Insulin–Like Growth Factor Binding Protein 4 Enhances Cardiomyocytes Induction in Murine-Induced Pluripotent Stem Cells

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ABSTRACT

Insulin-like growth factor binding protein 4 (IGFBP4) has been reported to play critical role in cardiomyocytes differentiation of embryonic stem cells (ESCs). But whether it promotes cardiomyocytes induction of iPSCs is unclear. In the present study, we aim to explore the role of IGFBP4 in the cardiogenesis of mouse iPSCs. We observed that IGFBP4 treatment at late stage during differentiation process of mouse iPSCs greatly enhanced the beating frequency of embryoid bodies (EBs). The expressions of Nkx2.5 (cardiac-specific transcription factor), α-MHC, α-actinin, and Troponin I (cardiac-specific protein) were significantly enhanced by IGFBP4 treatment. Immunostaining analysis showed that α-MHC, TNNT2 and connexin 43, typical cardiac markers, were obviously expressed in isolated cardiomyocytes from iPSCs with or without IGFBP4 treatment. Further study revealed that IGFBP4 had little effect on the apoptosis of EBs, but it significantly promoted the proliferation of cardiomyocytes from iPSCs characterized by higher ratio EdU positive cells in differentiated cardiomyocytes. We next observed that IGFBP4 inhibited β-catenin expression in cytosol of EBs at late stage during differentiation of iPSCs. Knockdown of β-catenin using a siRNA technique promoted the proliferation of differentiated cardiomyocytes and enhanced cardiomyocytes induction of iPSCs, suggesting that the effect of IGFBP4 on cardiomyocytes differentiation of iPSCs has relationship with β-catenin signaling pathway. In conclusion, IGFBP4 promotes cardiogenesis of iPSCs by enhancing the proliferation of differentiated cardiomyocytes through inhibiting β-catenin signaling. J. Cell. Biochem. 115: 1495–1504, 2014. © 2014 Wiley Periodicals, Inc.

KEY WORDS: CARDIOMYOCYTES; DIFFERENTIATION; PROLIFERATION; MOUSE IPSCS; IGFBP4

Terminally differentiated adult cardiomyocytes lack the ability to regenerate. Their malfunction or significant loss due to disease or aging can lead to lethal consequences such as heart failure. Heart transplantation for patients with end-stage heart failure may be an efficient way to solve this problem. But it is limited by the number of donor organs available. One of the most promising strategies to resolve the shortage of organs is cell replacement therapy. Recent reports describing cardiomyocytes induction from induced pluripotent stem cells (iPSCs) has raised the possibility [Mauritz et al., 2008; Zhang et al., 2009]. However, the low efficiency and yield of cardiomyocytes from iPSCs currently limits the application of this technology [Nsair and MacLellan, 2011]. As

such, in vitro, improving generation of autologous cardiac cells from iPSCs for transplantation and the effective treatment of heart disease is a key area of study.

Thus, we sought to identify factors that may efficiently induce cardiomyocytes from iPSCs differentiation. Insulin-like growth factor binding protein 4 (IGFBP4) usually acts by binding to IGFs thereby modulates the actions of insulin-like growth factors (IGFs) [Firth and Baxter, 2002; Durai et al., 2005]. Recently, it was reported to strongly promote cardiomyocytes differentiation of embryonic stem cells (ESCs) in the late phase after embryoid body (EB) formation [Zhu et al., 2008]. However, the cardiogenic effect of IGFBP4 was independent of its IGF-binding activity but was mediated by the inhibitory effect on canonical Wnt signaling [Zhu et al., 2008]. Knockdown of IGFBP4 by two different morpholino constructs resulted in cardiac defects in *Xenopus embryos* [Zhu et al., 2008]. Therefore, promotion of cardiomyocytes differentiation of ESCs using IGFBP4 is physiologically relevant. However, whether it could enhance cardiomyocytes induction in iPSCs is unclear.

Here, we study the role of IGFBP4 on cardiogenesis of mouse iPSCs and explore potential mechanisms. The present study would gain insights into enhancing cardiomyocytes differentiation in iPSCs and may lead to an improvement in the future application of iPSCs in cell therapy for cardiovascular diseases.

MATERIALS AND METHODS

CULTURE AND DIFFERENTIATION OF MOUSE iPSCs (miPSCs)

The mouse iPS cell line 20D-17 (APS0001, RIKEN BioResource Center, Japan), with an EGFP transgene targeted to Nanog was used in the present study. The cell was reprogrammed by the transduction with retroviral vectors encoding the four transcription factors Oct4, Sox2, c-Myc, and Klf4. The iPSCs used in this study were routinely maintained in standard Knockout DMEM (KSR, Invitrogen, USA) medium containing 15% knockout fetal bovine serum (FBS) (Invitrogen), 1% non-essential amino acids (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma-Aldrich), 0.2 mmol/L L-glutamine (Invitrogen), 50 U/ml penicillin, and 50 mg/ml streptomycin on mitomycin-C (Roche)-treated mouse embryonic fibroblast (MEF) feeder layers in the presence of leukemia inhibitory factor (LIF) (Millipore, Billerica, MA, 1,000 U/ml). Cells were passaged when confluence reached 50–60% to preserve the undifferentiated phenotype.

For differentiation, iPSCs were trypsinized and EBs were generated by the three-dimensional hanging-drop method. EBs were grown in hanging drops for 3 days (day 0 to day 3), each drop initially consisted of 1,000 cells in 30 μl of differentiation medium (growth medium without LIF). On the third day, individual EBs were transferred to gelatin-coated 96-well culture plates, allowed to attach, and incubated in differentiation media according to the need. IGFBP4 (Sino Biological, Inc.) was added at day 3–8, day 3–6, and day 6–8 period under 1 μg/ ml to determine the effective time-window. Later, IGFBP4 was added from 0.5 to 2 μg/ ml during day 6–8. The FBS concentration was reduced to 5% at day10. Medium was renewed every 2–3 days. These cells were grown at 37°C in a 5% CO_2 humidified atmosphere.

QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

Total RNA was extracted from iPSCs using Trizol reagent (Invitrogen, Carlsbad, CA) and treated with DNAse I (Promega, Madison, WI) for 15 min to eliminate the potential contamination of genomic DNA. cDNA was generated by reverse transcribed 1 μg of RNA using oligo (dT) primer and Rever-Tra Ace reverse transcriptase (Toyobo, Osaka, Japan) according to the manufacturer's recommendations. For quantitative PCR, 50 ng of cDNA was amplified with the iQ SYBR Green Supermix kit on an iCycler System (Bio-Rad) and Power SYBR Green PCR Master Mix (TaKaRa) for relative quantification of the indicated genes. Primer sequences used were included in Table I. The expression of each target mRNA was quantified relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

APOPTOSIS ANALYSIS

Cells were dissociated into single cells and stained by Propidium Iodide Solution/Annexin V FITC Conjugate (Invitrogen), and apoptosis was analyzed and quantified by flow cytometry analysis (EPICS ALTRA, Beckman Coulter) using Expo32 software (Expo32 Multi COMP and V1.2 Analysis).

