



Cardiotrophin-1 promotes cardiomyocyte differentiation from mouse induced pluripotent stem cells via JAK2/STAT3/Pim-1 signaling pathway

Tong LIU¹, Ran ZHANG², Tao GUO¹, Sai MA¹, Dong HAN¹, Xiu-Juan LI¹, Yan JIN³,
Miao-Miao FAN¹, Ya-Bin WANG², Yun-Dai CHEN², Feng CAO^{1,2}

¹Department of Cardiology, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi, China

²Department of Cardiology, Chinese PLA General Hospital, Beijing, China

³Research and Development Centre of Tissue Engineering, Fourth Military Medical University, Xi'an, Shaanxi, China

Abstract

Background The induced pluripotent stem cell (iPSC) has shown great potential in cellular therapy of myocardial infarction (MI), while its application is hampered by the low efficiency of cardiomyocyte differentiation. The present study was designed to investigate the effects of cardiotrophin-1 (CT-1) on cardiomyocyte differentiation from mouse induced pluripotent stem cells (miPSCs) and the underlying mechanisms involved. **Methods** The optimal treatment condition for cardiomyocyte differentiation from miPSCs was established with ideal concentration (10 ng/mL) and duration (from day 3 to day 14) of CT-1 administration. Up-regulated expression of cardiac specific genes that accounted for embryonic cardiogenesis was observed by quantitative RT-PCR. Elevated amount of α -myosin heavy chain (α -MHC) and cardiac troponin I (cTn I) positive cells were detected by immunofluorescence staining and flow cytometry analysis in CT-1 group. **Results** Transmission electron microscopic analysis revealed that cells treated with CT-1 showed better organized sarcomeric structure and more mitochondria, which are morphological characteristic of matured cardiomyocytes. Western blot demonstrated that CT-1 promotes cardiomyocyte differentiation from miPSCs partly via JAK2/STAT3/Pim-1 pathway as compared with control group. **Conclusions** These findings suggested that CT-1 could enhance the cardiomyocyte differentiation as well as the maturation of mouse induced pluripotent stem cell derived cardiomyocytes by regulating JAK2/STAT3/Pim-1 signaling pathway.

J Geriatr Cardiol 2015; 12: 591–599. doi:10.11909/j.issn.1671-5411.2015.06.002

Keywords: Cardiac differentiation; Cardiotrophin-1; Mouse induced pluripotent stem cells; Pim-1

1 Introduction

Stem cell therapy shows promising potential for cardiac tissue regeneration.^[1,2] Because of the self-renewal and multi-lineage differentiation potential, pluripotent stem cells, including embryonic stem cell (ESCs) and induced pluripotent stem cells (iPSCs), are ideal donor cells.^[3–5] The iPSCs can be utilized in drug screening, disease modeling and cell therapy.^[6–8] When administered in myocardial infarction (MI) animals, iPSC-derived cardiomyocytes (iPS-CMs) showed improvement in cardiac function.^[3] However, one important prerequisite for the scientific and therapeutic application of iPSCs in patients with coronary heart disease (CHD) is to generate highly-purified iPS-CMs.

The optimization of cardiomyocyte differentiation from

iPSCs has been well studied and the roles of cytokines during this process received much attention in these years,^[9–13] such as bone morphogenetic proteins (BMPs), fibroblast growth factor (FGFs), ascorbic acid (AA), apelin (APL) and dickkopf-1 (Dkk-1). These cytokines were proved to be effective in inducing cardiogenesis of iPSCs. However, the underlying mechanisms are still not fully understood.

Another obstacle that prevent the application of iPSCs is that iPS-CMs is less mature than their counterparts from ESCs or adult heart, which is characterized by delayed and scrambled myofibril formation and less sensitivity to β -adrenergic stimulus.^[14,15] It poses a potential risk that these immature iPS-CMs cause ineffective contractile force, even arrhythmia following cell transplantation.^[16] Therefore, generation of matured iPS-CMs is an important prerequisite for potential clinical application.

Cardiotrophin-1 (CT-1) is a heart-targeting cytokine and a member of interleukin-6 (IL-6) family, which is highly expressed during early embryogenesis.^[17] The proper expression of CT-1 is essential for both morphogenesis and

Correspondence to: Feng CAO, MD, PhD, Department of Cardiology, Chinese PLA General Hospital, 28 Fuxing Road, Beijing, China.

E-mail: wind8828@gmail.com

Received: July 23, 2015

Revised: October 19, 2015

Accepted: November 2, 2015

Published online: November 6, 2015

cardiogenesis of the embryonic heart. Recently, CT-1 was proved to facilitate the cardiogenesis in ESCs and BM-MSCs. However, whether it could enhance cardiomyocytes differentiation of miPSCs is unclear.

CT-1 shares common membrane receptors gp130 with other members of IL-6 family.^[18] Homo- and hetero-dimerization of gp130 results in the activation of Janus kinase signal transducer (JAK), which in turn activates signal transducer and activator of transcription (STAT) family.^[19] JAK2/STAT3 pathway has been proved to be the most highly correlated with the regulation of cardiomyocyte differentiation.^[20] Pim-1, a target gene which is activated by STAT3, has been reported to promote the proliferation of cardiac progenitor cells.^[21] However, the exact role of Pim-1 in cardiomyocyte differentiation from miPSCs has not been elucidated. Therefore, we hypothesis that Pim-1 might be a target molecule for JAK2/STAT3 to take effect in the CT-1 mediated cardiomyocyte differentiation from miPSCs.

The aims of the present study were to investigate the effects of CT-1 on cardiomyocyte differentiation from miPSCs and the underlying mechanisms involved.

2 Methods

2.1 Culture and differentiation of miPSCs

The iPS cell line used in this study was maintained in Knockout Dulbecco's Modified Eagle Medium (KO-DMEM; Invitrogen, Boston, MD, USA) containing 15% fetal bovine serum (FBS, GE, Pittsburgh, PA, USA), 1 $\mu\text{mol/L}$ non-essential amino acid (NEAA, GE), 1 mmol/L Glutamax (Invitrogen), 100 $\mu\text{mol/L}$ β -mercaptoethanol (Invitrogen), 50 U/mL penicillin, and 50 mg/mL streptomycin (both purchased from GE) on irradiation inactivated mouse embryonic fibroblasts (MEFs) feeder layers in the presence of 1,000 U/mL leukemia inhibitory factor (LIF, Millipore, Billerica, MA, USA). Differentiation medium contains high glucose DMEM (Hyclone, Logan, UT, USA), 20% FBS, 1 mmol/L Glutamax, 100 $\mu\text{mol/L}$ β -mercaptoethanol, 50 U/mL penicillin, and 50 mg/mL streptomycin. Differentiation of miPSCs was initiated using hanging drop method as previously described.^[22] Formed embryoid bodies (EBs) were transferred to ultra-low attachment plates (Corning, Tewsbury, MA, USA) for another three days of suspension culture. Then EBs were seeded onto 0.1% gelatin (Millipore)-coated plates for adherent culture and further examination.

