Tbx1 Modulates Endodermal and Mesodermal Differentiation from Mouse Induced Pluripotent Stem Cells

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The T-box transcriptional factor (Tbx) family of transcriptional factors has distinct roles in a wide range of embryonic differentiation or response pathways. Tbx1, a T-box transcription factor, is an important gene for the human congenital disorder 22q11.2 deletion syndrome. Induced pluripotent stem cell (iPSC) technology offers new opportunities for both elucidation of the pathogenesis of diseases and the development of stem-cell-based therapies. In this study, we generated iPSCs from *Tbx1*^{-/-} and *Tbx1*^{+/+} fibroblasts and investigated the spontaneous differentiation potential of iPSCs by detailed lineage analysis of the iPSC-derived embryoid bodies. Undifferentiated *Tbx1*^{-/-} and *Tbx1*^{+/+} iPSCs showed similar expression levels of pluripotent markers. The ability of the *Tbx1*^{-/-} iPSCs to generate endodermal and mesodermal lineages was compromised upon spontaneous differentiation into embryonic bodies. Restoration of Tbx1 expression in the *Tbx1*^{-/-} iPSCs to normal levels using an inducible lentiviral system rescued these cells from the potential of defective differentiation. Interestingly, overexpression of Tbx1 in the *Tbx1*^{-/-} iPSCs to higher levels than in the *Tbx1*^{+/+} iPSCs again led to a defective differentiation potential. Additionally, we observed that expression of fibroblast growth factor (FGF) 10 and FGF8 was downregulated in the *Tbx1*^{-/-} iPSC-derived cells, which suggests that Tbx1 regulates the expression of FGFs. Taken together, our results implicated the Tbx1 level as an important determinant of endodermal and mesodermal lineage differentiation during embryonic development.

Introduction

INDUCED PLURIPOTENT STEM CELL (iPSC) technology is a ground-breaking advancement in biomedical research [1]. Similar to embryonic stem cells, iPSCs have the ability to self-renew and to differentiate into all three germ layer types [2]. Therefore, iPSCs provide a powerful in vitro model for the study of gene function and developmental biology.

T-box transcription factors have important roles in embryonic development, and their mutation is associated with developmental disorders in humans and mice [3]. *Tbx1*, a putative T-box transcription factor, is expressed in the pharyngeal endoderm, ectoderm, and core mesoderm [4–6]. Cell-type-specific inactivation in mice and analysis of *Tbx1* downstream targets indicate that it plays multiple roles in endoderm, mesoderm, and ectoderm cells during pharyngeal development [7–9]. In humans, *Tbx1* is involved in the DiGeorge syndrome, which is associated with cardiac malformations as well as other developmental anomalies of organs and structures derived from the pharyngeal apparatus [10]. Homozygous-null *Tbx1* mice were reported to have many features of DiGeorge syndrome, including a single

cardiac outflow tract and a cleft palate, but missing the thymus and parathyroid glands [11]. A progressive dose reduction of *Tbx1* mRNA has been shown to be associated with the nonlinear increase in phenotypic severity [12]. Overexpression of *Tbx1* also led to structural heart and thymus defects [13]. Thus, embryonic development is sensitive not only to reduced Tbx1 dosage but also deregulation of its gene expression. However, the full analysis of *Tbx1* function in vivo could not be performed because mice demonstrated embryonic or perinatal lethality following loss of *Tbx1* function or overexpression of *Tbx1*.

To further understand the roles of Tbx1 in embryonic development, we generated iPSCs from fibroblasts of *Tbx1*^{-/-} and *Tbx1*^{+/+} mice, and then analyzed their differentiation potential. We show here that the abilities to differentiate into endoderm and mesoderm in the *Tbx1*^{-/-} iPSCs are reduced. Although normalization of Tbx1 expression rescued *Tbx1*^{-/-} iPSCs from abnormal differentiation potential, overexpression of Tbx1 again inhibited their endodermal and mesodermal differentiation. Our results suggest that the expression level of Tbx1 plays an important role in lineage-specific differentiation during embryonic development.

