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RESEARCH ARTICLE

# Gelatin-based hydrogels combined with electrical stimulation to modulate neonatal rat cardiomyocyte beating and promote maturation

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## Abstract

Cardiovascular diseases are the leading cause of morbidity and mortality throughout the world underlining the importance of efficient treatments including disease modeling and drug discovery by cardiac tissue engineering. However, the predictive power of these applications is currently limited by the immature state of the cardiomyocytes. Here, we developed gelatin hydrogels chemically crosslinked by genipin, a biocompatible crosslinker, as cell culture scaffolds. Neonatal rat cardiomyocytes appear synchronous beating within 2 days after seeding on hydrogels. Furthermore, we applied the electrical stimulation as a conditioning treatment to promote the maturation of cardiomyocytes cultured on the hydrogels. Our results show that electrical stimulation improves the organization of sarcomeres, establishment of gap junctions, calcium-handling capacity and propagation of pacing signals, thereby, increase the beating velocity of cardiomyocytes and responsiveness to external pacing. The above system can be applied in promoting physiological function maturation of engineered cardiac tissues, exhibiting promising applications in cardiac tissue engineering and drug screening.

**Keywords** Gelatin hydrogel · Electrical stimulation · Cardiomyocyte maturation · Cardiac tissue engineering

## Introduction

Cardiovascular diseases are a major cause of death worldwide due to the very limited self-regeneration capacity of the adult heart, thus new strategies are needed for in vitro production of functional heart tissue for implantation or drug screening, or to study the development and function of heart tissue [1, 2]. Cardiac tissue engineering has led to the development of engineered heart tissue models, intending to simulate the human heart and methods that are needed to generate the environmental signals in the developing heart [3]. In this regard, hydrogel materials have been shown to be suitable for cardiac regeneration and repair due to their ability to simulate the physical and chemical properties of native heart tissues [4, 5]. So far, several synthetic and natural hydrogels, such as poly(N-isopropyl acrylamide), collagen, chitosan, hyaluronic acid, and alginate, have been used to provide a microenvironment mimicking extracellular matrix to support the function of cardiac cell [6–9]. However, despite showing good biocompatibility, the characteristics of these developed hydrogels are inferior to the native myocardium, mainly due to the limited stiffness and relatively flat surfaces. In cardiac tissue engineering, biomaterials serve predominately as scaffolds for tissue formation, requiring the optimization of scaffolds in terms of electrical, mechanical, and topographic characteristics [10–12]. Gelatin is a derivative of collagen and can be used to promote cell adhesion and proliferation [12]. Gelatin has been widely used in tissue engineering for its low cost, low immunogenicity, and good biocompatibility. However, hydrogels made of pure gelatin are too soft and unstable

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and therefore need to be reinforced with a cross-linking agent. Genipin is naturally found in the Gardenia jasminoides fruits. It can crosslink macromolecules by binding amine groups between adjacent molecules, and has been used as a non-toxic and water-soluble crosslinker for gelatin, showing great advantages compared with traditional crosslinkers [13]. In addition, genipin itself may also provide a therapeutic benefit due to its anti-inflammatory property which has been verified by in vivo experiments [14]. We previously showed that the gelatin-based hydrogels chemically crosslinked by genipin are favorable for the growth of cultured neonatal rat ventricular cardiomyocytes, including rapid development of cell size, actin cytoskeleton, gap junction, and synchronous contraction within 2 days after cell seeding, showing promising applications for cardiac tissue regeneration [15].

Generally, the maturation of cardiomyocytes can be summarized in the following aspects: (1) Morphology, proliferation and structural properties; (2) Metabolism; (3) Genetic profile; (4) Functional properties [16]. Adult ventricular myocytes possess densely packed sarcomeres, mitochondria, transverse tubules, and sarcoplasmic reticulum (SR), and mainly obtain energy through fatty acid oxidation. Myocardial tissue models constructed in vitro from neonatal rat cardiomyocytes or stem cell-derived cardiomyocytes often have limited ability to emulate all the functional features of adult myocardium, which limit their application in transplantation and cardio-toxicity screening because of the immature phenotype [17, 18]. Many recent studies have demonstrated that employing mechanical and electrical stimulation can enhance the maturation of cardiomyocytes [19, 20]. It has been proved that dynamic mechanical stimulation can promote the contraction and maturation of engineered myocardium under certain elastic load [21]. Similarly, the optimization of electrical field stimulation improved the contraction behavior and enhanced hypertrophic growth of cardiomyocytes [22]. Electrical stimulation can also promote the cardiac differentiation potential of stem cells such as human induced pluripotent stem cells and human embryonic stem cells [23, 24]. Additionally, the preconditioning of electrical stimulation could enhances the therapeutic efficacy of stem cells in the infarcted heart [25]. These studies suggest that the exogenous electrical stimulation exerts crucial effects during cardiogenesis and subsequent maturation.

In this study, for the purpose of producing cardiac tissue with mature functions in vitro, we developed the gelatin-based hydrogel scaffolds crosslinked by genipin, offering the desirable mechanical property and good biocompatibility for generating functional cardiac tissue in vitro. Moreover, we applied the electrical stimulation as a conditioning treatment for the cardiomyocytes cultured on the gelatin-based hydrogels. We further conducted substantial biological studies to investigate the development of cardiac cells, including cytoskeletal organization, expression of specific cardiac markers, as well as beating velocity and calcium dynamics after electrical stimulation, aiming to bring new strategies into the process of myocardial development and maturation, facilitate and accelerate translational studies of disease modeling, drug discovery, and eventually for therapy using cardiac tissue engineering.

## Materials and methods

### Fabrication and characterization of gelatin hydrogels

Gelatin hydrogels were prepared according to our previously established protocol [15]. Briefly, 0.6 g of gelatin powder (molecular weight 10–70 kDa, extracted from bovine skin, Shengxing Biotechnology, China) was dissolved in 10-mL ultrapure water at 45 °C. The genipin was dissolved (Zhixin Biotech Ltd, Linchuan, China) in 10-mM HEPES at pH 7.4 (HEPES, ShengXing Biotechnology, China), yielding 0.2% (w/v) solution. To fabricate the scaffolds, a 30-μL drop of the gelatin solution was poured on the smooth surface of a PDMS layer and then covered with a 10-mm diameter circular glass coverslip, gelling for 30 min at 4 °C to obtain the hydrogel scaffolds, as illustrated in Fig. 1a. After removal of the PDMS layer, the hydrogels on the glass were crosslinked in the genipin solution at 25 °C for 24 h and then soaked in ultrapure water for later use.

