperphosphorylation and protein stabilization via activation of PKC (as reviewed elsewhere).[18, 26-28] This simulates the embryonic condition and enhances expression of embryonic splicing profiles of MBNL and CELF1-targeted transcripts in adults.[29-32] The aberrant splicing of insulin receptor (exclusion of exon 11), chloride channel-1 (inclusion of exons containing stop codons) and cardiac troponin (exclusion of exon 5) have been correlated to clinical manifestations of insulin resistance, myotonia and cardiac conduction block/cardiomyopathy.[33-35]

The underlying molecular mechanism for CNS involvement might also be related to aberrant splicing. MAPT aberrant splicing has been found in DM1 brains. Different from other neurodegenerative disorders, pathological tau is mainly the shortest isoform with reduction of exon 2 inclusion.[36-39] Exon 3, 6 and 10 inclusion are also reduced but not as consistently detected as exon 2.[18, 38-41] Interestingly, the inclusion or exclusion of the exons is not induced by a one specific splicing factor. For example, the inclusion of

exons 2/3 is repressed by ETR-3[39] while the inclusion of exon 10 is repressed by CELF2[36]. MBNL1 seems to be less important in the brain than it is in the muscle tissues.[38, 42] Other aberrantly spliced genes in human brain include APP, and SORBS1, DCLK1, CAMK2D, CACNA1D, NMDAR which were also identified in MBNL1 and MBNL2 knockout mice.[42, 43] However, the above findings are only isolated reports and were mainly restricted to animal studies or autopsy tissues which are from patients with advanced stage disease and multiple confounding factors from age and comorbidities prior to death.

The therapeutic modalities for DM1 are limited to symptomatic and supportive care. Multidisciplinary care has greatly increased the patient quality of life. In particular, prophylactic pacemaker placement has dramatically reduced sudden cardiac death. However, no treatment has been able to prevent the progression of this daunting disease. Recently, therapeutic development targeting neutralization or elimination of expanded RNA foci has provided hope. Multiple approaches, including ribozymes, antisense oligonucleotides (ASO) and chemicals (pentamidine and small molecules), have been developed and show promising results.[44-50] ASO[49], pentamidine[47] and small molecules[50] are the most promising approaches. However, these studies have been limited to animal models.

Human induced pluripotent (hiPS) cells are generated by direct reprogramming of human somatic cells. These hiPS cells possess many of the properties of human embryonic stem cells (hESC) and have the potential to differentiate into most tissue types in the human embryo, including cardiomyocytes and neural cells.[**51-56**] The major advantage of human iPS cells over hESC is that they overcome most of the limitations

of hESC. The patient-specific iPS cell lines eliminate the ethical and immune rejection concerns. The most anticipated clinical application of iPS cell technology is for personalized cell therapy. Other potential applications of iPS cells are mechanistic studies and therapeutic drug development. Disease-specific iPS cells will preserve the genetic mutation carried by the patient on the functional human genomic background, which cannot be accomplished in animal models.

In this study, we proposed to establish two DM1 hiPS cell lines and one normal hiPS cell line. The human iPS cells and differentiated progenies will contain the pathognomonic features of DM1 cells. These cells will be ideal for mechanistic studies, therapeutic drug development and studies for future cell replacement therapy.

CHAPTER 2
METHODS

Reagents and Cells

Culture medium: Media for iPS cells culture (DMEM/F12, 20% KSR, Glutamax, 2-Mercaptoethanol, Sodium Pyruvate, MEM NEAA, penicillin/streptomycin), recombinant human fibroblast growth factor-basic (FGFb)(#PHG0021), recombinant human epidermal growth factor (Hu EGF) (#PHG0311L), and SuperScript III Reverse Transcriptase (#18080) were purchased from Invitrogen (Eugene, OR). Defined Cryopreservation Medium for hESC and hiPSCs (#05854), ACCUTASETM (#07920), Anti-Oct-3/4 (#01550), Anti-SSEA-4 (#01554), FITC-conjugated goat anti-mouse IgG (#10210), Heparin (#07980), Y-27632 ROCK inhibitor (#07171), AggreWellTM 800 (#27865), STEMdiffTM Neural Induction Medium (#05831), STEMdiffTM Neural Rosette Selection Reagent (#05832), NeuroCultTM NS-A proliferation kit (#05751) and NeuroCultTM NS-A Differentiation kit (#05752) were purchased from STEMCELL Technologies (Vancouver, BC, Canada). Poly-L-Ornithine (#P4957), Laminin (#L2020), and Mitomycin (M4287) were purchased from Sigma-Aldrich (St. Louis, MO). The following antibodies were purchased from the companies indicated: Nestin and alpha fetoprotein (AFP), R&D, Minneapolis, MN; Neurofilament H (#2836), Cell Signaling, Boston, MA; GFAP (#NB300-41A), Novus Biologicals, Littleton, CO; beta-Tubulin III (#CBL 412 X), Millipore, Billerica, MA; and Desmin,Lab-Vision, Kalamazoo, MI. IbidiTreat µ-Slides were purchased from ibidi GmbH, Martinsried, Germany.

Clinical Information of Study Subjects

This study was approved by the University of Florida Institutional Review Board. All subjects provided written consent. Nine subjects were recruited (Table 2-1).

Fibroblasts were collected as described below for all of them. Two DM1 and one normal control were selected for reprogramming. DM1-03 is a 46-year old Caucasian male with symptoms onset at the age of 25. At the time of biopsy, he had severe myotonia, cataracts (removal surgery at the age of 30), dysphagia (percutaneous endoscopic gastrostomy (PEG) placed at age 43), conduction block (pacemaker placed at age of 45), hypersomnia and cognitive impairment, but still functioned independently with his activities of daily living. DM1-05 is a 45-year old Caucasian female with symptom onset at the age of 26. She suffered from myotonia, mild dysphagia, no conduction block, no cataracts, and no diabetes, some cognitive impairment, but functioned well both socially and occupationally. The normal control subject was a 51-year old Caucasian male with no medical problems.

Skin Biopsy and Culture of Human Dermal Fibroblasts

Skin biopsies were performed under local anesthesia by once time punch biopsy (6mm in diameter) in the lateral thigh at the middle level between gluteal fold and popliteal fossa. Biopsy specimens were processed into 0.5mm cubes with scissors and scaples in culture dishes and placed into duplicate 25cm2 flasks and cultured in primary culture medium (DMEM with 20% FBS). When fibroblast cells from adjacent explants started merging (after a mean 2 weeks), the flasks were treated with 0.05% Trypsin/EDTA and passed to a 75cm^2 flask and further expand when cells get 90% confluent. Cells at passage 3 were used for reprogramming.

