Journal of Advanced Veterinary and Animal Research
ISSN 2311-7710 (Electronic)
http://doi.org/10.5455/javar.2017.d196
A periodical of the Network for the Veterinarians of Bangladesh (BDvetNET)

March 2017
Vol 4 No 1, Pages 88-96.

JAVAR Journal of Advanced Veterinary and Animal Research

Original Article

Characterization exploration of endothelial progenitor cells from bovine bone marrow

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• **Received:** Dec 5, 2016 • **Revised:** Jan 1, 2017 • **Accepted:** Jan 5, 2017 • **Published Online:** Jan 5, 2017

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ABSTRACT

Objective: This research is designed to explore the methods of isolation and culture for endothelial progenitor cells from bovine bone marrow, characteristic, induced differentiative capacity *in vitro*.
Material and methods: Main experimental reagents contain DMEM/F12, fetal bovine serum, percoll lymphocyte separating, Trypsin 1: 250, VEGF, bFGF, GF-1, EDTA and so on. Cultivation system is DMEM/F12 with 10% FBS and VEGF 10 ng/mL, cultured under 37°C, 5% CO_2, saturated humidity. Cell viability is measured by trypan blue solution exclusion test. Immunofluorescent detection is used to detected cell surface markers and double swallows, while bovine chromosome is analyzed by karyotyping.
Results: We find that the majority of bovine endothelial progenitor cells (EPCs) are fibrous shaped. Frozen survival of bovine EPCs before and after cryopreservation is 95.2±0.14% and 80.9±0.30% respectively; cryopreservation affects little on the viability of bovine EPCs. Immunofluorescent detection of the cell surface markers CD34, CD133 and flk present positive, which can confirm that the cell cultured *in vitro* are EPCs. Then Dil-ac-LDL and FITC-UAE-1 uptake assays are carried out. Eventually, bovine EPCs are induced to differentiate into endothelial cells and smooth muscle cells respectively, demonstrating the multi-lineage differentiation potential of bovine EPCs *in vitro*.
Conclusion: EPCs can be got with proper culture system. The little cell cryopreservation effect and stronger induced differentiation potential *in vitro* imply that EPCs can be applied in genetic resources conservation and reuse.

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KEYWORDS

Bovine; Bone marrow; EPCs; Culture

How to cite: Ning M, Bai C, Sun Y, Li X, Guan W (2017). Characterization exploration of endothelial progenitor cells from bovine bone marrow. Journal of Advanced Veterinary and Animal Research, 4(1): 88-96.

INTRODUCTION

Endothelial progenitor cells (EPCs) are also known as precursor cells used in the treatment of atherosclerosis, which can differentiate into mature endothelial cells that participate in the repair of endothelial injury (Talbot and Blombergie, 2008; Spadaccio et al., 2010). In recent years, EPC transplantation display a wide application prospect in the field of vascular diseases therapy such as ischemic disease of heart head blood-vessel and wound healing (Marsboom and Janssens, 2008; Parolini et al., 2009), which has opened up a new field of cell therapy. But on account of the difference between sample source and separation methods, EPCs are gained little. So, new isolation and cultivation method of EPC is needed urgently.

The endothelial cells play an important role in maintaining the stability of vascular intima, the damage of endothelial cells will cause the diseases of the cardiovascular system (George et al., 2004). Scientists have been looking for the method to repair vascular endothelium via amplification of endothelial progenitor cells *in vitro* (Schmeisser et al., 2001; Gao et al., 2008; You et al., 2008). The discovery of EPC provides premise to achieve this desire. Asahara isolated the bone marrow sources of cells that CD34 and KDR positive from peripheral blood, which is called EPC now. later (Asahara et al., 1997; Kawamura et al., 2006), scientists also isolated EPC from umbilical cord blood, liver, marrow, arterial outer membrane and fat tissue (Eggermann et al., 2003; Bonello et al., 2006). They all can directionally home to the ischemic area and differentiate into mature endothelial cells with delayed high proliferation potential, not only can they participate in the embryonic development of angiogenesis, but also promote restoration of the damaged endothelial capillaries (Peichev et al., 2000; De Falco et al., 2009), apply to endangered species resources conservation. This research will explore the methods of isolation and culture, characterization and induced differentiation of EPC from bovine bone marrow.