2.2 CT-1 administration and reagents interfering with JAK2/STAT3/Pim-1 signaling cascade

Cells were treated with recombinant human CT-1

(Sigma-Aldrich, St. Louis, MO, USA) and normal differentiation medium was used as control. To determine the temporal effect of CT-1 on cardiogenesis of miPSCs, cells were systematically treated with 5 ng/mL CT-1 at early stage (day 0-2), mid-stage (day 3-6) or late stage (day 7-14) individually or with diverse combination as indicated. To evaluate the dosage effect of CT-1, cells were treated with CT from 0.3 to 30 ng/mL. For both groups, medium was changed every other day. For interfering JAK2/STAT3/Pim-1 signaling cascade, AG490 (50 $\mu\text{mol/L}$, JAK2 inhibitor), S3I-201 (100 $\mu\text{mol/L}$, STAT3 inhibitor), SGI-1776 free base (10 $\mu\text{mol/L}$, Pim-1 inhibitor) were applied 1 h before CT-1 treatment. All inhibitors were purchased from Selleckchem (Selleckchem inhibitor, Boston, MA, USA).

2.3 Reverse transcription-PCR and quantitative RT-PCR

Total RNA was extracted from cells using TRIzol (Invitrogen) and was reverse-transcribed into cDNA using ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan). PCR was performed using Taq DNA Polymerase (Takara, Shiga, Japan). QRT-PCR was carried out using ABI Step-One Plus Real-Time PCR Detection system (Applied Biosystems, Grand Island, NY, USA) with SYBR Premix Ex Taq™ II (Takara, Shiga, Japan). All genes were amplified for 40 circles. The relative level of each mRNA transcript were calculated by normalizing to β -actin and expressed as a relative ratio. The (q) RT-PCR primers are listed in Table 1.

2.4 Immunostaining and confocal microscopy analysis

Cells were fixed with phosphate buffer saline (PBS) containing 4% paraformaldehyde at room temperature for 30 min. After washing with PBS, cells were permeated with 0.05% Triton X-100 (Sigma-Aldrich) for 15 min, then blocked with 1% BSA (Sigma-Aldrich) plus 4% normal goat serum (Sigma-Aldrich) at room temperature for 1 h. The cells were incubated with primary antibodies overnight at 4°C: anti-octamer-binding transcription factor 4 (Oct-4, dilution 1:100, Santa Cruz, Dallas, TX, USA), anti-cardiac troponin I (cTn I, dilution 1:200, Abcam, Cambridge, MA, USA), anti- α -myosin heavy chain (α -MHC, dilution 1:100, Abcam). After washing with PBS, The cells were incubated with secondary antibodies (dilution 1:500, Abcam) at room temperature for 1 h and then counterstained with 4',6-diamidino-2-phenylindole (DAPI, dilution 1:1,000, Sigma-Aldrich) for 5 min. Samples were examined using Olympus FV1000 confocal laser scanning microscope (Olympus, Japan). For manual quantification, samples from each group were examined for α -MHC and cTn I⁺ cells in 5 random fields with each field contained 50 cells.

Table 1. Primer sets for quantitative RT-PCR.

Gene	Sequences
Oct4	F: 5' – CACGAGTGGAAAGCAACTCA – 3' R: 5' – AGATGGTGGTCTGGCTGAAC – 3'
Brachyury	F: 5' – CCAAGGACAGAGAGACGGCT – 3' R: 5' – AGTAGGCATGTTCCAAGGGC – 3'
Isl1	F: 5' – GATGCGCTCATGAAGGAGCAACTA – 3' R: 5' – GTTTCTGTCTTCACCCGCTTGT – 3'
Gata4	F: 5' – CCCTACCCAGCCTACATGG – 3' R: 5' – ACATATCGAGATTGGGGTGTCT – 3'
Nppa	F: 5' – GCCCTCGGAGCCTACGAA – 3' R: 5' – GGTACCGGAAGCTGTTGCA – 3'
Mef2c	F: 5' – GGTATGGCAATCCCCGAACTCAC – 3' R: 5' – GCCAGCCAGTACTGACCCAAGAT – 3'
β -actin	F: 5' – TGACAGGATGCAGAAGGAGA – 3' R: 5' – GTAAGTTCGCTCAGGAGGAG – 3'
Flk-1	F: 5' – GCTTGCTCCTTCTCATCTC – 3' R: 5' – CCATCAGGAAGCCACAAAGC – 3'
Nkx2.5	F: 5' – GACAAAGCCGAGACGGATGG – 3' R: 5' – CTGTCGCTTGCACTGTAGC – 3'
Tbx5	F: 5' – CGTTTGGACACATTATCCTGAACT – 3' R: 5' – TGAACCGAACCCATTATTTTCG – 3'
cTn T	F: 5' – GAGGAGGTGGTGGAGGAGTA – 3' R: 5' – GGCTTCTCATCAGGACCAA – 3'

cTn T: cardiac troponin T; Flk-1: fetal liver kinase1; Gata4: GATA binding protein 4; Isl1: Islet-1 transcription factor; Mef2c: Myocyte enhancer factor 2c; Nppa: Natriuretic peptide type A; Nkx2.5: NK2 transcription factor related, locus 5; Oct-4: octamer-binding transcription factor 4; Tbx5: T-box transcription factor 5.

2.5 Flow cytometry analysis

Mouse EBs (mEBs) were dissociated with TryLE (Invitrogen) for 10 min. Cells were fixed and permeabilized with PharMingen Perm/Fix solution (BD Bioscience, San Jose, CA, USA) for 30 min at 4°C, and then incubated with primary antibodies against mouse cTn I (dilution 1:200, Abcam) overnight at 4°C. Then cells were stained with FITC-conjugated antibody (1:500, Acam) for 1 h at room temperature. The data was analyzed using FACS Calibur (BD bioscience) and FlowJo software to quantify the percentage of cTn I⁺ cells.