Three-dimensional topographic images of hydrogels were recorded on a BioScope Resolve atomic force microscope by using peak force tapping mode (AFM, Bruker Corporation, USA). Surface morphology was recorded using a probe with a spring constant of 0.1 N/m (MLCT-A, Bruker Corporation, USA). The Young's modulus of hydrogels in phosphate-buffered saline (PBS) was directly collected using a cantilever with a calibrated spring constant, k, of 0.133 N/m (PFQNM-LC Probe, value given by the manufacturer) at 0.5 Hz scan rate and 2 kHz peak force frequency. The mean values of Young's modulus were obtained by Nasoscope Analysis 1.7 (Bruker Corporation, USA).

Hydrogel structure was evaluated by means of a field-emission scanning electron microscopy (FE-SEM, Ultra Plus, Carl Zeiss, Germany). Prior to the observation, the hydrogels were fast frozen in liquid nitrogen and then freeze-dried for 48 h.

### Cardiomyocytes harvest, seeding, and culture

All procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) at Southeast University. Cardiomyocytes were extracted from ventricles of two-day-old Sprague—Dawley rats using previously described protocols [26]. After

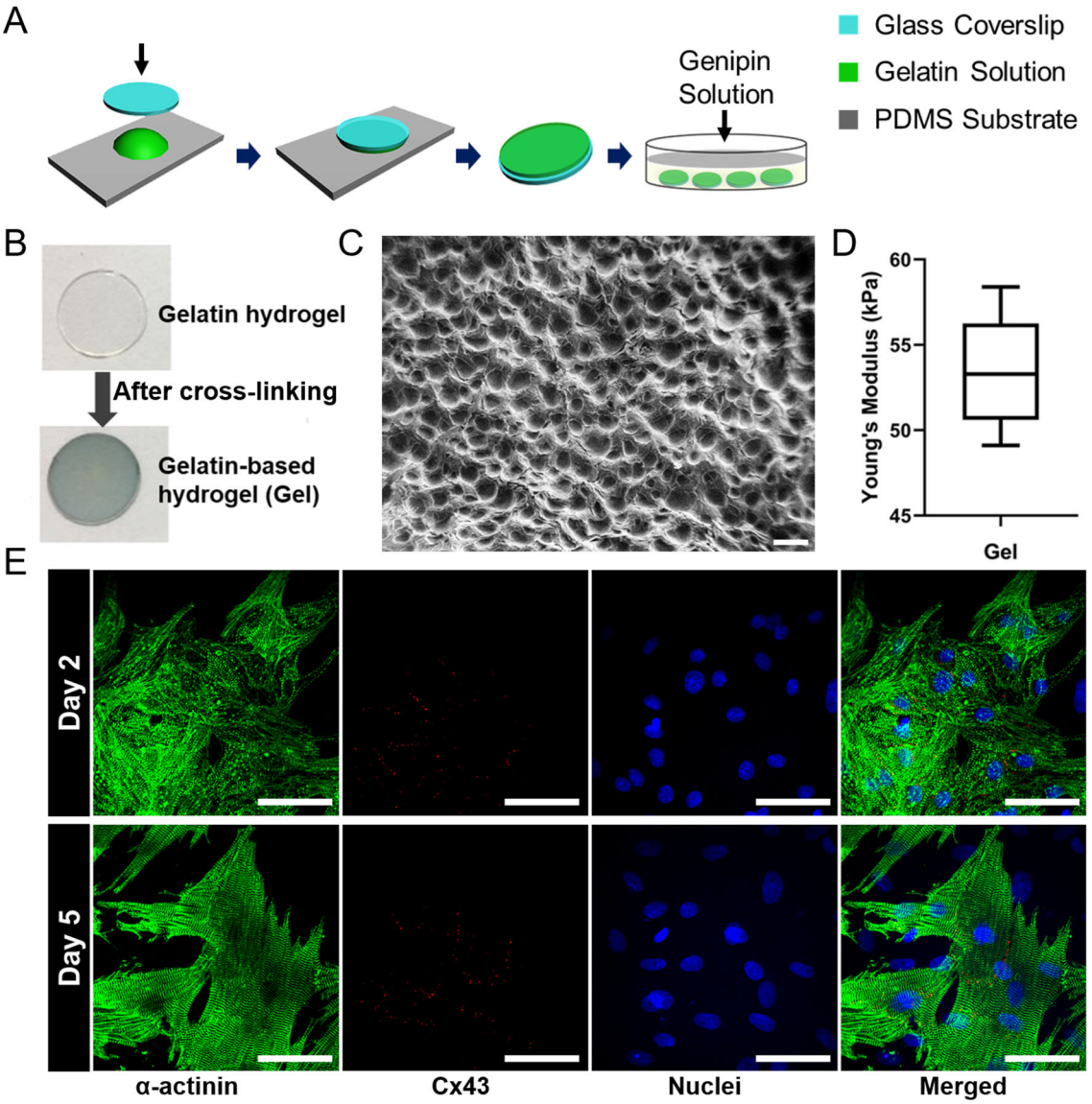


**Fig. 1** Fabrication and characterization of gelatin-based scaffolds. **a** Schematic diagram of fabrication process of gelatin-based hydrogel scaffolds. **b** Physical picture of gelatin hydrogels before and after crosslinking with genipin. **c** SEM image of the surface of gelatin-based hydrogels. Scale bar: 50 μm. **d** Young's modulus of gelatin-based hydrogels after crosslinking measured by AFM. **e** Immunofluorescent staining of cardiac-specific proteins in cardiomyocytes cultured on gelatin-based hydrogels, sarcomeric α-actinin (green), connexin 43 (red), nucleus (blue). Scale bars: 50 μm

isolation, cells were pre-plated for 1 h to enrich for cardiomyocytes. The number of cells was then counted using hemocytometer and flow cytometry. Before seeding cardiomyocytes, hydrogels were sterilized overnight with 75% (v/v) ethanol and soaked in 37 °C culture media for 2 h. Cells were cultured on scaffolds in 48-well plates at a cell density of 20,000/cm$^2$ in α-MEM supplied with 15% fetal bovine serum (Gibco, cat: 10099141) and 1% penicillin/streptomycin (Gibco, cat: 15070063) for 1 day. After attachment to the hydrogels, cells were washed with PBS and incubated in fresh culture medium to remove the non-adherent cell. On the second day, the serum concentration was reduced to 10% and media was changed every second day. Besides, cardiomyocytes were also seeded in conventional cell culture dishes and flasks as control.

