Wang et al.: Puerarin and Structural Maturation of Cardiomyocytes

Cellular Physiology and Biochemistry

Puerarin Facilitates T-Tubule Development of Murine Embryonic Stem Cell-Derived Cardiomyocytes

Lu Wang\textsuperscript{a}  Yurong Cui\textsuperscript{a}  Ming Tang\textsuperscript{a}  Xinwu Hu\textsuperscript{a}  Hongyan Luo\textsuperscript{a}  Jürgen Hescheler\textsuperscript{b}  Jiaoya Xi\textsuperscript{a}

\textsuperscript{a}Department of Physiology and Chinese-German Stem Cell Center, Tongji Medical College, Huazhong University of Science and Technology, The Key Laboratory for Drug Target Researches and Pharmacodynamic Evaluation of Hubei Province, Wuhan, China; \textsuperscript{b}Institute for Neurophysiology, University of Cologne, Cologne, Germany

Key Words
Puerarin • Murine embryonic stem cells • Cardiomyocytes • Ultrastructure • T-tubule

Abstract
Aims: The embryonic stem cell-derived cardiomyocytes (ES-CM) is one of the promising cell sources for repopulation of damaged myocardium. However, ES-CMs present immature structure, which impairs their integration with host tissue and functional regeneration. This study used murine ES-CMs as an in vitro model of cardiomyogenesis to elucidate the effect of puerarin, the main compound found in the traditional Chinese medicine the herb \textit{Radix puerariae}, on t-tubule development of murine ES-CMs. Methods: Electron microscope was employed to examine the ultrastructure. The investigation of transverse-tubules (t-tubules) was performed by Di-8-ANEPPS staining. Quantitative real-time PCR was utilized to study the transcript level of genes related to t-tubule development. Results: We found that long-term application of puerarin throughout cardiac differentiation improved myofibril array and sarcomeres formation, and significantly facilitated t-tubules development of ES-CMs. The transcript levels of caveolin-3, amphiphysin-2 and junctophilin-2, which are crucial for the formation and development of t-tubules, were significantly upregulated by puerarin treatment. Furthermore, puerarin repressed the expression of miR-22, which targets to caveolin-3. Conclusion: Our data showed that puerarin facilitates t-tubule development of murine ES-CMs. This might be related to the repression of miR-22 by puerarin and upregulation of Cav3, Bin1 and JP2 transcripts.
Introduction

Cell replacement is a novel therapeutic strategy for rational treatment of myocardium infarction. This strategy is transplanting suitable cells into the infarcted area of heart which can functionally integrate into the host tissue, and thus rescuing cardiac function [1]. Embryonic stem (ES) cells generated from blastocysts inner cell mass can differentiate into beating cardiomyocytes (CMs) in vitro, which have been widely accepted as a promising source for cell replacement therapy [1-4]. The typical structure of adult CMs is characterized by bundles of parallel myofilaments, sarcomeres with Z-disc, A-, I-, and H-band as well as the M-line, which are the structural basis of cardiac muscle contraction. Moreover, the transverse tubules (t-tubules), which emerge into deep invaginations of the plasma membrane of CMs and are rich in ion channels, are crucial for excitation-contraction coupling (ECC) of CMs [5]. Therefore, the mature structure of sarcomeres and t-tubules in ES cell-derived CMs (ES-CMs) are prerequisite for their function and integration in the host tissue after transplantation [6].

However, accumulated transplantation studies have shown that only partial or transient restoration of electrophysiological and contractile function is observed after transplantation of ES-CMs or induced pluripotent stem cell derived CMs [7, 8]. One of potential reasons might be related to the underdeveloped structure of ES-CMs and immature contractile properties. It has been reported that comparing with adult CMs, both human and murine ES-CMs (hES-CMs and mES-CMs) exhibit immature structural features, such as irregularly organized myofibril, early-stage sarcomeres with nascent Z-discs and I-band, but lack of M-line [9-11]. In addition, the t-tubules formation-related genes caveolin-3 (Cav3) and amphiphysin-2 (Bin1) are absent in both h and mES-CMs [12], thus resulting in the absence of organized t-tubules and unsynchronized Ca\(^{2+}\) transient [10, 12] and impaired contractile properties. Obviously, the immature structure of ES-CMs will lead to poor contractile function, thus hampers their integration with the host cells.

