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The role and mechanism of NRG1/ErbB4 in inducing the differentiation of induced pluripotent stem cells into cardiomyocytes

Xiaoou Li[1†], Heng Zhang[1†], Wenjing Li[1], Hu Tuo[1], Bing He[1*] and Hong Jiang[2*]

Abstract

Background We aimed to investigate the effect and potential mechanism of enhancing Neuregulin1 (NRG1)/v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 4 (ErbB4) expression on the differentiation of induced pluripotent stem cells (iPSCs) into cardiomyocytes.

Methods We utilized CRISPR-CAS9 technology to knock in ErbB4 and obtained a single-cell clone IPSN-AAVS1-CMV-ErbB4 (iPSCs-ErbB4). Subsequently, we induced the differentiation of iPSCs into cardiomyocytes and quantified the number of beating embryoid bodies. Furthermore, quantitative real-time PCR assessed the expression of cardiomyocyte markers, including ANP (atrial natriuretic peptide), Nkx2.5 (NK2 transcription factor related locus 5), and GATA4 (GATA binding protein 4). On the 14th day of differentiation, we observed the α-MHC (α-myosin heavy chain)-positive area using immunofluorescent staining and conducted western blotting to detect the expression of cTnT (cardiac troponin) protein and PI3K/Akt signaling pathway-related proteins. Additionally, we intervened the iPSCs-ErbB4 + NRG1 group with the PI3K/Akt inhibitor LY294002 and observed alterations in the expression of cardiomyocyte differentiation-related genes.

Results The number of beating embryoid bodies increased after promoting the expression of NRG1/ErbB4 compared to the iPSCs control group. Cardiomyocyte markers ANP, Nkx2.5, and GATA4 significantly increased on day 14 of differentiation, and the positive area of α-MHC was three times that of the iPSCs control group. Moreover, there was a marked increase in cTnT protein expression. However, there was no significant difference in cardiomyocyte differentiation between the iPSCs-ErbB4 group and the iPSCs control group. Akt phosphorylation was significantly increased in the iPSCs-ErbB4 + NRG1 group. LY294002 significantly reversed the enhancing effect of NRG1/ErbB4 overexpression on Akt phosphorylation as well as the increase in α-MHC and cTnT expression.

Conclusions In conclusion, promoting the expression of NRG1/ErbB4 induced the differentiation of iPSC into cardiomyocytes, possibly through modulation of the PI3K/Akt signaling pathway.

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Keywords NRG1, ErbB4, iPSC, Cardiomyocyte, Differentiation

Background

Induced pluripotent stem cells (iPSCs) are a type of pluripotent stem cells similar to embryonic stem cells obtained through reprogramming of somatic cells in recent years, bringing unprecedented hope for basic research and clinical disease treatment [1]. In theory, iPSCs can differentiate into any cell of cardiac tissue, avoiding ethical controversies and immune rejection, and becoming the most ideal and abundant cell source in the field of cardiac tissue engineering [2]. However, a large number of studies have found that the efficiency of spontaneous differentiation of iPSCs into cardiomyocytes is very low, and the differentiated cells exhibit significant heterogeneity, thus limiting the clinical application of iPSCs [3, 4]. Therefore, it is necessary to explore an efficient induction strategy to improve the efficiency of directed differentiation of iPSCs into cardiomyocytes.

There are four members in the epidermal growth factor Neuregulins (NRGs) family, namely NRG1, NRG2, NRG3, and NRG4. Among these, NRG1 plays a major role in the heart [5, 6]. The v-erb-b2 avian erythroblastic leukemia viral oncogene homologs (ErbBs) belong to the tyrosine kinase transmembrane receptor family, including four known subtypes: ErbB1, ErbB2, ErbB3, and ErbB4 [7]. The NRG1/ErbBs signaling pathway plays an important role in the development and function of the heart and has attracted much attention in gene therapy for heart disease [8]. NRG1, as an extracellular messenger, binds to the extracellular segments of ErbBs, activating intracellular signaling pathways and regulating cell proliferation, apoptosis, migration, differentiation, and adhesion [8, 9]. In the cardiovascular system, the NRG1/ErbB4 signaling pathway also plays an important role. Firstly, it is involved in the regulation of cardiac growth and development, playing a leading role in the formation and differentiation of cardiomyocytes [10]. Secondly, it increases myocardial contractility and improves heart function [11]. Researchers have attempted to use NRG1/ErbB4 to induce directed differentiation of bone marrow mesenchymal stem cells into cardiomyocytes [12]. Eldridge S et al. found the activation of the NRG1/ErbB4 signaling pathway and downstream signaling networks in the spontaneous differentiation of iPSCs into cardiomyocytes [13]. However, the exact role of NRG1/ErbB4 in the differentiation of iPSCs into cardiomyocytes is not yet clear. PI3K, a vital intracellular protein kinase, is essential for intracellular signal transduction [14]. Its activation triggers the phosphorylation of Ser473 and Thr308 on the downstream Akt protein, thereby initiating the Akt signaling pathway [15]. It has been shown that NRG1/ErbB4 promotes mesenchymal stem cell survival through the activation of the PI3K/Akt pathway [10]. However, limited research has explored the relationship between NRG1/ErbB4 and PI3K/Akt in the differentiation of stem cells into cardiomyocytes.

Here, this study aims to study the potential roles of the NRG1/ErbB4 signaling pathway in the differentiation of iPSCs into cardiomyocytes and to preliminarily explore the underlying mechanisms. Our findings may lay the foundation for subsequent in-depth mechanistic research and in vivo experiments.

Methods

Ethics statement

The study did not include any human or animal experimentation for which ethical approval was required.