## External electrical stimulation

On day 2 after cell seeding, external electrical stimulation was applied to investigate the response of the cultured cardiomyocytes according to previously established protocol [27]. Primarily, a chamber was assembled using a 100-mm

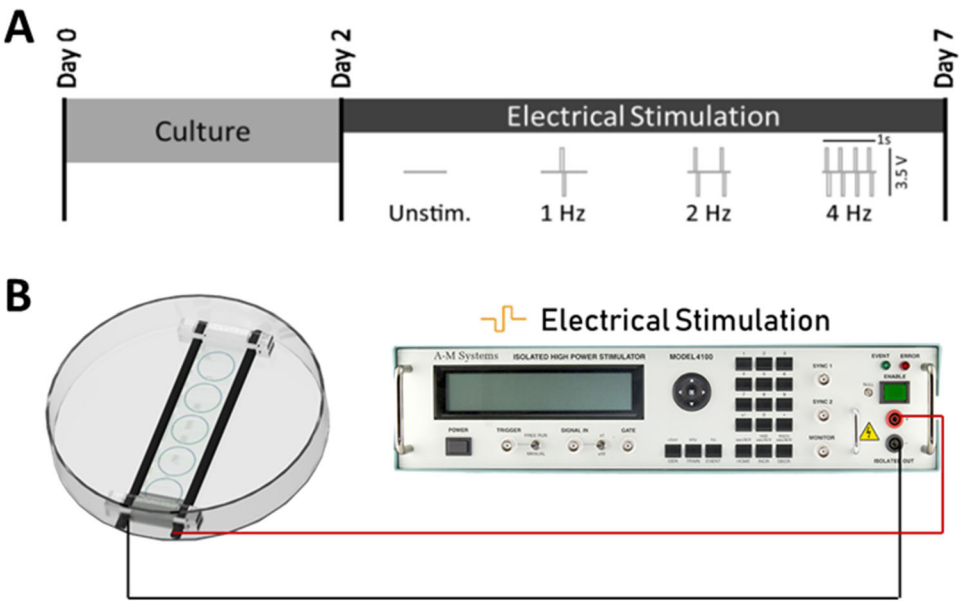


**Fig. 2** Experimental setup for applying electrical field stimulation to cardiomyocytes. **a** CMs were cultured on hydrogels within 2 days after seeding, followed by a 5-day electrical stimulation (rectangular, 2 ms, 3.5 V biphasic pulses at 1, 2, or 4 Hz) or cultured without electrical stimulation (unstimulated controls). **b** Schematic diagram of the electrical stimulation device (modified 100-mm Petri dish with carbon rod electrodes), the hydrogels are placed in the circular glass groove between the two electrodes

diameter plastic petri dish fitted with two 2.5-mm diameter rod-shaped electrodes, 8.5 cm in length, with 1.5 cm spacing. A 7 cm long and 1.5 cm wide glass plate with five circular grooves is placed between the two electrodes and both ends of the electrodes are fixed with Acrylic board. The electrodes were connected to the stimulator (MODEL 4100, A-M Systems, USA) using platinum wires and all connections were firmed using epoxy resin AB glue (Deli, China) (Fig. 2b). Rectangular electrical pulses with 2 ms durations at three different frequencies (1, 2, or 4 Hz) were applied continuously for an additional 5 days (Fig. 2a). Cardiomyocytes on gelatin hydrogels cultured in Petri dishes without electrical stimulations served as 'unstim.' controls.

## Immunostaining

On day 7 after seeding, cardiomyocytes on hydrogels were fixed with 4% (w/v) paraformaldehyde (PFA, Sigma, Cat: P6148) for 15 min followed by treatment with 0.2% Triton X-100 (Sigma, Cat: 100X) for 10 min at room temperature. Then, 3% bovine serum albumin (BSA, SunShine Biotechnology, China, Cat: S475) in PBS was used to prevent nonspecific binding of the antibody. Afterward, cardiomyocytes were incubated with primary antibodies (anti-α-actinin (Sarcomeric), mouse monoclonal, 1:200 in PBS, Sigma, Cat: A2543; anti-connexin 43, rabbit polyclonal, 1:75 in PBS, Cell Signaling, Cat: 3512S) for 1 h at 37 °C, followed by incubation with corresponding secondary antibodies for 1 h at 37 °C (1:200 in PBS; Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen, Cat: A11037), Alexa Fluor 488 goat anti-mouse IgG(Invitrogen, Cat: A28175). Eventually, cells were immersed in Hoechst 33342 (Sigma, Cat: B2261) at a concentration of 10 μg/ml for 15 min at room temperature to stain the nucleus. Immunostaining was observed with a Revolution XD confocal laser scanning microscope (Andor, Northern Ireland).

## Intracellular calcium transient measurement

Calcium indicator Fluo-4 AM was utilized to assess intracellular calcium transient of cardiomyocytes on hydrogels. All samples (unstim. and stimulated) on day 7 were gently washed three times with PBS and incubated in a 2-μM Fluo-4 AM solution (Invitrogen, Cat: F14201) for 20 min. The cells were gently washed three times and then incubated for another 25 min in Hank's Balanced Salt Solution (HBSS, Gibco, Cat: 14185052) solution without calcium and magnesium followed by calcium transient imaging at 1-Hz stimulation. Intracellular calcium fluorescence was analyzed under a fluorescent microscope at 488-nm wavelength. Non-compressed videos in*.avi format were recorded with a high-frame speed charged coupled device (CCD) digital camera. Fluorescence changes were measured as the ratio of the fluorescent dye intensity (F) during cells' contractions over the initial background fluorescence intensity (F0) and plotted over time.