Recently, transgenic technique [13], electrical stimulation [14] and cytokines application [7] had been used to promote in vitro maturation of ES-CMs, but most of these studies had focused mainly on contractile and electrophysiological properties. It is still unknown whether the methodological modifications mentioned above also have parallel effect on the structural development of ES-CMs. Additionally, the safety and convenience of these methods need to be fully considered. Safer and more economical approaches to drive structural maturation of ES-CMs in vitro are still needed.

Puerarin (7, 4′-dihydroxy-8-β-D-glucosylisoflavone, C\(_{21}\)H\(_{20}\)O\(_{9}\)) is a traditional Chinese medicine extracted from the herb *Radix puerariae*, and wildly used for treatment cardiovascular diseases such as myocardial infarction [15], arrhythmic [16] and ischemic [17] in China. Our previous study found that puerarin enhances the cardiac differentiation and ventricular specialization of mES cells [4]. In this study, we tested the hypothesis that structural organization of mES-CMs would be improved by long-term application of puerarin throughout cardiac differentiation in vitro. Our findings suggest that puerarin facilitates t-tubule development of mES-CMs.

Method and Materials

Culture of mES-CMs

The mES cell line D3 (ATCC, USA) and its transgenic cell line αPIG (clone 44) were cultured and differentiated into spontaneously beating CMs as previously described [3]. Briefly, the embryoid bodies (EBs) were generated using the hanging drop method in Iscove’s Modified Dulbecco’s Medium supplemented with 20 % fetal bovine serum, 1 % non-essential amino acids, 2 mM L-glutamine, 100 units/mL penicillin-streptomycin, and 0.1 mM β-mercaptoethanol.

For cardiac differentiation of transgenic mES cells, puromycin (10 μg/mL) was added into the medium for purification of CMs at day 9 and day 12 of differentiation. Puerarin (100 μM, National Institutes For Food and Drug Control, China), which dissolved in 0.05 % dimethyl sulfoxide (DMSO, Sigma, USA), was applied
from day 0 to day 20 as previously described [4]. EBs cultured with differentiation medium containing 0.05% DMSO were served as controls. Medium was changed every two days. All the following experiments were performed by using wild type mES cells (D3) if not otherwise indicated. All cultivation medium and reagents were purchased from Gibco if not otherwise indicated.

**Isolation of mES-CMs and embryonic ventricular CMs**

Spontaneous beating areas were mechanically microdissected at day 16 and 20 of differentiation, and were dissociated into single cells by incubation in collagenase II (1 mg/ml, Roche, Germany) at 37 °C for 30 min. Cells were then plated onto gelatin-coated glass coverslips, and cultured with culture medium without puerarin and DMSO for at least 24h for further experiments. For preparation of embryonic ventricular CMs, pregnant mice (Kunming mice provided by the Center of Animal Experimentation of Tongji Medical College, Huazhong University of Science and Technology, China) were sacrificed after coitum 16.5 days (E16.5). The ventricles of the embryonic hearts were isolated, dissected, and enzymatically dissociated into single cells as previously described [18].

**Transmission electron microscope (TEM)**

Clusters of mES-CMs purified by puromycin were collected at day 16 and 20 of transgenic mES differentiation, and immediately fixed by 2.5% glutaraldehyde in 0.1 M PBS. They were then incubated in buffered 1% osmium tetroxide for 2 h, dehydrated in graded ethanol series and embedded in Epon. Ultrathin sections were cut and stained with uranyl acetate and lead citrate. The sections were observed under a transmission electron microscope (FEI, Hillsboro, USA).

**T-tubules fluorescent staining and score**

Fluorescent t-tubule staining was employed to further study the morphology of t-tubule according to previous reports [12, 13]. Single beating CMs with or without puerarin treatment at day 16 and 20 of differentiation were acquired from 7 and 8 independent differentiations, respectively. Then cells were incubated with the lipophilic fluorescent indicator di-8-aminonaphthylethenylpyridinium (Di-8-ANEPPS, 5 μM; Invitrogen, USA) for 10 min at 37 °C and then washed by PBS for 15 min. Images were then randomly taken at relative midplanes of cell height by screening through Z axis of fluorescence microscopy (Olympus, Japan) using 488 nm excitation light with detection at > 505 nm.