Culture and differentiation induction of iPSCs

Human iPSC IPSN0005 derived from umbilical cord mesenchymal stem cells was provided by Shenzhen Sino-Bio Biotechnology Co., Ltd (China). The differentiation induction of iPSCs into cardiomyocytes included the following steps: (1) iPSCs training stage: We plated the iPSCs cell line at a density of (2.5–3.5)×10^4 cells/cm^2, with 3 replicates in each condition. The cells were digested with Accutase (Sigma, St. Louis, MO, USA), counted, and passaged to 12-well culture plates and cultured in mTeSR1 medium (Stemcell, Vancouver, British Columbia, Canada) containing Y27632 (Sigma). Every day, 2 mL of mTeSR1 medium was replaced. (2) Cardiomyocyte differentiation stage (Day 0 - Day 16): On Day 0, the cells were washed once with RPMI1640 (Life, USA) and then incubated with 2 mL of RPMI1640 supplemented with B27 (Life Technologies, Carlsbad, CA, USA) and CHIR99021 (Selleckchem, Houston, TX, USA) but without insulin. The medium replacement time was accurately recorded. On Day 1, precisely 24 h later, the medium was replaced with RPMI1640 containing B27 without insulin, 2 mL per well. On Day 3, 1 mL of the old medium was aspirated from the culture well. The remaining 1 mL of the old medium was discarded. Then, the cells were incubated with 1 mL of the aspirated old medium and 1 mL of the freshly prepared RPMI1640 medium supplemented with B27 and IWP2 (Sigma) but without insulin. On Day 5, the medium was replaced with RPMI1640 medium supplemented with B27 but without insulin, 2 mL per well. On Day 7, the medium was replaced with RPMI1640 medium supplemented with B27, 2 mL per well. On Day 8, the GFP fluorescence in the cells was assessed and recorded. On Day 10, beating cardiomyocytes were visually confirmed, followed by a medium change to RPMI1640 supplemented with B27

(2 mL per well). Subsequently, the medium was changed every two days, 2 mL per well, until Day 16.

Construction of IPSN-AAVS1-CMV-ErbB4

Cell culture

The IPSN0005 iPSCs were seeded onto Matrigel-coated culture plates and maintained in mTeSR1 medium at 37 °C with 5% CO_2. When the cell confluence reached approximately 80%, they were prepared into single cells using Accutase and then subcultured at a density of (2.5–3.5)×10^4 cells/cm^2. During subculturing, 10 μM Y27632 was added for 24 h, followed by its removal, and continuation of culture in mTeSR1 medium.

Gene editing with CRISPR-CAS9 technology

The ErbB4 gene was introduced into the IPSN0005 iPSCs using CRISPR-Cas9 technology. Briefly, single guide RNAs targeting the ErbB4 locus were designed using the online tool CRISPOR (https://crispor.tefor.net/and). The ErbB4 CRISPR/Cas9 system was constructed following a published protocol [16]. The XhoI-CMV-ErbB4-NotI sequence was synthesized by Sangon Biotech (Shanghai, China) and then ligated into the AAVS1 targeting vector (T-AAVS1) to generate the donor plasmids T-AAVS1-CMV-ErbB4. Subsequently, the donor plasmids T-AAVS1-CMV-ErbB4 and ErbB4 CRISPR/Cas9 were co-transfected into iPSCs using the Human Stem Cell Nucleofector® Kit 2 (Lonza). The transfected cells were seeded onto a Matrigel-coated 12-well plate in mTeSR1 supplemented with 10 μM Y27632 and the medium was refreshed daily. On the third day, puromycin (1 μg/mL) was introduced to screen the target cells. To promote the expansion of targeted cells, the cells were incubated with G418 (0.5 mg/mL) in mTeST1 supplemented with 10 μM Y27632 for 24 h. Then, the culture medium was replaced with mTeSR1. Subsequently, the target clone IPSN0005-AAVS1-CMV-ErbB4-26#, which was named iPSCs-ErbB4, was selected using the single-cell cloning method.

Identification of iPSCs-ErbB4

STR analysis

Genomic DNA was extracted from the original cell line IPSN0005 iPSCs and the gene-edited cell line iPSCs-ErbB4. The multiplex PCR amplification system (CELL STR IDTM) was utilized to amplify 20 STR loci and 1 gender locus. Subsequently, the PCR products were analyzed using the ABI 3130xl DNA Analyzer (Applied Biosystems®), and the results were evaluated with GeneMapper ID-X v1.2 software (Applied Biosystems®).

Karyotype analysis

When the confluence of iPSCs-ErbB4 cells reached 30%, they were treated with 100 μg/mL colchicine for 10 min, followed by Karyotype analysis using the G-banding method. At least 20 metaphases were analyzed.

Mycoplasma detection

The culture supernatant of iPSCs-ErbB4 cells was collected for mycoplasma detection using the MycoAlert™ Mycoplasma Detection Kit (Lonza).

Junction PCR

Generally, the junction PCR product spans homologous arms. For the junction PCR of the left arm (Junction L, 1826 bp), the genomic sequence outside the homologous arm was selected as the upstream primer (F2: TCGAC CTACTCTCTTCCGCA) and the integration sequence was used as the downstream primer (R2: GGCTTCAT GGTGGCAGATCT). For the junction PCR of the right arm (Junction R, 1774 bp), the integration sequence was chosen as the upstream primer (F3: CACAGGGTGCT ACTGCTGAG), and the genomic sequence outside the homologous arm was selected as the downstream primer (R3 GGAACGGGGCTCAGTCTG). A junction PCR amplification product size consistent with the theoretical value indicates a positive clone.

Cell culture and treatment

According to different treatments, the cells were divided into the iPSCs control, iPSCs-ErbB4, iPSCs+NRG1, and iPSCs-ErbB4+NRG1 groups. In the iPSCs control and iPSCs-ErbB4 groups, iPSCs and iPSCs-ErbB4 were induced for 14 days for cardiomyocyte differentiation. In the iPSCs+NRG1 and iPSCs-ErbB4+NRG1 groups, NRG1 (20 ng/ml; Sigma) was added from day 0 induction until day 14. The percentage of beating embryoid bodies (EBs) (1×10^3 cells/EBs) was quantified at different time points during differentiation. Additionally, the PI3K/Akt inhibitor LY294002 was given from day 0 to day 6 during the differentiation of iPSCs.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using the Ultrapure RNA total RNA extraction kit (CW0580S, CWBIO, Beijing, China). Subsequently, 1 μg of RNA was reverse transcribed into cDNA using the HiScript II Q RT SuperMix for qPCR (+gDNA wiper) (R223-01, Vazyme). The qRT-PCR reaction was performed using 2×SYBR Green PCR Master Mix (A4004M, Lifeint). GAPDH served as the reference gene. The PCR system consisted of upstream and downstream primers (0.8 μL each), cDNA (2.0 μL), and ddH2O (6.4 μL). The PCR procedures were 94 °C for 15 s, 55 °C for 15 s, and 68 °C for 30 s, with a total of 45 cycles. The primer sequences are presented in Table 1. The relative expression of mRNAs was determined after normalization with reference gene using the $2^{-\Delta\Delta Ct}$ method.

