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RESEARCH ARTICLE

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TMEM182 inhibits myocardial differentiation of human iPS cells by maintaining the activated state of Wnt/β-catenin signaling through an increase in ILK expression

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Abstract

Transmembrane protein 182 (TMEM182) is notably abundant in muscle and adipose tissue, but its role in the heart remains unknown. This study examined the contribution of TMEM182 in the differentiation of human induced pluripotent stem cells (hiPSCs) into cardiomyocytes. For this, we generated hiPSCs overexpressing TMEM182 in a doxycycline-inducible manner and induced their differentiation into cardiomyocytes. On Day 12 of differentiation, expression of the cardiomyocyte markers, *TNNT2* and *MYH6*, was significantly decreased in TMEM182-overexpressing cells. Additionally, we found that phosphorylation of GSK-3β (Ser9) and β-catenin (Ser552) was increased during TMEM182 overexpression, suggesting activation of Wnt/β-catenin signaling. We further focused on integrin-linked kinase (ILK) as the mechanism by which TMEM182 activates Wnt/β-catenin signaling. Evaluation showed that ILK expression was increased in cells overexpressing TMEM182. These results suggest that TMEM182 maintains Wnt/β-catenin signaling in an activated state after mesoderm formation by increasing ILK expression, thereby suppressing hiPSCs differentiation into cardiomyocytes.

KEYWORDS

hiPSCs, myocardial differentiation, TMEM182

1 | INTRODUCTION

Proteins can be classified in different ways according to their characteristics. When proteins are considered in relation to the biological membrane, those that adhere to the membrane are specifically called membrane proteins, while those that completely penetrate the membrane are called transmembrane proteins. Transmembrane proteins play a very important role in intercellular communication and transport of molecules and ions, and they reportedly account for about 30% of the mammalian proteome.[1]

Among various transmembrane proteins, this study focused on transmembrane protein 182 (TMEM182). The TMEM182 gene encodes an entirely 229-amino-acid protein, which is predicted to consist of four putative membrane-spanning regions.[2] Moreover, TMEM182

is known to be highly expressed in muscle tissue.[3,4] Indeed, when we examined the protein expression of TMEM182 in various mouse organs, we found that its expression was higher in the heart and skeletal muscle than in other organs (Figure S1). Previous studies have demonstrated that the TMEM182 transcript is enriched in muscle tissue and significantly upregulated during the differentiation of C2C12 myoblasts into myocytes in vitro.[3] Additionally, TMEM182 inhibits skeletal muscle development, growth, and regeneration, suggesting that the TMEM182 gene plays an important role in skeletal muscle development.[4] Thus, although the role of TMEM182 in skeletal muscle is gradually being clarified, its function and significance in the heart, where it is also highly expressed, is not yet known. In this study, we decided to investigate the involvement of TMEM182 in cardiomyogenesis first.

Human induced pluripotent stem cells (hiPSCs) are capable of differentiating into cells of various tissues and organs, including cardiomyocytes that are the focus of this study. HiPSCs can be converted into functional cardiomyocytes along the mesodermal lineage, making them valuable models for understanding the mechanisms of human mesoderm induction and initiation of cardiomyogenesis.[5–8] Clarifying the role of TMEM182 in the myocardial differentiation of hiPSCs would be a useful step toward investigating the role of TMEM182 in the development and physiology of the heart in vivo. Furthermore, if the expression and functional regulation of TMEM182 are determined as leading to improved myocardial differentiation efficiency and proliferative capacity, this finding could be applied to regenerative medicine and drug discovery research using hiPSC-derived cardiomyocytes. Therefore, the purpose of this study was to clarify the involvement of TMEM182 in the early stages of myocardial development.

2 | MATERIALS AND METHODS

2.1 | Molecular biology

Complementary DNA (cDNA) coding for human TMEM182 (NM_144632) was obtained by RT-PCR using forward (5′-ATGAGCTAGGACAGCCTTCTC-3′) and reverse (5′-TACTAACTTTATGCTGGGATC-3′) primers, and RNA prepared from hiPSCs as a template. For the plasmid construction of human influenza hemagglutinin (HA)-tagged TMEM182, 11 amino acids (GGYPYDVPDYA) containing an HA-tag and two glycine residues as a flexible linker were inserted at the carboxyl terminus using a PCR-based strategy. The protein-coding region of cDNA was amplified by PCR using the first RT-PCR product, and inserted into the EcoRI site of the expression vector KT2-014-PB-TW-KRAB-dCas9-CRNKB by exchanging the dCas9 region with HA-tagged TMEM182.

2.2 | Culture of human iPS cells

In the present study, hiPSCs were passaged every 7 days using Accutase (AT104, Innovative Cell Technologies, San Diego, CA, USA). StemFit AK02N medium (RCAK02N, Ajinomoto, Tokyo, Japan) supplemented with 10 μM Y-27632 (10–2301, Focus Biomolecules, Plymouth Meeting, PA, USA) and 1.67 μg/mL iMatrix-511 silk (892021, Nippi, Tokyo, Japan) was used to seed hiPSCs at each passage. After 24 h, the medium was changed to freshly prepared StemFit AK02N medium without Y-27632, and the medium was changed every other day. The plating density was 14,000 cells/1.5 mL per well of a 6-well plate. Cells were counted using a TC20 Automated Cell Counter (Bio-Rad Laboratories, Hercules, CA, USA).

2.3 | Differentiation of human iPS cells into cardiomyocytes

To differentiate hiPSCs into the cardiac lineage, we used a well-defined protocol.[6,9] Briefly, hiPSCs were seeded at 30,000–60,000 cells/mL per well of a 12-well plate 4 days prior to the initiation of differentiation and then cultured in StemFit AK02N medium until differentiation initiation (Day 0). From Day 0 to Day 5 of differentiation, the cells were cultured in RPMI 1640 Medium (11875093, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with B-27 Supplement, minus insulin (A1895601, Thermo Fisher Scientific, Waltham, MA, USA). At the start of differentiation, the cells were treated with 12 μM CHIR99021 (034-23103, FUJIFILM Wako Pure Chemical, Osaka, Japan) for 24 h and, on Day 2, with 5 μM IWP-2 (3533, Tocris Bioscience, Bristol, UK) for 48 h. From Day 6 of differentiation, the cells were maintained in RPMI 1640 Medium supplemented with B-27 Supplement (50X), serum free (17504044, Thermo Fisher Scientific, Waltham, MA, USA), and the medium was changed every 3 days. Doxycycline (DOX, final concentration; 1 μg/mL) (D9891, Merck KGaA, Darmstadt, Germany) was applied simultaneously with the initiation of differentiation. A schematic diagram of the protocol is shown in Figure 2A.

2.4 | Transfection of siRNA

Twenty-four hours after hiPSC seeding, the cells were transfected with siRNA using the *Trans*IT-X2 Dynamic

Delivery System (MIR6000, Mirus, Madison, WI, USA) for 72 h according to the manufacturer's protocol. The siRNA sequences used in this study are listed in Table S1.

2.5 | Isolation of total RNA

Total RNA was isolated using a standard procedure after solubilization with RNAzol RT (RN190, Molecular Research Center, Cincinnati, OH, USA). The absorbance of RNA at 260 and 280 nm was measured using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.6 | Real-time PCR

Total RNA (1 μg) was reverse-transcribed into a cDNA template using the PrimeScript RT reagent kit (RR037A, Takara Bio, Shiga, Japan) according to the manufacturer's protocol. The expression levels of mRNA for the target genes relative to the internal control GAPDH were measured by the StepOne Plus Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA) using Probe qPCR Mix (RR391A, Takara Bio, Shiga, Japan) and TaqMan probes (Thermo Fisher Scientific, Waltham, MA, USA). The TaqMan probes used in this study are listed in Table S2.