To describe the effect of puerarin on morphology of t-tubule, a score was defined. Murine embryonic ventricular CMs at E16.5 served as positive cells, which presented an organized array of bright red spots in the cellular periphery and mideplane. As shown in Table 1, CMs presenting similar pattern of bright red spots as positive cells were scored as “CMs with developing t-tubule”, otherwise scored as “CMs without t-tubule”. T-tubules score of total 1782 mES-CMs with or without puerarin treatment, which acquired from 7-8 independent differentiations, was performed independently by two operators and the percentage of “CMs with developing t-tubule” was calculated.

### Table 1. The definition of t-tubule score

| Score                      | Pattern                                      | Representative image |
|----------------------------|----------------------------------------------|----------------------|
| CMs without t-tubule       | CMs without any bright red spots in the cellular periphery and mideplane |
| CMs with developing t-tubule| CMs with bright red spots in the cellular periphery and mideplane |
Quantitative real-time PCR analysis

Total mRNA was extracted from EBs using TRizol Reagent (Invitrogen, USA) according to the manufacturer’s instructions. cDNA was synthesized. Real-time PCR was performed in 96-well plates in triplicates using MxPRO3000 detector (Strata gene Technology Company, USA) with SYBR Green Real-time PCR Master Mix plus (Toyobo, Japan) for relative quantification of genes. The transcript level of GAPDH was used for internal normalization. The primers are listed in Table 2. Bulge-Loop™ microRNAs (miRs) primer for U6 and miR-22 (RIBOBIO, China) were used according to the manufacturer’s protocols. U6 was used as a normalization control. The relative quantification of PCR products was performed according to the 2^(-ΔΔct) method and normalized by control.

Statistics

Results are expressed as mean ± SEM. Statistical significance of difference was analyzed using the unpaired t test if not otherwise indicated. Statistical significance was accepted when p < 0.05.

Results

Puerarin improves the myofibrillar alignment and sarcomere development of mES-CMs

We firstly observed total 72 cells by TEM to investigate whether puerarin influences the ultrastructural features of mES-CMs. At day 16 of differentiation, myofilaments in the majority of puerarin-treated mES-CMs were well organized. Sarcomeric pattern showed relatively mature Z-discs and clear I-bands (Fig. 1B). However, the majority of untreated cells displayed an early-stage ultrastructural phenotype [9-11], which were characterized by misaligned myofilbrils at low density and dispersive nascent Z-discs (Fig. 1A). Moreover, the percentage of cells with Z-discs was almost doubled in the puerarin treatment group compared with the control group. 37.5 % of control cells (9 out of 24 cells) presented misaligned myofilbrils at low density and dispersive nascent Z-discs, 62.5 % of control cells (15 out of 24 cells) only had low density of misaligned myofilbrils without Z-discs. However, 66.7 % of puerarin-treated cells (10 out of 15 cells) presented well-organized myofilaments and relatively mature Z-discs (Fig. 1E). In addition, the percentage of cells with sarcomeres was also higher in the puerarin treatment group compared to the control group. Sarcomeres were present in 8.3 % of control cells (2 out of 24 cells), while present in 53.3 % of puerarin-treated cells (8 out of 15 cells) (Fig. 1F).

At day 20, further developed structure of mES-CMs was observed. More cells presented sarcomeres, and similar sarcomeres with better-organized myofilbrils, Z-discs, and I-bands were presented in both two groups of cells (Fig. 1C and D). Besides, H-bands were faint, and observed frequently in the puerarin-treated cells, but observed occasionally in untreated cells. In the control group, 63.2 % of cells (12 out of 19 cells) presented better-organized myofilbrils and mature Z-discs (Fig. 1E), and 31.5 % of cells (6 out of 19 cells) presented better organized sarcomeres (Fig. 1F). In the puerarin treatment group, 71.4 % of cells (10 out of 14 cells) presented better organized myofilbrils and mature Z-discs (Fig. 1E), and 57.1 % of cells (8 out of 14 cells) presented better organized sarcomeres (Fig. 1F).
We also detected the intercellular junction development of mES-CMs in two groups. As shown in Fig. 2, similar intercellular junctions in the both groups were observed. Differentiated CMs of the both groups were mainly connected by desmosomes at day 16 (Fig. 2 A and B). At day 20, the nascent intercalated discs formed.
by desmosomes, fascia adherens and gap junctions were observed in mES-CMs of the both groups (Fig. 2C and D).

Taken together, these data suggest that puerarin facilitates the development of myofibril and sarcomeres of mES-CMs, but might not affect the intercellular junction.