Southern Blot

High molecular weight DNA was extracted from DM1 fibroblasts cells using conventional methods. Five micrograms of DNA was digested by NcoI and separated by 0.5% agarose gel before transferring to a Nitran supercharged membrane, which was

hybridized overnight at 74°C with a DM1 probe. This probe is 777 base pair long PCR product amplified from a region of the DMPK gene located upstream of the CTG repeat (Forward primer 5'-TGCCTCAGACCTGCTGCCCA-3', reverse primer 5'-AACCCAATGCAGCCCAGGGC-3').

Generation of iPS Cell Lines

Reprogramming was performed with the four traditional Yamanaka factors using retroviral vectors[53]. Retroviruses, one for each of the four reprogramming factors (i.e., Oct4, Sox2, Klf4, and c-Myc) and enhanced green fluorescent protein (EGFP), were individually packaged using a 293FT human embryonic kidney cell line (Invitrogen). After 72 hours of virus production, cell supernatant was filtered through a 0.45µM pore size filter, and the virus was concentrated by centrifugation for 16 hours at 8,000 xg at 4°C. Oct4, Sox2, Klf4, and c-Myc viruses were combined and used for transduction. One day prior to transduction, fibroblasts were plated at a density of $1x10^5$ cells per $9.5cm^2$ cell culture dish in DMEM containing 15% FBS. Transduction efficiency was monitored using additional fibroblasts retrovirally transduced with EGFP virus alone. On Day 5 after transduction, each $9.5cm^2$ plate was passaged to a $56cm^2$ dish with feeders ($6.7x10^5$ mitomycin-C mitotically inactivated mouse embryonic fibroblasts (MEFs). On Day 6, the medium was changed from DMEM containing 15% FBS to iPS medium. Half of the media was removed and replaced with fresh iPS medium every other day (Days 8, 10, 12, etc.) until colonies were ready to be picked up. mFreSR freezing medium (STEMCELL Technologies) was used to prepare iPS cells frozen stocks.

Characterization of iPS Cells Lines

Morphology. iPS cells were cultured with MEF feeders. The morphology of iPS cell clones was examined and compared to that of human embryonic stem cells (hESCs) by growing pattern, nuclear/cytoplasm ratio.

RT-PCR of stem cell markers. Total RNA was extracted from fibroblasts (passage 4) and iPS cells (passage 13 of DM1-03 and DM1-05, passage 15 of normal control) using RNaeasy Micro Kit (Qiagen, Valencia, CA). cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen) from 1µg total RNA in a final volume of 20µl. 1µL was used for subsequent PCR. Stem cell marker RT-PCR was performed using primers targeting endogenous human OCT4, SOX2, NANOG and MYC. Electrophoresis of the PCR products was carried out on a 1.6% agarose gel.

Alkaline phosphatase activity assay and immunocytofluorescence staining of stem cell markers. The assays were performed on ibidiTreat µ-Slides. Three to five iPS clumps (around 100 cells) were seeded into chambers with feeders and cultured for 2-3 days to allow the colonies to expand. For the alkaline phosphatase activity assay, the chamber was incubated with a Liquid Fast-Red Substrate System (Thermo Scientific, #TA-060-AC) overnight at 4°C. Nuclear transcription factor Oct4 and cell surface marker SSEA4 were examined by immunocytofluorescence staining. The slides were washed with phosphate buffered saline (PBS) and fixed in 4% paraformaldhyde for 5 minutes, permeabilized with 0.3% Triton X-100 for 15 minutes, blocked with 10% normal goat serum (Vector Laboratories) for 30 minutes and incubated with Oct4 or SSEA4 (1:100) overnight at 4°C. The slides were washed and incubated with goat anti-mouse FITC-conjugated secondary antibody (1:500) (StemCell Technologies) for 30 minutes. Slides were washed and mounted with VECTASHIELD mounting medium with DAPI

(Vector Laboratories). Pictures were taken using an Olympus IX81-DSU Spinning Disk confocal microscope.

Prolonged self-renewal. iPS cells were passaged at a 1:4 ratio approximately every 4-5 days and were maintained to passage 20 for DM1-03 and DM1-05 and passage 21 for normal control iPS cells. Over this time, iPS cell growth pattern and morphology did not change.

Embryoid Body Formation

Undifferentiated colonies (passage 17 of DM1-03, passage 16 of DM1-05 and passage 17 of normal control iPS) were collected and resuspended in 3 mL accutase and incubated for 25 min at 37°C to make a single cell suspension. Cells were then centrifuged at 300 xg for 5 minutes. The cell pellet was resuspended in embryoid body (EB) medium to generate $2.5X10^5$ cells/ml and 2 mL was added to each well of an AggreWell 400 plate (STEMCELL Technologies). The plate was centrifuged at 100 xg for 3 minutes to capture the cells in the microwells. This generated roughly 500 cells per EB. Twenty-four hours later, EBs were detached by flushing the microwells with induction medium and transferred to a low attachment 6-well plate for further culture of five days. The EBs were transferred to ibidiTreat µ-Slides (coated with Poly-Orthnine-Laminin) for further differentiation.

Neural Differentiation

Neural differentiation using the AggreWell 800 (Stemcell Technologies) was performed following manufacturer's protocol. Briefly, a single cell suspension was prepared, and 1.2 to 2.5 $X10^6$ cells were seeded to each well of the AggreWell 800. The cells were captured in the microwells by centrifugation and cultured for five days. Over this time, the iPS cells collected at the bottom of the well aggregated to form

neurospheres. The spheres were flushed from the microwells using neural induction medium and were transferred to a Poly-Ornithine-Laminin coated 6-well plate. On Day 7, neural rosettes were detached using STEMdiff Neural Rosette Selection Reagent (Stemcell Technologies) and transferred to a 6-well plate coated with Poly-Ornithine-Laminin. Neural stem cells (NSC) started growing out of neural rosettes after 12 hours. Cells were cultured for seven days. NSCs were detached with accutase to make a single cells suspension and 4 X 10^4 cells were seeded to ibidiTreat µ-Slides and cultured in Neural Induction medium (Stemcell Technologies) for observation and staining of neural markers. For further propagation and differentiation, NSC were detached using accutase and grown either in suspension or as attached cells using NeuroCult NS-A proliferation medium (Stemcell Technologies).

