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RESEARCH ARTICLE

Putative Endothelial Progenitor Cells Derived from Chicken Bone Marrow Cells *in Vitro*: Effect of Basal Culture Media on their Morphological, Phenotypic and Functional Properties

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ABSTRACT

Endothelial progenitor cells (EPCs) in circulation are originally thought to be mobilized from bone marrow (BM) and bone marrow mononuclear cells (BMMNCs) are extensively used for the induction of EPCs *in vitro*. In literature, various basal media have been employed for support the differentiation and growth of putative EPCs from BMMNCs. However, it remains unknown whether the basal culture medium affects the biological behaviors of EPCs. The aim of this work was to assess the characteristics and angiogenic activity of the putative EPCs induced from BMMNCs in two basal media: endothelial growth medium-2 (EGM-2) and Dulbecco's modified eagle medium (DMEM). Unfractioned BMMNCs from chicken were cultured in either EGM-2 or DMEM medium containing identical supplements. Cell morphology was constantly monitored using light microscope. The expression of progenitor marker CD133 and endothelial makers CD31 and VEGFR-2, ability of the cells to uptake Dil-labeled acetylated low density lipoprotein (Dil-ac-LDL) and to bind lectin *Ulex europaeus* agglutinin 1, migration capacity and angiogenic activity was determined on day 14 of plating. BMMNCs cultured in EGM-2 were morphologically different from those in DMEM and had higher mRNA level of CD133 and CD31. The Dil-ac-LDL/lectin dual-positive cells in EGM-2 did not differ from that in DMEM. However, the cells in EGM-2 had increased migration capacity and also formed tubular networks on Matrigel, whereas those in DMEM did not. Taken together, these results suggest the choice of basal culture medium has significant influence on the differentiation of BMMNCs to EPCs.

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INTRODUCTION

Endothelial progenitor cells (EPCs) are bone marrow-derived immature cells having the ability to migrate to peripheral blood and to differentiate into mature endothelial cells (Rafii, 2000), and are commonly defined as cells expressing endothelial markers (e.g., VEGFR-2, CD31) along with progenitor makers (e.g., CD34, CD133) despite none of these markers are fully specific (Timmermans *et al.,* 2009). So far, numerous studies have explored the role of EPCs in maintaining endothelial integrity and mediating repair of damaged endothelium (Zhang *et al.,* 2014; Ikutomi *et al.,* 2015). Bone marrow-derived EPCs were shown to promote vascular repair by migration, homing into damaged tissue and incorporate into neovascularization (Takahashi *et al.,* 1999). Because of their demonstrable regenerative capacity, EPCs have continuously received attention in regenerative medicine and are regarded as a promising treatment option for patients suffering from cardiovascular disorders (Chong *et al.,* 2016). The EPCs can also serve as tumor-selective targeting vectors for the delivery of therapeutic genes (Muta *et al.,* 2003; Debatin *et al.,* 2008).

Although the pathophysiological role and therapeutic application of EPCs have been the subjects of intense experimental and clinical investigation, definition of EPCs is still difficult due to a lack of consensus regarding the EPC source, the optimal isolation and culture techniques. Unfractionated BMMNCs have been widely employed for in vitro isolation and expansion of EPCs (Liu *et al.,* 2013). However, in literature, different basal media such as endothelial growth medium (EGM)-2 (Hur *et al.,* 2004;

Seemann *et al.,* 2014) and Dulbecco's modified eagle *medium* (DMEM) (Sekiguchi *et al.,* 2011; Bai *et al.,* 2012; Lu *et al.,* 2014) have been applied by investigators. The EGM-2 medium is optimized for the culture of endothelial cells from large blood vessels and various endothelial cell lines, whereas the DMEM is designed to preserve and maintain the growth of a broad spectrum of cell types. It is noteworthy that DMEM is used for cultivation of BMMNC-derived mesenchymal stromal cells (MSCs) as well (Soleimani and Nadri, 2009; Montzka *et al.,* 2010).

It is found that choice of expansion medium can have a substantial influence on the characteristics of BMMNCs-derived MSCs (Hagmann *et al*., 2013). However, to date, very little is known about the impact of basal medium on EPC differentiation from BMMNCs. Our previous studies have shown that EPCs from broiler chickens share the same characteristics and function with EPCs in mammals (Bi *et al.,* 2014; Shah *et al*., 2014), providing a potential avian model for biomedical research in EPC-mediated tissue regeneration and EPC-based cell therapy. The present study was conducted to compare the biological features of the putative EPCs derived from chicken BMMNCs in EGM-2 and DMEM media.