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Materials and Methods

Generation of iPSCs and cell culture

Early passage primary mouse embryo fibroblasts (MEFs) were prepared from the *Tbx1*^{+/+} and *Tbx1*^{-/-} embryos. *Tbx1*^{+/+} and *Tbx1*^{-/-} iPSCs were generated by using the STEMCCA Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit (Millipore, Billerica, MA), according to the manufacturer's instructions. After the first passage, mouse iPS cell colonies were directly adapted to serum-free, feeder-free expansion ESGRO Complete Plus Medium (Millipore). For cell passage, the iPSCs were dissociated with Accutase (Millipore) and placed into 0.1% gelatin-coated plates. The medium was replaced every other day.

Genotyping

Genotyping of iPSCs was determined by polymerase chain reaction (PCR). Genomic DNA was isolated, and primers specific for wild-type (*Tbx1*^{+/+}) and the knock-out (*Tbx1*^{-/-}) allele were used for the PCR analysis. The following primers were used: 1 (5'-TGCATGCCAAATGTTTCCCTG-3'), 2 (5'-GATAGTCTAGGCTCCAGTCCA-3'), and 3 (5'-AGGGCCAGCTCATTCTCCAC-3').

Teratoma formation

The iPSCs were suspended at 1×10^6 cells/100 μ L in phosphate-buffered saline and injected subcutaneously into the dorsal flank of 4-week-old female nude mice. After 4 weeks, the tumors were dissected, fixed in a 10% formaldehyde neutral buffer solution (Fisher Scientific, Detroit, MI), embedded in paraffin, and sliced. The sliced sections were stained with hematoxylin and eosin.

Generation of inducible *Tbx1* expression in iPSCs

An inducible Lentiviral Tet-on system was used (Clontech, Mountain View, CA). The *Tbx1* gene was amplified by reverse transcription-polymerase chain reaction (RT-PCR) from total RNA isolated from mouse cells and subcloned into the pLVX-TRE3G vector using the In-Fusion HD system (Clontech). Viruses for the pLVX-Tet3G and pLVX-TRE3G-*Tbx1* were generated by transfecting 293T cells using the Lenti-X HTX Packaging System (Clontech). The *Tbx1*^{-/-} iPSCs were co-infected with the pLVX-Tet3G and pLVX-TRE3G-*Tbx1* viruses and selected with G418 (1 mg/mL) and puromycin (25 μ g/mL). To induce appropriate *Tbx1* expression during embryoid body (EB) differentiation, doxycycline (Clontech) was added to the cultures at 50 or 500 ng/mL.

Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde, permeated with 0.25% Triton X-100 (Sigma, St. Louis, MO), and blocked with 10% goat serum (Gibco, Carlsbad, CA). The cells were incubated with primary antibody at 4°C overnight. The following primary antibodies were used: mouse polyclonal anti-SSEA-1 antibody (1:200; Cell Signaling, Beverly, MA), rabbit polyclonal anti-Oct4 antibody (1:200; Cell Signaling), rabbit polyclonal anti-alpha 1 Fetoprotein antibody (1:200; Abcam, Cambridge, MA), rabbit polyclonal anti-alpha smooth

muscle actin (α -SMA) antibody (1:200; Abcam), and rabbit polyclonal anti- β III tubulin antibody (1:200; Abcam). The cells were then incubated with Cy3-conjugated anti-rabbit secondary antibody (1:1,000; Invitrogen, Carlsbad, CA), or Cy3-conjugated anti-mouse secondary antibody (1:1,000; Millipore) at room temperature for 1 h. After the cells were rinsed, they were counterstained with 4',6'-diamidino-2-phenylindole (DAPI; Sigma), and analyzed under fluorescence microscope (Nikon, Kanagawa, Japan) or confocal microscope (Nikon). To quantify the percentage of positive cells, computer-assisted morphometric analysis was performed. More than 20 fields of immunofluorescently stained specimens at a magnification of $\times 100$ were chosen at random and scanned. The positive and negative signals were transferred to digital images. The percentage of the total area showing positive signals was automatically calculated using Adobe Photoshop 7.0 software (Adobe Systems, Inc., San Jose, CA).