2.7 | Western blot analysis

Cultured cells were lysed in SDS-PAGE sample buffer (0.1 M Tris–HCl, 4% SDS, 12% β-mercaptoethanol, 20% glycerol, and bromophenol blue). The lysates were stored at −80°C until use. The protein concentration of the lysates was determined using Coomassie brilliant blue staining. Equivalent amounts of protein for each condition were resolved by SDS-PAGE. The gel was placed into a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, Hercules, CA, USA), electrophoretically transferred to polyvinylidene fluoride membrane (Merck KGaA, Darmstadt, Germany), and blocked with 2% skim milk (31149–75, Nacalai Tesque, Kyoto, Japan) in Tris-buffered saline containing 0.05% Tween 20 (TBS-T). Subsequently, the membrane was probed overnight at 4°C with primary antibodies against Na,K-ATPase (#3010), Phospho-β-catenin (Ser552) (#9566), β-catenin (D10A8) (#8480), Phospho-Akt (Ser473) (D9E) (#4060), Akt (#9272), Phospho-GSK-3β (Ser9) (#9336), and GSK-3β (27C10) (#9315), which were purchased from Cell Signaling Technology, Collagen I (ab34710), Integrin linked ILK (EPR1592) (ab76468), and Cardiac Troponin T (EPR3695) (ab91605), which were purchased from Abcam, GAPDH (M171-3) and α-tubulin (PM054), which were purchased from Medical & Biological Laboratories, HA (3F10) (11867423001) and EGFR (A204), which were purchased from Merck KGaA, TMEM182 (25366-1-AP), which was purchased from Proteintech, and PLN (1D11), which was a generous gift from Dr. Robert Johnson (Merck Research Laboratories, West Point, PA, USA). The membrane was washed in TBS-T thrice for 7 min and was incubated with horseradish peroxidase-conjugated anti-rabbit (4050-05), anti-mouse (1030-05), anti-rat (3030-05), or anti-goat (6460-05) IgG antibodies (SouthernBiotech, Birmingham, AL, USA) in TBS-T supplemented with 2% skim milk at room temperature for 1 h. Finally, the membrane was washed in TBS-T thrice for 7 min. The blots were developed using a Luminata Western HRP substrate (Merck KGaA, Darmstadt, Germany). Signals were detected using the densitometry system Fusion FX7 (Vilber Lourmat, Eberhardzell, Germany). Densitometric analyses of the detected bands were performed using Image-J software.

2.8 | Cell fractionation

Cultured cells were harvested using PBS containing Protease Inhibitor Cocktail Set V (PI) (162-26031, FUJIFILM Wako Pure Chemical, Osaka, Japan) and centrifuged at 15,000 rpm, 4°C for 5 min. The pellets were suspended in 300 μL of fractionation buffer (a mixture of 20 mM HEPES pH 7.4, 10 mM KCl, 2 mM $MgCl_2$, 2 mM dithiothreitol and PI). Cells were homogenized using Bioruptor II (BM Equipment, Tokyo, Japan) (25 cycles of a 10 s pulse with a 30 s interval) and centrifuged at 10,000 × *g*, 4°C for 5 min. The supernatant was further centrifuged at 100,000 × *g*, 4°C for 30 min. The resultant supernatant and precipitate after centrifugation were retained as cytosolic and membrane fractions, respectively.

2.9 | Immunofluorescence staining

DOX-treated or untreated cells on olefin film-bottomed dishes (FD10300, Matsunami Glass Ind., Ltd., Osaka, Japan) were fixed with 4% paraformaldehyde for 15 min and permeabilized with PBS containing 0.1% Triton X-100 for 10 min. After blocking them in PBS with 5% bovine serum albumin (BSA) (013-27,054, FUJIFILM Wako Pure Chemical, Osaka, Japan) for 2 h, the cells were treated overnight with anti-α-Actinin (A7811, Merck KGaA, Darmstadt, Germany), anti-HA (11867423001, Merck KGaA, Darmstadt, Germany), anti-Vimentin (D21H3) (#5741, Cell Signaling Technology, Danvers, MA, USA) and anti-β-catenin (E247) (ab32572, Abcam, Cambridge, UK) antibodies (1:100) at 4°C.

Subsequently, the cells were incubated with Alexa Fluor 488–labeled donkey anti-rabbit IgG (ab150061, Abcam, Cambridge, UK), Alexa Fluor 488–labeled donkey anti-rat IgG (712-545-150, Jackson ImmunoResearch, West Grove, PA, USA) and Alexa Fluor 647–labeled donkey anti-mouse IgG (ab150111, Abcam, Cambridge, UK) secondary antibodies (1:100) at room temperature for 2 h. After counterstaining with DAPI (4′,6-diamidino-2-phenylindole; 1 μg/mL) (D1306, Thermo Fisher Scientific, Waltham, MA, USA), the fluorescent labeled cells were observed using a Leica TCS-SP8 confocal laser scanning microscope (Leica, Wetzlar, Germany).

2.10 | Immunoprecipitation

The membrane fraction lysates of hiPSCs were added to magnetic agarose beads conjugated with an anti-HA-tag monoclonal antibody (M180-10, Medical & Biological Laboratories, Tokyo, Japan) and incubated overnight at 4°C with gentle rotation. The agarose beads were then washed thrice with PBS-T, and the protein complexes were eluted with 25 mM Glycine. Finally, 1 M Tris–HCl (pH 7.4) was added and adjusted with 2×SDS-PAGE sample buffer.

2.11 | Mouse dissection and tissue sampling

After euthanasia, the abdominal cavity of the mice was carefully opened, and the target organs (liver, spleen, heart, brain, lung, skeletal muscle, and testis) were rapidly excised. Each organ was immediately placed into a tube containing pre-chilled lysis buffer (50 mM HEPES (pH 7.4), 5 mM sodium pyrophosphate, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, and 1 mM phenylmethylsulfonyl fluoride). The tissues were then mechanically disrupted using the TissueLyser II (QIAGEN, Hilden, Germany) to ensure efficient cell lysis. The lysates were centrifuged at 4°C, and the supernatants were collected for the analysis of TMEM182 protein expression. All animal procedures were performed according to the guidelines of the Osaka Medical and Pharmaceutical University Animal Care and Use Committee (approval protocol number: AM23-081).

2.12 | Statistical analysis

Data are presented as mean ± standard deviation (SD) values. Comparisons between the two groups were performed with Student's *t*-test. One-way analysis of variance (ANOVA) followed by Tukey–Kramer test was used for multiple comparisons. Statistical analyses were performed using Statcel4 (an add-in software for Excel). Differences were considered statistically significant when the calculated (two-tailed) p value was <0.05.

3 | RESULTS

3.1 | Pluripotency and proliferative potential of hiPSCs are not affected by doxycycline-induced overexpression of TMEM182

To investigate the effect of TMEM182 on the myocardial differentiation process of hiPSCs, we generated hiPSCs overexpressing hemagglutinin (HA)-tagged TMEM182 induced by doxycycline (DOX). Evaluation showed that DOX treatment resulted in an approximately 100-fold increase in *TMEM182* expression (Figure 1A). We also observed DOX-induced expression of the HA protein in the plasma membrane fraction of hiPSCs (Figure 1B). Expression of the pluripotency markers, *NANOG* and *POU5F1*, was not altered by TMEM182 overexpression (Figure 1C). Next, we examined the effect of TMEM182 on the cell proliferation rate in hiPSC cultures 7 days after seeding. The results showed no effect of TMEM182 overexpression on the hiPSC proliferation rate (Figure 1D). These results suggest that overexpression of TMEM182 in the membrane fraction of hiPSCs did not affect the pluripotency or proliferative potential of hiPSCs.

