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## Abstract

The renoprotective effects of endothelial progenitor cells (EPC) have been clearly identified and many results have shown their successful use in neovascularization of ischemic tissues associated with blood vessel diseases. However, the availability of a sustainable source of EPC for large therapeutic applications remains critical. The aim of this work is to ensure the production of a specific EPC profile from mesenchymal stem cells (MSC). Non-adherent EPC were significantly amplified after 6 days of angiogenic growth factor activation. Three day-EPC showed an over expression of the hematopoietic markers CD34 and CD45 with a commitment to endothelial lineage with the expression of CD31 and CD146 cell markers. MSC lost their mesenchymal profile by down-regulating the CD90 and CD105 expression.

## Human Umbilical Cord Blood Mesenchymal Stem Cell Differentiation to Endothelial Progenitor Cells *in vitro* Results in a Population with an Endothelial and Monocytic Profile

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Cytokeratin 8/18 and von Willebrand Factor VIII were progressively expressed on EPC as well as the monocytic CD14. In six-day EPC, all of the CD31+ cells expressed CD14 and CD146. CD54, CD11a, and CD49d adhesion molecules were also strongly expressed in both 3-day and 6-day EPC. These findings suggest that MSC-derived EPC might be a unique population of CD34+CD45+CD14+CD31+CD146+ cells sharing both stemness and progenitor characteristics of endothelial cells

and monocytes, and that this culture model improved EPC production and expansion for a potential future use in vascular diseases.

**Keywords:** Endothelial Progenitor Cells, Expansion Culture, Mesenchymal Stem Cells, Monocytes, Neovascularization.

## 1. Introduction

*In vivo* life-saving of human organs strongly suggested the presence of residual stem cells involved in the reconstitution of tissue damages and organ functions (Herrera *et al.*, 2006; Gupta *et al.*, 2006; Chen *et al.*, 2008; Bruno *et al.*, 2009). These stem cells are mainly identified as mesenchymal stem cells (MSC) (Bruno

*et al.*, 2004; Chen *et al.*, 2008; Pasquinelli *et al.*, 2010) and act in a paracrine manner (Kinnaird *et al.*, 2004; Hoch *et al.*, 2012; Dorronsoro and Robbins, 2013) to repair injury. Over the last decade, such physiological aspects of MSC sparked great interest for advanced therapeutic applications in vascular diseases (Wang *et al.*, 2004; Herrera *et al.*, 2006; Pasquinelli *et al.*, 2010; Dorronsoro and Robbins, 2013; Peng *et al.*, 2013).

In animal subjects with renal injury, the transplantation of MSC induced angiogenesis, proliferation of renal cells and recovery of renal function improved by cell recruitment into ischemic sites (Asanuma *et al.*, 2010; Fang *et al.*, 2012; Peng *et al.*, 2013; Dorransoro and Robbins., 2013; Liu *et al.*, 2013(1); Liu *et al.*, 2013 (2); Zhu *et al.*, 2013). This cell migration resulted in neovascularization of the renal tubules, and was achieved by the differentiation of circulating MSC under local trophic preconditioning and changes in their morphology, size and function (Gang *et al.*, 2006; Wu *et al.*, 2007).

In addition, circulating endothelial progenitor cells (EPCs) have been identified (Asahara *et al.*, 1997; Shi *et al.*, 1998; Yamaguchi *et al.*, 2003; Smadja *et al.*, 2007) but were less abundant than MSC (Aguirre *et al.*, 2010). These cells were able to form new blood vessels both *in vitro* and *in vivo*, to migrate to injured sites and to differentiate into endothelial cells inducing tissue repair and neovascularization (Shi *et al.*, 1998; Yamaguchi *et al.*, 2003; Hur *et al.*, 2004; Asahara *et al.*, 2005; Blann and Pretorius, 2006; Duan *et al.*, 2006). Furthermore, the angiogenic potency of these EPC was more important compared to MSC (Chade *et al.*, 2009; Aguirre *et al.* 2010; Zhu *et al.*, 2013). EPC hold great interest for renal tissue repair and efforts have to be doubled up to guarantee a sustainable specific cell source and profile for future therapeutic potential use.