Flow cytometric analysis

EBs were made into single-cell suspensions and then stained with the fluorochrome-conjugated antibodies. For intracellular staining, the cells were first permeabilized with a BD Cytofix/Cytoperm solution for 20 min at 4°C. Indirect staining of fluorochrome-conjugated antibodies included rabbit polyclonal anti-alpha 1 Fetoprotein antibody, rabbit polyclonal anti- α -SMA antibody, and rabbit polyclonal anti- β III tubulin antibody (Abcam). The cells were then incubated with PE-conjugated goat anti-rabbit IgG secondary antibody (Santa Cruz Biotech, Inc., Santa Cruz, CA). The samples were analyzed on an FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

RNA isolation and RT-PCR

Total RNA was isolated from iPSCs, and their derivatives using the Nucleo Spin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. cDNA was synthesized using 1 μ g of total RNA with the High Capacity cDNA Reverse Transcription Kit (Invitrogen). Real-time quantitative RT-PCR (qRT-PCR) was performed using the 7500 real-time PCR system (Applied Biosystems, Paisley, United Kingdom) using the Power SYBR green mastermix (Applied Biosystems). The primer sequences used in this study are described in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/scd).

Spontaneous differentiation of iPSCs

On day 0 of differentiation, undifferentiated iPSCs were dissociated with Accutase and resuspended in differentiation medium. A total of 2.4×10^6 cells/well were added into an AggreWell™ 400 plate (Stemcell, BC, Canada) to form EBs and incubated at 37°C with 5% CO₂ and 95% humidity for 48 h. After 2 days, the EBs were plated at $\leq 1,000$ EBs per well in an ultralow adherence six-well plate for an additional 5 days. Attached cultures of differentiating iPSCs were initiated by plating the EBs onto 0.1% gelatin-coated 24-well tissue culture plates at a density of one to two EBs per well, and the growth medium was renewed every 2 days. The plates with attached EB clusters were carefully observed under a phase-contrast microscope. The numbers of

beating EBs were counted, and the percentage of beating EBs was calculated. Four independent experiments were done for each group, and at least 100 EBs per group were counted in each experiment. The differentiation medium used in this study consisted of 0.1 mM β -mercaptoethanol, 1 mM L-glutamine, 1% nonessential amino acid stock, and 1% penicillin–streptomycin (all from Gibco except for the first from Sigma) in Knockout™ Dulbecco's modified Eagle's medium (high-glucose with sodium pyruvate formulation; GibcoA) supplemented with 15% Knockout serum replacement (serum replacement for ES cells; Gibco).

Statistical analysis

All data were reported as mean $\pm$ SEM. The Student's *t*-test was used to determine the significance of differences

in comparisons. Values of $P < 0.05$ were considered to be statistically significant.

Results

Generation and characterization of iPSCs from MEFs

We generated *Tbx1*^{-/-} and *Tbx1*^{+/+} MEFs from embryos of pregnant female *Tbx1*^{+/-} mice (after mating with male *Tbx1*^{+/-} mice). We then generated iPSCs by transducing *Tbx1*^{-/-} and *Tbx1*^{+/+} MEFs with a polycistronic lentivirus containing oct4, klf4, sox2, and c-myc (OKSM). Cell colonies started to emerge 8 days after transduction. The iPSCs were transferred to MEFs first, and then adapted to serum-free, MEF-free medium. We established two *Tbx1*^{-/-} and