MATERIALS AND METHODS

This study was approved by the Animal Care and Use Committee of Zhejiang University.

Isolation and primary culture of bone marrow cells: Bone marrow cells were hygienically extracted from the femurs of 1-week-old clinically healthy broiler chickens (Cobb 500). Mononuclear cells were obtained by density-gradient centrifugation using Ficoll medium (1.078g/mL, Tianjin Haoyang Biological Manufacture, China), distributed into two equal samples and resuspended in either DMEM (Invitrogen) or EGM-2 medium (Lonza).

Cells were plated onto 6-well plates at a density of 1 × 10^7 cells/well and maintained in 1 mL of complete DMEM or EGM-2 containing identical amount of penicillin (100IU/mL), streptomycin (100μg/mL) and FBS (2%) with supplementation of insulin-like growth factor 1 (IGF-1), fibroblast growth factor-β (FGF-β), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), ascorbic acid and heparin as suggested by Lonza. Cells were incubated at 39ºC with 5% CO_2. After 48 hrs of plating, the non-adherent cells in the cultures were removed and the medium was changed every 2 days.

Cell characterization: Cell morphology was monitored using phase contrast microscopy. EPC phenotype were further characterized for their ability to incorporate acetylated low density lipoprotein labeled with fluorescent Dil dye (Dil-ac-LDL, Invitrogen) and uptake of fluorescein isothiocyanate-conjugated lectin *Ulex europaeus* agglutinin (Sigma) as previously described (Shah *et al.,* 2014). Cell nuclei were visualized using 4′,6-diamidino-2-phenylindole (DAPI). The Dil-ac-LDL/lectin double-positive cells were counted and the percentage of these cells in the cultures was quantified as described by Shah *et al.* (2014).

Real-time quantitative PCR (qPCR): On day 14 of plating, cells were harvested and total RNA was extracted using TRIzol reagent (Takara). cDNA was synthesized with 500ng RNA using PrimeScript RT reagent/gDNA eraser kit (Takara). The primer sets for CD133, VEGFR-2, CD31 and GADPH (reference gene) were designed according to our previous study (Bi *et al.,* 2014). qPCR was performed in a 20μL volume containing 2μL of 1:20 diluted cDNA template, 0.4μM of sense and anti-sense primers, 7.2μL ddH_2O and 10μL SYBR green Master Rox (Roche, Diagnostic). PCR was performed using ABI Prism 7500 Sequence Detection System (Applied Biosystems) with the following thermal conditions: 95°C for 10 min, 40 cycles of 95°C for 15s, 60°C for 30s, and 72°C for 30s. qPCR values were normalized against GADPH expression, and the fold change of the gene of interest in the cells cultured in EGM-2 over that in DMEM was calculated by using $2^{-\Delta\Delta Ct}$ method.

Cell migration: Cell migration assay was performed in a Boyden chamber separated by a polycarbonate membrane with pore size of 5μm. Briefly, a total of 3 × 10^4 cells was suspended in 150 serum-free medium and added into the upper chamber. Medium (200μL) containing 5% FBS was added into the lower chamber. After migrating for 04 hrs at 37°C, the membranes were removed, fixed in 4% paraformaldehyde for 10 min, and then stained with Giemsa reagent. Cells had passed through the membrane were counted (×100 power). A minimum of 4–5 fields (2.54mm^2/field) per membrane (33.2mm^2/filter) were quantified. Cell migration ability was expressed as total number of migrated cells/total number of input cells×100%.

***In vitro* tube formation:** *In vitro* tube formation assay was performed as previously described (Shah *et al.,* 2014).

Statistical analysis: Data are presented as mean±SD. Intergroup comparison was done by student's *t*-test with SPSS 16.0 (SPSS Inc. Chicago, IL, USA) and $P<0.05$ was considered as significant.