## Beating velocity analysis

Non-compressed videos in *.avi format were directly processed in a custom-written software as previously described and then counted [15]. Briefly, the beating velocity was calculated by matching a block of pixels to an identically-sized block of pixels in the i + dth frame, where d is a delay in frames that the user selects. Then the velocity is calculated

**Table 1** The primer sequences for reverse transcription quantitative polymerase chain reaction (RT-qPCR)

| Gene | Primer sequence (5′–3′) |
|---|---|
| *Cx43* | Forward: TCCTTGGTGTCTCTCGCTTT |
| | Reverse: GAGCAGCCATTGAAGTAGGC |
| *Atp2a2* | Forward: TGCTGGAACTTGTGATCGAG |
| | Reverse: AGCGTTTCTCTCCTGCCATA |
| *Ryr2* | Forward: TTTCGTGAGCATTAGCAACG |
| | Reverse: GAGGCACAAAGAGGAACTCG |
| *Actn1* | Forward: CGAGTGCACAAGATCTCCAA |
| | Reverse: CTCTGACACCACAGGAGCAA |
| *GAPDH* | Forward: GGCATTGCTCTCAATGACAA |
| | Reverse: TGTGAGGGAGATGCTCAGTG |

based on the distance traveled by the pixel and the known time.

### Gene expression

Gene expressions related to contractile protein, gap junction and calcium channel proteins, including α-actinin (*Actn1*), connexin 43 (*Cx43*), sarcoplasmic reticulum Ca2 + -ATPase2a (*Atp2a2*), and ryanodine receptor 2 (*Ryr2*), were assessed by RT-qPCR. Cells were lysed in Trizol (Invitrogen) and RNA extraction was performed according to the manufacturer's protocol. 5 scaffolds of each type were pooled to obtain enough RNA, then aliquots of 500-ng total RNA from each group were reverse transcribed into cDNA, using a cDNA synthesis kit (TaKaRa, Japan). Subsequently, the cDNAs were purified utilizing the spin columns and buffers provided with the cDNA synthesis kit. RT-qPCR was performed with prepared cDNA using an Applied Biosystem 7500 Real-Time PCR System (ABI, USA), according to the manufacturer's instructions. Gene-specific primers were obtained from (Jinsirui, Biotechnology, China) and all samples were run in duplicates. RT-qPCR data were analyzed according to the ΔΔCt method using the mean Ct value of the housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Primer sequences used are showing in Table 1.

### Statistical analysis

GraphPad Prism was used for statistical analysis of all groups with one-way or two-way analysis of variance. Error bars represent the mean ± standard deviation (SD) of measurements. Significance levels were determined by $*p < 0.05$, $**p < 0.01$, and $**p < 0.001$.

## Results and discussion

### Fabrication/characterization of gelatin-based scaffolds and cardiomyocyte behavior on scaffolds

The gelatin-based hydrogels were fabricated using a flat PDMS mold. The gelatin solution gelled at 4 °C and then easily peeled from the PDMS mold. During the cross-linking process, genipin reacted with the amino acids of gelatin to form monomers and further dimerize the copolymer [13]. The copolymers formed during the cross-linking reaction caused the hydrogel to change from transparent to blue but did not induce changes in morphology or size (Fig. 1b). According to our experiment, the genipin-crosslinked gelatin hydrogels have no obvious morphological changes and remain stable even after 3 months of incubating under cell culture conditions.

The SEM image demonstrated highly porous surfaces of the gelatin-based hydrogel with a uniform pore size (Fig. 1c). Moreover, the hydrogel also showed a regular pore structure inside, with pore sizes ranging from 20 to 50 μm (Figure S1A). Atomic force microscopy images were taken to observe the surface nanotopography of hydrogels. The 500 × 500 $nm^2$ area of 3D image showed uniform nanostructures on the gelatin-based hydrogel surface (Figure S1B). The adhesion and spreading of the cells on the matrix surface generally occur through the cell-substrate interactions, mainly through integrins on cell membranes [28]. Integrins firstly bind to the matrix surfaces and cluster together to form focal complexes, which can further mature into focal adhesions for cell spreading in appropriate interfacial environments [28]. Therefore, interfacial environments, including cell-adhesive proteins, topography, roughness, and stiffness, would play essential roles in cell adhesion on the scaffolds. In this study, the gelatin-based hydrogel surfaces contain a large amount of gelatin nanostructures and provide plenty of "adhesive anchors" for cell attachment, which promotes the adhesion and rapid spreading of cardiomyocytes (cell area varies from 1393 ± 169 $\mu m^2$ on day 1 to 5178 ± 310 $\mu m^2$ on day 2 after cell seeding).

In addition to the physical structure and chemical compatibility, the mechanical properties of biomaterials are also very important to cell proliferation and phenotype determination [29]. Mimicking the stiffness of the native heart is an effective strategy when designing scaffolds for cardiac tissue engineering. The Young's modulus of the adult human myocardium ranges non-linearly from 10–20 kPa (start of diastole) to 200–500 kPa (end of diastole) [30]. The mechanical properties of wet hydrogels were characterized to evaluate their ability to accommodate strain from cardiac beating using AFM. The results showed that Young's modulus of the hydrogels (53.4 ± 3.0 kPa) falls in the range of the stiffness of the native heart, demonstrating an acceptable mechanical prop-

erty for cardiac cell growth and tissue development (Fig. 1d) [30].

