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MFN2 silencing promotes neural differentiation of embryonic stem cells via the Akt signaling pathway

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Abstract

Mitofusin 2 (MFN2) is a regulatory protein participating in mitochondria dynamics, cell proliferation, death, differentiation, and so on. This study aims at revealing the functional role of MFN2 in the pluripotency maintenance and primitive differetiation of embryonic stem cell (ESCs). A dox inducible silencing and routine overexpressing approach was used to downregulate and upregulate MFN2 expression, respectively. We have compared the morphology, cell proliferation, and expression level of pluripotent genes in various groups. We also used directed differentiation methods to test the differentiation capacity of various groups. The Akt signaling pathway was explored by the western blot assay. MFN2 upregulation in ESCs exhibited a typical cell morphology and similar cell proliferation, but decreased pluripotent gene markers. In addition, MFN2 overexpression inhibited ESCs differentiation into the mesendoderm, while MFN2 silencing ESCs exhibited a normal cell morphology, slower cell proliferation and elevated pluripotency markers. For differentiation, MFN2 silencing ESCs exhibited enhanced three germs' differentiation ability. Moreover, the protein levels of phosphorylated Akt308 and Akt473 decreased in MFN2 silenced ESCs, and recovered in the neural differentiation process. When treated with the Akt inhibitor, the neural differentiation capacity of the MFN2 silenced ESCs can reverse to a normal level. Taken together, the data indicated that the appropriate level of MFN2 expression is essential for pluripotency and differentiation capacity in ESCs. The increased neural differentiation ability by MFN2 silencing is strongly related to the Akt signaling pathway.

KEYWORDS
Akt signaling pathway, embryonic stem cells (ESCs), Mitofusin 2, neural differentiation

1 | INTRODUCTION

Embryonic stem cells (ESCs) derived from the inner cell mass can self-renew infinitely and differentiate into all three germ cells (Thomson et al., 1998; J. Yu et al., 2007). The balance between preservation of self-renewal and regeneration relied differentiation is under precise control by various biological systems, including cellular transcription factors, growth factors, the extracellular environment, cell–cell signaling and cellular metabolism (Huang, Ye, Zhou, Liu, & Ying, 2015). Mitochondria, as bioenergetic organelles, can participate in the balanced process via energy metabolism, mitochondrial proteostasis, mitophagy and key mitochondrial signaling events (Wanet, Arnould, Najimi, & Renard, 2015; X. Xu et al., 2013). However, less attention has been paid to the morphological changes in the ESCs self-renewal and differentiation process.

In ESCs, mitochondria are granular and spherical dots and gradually turn into developed interconnected tubules during the differentiation process, and are controlled by two opposing

processes: fusion and fission (Chan, 2006; Prigione & Adjaye, 2010). The balance between fusion and fission is mainly regulated by mitofusins (MFN1/2) and dynamin-related protein 1 (Drp1)(Smirnova, Griparic, Shurland, & van der Bliek, 2001; F. Yu et al., 2018). Various studies have demonstrated MFN1/2 and Drp1 regulate reprogramming and differentiation. Drp1 mediated mitochondrial fission has been shown to be necessary for cell reprogramming (Prieto et al., 2016). In the same manner, MFN1/2 has been shown to be the main obstacle of cell reprogramming (Son et al., 2015). However, another study has demonstrated Drp1 has no effects in reprogramming but is necessary for ESCs neural differentiation (Wang et al., 2014). Paradoxically, MFN2 was shown to be indispensable for ESCs cardiac differentiation by increasing Ca^{2+}-dependent calcineurin activity and Notch1 signaling (Kasahara, Cipolat, Chen, Dorn, & Scorrano, 2013). In hematopoietic stem cells, MFN2 is specifically responsible for maintenance of stemness with extensive lymphoid potential by increasing buffering of intracellular Ca^{2+} and then negatively regulating the transcriptional activity of the nuclear factor of active T cells (Luchsinger, de Almeida, Corrigan, Mumau, & Snoeck, 2016). In spite of the several reports above, how MFN2 regulate ESCs self-renewal and primitive differentiation is still unclear.

The mammalian target of rapamycin(mTOR) is a serine/threonine kinase of the phosphoinositide 3-kinase (PI3K)-related kinases. mTOR nucleates two distinct complexes called the mammalian target of rapamycin complex 1 (mTORC1) and mTORC2 (Parker, Knox, Zhang, Wakelam, & Semple, 2015). Activation of Akt can be initiated by PI3K, which in turn activates mTORC1 and further downstream targets such as p70S6 kinase. The mTOR/Akt signaling pathway is also a key regulator of the homeostasis of several stem cell pools in which it finely regulates the balance between self-renewal and differentiation in stem cells. Several studies have shown that mTOR activation causes enhanced generation of neural stem cells (NSCs)/neural progenitor cells (NPCs), followed by neuronal differentiation (Endo, Antonyak, & Cerione, 2009; Marfia et al., 2011), and the inhibition of mTOR abrogates the increase of differentiated neurons (Han et al., 2008). In the central nervous system, the mTOR/AKT pathway is also involved in axon regeneration, neuronal activity, and dendritic arborization (S. Li et al., 2016). A previous study demonstrated that MFN2 acting as a mTORC2-interacting protein, negatively regulated cancer cell survival through inhibiting mTORC2 and Akt (K. Xu et al., 2017). Another similar study also demonstrated the mTOR/Akt signaling pathway was involved in MFN2-regulated smooth muscle cell proliferation (D. Zhang et al., 2012). So far, whether or not the reaction of MFN2 and mTOR/Akt is involved in the self-renewal and differentiation of ESCs is largely unknown.

In this study, we explored the function of MFN2 in ESC fate determination, revealing an essential role of MFN2 in ESCs' pluripotency and differentiation. Our data indicates overexpressing of MFN2 does not affect the self-renewal, proliferation and neural differentiation of ESCs, but inhibits the meso-endoderm differentiation. MFN2 silencing not only promotes the neural differentiation of ESCs but also increases their pluripotency and the three germs layers' differentiation ability. We first identified that MFN2 downregulation in ESCs increases the phosphorylation level of Akt during its neural differentiation, suggesting MFN2 regulates the stem cells fate by regulating the Akt signaling pathway.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Human WA01 ESCs were maintained and expanded on plates coated with growth factor reduced Matrigel (Corning) in serum-free, defined TeSR-E8 medium (Stemcell Technologies). The cells were passaged by nonenzymatic dissociation using 0.5 mM ethylenediaminetetraacetic acid. The ESCs' spontaneous differentiation was carried out by culturing ESCs in Dulbecco's modified Eagle's medium supplement with 15% fetal bovine serum for 6 days.

HEK293t cells were maintained and expanded in Dulbecco's modified Eagle's medium supplement with 10% fetal bovine serum and 1% P/S. All the cells were cultured at 37°C in humidified incubators with 5% CO_2 and 95% air.

2.2 | MFN2 knockdown by inducible shRNA system

A pair of 59-nt long oligonucleotides, encoding a 21-nt (CATTGATCACGGTGCTCTTCC) long shRNA against human MFN2 were designed. Additional *Age*I and *Eco*RI restriction sites were incorporated in the sequences to facilitate cloning. A BLAST search was performed using the National Center for Biotechnology Information (NCBI) Expressed Sequence Tags database to confirm that the shRNA construct specifically targeted human MFN2. A scrambled shRNA sequence (TTCTCCGAACGTGTCACGT), exhibiting no homology to the human sequence database, was used as a negative control. The oligonucleotides were annealed and cloned into dox inducible pLKO.1 plasmid (Sigma).