EPC were primarily isolated from circulating blood, but this isolation appeared to necessitate more labor to collect the specific cellular profile at the suitable needed concentration. To overcome this, some authors have suggested that EPC be obtained from MSC when cultured in a conditioned media supplied with additional growth factors or by MSC-secreted proangiogenic factors media (Wang *et al.*, 2004; Song and Tuan, 2004, Wu *et al.*, 2007; Hoch *et al.*, 2012). In the present study, angiogenic growth factors were applied to MSC in order to obtain a sustained EPC production with an endothelial profile presenting abilities for proliferation, migration and homing properties.

## 2. Materials and Methods

### 2.1. Collection of human umbilical cord blood (hUCB) cells

All patients were informed about UCB collection, and signed informed consents were obtained before performing the collection. The institutional ethical committee requirements were respected from notification of patients to actual collection. UCB samples were obtained from full-term healthy and consenting women after normal vaginal and/or cesarean deliveries using a sterile syringe from the cord after placenta delivery and flowed into flasks containing a conservation medium composed of RPMI (Gibco, Life Technologies) at 10% EDTA (Gibco, Life Technologies), 5% Bovine Foetal Serum (BFS, Promega, Promega Corporation), 5% penicillin-streptomycin (Gibco, Life Technologies) and 2% fungisone (Gibco, Invitrogen).

### 2.2. MSC separation and expansion culture

Human umbilical cord blood (hUCB)-derived MSC were obtained as previously described (Mazini *et al.*, in press). MSC were then cultured in DMEM supplemented with 10% BFS, 2% penicillin-streptomycin and 1% fungisone, and seeded at  $2 \times 10^6$  cells into 75 cm<sup>2</sup> flasks and maintained at 37°C and 5% CO<sub>2</sub>. The culture medium was changed twice a week. When 90% flask confluence was reached, 5% trypsin EDTA (Gibco, Life Technologies) was added to the adherent MSC and incubated at 37°C for 10 min. Cells were washed twice in RPMI supplemented with 5% BFS. The final pellet was counted and tested for viability by Trypan Blue dye exclusion (Gibco, Invitrogen) before seeding in new flasks for an additional passage.

### 2.3. EPC production

When total MSC flask confluence was reached, basic-fibroblasts growth factors (b-FGF, Gibco, Life Technologies) and vascular endothelial growth factor (VEGF, (Gibco, Invitrogen) were added to the culture medium at 10µg/ml and 40µg/ml, respectively. EPC were produced in the non-adherent supernatant of the culture. EPC were then collected and tested for viability by Trypan Blue dye exclusion.

## 2.4. Immunophenotyping of MSC and EPC

The immuno-phenotypic characterization of cells was performed by flow cytometry, using the cell markers monoclonal antibodies (mAbs) CD11a (Beckman Coulter), CD14 (Invitrogen), CD25 (Invitrogen), CD31 (Invitrogen), CD34 (Invitrogen), CD45 (Beckman Coulter), CD49d (Beckman Coulter), CD54 (Beckman Coulter), CD90 (Beckman Coulter), CD105 (Beckman Coulter) and CD146 (Beckman Coulter). According to the manufacturer's instructions, a suspension of  $1.10^6$  MSC or EPC was incubated with 10  $\mu$ l of mAb for 30 min at +4°C. MSC were collected by trypsinization of confluent flasks and EPC from the supernatant of 3 and 6 days of activated culture. Cells were washed before being tested by the cytometer.

## 2.5. Immunocytochemistry

Suspensions of MSC and EPC were seeded on slides using Lab-Tek Chamber Slide System (Rotenta R 300, Hettich), and centrifuged for 30 min at 300g. Once the slides were dry, immunostaining with Cytokeratin 8/18 (CK18/8) (Invitrogen, Life Technologies) and von Willebrand Factor VIII (Factor VIII) (Invitrogen, Life Technologies) mAbs was performed using the Histostain sp Broad Spectrum kit (DAB) (Invitrogen). The reaction color was visualized via a DAB reaction. The cells were then lightly counter-stained with hematoxylin and examined under optical microscope.