SIRNA AND IN VITRO TRANSFECTION

The endogenous expression of β-catenin of EBs was knocked down by transfection with three β-catenin-specific siRNAs (si1#5′-GCACCAUGCAGAAUACAAATT-3′, si2#5′-GCCUCUGAUAAAGG-CAACUTT-′, and si3#5′-GCCUUAGUAAACAUAAUGATT-3′) using Lipofectamine 2000 according to the manufacturer's instructions. The effects of the three siRNA were measured using Western blotting. A non-silencing si-C with the sequence 5′-UUCUCCGAACGUGU-CACGUTT-3′ (150 nM, GenePharma, China) was used as a control.

THE PROLIFERATION ANALYSIS OF DIFFERENTIATED CARDIOMYOCYTES

The proliferation of differentiated cardiomyocytes from iPSCs was analyzed by flow cytometry (FACS) with TNNT2/EdU double-staining. The protocol is based on Click-iT Edu chemistry, following the instruction of Click-iT-EdU 647 Flow Cytometry kit (Molecular Probes/Life Technologies C10424). Cardiomyocytes are detected from whole cells using an antibody specific for TNNT2 antibody (1:200, Abcam) and FITC-conjugated anti-rabbit secondary antibody (1:1,000, Invitrogen) by FACS. At day 12, 30 min before the end of the culture period add 10 μM EdU to one well of a 12-well plate containing differentiated cells and incubate at 37°C. After 30 min of EdU treatment, remove all culture medium and replace with PBS, singularize the cells with 0.25% Trypsin–EDTA, resuspend the cells in 100 μl of fixative (4% paraformaldehyde in PBS) for 15 min at

TABLE I. Primers Used for Real-Time PCR

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
IGFBP4	GGCATGGAAGGAATCAAGGTG	TTCGTGGAACTTCAGCCACAG
Nkx2.5	GCCCAGTACCTCCGAAAGTC	GCCTTAACATACTCCTCCTTGTC
α-*MHC*	CCACTCTTCCACCTTCGATG	TCCACCACCCTGTTGCTGTA
GAPDH	CCACTCTTCCACCTTCGATG	TCCACCACCCTGTTGCTGTA

A iPSCs culture and differentiation protocol

iPS Media (plus LIF)	iPS Media (no LIF)		
15% FBS	15% FBS	10% FBS	5% FBS

Days: -3   0   3   10   15

B Undifferentiation

DAPI — Oct3/4

DAPI — SSEA-1

DAPI — AKP

C Differentiation

2d — GFP-Nanog

4d — GFP-Nanog

6d — GFP-Nanog

8d — GFP-Nanog

10d — GFP-Nanog

D

Beating EBs(%) vs Day (8, 10, 12, 15, 17)

E IGFBP4 relative expression
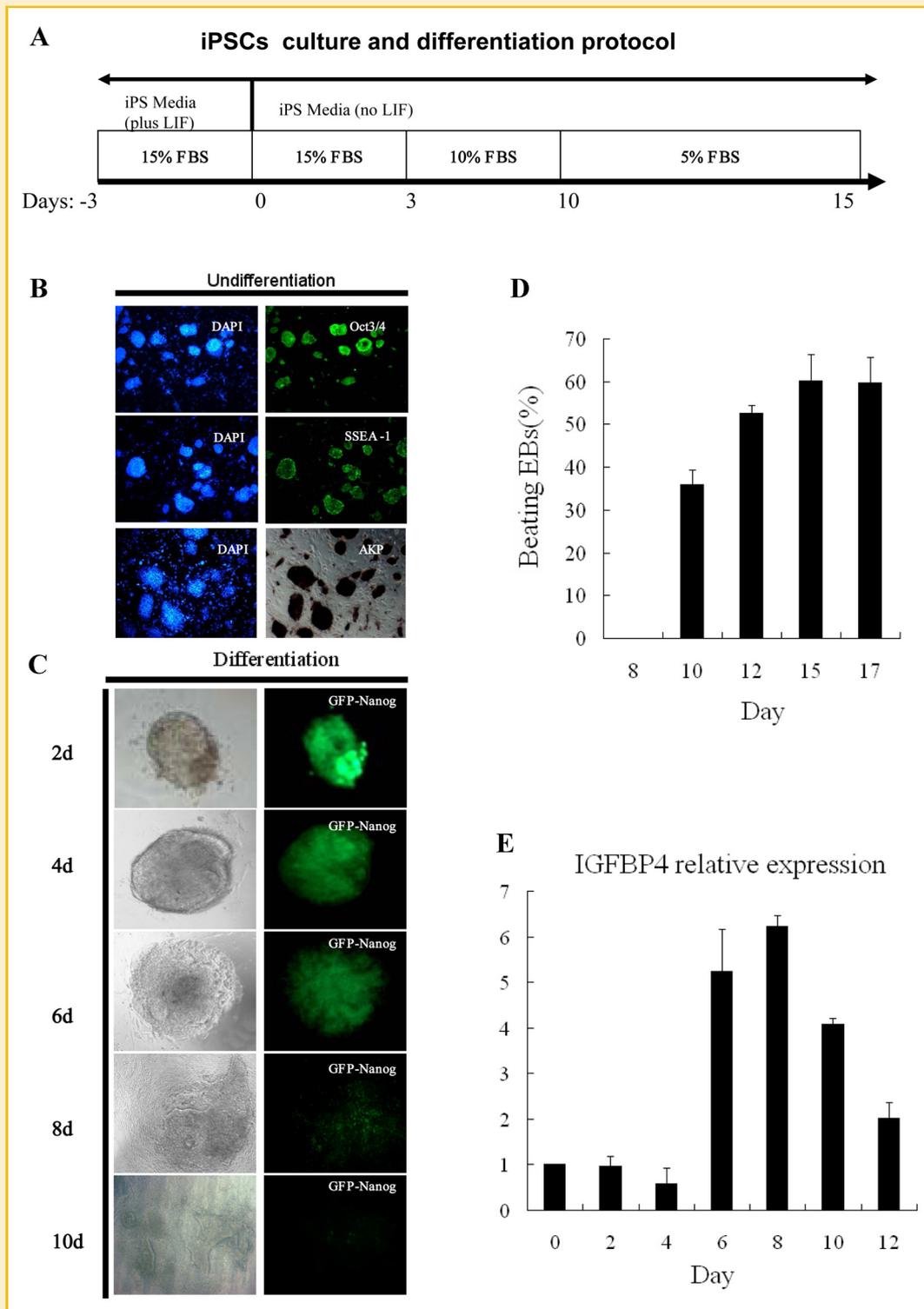
Day (0, 2, 4, 6, 8, 10, 12)

