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Comparative Study of Myocytes From Normal and mdx Mice iPS Cells

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ABSTRACT

Recently, induced pluripotent stem cells (iPS cells) have been derived from various techniques and show great potential for therapy of human diseases. Furthermore, the iPS technique can be used to provide cell models to explore pathological mechanisms of many human diseases in vitro, such as Duchenne muscular dystrophy (DMD), which is a severe recessive X-linked form of muscular dystrophy without effective treatment. In this study, we try to determine whether there are different characteristics of myocytes from mdx iPS cells and C57BL/10 iPS cells. Our results showed that both of mdx and C57BL/10 cells could be induced into iPS cells in vitro, whereas colony-forming ability of mdx iPS cells was much weaker than that of C57BL/10 iPS cells. Meanwhile, mdx iPS cells could be induced to differentiate into myocytes, whereas their differentiation efficiency was much lower than that of C57BL/10 iPS cells. And, the number of apoptotic cells in differentiated myocytes from mdx iPS cells was significantly higher than that from C57BL/10 iPS cells. More importantly, treatment of a pan-caspase inhibitor (Z-VAD) produced a significant decrease in apoptotic cells. This study might add some insight to the biology study of dystrophin gene. J. Cell. Biochem. 113: 678–684, 2012.

KEY WORDS: iPS; MYOGENIC DIFFERENTIATION; MDX MICE

Induced pluripotent stem (iPS) cells are generated from somatic cells by forced expression of four transcription factors (Oct3/4, Sox2, Klf4, and c-Myc) [Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007], these iPS cells from somatic cells are similar to embryonic stem (ES) cells, expression of pluripotency markers, and ability to differentiate into the three germ layers both in vitro and in vivo. Therefore, iPS cells provide a powerful in vitro model system for the study of the molecular mechanisms of diseases [Nishikawa et al., 2008].

Duchenne muscular dystrophy (DMD) is one the most common genetic disorders among children and caused by mutations in the dystrophin gene [Hoffman et al., 1987]. Dystrophin associates with the subunit of dystroglycan on the intracellular side of the sarcolemma and is an essential component of the DGC, which links the extracellular matrix to the actin cytoskeleton in skeletal muscle myotubes [Ervasti and Campbell, 1993]. The absence of the cytoskeletal protein dystrophin leads to muscle membrane instability, progressive skeletal muscle wasting, cardiomyopathy, and finally premature death from respiratory complications and/or cardiac failure [Hardiman, 1994; Fayssoil et al., 2010]. Despite the understanding of the molecular genetics of DMD, the molecular and cellular mechanisms that underlie the loss of muscle mass in this disease are not fully understood. One possibility is that apoptosis plays a significant role in pathogenesis, because histological studies have found apoptotic myonuclei in DMD. However, the extent to which apoptosis controls the progression of this disease has not been fully understood.

The mdx mouse, which carries a nonsense mutation in exon 23 of the dystrophin gene, is a widely used model for DMD, exhibiting many features of DMD, including cardiomyopathy; skeletal muscular dystrophy; fibrosis; and apoptosis of skeletal muscle myoblasts [Im et al., 1996]. In this study, iPS cells were generated from mdx mice and C57BL/10 mice cells, and their characteristics were further detected to determine whether iPS cells from mdx mice were prone to apoptosis in the process of myocyte differentiation.

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MATERIALS AND METHODS

MICE AND CELL CULTURE

The mdx (C57BL/10ScSnJ) adult mouse was purchased from the Jackson Laboratory (ME) and the C57BL/10 control mouse was purchased from the Laboratory Animal Center of Sun Yat-Sen University. All animal studies were performed following Sun-Yet university guidelines for animal care and approved by the local ethics committee.

The tail-tip fibroblasts (TTFs) were prepared from tail tips of adult mdx and C57BL/10 mouse as reported previously [Takahashi et al., 2007]. Fibroblasts were maintained in DMEM containing 10% fetal bovine serum (FBS) and expanded in 1:5 ratio. TTFs at passage three used for iPS cell induction [Takahashi et al., 2007].

Murine ES cells (E14Tg2a, from American Type Culture Collection, Rockville, MD) and iPS cells were cultured in DMEM medium supplemented with 15% FBS, 2 mM glutamine, 1% nonessential amino acids, 50 U/50 μg/ml penicillin–streptomycin, 0.1 mM β-mercaptoethanol (all from Invitrogen, Carlsbad, CA), and 1,000 U/ml LIF (Chemicon, Temecula, CA) on irradiated mouse embryonic fibroblast (MEF) feeders at 37°C with 5% CO_2.