RESULTS

As shown in Fig.1, BMMNCs in EGM-2 and DMEM showed significantly different morphologies and growth patterns. After 48 hrs of culture, more cells in EGM-2 were adhered to the well bottom compared to those in DMEM. While the BMMNCs in EGM-2 were still round shaped on day 2, some of their counterparts in DMEM had already changed to a rod-like shape. After 4–5 days, the cells in EGM-2 differentiated to small clusters composed of polygonal or short spindle-like cells, matching the typical morphology of early EPCs as Shao *et al.* (2011) reported. In contrast, cells in DMEM exhibited out-growth colonies surrounded by elongated fusiform-shaped cells similar to that of the BMMNC-derived MSCs (Li *et al.,* 2015). After 14 days of culture, cells in EGM-2 appeared to have both cobblestone- and spindle-like morphologies, whereas the cells in DMEM were still elongated fusiform-shaped. Notably, the cell number in DMEM culture was significantly reduced as compared to that in EGM-2 culture.

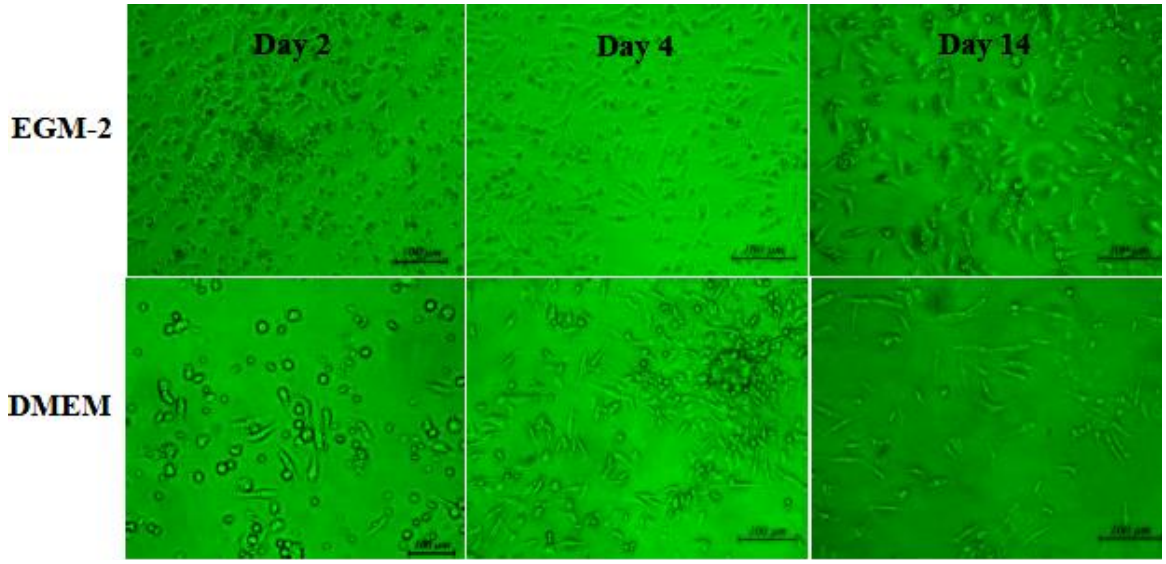
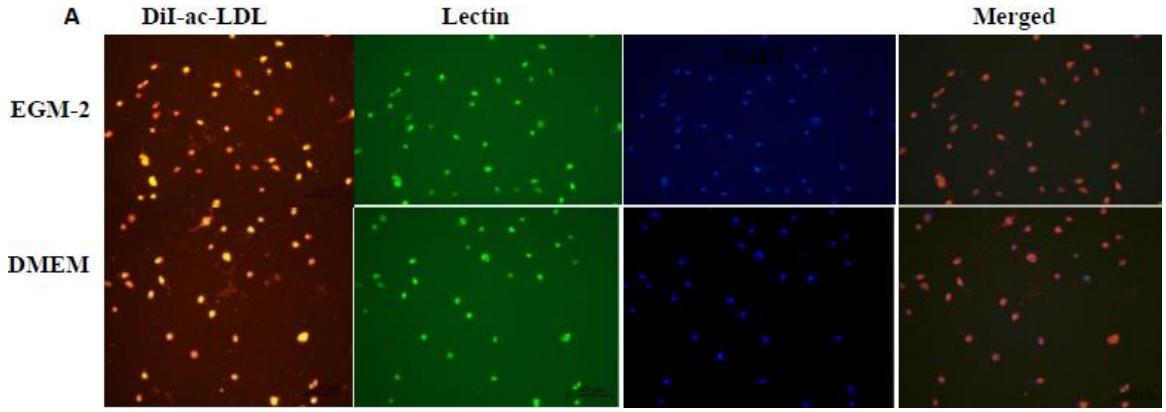


Fig. 1: Morphological characteristics of BMMNC-derived putative EPCs in either EGM-2 or DMEM medium. Non-adherent cells were removed after 48 hrs of culture. Note the significantly morphological difference of the cells in the two different basal media.