2.3 | Cloning of human MFN2

Total RNA from human peripheral blood mononuclear cells was extracted using a total RNA extraction kit (Promega), and reverse transcripted. Full-length cDNA for human MFN2 was obtained by polymerase chain reaction (PCR) using PrimeSTAR DNA polymerase (Takara) and the primers forward: 5-GCTCTAGAATGTCCCTGCTCTTCTCTCGATGCA-3 and reverse: 5-CGGGATCCCTATCTGCTGGGCTGCAGGTACTGG-3, *Xba*I and *Bam*HI restriction sites were incorporated in the primers to facilitate cloning. Purified PCR products were subsequently cloned into the pEF1α-Puro-GFP plasmid (Clonetech).

2.4 | Virus production and infection of hESCs

Lentiviral particles for gene knockdown and overexpression were packaged using pspax2 and pmd2g third transfection of 293t cells. The virus was concentrated by using a Beckman supercentrifuge. For

infection, ESCs was cultured in mTeSR1 medium supplemented with a concentrated virus for 12 hr. To improve the efficiency of infection, 8 μg/ml polybrene was added to the infection medium. 48 hr postinfection, the positive ESCs were screened by puromycin (0.5 μg/ml, thermos fisher).

2.5 | Immunofluorescence

For detection of MFN2 expressing and the pluripotency markers in ESCs, immunofluorescence was carried out, as previously described. Briefly, ESCs in growth medium were seeded in a confocal dish at a density of 2×10^5 cells/cm^2. After incubation for 24 hr, the ESCs were fixed in 4% formaldehyde at room temperature and then washed using phosphate buffered saline (PBS) solution. The cells were then treated with 0.5% Triton X-100 for 15 min at room temperature, followed by incubation with 5% NGS in PBS for 1 hr. Primary antibodies and the secondary conjugated antibody were used in the following process. For detection of mitochondria morphology in ESCs, living cells were cultured in the mTeSR1 medium supplemented with Hochest33342 and Mitotracker Red. The cells were observed and photographed by using a confocal fluorescence microscope.

2.6 | ESCs hematopoietic differentiation via EB formation

To induce ESCs hematopoietic differentiation, EB was generated by treating ESCs with collagenase B for 20 min. The cells were gently scraped with a cell scraper to form small aggregates (10–20 cells). The aggregates were resuspended in StemPro34 (Invitrogen), supplemented with L-glutamine (2 mM), ascorbic acid (1 mM), monothioglycerol (4×10^{-4} M; Sigma-Aldrich), and transferrin (150 mg/ml; Roche). BMP4 (10 ng/ml; R&D), basic fibroblast growth factor (5 ng/ml; Peprotech), SB431542 (6 μM; Tocris), vascular endothelial growth factor (15 ng/ml; R&D), interleukin-6 (IL-6; 10 ng/ml; R&D), insulin-like growth factor 1 (25 ng/ml; R&D), IL-11 (5 ng/ml; R&D), stem cell factor (50 ng/ml; Miltenyi), erythropoietin (2 U/ml), thrombopoietin (30 ng/ml; R&D), IL-3 (30 ng/ml; R&D), and FMS-like tyrosine kinase 3 ligand (FLT3L; 10 ng/ml; Miltenyi) were added as indicated. The cultures were maintained in a 5% CO_2/5% O_2/90% N_2 environment. On the day of the assay, EBs were harvested and dissociated into single cells by a 40-min treatment with 0.2% collagenase IV. Afterward, 1 ml of medium with serum was added and the EBs were dissociated into single cells by passaging six times through a 20-gauge needle.

2.7 | ESCs neural differentiation

To effectively induce ESCs neural differentiation, the neural differentiation kit (Stemcell Technologies) was used, as per the manufacturer's instructions. Briefly, the cells were seeded at a density of 2×10^5 cells/cm^2 in a 24-well plate. A daily full medium change was performed with warm STEMdiff Neural induction medium until the cultures were ready to be passaged.

2.8 | Western blot analysis

The cells were harvested and lysed on ice using lysis buffer (Pierce). The protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred onto polyvinylidene difluoride membranes, and incubated overnight with anti-MFN2 (ab124773; Abcam), NANOG (ab218524; Abcam), SOX2 (AB5603; Millipore), OCT4 (sc-8629; Santa Cruz), SSEA4 (sc-21704; Santa Cruz), NESTIN (sc-23927; Santa Cruz), pAkt (Thr308, pAkt (ser473), Akt and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Cell Signaling Technology, Danvers, MA) antibodies. The membranes were visualized using an enhanced chemiluminescence kit (Beyotime Institute of Biotechnology, Suzhou, Jiangsu, China).

2.9 | Quantitative real-time PCR (qPCR) analysis

Total RNA from culture cells was extracted with an RNA extraction kit, according to the protocols, and reverse transcription was performed with a PrimeScript RT reagent kit (Takara, Kyoto, Japan), following the manufacturer's instructions. qPCR analysis was performed on a Bio-Rad CFX manager instrument using SYBR Green Real-time PCR master mixes (Takara, Kyoto, Japan). The amount of mRNAs was all normalized to GAPDH. The primer sequences are shown in Table 1.

2.10 | Cell counting kit 8 (CCK8) assay

When the cell density reached 80%, ESCs was dissociated with Accutase to form a single cell suspension. After counting, the cells were seeded in a matrigel pre-coated 96-well with a cell density of 1.5×10^3/well. After 24, 48, and 72 hr of incubation, 10% CCK8 (Dojindo, Kyushu, Japan) was added into the culture medium for another 2-hr incubation. The optical density values at 490 nm were measured using a microplate reader.

2.11 | Electron microscopy

The cells were fixed in 2.5% glutaraldehyde, washed with PBS three times, then were post-fixed in 1% osmium tetroxide for 2 hr, dehydrated in graded alcohol and acetone, and embedded in epoxy resin. The ultrastructures of the mitochondria were obtained and viewed under an electron microscope (JEOL, Tokyo, Japan).

2.12 | Flow cytometry

The differentiated cells were dissociated by Accutase forming a single cell suspension. Then, the cells were fixed, permeabilizated and washed by using an intracellular staining kit (Biolegened, 424401). The stained cells were analyzed using a BD caliber flow cytometer at the indicated time points. Data analysis was carried out using Flowjo software. The detecting antibody used for these studies: CD34-PE (BD Biosciences, 550761), SOX1-PE (BD Biosciences, 561595), and PAX6 (Abconal, A4003).

TABLE 1 The qPCR primer sequences used in the study

Gene	Primer forward	Primer reverse
MFN2	CACAAGGTGAGTGAGCGTCT	CGTTGAGCACCTCCTTAGCA
T	TATGAGCCTCGAATCCACATAGT	CCTCGTTCTGATAAGCAGTCAC
MESP1	TCGAAGTGGTTCCTTGG	TGCTTGCCTCAAAGTGTC
OCT4	CTTGAATCCCGAATGGAAAGGG	GTGTATATCCCAGGGTGATCCTC
NANOG	ACAACTGGCCGAAGAATAGCA	GGTTCCCAGTCGGGTTCAC
SOX2	CCGTTCATCGACGAGGCTAA	ATGTGCGCGTAACTGTCCAT
GATA6	CTGCGGGCTCTACAGCAAG	GTTGGCACAGGACAATCCAAG
SOX17	CCTTCACGTGTACTACGGCG	GTTCAAATTCCGTGCGGTCC
SOX1	CAACCAGGACCGGGTCAAAC	CCTCGGACATGACCTTCCACT
PAX6	CGAGACTGGCTCCATCAGAC	CTTTTCGCTAGCCAGGTTGC
NESTIN	AAGAGACTCAACAGCGACGG	TCTTGTCCCGCAGACTTCAG
GAPDH	TCTCCTCTGACTTCAACAGCGAC	CCCTGTTGCTGTAGCCAAATTC

2.13 | Statistical analysis

Statistical analysis was performed using one-way analysis of variance and the Student-Newman-Keuls test, and statistical significance was considered at $p < .05$. All experiments were performed in triplicate and from three independent experiments. All data are expressed as the mean ± *SD*.