MATERIALS AND METHODS

Ethical statement: All animal bodies are treated in accordance with NIH and USDA guidelines for the use of animals in research and all experimental procedures involving bovine are conducted in accordance with the protocols and guidelines for agricultural animal research codified by the Committee for Ethics of Beijing, China.

Experimental reagents: DMEM/F12 (Gibco, USA), fetal bovine serum (Biochrom, Germany) Percoll lymphocyte separating (Pharmasha), Trypsin 1: 250 (Amresco), VEGF, bFGF, IGF-1, PDGF-BB (Invitrogen, USA), rabbit anti chicken CD34, flk and CD133 polyclonal antibody (MBL, Japan), EDTA, FITC conjugated goat anti rabbit secondary antibody IgG (Zhongshan Golden Bridge, China).

Isolation and culture of bovine EPCs: Bovine fetus is putted to execution and soaked with 75% ethyl alcohol for disinfection immediately. Thighbone and shinbone are isolated from body, marrow cavities are washed with 3 mL PBS. Beating together of the liquid and adding up to 3 mL stem cell separation fluid of 1.077 gm/mL density 1500 r/min. Afterwards drawing monocyte present white clouds at the interface. Washing with PBS for 2 times. Finally aseptic operation is needed to pay attention all the whole process.

Cells are inoculated in terms of $5.0 x 10^6$/mL density in DMEM/F12 with 10% FBS and VEGF 10 ng/mL, cultured under 37°C, 5% CO_2, saturated humidity. Changing medium after 1 day of inoculation, then changing medium every 2 days. Cells can be passaged when going to 70-80% healing.

Estimation of cell viability: EPC viability before and after cryopreservation is detected using the Trypan Blue Solution exclusion test (Liu et al., 2010). Collecting cells when cell confluence degrees reach 80-90%, making into cell suspension of 2.0×10^6 density. Mixing 100 μL cell suspension and 100 μL Trypan Blue Solution, calculating total number of cells and cell number does not shade with blood count plate. Three lines are drawed after the data sorting table.

Immunofluorescence: EPC of passages 5 which go to 60-70% healing are selected. Cells are washed with PBS and then fixed in 4% (m/v) paraformaldehyde for 15 min then washed thrice with PBS. Cells are permeabilized using 0.2% (v/v) Triton X-100 for 20 min and washed a further three times with PBS. The cells are blocked in 10% (v/v) goat serum for 30 min, and subsequently incubated in following antibodies: Rabbit anti Chicken CD34; Rabbit anti Chicken flk; Rabbit anti Chicken CD133 for 8 h at 4°C. After that, cells are washed thrice with PBS, and incubated in appropriate fluorescein isothiocyanate (FITC)-conjugated secondary antibody for 1 h at room temperature. After incubation, cells are washed thrice with PBS. Nuclear staining is carried out with DAPI dye for 15 min, then cells are washed thrice with PBS. The cells are observed using the Nikon TE-2000-E confocal microscope, images are acquired and used to calculate positive ratios.

Karyotyping analysis: EPC that are passaged for 5 h are selected, most cells are just attached and individually exist. (1) Adding up 20 µL colchicine of 10 µg/mL to 3 mL medium, cultured at 37°C, 5% CO_2 and saturated humidity for 72 h; (2) Cells are collected and added to centrifuge tube, centrifuge for 2000 rpm, 8 min; (3) Abandoning supernatant, adding up 10 mL KCl of 0.075 mol/L, water bath for 37°C, 30 min. (4) Adding to 1 mL stationary liquid and blending, centrifuge for 2000 rpm, 8 min; (5) Abandon supernatant, adding up 7 mL stationary liquid and blending, standing at room temperature for 15 min, centrifuge for 2000 rpm, 8 min; (6) Dropping then staining with Giemsa. (7) Images are taken from laser scanning confocal microscope (LSCM).