RETROVIRAL TRANSFECTION AND iPS CELL GENERATION

Four pMXs-based retroviral vectors encoding Oct-4, Sox2, c-Myc, or Klf-4 were purchased from Addgene. Plat-E packaging cells (Cell Bio labs, Inc., San Diego, CA) were seeded at 1×10^6 cells per well in six-well plate. On the next day, retroviral vectors were introduced into Plat-E cells by Lipofectamine 2000 (Invitrogen) according to manufacturer's recommendations. After 12 h, the medium was replaced with fresh medium. Virus-containing supernatants were collected and filtered through a 0.45 μm cellulose acetate filter (Millipore) at 72 h post-transfection. Equal volumes of the supernatants were mixed and supplemented with 4 μg/ml polybrene. TTFs were seeded at a density of 5×10^4 cells per well in six-well plate and incubated in the virus/polybrene-containing supernatants for 24 h.

Four days after infection, transfected fibroblasts were harvested by trypsinization and replanted at 1.5×10^5 cells per well in six-well plates on irradiated MEFs in mouse ES medium containing LIF. The medium was changed every other day. About 2 weeks after infection, the colonies were mechanically picked, and propagated under ES culture conditions. The iPS cells derived from mdx or C57BL/10 mice TTFs were termed as mdx-iPS or ctrl-iPS, respectively.

IMMUNOFLUORESCENCE AND ALKALINE PHOSPHATASE (AKP) ANALYSIS

Cells were fixed in 4% paraformaldehyde for 20 min, washed twice with PBS, and permabilized with 0.2% Triton-X-100. Cells were then blocked with 5% normal goat serum and incubated overnight at 4°C with primary antibodies against Oct4 (1:100; Santa Cruz), SSEA-1 (1:100; DSHB). After wash, cells were incubated with Cy3-conjugated secondary antibodies (1:100; Sigma) for 1 h at room temperature. The cell nucleus was labeled with Hoechst 33342 (Sigma). Cells were then washed with PBS and mounted for examination under a fluorescence microscope.

The activity of alkaline phosphatase was performed by histochemical staining. Induced pluripotent stem cells were fixed in 4% paraformaldehyde at room temperature for 15 min and washed twice with PBS, and then stained with the alkaline phosphatase substrate BCIP/NBT (Sigma) for 20∼30 min. The cells were observed under microscopy.

RNA EXTRACTION AND REVERSE TRANSCRIPTION-PCR ANALYSIS

Total mRNA was isolated from cultured cells by using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. First strand of cDNA was synthesized by using 2 μg of total RNA with MMLV-RT (Fermentas) and an oligo (dT) primer (Fermentas). For PCR, the samples were subjected to amplification with mouse specific primers. β-actin was used as positive control. The PCR products were analyzed by 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining. Primer sequences are listed in Table I.

EMBRYOID BODY (EB) FORMATION AND IN VITRO DIFFERENTIATION

To induce EB formation, undifferentiated ES cells and iPS cells were treated with 0.05% trypsin–EDTA and transferred to low adherence bacterial Petri dishes (approximately 10^7 cells/10 cm dishes) in ES cell medium without LIF. The medium was changed every other day. After 7 days culture, the EBs were plated in gelatin-coated plate and cultured for another 7–9 days. At the end, differentiated cells were

TABLE I. Sequences of the Primers Used for PCR Amplification

Gene	Forward	Reverse
Oct4	CTGAGGGCCAGGCAGGAGCACGAG	CTGTAGGGAGGGCTTCGGGCACTT
Nanog	AGGGTCTGCTACTGAGATGCTCTG	CAACCACTGGTTTTTCTGCCACCG
AFP	TGCTGCAAATTACCCATGAT	AAGGTTGGGGTGAGTTCTTG
ALB	GTCTTAGTGAGGTGGAGCAT	ACTACAGCACTTGGTAACAT
BMP4	CTGCCGTCGCCATTCACTAT	TGGCATGGTTGGTTGAGTTG
Nestin	TGCATTTCCTTGGGATACCAG	CTTCAGAAAGGCTGTCACAGGAG
GFAP	TGCCACGCTTCTCCTTGTCT	GCTAGCAAAGCGGTCATTGAG
Pax3	AACACTGGCCCTCAGTGAGTTCTAT	ACTCAGGATGCCATCGATGCTGTG
Pax7	CATCCAGTGCTGGTACCCCACAG	CTGTGGATGTCACCTGCTTGAA
Myf5	GAGCTGCTGAGGGAACAGGTGG	GTTCTTTCGGGACCAGACAGGG
MyoD	AGGCTCTGCTGCGCGACCAG	TGCAGTCGATCTCTCAAAGC
Myogenin	TGAGGGAGAAGCGCAGGCTCAAG	ATGCTGTCCACGATGGACGTAAGG
β-actin	AGAAGATCTGGCACCACACC	TACGACCAGAGGCATACAGG