Fig. 1. IGFBP4 mRNA expression profile of mouse iPSCs under natural differentiation. A: Representative schema of the culture protocol of iPSCs and EBs (LIF: leukemia inhibitory factor). B: Undifferentiated colonies on mitotically inactivated MEFs. Colonies were immunopositive for Oct3/4, SSEA-1, and AKP staining. C: Differentiated EBs on gelatin-coated dishes during the differentiation (2, 4, 6, 8, and 10 days). GFP targeted to Nanog was analyzed under fluorescence microscope (40×). D: The frequency of spontaneously beating EBs were analyzed from at least 70 EBs for each day during natural differentiation (from day 8 to day 17). E: Real-time-PCR analysis showed the expression profile of IGFBP4 mRNA during natural differentiation process (from day 0 to day 12) of mouse iPSCs. The data were quantified to day 0. Values are means ± SEM. Any experiment was repeated independently at least three times.

room temperature (RT). Wash cells in permwash (supplied as part of the Click-iT-EdU 647 Flow Cytometry kit) for three times. Spin down and then resuspend again in 50 μl permwash with 10% BSA. Incubate for 10–20 min at RT. Collect cells at 5,000 rpm 10 s. Rotate tubes 180° and spin again for 10 s. Resuspend in 25 μl permwash containing anti-TNNT2 antibody. Incubate for 30–45 min at room temperature (RT). Wash cells in 100 μl of permwash for three times. Resuspend in 25 μl permwash containing FITC secondary antibody (1:1,000). Incubate for 30–45 min at RT in the dark. Wash cells in 100 μl of permwash for three times. Add 200 μl of Click-iT reaction cocktail to each tube and mix well. Incubate for 30 min at RT in the dark. Wash cells once in 100 μl of permwash for three times, then tested by flow cytometry. Cardiomyocyte differentiation was quantified as the fluorescent intensity of TNNT2 staining, then TNNT2/EdU co-stained population was considered as prolifered cardiomyocytes [Wakeling, 2013].

ALKALINE PHOSPHATASE STAINING

To assess alkaline phosphatase (AKP) activity, iPSCs colonies were fixed in 4% paraformaldehyde and then treated according to the protocol by Alkaline Phosphatase Assay Kit (Beyotime Biotechnology).

IMMUNOSTAINING AND CONFOCAL MICROSCOPY

The iPSCs colonies and EBs treated with IGFBP4 or PBS were plated on glass coverslip culture chambers coated with 1% gelatin at a low density. At day 15, EBs were fixed in 4% paraformaldehyde at room temperature for 30 min, and then permeabilized with 0.25% Triton X-100 (Sigma-Aldrich). After blocking in 5% bovine serum (Gibco), cells were incubated with primary antibodies: anti-Oct4 antibody (1:200 Boster Biotechnology), anti-SSEA1 antibody (1:200, Millipore), anti-α-actinin antibody (1:300, Sigma-Aldrich), anti-α-MHC antibody (1:200, Abcam), anti-TNNT2 antibody (1:200, Proteintech), and anti-connexin 43 antibody (1:200, Proteintech) at 4°C overnight and then incubated with FITC-conjugated anti-rabbit secondary antibody (1:1,000, Invitrogen) and Cy3-conjugated anti-mouse secondary antibody (1:1,000, Jackson ImmunoResearch). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, 500 ng/ml in distilled water) for 5 min. The positive signals were analyzed under Axio Observer A1 fluorescence microscope (Olympus, Japan) and confocal microscope (Zeiss, Germany).

WESTERN BLOTTING

Cells were lysed in the presence of phosphatase inhibitor cocktail (Pierce). Proteins were separated by 10% (wt/vol) Tris glycine SDS/PAGE (Invitrogen) and transferred to a PVDF membrane (Millipore). After blocking with 5% (wt/vol) milk in Tris-buffered saline with Tween 20, the membrane was incubated with primary antibody overnight at 4°C. The antibodies were used in the study: anti-α-MHC antibody (1:1,000, Abcam), anti-Troponin I antibody (1:1,000, Abcam), anti-α-actinin antibody (1:1,000, Sigma-Aldrich), anti-β-catenin antibody (1:1,000, Abcam), anti-GAPDH antibody (1:5,000, Santa Cruz), anti-α-tubulin antibody (1:5,000, Proteintech), and horseradish peroxidase-conjugated secondary antibody from Santa Cruz Biotechnology at room temperature for 1 h, and developed by an enhanced chemiluminescence immunoblotting detection kit (Tian Gen).

STATISTICAL ANALYSIS

All data were reported as mean ± 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

IGFBP4 WAS INCREASED AT LATE STAGE OF THE DIFFERENTIATION PROCESS OF iPSCs

The mouse iPSCs and EBs were cultured as described in Figure 1A. Grown on MEFs, all the mouse iPSCs showed immunopositive for Oct3/4, SSEA-1, and AKP, markers of pluripotency (Fig. 1B). The iPSCs can differentiate in vitro by forming aggregates, called EBs. After 3 days of hanging drop culture and later placed on gelatin-coated
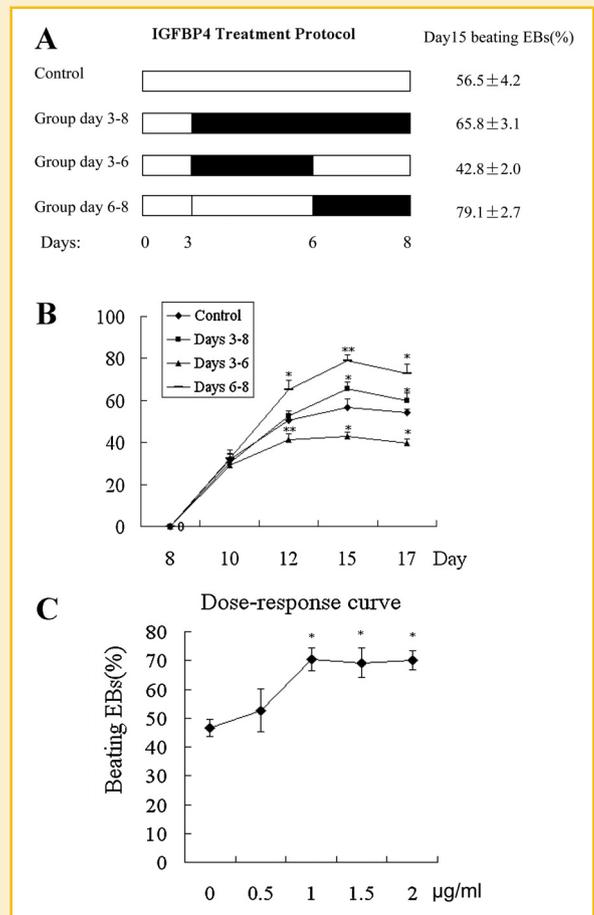

