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Regulation of human umbilical cord blood-derived multi-potent stem cells by autogenic osteoclast-based niche-like structure

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Abstract

Stem cell niches provide the micro-environment for the development of stem cells. Under our culturing regimen, a kind of osteoclast-centralized structure supports the proliferation of MSCs, derived from human cord blood, once they reside on osteoclasts. MSCs in this structure expressed Oct4 which is a marker of embryonic stem cells. Floating daughter cells of MSCs colony showed abilities to differentiate into osteocyte, adipocyte, and neuronal progenitor cells. Compared with the easy senescence of MSCs without this niche-like structure in vitro, these results suggested that osteoclasts might play an important role the development and maintenance of Umbilical cord blood (UCB)-derived MSCs and might provide a means to expand UCB-MSCs in vitro, more easily, through a stem cell niche-like structure.

Keywords: Stem cell niche; Umbilical cord blood; Stem cells; Osteoclast

Stem cells play an important role in developing and maintaining the body. In adult body, adult stem cells or progenitor cells maintain and repair tissue in which they reside by replacing the old, wornout somatic cells. Stem cell niches provide a special local microenvironment governing the fate of stem cells: self-renewal, proliferation or differentiation [1]. Tissue maintenance could be dramatically affected by the defect of stem cell niche [2]. Stem cell niche is now a very important issue in maintaining adult stem cells without differentiation, because, not like embryonic stem cells, adult stem cells including UCB-MSCs can easily terminally differentiate and are difficult to maintain in vitro, when these cells are taken from the body. One of the well-studied stem cell niches is the hematopoietic stem cell niche, in which the osteoblasts are believed to play a critical role. Those studies have been well-reviewed by Taichman [3] and more recently, by Wilson and Trumpp [4].

Though umbilical cord blood transplantation has been accepted as a practical approach for some diseases [5], especially the disease related to the hematopoietic system [6], research on the clinical application of UCB-MSCs is still limited. Recently, more interestingly, the use of UCB-MSCs has been tried clinically for intractable and incurable diseased patients. Previous successful reports have appeared on a spinal cord-injured female [7] and Buerger's disease [8] patients.

However, it is very difficult to culture UCB-MSCs without differentiation in vitro, even though there are several successful cases reported. Little is known as to how to culture and expand these cells. Presently, under the widely accepted method for adult stem cells, including UCB-MSCs, cells usually grow on a plastic surface. This may lead to many unpredictable changes in the character of stem cells. In our previous study, a kind of ES cell like stem

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cell population was obtained and was successfully induced into insulin producing cells [9]. However the environment, which contributes to maintain the ES cell like stem cells, was not well known. In this study, we report a self-renewing type niche, similar to the structure described by Taichman [3], in which osteoclasts played a main role in forming a stem cell niche-like structure. This study could provide a model for research on the interaction between stem cells and their niche, as well as a method for culturing and expanding stem cells derived from human umbilical cord blood.

Materials and methods

UCB-MSC isolation and primary culture

Full term UCB samples ($n = 11$) were obtained from the umbilical vein immediately after vaginal delivery with the informed consent of the mother approved by Borame Hospital Institutional Review Broad (IRB). And this work was also approved by Seoul National University IRB. Mononuclear cells were separated on Ficoll gradient as described from bone marrow (BM) [10].

Previously, we explored several culturing conditions and the most effective setting consisting of complete Dulbecco's modified Eagle's medium-low glucose (DMEM-lg; Gibco, Grand Island, NY, USA) at 5% CO_2 and 20% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), supplemented with 10 ng/mL basic Fibroblast Growth Factor (bFGF; R&D Systems, Minneapolis, MN, USA), 100 U penicillin, 1000 U streptomycin, and 2 mM/L glutamine (Gibco, Grand Island, NY, USA), were facilitated in this study.

We seeded 5×10^6 cells/ml mononuclear cells in the culture medium. Once primary cultured MNCs reached confluence, the attached cells were divided equally and subcultured in two different regimes (the niche-independent regime and the niche-like structure-dependent regime) by trypsinization at 37 °C for 10 min, after three times washing by phosphate buffered-saline (PBS, Gibco).