3 | RESULTS

3.1 | Mitochondria morphology and MFN2 expression in ESCs

During the ESCs spontaneous differentiation process, we used Mitotracker Red and a transmission electron microscope to visualize the morphology of the mitochondria. In ESCs, the mitochondria demonstrated to be fewer, with a spherical, and immature morphology, whereas in differentiated ESCs, the mitochondria exhibited to be more tubular and mature with many cristae (Figure 1a,b). To assess the expressing pattern of MFN2, we used the co-immunofluorescence of MFN2 and SOX2 in ESCs. MFN2 appeared partially and weakly expressed in the ESCs' nuclei, which was colocalized with the pluripotency marker SOX2 (Figure 2). Moreover, MFN2 expression was increased during first 3 days and decreased in the subsequent 3 days, which was demonstrated by western blot analysis (Figure 1c). The result above may provide a hint that MFN2 is indispensable for maintaining the pluripotency of ESCs.

3.2 | MFN2 overexpression partially decreased expression levels of pluripotency markers

To investigate the function of MFN2 in ESCs, we overexpressed MFN2 in ESCs via the lentivirus-based system. The overexpression efficiency of MFN2 was tested 48 hr postinfection by western blot and qPCR. Both MFN2 mRNA and the protein level were increased approximately 4-fold compared with the control group (Figure 3e,f). We, then, studied the effect of MFN2 overexpression in ESCs pluripotency. Compared to the control group, the MFN2 overexpressing ESCs exhibited no distinct influence on morphology but relatively less AP activity (Figure 3a,b). The CCK8 assay demonstrated similiar proliferation ability between MFN2 overexpression group and control group (Figure 3c). We then assessed the expression of the pluripotency marker in MFN2 overexpressing ESCs by immunofluorescence and western blot. Even though the MFN2 overexpressing ESCs were still able to maintain the morphology and the expression patterns of the pluripotency markers OCT4, SOX2, and SSEA4, which is demonstrated by the immunofluorescence (Figure 3d), the western blot and qPCR analysis demonstrated that upregulation of MFN2 in ESCs led to partially decreased expression levels of the pluripotency markers OCT3/4, SOX2 (Figure 3e,f).

3.3 | MFN2 overexpression inhibited ESCs mesendoderm differentiation

To assess the functional role of MFN2 in the ESCs triploblastic differentiation ability, we conducted the hematopoietic differentiation and neural differentiation in MFN2 overexpressing ESCs. For neural differentiation, the control group and MFN2 overexpressing cells are differentiated in neural differentiation medium via the monolayer protocol. The population of SOX1 and PAX6 positive cells were used as markers of neural differentiation ability. Compared to the control group, the MFN2 overexpressing group produced almost the same proportions of SOX1 and PAX6 positive cells (Figure 4a,b). In addition, the mRNA level of neural markers did not demonstrate a significant difference between the control and MFN2 overexpression group (Figure 4c). For mesendoderm differentiation, the control group and MFN2 overexpressing group cells were differentiated into the embryoid body (EB) using a protocol that allowed the generation of definitive hematopoietic precursors (Y. Li et al., 2017). The population of CD34-positive cells were assessed as a marker of mesendoderm differentiation ability. Compared to 4.67% positive

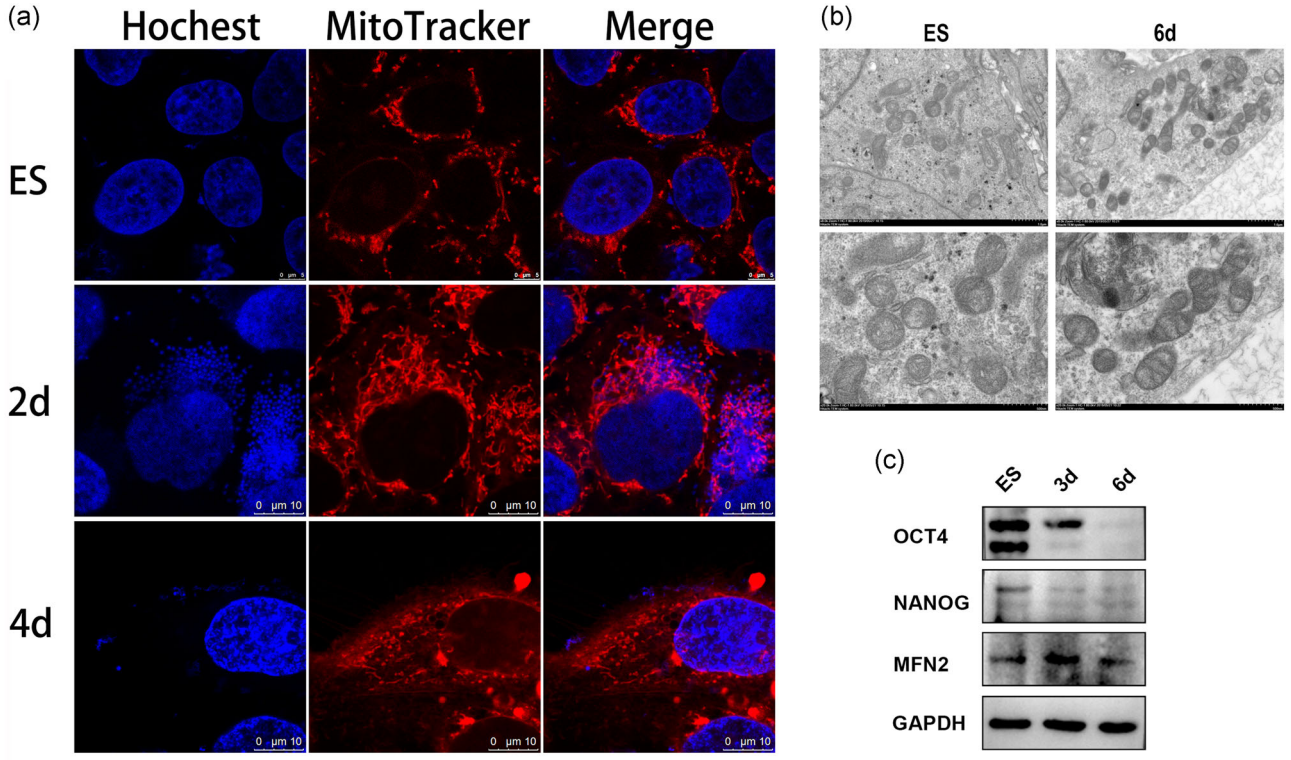

FIGURE 1 The morphological changes of mitochondria and expression level of MFN2 during ESCs' spontaneous differentiation process. (a, b) The fluorescence and transmission electron microscope images of mitochondria during ESCs' spontaneous differentiation process. (c) The expression level of MFN2 during ESCs' spontaneous differentiation process. ESC, embryonic stem cell; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MFN2, Mitofusin 2 [Color figure can be viewed at wileyonlinelibrary.com]