**Puerarin promotes the development of t-tubules of mES-CMs**

By TEM, t-tubules occurring at Z-line were observed occasionally in mES-CMs of both groups at day 20 (Fig. 3A). To further indentify the presence of t-tubules, the membrane-selective lipophilic dye Di-8-ANEPPS was used to stain living 16-days-old and 20-days-old mES-CMs. Total 1782 mES-CMs with or without puerarin treatment were scored. As shown in Fig. 3B, murine embryonic ventricular CMs (E16.5) showed a relative organized array of bright spots in the cellular periphery and mideplane was observed. (C) The statistic analysis of effect of puerarin on the percentage of CMs with t-tubule by t-tubule score at day 16 (n = 7 of independent differentiations) and day 20 of differentiation (n = 8 of independent differentiations). Total 478 of 16-days-old control cells and 716 of puerarin-treated cells were scored. Total 254 of 20-days-old control cells and 334 of puerarin-treated cells were scored. Results were expressed as means ± SEM. **, p < 0.01 vs. control. Scale bars: 0.5 μm (A), 20 μm (B).
bright dots in both mideplane and periphery (lower), indicating the presence of developing t-tubules. In the control group, 25.28 ± 1.27% (n = 7 of independent differentiations) mES-CMs at day 16 and 29.57 ± 1.54% (n = 8 of independent differentiations) at day 20 were scored as "CMs with developing t-tubule". In the puerarin treatment group, we found a significantly higher percentage of mES-CMs scored as "CMs with developing t-tubule" at both day 16 (39.56 ± 1.02%, n = 7 of independent differentiations, \( p < 0.01 \) v.s control) and day 20 (39.93 ± 2.35%, n = 8 of independent differentiations, \( p < 0.01 \) v.s control) (Fig. 3C). These data imply that puerarin improves the development of t-tubules of mES-CMs.

**Puerarin upregulates transcript levels of Cav3, Bin1 and junctophilin-2 (JP2)**

To find out the possible reason how puerarin affects the t-tubule development in the present study, we next focused on the expressions of t-tubules formation-related gene Cav3 and Bin1 [19], and t-tubules development-related gene JP2 [20]. The transcript levels of Cav3 (\( p < 0.05, n = 5 \)) and JP2 (\( p < 0.01, n = 5 \), Fig. 4) were significantly increased in the puerarin-treated cells at day 16, and were upregulated about 2-3 folds at day 20. Bin1 transcript was increased at all three observed time points, with about 2.5 folds upregulation at day 10 (\( p < 0.01, n = 5 \), Fig. 4). These data suggest that puerarin might facilitate the development of t-tubules of mES-CMs via upregulation of Cav3, Bin1 and JP2 transcripts.

**Puerarin represses expression of miR-22 at day20**

Many data showed that posttranscriptional regulated by miRs also quantitatively affects the maturation of ES-CMs *in vitro* and *in vivo*. Cav3 gene has been proved to be one of the target genes of miR-22 [21]. We hypothesized that miR-22 might be involved in puerarin-induced upregulation of Cav3 in mES-CMs. As shown in Fig. 5, the expression pattern of miR-22 showed a development-dependent downregulation from day 16 to day 20 in both groups (\( n = 3, p < 0.01 \) v.s day16, Fig. 5). Moreover, the expression of miR-22 was significantly repressed in the puerarin treatment group at day 20 (\( n = 3, p < 0.05 \) v.s control, Fig. 5).
Discussion

In this study, our major findings are: (1) puerarin promotes the myofibrils and sarcomeres development of mES-CMs; (2) puerarin facilitates the t-tubule development of mES-CMs; (3) puerarin significant upregulates the t-tubules biogenesis-related genes Cav3, Bin1 and JP2; (4) puerarin downregulates miR-22 of mES-CMs, suggesting miR-22 might involve in the effect of puerarin on t-tubule development.