Table 1 The primer sequences

Gene	Forward 5′-3′	Reverse 5′-3′
ErbB4 (iPSCs)	GTCCAGCCCAGCGATTCTC	AGAGCCACTAACACGTAGCCT
ErbB4 (mouse)	AGCCCGCAATGTCTTAGTGAA	GTTCCCATATAGTGACGCCAT
cTnT	GGAGGAGTCCAAACCAAAGCC	TCAAAGTCCACTCTCTCTCCATC
ANP	TGACAGGATTGGAGCCCAGAG	AGCTGCGTGACACACCACAAG
Nkx2.5	AAGTGCTCTCCTGCTTTCCCAG	TTGTCCAGCTCCACTGCCTTC
GATA4	CTCCAGCGGTAACTCCAGCAA	AGCAGACAGCACTGGATGGATG
GAPDH	TGTGTCCGTCGTGGATCT	TTGCTGTTGAAGTCGCAG

Note ErbB4, v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 4; cTnT, cardiac troponin; ANP, atrial natriuretic peptide; Nkx2.5, NK2 transcription factor related locus 5; GATA4, GATA binding protein 4

Western blot

After lysing the cells, proteins were extracted, followed by electrophoresis with 12% SDS-PAGE. Subsequently, the proteins were transferred onto a PVDF membrane with a pore size of 0.2 μm. Following washing, the membrane was blocked with BSA for 1 h and then incubated overnight at 4 °C with the primary antibodies of rabbit polyclonal anti-cardiac troponin (cTnT) antibody (1:500, Abcam, Cambridge, MA, USA), rabbit polyclonal anti-Sox2 antibody (1:500, Proteintech, Rosemont, IL, USA), rabbit anti-Nanog antibody (1:500, Proteinteh), rabbit anti-ErbB4 antibody (1:500, Abcam), and rabbit anti-phosphorylated AKT antibody (1:500, Proteintech). On the next day, the *s*econdary HRP-labeled goat anti-rabbit antibody (1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was added and incubated at room temperature for 1 h. Chemiluminescent detection using ECL was then performed. The grey value was analyzed using Quantity One software, with GAPDH serving as the internal reference.

Immunofluorescence staining

After induction for 14 days, the differentiated cells were fixed with 2 mL of 4% paraformaldehyde for 30 min at room temperature. After fixation, the cells were washed three times with a pre-warmed PBS solution. Subsequently, permeabilization was carried out using 0.3% Triton X-100 for 10 min, followed by another three washes with pre-warmed PBS solution. Afterward, 2 mL of 5% BSA was added for incubation at room temperature for 30 min. Following incubation, the diluted primary antibodies (Abcam, USA) were added and incubated at 4 °C in a humid chamber. The following primary antibodies were used: mouse anti-α-myosin heavy chain (α-MHC) antibody (1:200; Abcam), rabbit anti-octamer-binding transcription factor-4 (OCT4) (1:200; Abcam), and rabbit polyclonal anti-stage-specific embryonic antigen-1 (SSEA-1) antibody (1:200; Abcam). The next day, the cells were incubated with FITC-conjugated anti-rabbit secondary antibody (1:1000, Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature, followed by incubation with DAPI (Sigma-Aldrich) for 5 min. Subsequently, the cells were observed under an Axio Observer A1 fluorescence microscope (Zeiss, Munich, Germany) and the positive area of α-MHC was analyzed by ImageJ software.

Statistical analysis

Statistical analysis was performed using SPSS 11.0 software. All analyses were performed by using JMP 7 (SAS Institute. Inc.) and GraphPad Prism 7 (GraphPad Software. Inc.). All counting data are expressed as mean ± S.E.M. A paired t-test was used for within-group comparisons when variances were homogeneous, and ANONA was used for between-group comparisons. For comparisons of three or more experimental conditions, repeated-measures ANOVA was performed followed by a Tukey's post hoc test. All experiments were repeated at least three times. A P-value less than 0.05 indicates statistically significant differences.

Results

Construction of iPSCs-ErbB4

The process of inducing stem cell differentiation into cardiomyocytes in vitro and the expression changes in cardiac-specific genes and proteins mimic those in embryonic development [17]. In this study, we utilized qRT-PCR to measure *ErbB4* mRNA expression during heart development. The results revealed a substantial increase in *ErbB4* mRNA expression in the mature heart compared to the embryonic stage (supplementary Figure S1). Subsequently, we evaluated the expression of the cardiomyocyte marker *cTnT* mRNA during the 14-day induction of iPSCs into cardiomyocytes. We found that there was a sustained enhancement of *cTnT* mRNA expression throughout the cardiomyocyte differentiation process (Fig. 1A). Meanwhile, we dynamically monitored *ErbB4* mRNA expression, which exhibited a significant increase on the 7th day of iPSC differentiation and was about 20 times higher on the 14th day compared to the undifferentiated state (Fig. 1A). These results not only confirm the successful differentiation of iPSCs into cardiomyocytes but also strongly imply that ErbB4 may play a role in iPSC cardiomyocyte differentiation.