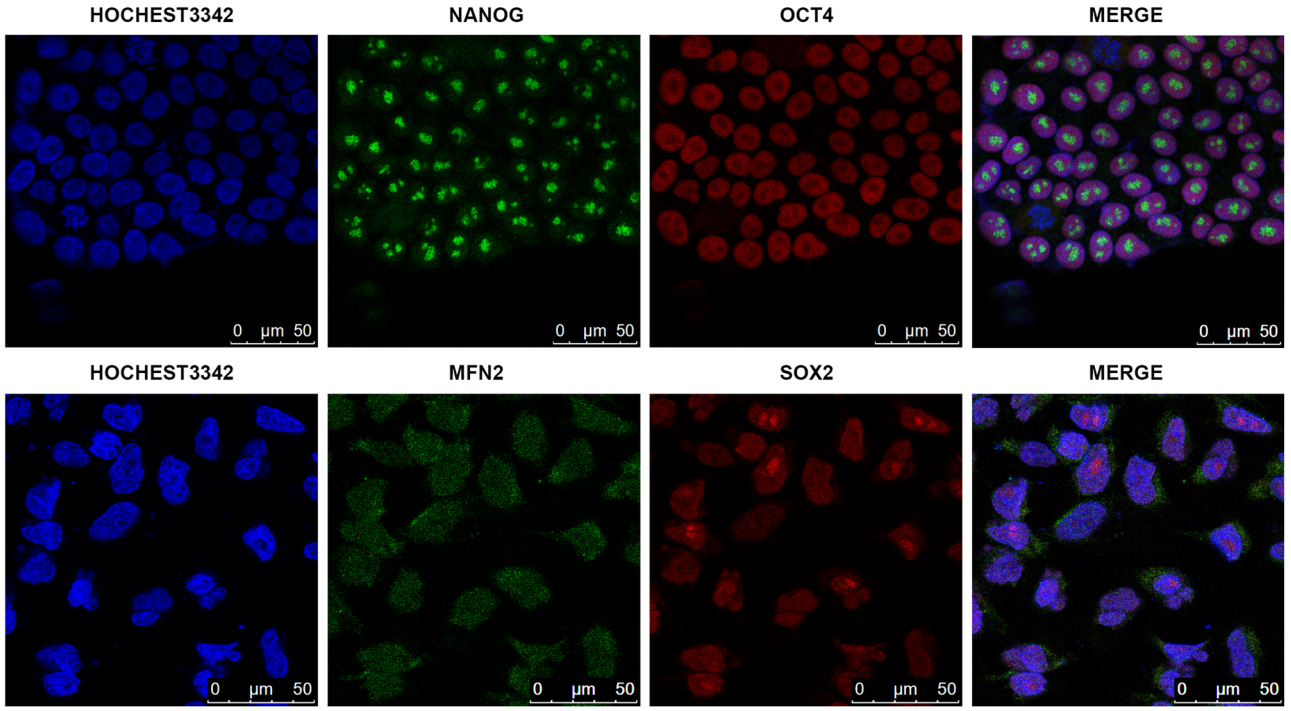

FIGURE 2 The expression pattern of Mitofusin 2 (MFN2) in embryonic stem cells (ESCs)s [Color figure can be viewed at wileyonlinelibrary.com]

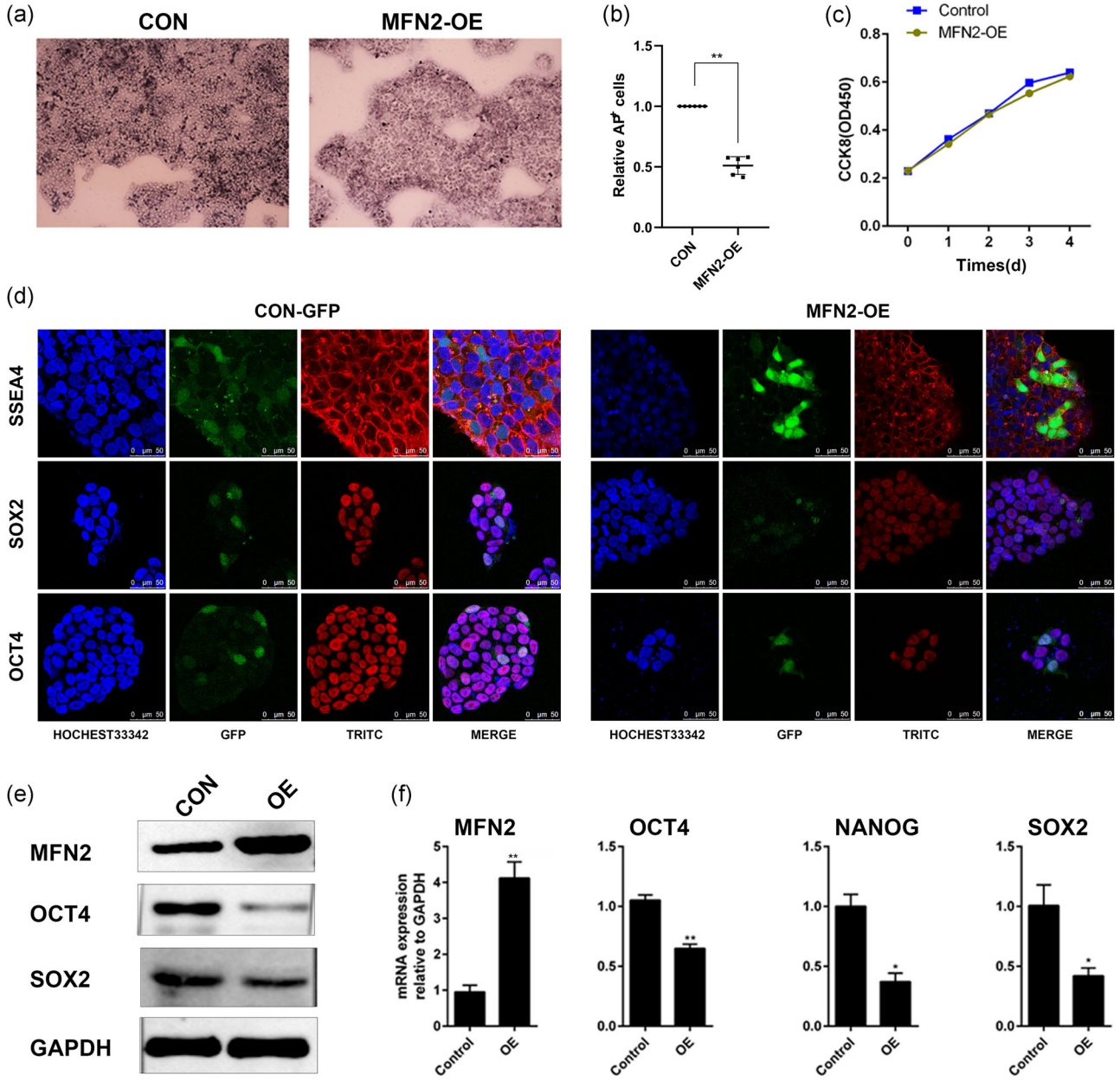

FIGURE 3 The effects of MFN2 overexpression in the pluripotency of ESCs. (a, b) ALP activity and scale bar of control and MFN2 overexpressing ESCs. (c) The CCK8 assay for control and MFN2 silencing ESCs at 0, 1, 2, 3, 4 days. (d) The immunofluorescence of pluripotency markers OCT4, SOX2, and SSEA4 in control and MFN2 overexpressing ESCs. (e) Western blot analysis of pluripotency markers in control and MFN2 overexpressing ESCs. (f) Real-time PCR analysis of pluripotent genes in control and MFN2 overexpressing ESCs. CCK8, cell counting kit 8; CON, control; ESC, embryonic stem cell; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; MFN2, Mitofusin 2; PCR, polymerase chain reaction. $^{*}p < .05$, $^{**}p < .01$. [Color figure can be viewed at wileyonlinelibrary.com]

cells in the control group, the MFN2 overexpressing group only produced 2.96% CD34-positive cells (Figure 4d,e). We also collected the control and MFN2 overexpression cells at Day 4 of the hematopoietic differentiation process and assessed the expressing level of the mesendorderm markers. The western blot and qPCR analysis demonstrated significant decreases in the expression levels of CALPONIN, T, CXCR4, and SOX17 (Figure 4f,g). Taken together, MFN2 overexpression did not influence hESCs' neural differentiation but inhibited mesendoderm differentiation.