Dil-ac-LDL and FITC- UEA-l uptake by EPCs: EPC of passages 5 which go to 60-70% healing is selected. Washed thrice using PBS, then incubated in media containing 12 µg/mL Dil-ac-LDL for 4 h at 37°C, 5% CO_2. Then cells are washed thrice more, fixed with 2% paraformaldehyde, and incubated for 1h with FITC-UEA-1 of 10 µg/mL concentration at room temperature. Images are got from laser scanning confocal microscope.

Endothelial differentiation: EPC attaining 60-70% confluence and in good growth status are selected. There are two groups divided. EPC of the experimental group is cultured with endotheliocyte medium containing 30 ng/mL VEGF, while EPC of control group remain in complete medium. Medium are refreshed every three days and immunofluorescence histochemical detection are carried out 20 days later, endothelial cells surface marker calpain, CD31 and PCDH12 are detected. EPC of control group used as a comparation.

Smooth muscle cell differentiation: EPC attaining 60-70% confluence and in good growth status are selected. There are two groups divided. Cells of the induced group is incubated in endotheliocyte medium containing 10 ng/mL PDGF-BB, while control cells remain in complete medium. The medium are refreshed every three days. Immunofluorescence histochemical detection are carried out 21 days later, smooth muscle cells marker SM-MHC and α-actin are detected. EPC of control group is used as a comparation.

RESULTS

EPCs morphological observation: Morphological feature is one of the important characters of cells.The isolated EPC exactly adhered are smaller and irregularly shaped (**Figure 1A**). The cells proliferate quickly within 48 h with blood island structure and cell clusters begin to shape and display fusiform, triangular and irregular shapes. Cells connect fore and aft, a linear structure form. A small amount of cells connect into the structure of the blood sample after 4 days culture. Then EPCs are passaged and we find that they develop fibrosis gradually (**Figure 1C**). The cells attaining 70-80% confluence can be passaged,the average passage time is 4-6 days, and the cells can be cultured up to P10 at most (**Figure 1**). Upon reaching P10, most cells display features representatively of senescence such as blebbing and karyopyknosis.

Estimation of cell viability: The cells begin to adhere at 24 h after resuscitation, grow in good condition and passage normally as former. No significant differences have been found between cells before and after cryopreservation (**Table 1**).

Table 1. The viability of EPCs before and after cryopreservation was determined using the Trypan blue exclusion test

Category	Endothelial Progenitor Cells(%)
Before Frozen	95.2±0.14
After Reviving	80.9±0.30

Immunofluorescence: EPC specific markers CD133, CD34 and flk are detected and images are captured by LSCM. We found that the cells expressed the three specific markers finally (**Figure 2**).

Karyotyping analysis: We find that bovine diploid chromosome is 2n=60. It is a total of 29 pairs of chromosomes and 1 on the sex chromosomes.

Dil-ac-LDL and FITC- UEA-l uptake by EPCs: EPC has the ability to uptake Dil acetylated low density lipoprotein (Dil-ac-LDL) and FITC-Oxytropis lectin 1 (FITC-UEA-I). Thus the capacity of bovine EPCs to uptake these two proteins is tested. It can display RFL red fluorescence under laser scanning confocal microscopy (LSCM) when the EPCs phagocytose Dil-ac-LDL and GFL green fluorescence, at the same time EPCs phagocytose FITC-UEA-1. The EPCs which is in the period of differentiation can not only Dil-ac-LDL but FITC-UEA-1 then display dual-fluorescence (yellow) under LSCM (Kalka et al., 2000; Hofstetter et al., 2002).