2.6 Western blot analysis

Cells were homogenized in cell lysis buffer. Samples were separated by SDS-PAGE. Separated proteins were transferred onto nitrocellulose membranes (Hybond-P, Amersham Biosciences; Piscataway, NJ, USA) that were incubated overnight at 4°C using the following antibodies: JAK2 (dilution 1:1,000, Cell signal, Danvers, MA, USA), p-JAK2 (dilution 1:1,000, Cell signal), STAT3 (dilution 1:1,000, Cell signal), p-STAT3 (1:200,000, Abcam), Pim-1

(dilution 1:10,000, Abcam). Membranes were then washed and incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at room temperature. Blot bands were visualized with an enhanced chemiluminescence system (Amersham Bioscience, Fairfield, Connecticut, USA). The protein bands were analyzed using VisionWorks LS, version 6.7.1.

2.7 Ultrastructure observation of induced iPS-CMs with transmission electron microscopy (TEM)

At day 15, mEBs were harvested and fixed in 3% glutaraldehyde, then post-fixed in 1% osmium tetroxide and embedded in osmium tetroxide. Ultra-thin sections were prepared and double stained with uranyl acetate and lead citrate. The images were obtained by JEM-1230 transmission electron microscope (JEOL, Japan).

2.8 Statistical analysis

The results are expressed as mean \pm SE. Statistics were calculated and performed using GraphPad Prism 5.0 (GraphPad Software Inc, San Diego, CA, USA). Statistical evaluation was performed using either the Student's *t* test or one-way analysis of variance (ANOVA) and a *P* value < 0.05 was considered statistically significant.

3 Results

3.1 Characterization of miPSCs and optimization of CT-1 administration

As shown in Figure 1, miPSCs exhibited a typical ES-like clonal, compact morphology (Figure 1A) and immunofluorescence staining detected strong expression of stem cell marker Oct4 (Figure 1B-D). We then set various combination of treatment duration time and different CT-1 concentration gradient to optimize CT-1 administration (Figure 2A). Our results indicated that CT-1 significantly promoted cardiomyocyte differentiation and showed evident synergistic effect at both mid-stage and late stage (Figure 2A). The maximum percentage of contracting EBs was 28.4% \pm 4.5% (*vs.* 6.2% \pm 0.72% in control, *P* < 0.01) and the level of cTn T mRNA increased by 3.4 \pm 0.43 fold following CT-1 treatment, which showed a concentration-dependent manner and reached its peak at 10 ng/mL (Figure 2B).

3.2 CT-1 facilitated cardiomyocyte differentiation of miPSCs by modulating the expression of genes that regulate cardiogenesis

EBs from both groups were subjected to beating area observation from day 6. Contracting EBs were not detected

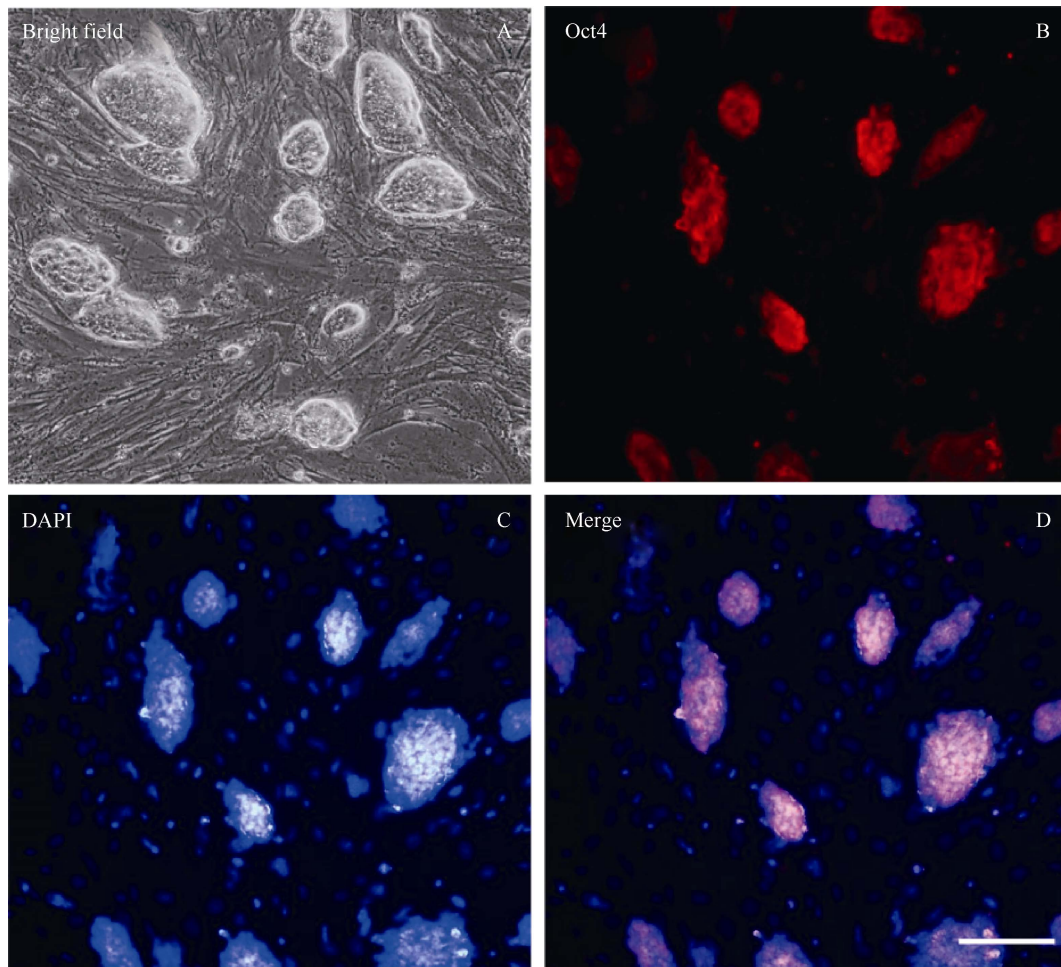


Figure 1. Characterization of miPSCs. (A): The miPSCs used in the present study, which are stained positive for pluripotent stem cell marker Oct-4 by immunofluorescence staining (B-D). Scale bar = 50 μ mol/L. DAPI: 4', 6-diamidino-2-phenylindole; miPSCs: mouse induced pluripotent stem cells; Oct-4: octamer-binding transcription factor 4.

until day 9 in control group but at day 7 in CT-1 group (Movie 1 in the supplemental material). The percentage of beating EBs were peaked at $24.5\% \pm 4.25\%$ at day 13 in CT-1 group, compared with $6.4\% \pm 1.22\%$ in control group ($P < 0.01$) (Figure 3A). To elucidate the exact roles of CT-1 in cardiomyocyte differentiation from miPSCs, we detected the expression of several pluripotent and cardiac genes by RT-PCR or qRT-PCR). CT-1 decreased and increased the expression of Oct4 and *Nppa/Mef-2c*, respectively. The expression of *Isl1* and *Gata-4* were also moderately augmented but mesoderm markers *Brachury* remained unchanged (Figure 3B). CT-1 showed a robust activation of cardiogenic mesoderm marker *Flk-1* (also known as *Vegfr2*) (Figure 3C), cardiac progenitor markers *Nkx2.5* (Figure 3D), *Tbx5* (Figure 3E), and sarcomeric marker *cTn T* (Figure 3F).

