Identification and biological characterization of chicken embryonic cardiac progenitor cells

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Abstract

Objectives: Many kinds of cardiac progenitor cell populations have been identified, including c-kit⁺, Nkx2.5⁺s and GATA4⁺ cells. However, these progenitors have limited ability to differentiate into different cardiac cell types. Recently, a new kind of cardiac progenitor cell named the multipotent Isl1⁺ cardiovascular progenitor (MICPs) has been identified, which also expresses Nkx2.5, GATA4, CD34 and Flk1.

Materials and methods: In this study, we have isolated and characterized MICPs from chicken embryonic heart tissues using immunofluorescence and PCR.

Results: Results shown that they express markers of cardiac progenitor cells, with high clonality. They have the ability to self-renew and can give rise to three types of heart cell *in vitro*.

Conclusions: Myocytes, smooth muscle cells and endothelial cells. Our work provides evidence for a developmental paradigm of the heart, that endothelial and muscle lineage diversification arises from multipotent cardiac progenitor cells. Existence of these cells provides a new opportunity for myocardial injury repair.

Introduction

In recent years, cardiac disease has emerged to be the leading cause of death worldwide, particularly in developed countries. Although wide ranges of both drug treatment and surgical procedures are used to either prevent or treat cardiac disease, the high risk and low success of

these therapies have limited their applications. Due to its unique characteristics, stem-cell transplantation is considered to be a potentially effective treatment for heart disease. Both embryonic and adult stem cells are being studied for clinical applications, and at least four types of autologous cell (skeletal myoblasts, bone marrow mononuclear cells, mesenchymal stem cells and endothelial progenitor cells) are being tested in early-stage clinical trials (1–4). Embryonic stem cells have two specific properties, self-renewal and differentiation, that make them particularly well suited for therapy; however ethical issues, and potential immunogenic and neoplastic transformation limit their current use. Transplantation of autologous stem cells has been considered because of their commitment to differentiate into many cell lineages, their high proliferative efficiency, and their origins, which overcome ethical, availability, and immunological problems (5). However, several problems related to arrhythmia (which is potentially life-threatening), must be taken into account. Furthermore, although mature cardiomyocytes can be formed from different adult stem cells *in vitro*, ability of these stem cells to cross lineage boundaries has caused heated debate in the scientific community.

Traditionally, the heart is regarded as a non-renewable organ as mature cardiomyocytes do not have the capacity for regeneration. In recent years, however, many kinds of cardiac progenitor cell have been found in the embryonic or post-natal heart, suggesting that the heart does have some regenerative potential (6–9).

Recent work in our laboratory has demonstrated existence of MICPs, cells which are marked by the transcriptional signature of Isl1, Nkx2.5, GATA 4 and Flk1. These cells can generate cardiac muscle, smooth muscle and endothelial cells, which represent the three major cell types of the heart tissue (8). MICPs have been cloned from both mouse ES cells and mouse embryos, and can ‘make the decision’ to enter muscle or endothelial differentiation pathways, at the single-cell level, hinting at a haematopoietic-like system for how diverse cardiovascu-

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lar lineages can be generated (8). In this study, we have explored methods for isolation, culture and identification of chicken embryo cardiac progenitor cells (CEPCs). Furthermore, these cells were induced to differentiate into myocardial cells and smooth muscle cells. This research may provide novel insights into *in vitro* culture and characterization of CEPCs, and contribute to development of tissue reconstruction procedures.

Materials and methods

Sample preparation

Embryonic chicken heart tissue samples (Fig. 2a) were obtained from 60 to 100 Beijing Fatty Chicken embryos at ED (embryonic day) 3–4, which were incubated in our laboratory. All eggs were cleaned using 75% alcohol, and heart tissues were then isolated from embryos and rinsed five times in PBS containing 100 U/ml penicillin and 100 mg/ml streptomycin.