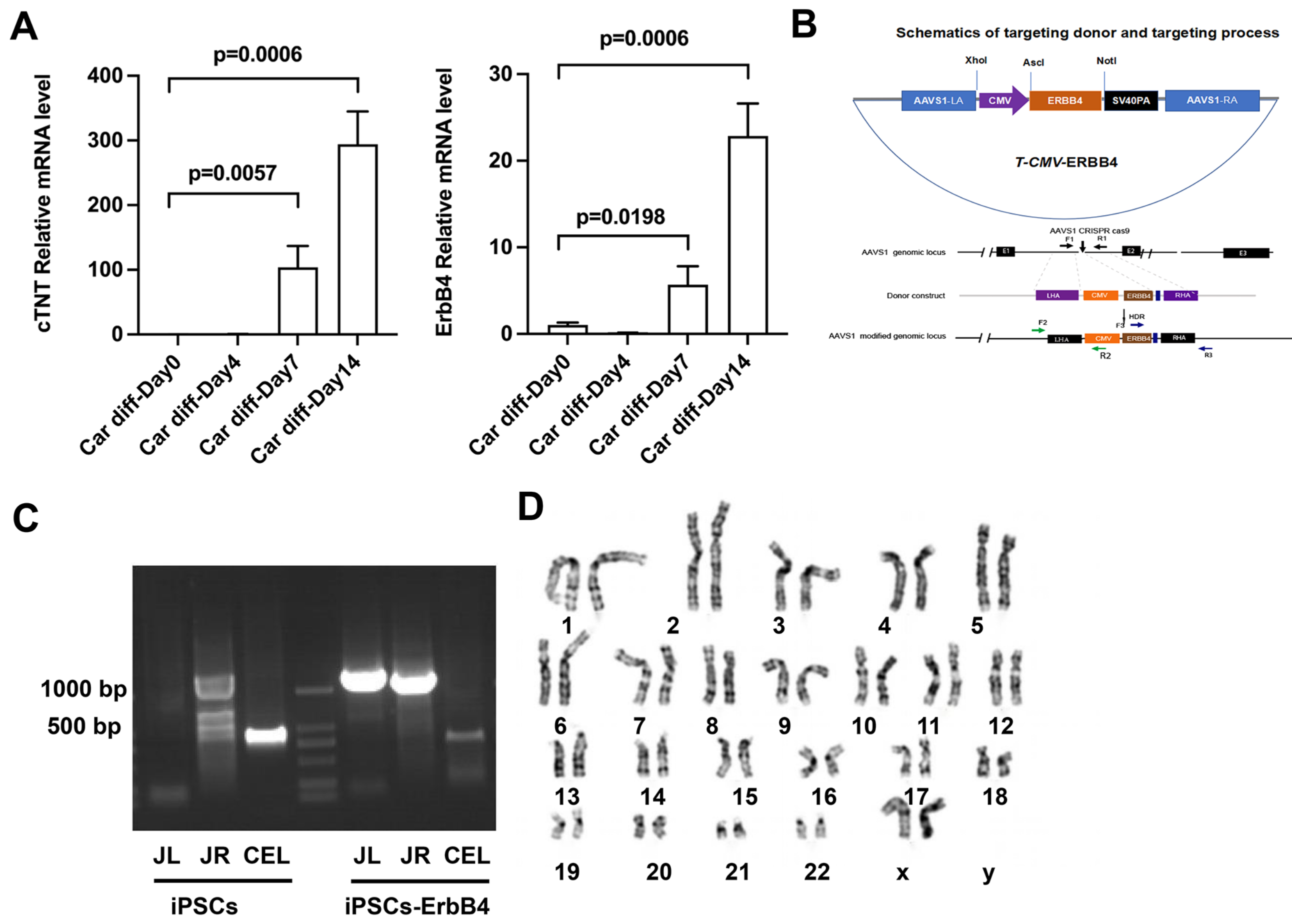

Fig. 1 The expression of *ErbB4* in iPSCs and the construction of the target genotype cell line iPSCs-ErbB4. (**A**) Analysis of *cTnT* mRNA and *ErbB4* mRNA by qRT-PCR. (**B**) The construction of IPSN-AAVS1-CMV-ErbB4 (iPSCs-ErbB4). (**C**) The Junction PCR sequencing results. (**D**) The results of Karyotype analysis

In this study, we knocked in the ErbB4 gene at the AAVS1 site in iPSCs by using CRISPR-CAS9 technology (Fig. 1B). We obtained a single-cell clone, IPSN0005-AAVS1-CMV-ErbB4-26# (iPSCs-ErbB4). The junction PCR sequencing of the clone genome showed that the product size (left arm 1826 bp and right arm 1774 bp) was consistent with the theoretical value (Fig. 1C), confirming the targeted gene integration and ensuring the correctness of the genotype. Karyotype analysis showed a result of 46, XX (Fig. 1D), indicating a normal karyotype. STR analysis demonstrated complete consistency between the gene-edited iPSCs single clone cell line and the donor cells at 20 STR loci and one gender locus (supplementary Table S1). The cells were free from bacterial or fungal contamination, and the mycoplasma test result was negative (supplementary Table S2). These results collectively demonstrate the genomic stability, authenticity, and absence of mycoplasma contamination in iPSCs-ErbB4.

Analysis of the pluripotency of iPSCs-ErbB4

Western blot results showed that compared to the iPSCs control group, the expression level of ErbB4 protein in the iPSCs-ErbB4 group significantly increased (Fig. 2A), indicating abundant expression of ErbB4. Next, we detected the pluripotency of the iPSCs-ErbB4 cell line by using Western blot. The results revealed that the expression levels of the pluripotency marker proteins Sox2 and Nanog showed no significant difference between the iPSCs-ErbB4 and the iPSCs control groups (Fig. 2B). Further immunofluorescence staining revealed consistent expression of OCT4 and SSEA-4 in the iPSCs-ErbB4 group and the iPSCs control group (Fig. 2C). The above results indicate that the ErbB4 gene overexpression did not affect the pluripotency of iPSCs.

Effects of the NRG1/ErbB4 signaling pathway on the differentiation of iPSCs into cardiomyocytes

Numerous studies [10, 11] support the crucial role of the NRG1/ErbB4 signaling pathway in the development, maintenance, and repair of the cardiovascular system. Consequently, NRG1 was introduced during the