Sarcomeric α-actinin and Cx43 were immunostained to assess the phenotype of the cardiomyocytes seeded on gelatin-based hydrogels. As shown in Fig. 1e, at day 2 of culture, α-actinin was expressed in abundance but the sarcomere structure was not in good organization. At day 7 of culture, cardiomyocytes grown on the hydrogels showed uniform and highly organized α-actinin structures. Although the immunostaining results of Cx43 show a small amount of expression and scattered around the cell, the synchronously beating behavior and calcium transient of the individual cardiomyocytes at day 2 has already demonstrated a delicate coupling of both impulse propagation and contractile behavior (Movie S1). On the contrary, after seeded on the culture dishes/flasks, cardiomyocytes gathered in small clusters and could not contract synchronously even after 7 days of culture. Actually, the proliferation of fibroblasts gradually squeezed the space of cardiomyocytes, resulting in a great decrease of cardiomyocyte number within 7 days (Movie S2). In addition, to further demonstrate the positive effect of the gelatin-based hydrogels, we modified the circular glass coverslips with a thin film of gelatin by immersing the coverslips in 0.2%(w/v) gelatin solutions for 2 h followed by rinse with ultrapure water. Then cardiomyocytes were seeded on the gelatin-modified coverslips. Although the results showed similar cell adhesion and spreading behavior to that observed on gelatin-based hydrogels, the beating amplitude of cardiomyocytes was significantly lower than that on the hydrogels at day 5 of culture (Movie S3). Furthermore, when electrical stimulation was applied, cardiomyocytes on gelatin-modified coverslips easily fell off the slides and it is difficult to continue electrical stimulation, which did not occur to cardiomyocytes on the gelatin-based hydrogels. Overall, cardiomyocytes in undesirable condition cannot tolerate the external electrical stimulation even at a lower frequency. The desirable cell behavior on gelatin-based hydrogels compared to that on cell culture dishes or gelatin-modified coverslips, is mainly due to the cell-adhesive gelatin surface with nanostructure anchors and a suitable substrate stiffness close to native myocardium tissue. The above results confirmed that our gelatin-based hydrogels are favorable for cardiac cell growth and tissue development.

As we know, the applications of most in vitro myocardial models are currently limited by the immature state of the cardiomyocytes. The single stimulation method is difficult to simulate the physiology of adult myocardium. The hydrogel should have great potential to be constructed as heart scaffolds when associated with other stimulation methods such as electrical, hydrodynamic, and mechanical stimulation. Electrical field stimulation can induce cell alignment and coupling, increase the amplitude of synchronous contractions to promote the maturation of cardiomyocytes [18]. The combination of our gelatin-based hydrogel and electrical stimulation may provide an ideal strategy for developing functional engineered cardiac tissue, which will be further investigated by following in vitro cell experiments.

## Enhanced organization and interaction of cardiomyocytes by electrical stimulation

A custom-made electrical stimulation device was set up to evaluate the ability of cardiomyocytes on hydrogels to adapt to external electrical stimulation (Fig. 2 and Figure S1C). At day 2 after seeding, cells developed their excitation–contraction coupling machinery, which could be demonstrated by the synchronous contraction of cardiomyocytes on the hydrogels (Movie S1). The application of electrical stimulation can immediately induce cardiomyocytes on different samples to beat at the same frequency (e.g., 1 and 2 Hz) by responding to the external stimulation (Movie S4). However, neonatal rat cardiomyocytes are difficult to catch the beating frequency at 4 Hz under the 4 Hz stimulation, mainly due to the immature contractile behavior of cardiomyocytes. After 5 days of continuous stimulation at a certain frequency (1, 2, or 4 Hz), cell elongation and aligned cell organization can be observed (Movie S5). Meanwhile, electrical stimulation induced higher expression of Cx43 and the rearrangement of cardiac fibroblasts and gap junctions (Figure S2). Nevertheless, a continuous stimulation at 6 Hz on neonatal rat cardiomyocytes was found beyond cell tolerance and caused cell death within 24 h.

We examined the expression of cardiac-specific proteins including α-actinin, a Z-band protein of sarcomere, and Cx43 by immunostaining after electrical stimulation (Fig. 3a). At day 7, the quantified surface coverage of α-actinin, on cardiomyocytes electrically stimulated at 1 Hz, 2 Hz, 4 Hz, and non-stimulated were $37.1 \pm 1.9\%$, $38.0 \pm 4.6\%$, $41.0 \pm 3.7\%$, and $37.1 \pm 1.5\%$, respectively (Fig. 3b). Due to the good biocompatibility and suitable mechanical property of the hydrogel, cardiomyocytes were well developed and have good cytoskeleton organization. The immunostaining and quantified surface coverage results demonstrated that the α-actinin expression on cardiomyocytes from the stimulated and non-stimulated groups have no significant difference, except for a slight improvement in the 4 Hz stimulation group. Conversely, the Cx43 expression of cardiomyocytes increased significantly after electrical stimulation. As a constitutive protein for the formation of cardiac gap junctions, Cx43 is essential for both cell–cell coupling and the reconstruction of functional tissues. The surface coverage of Cx43 of the non-stimulated group were $1.13 \pm 0.36\%$ while after electrical stimulation, the surface coverage of Cx43 significantly increased ($2.49 \pm 0.42\%$ at 1 Hz, $3.35 \pm 1.43\%$ at 2 Hz, and $4.20 \pm 0.76\%$ at 4 Hz) (Fig. 3c). Additionally, Cx43 was observed mainly in the extracellular membrane of car-