Immunohistochemical staining of chicken embryonic heart tissues

All incubations were performed in a humidified chamber. Sections of paraffin wax embedded heart tissue were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide, diluted in PBS (pH 7.4), for 10 min. After rinsing three times in PBS (3 min each), sections were incubated in 5% normal goat serum at room temperature for 20 min to block non-specific binding sites, and then incubated overnight at 4 °C in the presence of primary polyclonal antibodies [Isl1 (Abnova, Walnut, CA, USA), GATA 4 (Abcam, Cambridge, MA, USA), Nkx2.5 and Flk1 (B&M custom-made, Nagoya, Japan)] diluted 1:100 in PBS. To control for non-specific staining, primary antibodies were replaced with normal PBS. Sections were then rinsed in PBS (3 min) and incubated in secondary antibody (polymerized HRP conjugated goat-anti rabbit, mouse IgG) (Zhongshan Golden Bridge, Beijing, China) at 37 °C for 30 min, then washed three times in PBS (5 min each). Immunoreactivity was then visualized by incubating sections in 3,3'-diamino- benzidine (DAB) substrate. Samples were then counterstained with haematoxylin and coverslips were sealed using neutral balsam. Sections were examined using a Leica DMIRB (Leica, Wetzlar, Germany) computerized microscope with a Leica digital camera DFC320 attachment, and images were captured and stored for analysis. Results of immunohistochemical staining were described as negative (no specific staining), weak (light brown staining) or strong (deep brown staining) (10).

Isolation and *in vitro* culture of CEPCs

Chicken embryo cardiac progenitor cells were isolated according to the procedure for mouse cardiac progenitor isolation, as described previously, with some improvements (8,11). Briefly, the heart was isolated from each chicken embryo, main vessel was eliminated, and heart tissue was chopped into small pieces (about 1 mm³) under sterile conditions. Heart pieces were digested with 200 U/ml collagenase type II and collagenase type I (both from Invitrogen, Carlsbad, CA, USA) in Hank's balanced salt solution (HBSS) for 60 min at 37 °C, followed by 10-min treatment with 0.125% trypsin. Enzymatic activity was then neutralized with the same volume of FBS (Biocrom, Berlin, Germany). Cell suspension containing the majority of Isl1⁺ progenitor cells was obtained after being purified through FCM (flow cytometry). Antibodies anti-Isl1 and anti-Flk1, were used in cell purification. Cells were plated on plastic plates in DMEM/F12 containing 10% foetal bovine serum (FBS) for 3 h. Plates were then washed rigorously to remove non-adherent cells, and remaining cells were cultured in DMEM containing 15% FBS, 20 mM HEPES, 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. To stimulate Isl1⁺ progenitor cell growth, culture medium was changed to DMEM/F12 containing 2% B27 supplement (Invotrigen), 3.5% FBS, 20 ng/ml EGF (Peprotech, Rocky Hill, TX, USA), 20 ng/ml bFGF (Peprotech) and 10 ng/ml LIF (Sigma, Sigma-Aldrich, St. Louis, MO, USA) on the second day in culture, when CEPCS reached confluence. When cultured cells reached 70–80% confluence, they were subcultured at 1:3 ratio, using 0.1% (m/v) trypsin.

Colony-forming cell assay

Passage 3, passage 5 and passage 9 cells were seeded in 24-well microplates at 1×10^4 cell/well, cultured for 4 days, then numbers of colony-forming units (CFU) were counted, to calculate colony-forming level – formulated as CFU numbers/starting cell number per 24 well $\times 100\%$. This procedure was repeated six times for each passage number (12).

Cell population growth dynamics

Chicken embryo cardiac progenitor cells at passages 3, 5 and 9 were plated in 24-well plates at 1×10^4 cell/well, cultured for 8 days and counted every day (3 wells each time) afterwards. Mean cell counts at each time point were then used to plot their growth curve, based upon which PDT (population doubling time) was calculated. $PDT = (t - t_0) \log_2 / (\log N_t - \log N_0)$, t_0 : starting time of culture; t : termination time of culture; N_0 : initial cell number of culture; N_t : ultimate cell number of culture.

Table 1. Primer sequences used for RT-PCR

Gene	Primer sequence	T_m (°C)	Cycle	Fragment size (bp)
Isl1	F: 5'-TCCAAGGGATGACAGGAAC-3' R: 5'-AGGGTAAGGGCAGAAACAAC-3'	57.8	35	372
Nkx2.5	F: 5'-AATGTTTCCTAGCCCTGTGAC-3' R: 5'-GGTTTCCTCTCTCTCTGT-3'	59.0	35	366
GATA4	F: 5'-GTCACCTCGCTTCTCTTC-3' R: 5'-TAGTGCCCTGTGCCATCT-3'	61.0	35	360
Flk1	F: 5'-TCAGCCAACCCTTAGTATCCA-3' R: 5'-ATCACCAACCGAAGTCACAA-3'	59.5	35	304
GAPDH	F: 5'-TAAAGGCGAGATGGTGAAAG-3' R: 5'-ACGCTCCTGGAAGATAGTGAT-3'	60.0	35	244
cTnI	F: 5'-AAGAAGGGTGGCAAGAAGCA-3' R: 5'-CTGGTGGTCACTGACACGATTT-3'	60.0	35	231
sm-MHC	F: 5'-AGCACCACTGAATCCCAAAG-3' R: 5'-AGTCCAGGGCCACATAACAC-3'	58.0	35	243
CD34	F: 5'-GTGCCACAACATCAAAGACG-3' R: 5'-GGAGCACATCCGTAGCAGGA-3'	60.0	35	239
CD31	F: 5'-GGTCGCATGAACATGAAGAA-3' R: 5'-TTGGTAGGGTTTGTAAAGGAC-3'	58.0	35	248