3.2 | Overexpression of TMEM182 does not affect the differentiation of hiPSCs into cardiac mesoderm, but inhibits their differentiation into cardiac progenitor cells and cardiomyocytes

It is known that functional cardiomyocytes can be derived from hiPSCs.[10] When cardiomyocytes are induced to differentiate from hiPSCs, they first differentiate into mesoderm, then into cardiac mesoderm, and finally into cardiac progenitor cells (CPCs) and cardiomyocytes.[11,12] To investigate the effect of TMEM182 at each step of the induction of differentiation into cardiomyocytes, we differentiated TMEM182-overexpressing hiPSCs using existing differentiation protocols (Figure 2A).[6,9] On Days 2, 6, and 12 of differentiation, *TMEM182* expression was significantly increased in the DOX-treated group (Figure 2B). Furthermore, the mesoderm marker *BrachyuryT* and the cardiac mesoderm marker *MESP1* were expressed on Day 2, but their expression was not affected by the overexpression of TMEM182 (Figure 2C).

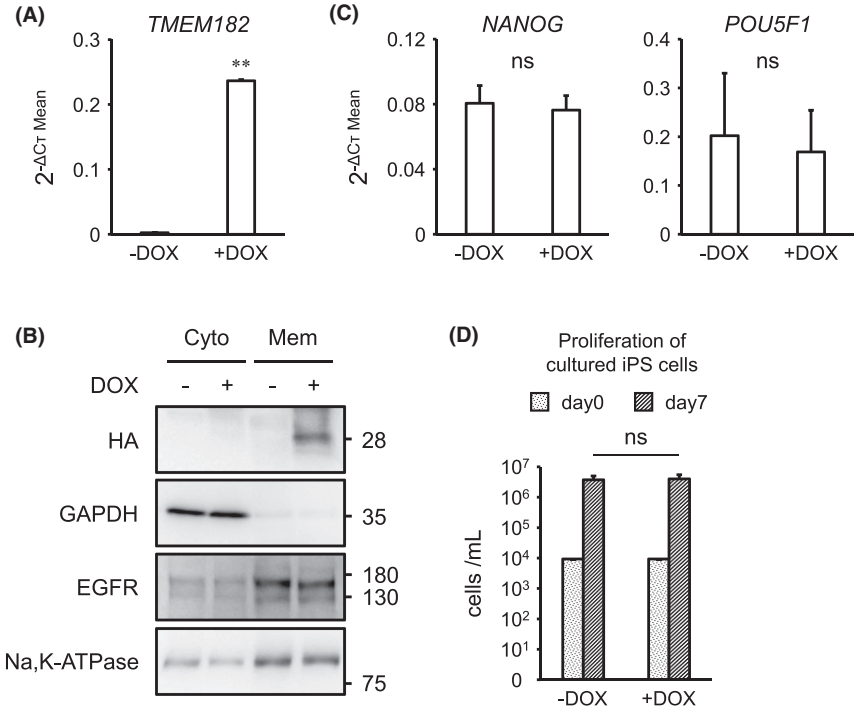

FIGURE 1 DOX-induced overexpression of TMEM182 did not affect the pluripotency or proliferative potential of hiPSCs. (A) Evaluation of *TMEM182* expression by qPCR in hiPSCs capable of overexpressing TMEM182 with DOX treatment. Values represent the mean ± SD of three independent experiments ($n = 3$). DOX, doxycycline. $^{**}p < 0.01$ versus −DOX treatment, by Student's *t*-test. (B) Detection of HA protein in the membrane fraction of hiPSCs overexpressing TMEM182 using western blotting. GAPDH antibody was used as a marker protein for the cytosolic fraction, while EGFR and Na,K-ATPase antibodies were used for evaluating the membrane fraction. Cyto, cytosolic fraction; Mem, membrane fraction. (C) Evaluation of *NANOG* and *POU5F1* expressions using qPCR in hiPSCs overexpressing TMEM182. Values represent the mean ± SD of three independent experiments ($n = 3$). There was no significant difference between the two groups, by Student's *t*-test. ns, non-significant. (D) Evaluation of cell proliferation over 7 days in hiPSCs during TMEM182 overexpression using cell counting. Values represent the mean ± SD of eight independent experiments ($n = 8$). There was no significant difference in cell numbers after culture for 7 days between the hiPSCs treated with DOX and those untreated with DOX, by Student's *t*-test. ns, non-significant.

However, on Day 12 of differentiation, the expression levels of *TBX5* and *GATA4*, CPC markers, and *TNNT2* and *MYH6*, cardiomyocyte markers, were significantly decreased in TMEM182-overexpressing cells (Figure 2D,E). Moreover, immunostaining with α-actinin indicated that cells overexpressing TMEM182 exhibited reduced cardiac sarcomere structure (Figure 2F). These results suggest that overexpression of TMEM182 did not affect the differentiation of hiPSCs into mesoderm, but suppressed their differentiation into CPCs and cardiomyocytes.

3.3 | Overexpression of TMEM182 promotes the induction of differentiation of cardiac fibroblasts during the myocardial differentiation process

POU5F1 is a pluripotency marker, indicating undifferentiated cells, and its expression was significantly reduced on Days 6 and 12 of differentiation compared to Day 2 in both groups, with and without TMEM182 overexpression (Figure 3A). This indicates that it was unlikely that undifferentiated cells remained. Since differentiation into CPCs and cardiomyocytes was suppressed in hiPSCs overexpressing TMEM182, we next examined the expression of cardiac fibroblast markers. Expression of *CDH18*,[13] an epicardial biomarker, tended to increase in cells overexpressing TMEM182 (Figure 3B). Interestingly, the expression of cardiac fibroblast markers, such as *POSTN*[14] and *DDR2*,[15] was significantly increased on Day 12 of differentiation in cells overexpressing TMEM182 (Figure 3B). At the same time, an increase in collagen type I alpha 1 chain (COL1A1) protein expression was also observed (Figure 3C). Furthermore, immunofluorescence staining using the cardiac fibroblast marker vimentin[16] showed a tendency for increased expression in cells overexpressing TMEM182 (Figure 3D). These results suggest that overexpression of TMEM182 facilitated the differentiation of hiPSCs into cardiac fibroblasts.