Endothelial differentiation of the EPCs: The capacity of bovine EPC to differentiate into endothelial cells is tested and then detected by subsequent morphological and phenotypic analysis. Cell morphology change from spindle to round or irregularly shaped after induction with VEGF for ten days. Immunofluorescence detection has been carried out after 20 days of induction to confirm that differentiation has occurred with the expression of

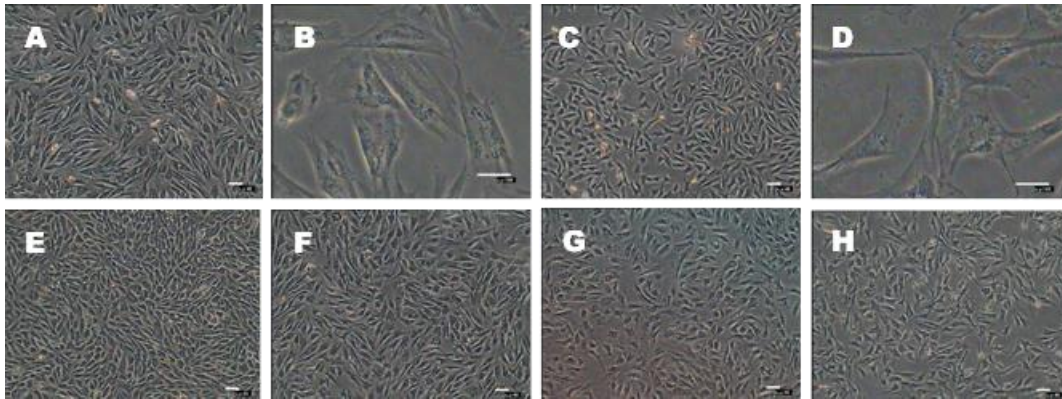

Figure 1. Morphology of bovine EPCs *in vitro*. A, B. Cells plated onto microplates after 0 h, scale bar is 40 µm and 100 µm respectively; C, D. P1 EPCs before passage with the fibrosis characteristic,scale bar is 40 µm and 100 µm respectively; E.P4 EPCs before passage (bar=40 µm); F.P6 EPCs before passage (bar=40 µm); G.P8 EPCs before passage (bar=40 µm); H.P10 EPCs before passage (bar=40 µm).