T-tubules of CMs are invaginations of the surface membrane occurred at the Z-discs, where amount of proteins involving in ECC such as L-type Ca\(^{2+}\) channel and sodium/calcium exchanger are localized [22]. T-tubules ensure the rapid spread of the electrical signal (action potential) to the cell central region triggering Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR), ultimately inducing myofilament contraction [23]. Studies from various groups have shown that t-tubules presented in adult ventricular CMs are absent in hES-CMs [12, 24]. HES-CMs lacking of t-tubules show poor Ca\(^{2+}\) handling properties, as reflected by smaller peak amplitude, slower rise, decay kinetics and nonuniform calcium dynamics across the cells [6, 25]. In addition, lack of t-tubules accounts for the spatial separation of L-type calcium channels located at t-tubule and RyRs located at SR, and results in the delay of calcium peak between cell periphery and center [12, 26]. By using TEM, Baharvand H [10] observed the presence of t-tubules at late stage of mES-CMs. Consistent with this report, we also detected the presence of t-tubules in mES-CMs at day 20 of differentiation. Furthermore, using Di-8-ANNEPPS live-staining, we confirmed that developing t-tubules were present in mES-CMs at both day 16 and day 20 of differentiation. Puerarin treatment significantly facilitated the development of t-tubules, which would benefit to the following contraction.

To further understand why puerarin facilitates t-tubules development, we measured the transcript levels of Cav3, Bin1 and JP2, which play crucial roles in the t-tubules formation and maturation. Cav3 is a key integral membrane protein with a hairpin structure involving in the biogenesis of t-tubule [27]. It plays an important role in cardiac calcium regulation [28]. Loss of Cav3 induces t-tubules abnormality in mice [29]. Bin1 localized in the t-tubules is another membrane-associated protein, which is thought to be an initiator in t-tubule formation [19]. Both of Cav3 and Bin1 play key roles in the biogenesis of t-tubules, and they are abundantly expressed in adult CMs [19], but absent in m and hES-CMs [12]. Emerging evidence indicates the important role of JP2, a protein anchoring the SR to t-tubules, in t-tubules development of CMs [20]. In the present study, puerarin upregulated Cav3, Bin1 and JP2 transcripts in mES-CMs differentiation stages, suggesting their contributions to the improvement of t-tubules development.

Recently, accumulated data showed that posttranscriptional control by miRs also quantitatively affects the development of ES-CMs in vitro and in vivo. MiR-22 is a muscle-enriched miR. Increasing evidence suggests that miR-22 integrates Ca\(^{2+}\) homeostasis and myofibrillar protein. Overexpression of miR-22 in mice has been shown to cause contractile dysfunction with reducing SR Ca\(^{2+}\) content and amplitude of Ca\(^{2+}\) transient as well as induced CMs hypertrophy [30]. Furthermore, the Cav3 gene is one of the target genes of miR-22 [21]. Overexpression of miR-22 leads to reduction of Cav3 gene expression level [21]. In the present study, we found that miR-22 dramatically decreased with development of mES-CMs. Consistent with the previous reports, we observed that puerarin repressed expression of miR-22, thereafter upregulated the transcript level of Cav3, demonstrating the role of miR-22 and Cav3 in puerarin-induced development of t-tubules.

It has been reported that during in vivo cardiogenesis, myofilaments were initially distributed in sparse, irregular myofibrillar arrays, then gradually matured into parallel arrays of myofibrils and eventually aligned into densely packed sarcomeres including Z-discs, I-, A-, H-bands and M-lines [31]. Recently, Lundy et al. reported that hES-CMs cultured for 3-4 month show greater myofibril density and alignment, and better organized sarcomeres compare with early-stage CMs [11]. The hES-CMs treated by combining three-dimensional cell cultivation with electrical stimulation exhibit maturer sarcomeric organization with H zones and I bands [14]. Here, we found that mES-CMs presented immature ultrastructure,
which is in agreement with previously reports, and puerarin-treated mES-CMs presented relatively maturer structure compared with control cells.

Of note, although signs of t-tubules formation induced by puerarin treatment were observed in this study, it is clear that further efforts are needed to drive full maturation. Further studies are also needed to know whether puerarin could affect the contraction function. Besides, the precise mechanisms underlying puerarin-induced ultrastructure and t-tubule development of mES-CMs remain unclear.

In conclusion, our results suggest that the long-term puerarin treatment facilitates structural maturation and t-tubule development of mES-CMs. These findings provide a new insight into the biological effects of puerarin on maturation of ES-CMs in vitro, and suggest the potential use of traditional Chinese medicine in driven structural maturation of functional CMs in vitro as well as in stem cell research.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (No.31100828), the Natural Science Foundation of Hubei Province (2011CDB363), the Project-sponsored by SRF for ROCS Jiaoya Xi, SEM, the Fundamental Research Funds for the Central Universities (HUST: 2013TS145).