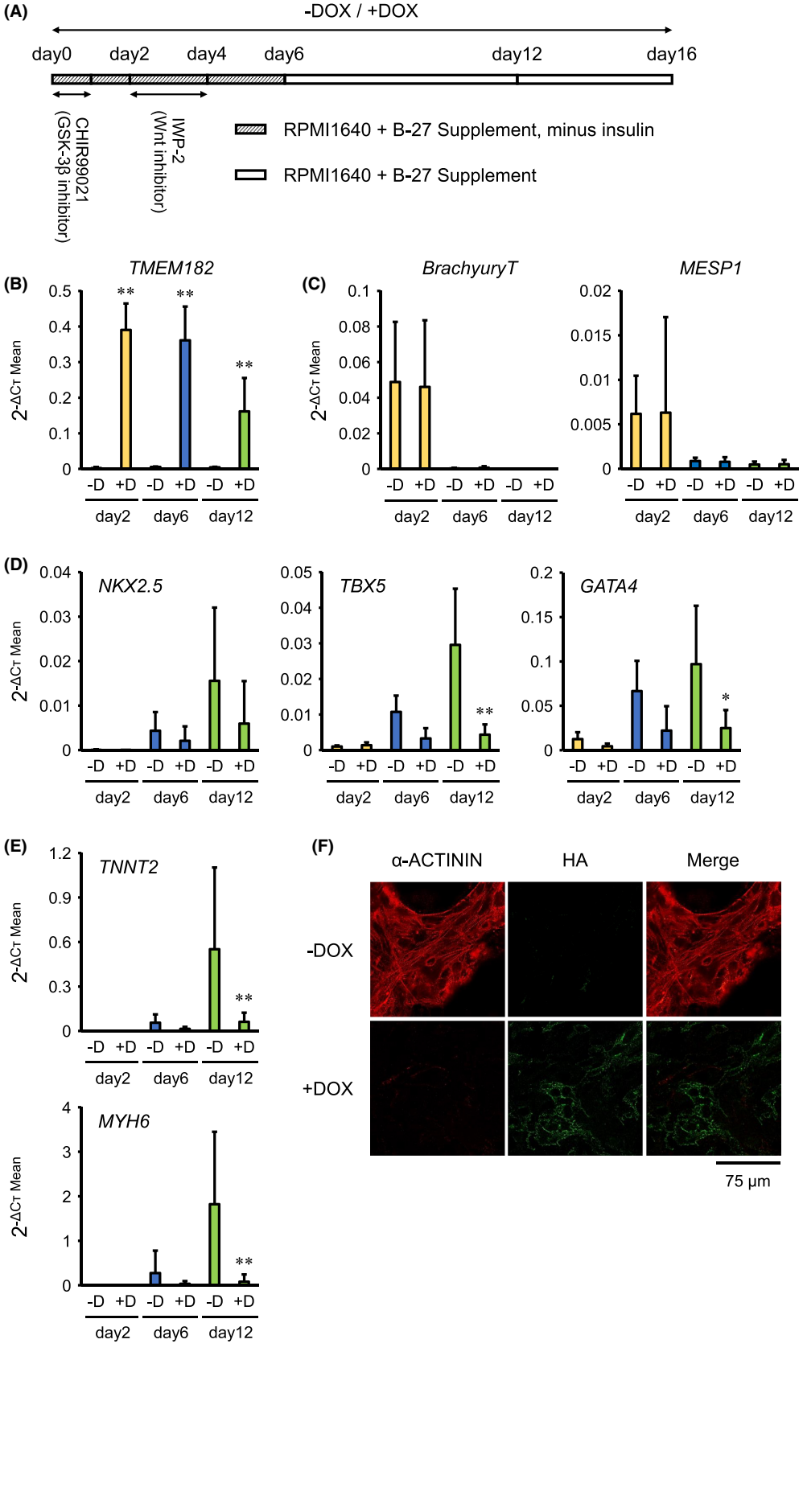
(A)
-DOX / +DOX
day0
day2
day4
day6
day12
day16
CHIR99021 (GSK-3β inhibitor)
IWP-2 (Wnt inhibitor)
RPMI1640 + B-27 Supplement, minus insulin
RPMI1640 + B-27 Supplement
(B)
TMEM182
(C)
BrachyuryT
MESP1
2-ΔCT Mean
-D +D -D +D -D +D
day2 day6 day12
(D)
NKX2.5
TBX5
GATA4
(E)
TNNT2
MYH6
(F)
α-ACTININ
HA
Merge
-DOX
+DOX
75 μm

FIGURE 2 Overexpression of TMEM182 had no effect on the differentiation of hiPSCs into cardiac mesoderm, although it did inhibit their differentiation into cardiac progenitor cells (CPCs) and cardiomyocytes. (A) Myocardial differentiation protocol of hiPSCs. Days 0–1: CHIR99021 (12 μM), Days 2–4: IWP-2 (5 μM), Days 0–16: DOX treatment (1 μg/mL). (B) Change in expression of *TMEM182* with DOX treatment during myocardial differentiation was confirmed using qPCR. Values represent the mean ± SD of five independent experiments ($n = 5$). $^{**}p < 0.01$ versus each day −DOX treatment, by one-way ANOVA followed by the Tukey–Kramer test. −D: without DOX treatment, +D: with DOX treatment. (C) Expression of *BrachyuryT* (mesoderm) and *MESP1* (cardiac mesoderm) during TMEM182 overexpression was confirmed using qPCR. Values represent the mean ± SD of five independent experiments ($n = 5$). There was no significant difference between the two groups on each day, by one-way ANOVA followed by the Tukey–Kramer test. (D) Expression of *NKX2.5*, *TBX5*, and *GATA4* (CPCs) during TMEM182 overexpression was confirmed using qPCR. Values represent the mean ± SD of five independent experiments ($n = 5$). $^{**}p < 0.01$, $^{*}p < 0.05$ versus each day −DOX treatment, by one-way ANOVA followed by the Tukey–Kramer test. (E) Expression of *TNNT2* and *MYH6* (cardiomyocytes) during TMEM182 overexpression was confirmed using qPCR. Values represent the mean ± SD of five independent experiments ($n = 5$). $^{**}p < 0.01$ versus each day −DOX treatment, by one-way ANOVA followed by the Tukey–Kramer test. (F) Immunofluorescence staining with α-ACTININ antibody and HA antibody in cells overexpressing TMEM182 on Day 16 of differentiation.

3.4 | TMEM182 inactivates GSK-3β via phosphorylation of AKT and promotes nuclear translocation of β-catenin

Since overexpression of TMEM182 by DOX treatment from the early stage of the differentiation process (Day 0 of differentiation) suppressed differentiation of hiPSCs into cardiomyocytes, we investigated how TMEM182 affected myocardial differentiation when DOX treatment was given at the middle stage of differentiation (Figure S2A). We found that TMEM182 expression was significantly upregulated in the DOX-treated group on Day 30 of differentiation when DOX treatment was administered from Day 9 of differentiation (Figure S2B). However, at this time point, there were no significant differences in the expression of various CPC markers or cardiomyocyte markers (Figure S2C,D), suggesting that TMEM182 is involved in the early stages of myocardial differentiation.

Next, to investigate through which intracellular signaling pathways TMEM182 regulates the myocardial differentiation of hiPSCs, we focused on Wnt/β-catenin signaling. The differentiation of hiPSCs into cardiomyocytes is regulated by the temporal and spatial dynamics of Wnt/β-catenin signaling. Specifically, activation of Wnt/β-catenin signaling in the early stages of differentiation promotes mesoderm formation. As differentiation progresses, suppression of Wnt/β-catenin signaling promotes the differentiation of CPCs into cardiomyocytes.[17] Moreover, prolonged activation of β-catenin induces cardiac progenitors to develop into cardiac fibroblasts.[18] Therefore, based on previous reports and our results, we hypothesized that overexpression of TMEM182 would activate Wnt/β-catenin signaling. As a result, in cells overexpressing TMEM182, Serine552 (Ser552) phosphorylation of β-catenin was increased (Figure 4A,B). Phosphorylation at Ser552 has been reported to induce nuclear accumulation of β-catenin and increase its transcriptional activity.[19–21] In fact, immunofluorescence staining with β-catenin and DAPI showed that the nuclear translocation of β-catenin was enhanced in cells overexpressing TMEM182 (Figure 4C). To further understand the upstream regulators of β-catenin activation, we investigated GSK-3β, which regulates β-catenin phosphorylation. We found that phosphorylation of Ser9, an inactive form of GSK-3β, was increased by overexpression of TMEM182 (Figure 4D,E). Since AKT has been reported to be involved in Ser9 phosphorylation of GSK-3β,[22] we next examined the phosphorylation of AKT. Consequently, Ser473 phosphorylation of AKT was also increased (Figure 4D,E). These results indicate that, in the situation of TMEM182 overexpression during myocardial differentiation of hiPSCs, phosphorylation of AKT (Ser473) is increased, which in turn increases phosphorylation of GSK-3β (Ser9). In turn, β-catenin escapes degradation by GSK-3β and increases its translocation to the nucleus, suggesting that Wnt/β-catenin signaling is in an activated state.