3.4 | Silencing MFN2 in ESCs promoted the expressions of pluripotency factors and cell proliferation

To uncover the functional role of MFN2 in ESCs pluripotency, we infected ESCs with the dox-inducible silencing plasmid system. Upon dox, ESCs sustainably expressed siRNA specifically targeted to MFN2. qPCR and western blot demonstrated MFN2 mRNA and protein levels remarkably declined upon dox (Figure 5c,d). After dox was added, hESCs lost their clonal morphology and began to spread

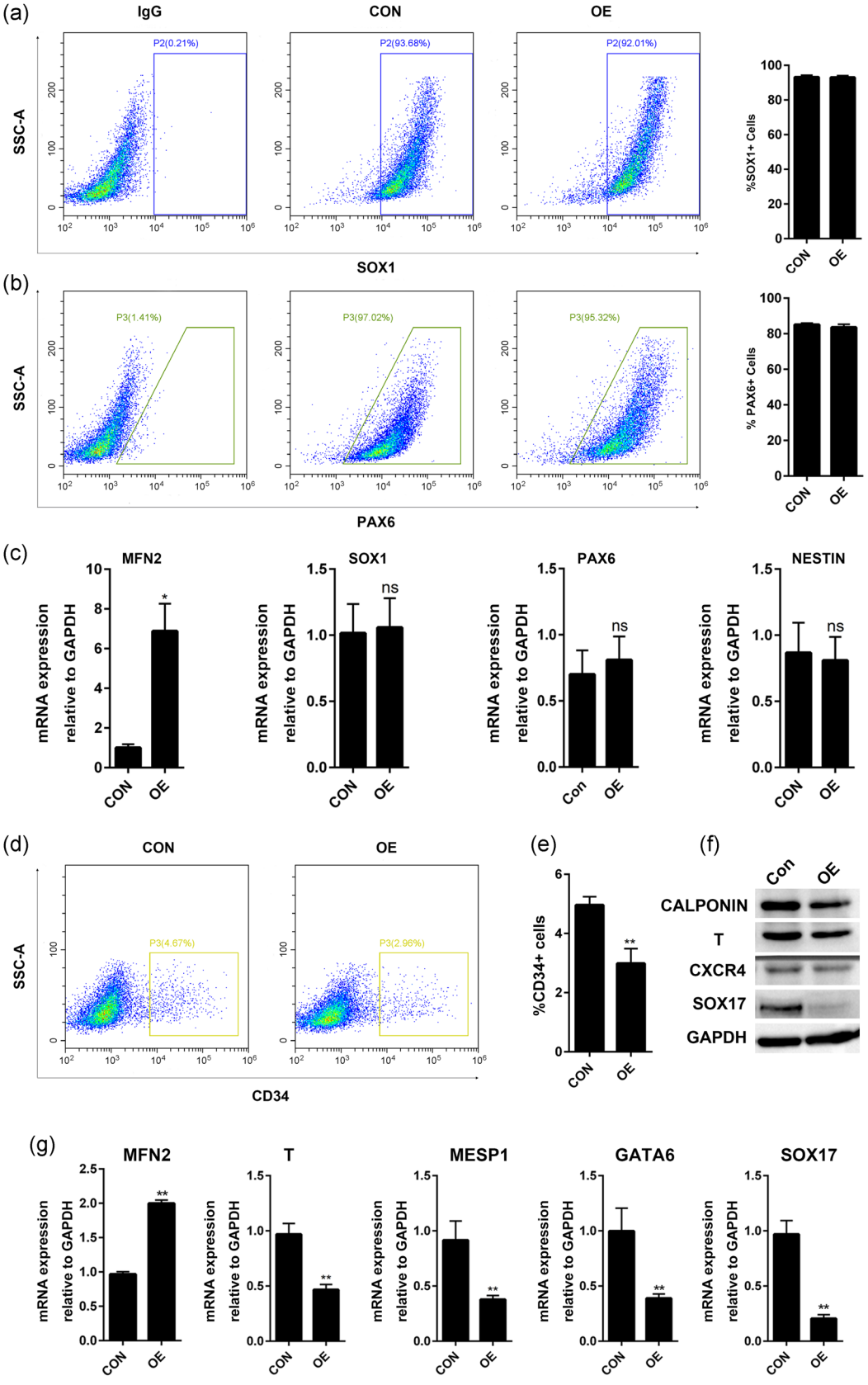

FIGURE 4 Continued.

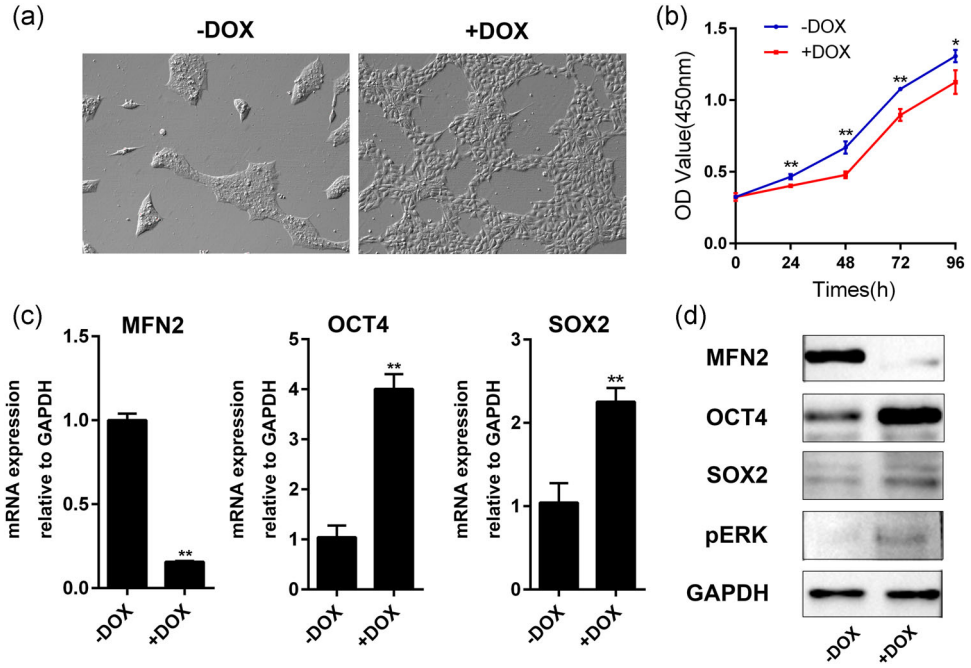

FIGURE 5 The effects of MFN2 silencing in the pluripotency of ESCs. (a) Cell morphology of control and MFN2 silencing ESCs. (b) The CCK8 assay for control and MFN2 silencing ESCs at 0, 24, 48, 72, 96 hr. (c) Real-time PCR analysis of pluripotent genes in control and MFN2 silencing ESCs. (d) Western blot analysis of pluripotency markers in control and MFN2 silencing ESCs. DOX, doxorubicin; ESC, embryonic stem cell; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MFN2, Mitofusin 2; PCR, polymerase chain reaction; pERK, phosphorylated extracellular signal-regulated kinase $^{*}p < .05$, $^{**}p < .01$ [Color figure can be viewed at wileyonlinelibrary.com]