The niche-like structure-independent culture regime. Cells were suspended in culture medium at 1×10^5/75 flask and culture medium was changed every 3 days. Cells were detached with 0.1% trypsin–EDTA (ethylenediaminetetraacetic acid, Sigma–Aldrich, St. Louis, MO, USA) when they reached their confluence and replated at a density of 1×10^4/ml in Falcon culture flasks. However, cells hardly reached confluence. In this case, we still passage them after 4 weeks.

The niche-like structure-dependent culture regime. Cells were re-seeded at the concentration of 1×10^5/75 flask. Three days later, the unattached cells were washed out. The same volume of medium was added every 7 days. Four weeks after subculture, the medium was collected and centrifuged. The cells, which have been suspended in the medium, were re-seeded in the second vessel at the concentration of 10^4/ml in DMEM-lg completed medium or underwent differentiation. The origin vessel, where the floating cells were collected, was maintained by adding medium and then suspended cells were obtained again two weeks later (see supplemented figure1).

In vitro differentiation

Floating cells collected from the medium were used for in vitro osteogenic, adipogenic, and neurogenic differentiation, as described by the work of Lee et al. [11].

Cytochemical staining

For oil-red O staining, cells were fixed with 4% formaldehyde and stained with oil-red O (Sigma–Aldrich) for 10 min. Tartrate-resistant acid phosphatase (TRAP) staining was performed as per the protocol from Sigma for TRAP staining kit.

Immunofluorescence

For immunofluorescence staining of surface proteins, cells, collected from the fourth and sixth weeks, were fixed overnight with 4% formaldehyde at 4 °C. For the intracellular proteins, cells were permeabilized with 0.1% Triton X-100 (Sigma–Aldrich) for 10 min after fixation. Slides and dishes were incubated with rabbit primary antibodies against human Oct4 (1:50), mouse anti-human SH4 (1:50), and mouse anti-human glial fibrillary acidic protein (GFAP; Santa Cruz Biotechnology, CA, USA) (1:50) for 1 h, followed by fluorescein- or phycoerythrin-coupled goat anti-mouse or -rabbit IgG secondary antibody (Dako; Carpinteria, CA, USA) for 1 h. Between incubations, slides and dishes were washed with PBS.

Western blotting

Cells were lysed and then proteins were extracted in high salt buffer (20 mM Hepes, pH 7.5; 25% glycerol; 0.42 M NaCl; 3 mM $MgCl_2$; 0.2 mM EDTA; 1 mM dithiothreitol; protease inhibitor cocktail (Sigma; St. Louis, MO, USA). Proteins were separated on 15% SDS–PAGE gels and transferred to nitrocellulose. Osteocalcin (Santa Cruz) pPAR gamma (Santa Cruz) and nestin (Santa Cruz) were detected with mouse monoclonal antibody and enhanced chemiluminescence reagents (ECL; Amersham; Piscataway, NJ, USA).

Flow cytometry

The suspended cells were collected and regarded as the floating round cell population. After 3 washes with PBS, the spindle-shaped cells were detached with trypsin (Gibco) for 10 min at 37 °C. For cell surface antigen phenotyping, floating and detached spindle-shaped cells were stained with fluorescein or phycoerythrin coupled antibodies, including CD4, CD20, CD29, CD31, CD34, CD44, CD51/61, CD73, CD105, and CD133 (all antibodies purchased from Becton–Dickinson, San Jose, CA, USA). The Oct4 (Santa Cruz Biotechnology) expression was also compared in these two populations by indirect staining method (following the protocol of BD Bioscience) after fixation with 4% PFA overnight at 4 °C and permeabilization with 70% ETOH for 20 min at 4 °C. Analyses were performed with FACS Calibur (Becton–Dickinson, NY, USA).

Statistical analysis

Statistical analysis was performed using ANOVA using statistical package SPSS 13.0 (SPSS Inc.; Chicago, IL).