3.5 | TMEM182 interacts with integrin-linked kinase and increases its expression

In a previous report, screening for proteins that bind TMEM182 using co-immunoprecipitation and mass spectrometry revealed that TMEM182 directly interacts with integrin beta 1 (ITGB1).[4] Furthermore, the cytoplasmic domain of ITGB1 has been shown to activate the canonical Wnt/β-catenin signaling pathway via integrin-linked kinase (ILK) in many cells (HEK293 and MDA231 human cell lines, ovarian cancer, and melanoma cells).[23–25] Moreover, ILK is known to regulate AKT Ser473 phosphorylation in a cell/tissue-dependent manner and can directly phosphorylate AKT on Ser473.[26] Based on these findings, we investigated the relationship between TMEM182 and ILK in this study. We found that ILK expression was increased in differentiated cells overexpressing TMEM182 (Figure 5A,B). The interaction between TMEM182 and ILK was further confirmed by immunoprecipitation using anti-HA antibody (Figure 5C). This suggests that TMEM182 interacts with ILK and activates Wnt/β-catenin signaling by increasing ILK expression. This interaction may

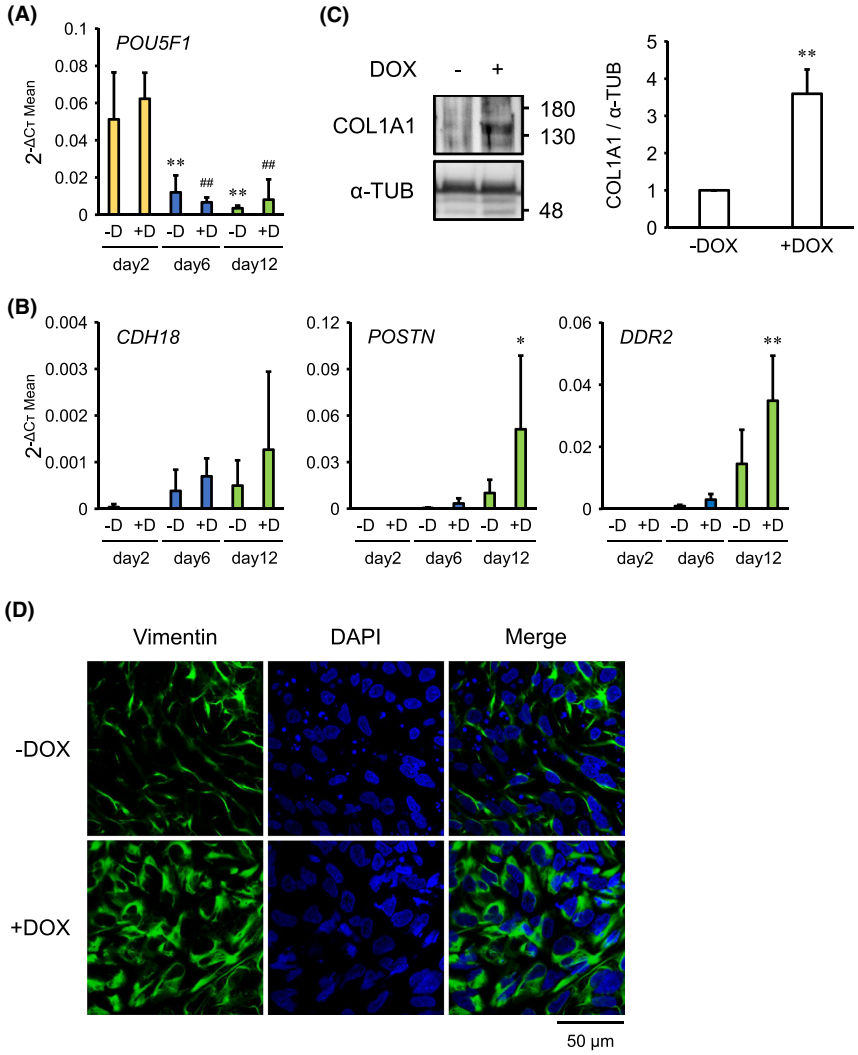

FIGURE 3 Overexpression of TMEM182 promoted the induction of differentiation of cardiac fibroblasts during the myocardial differentiation process. (A) Change in expression of *POU5F1* during TMEM182 overexpression was confirmed using qPCR. Values represent the mean ± SD of five independent experiments ($n = 5$). $^{**}p < 0.01$ versus −DOX treatment on Day 2, $^{\#\#}p < 0.01$ versus +DOX treatment on Day 2, by one-way ANOVA followed by the Tukey–Kramer test. (B) Expression of *CDH18* (epicardial) and *POSTN* and *DDR2* (cardiac fibroblasts) during TMEM182 overexpression was confirmed using qPCR. Values represent the mean ± SD of five independent experiments ($n = 5$). $^{**}p < 0.01$, $^{*}p < 0.05$ versus each day −DOX treatment, by one-way ANOVA followed by the Tukey–Kramer test. (C) Expression of COL1A1 during TMEM182 overexpression on Day 12 of differentiation was confirmed using western blotting. Quantification of COL1A1 protein expression in differentiated cells overexpressing TMEM182. Values represent the mean ± SD of three independent experiments ($n = 3$). $^{**}p < 0.01$ versus −DOX treatment, by Student's *t*-test. COL1A1, collagen type I alpha 1 chain; α-TUB, α-tubulin. (D) Expression of vimentin during TMEM182 overexpression on Day 12 of differentiation was confirmed using immunofluorescence staining.

disrupt the balance of the Wnt/β-catenin pathway during myocardial differentiation, which might suppress the differentiation of hiPSCs into cardiomyocytes (Figure 5D).

3.6 | Differentiation into cardiomyocytes is higher in the TMEM182 low-expression iPS cell line

Different clones of hiPSCs are known to exhibit varying degrees of myocardial differentiation.[27–29] Therefore, we examined the expression of TMEM182 in different hiPSC lines and happened to identify cells with both high and low levels of TMEM182 expression (Figures 6A and S3A). However, no differences were observed in the expressions of *NANOG* and *POU5F1* between these cells (Figure 6B). Additionally, when these hiPSC lines were subjected to differentiation into cardiomyocytes, the expression of cardiac progenitor and cardiomyocyte markers was significantly higher in the hiPSC lines with lower TMEM182 expression (Figure 6C). Moreover, protein expression of cardiac troponin T (cTnT) and

