Original Paper

Effects of Puerarin on Cardiac Differentiation and Ventricular Specialization of Murine Embryonic Stem Cells

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Key Words

Embryonic stem cells • Differentiation • Puerarin • Cardiomyocytes • Ventricle

Abstract

Aims: It is important to screen and identify chemical compounds to improve the efficiency of cardiac differentiation and specialization of embryonic stem (ES) cells. The objective of this study was to investigate the effect of puerarin, a natural phytoestrogen, on the *in vitro* cardiac differentiation and ventricular specialization of murine ES cells. **Methods:** Cardiac differentiation of murine ES cells was performed by embryoid body (EB)-based differentiation method. Quantitative RT-PCR, flow cytometry and immunofluorescence were employed to identify cardiomyocytes (CMs) derived from murine ES cells (mES-CMs). Patch clamp was used to study the electrophysiological properties of CMs. **Results:** We found that continuous puerarin treatment significantly increased the population of ES-CMs which express typical cardiac markers and are electrophysiological intact. Puerarin treatment shifted the cardiac phenotype from pacemaker-like cells to ventricular-like cells, which were Mlc2v-positive and present typical ventricular-like AP. Puerarin up-regulated transcripts involved in cardiac differentiation and ventricular specialization of ES cells. **Conclusion:** Our results suggest that puerarin promotes cardiac differentiation, and significantly enhances the specialization of mES cells into ventricular-like CMs. Puerarin may be used to increase the yield of ventricular mES-CMs during *in vitro* differentiation.

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Introduction

Currently, cardiomyocytes (CMs) derived from embryonic stem (ES) cells (ES-CMs) or from induced pluripotent stem cells generated by the reprogramming of somatic cells represent the most promising cell sources to treat incurable cardiac diseases [1]. These pluripotent stem cell-derived CMs have been proven to possess all electrophysiological properties of typical cardiac cell [2]. Since myocardium infarction high frequently takes place in the ventricles but not the atriums, it is crucial to drive the differentiation of pluripotent stem cells into ventricular-like CMs to restore the mechanical function of injured ventricles. However, pluripotent stem cell-derived CMs are the mixture of pacemaker-, ventricular-, and atrial-like cells [3]. Therefore, screening and identifying chemical compounds that induce high efficiency cardiac differentiation and ventricular specialization of pluripotent stem cells is one of major steps toward a cell-mediated replacement therapy.

It has been shown that multiple signaling events are involved in the cardiogenesis of ES cells, including the transforming growth factor-beta (TGF β), bone morphogenetic protein (BMP) and Wnt signaling pathways [4]. So far, proteins from TGF β , BMP, and Wnt families [4-6], cytokines [7], natural or synthetic chemical compounds [3, 8-11]; and several transgenic ES cell systems [12] have been applied to selectively enhance the efficiency of cardiac differentiation from ES cells. Among these reports, retinoic acid and transgenic ES cell system are highlighted by their effects on ventricular specialization of ES cells. However, the disadvantages of these methods must be carefully considered. The multiple potent biological effects of cytokines might lead to unexpected side effects, and the safety of transgenic ES cells is questionable. Therefore, nature, economical and safe chemical compound such as traditional Chinese medicine might be more advantageous for protection or differentiation of working myocytes.

Puerarin [7-hydroxy-3-(4-hydroxyphenyl)-1-benzopyran-4-one 8-(β -D-Glucopyranoside)] is the main isoflavone glycoside found in the herb radix pueraria or pueraria lobata (Willd.), which has been used in traditional Chinese medicine for thousands of years [13]. It has been demonstrated that puerarin has beneficial and protective effects on cardiovascular diseases, including unstable angina [14], acute myocardial infarction [15] and myocardial reperfusion injury [16]. It has been shown that puerarin promotes the *in vitro* osteoblasts differentiation in osteoblast-like UMR106 cells [17] and induces adipocyte differentiation of 3T3-L1 preadipocytes [18]. However, little is known about the pharmacological effects of puerarin on the cardiac differentiation of pluripotent stem cells.

In the present study, we investigated the effect of puerarin on the *in vitro* cardiac differentiation and ventricular specialization of murine ES cells. Our findings suggest that continuous application of puerarin increases the yield of functional ventricular CMs derived from murine ES cells. This study describes a new insight into the various biological effects of puerarin on cardiac differentiation of pluripotent stem cells.