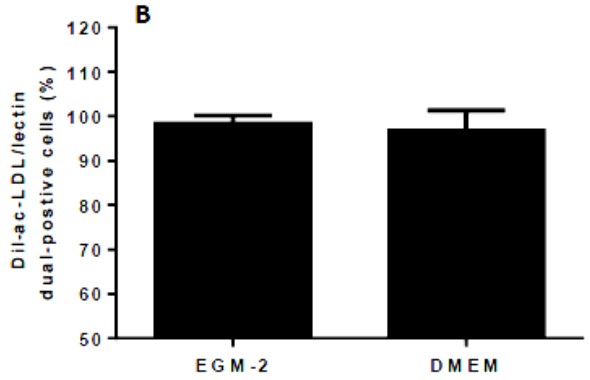


Fig. 2: Representative micrographs of Dil-ac-LDL intake and lectin binding by cells cultured in EGM-2 and DMEM (A). Cell's nuclei were visualized with DAPI. (B) Bar chart showing the portion of Dil-ac-LDL/lectin double-positive cells in the cultures (mean±SD of 5 replicates).

On day 14 of culture, the cells were used for the analysis of EPC phenotype by Dil-ac-LDL and lectin staining in addition to the detection of the mRNA expression of cell surface markers. No significant difference in the number of Dil-ac-LDL/lectin dual-positive cells was determined between BMMNCs cultured in EGM-2 and DMEM (Fig. 2). However, the gene expression of CD133, a typical marker for progenitor cells, as well as the endothelial markers CD31, were significantly increased in the cells cultured in EGM-2 than their counterparts in DMEM (Fig. 3).

As shown in Fig. 4, cells cultured in EGM-2 had significantly increased capacity in migration as compared to their counterparts in DMEM. After plating on a matrix gel surface, cells in both media attached to the matrix and then migrated to each others. After 40 hrs of culture, the cells in EGM-2 arranged into star-like shapes with omni-directional sprouts and established contacts to neighbor clusters, forming capillary-like structures. In contrast, the cells in DMEM were unable to form any tube-like structures but presented an apparent lack of differentiation during 40 hrs in culture (Fig. 5).

DISCUSSION

Despite extensive studies of EPCs on their biological properties and their therapeutic potential, there is still debate about the definition of an EPC because no specific surface markers have yet been identified for isolation of these cells (Fadini *et al.*, 2012). On more basic level, there is still a lack of uniform and standard method for EPC culture, making it difficult to compare the findings regarding EPC function in published studies. Although the efficiency of EPC isolation and induction from different sources of mononuclear cells (i.e., peripheral blood and bone marrow) has been evaluated (Amini *et al.*, 2012),