RNA FISH

Intranuclear foci containing (CUG)exp RNA were detected in DM1 fibroblasts, iPS cells, NSC, and neurons via RNA FISH using a Cy3-labeled $(CAG)_{10}$ DNA probe. Cells plated in ibidiTreat µ-slides were washed twice in sterile PBS (pH 7.4), fixed in 10% buffered formalin phosphate (Fisher Scientific) for 10 min at room temperature, washed three times in sterile PBS (pH 7.4), and dehydrated in pre-chilled 70% ethanol for 3 hr at 4ºC. The cells were washed in 40% formamide (EMD Chemicals) in 2X SCC buffer (300 mM sodium chloride, 30 mM sodium citrate, pH 7.0) for 10 min at room temperature, then blocked in hybridization buffer (40% formamide, 2X SCC buffer, 200 ug/mL BSA, 100 mg/mL dextran sulfate, 2 mM vanadyl sulfate, 1 mg/mL yeast tRNA (Invitrogen) for 15 min at 37ºC. The Cy3-labeled $(CAG)_{10}$ DNA probe was denatured for 10 min at 100ºC, chilled on ice for 10 min, then added to pre-chilled hybridization buffer for a final concentration of 500 pg/uL probe. Hybridization buffer containing the probe

was added to the cells and hybridization was performed in a humidified chamber for 2 hr at 37ºC. As a negative control, RNA FISH for $(CUG)_{exp}$ RNA was performed on control patient cells. As an additional negative control, the experiment was performed in parallel on all cells using hybridization buffer lacking the $(CAG)_{10}$ DNA probe. After hybridization, cells were washed three times in pre-warmed 40% formamide/2X SCC buffer for 30 min at 37ºC and once in sterile PBS (pH 7.4), followed by counterstaining with Vectashield containing DAPI (Vector Laboratories).

Immunocytofluorescence Staining and RNA FISH Plus Immunocytofluroscence

The studies were carried out on ibidiTreat µ-Slides. 40µl of neural stem cells at a density of 1 X 10^6 /ml were seeded to ibidiTreat µ-Slides and cultured in Neural Induction medium for observation and staining of neural markers. Cells were cultured for 3-5 days to allow for spontaneous differentiation. The slides were then fixed in 4% paraformaldhyde and processed for immunocytofluorescence staining. Primary antibody was incubated overnight at 4ºC with AFP (1:100), Desmin (1:100), Nestin (1:100), Neurofilament H (1:50), beta-tubulin III (1:250), or GFAP (1:500). The following day, slides were washed three times with PBS, incubated with appropriate secondary antibody conjugated with either Alexa Fluor 555 or Alexa Fluor 488 (Invitrogen) (1:500) for 30 minutes, washed with PBS and mounted with VECTASHILD Mounting Medium with DAPI. Pictures were taken using Olympus IX81-DSU Spinning Disk confocal microscope.

We combined the two studies in one slide to better illustrate the relationship. Briefly, the slides were first processed for intranuclear foci as described above then were incubated with primary antibody (Oct4, both AFP and desmin simultaneously, or neural markers) and staining was completed as described above.

Table 2-1. Summary of subjects

Subject ID	Age	Gender	DM type	Family history	Fibroblast	iPS cells
MD-iPS-01	63	F	1	Brother	Yes	
MD-iPS-02	46	F	1	Brother	Yes	
MD-iPS-03	47	M	1	Sister	Yes	Yes
MD-iPS-04	51	M	Control	None	Yes	Yes
MD-iPS-05	40	F	1	None	Yes	Yes
MD-iPS-06	50	F	2	Unknown	Yes	
MD-iPS-07	57	M	1	Daughter, granddaughter	Yes	
MD-iPS-08	33	F	1	Father, daughter	Yes	
MD-iPS-09	56	F	Control	None	Yes	
MD-iPS-10	65	M	2	Unknown	Yes	

CHAPTER 3
RESULTS

Confirmation of Expansion and *in vitro* Instability of CTG Repeats in DM1 Fibroblast Cells

Southern blot of genomic DNA isolated from cultured fibroblast cells showed the CTG repeat expansion size (Figure 3-1). In DM1 fibroblasts, the expanded alleles were shown as a smear representing a range in size of 976 to 1245 repeats for DM1-03 and 644 to 1051 repeats for DM1-05, suggesting repeat instability. In the normal control, the two normal alleles were detected as a single band by Southern blot. The CTG repeat size in DM1-03 tended to increase with passage while the DM1-05 CTG repeat size is relatively stable.

Outcomes

Reprogramming Efficiency is Similar for DM1 and Control iPS Cell Generation

The traditional retroviral method had similar transduction efficiency between DM1 and control cells as demonstrated by GFP fluorescence (Figure 3-2). Colonies with typical hESC morphology were ready to isolate around day 30 for both DM1 and control iPS cells. These cells were allowed to expand in culture, and 4-10 individual clones were further propagated. Clone number 7 of DM1-03, clone number 2 of DM1-05, and clone number 10 of control iPS cells were used for studies below.

DM1 and Control iPS Cells Have Stem Cell Features

Morphology. DM1 and control iPS cells grew as flat, well-circumscribed colonies with cells having high nuclear/cytoplasm ratio (Figure 3-3). There was no change in their morphology or growth through subsequent passages (at least through passage 20).

Stem cell markers. DM1 and control iPS cells but not the original fibroblasts expressed stem cell markers (Oct4, Nanog and Sox2) by RT-PCR (Figure 3-4).

Immunocytofluorescence analysis revealed positive staining of stem cell markers for alkaline phosphatase, Oct4, and SSEA4 in DM1 and control iPS (Figure 3-5).

DM1 iPS Cells are Pluripotent and Intranuclear RNA Foci are Present Within Stem Cells and Terminally Differentiated Cells

To examine the pluripotency of established iPS cell lines, embryoid bodies (EBs) were generated. All EBs showed a normal pattern of differentiation in culture chambers and differentiated into cells with various types of morphology. Small EBs have the tendency to differentiate more towards neural lineages (Figure 3-6). Endodermal and mesodermal cell differentiation was confirmed by immunohistochemical staining of AFP and desmin (Figure 3-7 A-C), respectively. Not surprisingly, we found intranuclear foci formed in both germ layer cells. No foci were found in the normal control cells at any stage (enlarged areas in Figure 3-7 A-C). Ectodermal differentiation was demonstrated by neural differentiation as described below. The specificity of the intranuclear foci assay was confirmed in the primary culture of fibroblasts. No signal was detected in normal fibroblasts in contrast to DM1 fibroblasts (Figure 3-7 D-F). Intranuclear foci were also found in DM1 iPS cells (Figure 3-8).

DM1 and Control iPS Cells Undergo Normal Process *in vitro* Lineage-directed Neural Differentiation

Neural differentiation of DM1 iPS cells was compared to control iPS cells. Both DM1 and control iPS cells underwent normal neural differentiation. iPS cells aggregated and formed neurospheres in the microwells (Figure 3-9 A). Neural rosettes started forming three days following attachment of neurospheres (Figure 3-9 B). Following re-plating of neural rosettes, NSCs began egressing from the edges and had the tendency to form loosely arranged rosettes (Figure 3-9 C) from which spontaneous differentiation

occurred (Figure 3-9 D). Eventually, the entire plate was dominated by NSCs. The NSCs have been passed up to passage 7 without losing neural differentiation potential.