FIG. 1. Analysis of generated *Tbx1*^{-/-} and *Tbx1*^{+/+} induced pluripotent stem cells (iPSCs). (A) Amplification with wild-type (*Tbx1*^{+/+})-specific primer pairs results in 216-bp products and with neomycin cassette-specific primer pairs results in 470-bp products for *Tbx1*^{-/-} iPSCs. (B) The morphology of *Tbx1*^{-/-} and *Tbx1*^{+/+} iPSCs. Scale bars = 200 μ m. (C) Reverse transcription–polymerase chain reaction (RT-PCR) analysis for isolated iPSCs using ES marker genes. TC-1 ES cells were used as a positive control and mouse embryo fibroblasts (MEFs) as a negative control. (D) iPSCs were examined for the expression of alkaline phosphatase (AP), Oct4, and SSEA-1. AP (dark red) expression was detected using immunocytochemistry; SSEA-1 and Oct4 (red) expression was detected by immunofluorescence staining. Nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI; blue). Scale bars = 200 μ m (AP). (E) In vitro differentiation of the iPSCs. Immunostaining images show all three germ layers, including AFP⁺ (endoderm), alpha smooth muscle actin (α -SMA)⁺ (mesoderm), and tubulin⁺ (ectoderm) cells. Scale bars = 50 μ m. (F) Teratoma formation in immunodeficient mice by the iPSCs. H&E staining was performed for the teratoma. The teratoma resulting from *Tbx1*^{-/-} and *Tbx1*^{+/+} iPSCs contained various tissues.

two *Tbx1*^{+/+} iPSC clones. Because identical results were obtained from the two *Tbx1*^{-/-} or two *Tbx1*^{+/+} iPSC clones, here we only showed the results from one *Tbx1*^{-/-} and one *Tbx1*^{+/+} iPSC clone. Figure 1A shows the genotyping of *Tbx1*^{-/-} and *Tbx1*^{+/+} iPSCs detected by genomic DNA PCR. The iPSC colonies demonstrated a bright cytoplasm and a rounded configuration (Fig. 1B). No morphological differences were observed between *Tbx1*^{-/-} and *Tbx1*^{+/+} iPSCs. We examined the expression of pluripotent markers in these iPSCs. RT-PCR analyses showed that both *Tbx1*^{-/-} and *Tbx1*^{+/+} clones expressed *Gdf3*, *zfp296*, *Rex1*, *Oct3/4*, *Nanog*, *fgf4*, *Dax1*, and *Cripto* genes (Fig. 1C). Their expression levels were similar to those in the mouse TC-1 ES cell line. In contrast, MEFs did not express these genes (Fig. 1C). We also evaluated the presence of alkaline phosphatase (AP) activity and the expression of other pluripotency markers SSEA-1 and Oct4 by immunofluorescence. Both *Tbx1*^{-/-} and *Tbx1*^{+/+} iPSCs were positive for AP, and expressed SSEA-1 and Oct4 (Fig. 1D).

To determine whether *Tbx1*^{-/-} and *Tbx1*^{+/+} clones could differentiate into the cell types from different germ layers in vitro, these cells were allowed to spontaneously differentiate in EB cultures. By staining the cells with antibodies against tissue-specific markers, such as the mesoderm marker α -SMA, the endoderm marker AFP, and the

ectoderm marker tubulin, we demonstrated that the EBs contained cell types derived from all three germ layers (Fig. 1E). We then determined in vivo differentiation ability of the cells and found that tumors formed 4–6 weeks after *Tbx1*^{-/-} and *Tbx1*^{+/+} iPS cell implantation. Hematoxylin and eosin staining of the tumor sections showed the presence of cell types from all the three germ layers (Fig. 1F). We concluded that *Tbx1*^{-/-} and *Tbx1*^{+/+} clones could spontaneously differentiate into derivatives of all three germ layers.