Fig. 2. Exogenous IGFBP4 promotes the frequency of spontaneously beating of EBs. A: Time windows of IGFBP4 treatment during differentiation of mouse iPSCs. IGFBP4 treatments from Day 3 to 8, Day 3 to 6 and Day 6 to 8 were represented by black bars. B: The beating frequency of EBs during the differentiation of iPSCs was analyzed among all the groups. C: Dose–response curve for cardiomyocyte induction by IGFBP4 treatment from day 6 to 8. Beating EBs at day 15 was calculated by IGFBP4 treatment from 0 to 2 μg/ml (results from at least three independent experiments involving over 60 EBs per condition). Values are means ± SEM; *$P < 0.05$, **$P < 0.01$ versus 0 μg/ml. Any experiment was repeated independently at least three times.
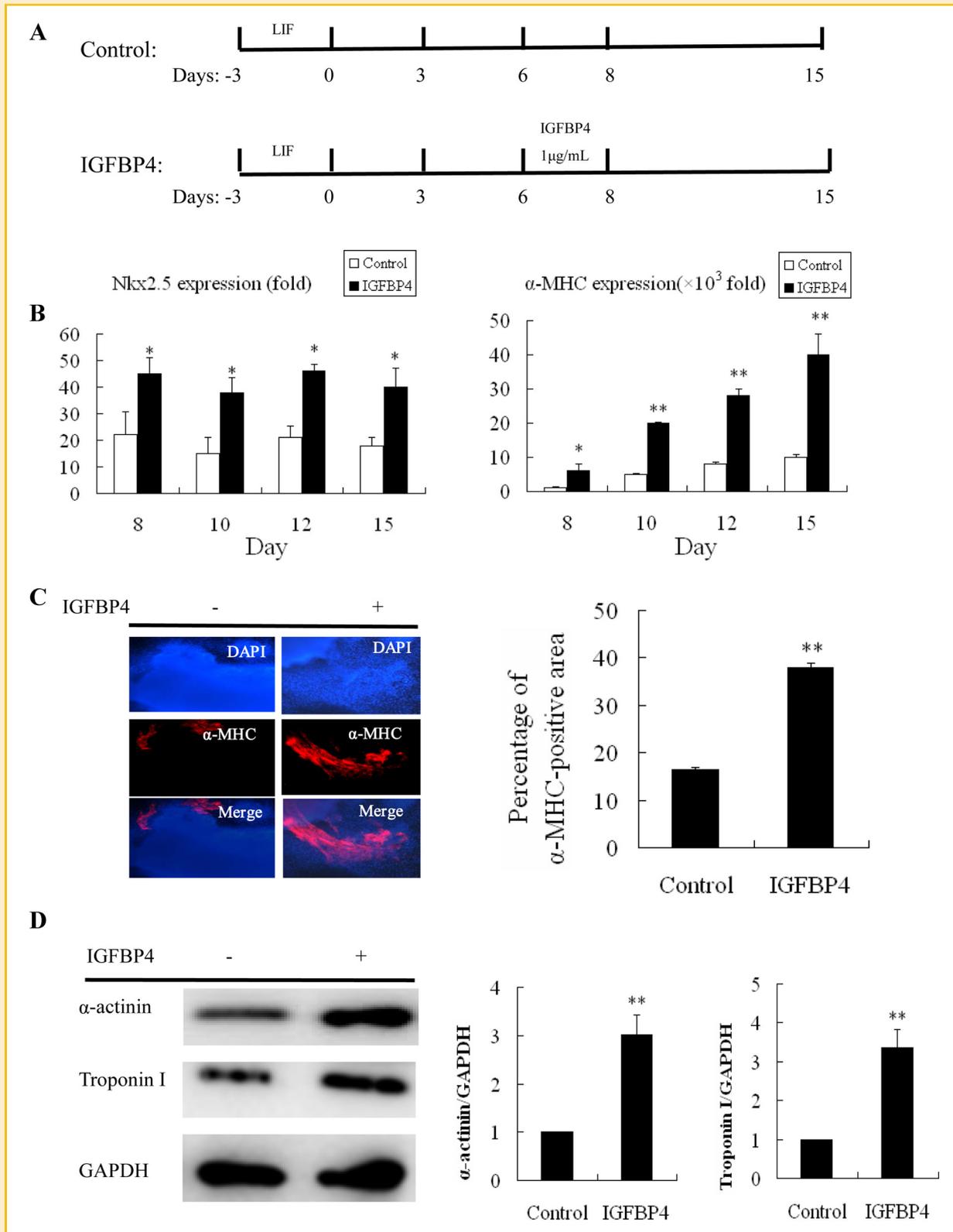
Fig. 3. Exogenous IGFBP4 treatment from day 6 to 8 enhances cardiac–specific genes and proteins expression in EBs. A: Representative schema of protocol for inducing cardiomyogenesis in mouse iPSCs without (Control) or with IGFBP4 treatment (IGFBP4). B: Real–time–PCR analysis of cardiac–specific transcription factor (Nkx2.5) and gene (α–MHC) in EBs during day 8–15 of differentiation. The data were normalized to day 0. Measurements were obtained from at least three independent experiments for each time-point, $*P < 0.05$, $**P < 0.01$ versus control. C: α–MHC positive area at day 15 in EBs was analyzed by immunostaining method. EBs were treated with IGFBP4 during day 6–8 at 1 μg/ml. Left panel: Representative pictures. Right panel: Quantitative analysis. D: Cardiac–specific Troponin I and α–actinin expression levels in EBs on days 15 of differentiation were determined by Western blot analysis. Left panel: Representative pictures. Right panel: Quantitative analysis. The data were normalized to GAPDH, and expressed as a ratio to control. Values are means ± SEM; $*P < 0.05$, $**P < 0.01$ versus control. Any experiment was repeated independently at least three times.

dishes, the number of GFP-Nanog positive cells in EBs was decreased gradually within the EBs differentiation from day 2 to day 10 (Fig. 1C). The EBs exhibited spontaneous contraction from day 10 and the incidence reached the maximum (60%) on day 15 (Fig. 1D).

To study the expression profile of IGFBP4 in the differentiation of mouse iPSCs into cardiomyocytes, total RNAs from cells at days 0, 2, 4, 6, 8, 10, and 12 were collected and extracted. Real-time-PCR analysis revealed that IGFBP4 mRNA level was increased from day 6 to day 8 during the differentiation process of mouse iPSCs (Fig. 1E). It suggests that IGFBP4 may exert its role at late stage of differentiation of iPSCs.