fixed in 4% paraformaldehyde, and performed by immunofluorescence staining. The primary antibodies used are alpha-fetoprotein (AFP) (1:100; R&D), desmin (1:1,000; Neomarker), and β-III-tubulin (1:100; Abcam). Total RNA derived from plated aggregated cells was collected for RT-PCR analysis.

MYOGENIC DIFFERENTIATION

Myogenic differentiations were obtained as previously described [Mizuno et al., 2010]. Briefly, undifferentiated ES cells and iPS cells were cultured in hanging drops for 3 days at a concentration of 800 cells per 20 μl drop in differentiation medium, which consisted of DMEM supplemented with 0.1 mM nonessential amino acids, 0.1 mM β-mercaptonethanol, 10 mM 5-azacytidine (Sigma), 5% horse serum (Hyclone), and 10% FBS. At day 4, the EBs were transferred to suspension cultures for an additional 3 days (Day 3 + 3). Finally, the EBs were plated in the same medium in 0.1% gelatin-coated six-well plates. The medium was changed every 2 days.

The stage-specific proteins in myogenic differentiation of ES and iPS cells are analyzed by immunofluorescence staining. Antibodies used in this study were Pax7 (1:200; Abcam), myosin heavy chain (MHC; 1:100; Santa), myogenin (1:100; Abcam) and MyoD (1:200; Abcam), and dystrophin (1:200; Abcam). Secondary antibodies were coupled with Cy3 (red) and used according to the manufacturer's instructions.

WESTERN BLOT ANALYSIS

Cells were harvested and washed twice with ice-cold PBS. Then cells were lysed in lysis buffer (Tris–HCl 50 mM, NaCl 150 mM, NaN_3 0.02%, Nonidet P-40 1%, sodium dodecyl sulfate 0.1%, sodium deoxycholate 0.5%, and 1% protease inhibitor cocktail) for 30 min at 4°C. Samples containing 30 μg of protein were separated by SDS–PAGE and transferred to nitrocellulose membranes (Millipore). Membranes were incubated with primary antibodies against Pax7, MyoD, myogenin, Bcl-2, Bax, and overnight at 4°C and then with the appropriate secondary peroxidase-conjugated antibodies for 1 hr at room temperature. Blots were visualized by exposure to Kodak X-ray film.

TUNEL ASSAY

To assess apoptotic cell death, the terminal deoxynucleotidyl transferase-mediated dUTP in situ nick end-labeling (TUNEL) method was used according to the protocol supplied by the manufacturer (In Situ Cell Death Detection Kit-POD distributed by Roche). Cells were double-labeled for the presence of apoptotic nuclei and muscle markers as previously described [Lorke et al., 2010]. Briefly, differentiated cells were first incubated with MHC primary antibodies as above described; after incubation with secondary antibody, the cells were rinsed in PBS and then labeled for TUNEL.

CELL COUNT AND STATISTICAL ANALYSES

To estimate the number of positive labeled cells, 10–20 microscopic fields were chosen from each group. The cell count is based on at least three independent experiments performed on duplicate samples. Data are presented as mean ± standard deviation. Statistical analysis was performed using independent sample *t* test. Differences were considered significant at $P < 0.05$.

RESULTS

GENERATION AND CHARACTERIZATION OF iPS CELLS

We generated iPS cells by retroviral expression of mouse Oct4, Sox2, c-Myc, and Klf4 from the mdx and C57BL/10 tail-tip fibroblasts (TTFs) (Fig. 1A,B). Four days after infection, cells were plated onto MEFs in ES cell culture medium. By 12 days after infection, small colonies became visible, and were picked and expanded on MEF feeder cells (Fig. 1C,D).

To confirm that these iPS cells exhibit ES-like properties, we performed staining of alkaline phosphatase (AKP) activity, as well as ES cell markers—SSEA-1 and Oct4. From the staining results, we showed that the iPS clones gained high AKP activity, and expressed SSEA-1 and Oct4 (Fig. 1E–J).