3.3 CT-1 enhanced the generation of iPS-CMs

Confocal microscopic analysis showed that CT-1 administration resulted in an increased number of *cTn I*⁺ cells

at day 14 (Figure 4A). Quantitative analysis showed that the percentage of cells that expressing α -MHC and *cTn I* in CT-1 group were $55.6\% \pm 4.22\%$ (vs. $26.3\% \pm 4.70\%$ in control, $P < 0.05$) and $50.3\% \pm 5.81\%$ (vs. $19.3\% \pm 1.45\%$ in control, $P < 0.05$), respectively. These results were further demonstrated by flow cytometry analysis (Figure 4D).

3.4 CT-1 promoted maturation of iPS-derived cardiomyocytes

Transmission electron Microscopic analysis demonstrated that samples from both groups showed ultrastructural features of early and immature cardiomyocytes containing myofibrils, large glycogen deposits, mitochondria and intercellular junctions. In CT-1 group, abundant primitive myofilaments, which indicate the myofibril formed at early stage of cardiomyocytes differentiation, were observed. In addition, mitochondria were also clearly visible. In contrast, only a few primitive myofibrils and immature mitochondria were found in control group (Figure 4E)

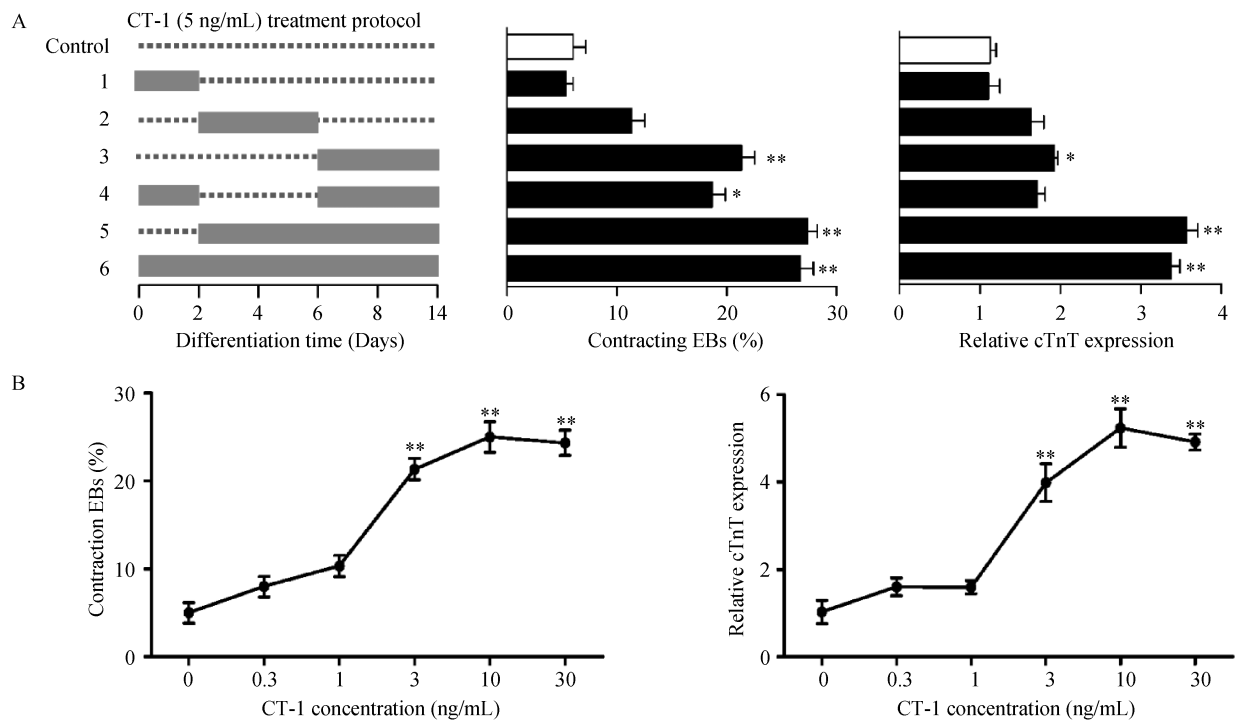


Figure 2. Optimization of CT-1 induced cardiomyocyte differentiation. (A): Time window for CT-1 promoted cardiomyocyte differentiation. Samples were collected at day 10. Left panel, representative diagram of CT-1 treatment protocol; middle panel, corresponding percentage of beating Ebs, results were triplicated with $n = 100$ in each group; right panel, corresponding relative cTn T gene expression, $n = 3$. (B): Concentration-dependent relationships of CT-1. Data are expressed as mean \pm SE. * $P < 0.05$, ** $P < 0.01$ vs. control. CT-1: cardiotrophin-1; cTn T: cardiac troponin T; EBs: embryoid bodies.