Differentiation of CECPCs

For cardio-myogenic differentiation, picked clonal cell mass of passage 3, passage 5 and passage 9 were digested to single cells and plated on plastic plates at $2 \times 10^4/\text{cm}^2$, then cultured for 10 days in DMEM/M199 (4:1 ratio) supplemented with 10% horse serum, 5% FBS and $10 \mu\text{M}$ 5-azacytidine. Smooth muscle cells were induced by culturing them on fibronectin-coated plates in DMEM/F12 supplement with 2% B27, 5% FBS, 10 ng/ml bFGF, 20 ng/ml EGF and 25 mM HEPES. For differentiation into endothelial cells, cells from the induced group were incubated in endotheliocyte medium containing 20 ng/ml VEGF, while control cells continued being cultured in complete medium, with no inducers.

Immunofluorescence staining

Cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h, followed by three rinses in PBS. They were then permeabilized with 0.25% Triton X-100 for 30 min. After rinsing three times in PBS, cells were blocked with 2% goat serum or 2% bovine serum albumin (BSA) in PBS for 45 min.

Cells were then incubated with antibodies to Isl1 (1:100, Isl1 monoclonal antibody, Abnova), to Flk1 and to CD34 (1:500, rabbit polyclonal antibody to Flk1, B&M, custom-made), GATA4 (1:100, rabbit polyclonal antibody to GATA4, Abcam), Nkx2.5 (1:500, rabbit polyclonal antibody to Nkx2.5, B&M, custom-made), cTnT (1:100, mouse monoclonal antibody to cTnT, Abcam) and α -actinin (1:100, alpha sarcomeric actin antibody (alpha Sr-1), Abcam) in a humidified chamber at 4°C overnight.

After three washes in PBS of 5 min each, cells were incubated in CY3 or FITC labelled secondary antibodies (1:200) at room temperature for 45 min. Finally, cells were rinsed three times in PBS for 5 min each time. Immunofluorescence results were observed and recorded using a confocal optical system (Nikon, Tokyo, TE2000).

SYBR Green real-time PCR analysis of gene expression

Total RNA was extracted from CECPC colonies at passages 3, 5 and 7 with Trizol (Invitrogen). cDNA was synthesized using an RNA PCR Kit (AMV) Ver 3.0 (Takara, China). Gene expression was detected using an ABI 7500 real-time PCR system (USA). Gene expression level was determined by specificity relative to expression of *GAPDH* gene, and specificity of PCR products was confirmed by melting curve analysis. Real-time PCR was performed in $20 \mu\text{l}$ mixture containing $10 \mu\text{l}$ SYBR premix Ex Taq buffer (Takara, Dalian, LN, China), $0.4 \mu\text{l}$ ROX Reference Dye, $0.8 \mu\text{M}$ each of forward and reverse primers (Table 1), $1 \mu\text{l}$ template cDNA and $7 \mu\text{l}$ ddH₂O. Cycling conditions were initial 10 s at 95°C followed by 40 cycles of two-temperature cycling: 5 s at 95°C (for denaturation) and 34 s at 60°C (for annealing and polymerization). Each experiment was performed with duplicates in 96-well plates and repeated three times. Expression level was calculated by the $2^{-\Delta\Delta C_t}$ method to compare the relative expressions.

RT-PCR

Total RNA was isolated from 2×10^6 cells. First-strand cDNA was synthesized from $5.0 \mu\text{g}$ of DNase-treated

Figure 1. Spatiotemporal distribution of CECPCs in the heart. Positive staining appears as a brown stain, nuclei are counterstained blue. The CECPCs were located in the atrium, ventricle and outflow during embryonic development. (a) Positive staining of Flk1 in heart tissue (cytoplasm); (b) Positive staining of Isl1 in heart tissue (nucleus); (c) Positive staining of Nkx 2.5 in heart tissue (nucleus); (d) Positive staining of Gata 4 in heart tissue (nucleus); (e and f) Positive staining of Isl1 in the outflow (bar = 100 μ m).