To further characterize the pluripotency of iPS cells, both mdx- and C57BL/10-derived iPS cells were subject to in vitro differentiation through embryo body (EB) formation. By immunofluorescence staining analysis, we confirmed that the differentiated cells were positive for markers of endoderm (alpha-protein, AFP), mesoderm (desmin) and ectoderm (β-tubulin III) (Fig. 1K–P). RT-PCR analysis showed the expression of the markers of three germ layers in the differentiated iPS cells (Fig. 1Q).

In our study, ESC-like colonies first appeared 7 days after infection in C57BL/10 TTFs, while colonies from mdx TTFs appeared on Day 9. In order to investigate whether there is difference between mdx and C57BL/10 on iPS colony generation, we performed alkaline phosphatase immunofluorescence on day 18 iPS cells, and counted the number of ESC-like colonies. Of 1.5×10^5 infected C57BL/10 TTFs, ~250 iPS colonies were obtained, representing a frequency of ~0.17%. While of 1.5×10^5 infected mdx-TTFs, ~80 colonies emerged.

Collectively, the above results demonstrated that the iPS clones exhibit the features of ES cells. However, the mdx-TTFs showed lower colony generation frequency than C57BL/10 TTFs.

MYOGENESIS DIFFERENTIATION OF iPS CELLS

The myogenic differentiation of ES and iPS cells were performed according to previous protocol with small modifications (see methods). Spindle-shaped cells appeared from the EBs 7 days after plating. During terminal differentiation, these spindle cells fused into multinucleated myotubes (Fig. 2A).

To characterize differentiated cells, we detected several muscle-specific marker expressions, including Pax7, MyoD, myogenin, MHC, and dystrophin by immunofluorescence staining at multiple time points. On Day 3 + 3 + 14, we detected the presence of Pax7+/MyoD− cells. Pax7 is a marker of early myogenic precursors, while MyoD indicates muscle fate determination [Chang et al., 2009]. Such result suggested the presence of more immature muscle precursor cells within these cultures. Upon terminal differentiation (Day 3 + 3 + 28), we observed the presence of additional markers of mature muscle fates, such as myogenin and MHC.

During myogenic differentiation, genes encoding the MRF: Pax3, Pax7, Myf5, myogenin, and MyoD were expressed in a sequential