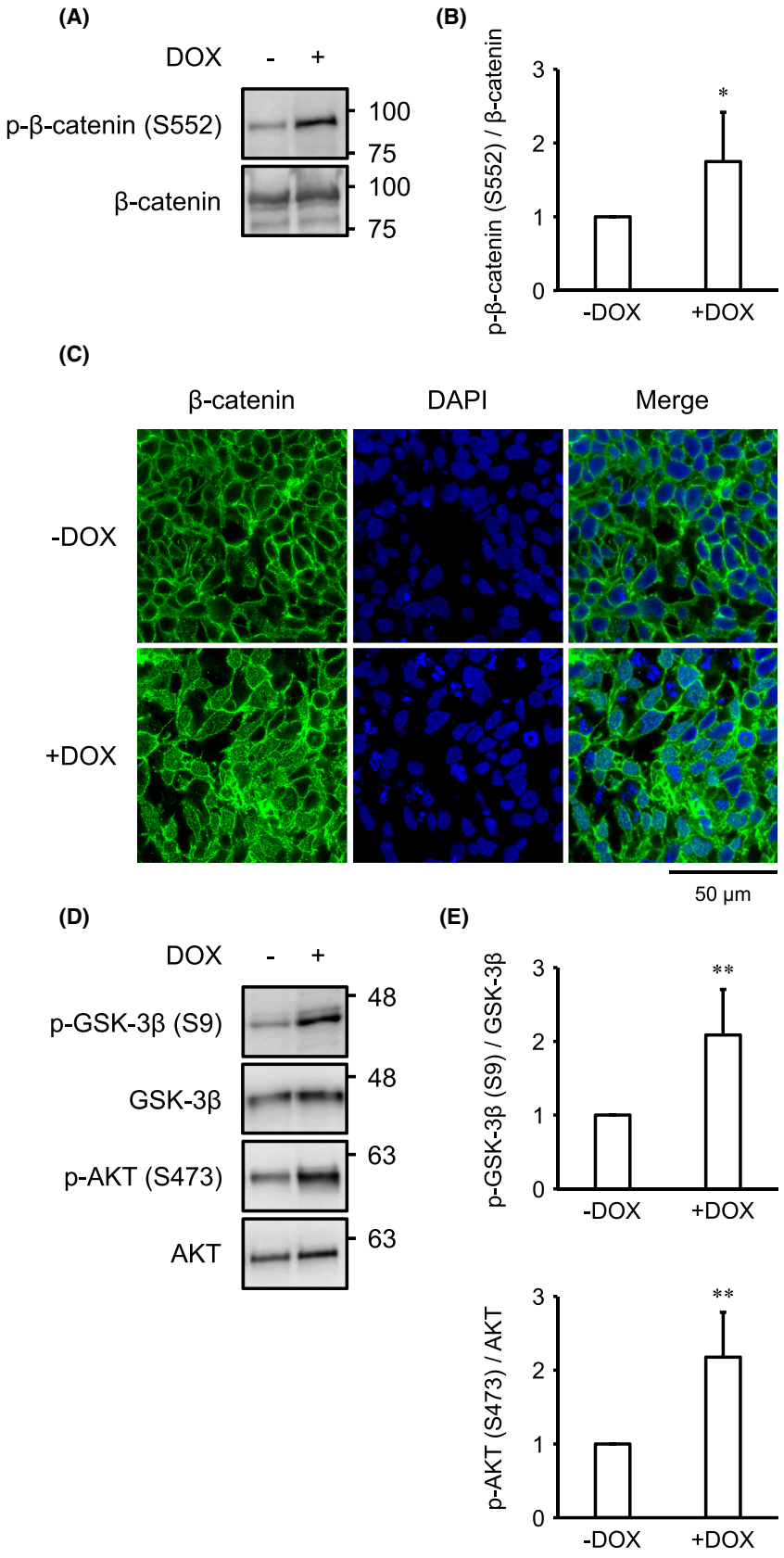

FIGURE 4 TMEM182 inactivates GSK-3β via phosphorylation of AKT and promotes nuclear translocation of β-catenin. (A) Protein expression of p-β-catenin (S552) and β-catenin was confirmed in cells overexpressing TMEM182 on Day 7 of differentiation by western blotting. (B) Quantification of p-β-catenin (S552) and β-catenin protein expression in differentiated cells overexpressing TMEM182. Values represent the mean ± SD of five independent experiments ($n = 5$). *$p < 0.05$ versus −DOX treatment, by Student's *t*-test. (C) Immunofluorescence staining of β-catenin and DAPI in cells overexpressing TMEM182 to observe the nuclear translocation of β-catenin. (D) Protein expression of p-GSK-3β (S9), GSK-3β, p-AKT (S473), and AKT was confirmed in cells overexpressing TMEM182 on Day 7 of differentiation by western blotting. (E) Quantification of p-GSK-3β (S9), GSK-3β, p-AKT (S473), and AKT protein expression in differentiated cells overexpressing TMEM182. Values represent the mean ± SD, p-GSK-3β (S9)/GSK-3β: $n = 5$, p-AKT (S473)/AKT: $n = 4$. **$p < 0.01$ versus −DOX treatment, by Student's *t*-test.

phospholamban (PLN) was assessed on Days 3, 6, 9, 12, and 15 of differentiation. These proteins were detected from Day 9 of differentiation, with higher expression in the hiPSC line with low TMEM182 expression (Figure 6D). Finally, we examined myocardial differentiation in a hiPSC line with high TMEM182 expression when TMEM182 expression was suppressed using siRNA. We confirmed that siRNA treatment of hiPSCs for 3 days resulted in an