out (Figure 5a). The alteration in hESCs morphology suggested a rapid expanding speed and good maintenance of pluripotency. Indeed, qPCR and western blot analysis revealed a rapid increase of pluripotency markers OCT4 and SOX2 after adding dox 48 hr (Figure 5c,d). Concomitant with the upregulation of pluripotency markers, the cell proliferation of hESCs was downregulated significantly after adding dox 48 hr (Figure 5b). When we detected the activity of ERK, the results showed that the level of phosphorylated ERK was increased dramatically by MFN2 silencing (Figure 5d). Taken together, downregulating MFN2 expression leads to good maintenance of pluripotency and reduced cell proliferation via inhibiting ERK phosphorylation.

3.5 | Silencing MFN2 in ESCs increased three germ layer differentiation ability of hESCs

We next asked whether MFN2 was required for the primitive differentiation of hESCs. We induced directed neural and mesendoderm differentiation using the protocols described above, and assessed the differentiation ability of the two groups. After adding dox 48 hr, we conducted neural differentiation. Strikingly, upon neural differentiation, the MFN2 silencing ESCs exhibited increased expression level of neural differentiation markers PAX6 and SOX1, which was assessed by flow cytometry. The quantification of positive values further confirmed these findings (Figure 6a,b). Additionally, the immunofluorescence of the neural differentiation markers PAX6 and NESTIN demonstrated a stronger expression level (Figure 6c). Consistent with this, the qPCR analysis revealed that the mRNA levels of PAX6, SOX1, Nestin and were also significantly increased in the dox induced MFN2 silencing group after 3 days' neural differentiation (Figure 6d). We also adopted the canonical hematopoietic differentiation method to evaluate the mesendoderm differentiation ability of the two groups. Unexpectedly, the dox-induced MFN2 silencing group exhibited an upregulated level of CD34-positive cells, which indicated MFN2 depletion promoted hematopoietic differentiation. The quantification of positive values further confirmed these findings (Figure 7a,b). We also collected the cells at Day 4 during hematopoietic differentiation and tested the mesendoderm differentiation markers by western blot and qPCR. Consistently, during differentiation, the cells depleted of MFN2

FIGURE 4 The effects of MFN2 overexpression in directed differentiation of ESCs. (a, b) The expression level of the neural differentiation markers PAX6 and SOX1 in control and MFN2 overexpressing differentiated ESCs. (c) Real-time PCR analysis of neural differentiation markers in control and MFN2 overexpressing differentiated ESCs. (d, e) Flow-cytometric analysis and quantitative analysis of CD34 in control and MFN2-overexpressing ESCs derived EBs at Day 8. (f) Western blot analysis of mesendoderm differentiation markers in control and MFN2-overexpressing ESCs derived EBs at Day 4. (g) Real-time PCR analysis of neural mesendoderm differentiation genes in control and MFN2 overexpressing ESCs derived EBs at Day 4. CON, control; EB, embryoid body; ESC, embryonic stem cell; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IgG, immunoglobulin G; MFN2, Mitofusin 2; PCR, polymerase chain reaction. $^{*}p < .05$, $^{**}p < .01$ [Color figure can be viewed at wileyonlinelibrary.com]

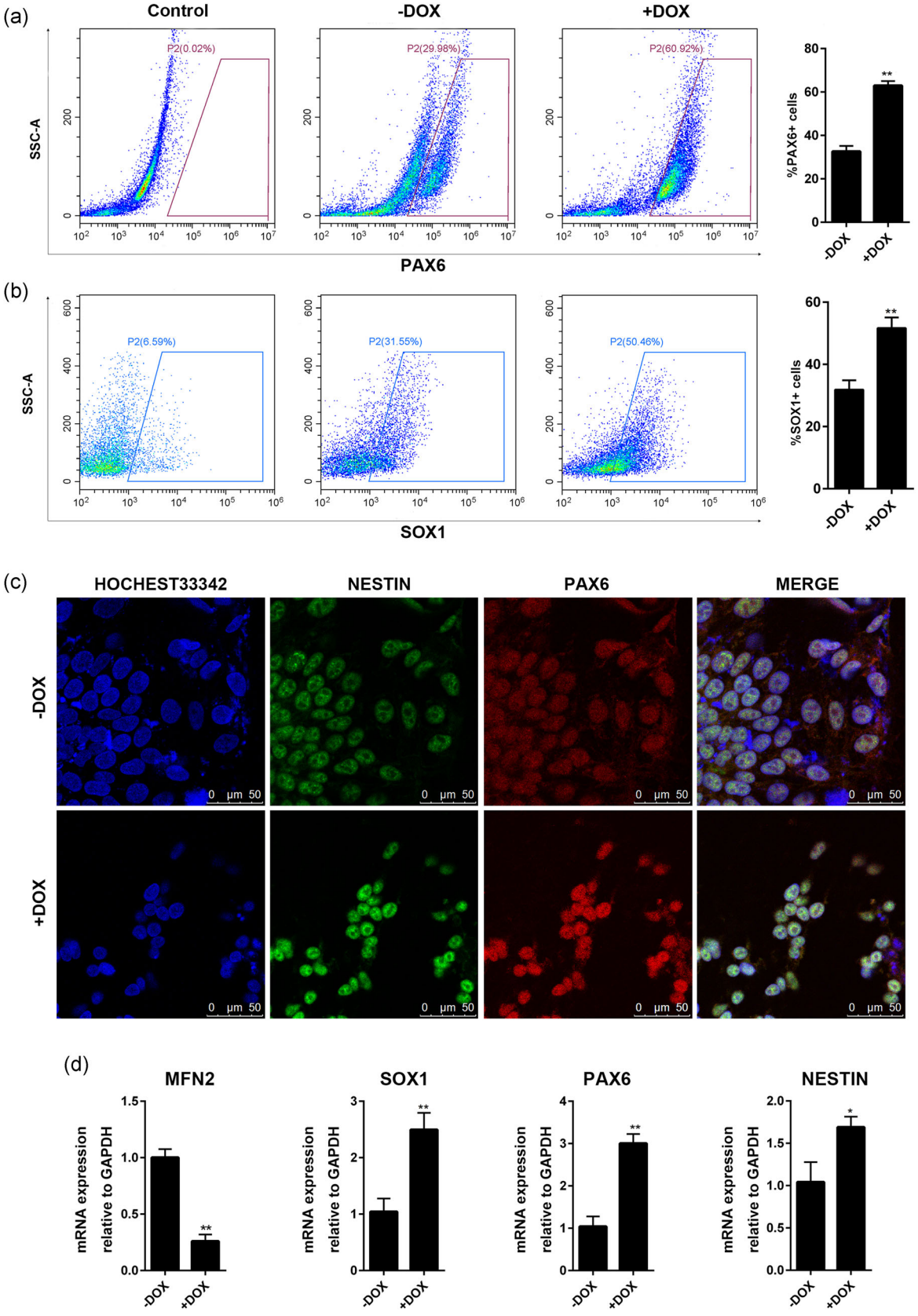

FIGURE 6 The effects of MFN2 silencing in directed differentiation of ESCs. (a, b) The expression level of the neural differentiation markers PAX6 and SOX1 in control and MFN2 silencing differentiated ESCs. (c) The immunofluorescence of PAX6 and NESTIN in control and MFN2 silencing differentiated ESCs. (d) Real-time PCR analysis of neural differentiation markers in control and MFN2 silencing differentiated ESCs. ESC, embryonic stem cell; MFN2, Mitofusin 2. $^{*}p < .05$, $^{**}p < .01$ [Color figure can be viewed at wileyonlinelibrary.com]