Intranuclear RNA Foci are Formed in Neural Stem Cell and Terminally Differentiated Neurons and Astrocytes

DM1 and control NSCs express Nestin and can further differentiate into neurons and astrocytes (confirmed by neuron and astrocyte markers). Intranuclear foci, a pathognomonic finding in DM1, are present in NSCs as well as terminally differentiated neurons and astrocytes (Figure 3-10). No intranuclear foci were observed in control cells (data not shown).

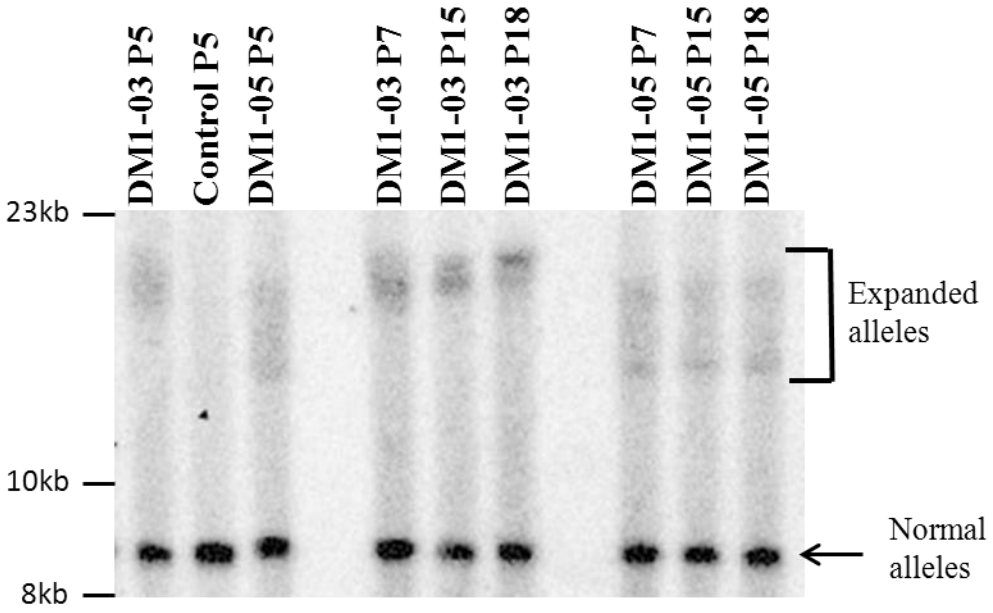

Figure 3-1. Southern blot of genomic DNA isolated from cultured fibroblast cells. Five micrograms of DNA from fibroblast cells was digested by NcoI for Southern blot and run in 0.5% agarose gel. Both DM1-03 and DM1-05 contain expanded allele. The control cell (DM1-04) has only one band showing overlapping of two alleles. The CTG repeat size in DM1-03 tends to increase with passage while the DM1-05 CTG repeat size is relatively stable.

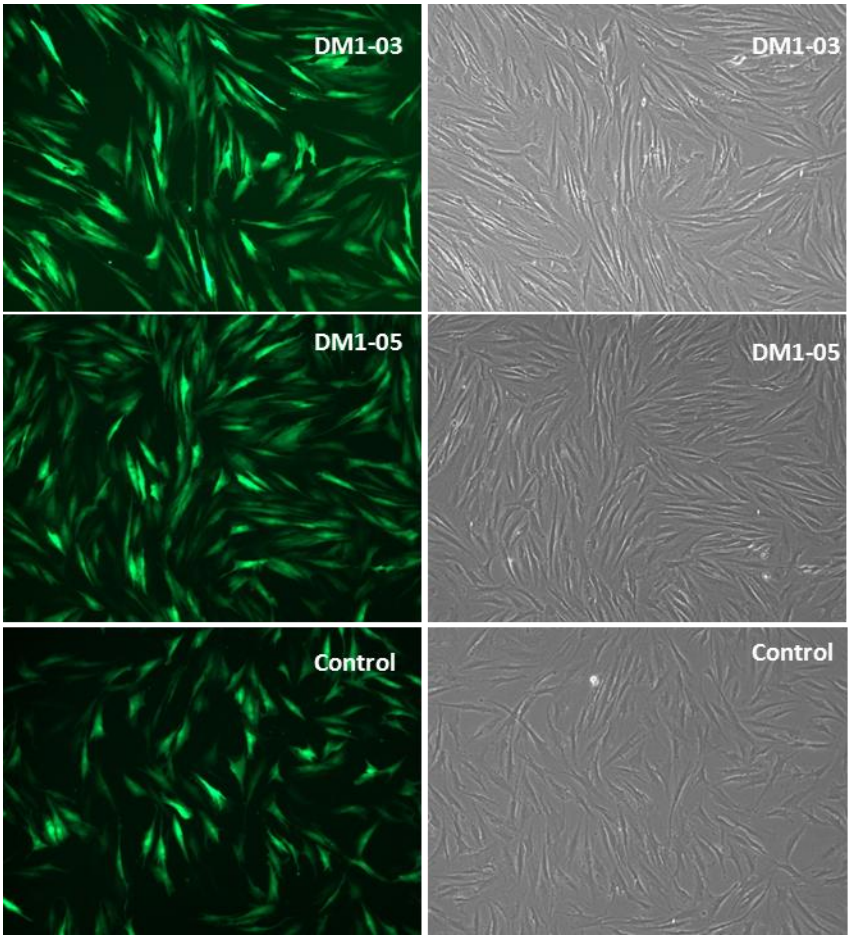

Figure 3-2. Retroviral infection. Retroviral infection was conducted on 6-well plate. EGFP was used as control to monitor infection efficiency. The infection efficiency is higher than 80%. No difference was seen between the two DM1 subjects and the normal control.

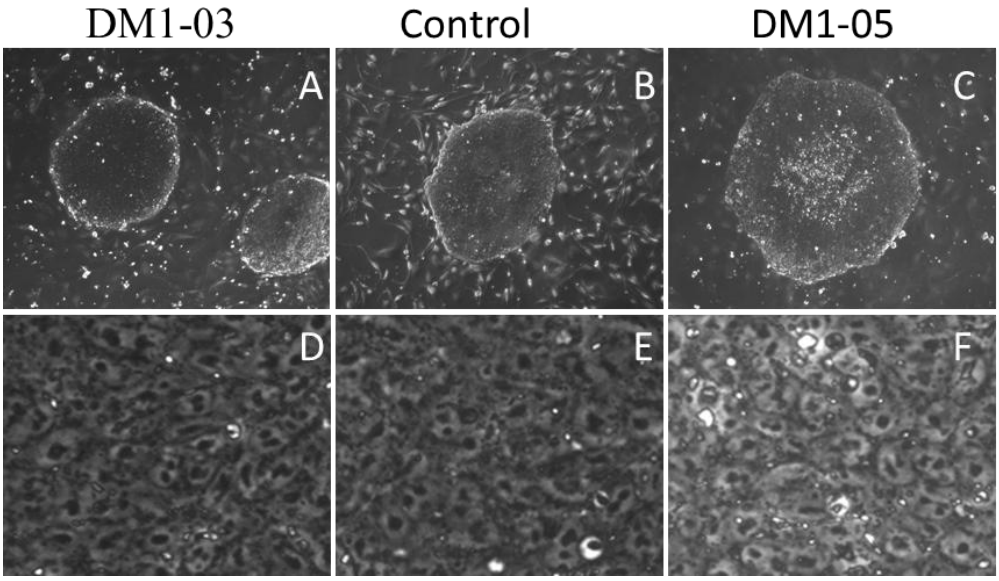

Figure 3-3. Typical DM1 and control iPS colonies growing on MEF. Typical DM1 and control iPS colonies growing on MEF are shown to be flat, well-circumscribed (A, B, C). High magnification shows tightly compacted cells with high nuclear/cytoplasm (D, E, F).