RNA with reverse transcriptase (Takara). RT products (cDNA) were used as PCR templates and amplified for 35 cycles. Primer sequences are listed in Table 1 and GAPDH was used as an internal control (Table 1). PCR was performed in a 50 μ l mixture containing 10 μ l 5 $\times$ PCR buffer (Takara), 28.5 μ l ddH₂O, 0.25 μ l Ex-Taq (Takara), 0.5 μ l each of forward and reverse primers, and 1.5 μ l template cDNA. Cycling conditions consisted of initial 2 min at 94 $^{\circ}$ C for one cycle, followed by 30 cycles of 30 s at 94 $^{\circ}$ C (for denaturation), 30 s at 50–60 $^{\circ}$ C (for annealing), and 2 min at 72 $^{\circ}$ C (for extension). PCR products were detected by 2.5% agarose gel electrophoresis.

Results

Spatiotemporal distribution of CECPCs in the heart

Immunohistochemical analysis demonstrated that Isl1, Flk1, GATA 4 and Nkx2.5 were extensively localized in the outflow tract, right ventricle, and part of the heart atrium (Fig. 1), but were not expressed in other cells.

There was clear difference between localization of CECPCs cells and other progenitor cells.

Morphology and population growth characteristics of CECPCs

In primary culture, CECPCs formed colonies 10 days after seeding, and many haemocytes were mixed with CECPCs; nevertheless, haemocytes and other cells were detached and eliminated from the population through FCM, the reason for which the cells were purified (Fig. 1i). Cells showed typical fibroblast fusiform morphology and maintained their expansion capacity and undifferentiated morphology up to the 9th passage (Fig. 2d–f). Cells began to adhere 1 h following passage and adhered completely after 24 h (Fig. 2b and 2c). Highest number of passages achievable for CECPCs was 12 in our experience, after which most cells had a somewhat senescent appearance, characterized by presence of vacuoles and karyopyknosis. Eventually, with increasing passage number, cells detached from the plates.

Figure 2. Morphology of chicken ED 8 heart and CECPCs. The morphology of CECPCs is varied, with the majority being oval or polygonal and showing clonal proliferation or a 'chain-like' structure during the culture period. (a) The structure of chicken ED 8 heart. (b) and (c) Primary cells after culture for 48 h. (d–h) Cell morphologies at passage 3, passage 5, passage 9, passage 11 and passage 12. (i) Purification of CECPCs through FCM.

Detection of CECPC markers

Specific surface antigen markers of CECPCs were detected by immunofluorescence and RT-PCR. Results demonstrated that CECPCs of different passage numbers were *Isl1*, *Flk1*, *GATA4*, *CD34* and *Nkx2.5* positive (Figs 3 and 4). Expression of *Isl1*, *Flk1*, *GATA4* and *Nkx2.5* CECPC marker genes was also analysed by real-time PCR (Fig. 5). Results revealed that CECPCs of different passage numbers expressed *Isl1*, *Flk1*, *GATA4*, *CD34* and *Nkx2.5*, but expression of *Isl1* was lower at passage 9.

Colony-forming cell assay

Colony formation was observed after 4 days, by microscopy. Colony-forming levels were 31.6 ± 0.2 , 30.6 ± 0.1 and $20.6 \pm 0.3\%$ for passage 3, passage 5 and passage 9, respectively, demonstrating capacity of cultured CECPCs for self-renewal (Fig. 5a).

Cell population growth kinetics

Population growth and proliferation of CECPCs were similar at passage 3, passage 5 and passage 9 according to the growth curves (Fig. 5b). After latency phase of 1–3 days, cell population growth entered the logarithmic phase, and reached plateau phase by approximately day 7.