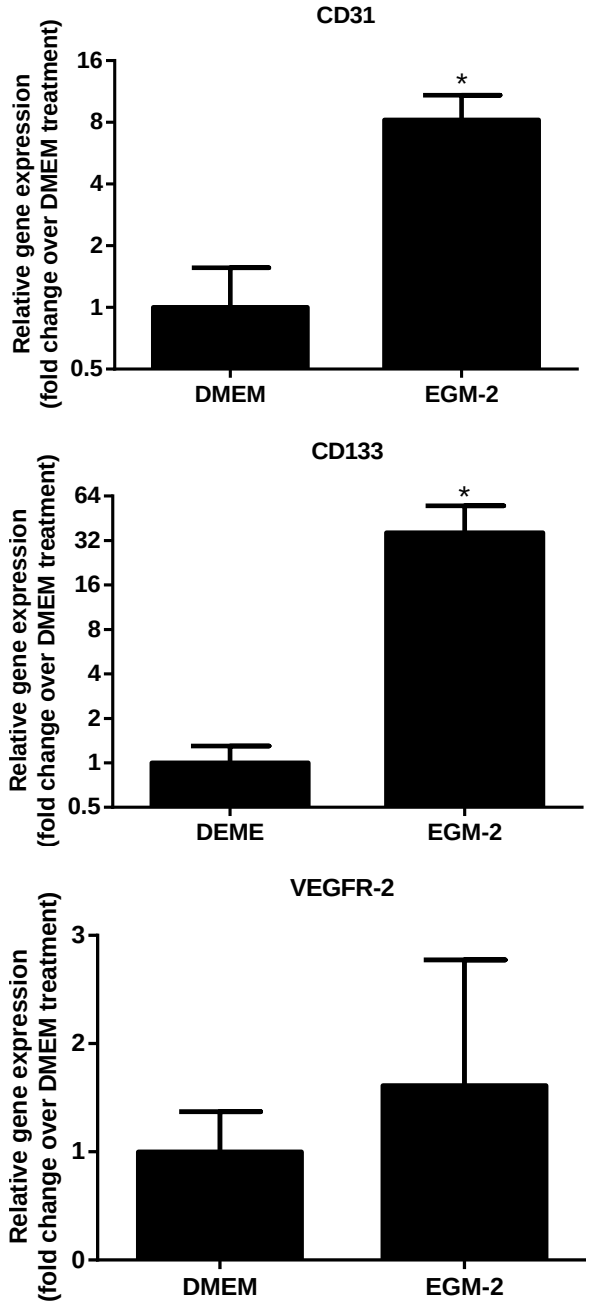


Fig. 3: Relative expression of progenitor marker CD133 and endothelial markers CD31 and VEGFR-2 in BMMNC-derived cells in EGM-2 and DMEM (mean±SD of 3 replicates). **P* <0.05.

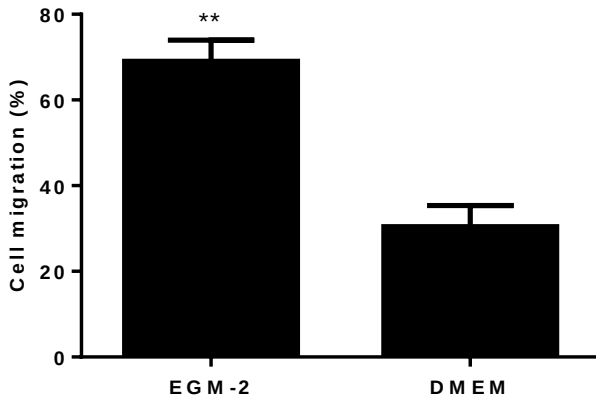


Fig. 4: Migration ability of BMMNCs-derived putative EPCs in EGM-2 and DMEM as measured using Boyden chamber assay (mean±SD of 4 replicates). ***P*<0.01.

few attempts have been made to address the influence of basal culture medium on the differentiation of mononuclear cells into EPCs. In this work, we isolated mononuclear cells from chicken bone marrow and cultured them in two different basal media to investigate the biological characteristics of the putative EPCs derived from these cells.

We found that BMMNCs in different culture media had strikingly different morphologies and showed different abilities to adhere, differentiate and proliferate. After plating the BMMNCs for 48 hrs, more adherent cells were found in EGM-2 than in DMEM. The cells in EGM-2, which differentiated into polygonal or short spindle morphology during the early stage of culture and subsequently into a mix of cobblestone-like and spindle-shaped morphologies, were different from those in DMEM, which formed outgrowth colonies and gave rise to elongated fusiform-shaped cells. BMMNCs in DMEM differentiated earlier than BMMNCs in EGM-2, but had reduced proliferative potential than the latter. In line with our observations, Yang *et al*. (2011) also demonstrated that rat BMMNCs cultured in different media displayed different morphologies and proliferative capacity.

The cells in EGM-2 and DMEM were then examined for EPC phenotype by evaluating their ability to uptake DiI-ac-LDL and bind lectin. Dual DiI-ac-LDL/lectin binding capacity has been widely employed in previous studies to define EPCs (Ahrens *et al.*, 2011; Zhi *et al.*, 2016). Although the cells cultured in EGM-2 and DMEM showed different morphological characteristics, we did not determine a significant difference in the number of DiI-ac-LDL/lectin dual-positive cells between the two populations. This is not surprising because the ability of DiI-Ac-LDL and lectin incorporation is not necessarily unique for EPCs (Rafii and Lyden, 2003).