Positive controls were realized by using primary specific mAbs without the secondary ones while in negative controls staining with primary specific mAbs was omitted. EPC produced in the culture supernatant were labeled with CK18/8 and Factor VIII mAbs after 3 and 6 days of activation, and compared to labeled MSC.

## 2.6. Statistical analysis

Mean EPC yield proliferation was tested by student's t-test for statistical differences. P value <0.05 was considered to be significant.

## 3. Results and Discussion

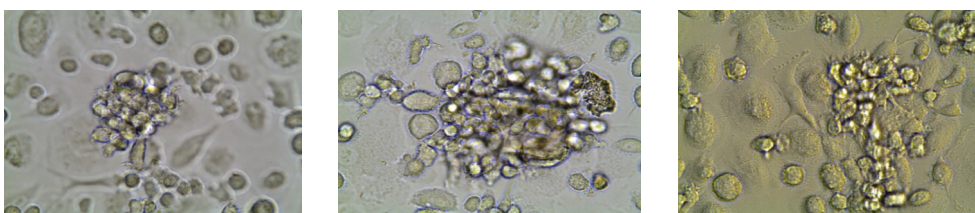
### 3.1. hUCB-derived MSC and differentiation culture

MSC are identified in culture as fibroblasts-like with a short spindle and are irregular in shape regardless of their origins. Previous findings suggested that hUCB-derived MSC were shown to have mesengenic activity *in vitro* as well as *in vivo* (Gang *et al.*, 2004; Peng *et al.*, 2012; Fang *et al.*, 2012; and Dorronsoro *et al.*, 2013). These cells promoted renal cell regeneration and enhanced angiogenesis through differentiation into endothelial cells promoting then new renal vessel formation.

In our culture, and when activated with b-FGF and VEGF, adherent MSC produce red colonies developing and spreading over the adherent layer and on the supernatant, as shown in *Figure 1*. After 3 days of activation, these colonies appear small with less than 50 cells, and increase in size and number until 9 days of activation. The colonies containing cells were isolated from the supernatant for identification, and cells were called thereafter EPC.

### 3.2. Kinetic of EPC production

In a conditioned media with growth factors or MSC-secreted proangiogenic factors, EPC can be obtained from MSC *in vitro* and expanded *ex-vivo*. Here, we report that colonies of EPC induced from hUCB-derived MSC can be obtained in culture after activation



**Figure 1.** MSC culture by optic microscope after 3 days (A), 6 days (B), and 9 days (C) of activation by b-FGF and VEGF (inverted phase contrast inverted optical microscope  $\times 40$ ).

with b-FGF and VEGF. These non-adherent EPC were collected after 3, 6 and 9 days of activation. The mean EPC yield increased progressively, as shown in **Table 1**, and presented a mean viability of 98% (unpublished data). EPC showed a significant production rate of more than 130 fold from 3 to 6 days ( $P < 0.05$ , **Table 1**). Thereafter, the mean EPC yield did not differ significantly. Seeded at  $2 \times 10^6$  per flask, MSC were able to produce more than  $1 \times 10^6$  fold EPC in 6 days.

Yield of EPC produced in activated MSC cultures

**TABLE 1. Kinetic of EPC production by mean value  $\pm$  SD (n= 25).\*:  $P < 0.05$  at 3-days versus 6-days**

Mean Yield of EPC ( $\times 10^6$ )	3-days EPC	6-days EPC	9-days EPC
	65.4 $\pm$ 21*	89.5 $\pm$ 25	91.1 $\pm$ 45

### 3.3. Surface markers of MSC and EPC

While differentiating to EPC, MSC have been reported to lose their mesenchymal stemness identified by their decreasing specific stromal markers expression (CD90, CD105) (Jin *et al.*, 2009). According to these findings, we also report that MSC overexpressed the

no-heamatopoietic CD105 and CD90 markers, but remained negative for the hematopoietic markers CD34 and CD45 (**Table 2**). Furthermore, CD146 and CD31 markers have already been reported as specific indicators of the endothelial lineage and vascular smooth muscle commitment (Bardin *et al.*, 2001, Kuwana *et al.*, 2006, and Espagnolle *et al.*, 2014). These markers were used to identify the EPC phenotype collected after 3 and 6 days of activation. Indeed, 3-day EPC presented a hematopoietic immature profile expressing the CD34 and CD45 with a clear direction toward the endothelial lineage by overexpressing the CD31 and CD146 cell markers. A markedly decreased expression of the stromal markers (CD105 and CD90) was observed in these 3-day EPC in contrast to the CD14 and CD105 whose expressions were slightly observed (**Table 2**).