Reduced endodermal development from *Tbx1*^{-/-} iPSCs in vitro

EBs are the in vitro development equivalent of the mouse embryo at early developmental stages [14]. Development of the endoderm is one of the earliest steps during embryonic development. Although the *Tbx1*^{-/-} and *Tbx1*^{+/+} iPSCs can spontaneously differentiate into an endodermal lineage in EB cultures, as shown in Figure 1E, the expression level of the endodermal marker AFP was lower in the *Tbx1*^{-/-} iPSCs than in *Tbx1*^{+/+} iPSCs. Therefore, we determined whether the expression of other endodermal markers was also affected. In these experiments, the iPSCs were grown in the absence of MEFs, and then differentiated by forming

FIG. 2. Role of *Tbx1* in the expression of endodermal lineage genes, in vitro. (A) Morphology of embryoid bodies (EBs) after 3 days of differentiation. (B–D) Real-time quantitative RT-PCR (qRT-PCR) was performed to detect the endoderm markers Sox17 (B), Foxa2 (C), and Gata4 (D). The expression level of genes in the *Tbx1*^{+/+} at day 1 is defined as 1. (E, F) EBs from *Tbx1*^{-/-} and *Tbx1*^{+/+} iPSCs on day 11 of differentiation were determined for the expression of AFP by flow cytometry. (E) A representative histogram of AFP expression. (F) Mean percentage of AFP⁺ cells. Values are means $\pm$ SEM; *P < 0.05. All experiments were independently repeated at least three times.

EBs with AggreWell 400 plate. As shown in Figure 2A, there was no obvious difference in size and morphology between the *Tbx1*^{-/-} and *Tbx1*^{+/+} EBs on day 3. However, Sox17, which is required for differentiation of the definitive endoderm [15], was downregulated in early stage EBs derived from *Tbx1*^{-/-} iPSCs as compared with those from *Tbx1*^{+/+} iPSCs (Fig. 2B). Foxa2, a forkhead transcription factor, which is initially expressed in the visceral endoderm [16], was also significantly decreased in the *Tbx1*^{-/-} EBs. Similarly, Gata4, a zinc finger transcription factor, which is required for visceral endoderm differentiation [17], was downregulated in *Tbx1*^{-/-} EBs (Fig. 2D). AFP is a late differentiation marker of the visceral endoderm. In addition to decreased AFP levels (Fig. 1E), the percentage of AFP-positive cells in *Tbx1*^{-/-} EBs was also decreased as determined by flow cytometric analysis (Fig. 2E, F). Taken together, these results indicate that development to the endodermal lineages is reduced in *Tbx1*-deficient cells.

Reduced mesodermal development of *Tbx1*^{-/-} iPSCs in vitro

To determine the effect of *Tbx1* deficiency on mesodermal differentiation potential, we first analyzed the expression of Brachyury and Flk1 genes. These encode proteins that are critical for mesoderm development. As shown in Figure 3A and B, the expression of both Brachyury and Flk1 in *Tbx1*^{-/-} EBs was significantly lower than that in *Tbx1*^{+/+} EBs, suggesting that *Tbx1* plays an important role in the development of mesodermal lineages. To further examine the mesoderm lineage gene expression, we examined genes related to cardiomyogenesis, which included Nkx2.5, Mef2c, alpha-MHC, and cTnT [18–21]. These genes play important roles in the specification of cardiac progenitor cells and cardiac development. The expression of Nkx2.5 and Mef2c transcription factor mRNAs was markedly reduced in *Tbx1*^{-/-} EBs during the early stages of differentiation compared with wild-type EBs (Fig. 3C, D). During the later stages of differentiation,

FIG. 3. Role of *Tbx1* in the expression of mesodermal lineage genes in vitro. The mRNA levels of the mesodermal markers Brachyury (A), Flk-1 (B), as well as early cardiogenic markers Nkx2.5 (C) and Mef2c (D) in *Tbx1*^{-/-} and *Tbx1*^{+/+} EBs were measured using qRT-PCR. The expression level of genes in the *Tbx1*^{+/+} at day 1 or 7 is defined as 1. (E) Terminal cardiac markers (α -MHC and cTnT) were measured on day 15 of the differentiation by qRT-PCR. The expression level of genes in the *Tbx1*^{+/+} is defined as 1. (F) The incidence of spontaneously beating EBs from the iPSCs was quantified at different time points during differentiation. (G, H) The expression of α -SMA was analyzed by flow cytometry 10 days after the differentiation. (G) A representative histogram of α -SMA expression. (H) Mean percentage of α -SMA⁺ cells. Values are means $\pm$ SEM; * P < 0.05. All experiments were independently repeated at least three times.