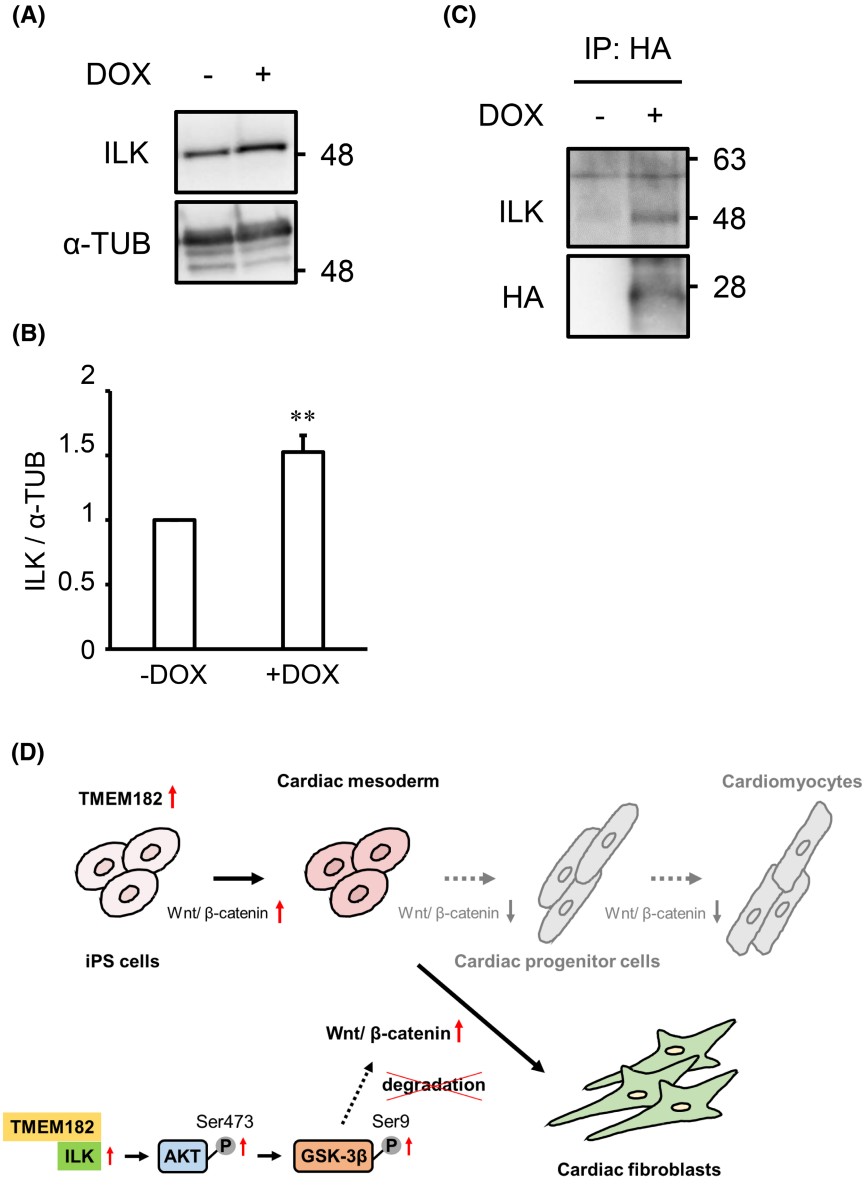

FIGURE 5 TMEM182 interacts with integrin-linked kinase (ILK) and increases its expression. (A) Protein expression of ILK was confirmed in cells overexpressing TMEM182 on Day 7 of differentiation. α-TUB, α-tubulin. (B) Quantification of ILK protein expression in differentiated cells overexpressing TMEM182. Values represent the mean ± SD of five independent experiments ($n = 5$). $^{**}p < 0.01$ versus −DOX treatment, by Student's *t*-test. (C) Membrane fractions of hiPSCs overexpressing TMEM182-HA in a DOX-induced manner were immunoprecipitated by HA antibody and western blotted with the ILK and HA antibody. (D) Schematic of intracellular signaling during TMEM182-overexpressing hiPSCs differentiation into cardiac fibroblasts. IP, immunoprecipitation.

approximately 70% reduction in TMEM182 expression (Figure S3B). The cells were then differentiated into cardiomyocytes, and *TNNT2* expression was assessed on Day 12 of differentiation. As a result, a significant increase in *TNNT2* expression was observed in differentiated cells in which TMEM182 expression was suppressed by siRNA (Figure 6E).

4 | DISCUSSION

The heart is the first organ to form during human development, and comprehending the formation of human cardiomyocytes is crucial for understanding cardiovascular diseases. However, the early events in mesoderm induction and cardiac specification are still not fully understood.[30] This study revealed that overexpression of TMEM182 from early stages of differentiation suppresses the differentiation of CPCs and cardiomyocytes in the process of hiPSCs differentiation into cardiomyocytes.

Initially, methods involving the formation of embryoid bodies were utilized for differentiating cardiomyocytes from human pluripotent stem cells (hPSCs). Currently, however, single-layer culture systems that more accurately induce differentiation into cardiomyocytes through the control of growth factors and small molecules are being employed.[31–33] Such methods for inducing myocardial differentiation in vitro mimic the stage-specific differentiation observed during cardiac development in embryos. Specifically, hPSCs are differentiated into cardiac mesoderm, followed by differentiation and proliferation into CPCs, with subsequent differentiation of the CPCs into mature cardiomyocytes.[34] Furthermore, temporal and spatial regulation of Wnt/β-catenin signaling plays a significant role in

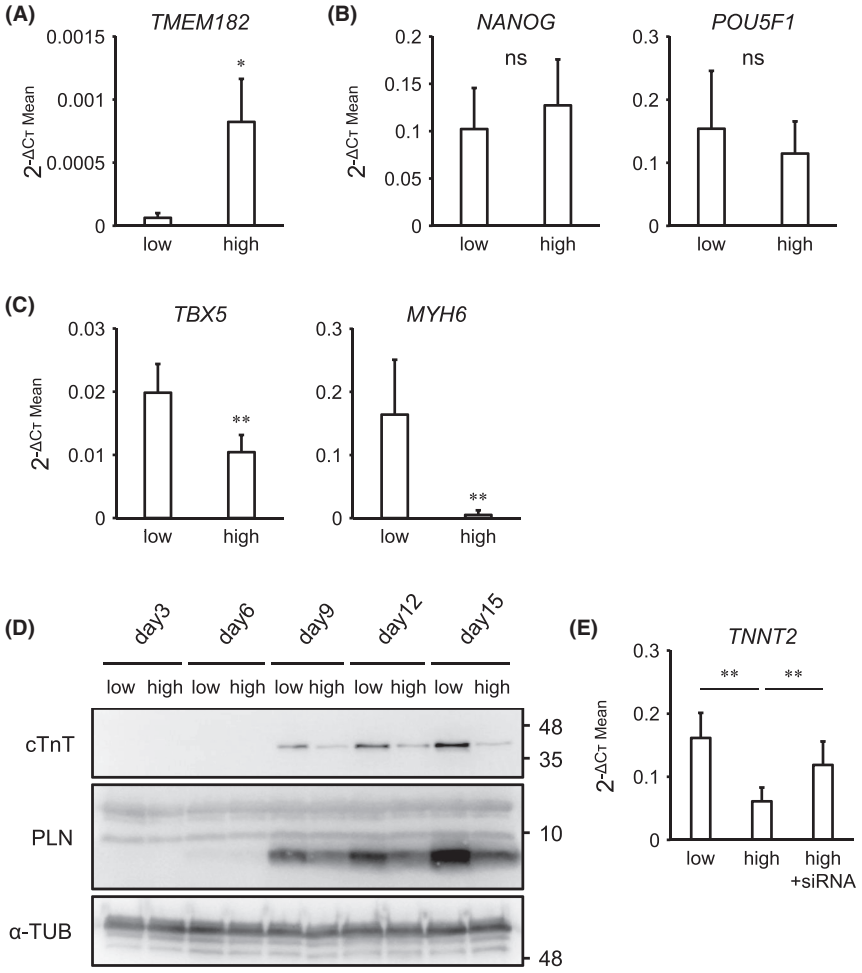

FIGURE 6 Differentiation into cardiomyocytes was higher in the low TMEM182-expression iPSC line. (A) Evaluation of *TMEM182* expression by qPCR in two hiPS cell lines with different TMEM182 expression levels. Values represent the mean ± SD of three independent experiments ($n = 3$). *$p < 0.05$ versus the low-expression cell line, by Student's *t*-test. (B) Expression of *NANOG* and *POU5F1* was confirmed using qPCR in hiPSCs with two different levels of TMEM182 expression. Values represent the mean ± SD of three independent experiments ($n = 3$). No significant difference was observed between the two groups by Student's *t*-test. ns, non-significant. (C) Expression of *TBX5* and *MYH6* was confirmed using qPCR in differentiated cells derived from hiPSCs with different expression levels of TMEM182. Values represent the mean ± SD, low: $n = 5$, high: $n = 4$. **$p < 0.01$ versus the low-expression cell line, by Student's *t*-test. (D) Changes in the expression of cardiomyocyte-associated proteins during the myocardial differentiation process of hiPSCs with different expression levels of TMEM182. cTnT, cardiac muscle troponin T; PLN, phospholamban; α-TUB, α-tubulin. (E) Changes in *TNNT2* expression were confirmed by qPCR when TMEM182 expression was suppressed by siRNA. Values represent the mean ± SD, low: $n = 6$, high: $n = 7$, high+siRNA: $n = 15$. **$p < 0.01$, by one-way ANOVA followed by the Tukey–Kramer test.