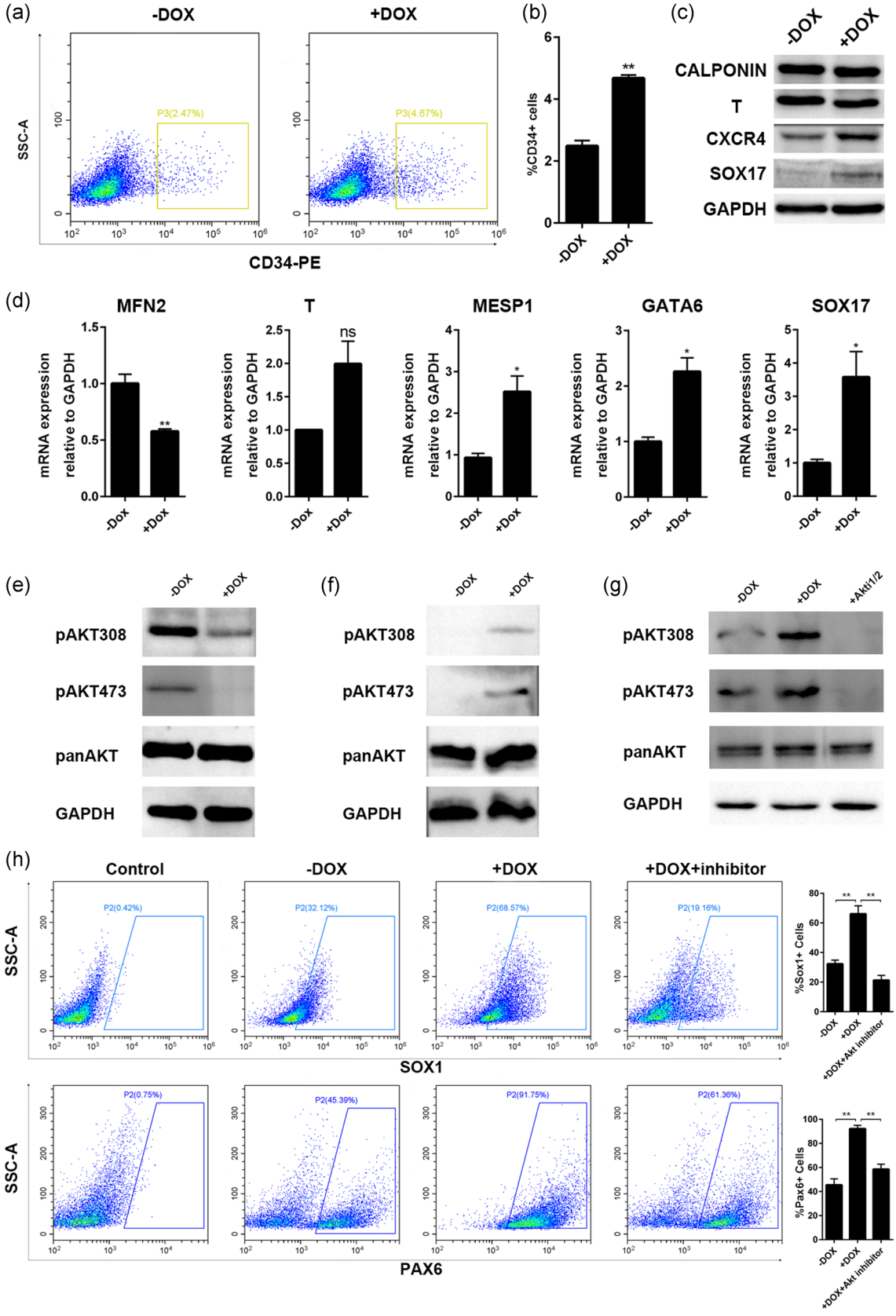

FIGURE 7 Continued.

exhibited much higher levels of endoderm markers CXCR4, SOX17, MESP1, T, and GATA6 in the MFN2 silencing group (Figure 7c,d). However, the mesendoderm differentiation marker T and CALPONIN remained unchanged (Figure 7c). In conclusion, downregulation of MFN2 promotes hESCs neural and mesendoderm differentiation and changes of MFN2 expression levels are related to the differentiation and pluripotency of hESCs.

3.6 | Silencing MFN2 ESCs boost neural differentiation via the Akt signaling pathway

The Akt signaling pathway was reported to promote ESCs neural differentiation, we then assessed the expressing level of phosphorylated Akt in MFN2 silencing ESCs and Day 3 post neural differentiation. Unexpectedly, MFN2 silencing ESCs exhibited decreased expression level of phosphorylated Akt (Thr308) and phosphorylated Akt (ser473) (Figure 7e), while elevated the expression level of phosphorylated Akt (Thr308) and phosphorylated Akt (ser473) 3 days post neural differentiation (Figure 7f). We adopted an Akt inhibitor Akti-1/2 (10 μM, Selleck) to inhibit the increase of Akt signaling activity (Figure 7g), and assessed the neural differentiation ability. As shown in Figure 7d, the increased SOX1 and PAX6 positive cells in the MFN2 silencing group can be recovered to the normal level (Figure 7h). In conclusion, MFN2 silencing ESCs boosted neural differentiation via the Akt signaling pathway.

4 | DISCUSSION

Human pluripotent stem cells (hPSCs) are able to proliferate indefinitely, which is called self-renewal and differentiate into any tissue of three germ layers. Due to their fundamental role in energy production, mitochondria have been traditionally known as the powerhouses of the cells (Dyall, Brown, & Johnson, 2004). Recent studies have demonstrated a strong and tight connection between mitochondria and pluripotency and differentiation of hPSCs. Compared to adult somatic cells, hPSCs depend mainly on anaerobic glycolysis, rather than aerobic metabolism based on oxidative phosphorylation for energy production (Folmes et al., 2011; Kondoh et al., 2007; Prigione, Fauler, Lurz, Lehrach, & Adjaye, 2010). Under self-renewing conditions, attenuating mitochondrial oxidative phosphorylation increases the expression level of the pluripotent markers Nanog, Oct4, and Sox2 (Mandal, Lindgren, Srivastava, Clark, & Banerjee, 2011). On the other hand, to meet the energetic demands and support cell proliferation, glycolysis is adapted to provide various biological factors, which are vital for maintaining pluripotency. Various studies have demonstrated inhibition and stimulation of glycolysis impairs and promotes stemness of PSCs, respectively (Prowse et al., 2012; Varum et al., 2009). As for differentiation, the transition process from glycolysis to oxidative phosphorylation can support cells with higher amounts of energy, elevated mtDNA copy number and more mature and developed mitochondria (Suhr et al., 2010). Studies have shown that inhibition of key glycolytic enzymes promotes differentiation while impairment of oxidative phosphorylation blocks differentiation (Chung et al., 2007; J. Zhang et al., 2011).