Results

Enhanced proliferation of MSCs on the niche-like structure

Seven out of the 11 samples were successfully attached and used for further study. Under the niche-like structure-dependent culture regime, all of the 7 samples showed a similar growth pattern. After subculture, cells reached 70% confluence, heterogeneously, 14 days later (Fig. 1A-a). Cell colonies were formed on large multinuclear cells, surrounded by spindle-shaped cells (Fig. 1A-b). As the colony enlarged (Fig. 1A-c), some cells escaped from the colony and became suspended in the medium. Four weeks after subculture, the cells, which have been suspended in the medium, were re-seeded in the second vessel. Repeatedly, the same structures were formed.

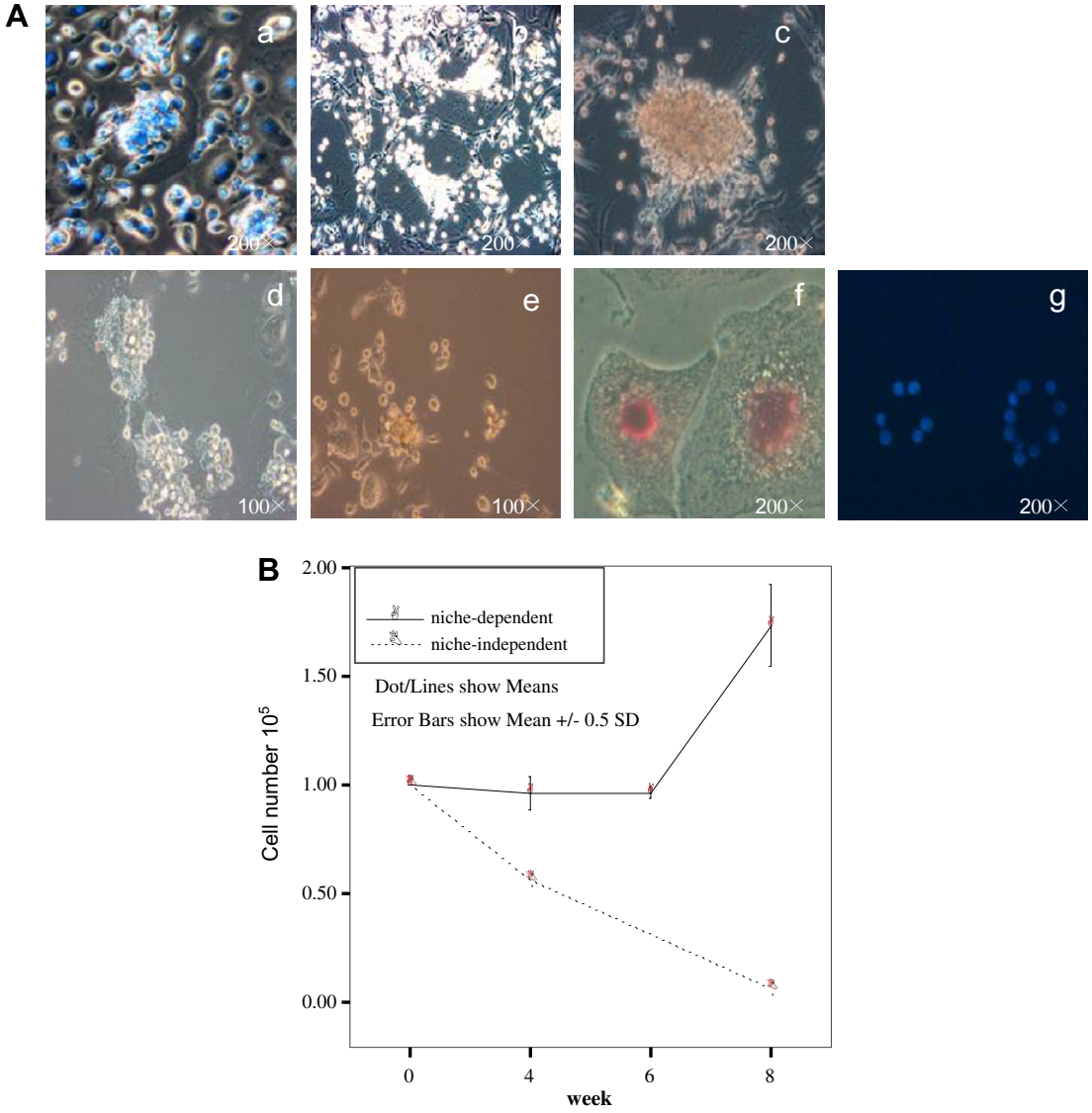

Fig. 1. The growth of MSCs with the osteoclast based structure. (A) a, b, and c indicate the morphology of cells after subculture on the fourteen day, twenty-first day and 28th day, respectively. Cell colonies usually set on large multinuclear cells which are surrounded by spindle or endothelial cell like cells. After removing the spindle-shaped cells by trypsinization (d), the multinuclear cells still could support a single cell-forming colony (e). But after a short time (10 days) the cell colony could not grow anymore. Those multinuclear cells (f), shown after Hoechst 33342, were positive for TRAP staining (g). (B) We tried to culture the cells in both niche-dependent and -independent regimen. For the cells with niche-independent regimen, cell number was counted after detached by trypsin. In case of niche-dependent regimen, the floating cells in each flask were collected and counted. After 8 weeks, the total number of cells, cultured with niche independent regiment, deceased dramatically. The total number of cells, under the niche dependent regiment, increased 2 times the initial seeding number (1×10^5) at the starting point.

Under the niche-independent regimen, Cell yield was dramatically decreased with passage. In passage 1, cells hardly reached confluence, though we still passage them after 4 weeks. Comparing these two culturing regimes, after 8 weeks, the cells cultured with niche-independent regimen decrease dramatically, whereas the cells under the niche-dependent regimen increase 2 times the initial seed number at the starting point (Fig. 1B).