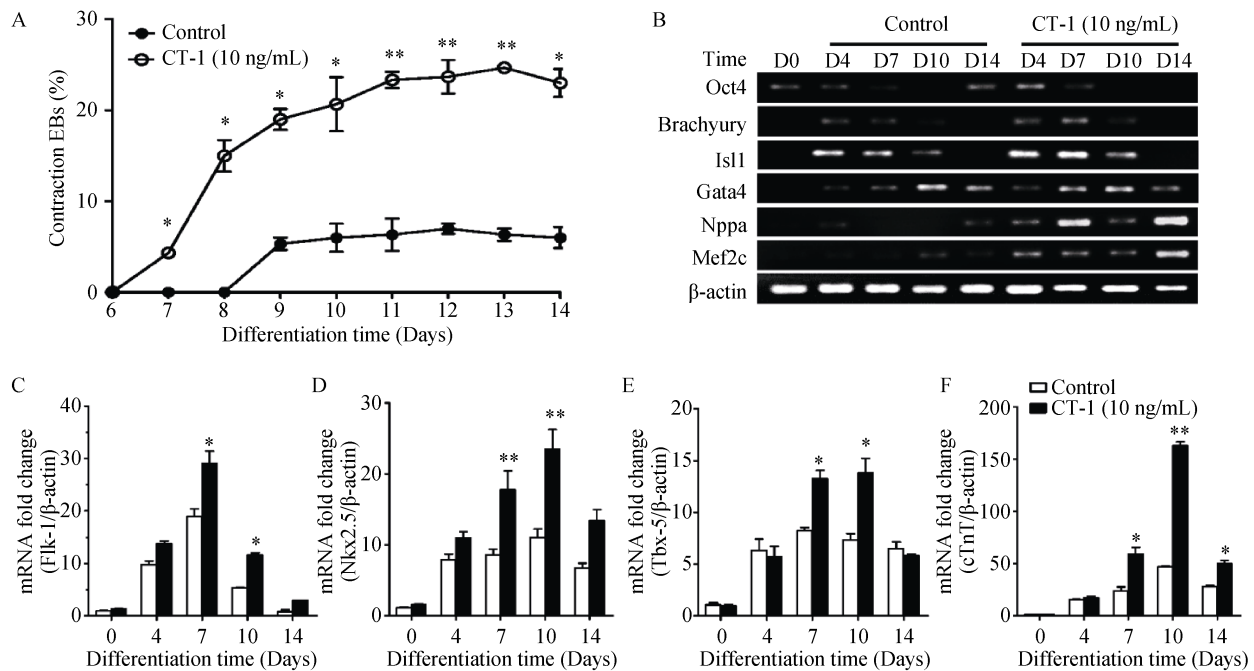


Figure 3. CT-1 facilitated cardiomyocyte differentiation of iPSCs by modulating the expression of genes that regulate cardiogenesis. (A): Percentage of beating EBs, $n = 100$. (B): RT-PCR analysis shows the upregulation of cardiac genes in CT-1 group compared with control. Quantitative RT-PCR demonstrates increased expression of cardiovascular progenitor cell marker Flk-1 (C), cardiac progenitor cell marker Nkx2.5 (D), Tbx5 (E) and cardiac-specific marker cTn T (F) in CT-1 group compared with control, $n = 3$. Data are expressed as mean \pm SE. * $P < 0.05$, ** $P < 0.01$ vs. control. CT-1: cardiotrophin-1; cTn T: cardiac troponin T; Flk-1: fetal liver kinase1; iPSCs: mouse induced pluripotent stem cells; Nkx2.5: NK2 transcription factor related, locus 5; Tbx5: T-box transcription factor 5.

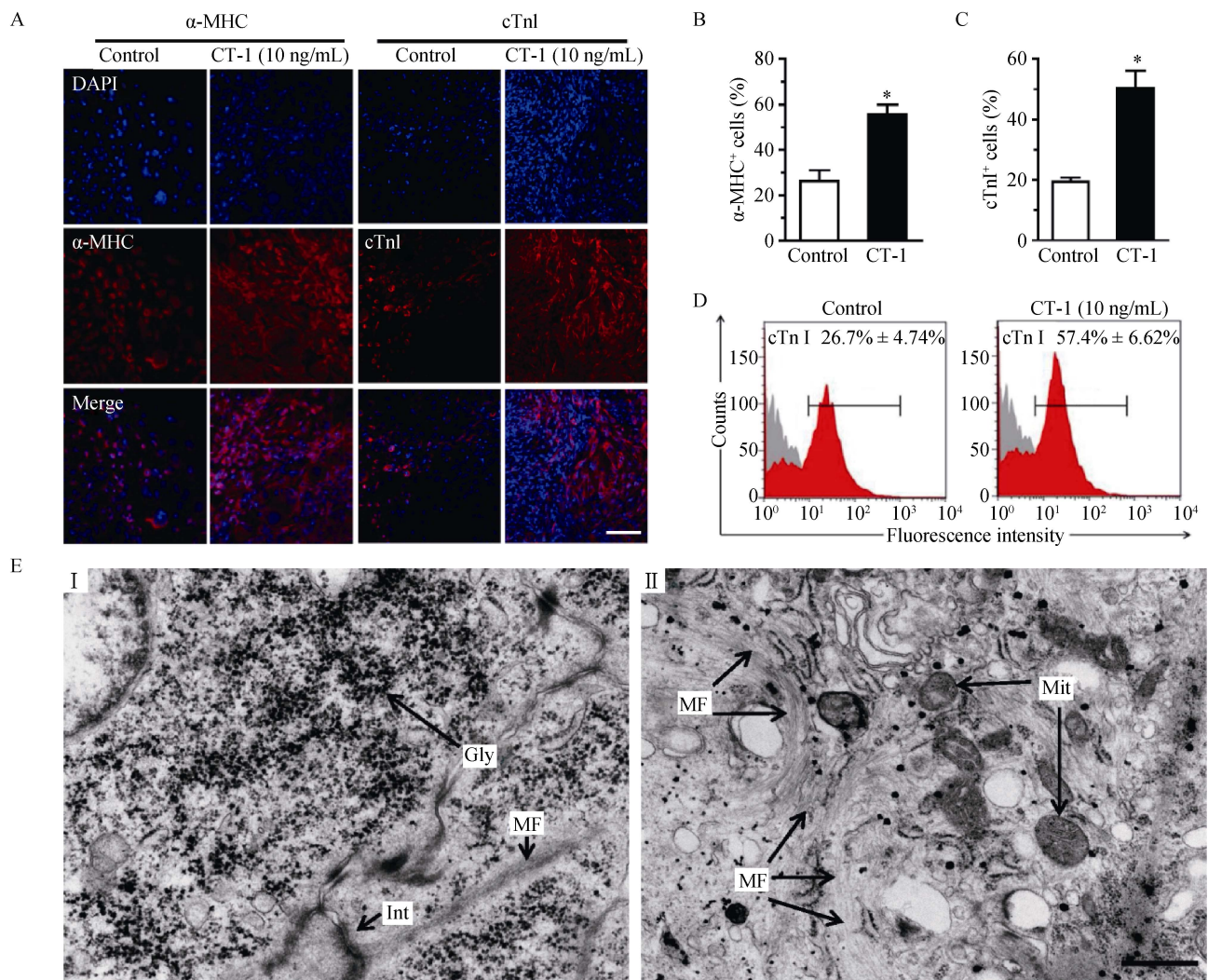


Figure 4. CT-1 enhanced the generation and maturation of iPS-CMs. (A): Confocal microscopic images of immunofluorescence staining for cardiac-specific protein cTn I and α -MHC. Scale bar = 50 μ m. (B, C): Semi-quantification of percentage of cells expressing α -MHC and cardiac cTn I, $n = 50$ in 5 random fields. (D): Flow cytometry analysis of cTn I⁺ cells, $n = 3$. (E): Ultrastructure of iPS-CMs in control group (I) and CT-1 group (II). Scale bar = 1 μ m. Data are expressed as mean \pm SE. * $P < 0.05$ vs. control. α -MHC: α -myosin heavy chain; CT-1: cardiostrophin-1; cTn I: cardiac troponin I; FCM: flow cytometry; Gly: glycogen deposits; Int: intercellular junctions; iPS-CMs: iPS-derived cardiomyocytes; MF: myofibrils; Mit: mitochondria.