EXOGENOUS IGFBP4 INCREASED THE BEATING FREQUENCY OF EBs FROM iPSCs

Previous study reported IGFBP4 was required at later stage of cardiogenesis [Zhu et al., 2008]. To explore the role of IGFBP4 on the cardiomyocytes differentiation of mouse iPSCs, we treated iPSCs with 1 μg/ml IGFBP4 at day 3–8, day 3–6, and day 6–8, respectively, during differentiation process of iPSCs (Fig. 2A). We observed that Group day 3–8, Group day 3–6, Group day 6–8 and Control group iPSCs all initiated beating activity from day 10 and reached the peak at day 15. The further analysis showed that the incidence of contracting EBs at day 15 was $56.5 \pm 4.2\%$ in control group, $65.8 \pm 3.1\%$ in Group day 3–8, $79.1 \pm 2.7\%$ in Group day 6–8, and $42.8 \pm 2.0\%$ in Group day 3–6, respectively. Group day 6–8 displayed the highest beating frequency from day 12 to day 17 (Fig. 2B). Finally, a dose–response relationship for IGFBP4 induced cardiomyogenesis was determined. We systematically added IGFBP4 from 0.5 to 2 μg/ml as soluble factors to these EBs during day 6–8 of iPSCs differentiation. The results revealed that IGFBP4 greatly increased the incidence of contracting EBs at day 15 compared to control EBs in dose-dependent manner. However, more than 1 μg/ml IGFBP4 failed to further increase the beating frequency of EBs (Fig. 2C). These results indicated that 1 μg/ml IGFBP4 treatment during day 6–8 of iPSCs differentiation was optical condition for promoting cardiomyocytes differentiation of iPSCs. Thus, we performed following experiments in iPSCs treated with IGFBP4 at this condition.

EXOGENOUS IGFBP4 ENHANCED CARDIAC–SPECIFIC TRANSCRIPTION FACTORS AND PROTEINS EXPRESSION IN iPSCS

To confirm IGFBP4 (day 6–8) treatment promotes cardiomyocytes differentiation of mouse iPSCs, we detected the expressions of cardiac-specific transcription factor in EBs during differentiation process of iPSCs (Fig. 3A). The IGFBP4 treatment (day 6–8) of EBs led to huge increases in mRNA expression of cardiac-specific proteins, Nkx2.5 and α-MHC, in comparison to control at day 15, as measured by real-time PCR (Fig. 3B). Moreover, IGFBP4-treated EBs and control EBs were fixed at day 15 and were immunostained with α-MHC, cardiac-specific protein. The α-MHC positive area was increased 2.3-fold in IGFBP4-treated EBs than that in control EBs ($P < 0.05$) (Fig. 3C). Consistent with the immunostaining results, Western blot analysis indicated the expressions of cardiac Troponin I and α-actinin were much higher (3.4- and 3.0-fold) in IGFBP4-treated EBs than that in control EBs at day 15 during differentiation (Fig. 3D). Finally, we examined morphology of cardiomyocytes from iPSCs with or without IGFBP4 treatment by immunofluorescence

analysis. The isolated cells from both group expressed cardiac markers (α-MHC and TNNT2) which displayed typical cardiomyocyte morphology under confocal laser microscopy. Connexin 43 positive signals were observed in the membrane of many cells, which indicated the presence of gap junctions among cardiomyocytes from iPSCs (Fig. 4).

IGFBP4 DOES NOT AFFECT APOPTOSIS BUT IT PROMOTE THE PROLIFERATION OF CARDIOMYOCYTES DERIVED FROM iPSCs

Previous study reported that IGFBP4 promoted apoptosis in colorectal cancer [Durai et al., 2007]. To explore whether IGFBP4 induces preferential apoptosis of non-cardiomyocytes cells which
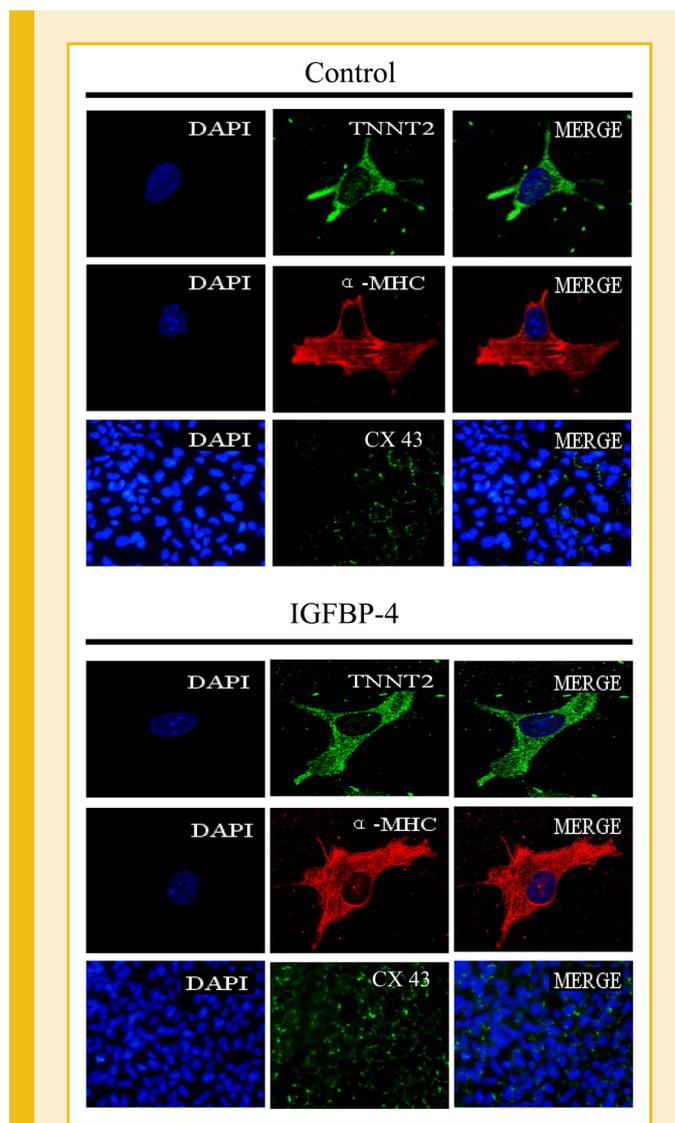