Materials and Methods

Cell culture and differentiation

Undifferentiated murine ES cells from line D3 (CRL-1934, ATCC, USA) were grown on mouse embryonic fibroblasts inactivated by mitomycin C treatment (Kyowa Hakko Kogy) in propagation medium composed of Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum (FBS), 100 units/mL penicillin-streptomycin, 1% non-essential amino acids (NEAA), 2 mmol/L L-glutamine, 0.1 mmol/L β -mercaptoethanol, and 1000 IU/mL leukemia inhibitory factor (ESGRO, Chemicon International). The differentiation of ES cells was induced by the generation of embryoid bodies (EBs) using the hanging drop method as previously described [11] with differentiation culture medium composed of Iscove's modified Dulbecco's medium (GIBCO), 20% FBS, 0.1 mmol/L β -mercaptoethanol, 1% NEAA, 2 mmol/L L-glutamine, 100 units/mL penicillin-streptomycin. Puerarin (1 μ mol/L, 10 μ mol/L, 100 μ mol/L, 1000 μ mol/L, Drug Biology Product Examination Bureau, Beijing, China) was added from day 0 to day 16. EBs that were cultured

Table 1. Primers for RT-PCR analysis. F: forward and R: reverse

Gene	Primer sequence (forward/reverse)	Ta (°C)	No. of cycles	Product size (bp)
α-MHC	F: ACCTGGGCAAGTCTAACAAAC R: CTGGATTCTGGTGATGATACG	58	30	704
GATA4	F: TCTCACTATGGGCACAGCAG R: GCGATGTCTGAGTGACAGGA	55	35	136
Nkx2.5	F: CAAGTGCTCTCCTGCTTTCC R: GGCTTTGTCCAGCTCCACT	55	35	137
Mef2c	F: GGCCATGGTACACCGAGTACAACGAGC R: GGGGATCCCTGTGTACCTGCACTTGG	63	30	381
Mlc2v	F: TGTGGGTACCTGAGGCTGTGGTTCAG R: GAAGGCTGACTATGTCCGGGAGATGC	63	35	189
HCN4	F: GCTGGTTGTGGTAGATTTC R: AAACATCTTGCCTTGGTAGC	55	35	674
Tbx5	F: CACAGCCCTTCAGCAGCGAGAC R: AGGGGCCCCGAGGTGAAATGAG	63	30	495
TGFβ1	F: GCAACATGTGGAAGCTTACCAGAA R: GACGTCAAAGACAGCCACTCA	55	35	106
TGFβ2	F: ATGGATGGCTTA AGG AGTTTGA R: AGATTGCCACTG ATT CCA AGTT	57	35	203
TGFβ3	F: GCTCTTCAGATACTTCGAC R: AGCAGTTCTCCTCCAGGTTG	55	35	442
Wnt3a	F: ATTGAATTTGGAGGAATGGT R: CTTGAAGTACGTGTAACGTG	54	40	318
Wnt11	F: GCCATGAAGGCCTGCCGTAG R: GATGGTGTGACTGATGGTGG	65	40	153
BMP2	F: GAATCAGAACAAGTCAGT R: GTTTGTGTTTGGCTTGACGC	55	30	272
BMP4	F: TGTGAGGAGTTTCCATCACG R: CAGCGAAGGACTGCAGGGCT	55	30	323
GAPDH	F: GTGTTCTTACCCCAATGTG R: CTTGCTCAGTGCCTTGC;TG	55	35	349

with differentiation medium containing 0.05% dimethyl sulfoxide (Sigma) served as negative controls. Medium was changed every two days. Rhythmically beating areas in EBs were considered to be beating CMs and were defined as the marker of successful cardiac differentiation. EBs containing rhythmically beating areas were labeled as beating EBs. The number of beating EBs was counted from day 7 to day 16 of differentiation. For the observation of EB-size, EBs were kept and in suspension cultivation till day 16 and images were taken every day. All cultivation medium and other substances for cell culture were purchased from Gibco BRL if not otherwise indicated.