very low levels α -Mhc and cTnT gene expression were detected in EBs derived from *Tbx1*^{-/-} iPSCs (Fig. 3E). Moreover, far fewer beating cardiomyocytes were observed in the *Tbx1*^{-/-} iPS-cell-derived EB cultures. Only 20% of *Tbx1*^{-/-} EBs were beating compared with 55% of beating EBs derived from *Tbx1*^{+/+} iPSCs (Fig. 3F). Flow cytometric analysis also showed that the expression of another mesodermal marker α -SMA was also significantly reduced (Fig. 3G, H). These data suggest that the Tbx1 is important for mesodermal differentiation, including the development of cardiac cells in vitro.

Normal ectodermal development from *Tbx1*^{-/-} iPSCs in vitro

We examined whether Tbx1 deficiency affected the development of the ectoderm in iPSC-derived EBs. When Fgf5, a marker for the primitive ectoderm, was measured, *Tbx1*^{+/+} EBs exhibited the typical transient existence pattern of this lineage. Fgf5 was expressed in *Tbx1*^{-/-} EBs at a level similar to that in *Tbx1*^{+/+} EBs (Fig. 4A). The expression levels of sox1 and nestin, markers of neuroectodermal development, were also not significantly different between the *Tbx1*^{-/-} and *Tbx1*^{+/+} EBs (Fig. 4B, C). Further, the expression level of β III tubulin (a pan neuronal marker) detected by immunofluorescence (Fig. 1E) and the percentage of β III tubulin-positive cells detected by flow cytometric analysis were similar between the *Tbx1*^{-/-} and *Tbx1*^{+/+} EBs

(Fig. 4D, E). These data suggest that Tbx1 requirement is not critically important for ectodermal differentiation.

Restoration and overexpression of Tbx1 in iPSCs

Because our data indicated that lack of Tbx1 disturbed endodermal and mesodermal lineage development in the iPSCs, we performed complementation analyses using an inducible lentiviral system to restore or to overexpress Tbx1 in *Tbx1*^{-/-} iPSCs. The expression of Tbx1 was controlled by a tetracycline inducible promoter in a lentiviral vector. EBs were differentiated and Tbx1 expression was induced from day 1 to 7. Tbx1 expression in *Tbx1*^{-/-} iPSCs was induced by doxycycline in a dose-dependent manner (data not shown). The expression level of Tbx1 in *Tbx1*^{-/-} iPSCs after 50 ng/mL doxycycline induction was comparable to that in *Tbx1*^{+/+} iPSCs, whereas 500 ng/mL of doxycycline induction led to Tbx1 expression in *Tbx1*^{-/-} iPSCs higher than that in *Tbx1*^{+/+} iPSCs (Fig. 5A). The restoration of Tbx1 to normal levels rescued *Tbx1*^{-/-} iPSCs from their defective differentiation potential, including gene expression and development of beating cardiomyocytes in culture (Figs. 5B–E and 6). However, when the Tbx1 was induced by the 500 ng/mL doxycycline, we observed the defective differentiation potential again (Figs. 5B–E and 6). To exclude the possible cell toxic effect of doxycycline, we analyzed the expression of endodermal and mesodermal genes Sox17, Foxa2, Gata4, Brachyury, Flk1, Nkx2.5, Mef2c, and

FIG. 4. Role of Tbx1 in the expression of ectodermal lineage genes in vitro. The mRNA levels of ectodermal markers Fgf5 (A), Sox1 (B), and Nestin (C) were measured by qRT-PCR during in vitro differentiation of *Tbx1*^{-/-} and *Tbx1*^{+/+} iPSCs. The expression level of genes in the *Tbx1*^{+/+} at day 1 is defined as 1. (D, E) The expression of β III tubulin was analyzed by flow cytometry 10 days after the differentiation. (D) A representative histogram of β III tubulin expression. (E) Mean percentage of β III tubulin⁺ cells. Values are means $\pm$ SEM. All experiments were independently repeated at least three times.