It was clear that the osteoclasts were necessary in this system because they, at least, provided the location where the MSCs anchored. We tried to remove the spindle-shaped cells by trypsinization in order to test if the spindle-shaped cells also contributed to the proliferation of MSCs. After trypsinization, spindle-shaped cells were removed and osteoclast-like cells still remained in the flask. Very few of round-shaped cells still attached to osteoclast-like cells (Fig. 1A-d; Fig. 1A-f and g show those multi-nuclear cells positive for TRAP staining). Within ten days, the round-shaped cells continuously proliferated to form small colonies and then stopped (Fig. 1A-e). This result indicated the spindle-shaped cells had some function for long-term culturing MSCs.

Characterization of the niche-like structure

In the niche-like structure (Fig. 2A-a; Fig. 2A-b as the model of Fig. 2A-a), Large multinuclear cells were positive for TRAP staining, demonstrating the property of osteoclast cells (Fig. 2A-c). The Oct3/4 gene, a POU family transcription factor, has been identified as a specific marker, expressed in undifferentiated stem cells containing high proliferating capacity, but not in cells of differentiated tissues [12]. Therefore, we used Oct4 as a marker for stemness. Oct4 staining was used for characterizing the cell colonies, which were the source of the suspended round-shaped cells, residing on the osteoclasts and the attached spindle-shaped cells, simultaneously. The positive staining for Oct4 indicated that round-shaped cells were in an earlier stage than the spindle-shaped cells (Fig. 2B-a and -d). The positive staining for Oct4 of the colonies in the fourth and sixth weeks indicated the stemness potentials of stem cells were maintained in those cells under our culture condition.

Characterizations of the suspended round-shaped cells and attached spindle-shaped cells

In order to characterize the suspended cells in the medium and the spindle-shaped cells, attached to plastic surface, which were usually regarded as mesenchymal stem cells, CD4, CD20, CD29, CD31, CD34, CD44, CD51/61, CD73, CD105, and CD133, were examined by Fluorescence Activated Cell Sorting (FACS) analysis. Interestingly, both spindle- and round-shaped cells had similar phenotypes. Both types of cells were negative for the lineage markers of CD4, CD20, CD34, and CD51. However, the round-shaped cells expressed CD29, CD44, CD31, CD 105 and, more importantly, CD73 and Oct4 (Table 1) in a larger proportion. Especially in the suspending cell population, the pluripotent stem cell marker, Oct4 (85.4%), was expected co-expressed with the lineage restricted adult stem cells marker CD73 (76.1%) or CD105 (45.6%). This may suggest that those cells represent an intermediate stage between pluripotent ES cells and lineage-restricted adult stem cells.