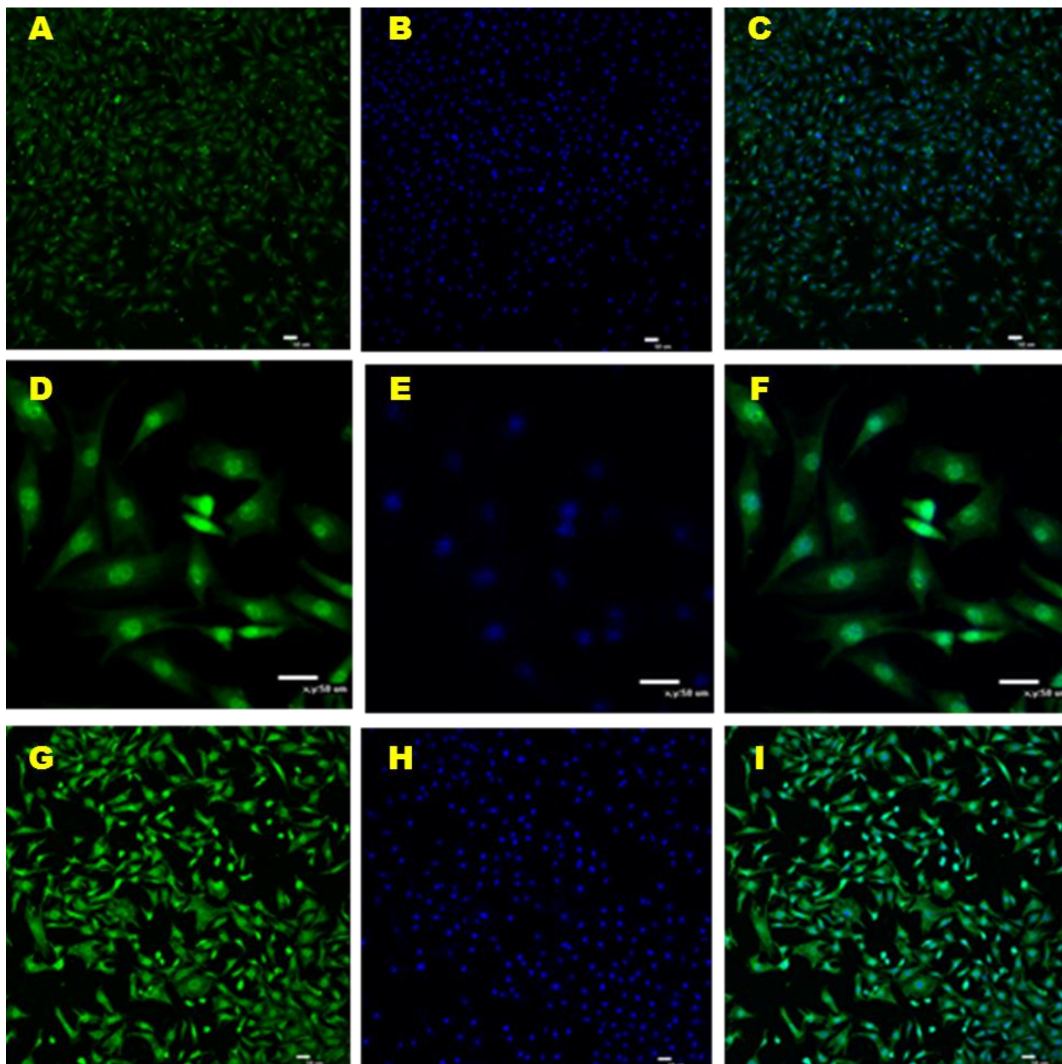

Figure 2. Expression of EPC surface markers in bovine EPCs. Expression of the EPC-specific markers CD133, CD34 and flk were examined by immunofluorescent labeling in bovine EPCs. CD133 marks both hematopoietic stem cells and EPCs. Its expression is gradually lost upon differentiation of EPCs into mature cells. A. CD34+; D.CD133+; G.flk+; B, E, and H.DAPI; C, F, and I Merged.

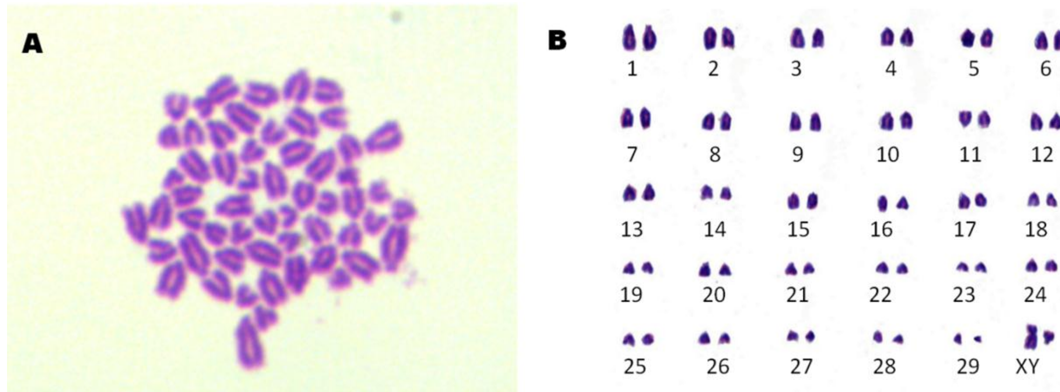

Figure 3. karyotyping analysis of bovine EPCs. A. Chromosome doubling in mitosis anaphase under microscope. B. Each pair of chromosomes after arrangement, a total of 29 to autosomal and a pair of sex chromosomes.