Reference

1. Passier R, van Laake LW, Mummery CL: Stem-cell-based therapy and lessons from the heart. Nature 2008;453:322-329.
2. Xi J, Khalil M, Shishechian N, Hannes T, Pfannkuche K, Liang H, Fatima A, Haustein M, Suhr E, Bloch W, Reppel M, Saric T, Wernig M, Janisch R, Brockmeier K, Hescheler J, Pillekamp F: 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.
3. Tang M, Yin M, Liang H, Yu C, Hu X, Luo H, Baudis B, Haustein M, Khalil M, Saric T, Hescheler J, Xi J: Baicalin maintains late-stage functional cardiomyocytes in embryoid bodies derived from murine embryonic stem cells. Cell Physiol Biochem 2013;32:86-99.
4. Cheng Y, Wang L, Tang M, Yin M, Cui Y, Liang H, Song Y, Hu X, Luo H, Gao Y, Wang J, Hescheler J, Xi J: Effects of puerarin on cardiac differentiation and ventricular specialization of murine embryonic stem cells. Cell Physiol Biochem 2013;32:789-800.
5. Orchard CH, Pasek M, Brette F: The role of mammalian cardiac t-tubules in excitation-contraction coupling: Experimental and computational approaches. Exp Physiol 2009;94:509-519.
6. Liu J, Fu JD, Siu CW, Li RA: Functional sarcoplasmic reticulum for calcium handling of human embryonic stem cell-derived cardiomyocytes: Insights for driven maturation. Stem Cells 2007;25:3038-3044.
7. Laflamme MA, Chen KY, Naumova AV, Muskheli V, Fugate JA, Dupras SK, Reinecke H, Xu C, Hassanipour M, Police S, O’Sullivan C, Collins L, Chen Y, Minami E, Gill EA, Ueno S, Yuan C, Gold J, Murry CE: Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. Nat Biotechnol 2007;25:1015-1024.
8. Halbach M, Peinkofer G, Baumgartner S, Maass M, Wiedey M, Neef K, Krausgrill B, Ladage D, Fatima A, Saric T, Hescheler J, Muller-Ehmsen J: Electrophysiological integration and action potential properties of transplanted cardiomyocytes derived from induced pluripotent stem cells. Cardiovasc Res 2013;100:432-440.
9. Hescheler J, Fleischmann BK, Lentini S, Malteev VA, Rohwedel J, Wobus AM, Addicks K: Embryonic stem cells: A model to study structural and functional properties in cardiomyogenesis. Cardiovasc Res 1997;36:149-162.
10. Baharvand H, Pirjoei A, Rohani R, Taie A, Heidari MH, Hosseini A: Ultrastructural comparison of developing mouse embryonic stem cell- and in vivo-derived cardiomyocytes. Cell Biol Int 2006;30:800-807.
Wang et al.: Puerarin and Structural Maturation of Cardiomyocytes

11 Lundy SD, Zhu WZ, Regnier M, Lafleamme MA: Structural and functional maturation of cardiomyocytes derived from human pluripotent stem cells. Stem Cells Dev 2013;22:1991-2002.

12 Lieu DK, Liu J, Siu CW, McNerney GP, Tse HF, Abu-Khalil A, Huser T, Li RA: Absence of transverse tubules contributes to non-uniform Ca^{2+} wavefronts in mouse and human embryonic stem cell-derived cardiomyocytes. Stem Cells Dev 2009;18:1493-1500.

13 Liu J, Lieu DK, Siu CW, Fu JD, Tse HF, Li RA: Facilitated maturation of Ca^{2+} handling properties of human embryonic stem cell-derived cardiomyocytes by calstabin expression. Am J Physiol Cell Physiol 2009;297:C152-159.

14 Nunes SS, Miklas JW, Liu J, Ascharch-Sobhi R, Xiao Y, Zhang B, Jiang J, Masse S, Gagliardi M, Hsieh A, Thavandiran N, Lafleamme MA, Nanthakumar K, Gross GJ, Backx PH, Keller G, Radisic M: Biowire: A platform for maturation of human pluripotent stem cell-derived cardiomyocytes. Nat Methods 2013;10:781-787.

15 Zhang S, Chen S, Shen Y, Yang D, Liu X, Sun-Chi AC, Xu H: Puerarin induces angiogenesis in myocardium of rat with myocardial infarction. Biol Pharm Bull 2006;29:945-950.