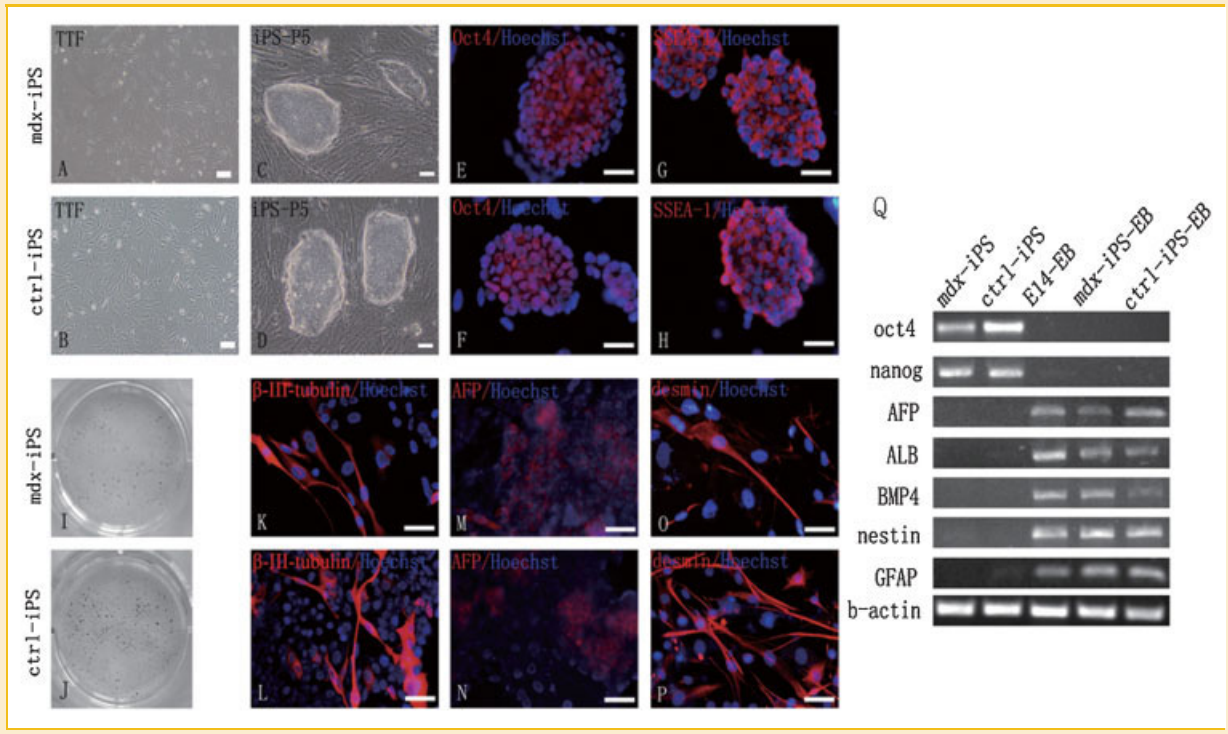

Fig. 1. Generation and characteristic of iPS cells from TTF of C57 and mdx mice. Tail-tip fibroblasts from 15-week-old mouse at passage 3 (A,B) were retroviral transduced with mouse Oct4, Sox2, c-Myc, and Klf4. Morphology of iPS cells (C,D). Immunofluorescence staining of Oct4 (E,F) and SSEA1 (G,H) of iPS clones. Hoechst 33342 was used as nuclear staining. iPS colony generation count from C57BL/10 (J) and mdx (I) fibroblasts by AKP staining. Immunofluorescence staining of three germ layer markers of iPS-derived cells: endoderm (alpha-protein, AFP) (K,L), mesoderm (desmin) (M,N) and ectoderm lineage (β-tubulin III) (O,P). Scale bar: 50 μm. Q: RT-PCR analysis of three germ layers markers of iPS-derived cells: endoderm (AFP, ALB), mesoderm (BMP4) and ectoderm (nestin, GFAP).

order closely resembling myogenesis in vivo [Haslett et al., 2002]. The time course of MRF expression was examined by RT-PCR (Fig. 2B). Expression of Pax3 and Pax7 both peaked on Day $3+3+14$, while Myf5, MyoD, and myogenin continued to be expressed after Day $3+3+14$. Western blot detection of Pax7, MyoD, and myogenin showed similar results (Fig. 2C).

The mdx-iPS cells showed less myogenic differentiation efficiency compared with the ES cell and ctrl-iPS. On Day $3+3+14$, the percentage of Pax7+ cells from E14 cells, ctrl-iPS cells, and mdx-iPS cells are $26.21 \pm 2.03\%$, $22.45 \pm 2.61\%$, $6.19 \pm 1.26\%$, respectively. On Day $3+3+28$, the percentage of myogenin+ cells from E14 cells, ctrl-iPS cells, and mdx-iPS cells are $20.50 \pm 2.42\%$, $20.12 \pm 2.14\%$, $3.25 \pm 1.18\%$, respectively, and the proportion of MHC+ cells is similar, are $19.81 \pm 2.02\%$, $18.99 \pm 2.25\%$, $4.46 \pm 1.17\%$, respectively.

Collectively, although we could successfully induce myogenic differentiation from ES and iPS cells, the mdx-iPS cells showed less differentiation efficiency than controls.

APOPTOTIC ASSAY OF DIFFERENTIATED MYOGENIC CELLS

According to the previous studies, deficiency of dystrophin gene is co-related with cell apoptosis, which results in muscle atrophy [Sandri et al., 1998]. We use TUNEL assay to study the apoptosis at different myogenic differentiation stages. On Day $3+3+14$, the proportion of TUNEL+ cells among the total cell number in E14 and ctrl-iPS was $2.23 \pm 0.13\%$ and $2.09 \pm 0.23\%$, respectively. And on Day $3+3+28$, the percentage of TUNEL+ cells in E14 and ctrl-iPS was $10.67 \pm 0.18\%$ and $10.42 \pm 0.69\%$, respectively. Comparatively, on Day $3+3+14$, the percentage of TUNEL+ cells in mdx-iPS derived cells was $7.22 \pm 0.24\%$, then increased significantly to $42.09 \pm 0.33\%$ on Day $3+3+28$, fourfold higher than that of the ctrl-iPS cells (Fig. 3A).

Next, we use double staining of MHC and TUNEL to study the apoptosis within muscle cells on Day $3+3+28$. The result showed among MHC+ population, up to $64.57 \pm 3.05\%$ cells were TUNEL+. While this proportion in E14 and ctrl-iPS was only $1.87 \pm 0.41\%$ and $2.09 \pm 0.49\%$, respectively (Fig. 3A). These data showed that during myogenic differentiation, mdx-iPS cells are more likely to undergo apoptosis than controls.