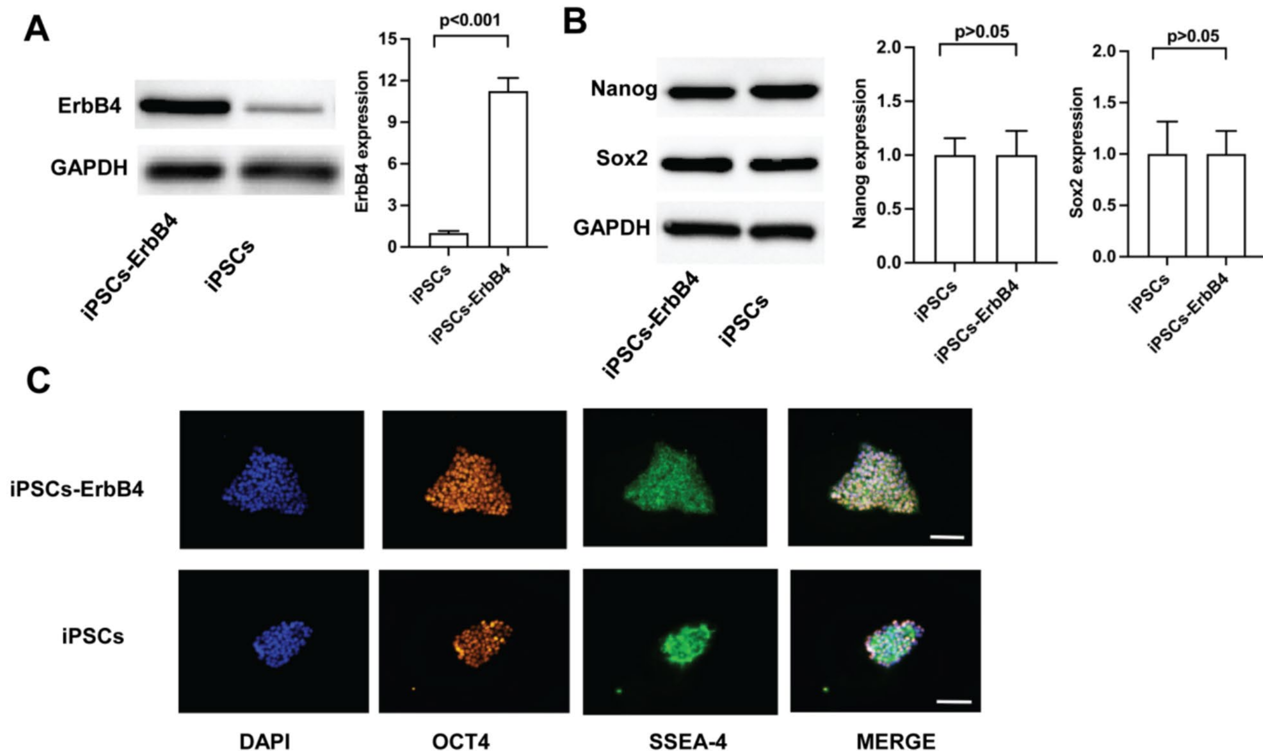

Fig. 2 Identification of target genotypic cell lines. (**A**) Western blot results of ErbB4 protein expression. (**B**) Western blot results of Sox2 and Nanog protein expression. (**C**) Immunofluorescence staining analysis of OCT4 and SSEA-4

differentiation in this study. To evaluate the effect of the NRG1/ErbB4 signaling pathway on iPSCs cardiomyocyte differentiation, we initially observed the EBs in each group under a microscope daily and quantified the number of beating EBs. Beatings in the iPSCs control group appeared by the 7th day and reached approximately 80% by the 14th day (Fig. 3A). Cell clusters with cardiac-like beating were observed in the iPSCs-ErbB4+NRG1 group on the 6th day, which rapidly increased, and by the 13th day, beating EBs accounted for over 95%, significantly higher than those in the iPSCs control group. The number and appearance time of beating EBs in the iPSCs-ErbB4 group increased compared to the iPSCs control group but without a significant difference. Moreover, the iPSCs+NRG1 group did not exhibit a significant difference compared to the iPSCs control group.

Next, the expression of various cardiomyocyte markers was tested with qRT-PCR. The results indicated that during the differentiation of iPSCs into cardiomyocytes, the expression of atrial natriuretic peptide (ANP), NK2 transcription factor-related locus 5 (Nkx2.5), and GATA4 was significantly increased in the iPSCs-ErbB4+NRG1 group compared to the iPSCs control group (Fig. 3B).

On the 14th day of cardiomyocyte differentiation, the expression of mature cardiomyocyte markers was examined with Western blot analysis. As shown in Fig. 3C, there was a significant increase in cTnT expression in the iPSCs-ErbB4+NRG1 group than in the iPSCs control group. Immunofluorescence staining revealed the presence of α-MHC-positive cells in all groups, with α-MHC localized in the cytoplasm emitting green fluorescence under the microscope (Fig. 3D). The number of α-MHC-positive cells in the iPSCs-ErbB4+NRG1 group was significantly higher, approximately three times that of the iPSCs control group (Fig. 3D). However, the fluorescence intensity of α-MHC in the iPSCs+NRG1 group showed no significant change compared to the iPSCs control group. These results suggest that the NRG1/ErbB4 signaling pathway promotes the cardiomyocyte differentiation of iPSCs.

Mechanism of NRG1/ErbB4 signaling pathway in promoting iPSC cardiomyocyte differentiation

To determine the mechanism of NRG1/ErbB4 signaling pathway in promoting iPSC cardiomyocyte differentiation, we detected the expression of various PI3K/Akt signaling pathway-related proteins on the 14th day of differentiation. Western blot showed that the Akt phosphorylation was significantly increased in the iPSCs-ErbB4+NRG1 group (Fig. 4A). This indicates that the PI3K/Akt signaling pathway may be involved in the