CEPCs gave rise to three distinct cardiovascular cell lineages

Prolonged and rhythmic contractions were observed in the CECPCs following myocardial induction for 12 days (Affix 1). To identify mature myocardial cells, cTnI was selected; this is a cardiac troponin I expressed by myofilaments. Cells in the control group, cultured in normal medium, did not display any differentiated appearance and did not express cTnI. However, cells of the induced group exhibited morphology

Figure 3. Detection of CECPC markers by immunofluorescence staining. The results show that Isl1, Flk1, Nkx2.5, CD34 and GATA4 are positively expressed. (b), Flk1+; (e), Isl1+; (h), Nkx2.5+; (k), GATA4+; (n), CD34+; (a, d, g, h and m) contrast; (c, f, l and o) merged (bar = 50 μ m).

of myocardial cells and were positive for cTnI expression, by immunofluorescence and PCR, but not Isl1 (Fig. 6).

Smooth muscle cells express sm-MHC, which is smooth muscle myosin heavy chain expressed mainly in the cytoskeleton. After inducing differentiation of smooth muscle cells, morphology of CECPCs became pleomorphic, and expression of sm-MHC was detected

by immunofluorescence and PCR, but not Isl1 (Fig. 7).

On day 10 after induction, cell morphology changed from spindle shaped to rounded or irregular shapes, until all cells became closely packed with cobblestone morphology. CD31 and CD34 were detected by immunofluorescence and RT-PCR (Fig. 8), indicating that CECPCs had differentiated into endothelial cells.

Figure 4. Detection of CECPC markers by RT-PCR. M: Marker; 1: GAPDH (244 bp); 2: Flk1 (304 bp); 3: Isl1 (372 bp); 4: GATA4 (360 bp); 5: Nkx2.5 (366 bp); 6: CD34 (239 bp).

Discussion

In embryology, the heart is one of the earliest organs to mature, and its morphological characteristics change significantly during early embryonic development. In our research, the time at which CECPCs would be isolated was an important consideration, as it was important to use embryonic heart tissue of the appropriate age, to isolate the highest number of CECPCs. According to isolation of mouse embryo cardiac progenitor cells, experiments demonstrated that between 3 and 4 days

Figure 5. CECPCs self-renewal assay and relative gene expression. (a) Colony-forming units in P3, P5 and P9 CECPCs were counted, indicating that the colony-forming rates decreased with increasing passage number. (b) Growth curve of different passages CECPCs, The growth curve of CECPCs appeared sigmoidal and the population doubling time (PDT) was about 49.2, 51.4 and 55.6 h respectively. (d–f) show the analysis of relative gene expression profiles in CECPCs of different passage numbers. The results indicate that the expression of Isl1 and Nkx2.5 genes decreased with increasing passage number, but Flk1 and GATA 4 gene expression increased with increasing passage number. These results demonstrate that the CECPCs possess the capacity for self-differentiation into endothelial cell lineages.

Figure 6. Identification of chicken myocardial cells. The cells polymerized to form myotubules after culture in myocardial medium for 6 days. About 10 days later, the myotubules increased and fused to form fascicles. There was no obvious metamorphosis in control. (a–f) Immunofluorescence detection of Isl1 and cTnI expression before (d–f) and after (a–c) myocardial differentiation (bar = 100 μ m). (g) RT-PCR detection of Isl1 (lines 1 and 3) and cTnI (lines 2 and 4) expression before (lines 1 and 2) and after (lines 3 and 4) myocardial differentiation. M: marker; Isl1 (372 bp); cTnI (231 bp). (h) Real time PCR detection of Isl1 and cTnI expression before and after myocardial differentiation.

was best time for isolating chicken ECPCs and the isolation method (8) was improved to obtain highest quantities of viable, differentiation-competent CECPCs. Morphologies of CECPCs varied, with the majority being oval or polygonal, and exhibiting clonal proliferation *via* a 'chain-like' structure. CECPCs were sensitive to pH value of the culture medium, its fluctuation led to vacuole formation and apoptosis.

Isl1, which belongs to the Islet subfamily of the LIM homeobox transcription factor gene family, was first discovered and cloned in rats by Karlsson *et al.* (13). Isl1 protein plays an important role in regulating the production of different endocrine cells during pancreatic development (14), and is involved in motor neuron generation in the nervous system (15). In addition, its expression is detected in the pituitary gland of the endocrine system (16). In recent years, Isl1 has been shown to participate in regulation of cardiac develop-

ment, and it is the only specific marker for heart progenitor cells (17). GATA4, which is no longer expressed after maturation of the heart, is an important transcription factor during cardiac development and is a marker of early cardiac progenitor cells (18). Nkx 2.5 is a further marker of early cardiac progenitor cells that belongs to the NK2-type family, and its expression declines after birth of the mammalian foetus (19). Flk1, which is also known as VEGFR2 (vascular endothelial growth factor receptor 2), is specifically expressed on the surface of a type of cardiac progenitor cell and associates with other markers to identify cardiac progenitor cells (9). Although Isl1 is the only specific marker of embryonic cardiac progenitor cells (ECPCs), other markers (Nkx2.5, GATA4, CD34 and Flk1) expressed on subprogenitors or mature cardiac cells are used in combination to identify ECPCs. In our experiments, Isl1, Nkx2.5, GATA4 and Flk1 were shown in CECPCs to