3.5 CT-1 facilitated cardiomyocyte differentiation of miPSCs partly via JAK2/STAT3/Pim-1 signaling pathway

As shown in Figure 5, we found that JAK2/STAT3/Pim-1 cascade was involved in the CT-1-mediated cardiomyocyte differentiation from miPSCs. AG490 significantly decreased the phosphorylation of JAK2 at Tyr 1007/1008 and STAT3 at Try 705 and in turn, attenuated the expression of Pim-1. The addition of SGI-1776 free base attenuated the abundance of Pim-1, while the phosphorylation of JAK2 and STAT3 were not affected. AG490 or S3I-201 significantly decreased the average beating area in EBs

(3.31% \pm 0.90% in AG490 group, 2.65% \pm 0.55% in S3I-201 group, vs. 5.97% \pm 1.55% in control, $P < 0.05$). Pim-1 inhibitor SGI-1776 free base also showed adverse effects on the generation of beating area in EBs (1.61% \pm 0.62% vs. 5.97% \pm 1.55% in control, $P < 0.05$) (Figure 5A). Taken together, these results suggested that CT-1 enhanced cardiomyocyte differentiation of miPSCs through JAK2/STAT3/Pim-1 cascade.

4 Discussion

Our findings extended the previous studies about CT-1 in enhancing cardiomyocyte differentiation from BM-MS-C

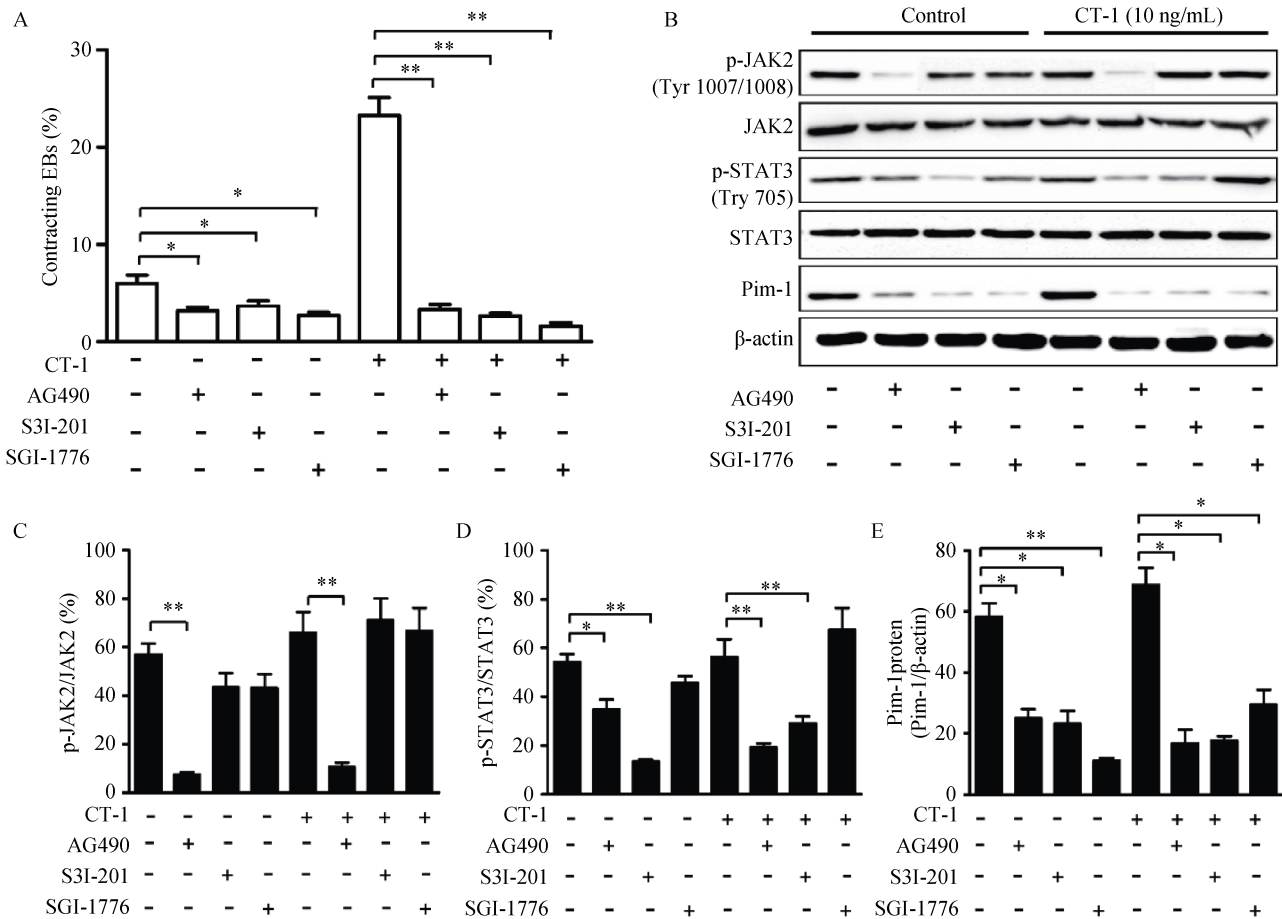


Figure 5. CT-1 facilitated cardiomyocyte differentiation of miPSCs partly via JAK2/STAT3/Pim-1 signaling pathway. (A): Percentage of beating EBs at day 14. Results were triplicated with $n = 100$ in each group. (B): Representative images of Western blotting of JAK2, STAT3 and Pim-1. Semi-quantification of protein expression of p-JAK2 (C), p-STAT3 (D) and Pim-1 (E). $n = 3$. * $P < 0.05$, ** $P < 0.01$ vs. control. AG490: Jak2 specific inhibitor; CT-1: cardiostrophin-1; JAK2: Janus kinase 2; miPSCs: mouse induced pluripotent stem cells; SGI-1776: Pim-1 specific inhibitor; STAT3: signal transducer and activator of transcription 3; S3I-201: STAT3 specific inhibitor.

and ESCs,^[9,23] indicating that CT-1 is a suitable inducer for cardiac differentiation of miPSCs. The important findings are that: (1) Mid-stage (day 3-6) and late-stage (day 7-14) of cardiomyocyte differentiation were crucial for CT-1 to take effect; (2) 10 ng/mL is a suitable concentration for CT-1 to facilitate cardiomyocyte differentiation of miPSCs; (3) CT-1 enhanced the differentiation as well as the maturation of iPS-CMs; (4) Pim-1 might play a vital role in JAK2/STAT3 mediated cardiomyocyte differentiation.