Quantification of cardiac differentiation by flow cytometry

EBs at day 12 of differentiation were dissociated to single cell suspensions by the addition of 0.05% Trypsin-EDTA (GIBCO) at 37 °C for 3 min and then 1 mg/mL collagenase B (Roche) at 37 °C for 20 min. One million cells per sample were fixed and permeabilized according to the protocol supplied by the manufacturer (BD Biosciences Pharmingen). Mouse IgG anti-α-sarcomeric-actinin antibody (A7811, 1:100, Sigma) served as the primary antibodies. The secondary antibody was sheep anti-mouse R-Phycoerythrin-conjugated IgG (P8547, 1:100, Sigma). Cells stained with primary isotype control antibody and secondary fluorescence-conjugated antibody served as a control. The percentage of α-actinin-positive cells was obtained by subtracting negative control after gating out the dead cells. Three independent experiments were performed with the Aria Cell Sorter (BD Biosciences).

Semi-quantitative RT-PCR and quantitative real-time PCR analysis

Total RNA was extracted from EBs using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions at day 4, 6, 8, 10 and 16 of differentiation. Reverse transcription was performed with ReverTra Ace reverse transcriptase (Toyobo, Japan). The primers, annealing temperature, product size, and number of RT-PCR cycles are depicted in Table 1.

Quantitative real-time PCR (qRT-PCR) was performed with 1.6 μL of cDNA template added to SYBR Premix Ex Taq (TaKaRa Biotechnology) containing specific primers on the MxPRO3000 Real-Time PCR machine (Stratagene Technology Company), as described before [11]. The primers and product size are depicted in Table 2. The expression of each gene was defined from the threshold cycle (Ct). After

normalization with reference to expression of the housekeeping gene GAPDH, relative expression levels were calculated by using the $2^{-\Delta\Delta Ct}$ method and normalized by control group.

Electrophysiological recordings

CMs isolated from EBs at day 16 of differentiation were plated on sterile, 0.1% gelatin-coated glass coverslips and incubated at 37°C for 24 to 36 h before the experiments. The action potentials (APs) of the ES-CMs were recorded from spontaneously beating ES-CMs using the whole-cell patch clamp under current clamp mode as described previously [11]. The data were acquired and analyzed off-line using the Clampfit 9.0 analysis software (Axon Instruments). The APs were

classified using previously described criteria [3, 11]. To detect the adrenergic and muscarinic regulation of CMs, isoproterenol (Iso, 1 μ M; Sigma) or carbachol (Cch, 1 μ M; Sigma) was added to the Tyrode's solution.

Immunofluorescent staining

Cells were fixed with 4% paraformaldehyde, and permeabilized with 1% Triton X-100 for 5 min. After being treated with 5% bovine serum albumin, specimens were incubated with monoclonal anti-sarcomeric-actinin (1:100, Sigma) or polyclonal rabbit anti-ventricular-specific gene myosin light chain-2v (Mlc-2v, 1:100, Abcam). The fluorescent antibody was Anti-Mouse-Ig-phycoerythrin for (1:100, Sigma) or FITC-conjugated goat anti-rabbit IgG (1:100, Proteintech Group). Nuclei were stained with DAPI (Vector Laboratories). Images were taken from randomly chosen field. The percentage of Mlc-2v-positive cells was calculated by dividing the number of Mlc-2v double positive cells to the total number of cells and normalized by control group. The counting was performed independently by three operators.

Statistical analysis

Unless otherwise stated, the results were given as the mean $\pm$ SEM for at least three independent experiments. The significance of differences between the means was evaluated by student's unpaired t-test or ANOVA. A value of $P < 0.05$ was used as the criterion for statistical significance.

Results

Puerarin enhances the percentage of ES-CMs in beating EBs

The percentage of beating EBs was significantly increased at day 12 of differentiation by application of high concentration (100 μ mol/L and 1000 μ mol/L) but not low concentration of puerarin (1 μ mol/L and 10 μ mol/L) (Fig. 1A). 100 μ mol/L of puerarin was chosen for the following experiments. The percentage of beating EBs progressively increased and reached a plateau from day 12 to day 16 in both groups, but was significantly higher in the puerarin treated group during the same period (Fig. 1B). As differentiation progressed, the strength of contraction of beating areas increased and peaked on day 16. At day 16 of differentiation, $81.4 \pm 3.5\%$ of EBs treated with puerarin exhibited spontaneous beating compared to $60.7 \pm 7.5\%$ in control group ($n = 5$ independent experiment, $p = 0.0380$). The diameters of EBs in both groups progressively increased and were comparable during the suspension culture period (Fig. 1C).