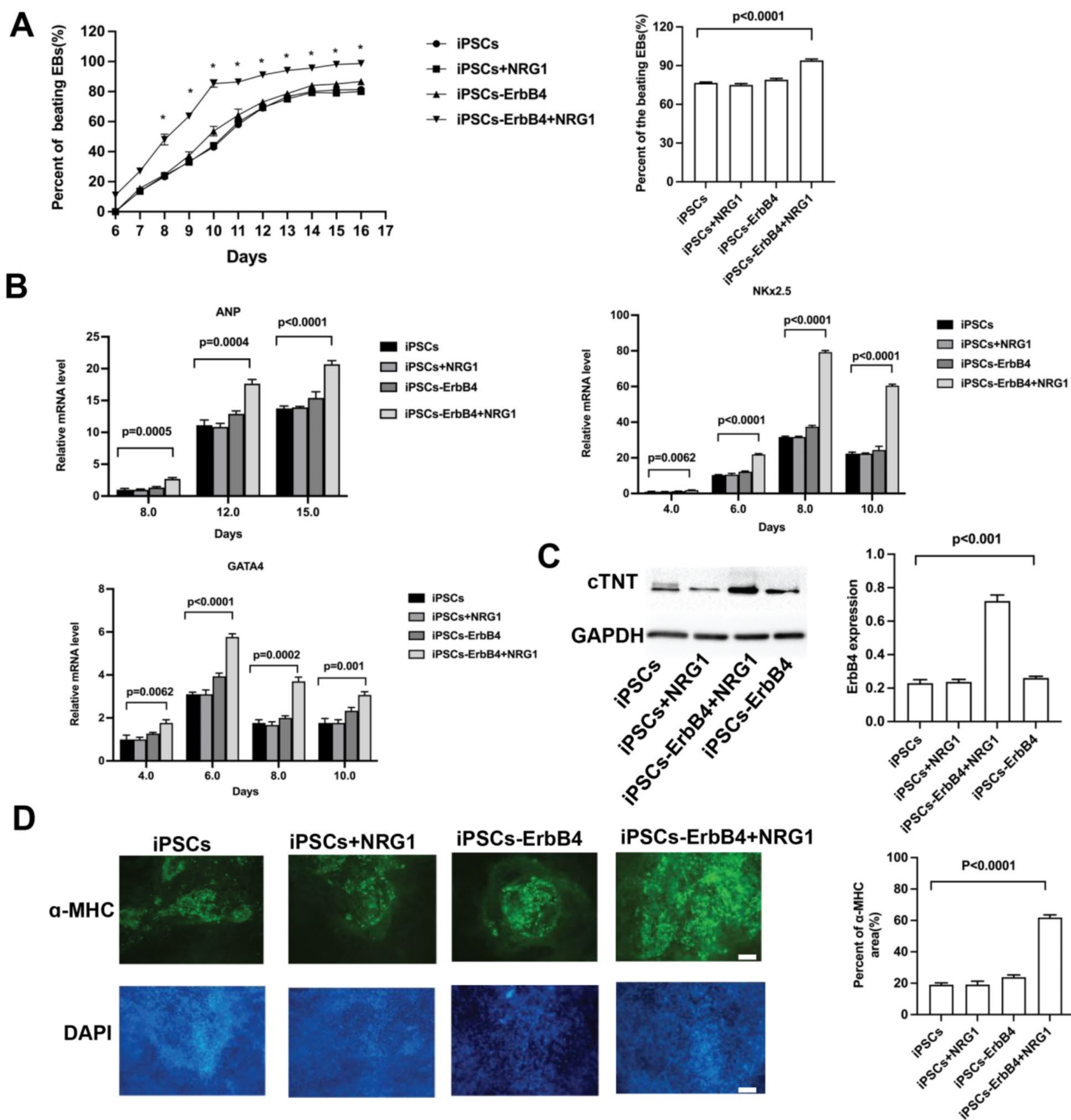

Fig. 3 Effect of NRG1/ErbB4 on myocardial differentiation of iPSC. (**A**) The percentage of beating EBs (1×10^3 cells/EBs) was quantified at different time points during differentiation. Values are means ± S.E.M; **p* < 0.05. (**B**) The mRNA expression of cardiomyocyte markers (ANP, Nkx2.5, and GATA4 during the cardiomyocyte differentiation of iPSCs was detected by qRT-PCR. (**C**) The expression of cTnT on day 14 of differentiation was measured by Western blot. Each column was normalized by GAPDH. (**D**) Immunofluorescent staining of α-MHC from each group on day 14 of differentiation. Left panels: the gross appearance of cardiomyocytes stained with α-MHC (green). Nuclei were stained with DAPI (blue). Scale bars = 100 μm. Right panel: quantitative evaluation of α-MHC positive area. Results are presented relative to the value obtained in iPSCs-ErbB4 + NRG1-control iPSCs

directed differentiation of iPSC into cardiomyocytes mediated by NRG1/ErbB4.

Subsequently, the PI3K/Akt inhibitor LY294002 was introduced during iPSC cardiomyocyte differentiation. We observed by Western blot that pretreatment with LY294002 significantly inhibited the enhancing effect of NRG1/ErbB4 overexpression on Akt phosphorylation (Fig. 4B) as well as the increase in α-MHC (Fig. 4C) and cTnT expression (Fig. 4D). These findings suggest that the positive regulatory effect of NRG1/ErbB4 during iPSC differentiation may be achieved through regulating the PI3K/Akt signaling pathway.