Embryonic cardiogenesis is subtly modulated by a series of developmental signals and microenvironment.^[24] CT-1, a member of IL-6 family, which was firstly discovered in 1995,^[17] is highly expressed in myocardial cells and cardiac fibroblasts during the early stage of embryonic cardiogenesis.^[18] It has been shown that CT-1 and its receptor glycoprotein 130 (gp130) play a key role in embryonic cardiomyocyte differentiation and morphogenesis.^[25] The deletion

of gp130 gene is lethal to embryonic development,^[26] whereas overexpression of CT-1 in mouse embryo resulted in reinforced cardiomyocyte differentiation.^[27] In addition, CT-1 is also reported to promote the cardiomyocyte differentiation of other stem cells such as MSCs and ESCs.

CT-1 has been reported to facilitate cardiomyocyte differentiation by promoting the expression of cardiac progenitor cell markers Gata4 and Nkx2.5 during the cardiac mesodermal specification.^[9] Our results demonstrated that CT-1 strongly up-regulated a series of cardiac progenitor cell markers including Gata-4, Nkx2.5 and Tbx5. Moreover, we also observed a moderate augment of the expression of Flk-1. Flk-1 is well documented markers of cardiovascular progenitor cells during the cardiogenic mesodermal specification,^[28] which is a cardiogenic phase prior to cardiac mesodermal specification. However, no detectable change of gene Brachyury, which is a marker for mesodermal spec-

specification, was observed in our study. These observations were further verified by our findings that CT-1 started to take effect as early as day 3 after the induction initiated, which is a representative time point of the beginning of cardiogenic mesodermal specification. Taken together, our findings suggest that CT-1 promote cardiomyocyte differentiation from miPSCs by facilitating cardiogenic mesodermal specification.

The maturity of iPS-CMs is another obstacle hampering its utilization. It has been proved that iPS-CMs were less matured compared with their counterparts derived from ESCs or fetal heart.^[14,15] Previous research indicated that CT-1 could facilitate the maturity of BM-MSC derived cardiomyocyte-like-cells.^[9] Our results showed that CT-1 facilitated the generation of matured myofilaments and mitochondrion. Moreover, up-regulation of gene expression of Mef2c and protein level of α -MHC was also observed when cells were treated with CT-1, indicating that CT-1 might promote the myogenesis of induced cardiomyocytes. It has also been reported that CT-1 showed a potential role in post-MI wound healing.^[29] Given that, further work is required to investigate the therapeutic effect of CT-1 on transplanting iPS-CMs into an ischemic heart disease model.

Embryonic cardiogenesis is a dynamic process that is modulated by the sequential expression of multiple signal transduction proteins and transcription factors.^[28] A number of signaling pathways were involved in the development of specialized cardiac subtype.^[30–32] It has been suggested that STAT3 plays an important role during cardiac differentiation of mouse embryonic stem cells.^[20] Compared with non-beating EBs, beating EBs shows higher protein abundance of phospho-JAK and phospho-STAT3; but a decreased ratio of beating EBs were observed when EBs are treated with AG490, a JAK2 inhibitor.^[20] STAT3 could also directly control the expression of Tbx5, Nkx2.5, and Gata-4 that are essential during embryogenesis.^[33] However, it is still unclear how JAK2/STAT3 is involved in detail during the process of cardiomyocyte differentiation from miPSCs. The oncogenic serine /threonine kinase Pim-1 was originally identified as a common proviral insertion site in T and B cell lymphomas,^[34] which shows a wide range of biological activities, including cell proliferation and differentiation. Overexpression of Pim-1 promoted the proliferation of cardiac progenitor cells.^[21] Tomohiko and coauthors also found that STAT3/Pim-1 pathway was essential in endothelial differentiation of cardiac resident Sca-1⁺ cells.^[35] In the present study, we found that the protein abundance of Pim-1 was increased by the activation of JAK2 and STAT3, and decreased by the inhibition of JAK2 with AG490 and STAT3 with S3I-201, respectively. Up-regulation of Pim-1

resulted in a promoted cardiogenesis, while inhibition of Pim-1 with SGI-1776 free base significantly abrogated CT-1 induced cardiogenesis. These results suggested that Pim-1 was an important target molecule which was essential for JAK2/STAT3 to take effect during CT-1 induced cardiomyocyte differentiation of miPSCs.

In conclusion, the current study demonstrated that CT-1 enhanced the cardiogenesis of miPSCs and facilitated the maturation of iPS-CMs via JAK2/STAT3/Pim-1 signaling pathway. These findings may benefit in the clinical application of iPSCs in drug screening and patient specific cardiac regeneration.

Acknowledgment

This work was supported by the National Funds for Distinguished Young Scientists of China (No. 81325009) and National Nature Science Foundation of China (No. 81270168, No. 81227901), (Feng Cao BWS12J037), Innovation Team granted by Ministry of Education PRC (IRT1053), National Basic Research Program of China (2012CB518101). Shaanxi Province Program (2013K12-02-03, 2014KCT-20). The authors declare no conflict of interest.

References

- 1 Li RA, Cardiovascular regeneration. *Stem Cell Res Ther* 2014; 5: 141.
- 2 Matar AA, Chong JJ. Stem cell therapy for cardiac dysfunction. *Springerplus* 2014; 3: 440.
- 3 Nelson TJ, Martinez-Fernandez A, Yamada S, *et al.* Repair of acute myocardial infarction by human stemness factors induced pluripotent stem cells. *Circulation* 2009; 120: 408–416.
- 4 Chong JJ, Yang X, Don CW, *et al.* Human embryonic-stem-cell-derived cardiomyocytes regenerate non-human primate hearts. *Nature* 2014; 510: 273–277.
- 5 Malecki M, Putzer E, Sabo C, *et al.* Directed cardiomyogenesis of autologous human induced pluripotent stem cells recruited to infarcted myocardium with bioengineered antibodies. *Mol Cell Ther* 2014; 2: pii: 13.
- 6 Takahashi K, Tanabe K, Ohnuki M, *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007; 131: 861–872.
- 7 Tse HF, Ho JC, Choi SW, *et al.* Patient-specific induced-pluripotent stem cells-derived cardiomyocytes recapitulate the pathogenic phenotypes of dilated cardiomyopathy due to a novel DES mutation identified by whole exome sequencing. *Hum Mol Genet* 2013; 22: 1395–1403.
- 8 Huang HP, Chuang CY, Kuo HC. Induced pluripotent stem cell technology for disease modeling and drug screening with emphasis on lysosomal storage diseases. *Stem Cell Res Ther* 2012; 3: 34.
- 9 Xinyun C, Zhi Z, Bin Z, *et al.* Effects of cardiotrophin-1 on