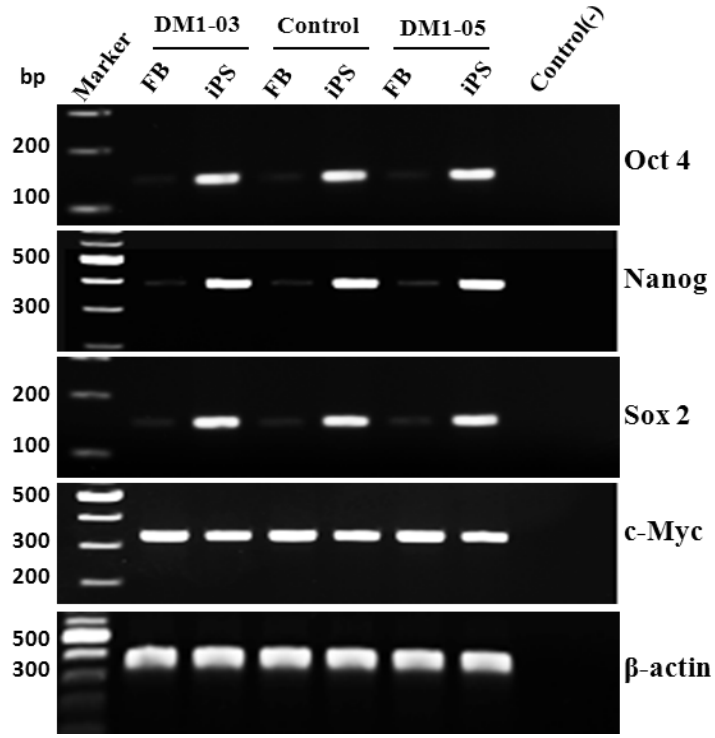

Figure 3-4. RT-PCR products of stem cell markers. RT-PCR products of stem cell markers show that DM1 and normal control iPS cells express stem cell markers (Oct4, Nanog and Sox2) which are not expressed in the original fibroblast cells (FB). In contrast c-Myc is ubiquitously expressed in somatic and stem cells. Control (-): no RNA control. β-actin: loading control.

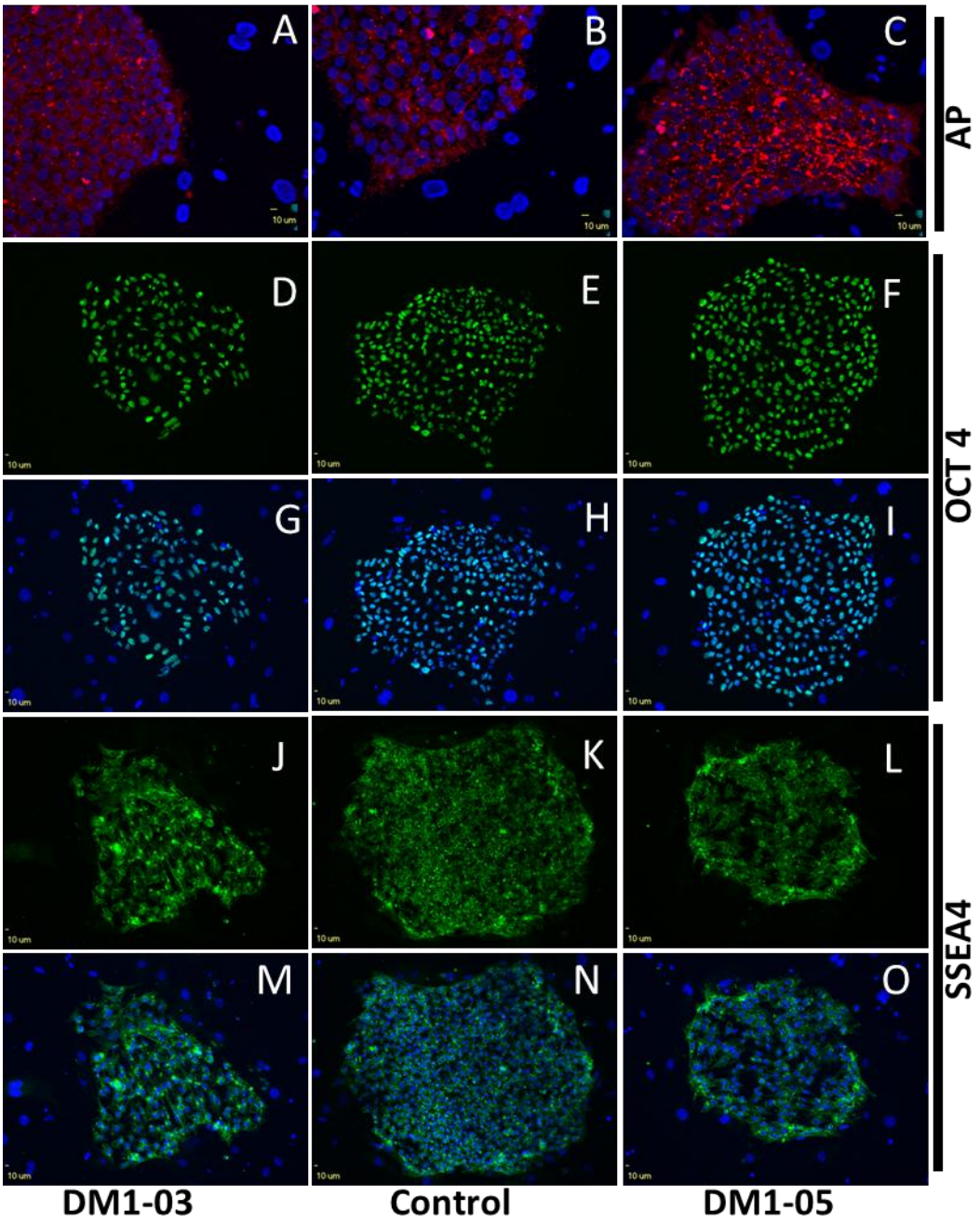

Figure 3-5. iPS stained colonies. All iPS colonies stained positive for alkaline phosphatase (A, B, C); intranuclear stem cell marker Oct4 (D, E, F and G, H, I merged with DAPI) and cell surface stem cell marker SSEA 4 (J, K, L and M, N, O merged with DAPI). The surrounding feeder cells are negative for stem cell markers.