The transition process was accompanied by a morphology change during the differentiation period, that is, from granular and spherical dots to developed interconnected tubules, which is tightly controlled by mitochondrial fusion and fission. MFN1 and MFN2 were the earliest identified to be involved in mitochondrial fusion by tethering of two adjacent mitochondrial outer membranes. Except for mitochondrial fusion, MFN2 is reported to participate in the various biological process such as cell metabolism, cell apoptosis and proliferation, autophagy, and so forth (El-Hattab, Suleiman, Almannai, & Scaglia, 2018). In the present study, we have shown the vital role of MFN2 in human ESCs' self-renewal and differentiation. Our data show that MFN2 is endogenously expressed in ESCs, which is evidenced by immunofluorescence and western blot (Figures 1 and 2). The expression pattern of MFN2 during ESCs' spontaneous differentiation also showed that MFN2 expression level was increased during the first 3 days and decreased in the subsequent 3 days (Figure 1). Collectively, these data indicate that MFN2 may have contrasting roles during self-renewal and differentiation.

So far, few studies have figured out the functional role of MFN2 in reprogramming and cardiomyocyte differentiation. However, MFN2 knockout mice are reported to die during the gastrulation period (Y. Chen & Dorn, 2013). Furthermore, MFN2 is reported to be related to cell proliferation and cell death (K. H. Chen et al., 2014; Marin-Garcia & Akhmedov, 2016). To obtain stable MFN2 silencing and overexpressing ESCs, a lentivirus-based dox inducible silencing and routine overexpressing experimental approach was used to downregulate and upregulate MFN2 expression, respectively.

Successful transduction of MFN2 shRNA and full-length cDNA into ESCs was evidenced by qPCR at mRNA level and Western blot

FIGURE 7 The effects of MFN2 silencing in mesendoderm differentiation and MFN2 silencing promote ESCs neural differentiation via Akt signaling pathways. (a, b) Flow-cytometric analysis and quantitative analysis of CD34 in control and MFN2-silencing derived EBs at Day 8. (c) Western blot analysis of mesendoderm differentiation markers in control and MFN2-silencing derived EBs at Day 4. (d) Real-time PCR analysis of neural mesendoderm differentiation genes in control and MFN2 silencing derived EBs at Day 4. (e) Western blot analysis of phosphorylated Akt (Thr308), phosphorylated Akt (Ser473) and pan-Akt in control and MFN2 silencing ESCs. (f) Western blot analysis of phosphorylated Akt (Thr308), phosphorylated Akt (Ser473), and pan-Akt in control and MFN2 silencing neural differentiated ESCs at Day 3. (g) Western blot analysis of phosphorylated Akt (Thr308), phosphorylated Akt (Ser473) and pan-Akt in control, MFN2 silencing and Akt inhibitor treated neural differentiated ESCs at Day 3. (h) Flow-cytometric analysis and quantitative analysis of PAX6 and SOX1 in control, MFN2 silencing and Akt inhibitor treated neural differentiated ESCs at Day 3. DOX, doxorubicin; EB, embryoid body; ESC, embryonic stem cell; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MFN2, Mitofusin 2; PCR, polymerase chain reaction. $^{*}p < .05$, $^{**}p < .01$ [Color figure can be viewed at wileyonlinelibrary.com]

at protein level. Upon MFN2 silencing, ESCs promoted the expression level of pluripotency markers, whereas MFN2 overexpression suppressed them. Furthermore, the MFN2 overexpression did not affect cell proliferation, while MFN2 silencing ESCs was shown to proliferate much slower than the control group. The results are consistent with the gene-trapped mESCs, which demonstrate low expression of MFN2 can maintain the pluripotency of ESCs (Kasahara et al., 2013). In addition, pluripotent gene expression is reported to be elevated when treating ESCs with MFN2 siRNA (Son et al., 2015). As for cell proliferation, our CCK8 data demonstrated MFN2 silencing inhibited cell proliferation, while MFN2 overexpression has no effect on it. The results are consistent with a study performed on human gastric cancer cells, which revealed low MFN2 expression suppressed cell proliferation and invasion (C. L. Fang et al., 2017).

To discuss the role of MFN2 in differentiation, we utilized the three germ directed differentiation methods. MFN2 were reported to participate in various differentiations, especially in neural differentiation. Studies have demonstrated that MFN2 suppression blocked the appropriate differentiation of neural stem cells (D. Fang, Yan, Yu, Chen, & Yan, 2016; Ribeiro, Genebra, Rego, Rodrigues, & Sola, 2018). Except for neural differentiation, MFN2 promoted hematopoietic differentiation and cardiomyocyte differentiation. However, in contrast, downregulating MFN2 in periodontal ligament stem cells and mesenchymal stem cells increased osteogenic and fibroblast differentiation respectively (Wu et al., 2017; Zhai et al., 2018). In our study, we demonstrated silencing MFN2 during differentiation promotes neural and hematopoietic differentiation, while MFN2 overexpression suppresses hematopoietic differentiation and does not affect neural differentiation. These results may be due to the diverse stem cell microenvironment and differentiation methods.

ESCs neural differentiation is promoted by various transcription factors, the signaling pathway and others, including Sox1, Pax6, Nestin, Akt signaling, Erk signaling, Tgf-β/BMP signaling pathway and so on (Chuang, Tung, & Lin, 2015). Among them, Akt signaling pathway plays an essential role in the commitment of ESCs into neural stem cells. Studies have demonstrated AKT signaling is involved during induction, migration, and differentiation of neural stem cells (Sittewelle & Monsoro-Burq, 2018). Blockage of the AKT signaling pathway may lead to defects in cytoskeleton changes, the stability of cell–cell junctions, and the nature of cell–substratum interactions in neural crest cells (Bahm et al., 2017; Wilson et al., 2016). MFN2 was reported to interact with the AKT signaling pathway via multiple methods. Mechanistic studies revealed that MFN2 suppressed mTORC2 through direct interaction by binding its domain HR1 (K. Xu et al., 2017). Furthermore, in various cancer cells, MFN2 can suppress AKT signaling to block the cell proliferation and progression (Ma, Chang, Yu, He, & Liu, 2015; Xue et al., 2018). Interestingly, in our study, we demonstrated the AKT signaling pathway was decreased in the MFN2 silencing group in ESCs, while it was increased in the MFN2 silencing group in NPCs. The results indicated the relationship between MFN2 and AKT signaling pathway was strongly dependent on cell type and cellular microenvironment.

In conclusion, this study found that MFN2 silencing helps to maintain the pluripotency and promote three germs' differentiation of ESCs. Our data indicated treating the cells with the AKT inhibitor can rescue the increased neural differentiation ability during the process, which reflects the close relationship between MFN2 and AKT signaling during neural differentiation. Therefore, these results enhanced our understanding of the balance between ESCs' pluripotency and differentiation, and altering MFN2 could be a promising strategy for directed differentiation.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Y.S.Q. made contributions to conception and design. Y.S.Q., C.C.H., Y.X.H., H.X.T., and L.Y. contributed to the performance of the experiments and data analysis. W.J.H. and L.Q.X. contributed to revising the manuscript.

DATA ACCESSIBILITY

All data generated or analyzed during the study are included in this published article.

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