Fig. 4. The sublocalization of cardiac markers in iPSCs–derived cardiomyocytes. iPSCs-derived cardiomyocytes were stained with anti-TNNT2 (green, 400×), anti-α-MHC (red, 400×), anti-connexin 43 (green, 200×); nuclei are stained with 4,6-diamidino-2-phenylindole (DAPI, blue). iPSCs were treated with IGFBP4 (1 μg/ml, day 6–8). CX43: Connexin 43. The cells at day 15 were isolated for immunostaining analysis. Any experiment was repeated independently at least three times.
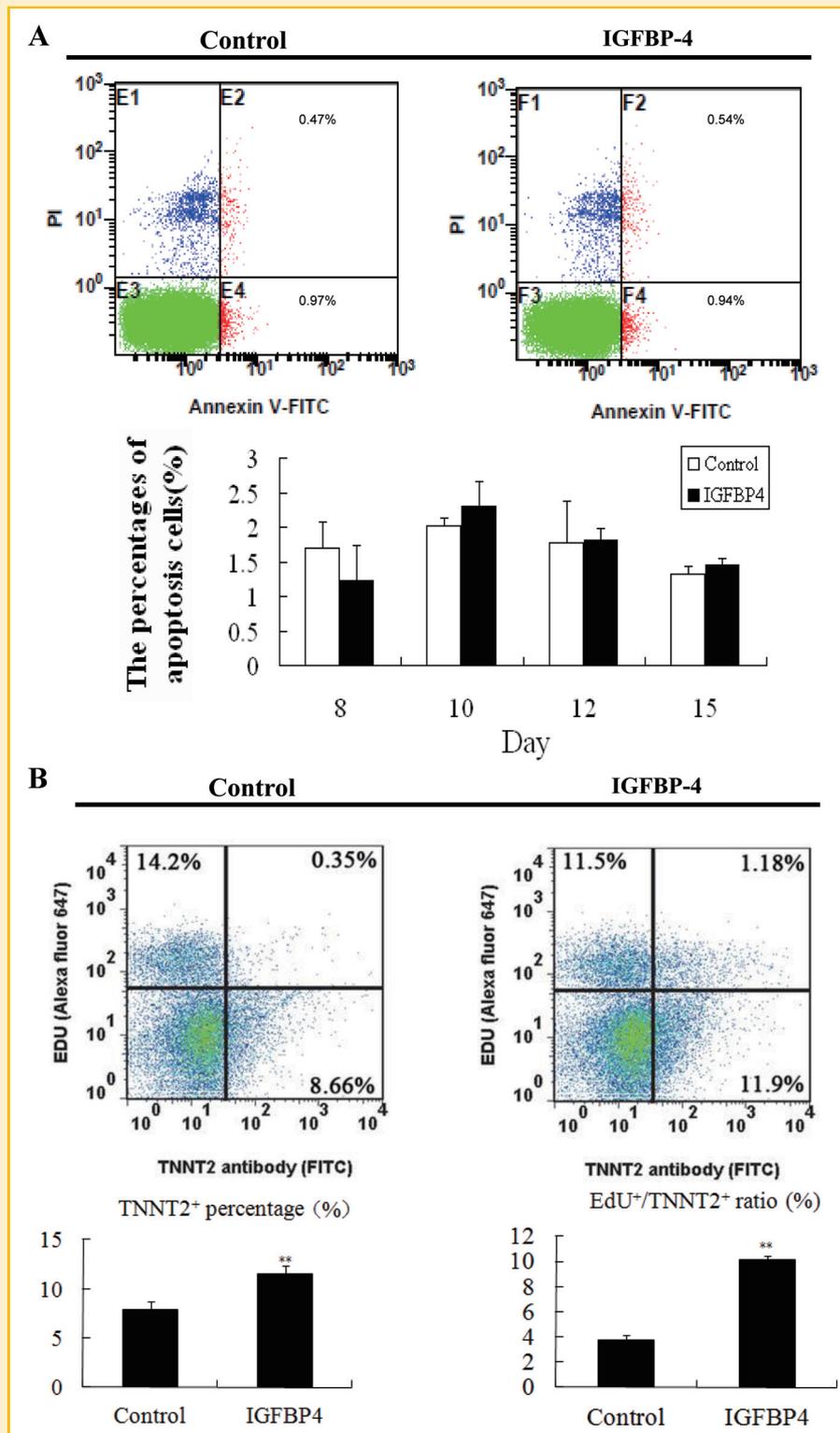
Fig. 5. IGFBP4 treatment does not affect the apoptosis but it promotes the proliferation of cardiomyocytes from mouse iPSCs. A: Apoptosis of EBs was analyzed from day 8 to day 15 by flow cytometry analysis with PI/Annexin V double staining. Up panel: Representative pictures at day 8. Down panel: Quantitative analysis. B: Proliferation of differentiated cardiomyocytes from iPSCs was analyzed by flow cytometry analysis of isolated cells from EBs with TNNT2 and EdU double-staining at day 12. Up panel: Representative pictures. Down panel: Quantitative analysis of the ratio of TNNT2 positive cells and TNNT2/EdU positive cells to TNNT2 positive cells. iPSCs were treated with IGFBP4 (day 6–8) at 1 μg/ml. Values are means ± SEM; *$P < 0.05$, **$P < 0.01$ versus control. Any experiment was repeated independently at least three times.

contributes to the relative enrichment of cardiomyocytes derived from iPSCs. We examined the apoptosis by Annexin V/PI double staining with flow cytometry analysis. However, there is no obvious difference in the ratio of Annexin V positive cells from day 8 to day 15 during the differentiation of iPSCs between control group and IGFBP4 group, suggesting that IGFBP4 has little effect on apoptosis of EBs (Fig. 5A).

We then examined whether IGFBP4 enhances the proliferation of cardiomyocytes derived from iPSCs. FACS analysis indicated that IGFBP4-treated EBs showed a markedly increased the percentage of TNNT2 + /EdU + cells in TNNT2 cells (control: $3.74 \pm 0.43\%$ and IGFBP4: $10.18 \pm 0.27\%$; $P < 0.01$) (Fig. 5B). It suggested that the IGFBP4 promotes cardiogenesis by enhancing the proliferation of cardiomyocytes derived from iPSCs.

IGFBP4 PROMOTES CARDIOMYOCYTES INDUCTION OF iPSCs BY INHIBITING β-CATENIN EXPRESSION

Previous study reported that inhibition of β-catenin protein level can help to enhance the cardiac differentiation of ES [Naito et al., 2006]. To explore the specific mechanism underlying IGFBP4 stimulated the proliferation of cardiomyocytes from iPSCs, we firstly investigated the expression of cytosol β-catenin during the differentiation of iPSCs. β-catenin protein level was increased at day 4 but it was decreased from day 6 (Fig. 6A). Further study showed that IGFBP4 inhibited β-catenin expression in cytosol of EBs at day 8 compared with control group (Fig. 6B). It suggests that IGFBP4-induced-inhibitory effect on β-catenin expression may contribute to the cardiomyocytes-promoting effect of IGFBP4 in iPSCs.