The cardiac specific genes α -myosin heavy chain (α -MHC) was up-regulated at differentiation day 6 and 8 in puerarin treated EBs (Fig. 1D). ES-CMs were further identified

Table 2. Primers quantitative real-time PCR analysis. F: forward and R: reverse

Gene	Primer sequence (forward/reverse)	Product size (bp)
α -MHC	F: GATTTCTCCAACCCAGCTGCGCC R: CGGATGTCAAAGGGCCGGGTC	201
Mlc2v	F: TCCCGAGGGCAAAGGGTCACT R: CGTCAGGGGGAAGGCTGCG	116
HCN4	F: CCCGCCTCATTGATACATTTCAT R: GAGGGCGTAGGAATACTGCTTC	233
Mef2c	F: TGTCCAGCCATAACAGTTTGG R: GGTGCGGTATCCATTCCCT	210
Tbx5	F: TGGCTGAAGTTCCACGAAGTG R: TTTGGGATTAAGGCCAGTCAC	187
TGF β 1	F: CCGCAACAACGCCATCTATG R: CCCGAATGTCTGACGTATTGAAG	80
TGF β 2	F: ATGGATGGCTTAAGGAGTTTGA R: AGATTGCCACTGATTCCAAGTT	203
TGF β 3	F: GGACTTCGGCCACATCAAGAA R: TAGGGGACGTGGGTCTATC	111
GAPDH	F: TGACCACAGTCCATGCCATC R: GACGGACACATTGGGGGTAG	204

Fig. 1. Puerarin enhances the percentage of ES-CMs in beating EBs. **A.** Effects of different doses of puerarin on the percentage of beating EBs at day 12 of differentiation (n = 3). **B.** Percentage of beating EBs during differentiation (n = 5). **C.** Statistics analysis of diameters of EBs without (n = 87) or with puerarin treatment (n = 87). No significant difference could be found. **D.** RT-PCR and real-time PCR analysis of cardiac α -MHC transcript level at day 6, 8, and 16 of differentiation. The data were from three independent experiments. **E.** Immunofluorescent staining of cardiac α -actinin (red) in control group (left) and puerarin treated group (right) at day 12. Nuclei were stained with DAPI (blue). Scale bar = 50 μm . **F.** The representative data of flow cytometric analysis at day 12 of differentiation. α -actinin-positive cells are shown in quadrant 1 (Q1). **G.** Flow cytometric analysis shows the percentage of α -actinin-positive cells in the total population of cells of EBs derived from the ES cells at day 12 of differentiation (n = 3). All Data are presented as mean $\pm$ SEM. *, p < 0.05 vs. control group. #, p < 0.01 vs. control group.

as cardiac α -actinin-positive cells (Fig. 1E). Flow cytometric quantification of the cardiac α -actinin-positive cells revealed that the percentage of α -actinin-positive cells was significantly higher in the puerarin treatment group ($15.3 \pm 1.9\%$) than in the control group ($8.2 \pm 1.2\%$) (n = 3, p = 0.0349) at day 12 (Fig. 1F and G). These data suggest that puerarin enhances the percentage of ES-CMs in beating EBs.

CMs derived from puerarin-treated ES cells are electrophysiological intact and their phenotypes are shifted from pacemaker-like cells to ventricular-like cells

We next investigated the electrophysiological properties of puerarin induced ES-CMs by patch-clamp. The APs of 16-day-old ES-CMs in both groups were recorded and characterized using patch-clamp analysis. Based on the criteria described previously [3, 11], the APs were classified as ventricular-like, atrial-like or pacemaker-like. Ventricular-like APs were characterized by a rapid up stroke velocity (about 50V/s), a longer duration (the action potential duration measured from the maximal depolarization to 90% repolarization (ADP90) is around 110 ms), a larger amplitude (around 95mV) and a pronounced plateau

Fig. 2. CMs derived from puerarin-treated ES cells are electrophysiological intact A. Three major types of APs were discriminated in the puerarin treatment ES-CMs: pacemaker-like, atrial-like and ventricular-like APs. B. The relative percentage of the AP types recorded from the control and puerarin treatment cultures. 54 of puerarin-induced ES-CMs and 46 of control ES-CMs from 6 independent differentiations were randomly selected and recorded. C and D. Typical response of mES-CMs in the control group (upper) and the puerarin treated group (low) to Iso (C) and Cch (D).