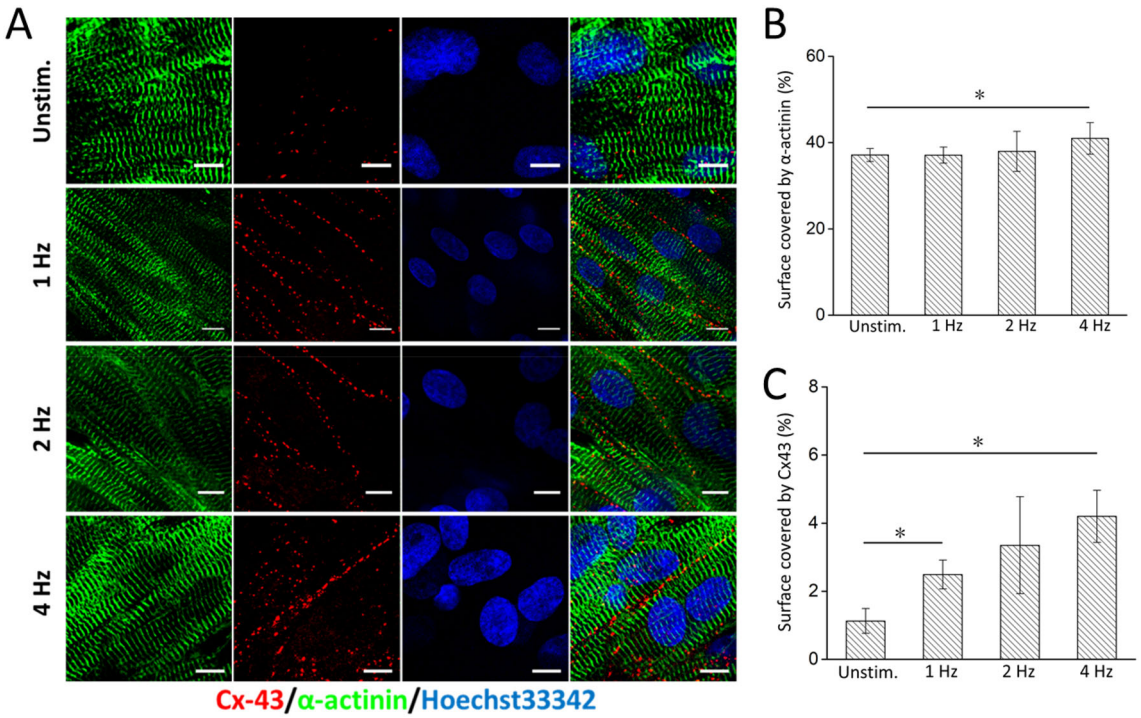


**Fig. 3** Electrical stimulation promotes the expression of cardiac-specific proteins. **a** Immunofluorescent staining of cardiac-specific proteins (Cx43: in red; α-actinin: in green; nucleus: in blue) with and without electrical stimulation. Scale bars:10 μm. **b–c** Quantification of the surface coverage by expressed α-actinin **b** and Cx43 **c**, $n = 8$. Asterisks (*) indicate statistical significance, $p < 0.05$

diomyocytes and arranged in a line after electrical stimulation (Fig. 3a), which promotes the establishment of gap junctions and propagation of pacing signals.

Overall, the electrical stimulation can promote the expression of contractile proteins and improve their arrangement. Cardiomyocytes showed stronger expression of the contractility related marker including α-actinin and Cx43 after electrical stimulation, particular at 4 Hz, when compared to the other treatment groups and the control group. In principle, electrical stimulation above the excitation threshold will cause a sequence of ion fluxes through specialized channels in the membrane (sarcolemma) of cardiomyocytes and lead to cardiac contraction. It has been previously reported that the change of stimulation rate is associated with changes in expression of contractile proteins, suggesting a dependence of cardiac maturation on stimulation rate [24]. Under a higher-frequency stimulation within the cell tolerance, cardiomyocytes express more related proteins to match the electrical stimulation frequency that is higher stimulation frequencies benefit cell maturation in vitro.

## Electrical stimulation matures calcium handling in cardiomyocytes

Calcium transient ($Ca^{2+}$) analysis was performed to investigate calcium signaling within cultured cardiomyocytes. Cardiomyocytes cultured on hydrogels in all groups (with or without electrical stimulation) exhibited synchronized calcium spikes, which confirmed good cell–cell communication between cardiomyocytes (Fig. 4b). Cardiomyocytes in the 4 Hz stimulation group displayed different calcium transients from those in the 1 Hz or 2 Hz stimulation and non-stimulated groups. The calcium ion transport rate of cardiomyocytes after 4 Hz stimulation was highest among all groups (Fig. 4a). More specifically, both non-stimulated and 1 Hz or 2 Hz stimulated cardiomyocytes displayed comparatively delayed calcium release (time-to-peak, non-stimulated: 318.4 ± 35.7 ms; 1 Hz: 307.2 ± 12.4 ms; 2 Hz: 298.5 ± 16.7 ms; 4 Hz: 236.7 ± 30.2 ms) and prolonged calcium re-uptake time (time-to-baseline[50%], non-stimulated: 393.2 ± 20.7 ms; 1 Hz: 341.1 ± 28.5 ms; 2 Hz: 326.5 ± 9.1 ms; 4 Hz: 215.9 ± 25.0 ms) (Fig. 4c and d). The results suggest that the electrical stimulation at a higher frequency within the cell tolerance can accelerate release and uptake kinetics of intracellular calcium. It has been known that 70% of the calcium influxes is a result

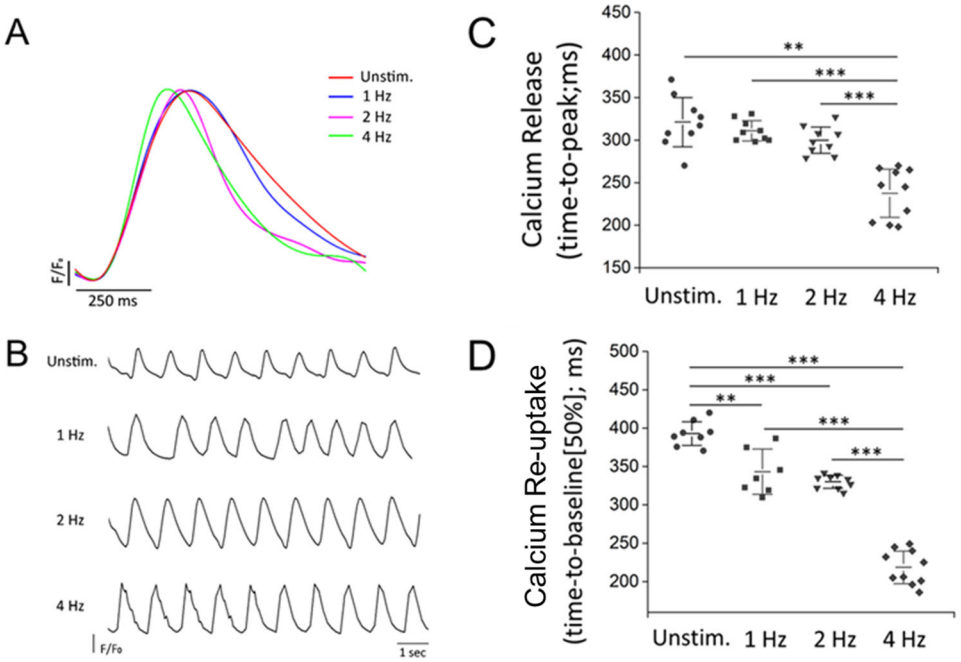


**Fig. 4** Electrical stimulation promotes the maturation of calcium handling in cardiomyocytes. **a–b** Representative calcium transients from cardiomyocytes recorded at 1 Hz field stimulation after loading with Fluo 4-AM; F/F0: change of fluorescent signal over background fluorescence. **c–d** Evaluation of the calcium transport rate. Time to peak: the process of calcium release from the SR ($n = 9/9/9/10$); Time to baseline: the process of re-uptake calcium ions from SR ($n = 8/7/9/10$). $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$