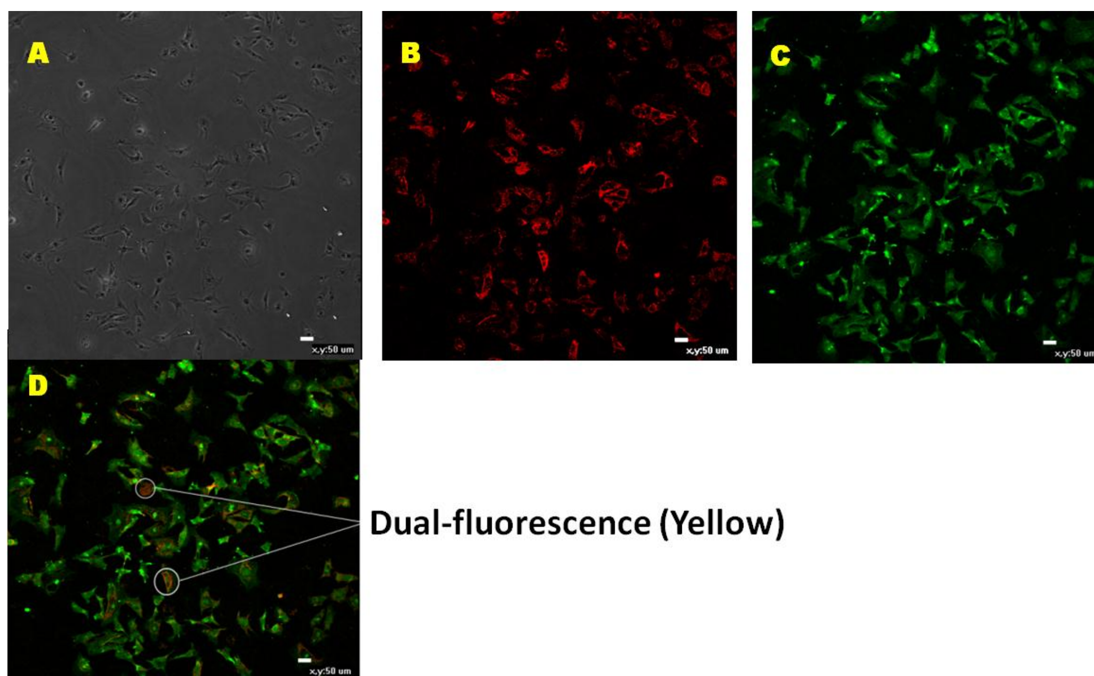

Figure 4. Bovine EPC uptake of DiI-ac-LDL and FITC-UEA-1. EPCs were incubated with DiL-ac-LDL and FITC-UEA-1, uptake was then assessed by immunofluorescent microscopy. DiL-acLDL (red); FITC-UEA-1 (green), differentiated EPCs (yellow). (bar=50 μm) A: Phase contrast; B: Dil-acLDL+; C: FITC-UAE-1+; D: Dil-acLDL & FITC-UAE1+ C.

endothelial markers examining. The endothelial markers of induced cells calpain, CD31 and PCDH12 are positive (**Figure 4**).

Smooth muscle cell differentiation of the EPCs: The ability of bovine EPC to differentiate into smooth muscle cells are also tested and then detected by subsequent morphological and phenotypic analysis. The cell morphology is changed at seven days after induction with becoming longer and forming structures typical of muscle cells. They express SM-MHC and α-actin which are specific markers for smooth muscle cells and detected by imunofluorescence (**Figure 5**).

DISCUSSION

Asahara et al. (1997) isolated EPCs from peripheral blood in 1997 firstly; the cells have the capacity to be differentiated into mature endothelial cells, and participate in vasculogenesis. EPCs derive from angioblasts as same as hematopoietic stem cells and form blood islands normally (Mauro et al., 2010; Roodhart et al., 2010). We find that EPCs are usually isolated from umbilical cord blood or peripheral blood in previous reports, nevertheless, 3% of EPCs can also be found in bone marrow mononuclear cells approximately (Boyer et al., 2005; Rotmans et al., 2006) and there are fewer researches paying close attention to it.