For understanding the mechanism of apoptosis, we thus investigated the expression of the anti-apoptotic protein (Bcl-2) and the pro-apoptotic protein (Bax) at different myogenic differentiation stages. Bcl-2 and Bax are members of the Bcl-2 family proteins, which are critical regulators of the apoptotic pathway [Hockenbery et al., 1993]. By Western Blot, we showed that there was no significant change of both protein expressions in the mdx-iPS group and ctrl-iPS group at different stages (Fig. 3B). This finding suggested that apoptosis was mediated by nonmitochondria signaling pathway.

Then, to test whether caspase activity is required for apoptosis during myogenic differentiation, we treated mdx-iPS cells with a pan-caspase inhibitor (z-vad). Interestingly, treatment with 50 μM

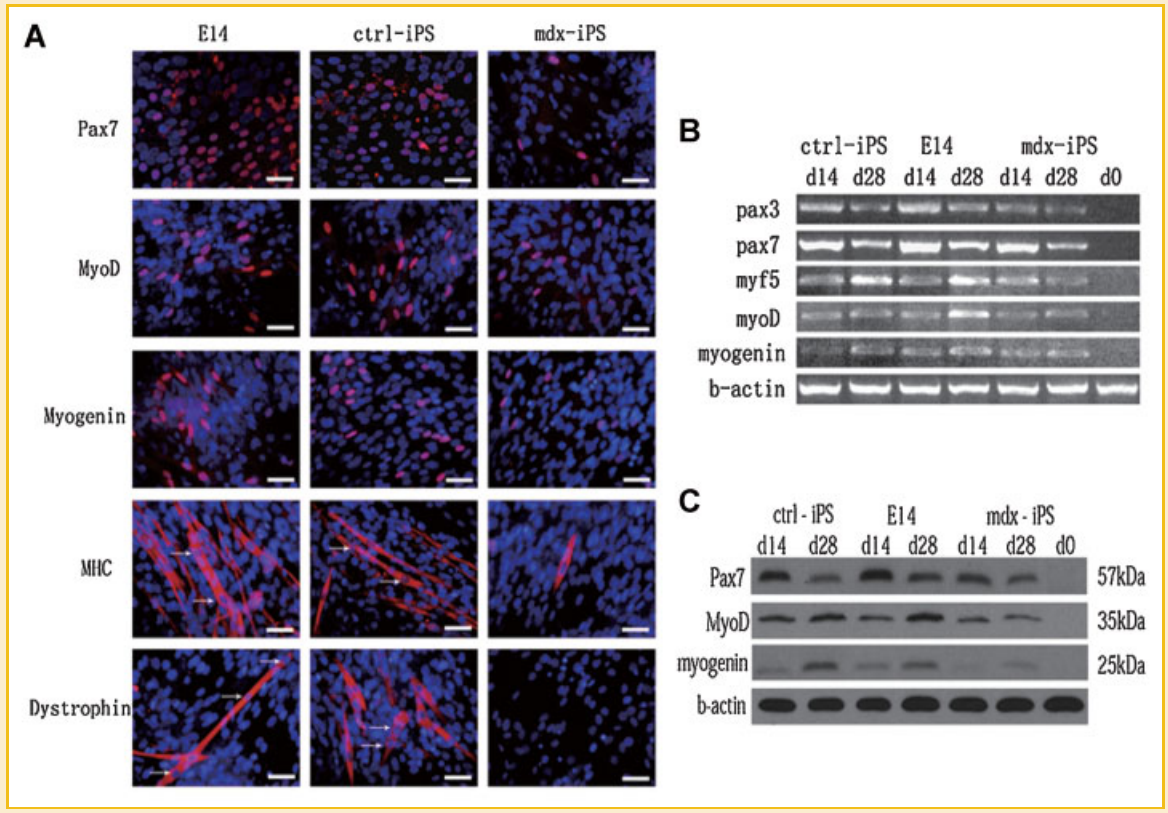

Fig. 2. Myogenesis Differentiation of ES and iPS Cells A: Immunofluorescence staining of muscle cell markers of ES and iPS cells. Cells were stained with Pax7, MyoD, myogenin, MHC, and dystrophin. Cell nuclei of multinuclei myotube-like cells are indicated by arrows. The mdx-iPS cells showed no dystrophin positive cells. Scale bar: 50 μm. B: RT-PCR analysis of muscle specific genes. RNA was isolated from differentiated cells from E14 cells, mdx-iPS cells and ctrl-iPS cells at Day 3 + 3 + 14, Day 3 + 3 + 28, and mdx-iPS cells at day 0 was used as negative control. RT-PCR of muscle specific genes including Pax3, Pax7, Myf5, MyoD, and myogenin from differentiated E14, mdx-iPS, ctrl-iPS. C: Western blot analysis of muscle specific protein. Protein was isolated from differentiated cells from mdx-iPS cells and ctrl-iPS cells at Day 0, Day 3 + 3 + 14, Day 3 + 3 + 28. The level of protein Pax7, MyoD and myogenin showed similar results with RT-PCR. β-actin was used as internal control.