Parameters	Control (n=46)			Puerarin (n=54)		
	p-like (n=18)	a-like (n=23)	v-like (n=5)	p-like (n=14)	a-like (n=28)	v-like (n=12)
APD90 (ms)	75.3±5.6	100.9±7.4	110.5±12.5	105.0±3.1*	102.8±5.3	136.4±21.4
APA (mv)	66.2±2.8	77.3±3.3	98.0±1.7	71.4±2.5	79.6±2.7	95.6±3.0
MDP (mv)	-52.4±2.1	-55.9±2.8	-60.1±1.5	-53.0±2.1	-55.3±1.4	-60.4±1.3

Table 3. Electrophysiological properties of puerarin-untreated (control) and puerarin-treated ES cell-derived cardiomyocytes at day 16 of differentiation. Data were expressed as mean ± SE. The electrophysiological parameters were used for the identification of the different cardiac cell types: APD90 is the action potential duration measured from the maximal depolarization to 90% repolarization in ms, APA is action potential amplitude and MDP is maximum diastolic potential. No statistical difference in the electrophysiological parameters between puerarin-treated and puerarin-untreated cells could be observed, with the exception of the ADP90 in pacemaker-like cells. *, $p < 0.05$ vs. control

phase. Cells with ventricle-like APs displayed a more negative maximum diastolic potential (MDP, around -60mV) compared to other cell types. Atrial-like APs were characterized by their short duration (ADP90 around 100ms), combined with a fast up stroke velocity (about 25 v/s) and a short plateau phase. Pacemaker-like APs were characterized by a prominent diastolic depolarization, a slow upstroke (about 8 v/s) and a smaller amplitude (about

Fig. 3. Puerarin shifts the phenotype of ES-CMs from pacemaker-like cells to ventricular-like cells. **A.** RT-PCR analysis of the mRNA levels of ventricular specific Mlc2v and pacemaker channel gene HCN4 in the EBs during differentiation. **B.** Real-time PCR analysis of Mlc2v mRNA levels in the EBs at days 6, 8 and 16 of differentiation. $n = 3$ independent experiments. **C.** Real-time PCR analysis of HCN4 revealed that puerarin up-regulated HCN4 transcript at day 8 but down-regulated it at day 16. $n = 3$ independent experiments. **D.** Representative immunofluorescent staining of Mlc2v (green) in control group (upper) and puerarin treated group (low) at day 16. Nuclei were stained with DAPI (blue). Scale bar = 50 μ m. **E.** The statistic analysis of normalized percentage of Mlc2v-positive cells by control group. The data are showed as mean $\pm$ SEM, *, $p < 0.05$. #, $p < 0.01$.

66mV). The MDP of pacemaker-like APs was about -52mV. Typical APs of three phenotypes of CMs could be recorded in both the control group and the puerarin treatment group (Fig. 2A). Interestingly, puerarin treatment doubled the percentage of ventricular-like cells, with a paralleled decrease of pacemaker-like cells. There was no effect on the percentage of atrial-like cells (Fig. 2B). In 54 of randomly selected puerarin-induced ES-CMs (from 6 independent differentiation experiments), 22.2% of cells presented ventricular-like APs, 51.9% atrial-like APs, and 25.9% pacemaker-like APs. In the control group, only 10.9% of ES-CMs (5 of 46) presented ventricular-like APs, 50.0% (23 of 46) atrial-like APs, and 39.1% (18 of 46) pacemaker-like APs. The ES-CMs in both groups had typical and comparable responses to Iso (1 μ M) (Fig. 2C) and Cch (1 μ M) (Fig. 2D), demonstrating their well established β -adrenergic

Fig. 4. The effect of puerarin on the expression of cardiogenesis signaling pathways and cardiac transcription factors. A: Representative data from RT-PCR for TGFβ1/β2/β3, Wnt3a/11, BMP2/4 and indicated cardiac transcription factors without (upper, C=control group) or with puerarin treatment (lower, P=puerarin treatment group). B: Real-time PCR analysis of TGFβ1/β2/β3, Tbx5 and Mef2c transcript levels in the EBs at day 6 of differentiation. C: Real-time PCR analysis revealed that the expression level of Mef2c was up-regulated by puerarin at day 8 of differentiation. D: Real-time PCR analysis showed that puerarin did not affect the expression levels of TGFβ1/β2/β3 and Mef2c at day 16 of differentiation. The data are shown as means ± SEM; n = 3 independent experiments, *, p < 0.05 vs. control group.

receptor and muscarinic acetylcholine receptor signaling pathways. Moreover, as shown in Table 3, the parameters of AP were unaffected by puerarin treatment, except for the APD90. The pacemaker-like ES-CMs had a significantly longer APD90 in puerarin treated group (105.0 ± 13.1 ms, n=14) compared with control group (75.3 ± 5.6 ms, n=18; p<0.05).