16 Zhang H, Zhang L, Zhang Q, Yang X, Yu J, Shen S, Wu Y, Zeng Q, Wang T: Puerarin: A novel antagonist to inward rectifier potassium channel (IK1). Mol Cell Biochem 2011;352:117-123.

17 Wu L, Qiao H, Li Y, Li L: Protective roles of puerarin and danshensu on acute ischemic myocardial injury in rats. Phytomedicine 2007;14:652-658.

18 Liu A, Tang M, Xi J, Gao L, Zhang Y, Luo H, Hu X, Zhao F, Reppel M, Hescheler J, Liang H: Functional characterization of inward rectifier potassium ion channel in murine fetal ventricular cardiomyocytes. Cell Physiol Biochem 2010;26:413-420.

19 Lee E, Marcucci M, Daniell I, Pypaert M, Weisz OA, Ochoa GC, Farsad K, Wenz MR, De Camilli P: Amphiphysin 2 (bin1) and t-tubule biogenesis in muscle. Science 2002;297:1193-1196.

20 Chen B, Guo A, Zhang C, Chen R, Zhu Y, Hong J, Kutschke W, Zimmerman K, Weiss RM, Zingman L, Anderson ME, Wehrens XH, Song LS: Critical roles of junctophilin-2 in t-tubule and excitation-contraction coupling maturation during postnatal development. Cardiovasc Res 2013;100:54-62.

21 Gurha P, Abreu-Goedder C, Wang T, Ramirez MO, Drumon AL, van Dongen S, Chen Y, Bartonicek N, Enright AJ, Lee B, Kelm RJ, Jr, Reddy AK, Taffet GE, Bradley A, Wehrens XH, Entman ML, Rodriguez A: Targeted deletion of mircoRNA-22 promotes stress-induced cardiac dilation and contractile dysfunction. Circulation 2012;125:2751-2761.

22 Brette F, Orchard C: T-tubule function in mammalian cardiac myocytes. Circ Res 2003;92:1182-1192.

23 Ibrahim M, Gorelik J, Yacoub MH, Terracciano CM: The structure and function of cardiac t-tubules in health and disease. Proc Biol Sci 2011;278:2714-2723.

24 Smolich J: Ultrastructural and functional features of the developing mammalian heart: A brief overview. Reprod Fertil Dev 1995;7:451-461.

25 Dolnikov K, Shilkrut M, Zeevi-Levin N, Gerecht-Nir S, Amit M, Danon A, Itskowitz-Eldor J, Binah O: Functional properties of human embryonic stem cell-derived cardiomyocytes: Intracellular Ca^{2+} handling and the role of sarcoplasmic reticulum in the contraction. Stem Cells 2006;24:236-245.

26 Lee YK, Ng KM, Lai WH, Chan YC, Lau YM, Lian Q, Tse HF, Siu CW: Calcium homeostasis in human induced pluripotent stem cell-derived cardiomyocytes. Stem Cell Rev 2011;7:976-986.

27 Carozzi AJ, Ikonen E, Lindsay MR, Parton RG: Role of cholesterol in developing t-tubules: Analogous mechanisms for t-tubule and caveolea biogenesis. Traffic 2000;1:326-341.

28 Vassilopoulos S, Oddoux S, Groh S, Cacheux M, Faure J, Brocard J, Campbell KP, Marty I: Caveolin 3 is associated with the calcium release complex and is modified via in vivo triadin modification. Biochemistry 2010;49:6130-6135.

29 Galbiati F, Engelman JA, Volonte D, Zhang XL, Minetti C, Li M, Hou H, Jr, Kneitz B, Edelmann W, Lisanti MP: Caveolin-3 null mice show a loss of caveolea, changes in the microdomain distribution of the dystrophin-glycoprotein complex, and t-tubule abnormalities. J Biol Chem 2001;276:21425-21433.

30 Gurha P, Wang T, Larimore AH, Sass M, Abreu-Goedder C, Ramirez MO, Reddy AK, Engelhardt S, Taffet GE, Wehrens XH, Entman ML, Rodriguez A: Microrna-22 promotes heart failure through coordinate suppression of ppar/err-nuclear hormone receptor transcription. PLoS One 2013;8:e75882.

31 Manasek FJ: Histogenesis of the embryonic myocardium. Am J Cardiol 1970;25:149-168.