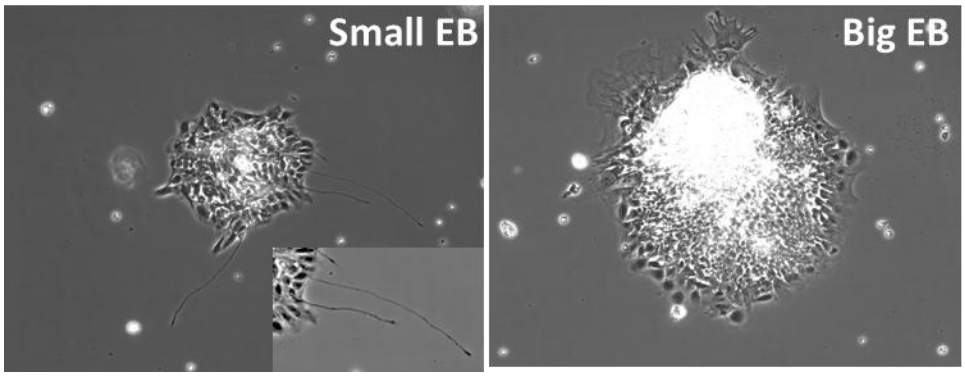

Figure 3-6. DM1-03 EBs. Microscopy of DM1-03 EBs demonstrate differentiation in cultured chamber into cells with different morphology. Spontaneous neural differentiation (with axons) can be seen in small EBs (insets).

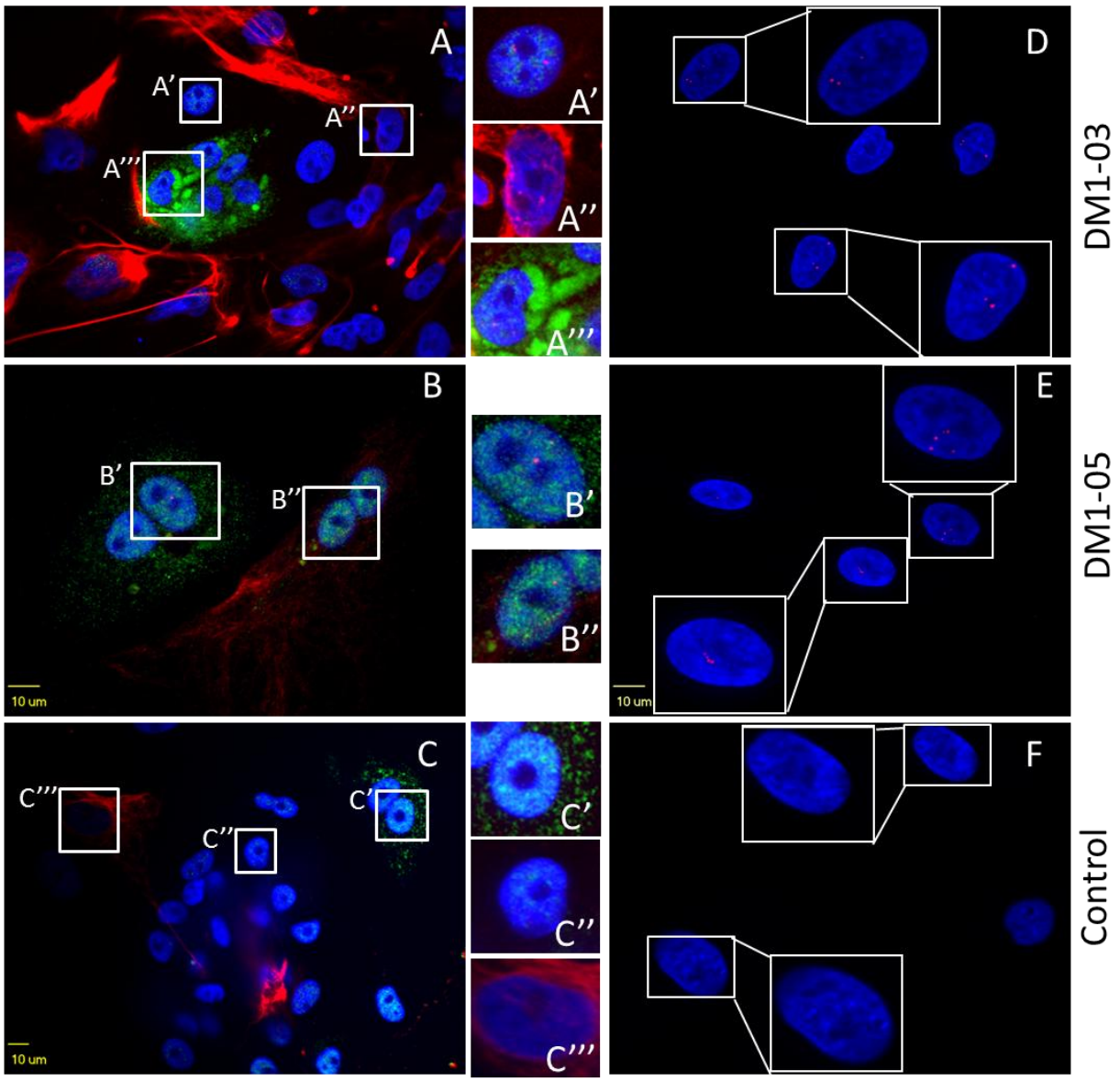

Figure 3-7. Endodermal and mesodermal cell differentiation. RNA FISH and double staining of endodermal (AFP) and mesodermal (Desmin) markers on EB were performed in the same IbidiTreat u-Slide. The endodermal and mesodermal cell differentiation was confirmed by positive AFP (green) and positive Desmin (red) cells. Cells that are negative for these markers are most likely ectoderm cells. Intranuclear foci were found in DM1 cells of three embryonic germ layers (A, B) but not in control cells (C). The corresponding primary culture of fibroblast cells show intranuclear foci in DM1 fibroblasts (D, E) but not normal control (F). Insets indicate areas of the image that are enlarged for greater detail.