z-vad-fmk (benzyloxycarbonyl-Val-Ala-Aspfluoromethylketone; Sigma) significantly decreased TUNEL+ cell numbers in mdx-iPS group, 30% decrease in number of apoptotic cells compared to the untreated cells. In the meanwhile, z-vad-fmk treatment would also improve myogenic differentiation (Fig. 3C). Taken together, our results suggested that apoptosis might occur via nonmitochondria caspase-dependent apoptotic pathway.

DISCUSSION

Although iPS cells can differentiate into muscle cells with electrophysiological characteristics and spontaneous contraction [Mizuno et al., 2010], very limited data are available concerning to the impact of mutated dystrophin gene on the in vitro differentiation potential of iPS cells into myogenic cell lineages. It was therefore the aim of this study to investigate the myogenic differentiation potential of mdx-iPS cells.

With the forced expression of four transcription factors, we successfully generated iPS cells from the tail tip fibroblasts of mdx-mouse and C57BL/10 mouse. These iPS cells, expressed typical pluripotent stem cell markers -alkaline phosphatase, SSEA-1, and Oct4. And RT-PCR analysis revealed that both the undifferentiated mdx-iPS and ctrl-iPS cells expressed pluripotent genes from the endogenous loci. To confirm the pluripotent characteristics of iPS cells, we generated embryo bodies (EBs) that were differentiated successfully to various cell types. These data clearly suggested that iPS cells derived from TTFs have ES cell-like properties.

In this study, we found less iPS generation rate in mdx fibroblasts than that in the control group, which might result from the dystrophin gene mutation. Previous reports have demonstrated that the generation efficiency of iPS cell is associated with potential transcript factors [Aasen et al., 2008; Banito et al., 2009; Tat et al., 2010]. The suppression or deletion of p53 could increase the efficiency of iPS cell generation [Hong et al., 2009]. Hugnot et al. [1993] found that the dystrophin gene expressed in fibroblasts and this could account for some abnormalities observed in patient's fibroblast cultures. Other research also found that higher activation of p53 in diaphragm muscle of mdx mice was associated with its increased phosphorylation and nuclear translocation [Dogra et al., 2008]. The lower efficiency of mdx-iPS cells may due to some high-expressed transcript factors, such as p53. Further research is necessary needed to clarify this possibility.

Differentiation assays showed that under appropriate culture conditions, both iPS cells derived from mdx TTFs and C57BL/10 TTFs could differentiate into the myocyte phenotype. RT-PCR