For verifying the above hypothesis, three β-catenin-specific siRNAs were used to knock down the expression of β-catenin of EBs.

Fig. 6. IGFBP4 inhibits β–catenin expression in cytosol of iPSCs. A: The β–catenin protein level in cytosol was measured by Western blotting during the differentiation of mouse iPSCs. B: The expression of β–catenin was analyzed at day 8 during the differentiation of mouse iPSCs with or without IGFBP4 treatment from day 6. Values are means ± SEM; *P < 0.05, **P < 0.01 versus control. Any experiment was repeated independently at least three times.

Two of these siRNAs (si1# and si2#) dramatically decreased the level of β-catenin compared to control (siC) in iPSCs (Fig. 7A). So we chose si1# and si2# for the following analysis. We transfected siRNA-β-catenin to EBs at day 6 during differentiation process and observed that Group β-catenin siC, Group β-catenin si1#, and Group β-catenin si2# all initiated beating activity at day 10 and reached the peak at day 15. The further analysis showed that si1# and si2# both greatly increased the incidence of contracting EBs at day 15 ($55.5 \pm 2.2\%$ in siC group, $78.1 \pm 3.0\%$ in Group si1#, and $85.1 \pm 1.1\%$ in Group si2#; Fig. 7B). Western blot analysis indicated that the expression of α-MHC (cadiomyocyte marker) of contracting EBs was significantly enhanced by siRNA-β-catenin in EBs compared with control (si1# 2.5-fold and si2# 2.6-fold). We then investigated the proliferation of differentiated cardiomyocytes from iPSCs at day 12 by FACS analysis with TNNT2 and EdU double-staining (Fig. 7C). Compared to siC group, siRNA-treated EBs showed a markedly increased percentage of TNNT2-positive cells (siC: $8.14 \pm 0.61\%$, si1#: $12.77 \pm 0.64\%$, si2#: $13.04 \pm 0.45\%$; Fig. 7D,E). Further FACS analysis revealed that either si1# or si2# sharply increased the ratio of $EdU^+/TNNT2^+$ cells to $TNNT2^+$ cells (siC: $4.52 \pm 0.44\%$, si1#: $11.64 \pm 0.54\%$ ($P < 0.01$) and si2#: $12.04 \pm 1.43\%$; Fig. 7D,F). It suggests that IGFBP4 promotes cardiogenesis by enhancing the proliferation of cardiomyocytes derived from iPSCs through inhibiting β-catenin expression.

DISCUSSION

Our findings here extend previous knowledge of IGFBP4 in promoting the cardiac differentiation of P19CL6 cells and ESCs [Zhu et al., 2008; Minato et al., 2012], indicating that IGFBP4 is an ideal cardiomyocytes inducer of mouse iPSCs, and reveal a novel cardiomyocytes-promoting mechanism. The important findings are that (i) IGFBP4 expression was increased at the late stage during differentiation of mouse iPSCs; (ii) Day 6–8 treatment was the most crucial period and 1 μg/ml concentration was suitable dose for IGFBP4 to take effect; (iii) Exogenous IGFBP4 promotes cardiomyogenesis of mouse iPSCs by inducing cardiomyocytes proliferation; and (iv) IGFBP4-induced-inhibitory effect on cytosol β-catenin expression may contribute to the cardiomyocytes-promoting effect of IGFBP4 in iPSCs. Therefore, our findings thus reveal a novel mechanism of IGFBP4 in cardiomyocytes induction of iPSCs, which would provide new insights to explore cardiac regeneration strategies.

The iPSCs display properties of self-renewal and pluripotency similar to ESCs, and yield germ line adult chimeras. This strategy provides an opportunity to generate patient-specific pluripotent stem cells, which could differentiate into cardiomyocytes in vitro. It will enable us to perform cell transplantation therapy for cardiac repair. However, the main hurdle of the exciting cell-based cardiac repair strategy for clinical application is the low generation of cardiomyocytes from the differentiated iPSCs. It is critical to identify the inductive effect of any growth factors to enhance the differentiation of iPSCs into cardiomyocytes. Here, we observed that IGFBP4 promoted cardiomyocytes differentiation of mouse iPSCs. Recently, it has been reported that IGFBP4 enhanced
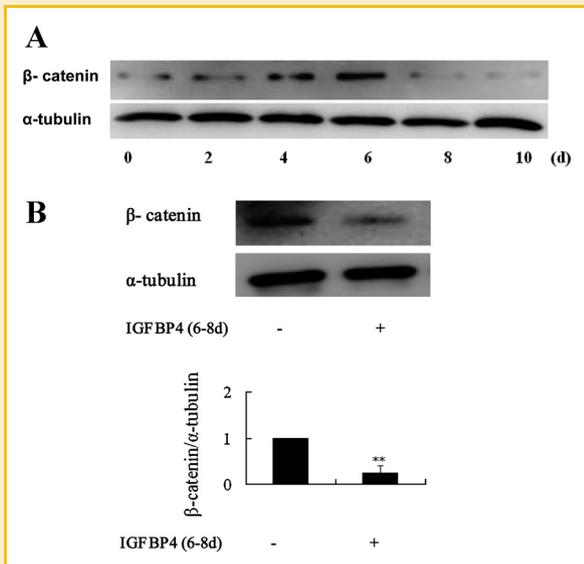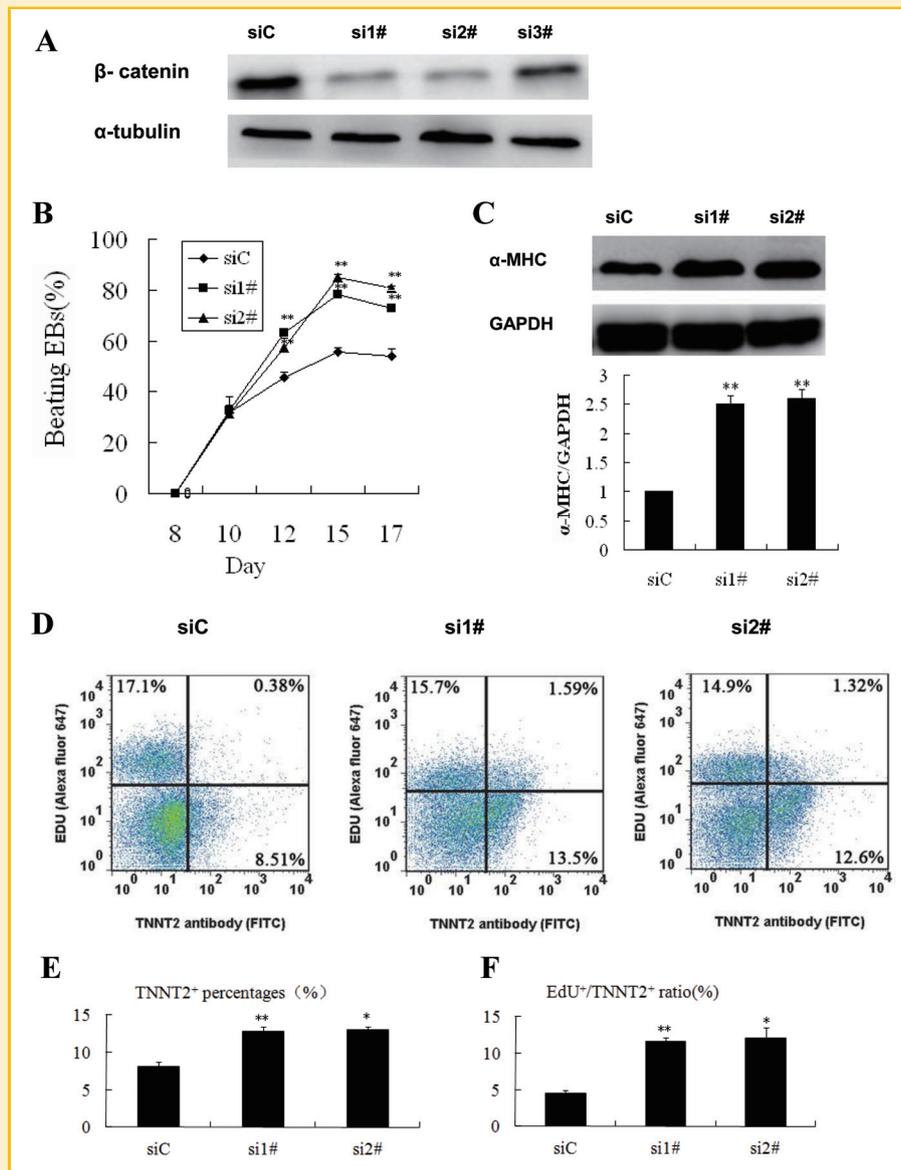
Fig. 7. Effect of β-catenin knockdown on cardiomyocytes induction of iPSCs. A: The knock-down efficiency of β-catenin (si1#, si2#, si3#) was detected by Western blotting. B: The beating frequency of EBs during the differentiation of iPSCs was analyzed among all the groups. C: Cardiac-specific α-MHC expression level in EBs at day 15 during differentiation was determined by Western blot analysis. D: Proliferation of differentiated cardiomyocytes from iPSCs was analyzed by flow cytometry analysis of isolated cells from EBs with TNNT2/EdU double-staining at day 12. E: Quantitative analysis of the percentage of TNNT2 positive cells. F: Quantitative analysis of the percentage of TNNT2/EdU positive cells to TNNT2 positive cells. siRNAs-β-catenin were transfected to EBs at day 6 during differentiation process of iPSCs. Values are means ± SEM; *$P < 0.05$, **$P < 0.01$ versus control. Any experiment was repeated independently at least three times.