We next studied the effect of puerarin on the expression levels of the Mlc-2v, and pacemaker channel gene potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 4 (HCN4) by RT-PCR and real-time PCR. Consistent with our patch clamp results, we found that puerarin treatment doubled the transcript level of ventricular-specific Mlc-2v at day 6 and day 16 of differentiation, but decreased that of pacemaker channel gene HCN4 at day 16 (Fig. 3A, B and C). Moreover, semi-quantitative analysis of immunofluorescent staining showed that Mlc-2v-positive cells at day 16 were almost doubled by puerarin treatment (Fig. 3D and E).

These data demonstrate that puerarin shifts the cardiac phenotype from pacemaker-like cells to ventricular-like cells, and facilitates ventricular specialization.

Puerarin up-regulates transcripts involved in cardiac differentiation and ventricular specialization of ES cells

The cardiac differentiation of ES cells is regulated by TGF-β, BMP and Wnt signaling pathways. These signaling pathways interact with each other by affecting downstream cardiac transcription factors including NK 2 transcription factor related locus 5 (Nkx2.5), GATA binding protein 4 (GATA4), T-box 5 (Tbx5) and myocyte enhancer factor 2c (Mef2c)

[4-6]. Therefore, we next focused on signaling pathways that regulate cardiac differentiation and downstream cardiac transcription factors by RT-PCR and qRT-PCR.

Puerarin treatment significantly affected the mRNA levels of TGF β family at day 6 of differentiation, but almost had no effects on Wnts and BMPs (Fig. 4A). QRT-PCR further confirmed that puerarin treatment significantly increased the mRNA level of TGF β 2 at day 6, but not TGF β 1 and TGF β 3 (Fig. 4B, C and D). Transcripts of the downstream cardiac transcription factor Tbx5 and Mef2c were up-regulated by puerarin at day 6 and day 8 of differentiation, respectively (Fig. 4A, B and C). GATA4 and Nkx2.5 showed no remarkable differences in transcript abundance between puerarin and control variants (Fig. 4A).

Discussion

Puerarin has been reported to possess antihyperglycemic, antihypercholesterolemia and antioxidant activities [19-21]. Key findings of the present study are: (1) continuous puerarin treatment significantly increases population of ES-CMs which express typical cardiac markers and are electrophysiological intact; (2) puerarin treatment shifts the cardiac phenotype from pacemaker-like cells to ventricular-like cells, which are Mlc2v-positive and present typical ventricular-like AP; (3) puerarin up-regulates transcripts involved in cardiac differentiation and ventricular specialization of ES cells.

So far, the effects of reported natural or synthetic cardiomyogenesis enhancers are quite different due to different culture conditions between lab to lab, different observation timepoints, and different cell lines. In the present study, a 20% increase in the percentage of beating EBs by puerarin treatment was observed from day 12 to day 16 of differentiation, which is comparable with effects of retinoic acid [3] and neuregulin 1 [7] on the D3 murine ES cells. However, the percentage of beating EBs is widely considered as a very crude index of the efficiency of *in vitro* cardiomyogenesis. Alternatively, the percentage of cardiac specific marker-positive CMs by flow cytometry analysis is a fairer criterion to demonstrate the effect of cardiomyogenesis agents. In our system, puerarin treatment harvested about 7% more α -actinin-positive CMs derived from D3 murine ES cells at day 12 of differentiation compared with control group. Comparable with our report, Yee-Ki Lee et al. found that ouabain facilitates cardiac differentiation with a 7% increase in the percentage of cardiac troponin I (cTnI)-positive CMs derived from D3 murine ES cells at day 9 of differentiation [9]. However, Wiese et al. reported that suramin has no effect on the percentage of cTnI-positive CMs derived from R1 murine ES cells at day 9 of differentiation, but increases the number of cTnI-positive CMs by 5.6 fold at day 22 [10]. Previously our group reported that baicalin treatment has no effect on the percentage of beating EBs and α -actinin-positive CMs derived from D3 murine ES cells at day 10 and 16 of differentiation. Nevertheless, baicalin results in a 70% increase in the percentage of beating EBs and a 10% increase in the percentage of α -actinin-positive CMs at day 20 of differentiation by inducing cell death of non-CMs in EBs [11]. With regard to synthetic chemical compounds, the reporters employed the quantification of the expressed EGFP from CMs derived from transgenic α MHC C123 murine ES cells to estimate their effects on cardiac differentiation. In their study, synthetic chemical compounds result in a 50 to 80% increase in cardiomyogenesis compared to untreated cells at day 14 of differentiation [8], which seems to be more efficient than puerarin.