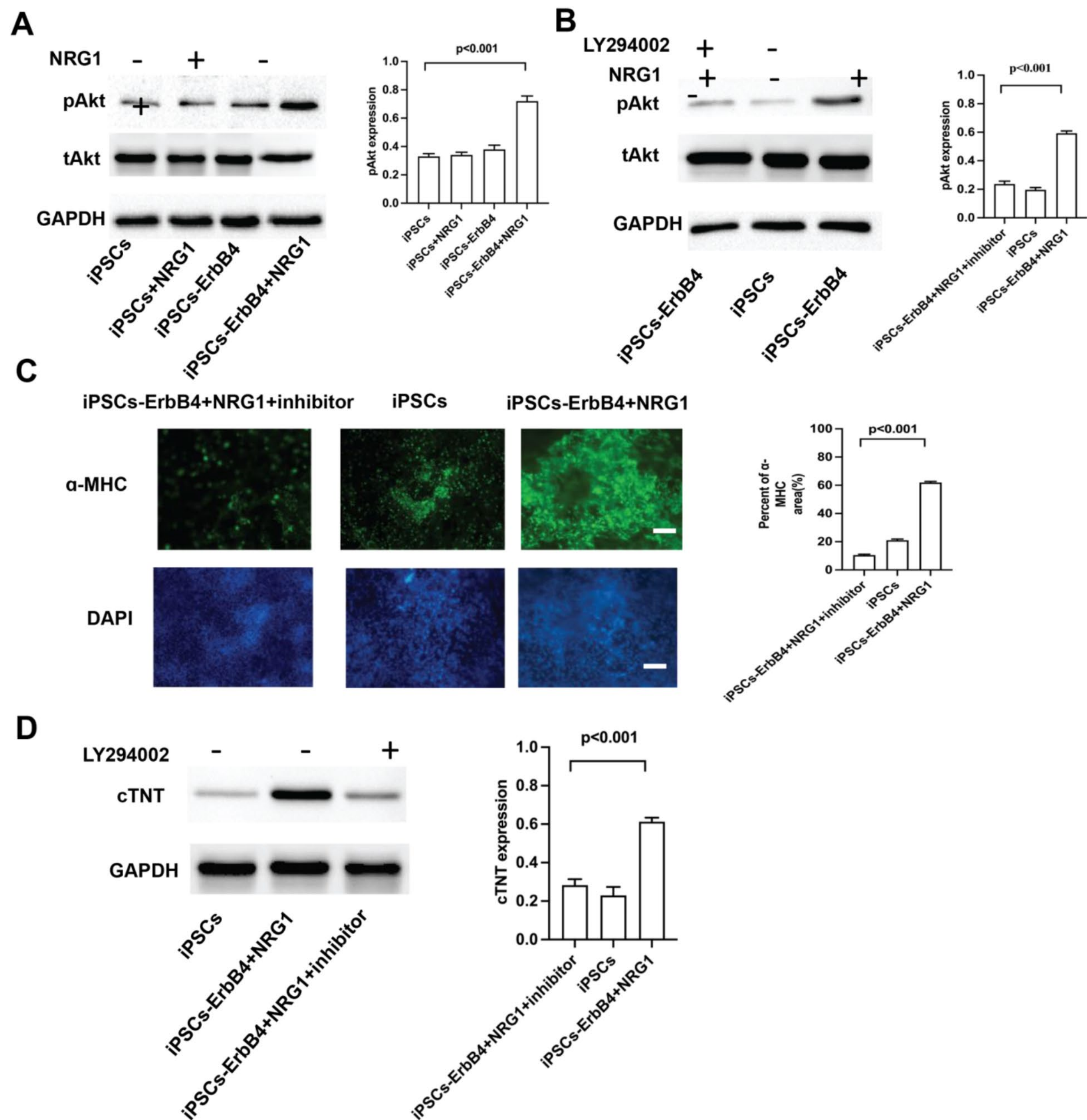

Fig. 4 NRG1/ErbB4 promotes cardiomyocyte differentiation of iPSCs by PI3K/Akt. (**A**) The expression of the total and phosphorylated Akt in each group by Western blot analysis on the 14th day of cardiomyocyte differentiation. (**B**) The expression of the total and phosphorylated Akt in each group pretreated with LY294002 by Western blot analysis on the 14th day of cardiomyocyte differentiation. (**C**) Immunofluorescent staining of α-MHC in EBs on day 14 of differentiation. Left panels: gross appearance of cardiomyocytes stained with antibody to α-MHC (green). Nuclei were stained with DAPI (blue). Scale bars = 100 μm. Right panel: quantitative evaluation of α-MHC positive area. (**D**) Western blot analysis of cTnT in each group. Each column was normalized by GAPDH

Discussion

This study was based on the relationship between iPSCs-derived cardiomyocyte differentiation and the NRG1/ErbB4 signaling pathway. We found that NRG1/ErbB4 promoted the gene expression of stage-specific cardiomyocyte differentiation markers, significantly increased the percentage of beating EBs, and enhanced the expression of mature cardiomyocyte markers. Furthermore, through mechanism analysis, we demonstrated that NRG1/ErbB4 may promote directed cardiomyocyte differentiation and improve the efficiency of cardiomyocyte differentiation by activating the PI3K/Akt pathway.

iPSCs, obtained through somatic cell reprogramming in recent years, are similar to embryonic stem cells and offer considerable promise for basic research and clinical disease treatment [1]. Recent studies have successfully demonstrated the differentiation of iPSCs into cardiomyocytes in mice and humans, providing opportunities for regenerative heart disease treatment and significant breakthroughs [3, 18]. However, the low efficiency of differentiation and the difference from normal cardiomyocytes hinder the clinical utility of iPSCs. Therefore, it is of great importance to enhance the efficiency and maturity of iPSCs' differentiation into cardiomyocytes. One recent study in cardiac pathology has revealed a close association between the abnormal expression and distribution of NRGs/ErbBs and cardiac development, particularly concerning NRG1/ErbB4 [19]. In our study, we focused on the effect of NRG1/ErbB4 on the differentiation of iPSCs into cardiomyocytes. We found that ErbB4 expression was rapidly upregulated during the cardiomyocyte differentiation of iPSCs, suggesting a potentially close association between the NRG1/ErbB4 signaling pathway and cardiomyocyte differentiation.

NRGs have been shown to bind to ErbB receptors, activating ErbB receptor tyrosine kinases and triggering a broad range of biological effects [20, 21]. Deletion of the NRG1 or ErbB4 gene during embryonic heart development in mice results in severe embryonic lethality [22]. Furthermore, the absence of ErbB4 during the embryonic period leads to atrial septal defects [22]. During ventricular development, NRG1 stimulates the proliferation of embryonic cardiomyocytes and the synthesis of the extracellular matrix, thereby promoting the development of myocardial trabeculae [23]. Targeted deletion of the ErbB4 gene in mouse ventricular myocytes leads to severe dilated cardiomyopathy in the second month after birth [24]. The overexpression of ErbB4 in bone marrow mesenchymal stem cells had no significant effect on the migration, apoptosis, and proliferation of induced cardiomyocytes [10]. In contrast, the introduction of NRG1 in the overexpression group markedly enhanced cell migration, promoted proliferation, and suppressed apoptosis [10]. The ErbB signaling pathway can be activated in human iPSC-derived cardiomyocytes by NRG1, indicating a crucial role of NRG1/ErbB4 signaling during the differentiation of iPSCs into cardiomyocytes [13]. However, the role of the NRG1/ErbB4 signaling pathway in the process of iPSC cardiac differentiation remains unclear. To further elucidate this, we established the cell line IPSN0005-AAVS1-CMV-ErbB4 (iPSCs-ErbB4) by using CRISPR-CAS9 technology, which is an efficient and precise strategy for target gene modification without compromising the pluripotency of iPSCs [16]. Subsequently, we evaluated the cardiomyogenic differentiation potential of this cell line in vitro. The results showed that activation of ErbB4 with NRG1 significantly enhanced the efficiency of iPSCs differentiation into cardiomyocytes as evidenced by an increased proportion of beating EBs and the upregulated expression of cTNT and α-MHC. Subsequent analysis revealed that NRG1/ErbB4 notably enhanced the expression of crucial cardiac transcription factors and specific markers such as ANP, Nkx2.5, and GATA4 mRNA, indicating that cardiomyocytes induced by NRG1/ErbB4 exhibit a high degree of differentiation and functionality. However, neither NRG1 nor ErbB4 affected the cardiomyocyte differentiation of iPSCs. These findings confirm that NRG1/ErbB4 significantly promotes iPSCs differentiation into functional cardiomyocytes. Nonetheless, the precise mechanism through which this signaling pathway regulates iPSC myocardial differentiation remains incompletely understood.