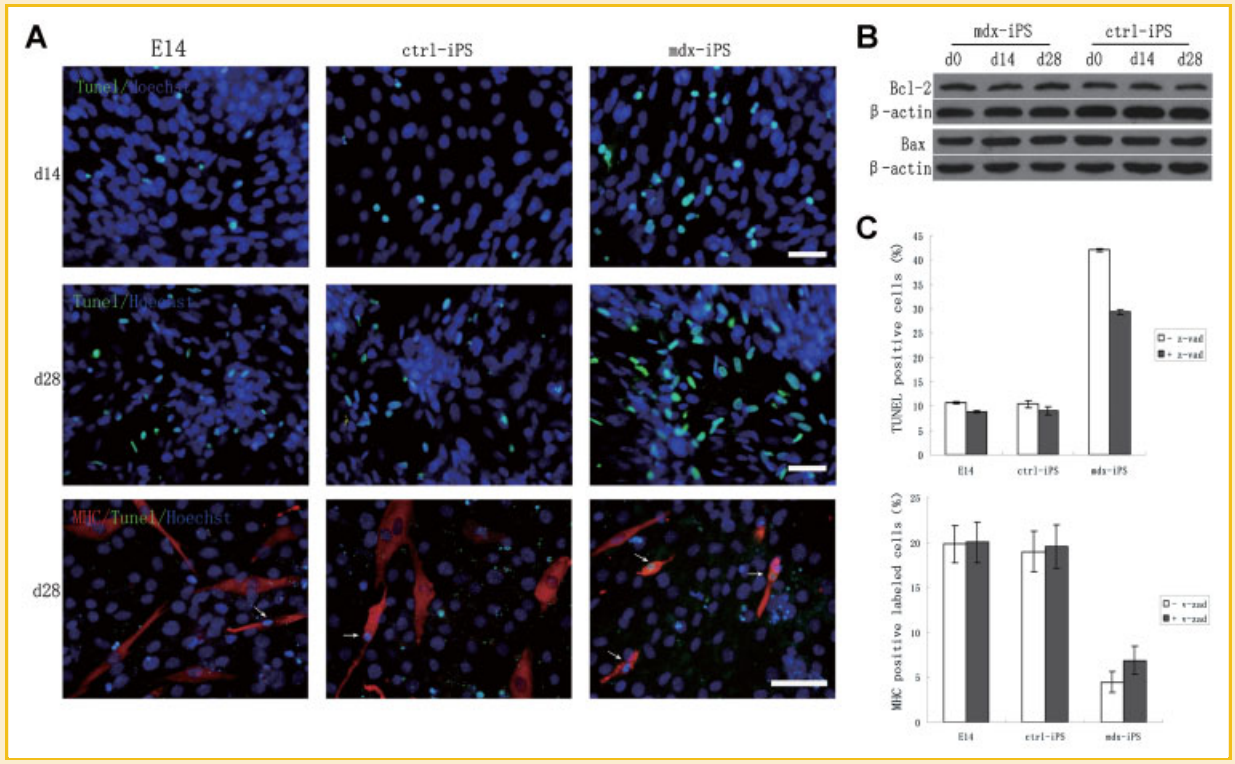

Fig. 3. Apoptosis of differentiated cells from ES and iPS cells A: TUNEL staining of E14, mdx-iPS and ctrl-iPS differentiated cells. Total nuclei are counterstained by Hoechst 33342 (blue). Double staining of MHC and TUNEL of muscle cells on Day 3 + 3 + 28. White arrows indicate TUNEL positive cells in MHC positive cells. Scale bar: 50 μm. B: Western blot analysis of the protein level of Bcl-2 and Bax. C: Quantitative analysis of TUNEL+ cells and MHC+ cells after z-vad-fmk treatment.

analysis demonstrated the expression of markers typical for muscle cells, including Pax3, Pax7, Myf5, myogenin, and MyoD. Immunofluorescence staining confirmed the expression of myocyte-typical proteins, including Pax7, MyoD, and myogenin. These properties are currently considered to be a critical requirement in identifying a putative myocyte population [Chang et al., 2009]. However in this study, the results of RT-PCR, Western-blot, and immunofluorescence staining indicated that the myogenesis ability of mdx iPS cells was much weaker than that of C57BL/10 iPS cells. This finding was in consistent with previous studies, some researches showed delayed myoblast fusion and poor myotube differentiation or impaired muscle differentiation in explanted cultures of Duchenne muscular dystrophy [Delaporte et al., 1984; Schuierer et al., 2005]. In a previous study, we found that the mdx-MSC displayed a myogenic defect compared to wildtype control animals [Li et al., 2008]. These reduced myotube formation may infer the important role of dystrophin protein in myotube maintenance. The dystrophin deficiency can lead to abnormal calcium homeostasis [Imbert et al., 1995] or elevated calpain proteolysis [Alderton and Steinhardt, 2000] in the myotubes, which reduce the myotube formation. These findings suggested that mdx-iPS cells have a reduced capacity for generating muscle fibers.

It has been reported that apoptosis cells increased in the DMD skeletal muscles [Haslett et al., 2002]. In this study, we found more apoptotic cells observed in mdx group than the control groups. Moreover, by double staining of MHC and TUNEL, we showed that most apoptosis occurred in myogenic lineage in mdx group. To better understand the mechanism of apoptosis, we detected the expression of Bcl-2 and Bax proteins, which showed no significant changes at different differentiation stages, suggesting a nonmitochondria signaling pathway involved. Then we treated mdx-iPS cells with a pan-caspase inhibitor–z-vad-fmk, which could rescue cell apoptosis as well as myogenic differentiation. These results showed that nonmitochondria caspase-dependent apoptosis pathways might play a significant role in mdx-iPS cells during myogenic differentiation.

In conclusion, our study successfully established iPS cells from mdx mouse. Compared with C57BL/10 TTFs, mdx TTFs showed less iPS generation rate. In addition, mdx-iPS cells showed less myogenic differentiation potential than controls. Moreover, during myogenic differentiation, more and more cells underwent apoptosis in mdx-iPS group. Finally, we showed the apoptosis might via nonmitochondria caspase-dependent apoptotic pathway. We believe these findings would result from the deficiency of dystrophin, and might add the insights to biology study of dystrophin.

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