cardiogenesis, whereas IGFBP4 gene silence inhibited the induced cardiomyocytes differentiation in P19CL6 cells and ESCs [Minato et al., 2012; Zhu et al., 2008]. However, whether IGFBP4 exerts the same effect in iPSCs is unclear. In the present study, we firstly studied the expression profile of IGFBP4 during the differentiation of iPSCs. The result showed that IGFBP4 was increased from day 6 to day 8 during the differentiation. IGFBP4 treatment during day 6–8 could promote differentiation of iPSC into cardiomyocytes in dose-dependent manner. However, IGFBP4 treatment during day 3–8 did not show obviously cardiogenetic effect while it treatment during day 3–6 displayed significantly inhibitory effect on cardiomyocytes

differentiation of mouse iPSCs. It suggests that IGFBP4 promotes differentiation of iPSCs into cardiomyocytes at late stage during differentiation process of iPSCs.

IGFBP4 has been reported to increase apoptosis via altering the expressions of Bcl-2 and Bax and decrease the angiogenesis in colorectal cancer [Durai et al., 2007]. We suspect whether IGFBP4 induces apoptosis of non-myocytes in EBs which contributes to the higher beating frequency of EBs from iPSCs. But flow-cytometry analysis showed that IGFBP4 had little effect on early and late apoptosis of the differentiated cells from iPSCs during day 8–15. The general morphology or size of EBs with or without IGFBP4 treatment

displayed no significant differences. It suggests that IGFBP4 enhanced the percentage of beating cells by increasing the number of cardiomyocytes, not by promoting apoptosis in non-cardiac lineages. Thus we examined the effect of IGFBP4 on the proliferation of cardiomyocytes derived from iPSCs. IGFBP4-treated EBs showed a markedly increased the percentage of $EdU^+/TNNT2^+$ cells in TNNT2 positive cells, which suggesting that IGFBP4 promotes cardiogenesis by enhancing the proliferation of differentiated cardiomyocytes. The results here may help to explain the phenomenon why the heart is initially formed but its subsequent growth is perturbed in the absence of XIGFBP-4 showed in previous work [Zhu et al., 2008].

In the previous study of Zhu, IGFBP4 has been described as an inhibitor of canonical wnt signaling required for cardiogenesis [Zhu et al., 2008]. Recent findings in ESCs suggest that the activation of canonical Wnt signaling at late stage during ESCs differentiation blocks cardiac induction and differentiation [Naito et al., 2006]. The present study revealed that cytosol β-catenin was decreased at late stage of differentiation of mouse iPSCs, and IGFBP4 greatly inhibited the expression of β-catenin in cytosol of EBs. Some studies reported that inhibition of Wnt/β-catenin signaling enhances the proliferation of ventricular cells during cardiac development [Rottbauer et al., 2002; Dohn and Waxman, 2012]. Our data revealed that knockdown of β-catenin by siRNA at late stage during differentiation process promoted the proliferation of differentiated cardiomyocytes and enhanced cardiomyocyte induction of iPSCs, which was similar to that of IGFBP4 treatment. Thus, IGFBP4 induced-proliferation of cardiomyocytes derived from iPSCs may relate to the inhibitory effect of IGFBP4 on the β-catenin signaling. However, the detailed mechanisms need to be clarified by further study.

In conclusion, we demonstrate IGFBP4 promotes cardiogenesis of mouse iPSCs at late stage by enhancing the proliferation of cardiomyocytes derived from iPSCs. IGFBP4-induced-inhibitory effect on the β-catenin signaling may be involved in the process. Our studies would gain insights into enhancing cardiomyocytes differentiation in iPSCs and may lead to an improvement in the future application of iPSCs in cell therapy for cardiovascular diseases.

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