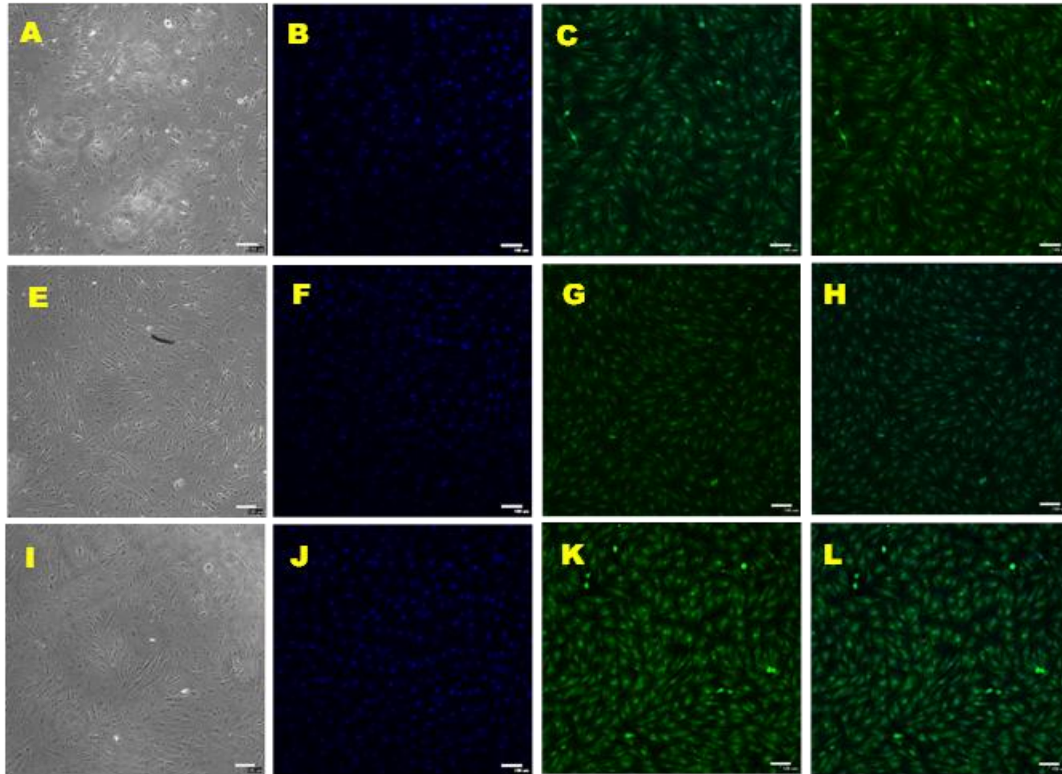

Figure 5. Identification of endothelial cells by immunofluorescent labeling. EPC morphology changed from spindle to rounded or irregular in shape, until the cells were very closely located and became cobblestone-shaped. A, E and I.Phase contrast; B, F and J.DAPI; C. calpain+; G.CD31+; K.PCDH12+; D, H and L merged.