- differentiation and maturation of rat bone marrow mesenchymal stem cells induced with 5-azacytidine *in vitro*. *Int J Cardiol* 2010; 143: 171–177.
- 10 Wang IN, Wang X, Ge X, *et al.* Apelin enhances directed cardiac differentiation of mouse and human embryonic stem cells. *PLoS One* 2012; 7: e38328.
 - 11 Kattman SJ, Witty AD, Gagliardi M, *et al.* Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. *Cell Stem Cell* 2011; 8: 228–240.
 - 12 Cao N, Liu Z, Chen Z, *et al.* Ascorbic acid enhances the cardiac differentiation of induced pluripotent stem cells through promoting the proliferation of cardiac progenitor cells. *Cell Res* 2012; 22: 219–236.
 - 13 Flaherty MP, Abdel-Latif A, Li Q, *et al.* Noncanonical Wnt11 signaling is sufficient to induce cardiomyogenic differentiation in unfractionated bone marrow mononuclear cells. *Circulation* 2008; 117: 2241–2252.
 - 14 Xi J, Khalil M, Shishechian N, *et al.* Comparison of contractile behavior of native murine ventricular tissue and cardiomyocytes derived from embryonic or induced pluripotent stem cells. *FASEB J* 2010; 24: 2739–2751.
 - 15 Kuzmenkin A, Liang H, Xu G, *et al.* Functional characterization of cardiomyocytes derived from murine induced pluripotent stem cells *in vitro*. *FASEB J* 2009; 23: 4168–4180.
 - 16 Kong CW, Akar FG, Li RA. Translational potential of human embryonic and induced pluripotent stem cells for myocardial repair: insights from experimental models. *Thromb Haemost* 2010; 104: 30–38.
 - 17 Pennica D, King KL, Shaw KJ, *et al.* Expression cloning of cardiotrophin 1, a cytokine that induces cardiac myocyte hypertrophy. *Proc Natl Acad Sci U S A*, 1995; 92: 1142–1146.
 - 18 Pennica D, Shaw KJ, Swanson TA, *et al.* Cardiotrophin-1. Biological activities and binding to the leukemia inhibitory factor receptor/gp130 signaling complex. *J Biol Chem*, 1995; 270: 10915–10922.
 - 19 Stahl N, Farruggella TJ, Boulton TG, *et al.* Choice of STATs and other substrates specified by modular tyrosine-based motifs in cytokine receptors. *Science* 1995; 267: 1349–1353.
 - 20 Foshay K, Rodriguez G, Hoel B, *et al.* JAK2/STAT3 directs cardiomyogenesis within murine embryonic stem cells *in vitro*. *Stem Cells* 2005; 23: 530–543.
 - 21 Del Re DP, Sadoshima J. Sadoshima, Enhancing the potential of cardiac progenitor cells: pushing forward with Pim-1. *Circ Res* 2012; 110: 1154–1156.
 - 22 Yoon BS, Yoo SJ, Lee JE, *et al.* Enhanced differentiation of human embryonic stem cells into cardiomyocytes by combining hanging drop culture and 5-azacytidine treatment. *Differentiation* 2006; 74: 149–159.
 - 23 Sauer H, Neukirchen W, Rahimi G, *et al.* Involvement of reactive oxygen species in cardiotrophin-1-induced proliferation of cardiomyocytes differentiated from murine embryonic stem cells. *Exp Cell Res* 2004; 294: 313–324.
 - 24 Vidarsson H, Hyllner J, Sartipy P. Hyllner, and P. Sartipy, Differentiation of human embryonic stem cells to cardiomyocytes for *in vitro* and *in vivo* applications. *Stem Cell Rev* 2010; 6: 108–120.
 - 25 Pennica D, Wood WI, Chien KR. Cardiotrophin-1: a multifunctional cytokine that signals via LIF receptor-gp 130 dependent pathways. *Cytokine Growth Factor Rev* 1996; 7: 81–91.
 - 26 Dyson E, Sucov HM, Kubalak SW, *et al.* Atrial-like phenotype is associated with embryonic ventricular failure in retinoid X receptor alpha $-/-$ mice. *Proc Natl Acad Sci U S A* 1995; 92: 7386–7390.
 - 27 Ateghang B, Wartenberg M, Gassmann M, *et al.* Regulation of cardiotrophin-1 expression in mouse embryonic stem cells by HIF-1 α and intracellular reactive oxygen species. *J Cell Sci* 2006; 119: 1043–1052.
 - 28 Rajala K, Pekkanen-Mattila M, Aalto-Setälä K. Cardiac differentiation of pluripotent stem cells. *Stem Cells Int* 2011. Article ID 383709.
 - 29 Freed DH, Moon MC, Borowiec AM, *et al.* Cardiotrophin-1: expression in experimental myocardial infarction and potential role in post-MI wound healing. *Mol Cell Biochem* 2003; 254: 247–256.
 - 30 Marvin MJ, Di Rocco G, Gardiner A, *et al.* Inhibition of Wnt activity induces heart formation from posterior mesoderm. *Genes Dev* 2001; 15: 316–327.
 - 31 Mima T, Ueno H, Fischman DA, *et al.* Fibroblast growth factor receptor is required for *in vivo* cardiac myocyte proliferation at early embryonic stages of heart development. *Proc Natl Acad Sci U S A* 1995; 92: 467–471.
 - 32 Winnier G, Blessing M, Labosky PA, *et al.* Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. *Genes Dev* 1995; 9: 2105–2116.
 - 33 Snyder M, Huang XY, Zhang JJ. Stat3 directly controls the expression of Tbx5, Nkx2.5, and GATA4 and is essential for cardiomyocyte differentiation of P19CL6 cells. *J Biol Chem* 2010; 285: 23639–23646.
 - 34 Hoover D, Friedmann M, Reeves R, *et al.* Recombinant human pim-1 protein exhibits serine/threonine kinase activity. *J Biol Chem* 1991; 266: 14018–14023.
 - 35 Iwakura T, Mohri T, Hamatani T, *et al.* STAT3/Pim-1 signaling pathway plays a crucial role in endothelial differentiation of cardiac resident Sca-1+ cells both *in vitro* and *in vivo*. *J Mol Cell Cardiol*, 2011; 51: 207–214.