FIG. 5. Effect of restoration and overexpression of Tbx1 in the endodermal differentiation in vitro. *Tbx1*^{-/-} iPSCs transduced with inducible lentivirus containing Tbx1 gene were induced to differentiate by EB cultures. The expression of *Tbx1* in the *Tbx1*^{-/-} iPSCs was induced by 50 or 500 ng/mL doxycycline. *Tbx1*^{+/+} iPSCs in the presence of phosphate-buffered saline (PBS) were used as positive controls. The expression level of *Tbx1* was determined by western blot (A). qRT-PCR was performed to detect genes of the endoderm markers Sox17 (B), Foxa2 (C), and Gata4 (D). The expression level of genes in the *Tbx*^{+/+} at day 1 is defined as 1. (E) Quantitative evaluation of endodermal induction by analysis of the AFP-positive area after immunofluorescent staining for EBs from iPSCs on day 11 of differentiation. Values are means $\pm$ SEM; *P < 0.05. All experiments were independently repeated at least three times.

alpha-MHC in *Tbx1*^{+/+} EBs in the presence or absence of 50 or 500 ng/mL doxycycline for 7–15 days, and found that the expression of these genes was not significantly different among each group (data not shown). Therefore, the defective differentiation potential in Tbx1-overexpressed *Tbx1*^{-/-} EBs induced by 500 ng/mL doxycycline was not due to the toxic effect of doxycycline.

Role of Tbx1 in the fibroblast growth factor signaling

Fibroblast growth factors (FGFs) have been shown to regulate organogenesis, tissue development, and stem cell differentiation [22,23]. A previous study demonstrated that Fgf8 and Fgf10 expression is altered in *Tbx1*^{-/-} mutants [24]. Consistent with this report, we found that Fgf10 and Fgf8 expression was significantly decreased in *Tbx1*^{-/-} EBs during differentiation (Fig. 7A, B), which suggested that Tbx1 affects differentiation of iPSCs likely through the regulation of FGF signaling.

Discussion

ES cells and iPSCs represent invaluable tools in developmental biology studies and have potential applications in regenerative medicine. For now, they may serve as valuable in vitro models of human diseases with unknown etiology

[25]. In this study, we generated *Tbx1*^{+/+} iPSCs and *Tbx1*^{-/-} iPSCs from *Tbx1*^{+/+} and *Tbx1*^{-/-} mouse MEFs, respectively. These iPSCs are pluripotent based on the similarities to murine ES cells, which included their gene expression pattern and ability to spontaneously differentiate into cells of different germ layers. Therefore, they provide a new model to study the role of Tbx1 during embryonic development.

The role of Tbx1 in cardiac development has been well established. Tbx1 has been shown to be a major genetic determinant of the 22q11.2 deletion syndrome (22q11DS) in humans [26]. Outflow-tract defects, such as persistent truncus arteriosus and tetralogy of Fallot, are characteristic cardiovascular features observed in patients with 22q11DS in addition to craniofacial defects [27]. Tbx1-null mice phenocopy the 22q11DS phenotype and Tbx1-hypomorphic mice display a milder phenotype with cardiovascular defects [11,28,29]. Consistent with these observations, we observed fewer beating cardiomyocytes in the *Tbx1*^{-/-} iPSCs compared with the *Tbx1*^{+/+} iPSCs. We also observed a decreased expression of the cardiac differentiation markers Nkx2.5, cardiac MHC, and cTnT in *Tbx1*^{-/-} EBs. Therefore, our data support the concept of a critical role for Tbx1 in cardiac development. Tbx1 has been reported as being required for sustained proliferation of mesodermally derived cardiac progenitors of the second heart field, a cardiac