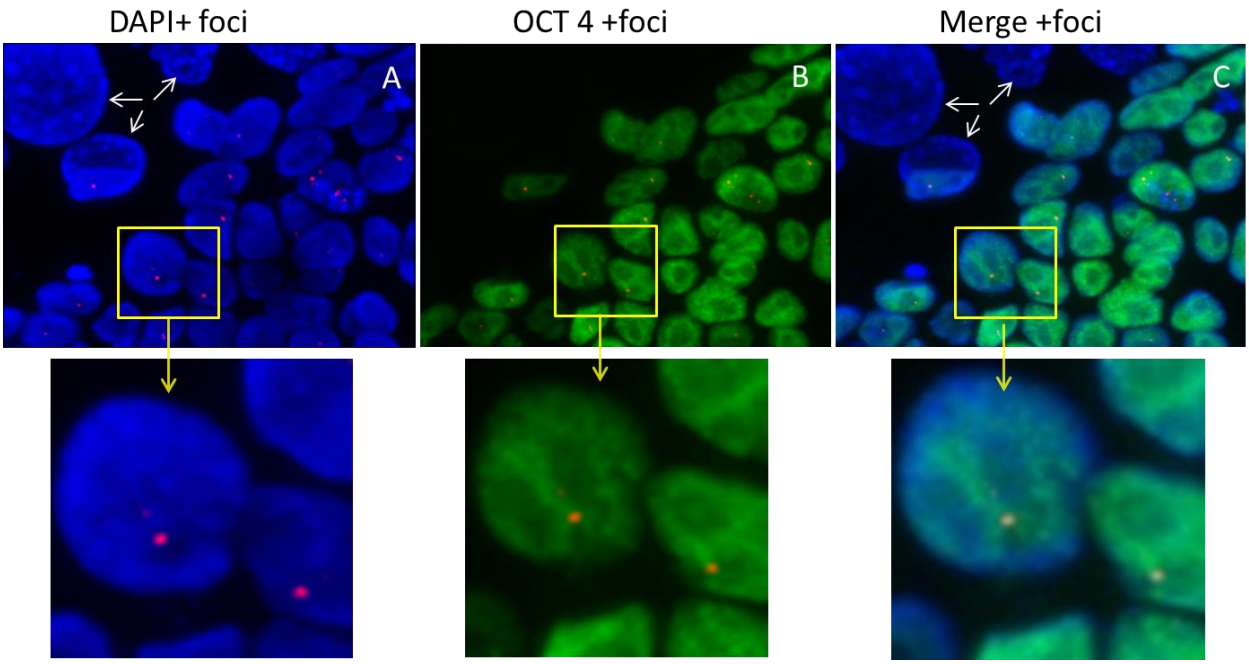

Figure 3-8. Intranuclear foci presence. Intranuclear foci were present in DM1iPS cell (only DM1-03 iPS is shown here). Stem cell marker (Oct4, green) and RNA FISH (red) were performed on the same IbidiTreat u-Slide. Feeder cells are negative for Oct4 and foci (arrows), which confirmed the specificity of both immunocytofluorescence and RNA FISH. A) DAPI (blue) and RNA foci (red). B) Oct4 (green) and RNA foci (red). C) DAPI (blue), RNA foci (red) and Oct4 (green) merge.

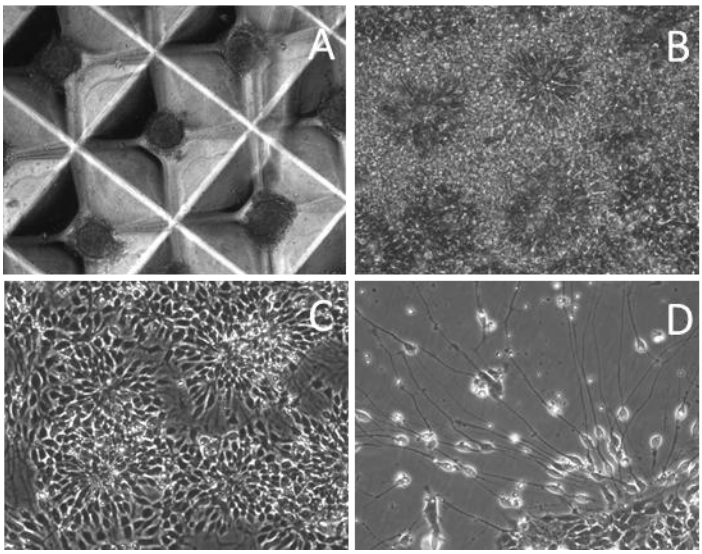

Figure 3-9. DM1 iPS and normal control iPS cells aggregate and form neurospheres normally in the microwells (only DM1-03 was shown here) (A). Neural rosettes start forming 3 days after neurosphere attachment (B). NSCs egress re-plated neural rosettes and re-form into small loosely arranged rosettes(C) from which spontaneous differentiation occurs (D).

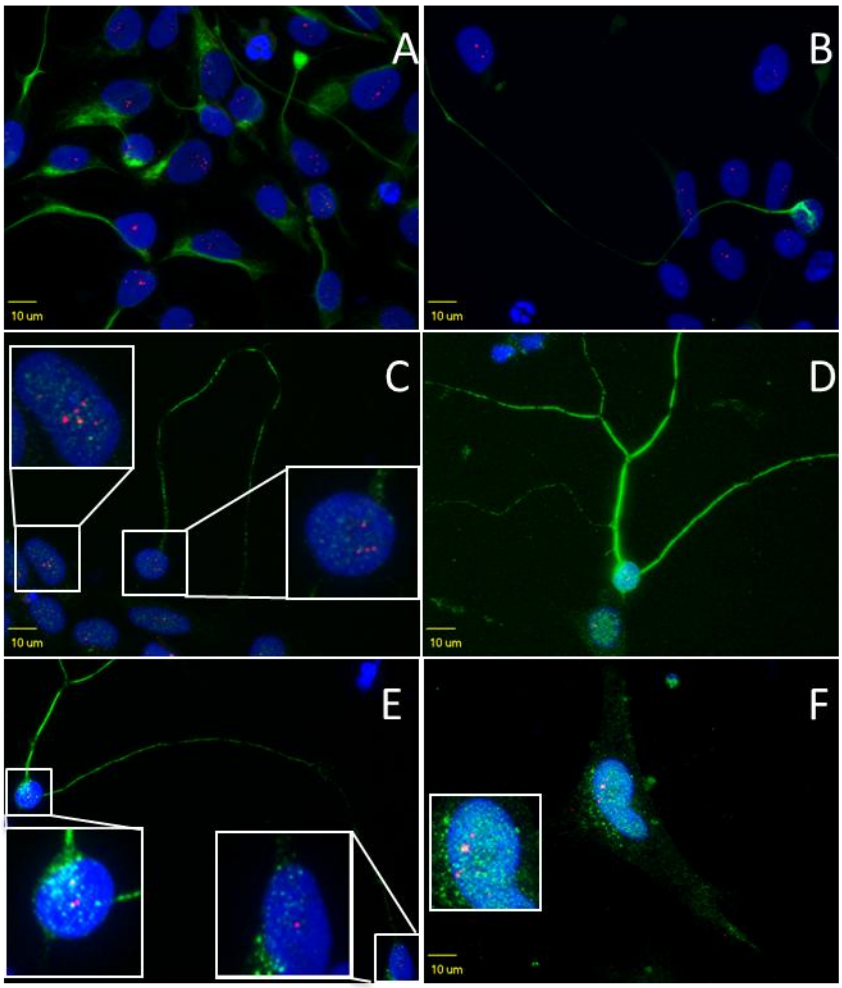

Figure 3-10. RNA FISH plus immunocytofluorescence staining of neural markers. DM1-03 NSC express Nestin (A, green) and can further differentiate into astrocytes positive for GFAP (F, green) and neurons positive for Neurofilament H (B, green) and beta-tubulin III (C, D, E, green). Intranuclear foci (red) are formed in NSCs, neurons and astrocytes (obvious in the enlarged boxes). DAPI is blue.