In 6-day EPC, CD14 and CD146 as well as CD31 were overexpressed while a down-regulation of CD34 expression was observed. This result suggested that colony-forming cells presenting a higher CD34 expression and appearing at 3 days seem to be more primitive than those obtained at 6 days, as already

**TABLE 2. Expression of surface markers on MSC and EPC detected by flow cytometry, -- negative <1%, + 1-25%, ++ 25-50%, +++ 50-75%, ++++ >75%. MSC: mesenchymal stem cells, EPC: endothelial progenitor cells.**

	Surface markers	MSC (%)	EPC (%) in 3-day activated culture (n=15)	EPC (%) in 6-day activated culture (n=10)
<b>Heamatopoietic markers</b>	CD34	--	++++	++
	CD45	--	+++	++++
	CD14	--	++	++++
	CD38	--	--	-
<b>Non-Heamatopoietic markers</b>	CD11a	+	++++	++++
	CD25	--	+	+
	CD31(endothelial cell marker)	-	++++	++++
	CD49d	+	++++	
	CD54	++	++++	++++
	CD90	++++	+	-
	CD105 (SH2)	++++	++	-
	CD146	++	+++	++++



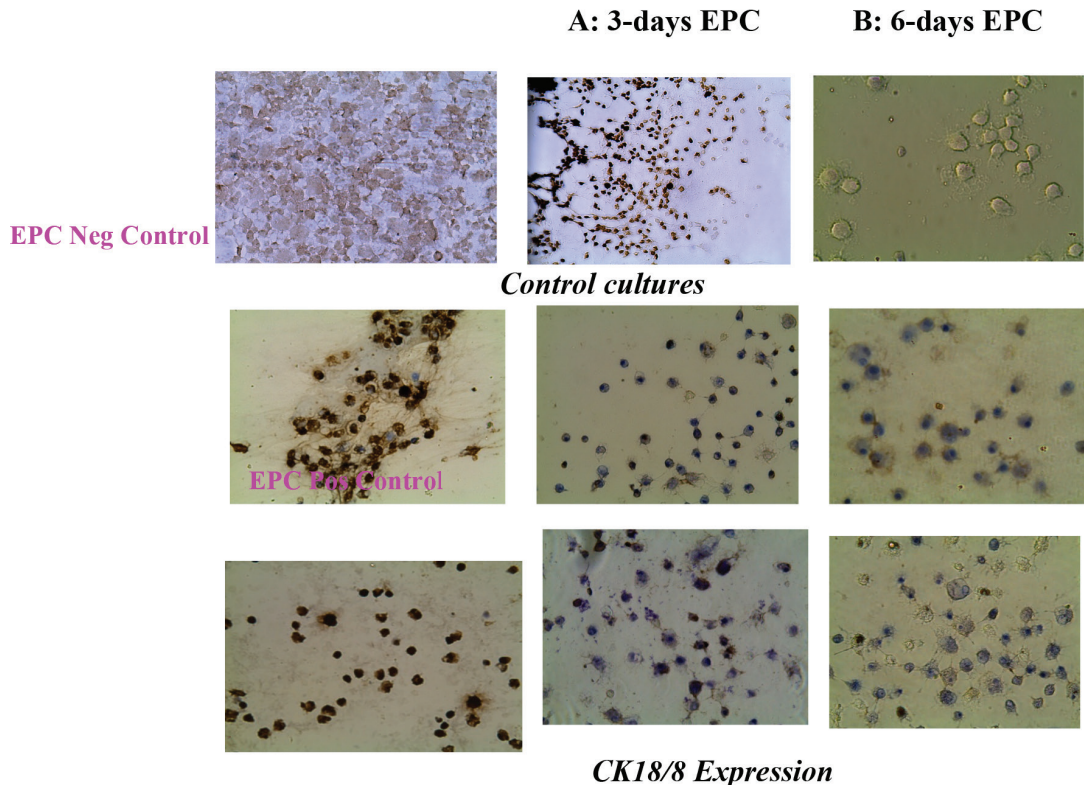
reported (Masuda *et al.*, 2011). This commitment was enhanced in 6-day EPC with an increase in CD14 expression relating to a monocytic cell profile. Similar EPC cell expression markers were observed regarding the expression of CD31, CD146, and CD144 (Asahara *et al.*, 1997, Bardin *et al.*, 2001, Kuwana *et al.*, 2006; Hoch *et al.*, 2012). Our results further show that cell adhesion molecules CD11a, CD49d, and CD54 were also strongly expressed in both 3 and 6-day EPC suggesting that the induced EPC presented homing and cell cohesion potentials. Elsewhere, EPC CD146 expression was reported to enhance their ability to adhesion and migration, and this expression was found to be related to vascular smooth muscle commitment (Kebir *et al.*, 2010; Espagnolle *et al.*, 2014).