of $Ca^{2+}$-induced-$Ca^{2+}$-release (CICR) from the sarcoplasmic reticulum (SR) through ryanodine receptor 2 (RyR2) in adult cardiomyocytes, and the intracellular calcium is transferred back to the SR by sarcoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) or removed from the cardiomyocytes by $Na^{+}/Ca^{2+}$ exchanger (NCX)0.[18] Therefore, the faster release and uptake of intracellular calcium is paralleled by maturation of the calcium-handling machinery with enhanced SERCA and RyR2 function, suggesting that the higher-frequency electrical stimulation promotes the maturation of calcium dynamics in cardiomyocytes.

## Beating velocity analysis

Beating velocity was calculated through computational motion-tracking information to quantitatively investigate the different cardiomyocyte beating behavior upon electrical stimulation. As illustrated in Fig. 5, the quantified beating velocity of 4 Hz stimulated cardiomyocytes on gelatin-based hydrogels increased approximately twofold compared to the non-stimulated groups, while the positive values represent the process of cell contraction and negative values represent the process of relaxation. Furthermore, cardiomyocytes from the 4 Hz stimulated group were enabled to generate similar beating pattern responding to the applied frequencies up to 5 Hz, while those from the non-stimulated group were only able to follow the applied frequencies up to 2 Hz, which indicates the maturation of contractile behavior of cardiomyocytes after electrical stimulation (Fig. 5a and b). The heat maps of the beating motions showed a higher beating velocity of 4 Hz stimulated cardiomyocytes compared to the non-stimulated cells during one representative myocardial beating cycle (Fig. 5c and d). Beating velocity is determined by the $Ca^{2+}$ handling of SR, as well as the developmentally-regulated myocardium fiber [31]. Such beating behavior of electrically stimulated cardiomyocytes can be attributed to the enhanced electrophysiological characteristics, highly organized α-actinin structures and improved excitation–contraction coupling of cardiomyocytes, which were consistent with the above immunostaining and calcium transient results.

## Quantitative PCR test

Cardiac genes in immature cardiomyocytes are expressed mostly similar to human fetal cardiac tissue instead of adult-cardiac tissue. The focus was placed on the relative expression levels of cardiac-specific and ion channel genes in cardiomyocytes in the absence or presence of electrical stimulation. We tested the genes for α-actinin (*Actn1*), connexin 43 (*Cx43*), sarcoplasmic reticulum $Ca^{2+}$-ATPase2a (*Atp2a2*), and ryanodine receptor 2 (*Ryr2*), using RT-qPCR analysis after 5 days of culture with or without electrical stimulation. The analysis data are summarized in Fig. 6. The expressions of *Actn1* showed significant upregulation for 2 Hz and 4 Hz stimulated cardiomyocytes compared to the non-stimulated group. As for the expression of *Cx43*, a similar tendency to *Actn1* was observed in all groups. Both suggest a more mature phenotype has developed after electrical stimulation. Electrical impulse propagation is regulated primarily through gap junctions, which synchronize action potentials and play