the process of myocardial differentiation from hPSCs.[35] Manipulating Wnt/β-catenin signaling in hPSCs can lead to a series of states similar to those occurring during early embryonic development, transitioning from an epithelial phenotype to the cardiac mesodermal lineage, and ultimately differentiating into functional cardiomyocytes.[30] Specifically, inhibition of GSK-3β activates Wnt/β-catenin signaling, leading to increased expression of BrachyuryT, a mesodermal marker, from undifferentiated iPSCs, initiating mesodermal induction.[9,36] In the present study, however, overexpression of TMEM182 did not affect the induction of BrachyuryT or MESP1 expression. It is conceivable that GSK-3β inhibition during myocardial differentiation could trigger a more robust activation of Wnt/β-catenin signaling compared to TMEM182 overexpression, potentially resulting in a plateau in Wnt/β-catenin signaling activation. After differentiation into BrachyuryT-positive cells, inhibition of Wnt/β-catenin signaling destines mesoderm cells to become cardiomyocytes.[17] Approximately 3–4 days after the inhibition of Wnt/β-catenin signaling, iPSC-derived T+ mesodermal cells begin to express cardiac transcription factors, such as NKX2.5, transitioning into a population of CPCs.[37] In the final stages of differentiation, CPCs proliferate and form immature cardiomyocytes. At this stage, several cardiac transcription factors,

including NKX2.5, TBX5, and GATA4, cooperatively activate the transcription of cardiac structural genes, such as cardiac troponin and myosin heavy chain.[32] Indeed, expression of these molecules was confirmed on Day 12 of myocardial differentiation, whereas cells overexpressing TMEM182 exhibited a significant reduction in the expression of *TNNT2* and *MYH6* (Figure 2E). The differentiation into cardiomyocytes is known to initially involve activation of β-catenin, followed by its subsequent inhibition, but sustained activation of β-catenin promotes differentiation into fibroblasts of CPCs.[38] Indeed, in the present study, overexpression of TMEM182 inhibited the differentiation of hiPSCs into cardiomyocytes, while increasing the expression of epicardial biomarkers, such as *CDH18*, and fibroblast biomarkers, such as *POSTN* and *DDR2* (Figure 3B).

In previous reports, TMEM182 was shown to play an inhibitory role in skeletal muscle development, growth, and regeneration. To elucidate the mechanism of the inhibitory effect of TMEM182 on skeletal muscle, screening for proteins that bind to TMEM182 was performed using co-immunoprecipitation and mass spectrometry in primary chicken myoblasts. As a result, ITGB1, an essential membrane receptor involved in cell adhesion and muscle development, was identified as a potential binding protein of TMEM182.[4] Therefore, in the present study, we focused on ILK, an adapter between integrins and the cytoskeleton. ILK serves as a scaffold for other proteins regulating several signaling pathways, is one of the crucial regulatory factors in the signaling cascade mediated by integrin.[39] Indeed, the cytoplasmic domain of ITGB1 has been shown to activate the canonical Wnt/β-catenin signaling pathway through ILK in many cell types.[23–25] Phosphorylation of GSK-3β at Ser9, indicating its inactivation, inhibits proteasomal degradation of β-catenin, leading to its accumulation in the cytoplasm and increased translocation into the nucleus, contributing to gene expression associated with cell proliferation and differentiation.[40,41] Furthermore, activation of ILK is known to induce phosphorylation of GSK-3β and translocation of β-catenin into the nucleus.[42,43] Indeed, in a previous study, knockdown of ILK in MC3T3-E1 osteoblasts resulted in decreased phosphorylation of Ser9 of GSK-3β.[44] Moreover, ILK is known to phosphorylate Ser473 of AKT,[26] and phosphorylated AKT is speculated to phosphorylate Ser9 of GSK-3β.[45] In the present study, we found that TMEM182 interacts with ILK and increases the expression level of ILK (Figure 5B,C). Consequently, there was an increase in phosphorylation of AKT and inactivation of GSK-3β (Figure 4E), leading to increased translocation of β-catenin into the nucleus (Figure 4B,C), assuming sustained activation of Wnt/β-catenin signaling. We hypothesized that the balance of Wnt/β-catenin signaling during myocardial differentiation from hiPSCs is disrupted by overexpression of TMEM182, resulting in suppression of myocardial differentiation and, instead, promotion of differentiation into cardiac fibroblasts (Figure 5D). Given this hypothesis, the question arises whether inhibiting the Wnt/β-catenin signaling pathway under TMEM182 overexpression could relieve the suppression of myocardial differentiation. However, in the present study, we were not able to perform experiments to test this question. One reason for this is that we were unable to pinpoint the exact stage during differentiation when TMEM182 has its strongest effect through sustained activation of Wnt/β-catenin signaling. Based on the observed results, though the overexpression of TMEM182 does not alter the expression of mesodermal markers on Day 2 of differentiation (Figure 2C), and the increased expression of TMEM182 from Day 9 onwards does not affect myocardial differentiation (Figure S2C,D), it is suggested that TMEM182 may exert its influence between Days 3 and 8 of differentiation. However, the precise timing at which TMEM182 has the most significant effect remains unclear, making it challenging to determine when exactly to inhibit Wnt/β-catenin signaling. Moving forward, it will be crucial to further investigate the specific stages of myocardial differentiation where TMEM182 exerts its influence.

TMEM182 is known to be highly expressed in the heart and muscle tissues,[3] and ILK is reported to be most abundant in the heart.[46] Given the interaction between TMEM182 and ILK shown in the present study, it is conceivable that these molecules might cooperate to play essential roles in the heart. Additionally, ILK has been implicated in various cardiac diseases,[47] suggesting the need for further investigation into the relationship between TMEM182 and cardiac diseases.

Finally, for the utilization of hiPSCs in regenerative medicine and drug discovery through the construction of cardiac disease models, a protocol to consistently and efficiently induce cardiomyogenesis and obtain homogeneous populations of differentiated cardiomyocytes is essential. However, it has become evident that various iPSC lines or different iPSC clones derived from the same fibroblasts exhibit different efficiencies in myocardial differentiation.[48–51] The reasons for such inter-strain variability are suggested to include differences in the initial pluripotent state, conditions for maintaining the strains, inherent differences in the production of endogenous growth factors among individual strains, and differences in the epigenetic states determined by the tissue of origin.[50,52,53] Consequently, selecting iPSCs that are less likely to differentiate into cardiomyocytes might result in a higher likelihood of undifferentiated iPSCs remaining within the transplanted cell population, posing a higher risk of tumor formation by persistence of these undifferentiated

iPSCs in the body. Therefore, for the clinical application of hiPSC-derived cardiomyocytes, it is necessary to select appropriate hiPSC strains that produce high-purity cardiomyocytes with minimal undifferentiated hiPSCs. Previous reports have comprehensively analyzed the gene expression of iPSC strains that are prone or resistant to myocardial differentiation using three gene expression analysis methods, revealing a correlation between the expression level of CXCL4/PF4, known as the platelet-derived antiheparin factor, and the propensity for myocardial differentiation.[29] In other words, by using the expression level of CXCL4/PF4 as a marker, iPSC strains suitable for cardiomyocyte production can be selected. In the present study, our results indicated that hiPSC lines with low expression of TMEM182 exhibit greater efficiency of differentiation into cardiomyocytes. Evaluation of TMEM182 expression suggests the ability to predict the cardiomyogenic differentiation potential of individual hiPSC lines.

In conclusion, TMEM182 has been suggested to function as a novel biomarker for selecting hiPSC lines expected to differentiate into cardiomyocytes, potentially contributing to its practical application in cardiac regeneration medicine as a quality control method for hiPSC lines.

AUTHOR CONTRIBUTIONS

H.M. and S.W. conceived the project. H.M., S.Y., and S.W. performed experiments and analyzed data. H.M., S.Y., S.W., and S.T. wrote the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Request for resources and reagents should be directed to and will be fulfilled by Hirofumi Morihara (hirofumi.morihara@ompu.ac.jp).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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