The lack of commercial chicken-specific antibodies against EPC-related surface antigens limited our attempts to immunohistochemically identify the EPC phenotype of the cells obtained in EGM-2 and DMEM. However, our qPCR results showed that both the cells in EGM-2 and DMEM expressed progenitor cell marker CD133 and endothelial markers VEGFR-2 and CD31. However, the cells in EGM-2 had significantly increased mRNA level of CD133 and CD31 compared to those in DMEM. These results, along with the findings that the morphology of cells in EGM-2 resembled the EPCs more closely than those in DMEM, suggest that BMMNCs have increased capacity of EPC differentiation in EGM-2 than in DMEM. It must be noted that we did not measure the mRNA level of CD34 because our previous studies showed that EPCs from broiler chickens lacked CD34 expression (Bi *et al.*, 2014; Shah *et al*., 2014).

EPC migration is thought to be important for angiogenesis (Schmidt *et al.*, 2007). It is found that the putative EPCs derived from mammal BMMNCs under different culture conditions had different migration abilities (Guan *et al.*, 2013). In line with this study, we also determined a significant difference in the migration capacity of chicken BMMNCs cultured in different basal media. Additionally, the cells derived from BMMNCs in EGM-2 in this work formed well-organized tubular structures on Matrigel, whereas those from BMMNCs in DMEM did not. These results allowed us to argue that the

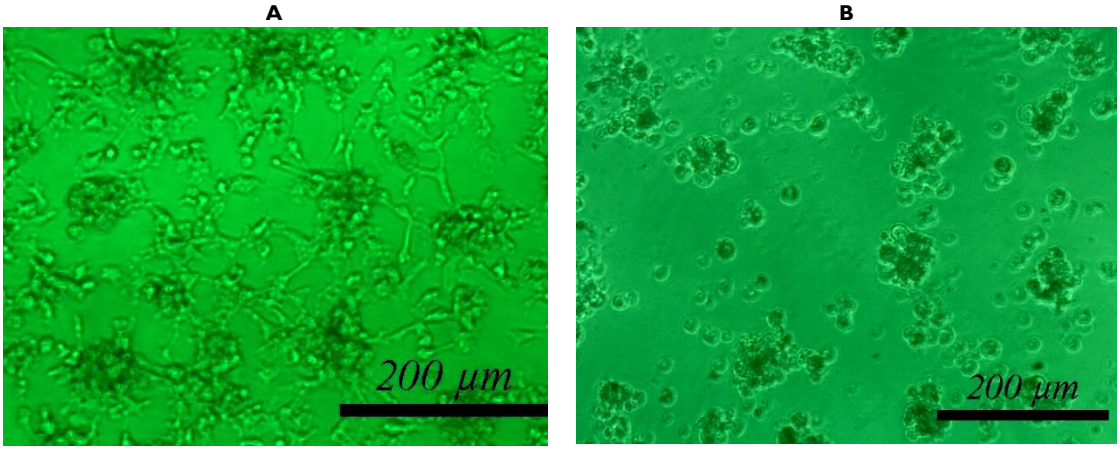


Fig. 5: Representative phase-contrast images of in vitro tube formation of the putative EPCs derived from BMMNCs in EGM-2 (A) and DMEM (B).

cells we obtained in EGM-2 were more likely EPCs, reproducing previously reported findings (Hur *et al.*, 2004; Seemann *et al.*, 2014). However, others have also shown that rat BMMNCs in DMEM can differentiate into typical EPC phenotype with the ability to form tube-like structures (Carneiro *et al.*, 2015). This discrepancy could be attributed to the differences in the culture time and the stimulatory factors employed. To this end, cautions must be taken when compare the findings between different laboratories.

Conclusions: This work demonstrates that basal culture medium has a substantial influence on the EPC differentiation of BMMNCs. Our results that the cells cultured in EGM-2 matched more closely the key features of putative EPCs currently defined suggest that EGM-2 may be more suitable than DMEM for the isolation and expansion of putative EPCs from BMMNCs. *In vivo* functionality of the cells derived from BMMNCs in these two media warrants further investigations. Moreover, our previous study has shown that the BMMNCs cultured in EGM-2 containing 10% FBS also express CD133, VEGFR-2 and CD31, but they display the characteristics of MSCs as well and have very limited ability to form tubular structures on Matrigel (Shah *et al.*, 2014). In context, findings in our previous study (Shah *et al.*, 2014) and in this work, suggest that the amount of FBS in EGM-2 may also influence EPC differentiation of BMMNCs. In this regard, further studies are still needed to optimize the culture conditions for production of EPCs from BMMNCs in EGM-2.

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Author's contribution: XT conceived and designed the experiment and QAS executed the experiment. XT and SH critically revised article for additional intellectual input and approved the present manuscript.

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