Our findings suggest that the population phenotype CD34+CD45+CD14+CD31+CD146+ we found might be a unique population sharing the characteristics of monocytes and endothelial progenitors. These EPC seemed to derive from hematopoietic progenitor cells induced by differentiation of MSC.

### 3.4. Expression of CK18/8 and Factor VIII in EPC

To identify the blue DAB reacting color concentrated in the cell cytoplasm, EPC positive (with primary mAb) and negative (with secondary mAb) controls were realized. Negative and positive MSC controls showed no specific labeling with CK8/18 and Factor VIII (data not shown).

EPC were found to specifically express CK18/8 after 3 and 6 days of activation when compared with the negative controls (in upper) and with their respective positive control (*Figure 2*). In the latter, some non-specific labeling was observed. After 6 days of activation, all EPC were labeled. Three-day EPC mostly expressed Factor VIII as compared to control cultures, and this expression increased at 6 days. Cell size increased progressively during activation. The increase in CK8/18 and Factor VIII expression is related to the epithelial phenotype acquired by the EPC. In addition, these changes were concomitant with the overexpression of CD14, CD31 and CD146, suggesting the acquisition of a functional profile of the induced EPC.



**Figure 2.** Differential expression of Cytokeratin 18/8 (CK18/8) and von Willebrand Factor VIII (Factor VIII) in MSC control cultures and in EPC after 3 and 6 days of activation. (Phase contrast inverted optical microscope x40).

## Conclusion

Despite their high proliferative and self-renewal potentials, MSC were reported to have a less angiogenic effect than that observed with infused EPC (Kinnaird *et al.*, 2004 and Dai *et al.*, 2005), and respiratory complications associated with engrafted MSC were also reported (Forslow *et al.*, 2012). Nonetheless, a successful use of EPC in the transplantation of ischemic tissues appears to be a more promising approach in the treatment of diseases associated with blood vessel disorders (Madebbu *et al.*, 2004; Kuwana *et al.*, 2004; Ichim *et al.*, 2008; Chade *et al.*, 2009). However, the direct isolation of EPC from bone marrow or peripheral blood is greatly limited by their relatively lower and insufficient numbers.

The culture system using angiogenic growth factors we used appeared appropriate for a quick generation of EPC populations sharing endothelial and monocytic profiles. Indeed, using the VEGF in the culture medium mimicked the pathological situations where this cytokine is highly secreted by ischemic tissues (Chade *et al.*, 2009). Other findings reported that early appearing EPC in culture were a combination of angiogenic T cells CD3+CD31+CXCR4+ and CD14+ monocytes (Urbich *et al.*, 2003; Hill *et al.*, 2003; Hur *et al.*, 2007), and that monocyte-derived multipotent cells also presented an endothelial potential (Kuwana *et al.*, 2006), implying the possible role of monocytes in inducing vasculogenesis. Furthermore, our findings suggest that hUCB-derived MSC might be a source of induced EPC with vasculogenesis potency, and that this culture model could be considered for EPC production as a future target in neovascularization in advanced therapeutics of blood vessel diseases. The use of such EPC for therapy may occur if a suitable model of standardized production and cryopreservation in a bank is realized under GMP regulatory conditions.

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