PI3K, an intracellular protein kinase, can stimulate cellular self-renewal. Akt, a serine/threonine protein kinase, mediates cell growth, proliferation, migration, differentiation, and the contractile force of cardiomyocytes through self-phosphorylation [25, 26]. Multiple studies have demonstrated the significant role of the PI3K/Akt signaling pathway in directing the differentiation of embryonic and adult stem cells into cardiomyocytes and in maintaining cell survival [27, 28]. Inhibition of the PI3K/Akt signaling pathway markedly suppresses cardiomyocyte differentiation [29]. Furthermore, the adipose mesenchymal stem cell cardiomyogenesis induced by 5-aza has been reported to be closely associated with the PI3K/Akt pathway and the adipose mesenchymal stem cell differentiation can be enhanced by activating the signaling proteins in this pathway [30]. It has been demonstrated that NRG1/ErbB4 signaling pathway, which is involved in cardiomyocyte differentiation, can activate PI3K/Akt [31, 32]. However, whether NRG1/ErbB4 signaling also regulates cardiomyogenesis through the PI3K-Akt pathway remains unclear. Here, we utilized western blotting to investigate the impact of the NRG1/ErbB4 signaling pathway on the downstream Akt activity of the PI3K/Akt pathway. The findings revealed a significant increase in phosphorylated AKT levels in the iPSCs-ErbB4+NRG1 group compared to the iPSCs control group. Pretreatment with the PI3K-specific inhibitor LY294002 markedly suppressed the NRG1/ErbB4 signaling pathway-induced elevation of phosphorylated AKT levels, suggesting a potential partial activation of the PI3K/Akt signaling pathway by the NRG1/ErbB4 signaling pathway. Additionally, inhibition of the PI3K/Akt signaling pathway with LY294002 hindered the promotion of iPSC cardiomyocyte differentiation by the NRG1/ErbB4 signaling pathway. These results suggest that the NRG1/ErbB4 signaling pathway may enhance the expression of cardiomyocyte-specific genes and facilitate the differentiation of iPSCs into cardiomyocytes by activating the PI3K/

Akt signaling pathway. The PI3K/AKT pathway performs multiple functions, such as inducing cardiomyocyte proliferation and differentiation from iPSCs, through the interaction with a complex network of downstream target proteins [33]. One of the primary substrates of AKT is mTOR (mammalian target of rapamycin), which plays a crucial role in regulating cardiac hypertrophy, contractility, and overall cardiac function. Research on mTOR has indicated that transient inhibition of the mTOR-signaling pathway can induce iPSC-derived cardiomyocytes to enter a quiescent state and enhance their maturation [34]. Additionally, GSK-3β (Glycogen synthase kinase-3β) is another vital downstream target involved in regulating cardiac tissue formation and cardiogenesis. Buikema et al. demonstrated a substantial expansion of hiPSC-cardiomyocytes in vitro (e.g., 100- to 250-fold) through GSK-3β inhibition [35]. However, there is currently a lack of relevant research on the mechanism through which the NRG1/ErbB4 signaling pathway activates PI3K/Akt during cardiomyocyte differentiation or the downstream targets, necessitating further exploration.

In addition to the PI3K/Akt pathway, multiple other pathways downstream of NRG1/ErbB4 have been identified. One such pathway is the RAF-MEK-ERK signaling cascade, which plays a critical role in cell proliferation and survival [36]. It has been demonstrated that in cardiomyocytes, the binding of NRG1 to ErbB4 leads to the activation of AKT and ERK (an important member of the RAF-MEK-ERK signaling cascade), thereby promoting cell growth and survival [37]. Moreover, Eldridge et al. [13] reported that NRG1-ErbB4 activation induced the downstream signaling proteins AKT and ERK1/2 in iPSCs. Importantly, the Ras/Raf/MEK/ERK and Ras/PI3K/Akt pathways interact with each other to regulate cell growth, apoptosis, and cell cycle [38]. However, the relationship between NRG1/ErbB4 and Ras/Raf/MEK/ERK pathways in the differentiation of iPSCs into cardiomyocytes warrants further investigation.

There are some limitations in this study. First, we only used the IPSN0005 iPSC line as a control, potentially limiting the generalizability of the results. Future studies should include multiple iPSC lines as controls for a more comprehensive assessment. Second, functional assessments of differentiated cardiomyocytes were not conducted in the current study, highlighting the need for contractility assays and calcium handling assays to evaluate cardiomyocyte functionality in future investigations. Third, the study also faces the challenge of potential off-target effects associated with CRISPR-Cas9 gene editing technology, which may introduce genetic alterations that might confound the interpretation of results. To mitigate this limitation, experimental validation of potential off-target sites using advanced sequencing techniques, such as whole-genome sequencing and targeted amplicon sequencing, is essential.

Conclusions

In conclusion, our results demonstrate that the NRG1/ErbB4 signaling pathway promotes the in vitro differentiation of iPSCs into cardiomyocytes, and this effect may be achieved through the activation of the PI3K/Akt pathway. This provides a feasible and effective strategy for efficiently inducing stem cell differentiation into cardiomyocytes, laying a theoretical foundation for the next step of iPSC transplantation for the repair of damaged myocardium. Given the potential exhibited by the iPSCs-ErbB4+NRG1 complex for differentiating into iPSCs cardiomyocytes, we plan to further investigate the therapeutic application of these cells in treating cardiac disorders, such as myocardial infarction. Our plan involves the transplantation of engineered iPSCs expressing NRG1 and ErbB4 into a myocardial infarction model, aiming to assess both cardiomyocyte regeneration and cardiac functionality.

Abbreviations

NRG1	Neuregulin1
ErbB4	Erythroblastic leukemia viral oncogene homolog 4
iPSCs	Induced pluripotent stem cells
qRT-PCR	Quantitative real-time PCR
cTnT	Cardiac troponin
α-MHC	α-myosin heavy chain
OCT4	Octamer-binding transcription factor-4
SSEA-1	Stage-specific embryonic antigen-1

Supplementary Information

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Supplementary Material 1

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Not applicable.

Author contributions

All authors contributed to the study conception and design. XOL, HZ, WJL, and HT acquired the data. XOL and HZ analyzed and interpreted the data. XOL, HZ, BH, and HJ prepared the paper. XOL, HZ, and BH searched the literature. BH collected the funds. All authors read and approved the final manuscript.

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Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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