FIG. 6. Effect of restoration and overexpression of *Tbx1* in the expression of mesodermal differentiation in vitro. *Tbx1*^{-/-} iPSCs transduced with inducible lentivirus containing *Tbx1* gene were induced to differentiate by EB cultures. The expression of *tbx1* in the *tbx1*^{-/-} iPSCs was induced by 50 or 500 ng/mL doxycycline. *Tbx1*^{+/+} iPSCs in the presence of PBS were used as positive controls. The mRNA levels of the mesodermal markers *Flk-1* (A), *Brachyury* (B), and the early cardiogenic markers *Mef2c* (C) and *Nkx2.5* (D) were measured by qRT-PCR during in vitro differentiation. (E) Terminal cardiac markers (α -MHC and cTnT) were measured on day 15 of differentiation by qRT-PCR. (F) The incidence of spontaneously beating EBs from iPSCs was quantified at different time points during differentiation. Values are means $\pm$ SEM; *P < 0.05. All experiments were independently repeated at least three times.

progenitor cell population that contributes to the development of most of the heart [30]. Thus, the defective cardiac development in the *Tbx1*^{-/-} iPSCs was possibly due to the reduced mesoderm, cardiac differentiation and/or decreased proliferation of the cardiac progenitor cells.

DiGeorge syndrome is also characterized by thymus and parathyroid aplasia/hypoplasia. *Tbx1*^{-/-} mice had no thymus and parathyroid glands and had a cleft palate, suggesting that *Tbx1* is also involved in the endoderm development. In our study, in addition to the effect on cardiac development, the expression of endoderm markers was significantly changed when *Tbx1* was silenced. We

observed a decreased expression of the visceral endodermal marker AFP in *Tbx1*^{-/-} EBs and the downregulation of regulatory genes, such as *Foxa2* and *Gata4*. These findings indicate that a normal *Tbx1* level is required for appropriate visceral endoderm formation.

To determine whether *Tbx1* deletion was the responsible phenotype in *Tbx1*^{-/-} iPSCs, we performed complementation experiments. We found that normalization of *Tbx1* expression rescued *Tbx1*^{-/-} iPSCs from the defective differentiation potential. Interestingly, when the *Tbx1* gene was overexpressed in iPSCs, they had the similar phenotype observed in the *Tbx1*^{-/-} iPSCs. Our results are consistent

FIG. 7. Role of *Tbx1* in the fibroblast growth factor signaling. The mRNA levels of *Fgf10* (A) and *Fgf8* (B) were measured by qRT-PCR during in vitro differentiation of *Tbx1*^{-/-} and *Tbx1*^{+/+} iPSCs. The expression level of genes in the *Tbx1*^{+/+} at day 1 is defined as 1. Values are means $\pm$ SEM; *P < 0.05. All experiments were independently repeated at least three times.

with previous reports that mice with extra copies of human *Tbx1* have many of the abnormalities observed in *Tbx1*^{+/-} and *Df1* mice [13,31] and that overexpression of *Tbx1* results in inhibitory effects upon *Mef2c* expression during muscle differentiation [32]. Taken together, these data suggest that the mouse embryonic development is sensitive to the *Tbx1* expression level.

A number of efforts have been made to identify *Tbx1*-target genes as a means to further explore its role during development. *Tbx1* has been shown to regulate, directly or indirectly, several major signaling systems, including the FGF [24,33,34], the retinoic acid [35], the Delta-Notch [36], and the BMP/Smad1 [37] pathways. In this study, we found that *Fgf8* and *Fgf10* were downregulated upon the loss of *Tbx1*, consistent with the previous study that showed that *Fgf10* and *Fgf8* expression was affected in *Tbx1*^{-/-} mice. Thus, FGF signaling possibly mediates the effects of *Tbx1* in the endoderm and cardiac development from iPSCs.

Various directions have been undertaken to reveal the molecular mechanisms underlying the role of *Tbx1* during development, which could lead to better insights into the pathogenesis in 22q11DS. In our study, we have generated iPSCs from the *Tbx1*^{-/-} mouse and provided evidence that *Tbx1* modulates the cardiac and endoderm development, probably through regulation of FGF signaling. Further studies on the functions of *Tbx1* in differentiation can be provided using this iPSC model.

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Author Disclosure Statement

The authors declare no competing financial interests.

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