Figure 7. Identification of chicken smooth muscle cells. The cell morphology was changed at day 7 after induction. The cells became longer and formed typical structure of muscle cells. The expression of sm-MHC (smooth muscle myosin heavy chain, special marker for smooth muscle cells) and IsI1 were detected by immunofluorescence and RT-PCR, and the results showed sm-MHC positive and IsI1 negative. (a–f) Immunofluorescence detection of α -actin expression before (d–f) and after (a–c) smooth muscle cell differentiation. (g) RT-PCR detection of IsI1 and sm-MHC expression before and after smooth muscle cell differentiation. 1: CECPCs, IsI1; 2: CECPCs, sm-MHC; 3: chicken smooth muscle cells, IsI1 (372 bp); 4: chicken smooth muscle cells, sm-MHC (243 bp). (h) Real time PCR detection of IsI1 and sm-MHC expression before and after smooth muscle cell differentiation.

be positively expressed, by immunofluorescence, real-time PCR and RT-PCR.

Cardiac progenitor cells can differentiate into three cell types *in vivo*, – cardiac muscle cells, smooth muscle cells and endothelial cells, which constitute the main tissue structure of heart (17). 5-azacytidine, as a cytosine analogue DNA demethylation agent, was previously usually used for differentiation of mature cardiac muscle cells from stem cells through genes related to DNA demethylation. It has been widely described in literature as an effective chemical stimulus used to promote cardiomyogenic differentiation, in various cell types (20,21). VEGF is also able to induce other stem/progenitor cells to mature into endothelial cells (22). The underlying mechanism is far from clear, except that the VEGFR-2 receptor is located on the cell membrane, that protein kinase B is activated by VEGF, and subse-

quently initiates different intracellular signalling pathways, promoting growth and differentiation of cells through activation of inositol triphosphate (23). Some scholars have suggested that *Hox* gene is a key factor for differentiation of stem/progenitor cells to mature into endothelial cells. Further research suggests that serine/threonine kinase Pim-1 is responsible for VEGF promotion towards endothelial cell differentiation (24). Growth and differentiation of smooth muscle cells can be controlled by EGF in serum or secreted from neighbouring cells (25–27). There are four types of EGF-receptor (EGFR) family in the cell membrane of ECPCs, heparin-binding EGF (HB-EGF), transforming growth factor alpha (TGF alpha), epiregulin (ER) and betacellulin (BTC). Among these four family members, EGFR was solely activated by EGF, resulting in induction of phenotypic modulation of SMCs. This effect was mediated

Figure 8. Identification of chicken endothelial cells. The cell morphology was changed after induction at day 10, from spindle to roundness or irregularity in shapes, until the cells were very closely located and showed cobblestone shape. (b) and (e) show positive expression of the endothelial cell markers, CD31 and CD34; (a) and (d) contrast; (c) and (f) merged; (g) RT-PCR detection of CD31 and CD34 expression before and after endothelial cell differentiation. 1: CECPCs, CD34 (239 bp); 2: CECPCs, CD31(248 bp); 3: endothelial cell, CD34 (239 bp); 4: endothelial cell, CD31 (248 bp). (h) Real time PCR detection of CD34 and CD31 expression before and after endothelial cell differentiation.

through co-ordinated activation of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (p38 MAPK) pathways (28). Differentiation results in our study showed that CECPCs could be induced into smooth muscle cells, endothelial cells and cardiac muscle cells capable of rhythmic contraction *in vitro*. Our results provide strong experimental evidence for the multi-potentiality of ECPCs. Thus, research on use and differentiation of cardiac progenitor cells will not only unveil further mysteries of heart development, but also provide promising cell sources for tissue engineering.

In conclusion, in this study, CECPCs were isolated from ED (embryonic day) 8–9 chicken embryos, and self-renewal ability and differential potential was evaluated *in vitro*. This work provides an important bearing on the potential application of CECPCs as a stem-cell source for regenerative therapies.

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