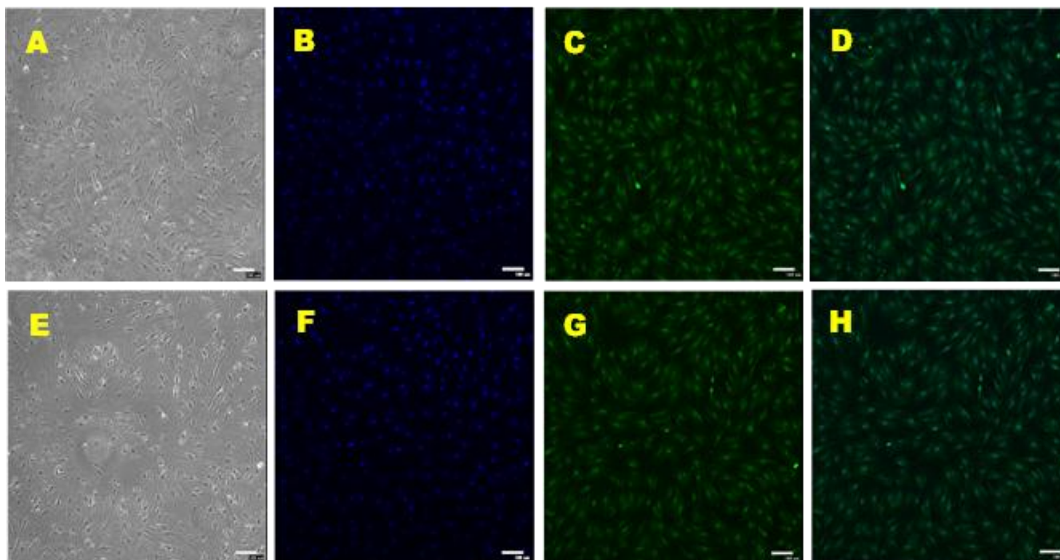

Figure 6. Smooth muscle cells were identified by immunofluorescence.EPC morphology changed after induction at day 7, whereby the cells became longer in shape and formed structures typical of muscle cells. A and E. Phase contrast; B and F.DAPI; C.SM-MHC; G.α-actin. D and H Merge.

So, we are characterizing EPCs isolated from bovine bone marrow in this study.

Nowadays, there are three current isolation methods of EPCs *in vitro* including flow cytometry, MASC and density centrifugation (Hummon et al., 2007; Bai et al., 2010; Chen et al., 2010). These are usually used for mononuclear cells such as hematopoietic cells and EPCs in blood. However, these are not the most suitable for bovine bone marrow-derived cell isolation. In

comparison to other mammal-derived EPCs, the difficulty lie in that bovine EPCs proliferate slower and not easy to rapidly passage.

EPCs are identified in the way of detection of the cell surface markers CD34, CD133 and flk by immunofluorescence (Takahashi et al., 1999; Vasa et al., 2001; He et al., 2006). Moreover, EPCs possess the specific ability to uptake both Dil-ac-LDL and FITC-UEA-1 (Gulati et al., 2003; Thomas et al., 2009; Heida et al., 2010), which can be used to identify EPC cell types. In this experiment, phagocytosis of labeled Dil-ac-LDL and FITC-UEA-1 demonstrate that the EPCs are double positive for these indicators. Weibel-Palade bodies are observed by laser scanning confocal microscope. Thus, bovine EPCs maintain the characteristics typical of EPCs *in vitro*.

CONCLUSION

In short, this study explores an optimized method for the isolation and culture of bovine EPCs cells. We gained that express specific surface markers CD133, CD34 and flk of EPC by immunofluorescence on LSCM. Karyotyping analysis confirms that these cells come from bovine. Cell cryopreservation has little effects on bovine EPCs, which contribute to genetic resources conservation and reuse. We also induce bovine EPCs to differentiate into endothelial and smooth muscle cells, which supports the bovine EPCs multipotency potential *in vitro*. This study not only provides technological means for the establishment of a bovine EPC line, but also suggests a new method to preserve the valuable genetic resources of bovine, which provide a seed resource for clinical application in the future.

ACKNOWLEDGEMET

This research is supported by The Agricultural Science and Technology Innovation Program (ASTIP) (cxgc-ias-01), project National Natural Science Foundation of China (Grant No 1472099) and the project National Infrastructure of Animal Germplasm Resources (2014 year).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS' CONTRIBUTION

MN carried out the isolation and culture of EPCs and participated in the immunofluorescence histochemistry, differentiation and drafting the manuscript. CB and YS polished English language of the manuscript. WG and XL conceived the study, and participated in its design and coordination. All authors read and approved the final manuscript.

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