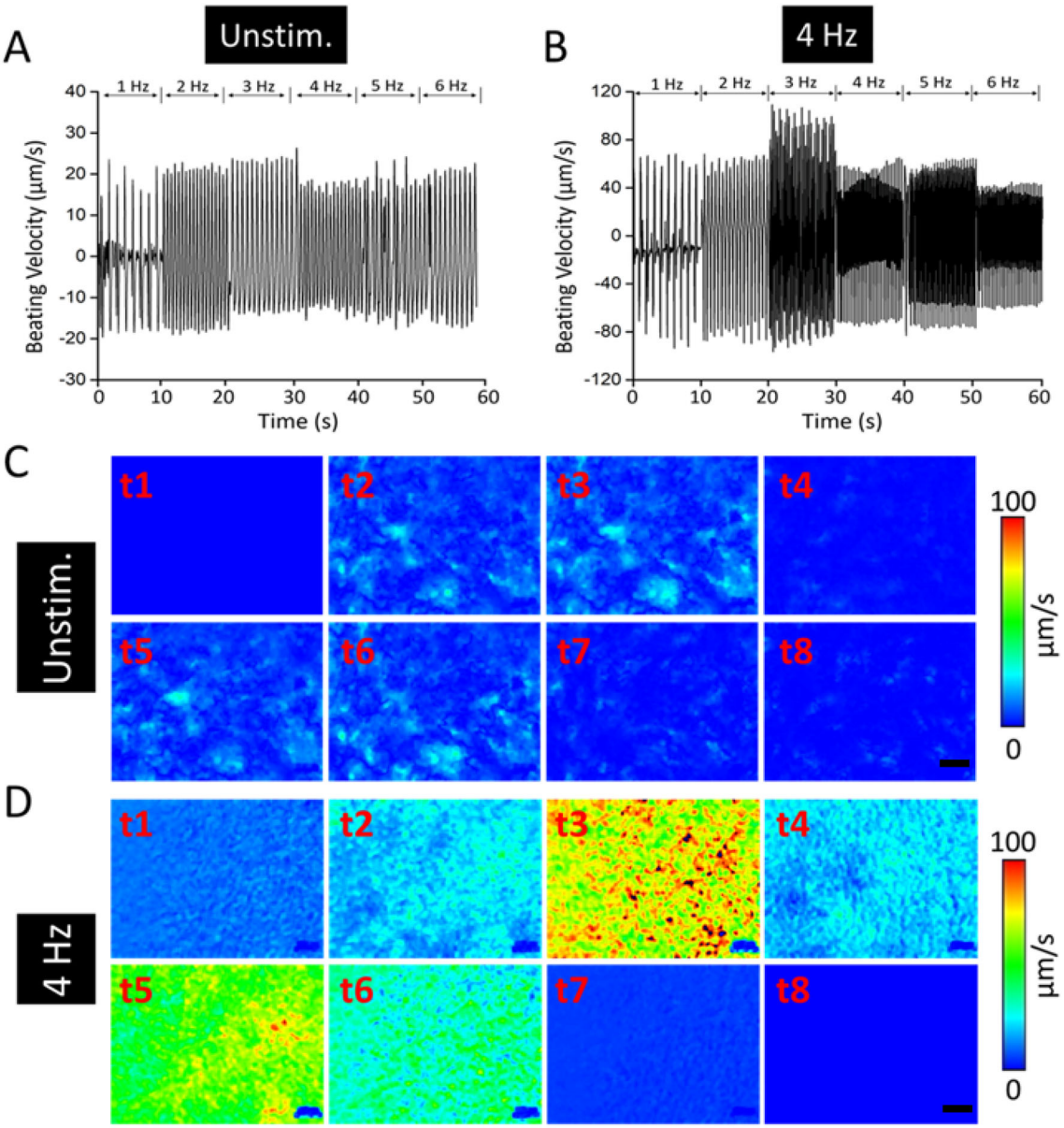


**Fig. 5** Evaluation of the beating velocity of cardiomyocytes. **a, b** Representative recordings from cardiomyocytes with or without electrical stimulation at the indicated stimulation frequencies (1–6 Hz field stimulation). **c, d** Representative heat maps of the beating motions of the unstimulated and electrically stimulated cardiomyocytes during one myocardial beating cycle. Scale bars:100 μm

an important role in the development of regular synchronous contractions [32]. The upregulation of *Actn1* and *Cx43* promoted α-actinin and Cx43 protein expression, respectively, further improving contractile apparatus organization, pacing signal conduction and contractile behavior, which have been shown in the above results.

The maturation of calcium handling involves the development of SR calcium channel and calcium storage in cardiomyocytes. The *Atp2a2* gene encodes for SERCA2 which functions in both contraction and relaxation as $Ca^{2+}$ transporter in the SR. The *Ryr2* gene encodes for the ryanodine receptor which serves to release $Ca^{2+}$ from the SR into the cytosol. As shown in Fig. 6, Cardiomyocytes in electrically stimulated groups showed distinct ascent in the expressions of *Atp2a2* and *Ryr2*. Particularly, these two gene expressions in the 4 Hz stimulated group was significantly higher than those in other groups, suggesting a more mature phenotype of calcium-handling properties. Notably, all the upregulation of maturation-related genes was most evident in the 4 Hz stimulation group, which was consistent with the previous report that high-stimulation frequencies benefit maturation in vitro [33, 34].

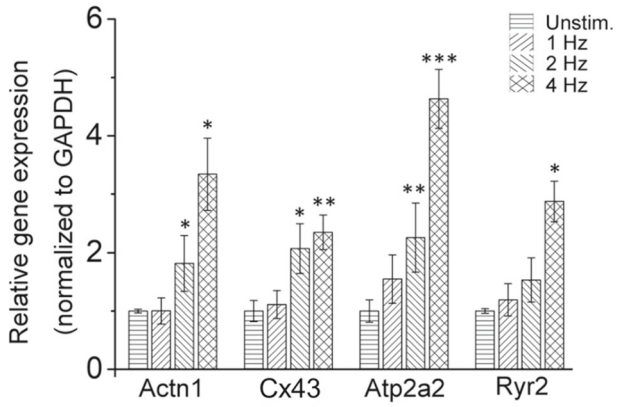


**Fig. 6** Gene expression analysis of α-actinin (*Actn1*), connexin 43 (*Cx43*), sarcoplasmic reticulum $Ca^{2+}$-ATPase2a (*Atp2a2*), and ryanodine receptor 2 (*Ryr2*) from cardiomyocytes with or without electrical stimulation. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ compared to unstimulation, $n = 3–6$

## Conclusion

In this study, we prepared gelatin-based hydrogels chemically crosslinked by genipin as scaffolds, which possess suitable mechanical properties and good biocompatibility and therefore are favorable for the development of cultured neonatal rat ventricular cardiomyocytes. Cardiomyocytes appeared synchronous beating within 2 days after seeding on hydrogels and maintained spontaneous contraction for months. Combining the advantageous properties of hydrogels with external electrical stimulation can modulate the beating behavior and induce remarkable enhancement of maturation of cardiomyocytes. It was observed that the electrical stimulation improved the organization of sarcomeres and promoted the expression of Cx43 protein, as well as the maturation-related gene expressions (*Actn1*, *Cx43*, *Atp2a2*, *Ryr2*). Moreover, electrical stimulation accelerated release and uptake kinetics of intracellular calcium, thereby, increasing the beating velocity of cardiomyocytes and responsiveness to external pacing. Therefore, our system can be favorably used for the maturation of cardiomyocytes and the achievement of functional mature cardiac tissue in vitro for application in cardiac tissue engineering and drug toxicity screening. Important areas of future work include quantifying the effect of electrical stimulation on other cell types like human iPSC-derived cardiomyocytes (hiPS-CMs), measuring other biophysical parameters, such as the conduction velocity and the contractile forces which are important in drug screening and toxicity testing.

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**Author contributions** FZ participated in the experimental research, data analysis, writing and editing of the manuscript. KYQ and XPL performed cardiomyocyte harvest and culture. CML and LSO established the experimental setup for electrical stimulation and developed softwares for data analysis. KHW and XWW provided guidance on cell study and helped to revise the manuscript. NPH conducted the design of the work as well as the deep review, editing, guidance, and supervision. All authors have read and approved the article for publication.

## Compliance with ethical standards

**Conflict of interest** The authors declare that there is no conflict of interest.

**Ethical approval** Cardiomyocytes were extracted from neonatal rat ventricles of two-day-old Sprague–Dawley rats. All related procedures were carried out as approved by the Institutional Animal Care and Use Committee (IACUC) of Southeast University.

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