Both human and murine pluripotent stem cells differentiate and develop into a heterogeneous population of CMs *in vitro* [3, 10]. Ventricular myocytes, atrial myocytes and pacemaker cells are different from each other on both morphological, molecular, and functional properties [22]. Mlc2v is highly expressed and spatially restricted to the ventricular portion of the murine heart tube and the outflow tract, with no detectable expression in the atrial or sinus venosus regions [23, 24]. Therefore, Mlc2v has been used as a molecular marker for the process of ventricular specialization during *in vitro* cardiogenesis [3]. In contrast, HCN4 is highly expressed in sinus node and is required for mature cardiac pacemaker activity [25]. Interestingly, in the present study, we found that puerarin treatment

shifts the cardiac phenotype from pacemaker-like cells to ventricular-like cells, which are Mlc2v-positive and present typical ventricular-like AP.

Numerous signaling events are involved in the cardiogenesis of ES cells [26]. These signaling pathways regulate cardiac differentiation by affecting downstream transcription factors and structural genes [27]. *In vivo*, development and formation of the chambered heart are determined by cooperation and interaction of many transcription factors and structural genes [22]. Tbx5 belongs to the Tbx superfamily and is necessary for proper septal [28], cardiac conduction system and ventricular chamber [27] formation. It specifies the identity of the left ventricle through tight interactions the heart-specific factors Nkx2.5, GATA4 and Mef2c [29]. Mef2c is another fundamental gene in ventricular differentiation [30]. It has been shown that functional interaction between Tbx5 and Mef2c is crucial in ventricular differentiation [31].

In the present study, we observed that puerarin up-regulated transcript level of TGFβ2 at differentiation day 6, but had no effect on expressions of TGFβ1, TGFβ3, Wnts and BMPs. Consistent with our results, previous report demonstrates that TGFβ2, but not TGFβ1 or TGFβ3, promotes cardiac myocyte differentiation from murine CGR8 ES cells [32] and enhances cytoprotective factors released from ES cells [33]. We only found that puerarin affected expression of Tbx5 and Mef2c, but not Nkx2.5 and GATA4. These results indicate that puerarin might affect cardiac differentiation via TGFβ signaling pathway. Up-regulation of TGFβ2, Tbx5 and Mef2c transcripts might play roles in puerarin-induced cardiac differentiation and ventricular specialization. Further studies are suggested to better understand the role of TGFβ2 and TGFβ signaling pathway in puerarin-induced ventricular specialization of ES cells.

In addition to transcriptional regulation, posttranscriptional control by micro RNA (miRs) also quantitatively affects the differentiation [34] and ventricular specification [35] *in vitro* and *in vivo* [36]. It has been shown that miR-1, miR-133, and miR-499 play crucial roles in cardiogenesis. MiR-1 and miR-133 are cardiac and skeletal muscle specific miRs, which are transcriptionally controlled by MyoD and Mef2c [37, 38]. MiR-1 promotes mesoderm formation from ES cells and promotes further differentiation into cardiac muscle progenitors [34]. *In vivo*, lacking miR-1 results in ventricular septal defects and cardiac dysfunction in mice [36]. MiR-499 has been proven to be crucial to cardiac differentiation and ventricular specification of human ES cells [35, 39]. Recently, it is reported that miR-27 plays a role in mouse ventricular maturation by modulating Mef2c expression [40]. Collectively, we hypothesize that these miRs might be involved in puerarin-induced cardiac differentiation and ventricular specification. Further studies of miRs in our system are needed.

This study provides the first report of the effect of puerarin on the cardiac differentiation of ES cells. Our results suggest that long-term puerarin treatment promotes cardiogenesis and enhances the specialization of mES cells into ventricular-like cells. Additional work is needed to further confirm its effects on human pluripotent stem cells.

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