CHAPTER 4
DISCUSSION

Since the first description of DM1 by Steiner in 1909 and identification of the gene mutation in 1992[57-60], much knowledge has been gained into the pathogenesis of DM1 using mouse models. However, these animal models only display certain aspects of the disease, and none of them can fully recapitulate the clinical phenomena. The pathogenesis of CNS involvement is particularly poorly understood. We recently generated MBNL2 knockout mice and found MBNL2 plays an important role in cognitive impairment in DM1.[61] However, there are some hurdles that mouse models cannot overcome. The biggest hurdle is that pathogenic mimicking is generated on a different genomic background. Thus, to overcome these limitations, we proposed DM1 patient-derived iPS cells as a cellular model system for DM1 disease studies.

To achieve this end, we generated a DM1 cellular model through reprogramming human skin fibroblasts isolated from DM1 patients. Once established, DM1 and control iPS cells grow in a similar pattern to hESCs with high nuclear/cytoplasm ratio, express stem cell markers and have stem cell features of self-renewal and pluripotency. These cells harbor the naturally mutated gene in the human genomic background, making this an ideal isogenic cellular model for DM1 studies.

For mechanistic studies, previous alternative splicing studies are restricted to autopsy brain tissues which may have masked some differences due to non-selective inclusion of different type cells and premortem confounding factors. In the current study, we have shown that iPS cells derived from reprogrammed DM1 fibroblasts can be differentiated into viable neural cells — cells that are otherwise difficult to obtain unlike the easily isolated myoblast or fibroblast cells by muscle or skin biopsy. Through lineage-

specific differentiation and cell sorting, future experiments can analyze the expression of DMPK, MBNL, CELF and alternative splicing of their target genes in a cell-type specific manner, which may further shed light on the pathogenesis of CNS involvement. Furthermore, these iPS cells represent an unlimited resource of cells for repeated studies and comparisons.

Intranuclear CUG RNA foci are pathognomonic findings in DM1. These RNA foci can be found in cultured human fibroblasts myoblasts, biopsy tissues[57], neuronal cells in the post-mortem brain[18] and transgenic mouse models.[21-23, 29, 62, 63] The foci contain mutant DM1 mRNA and sequestered splicing and transcription factors, among them MBNL 1, 2 and 3 are main factors that have been well studied in DM1 pathogenesis which is elegantly described in several reviews.[3, 5, 6, 64-66] In this study, we found these foci not only in terminally differentiated cells but also in pluripotent and multipotent cell stages. To our knowledge, ours is the first report to track the foci formation during the developmental course from stem cell to terminally differentiated neuron.

The presence of intranuclear foci in iPS cells creates a discrepancy between the findings of RNA foci and clinical presentation in these two patients. Both patients had no symptoms prior to early adulthood. Why the patients were protected in young age or why they became symptomatic as adults if the RNA foci exist throughout the course of embryonic development, childhood and adolescence is unknown. In addition, it is not known whether the components in the foci are different in different stages and whether any aberrant splicing occurs prior to onset of symptoms. It is also unknown whether DM1 is a developmental disorder or degenerative disorder, and whether these findings are only restricted to *in vitro* culture condition. It is reasonable to predict that the foci

exist in live body tissues from the time embryo forms even in adult-onset cases. The foci are always there, ready for trapping emerging proteins that are important for cell viability or anti-aging.

Intranuclear RNA foci are considered pathogenic and can be used as outcome measures for therapeutic intervention. Multiple approaches, including antisense oligonucleotides, ribozymes and chemicals, have been developed for the treatment of DM1.[44-48, 67, 68] However, all are based on animal models and restricted human cell cultures (myoblasts). Treatment outcomes from these studies can be further validated in these human disease-specific DM1 iPS cells and almost all cell types from lineage-directed differentiation by assessing changes in intranuclear foci. The *in vivo* efficacy assay can be analyzed in teratoma tissues derived from this iPS cells in nude mice. These cell lines represent an additional, more applicable model to further translate results from basic studies into clinical applications. However, the fundamental cure for DM1 will be correction of the mutation. Recent technological developments involving genomic editing with either zinc-finger nucleases (ZFNs) or transcription activator-like effector nucleases (TALENs) are very promising in this area. We think these disease-specific human iPS cell are ideal for testing these technologies and pave the way for future cell-based therapy.

In conclusion, we have established disease-specific human DM1 iPS cell lines. These mutant iPS cells harbor mutations in the native genomic background as DM1 patient and have the potential to differentiate into cells of the three embryonic germ layers. Neural cells differentiated form these iPS cells contain pathognomonic intranuclear RNA foci. These cell lines represent an invaluable tool for the study of DM1

CNS neuropathogenesis and can readily be exploited as a translational platform for therapeutic drug development.

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BIOGRAPHICAL SKETCH

Guangbin Xia graduated from the Second Military Medical University, Shanghai, China with a medical degree in 1990. He received his PhD in 2002 from Tokyo Medical and Dental University, Tokyo, Japan. He did his internship training in University of Virginia and completed his neurology residency in University of Florida in 2010. He then completed a year of fellowship training in Neuromuscular Medicine at the University of Florida in 2011. He also completed training in the Clinical Research Consortium for Spinocerebellar Ataxias/NIH. In 2012, he completed a Master of Science with a concentration in clinical and translational science while a scholar in the Advanced Postgraduate Program in Clinical Investigation.

Dr. Xia is board-certified by the American Board of Psychiatry and Neurology (ABPN), the Neuromuscular Medicine Board and the American Board of Electrodiagnostic Medicine (ABEM). He is a member of the American Academy of Neurology and International Society for Stem Cell Research (ISSCR). Dr. Xia's research interest is disease-specific induced pluripotent stem (iPS) cells in the application of neurodegenerative disorders. Myotonic dystrophy and spinocerebellar ataxias (SCAs) are his current research focus. He has successfully established disease-specific iPS cell lines for DM1, SCA2 and SCA3. These disease-specific iPS cell lines are ideal isogenic cellular models for mechanistic studies and function as translational platform for drug development and for the study of cell-replacement therapy.