In vitro differentiation of MSCs into adipocyte, osteocyte, and neuronal progenitor cells

Cells were collected from five flasks and treated with different medium for adipocyte, osteocyte, and neurocyte genesis. After treatment, von Kossa staining for the calcium accumulation around osteocytes; oil-red O staining for the lipid in adipocytes; and GFAP staining for the neuronal progenitor cells were used for characterization of the differentiation of UCB-MSCs. The cells, which had undergone adipocyte differentiation for 4 weeks, were shown with red color when stained by oil-red O (Fig. 3A), osteocyte accumulated calcium was shown with black blown color after cells were treated by differentiation medium for two weeks (Fig. 3C); and the cells which had undergone

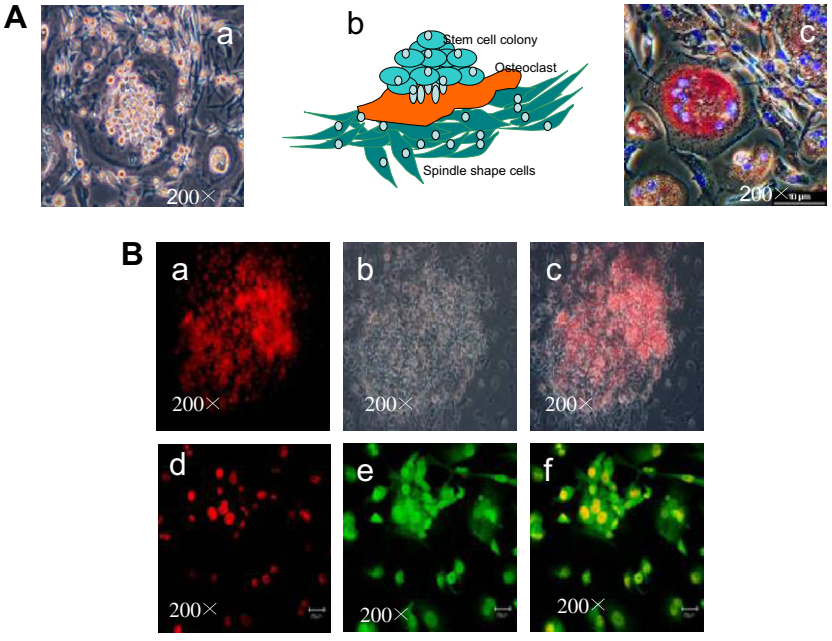

Fig. 2. Characterization of the stem cell niche-like structure. (A) a is an example of the niche like structure and b is its model. TRAP staining and Hoechst staining (c) were preformed after removing the suspending cells and round cells. The large multinuclear cells were TRAP positive cells and the single nuclear spindle-shaped cells were negative for trap staining. (B) Oct4 (a, c, d, f, red) staining was performed in order to prove the stemness of these cells. SH4 (e, f, green) was regarded as a stem cell marker for mesenchymal stem cells from umbilical cord blood. f indicates the cells could express oct4 and SH4 both. c is a merged view of Oct4 staining and optical image (b). (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

Table 1
The percentage of positive cells for CD antigens in the two different morphological cell populations

CD series antibodies	The positive staining cells/total acquisition cells (%)	
	Suspended round-shaped cells	Attached spindle-shaped cells
CON	3.7 ± 0.3	4.3 ± 0.2
Oct4	85.4 ± 3.7	55.09 ± 1.6
73	76.1 ± 8.1	77.18 ± 0.9
31	90.68 ± 6.9	56.49 ± 4.7
29	70.7 ± 2.4	46.42 ± 1.8
34	6.56 ± 0.5	2.2 ± 2.5
4	7.08 ± 0.7	12.3 ± 0.1
51/61	8.74 ± 3.0	6.22 ± 0.3
105	45.76 ± 0.2	17.72 ± 2.3
133	12 ± 4.1	11.4 ± 0.1
20	11.8 ± 0.1	15.24 ± 3.4
44	93.02 ± 2.6	73.82 ± 5.7

neuron differentiation for 4 weeks, expressing the neuronal progenitor marker GFAP, were also shown with the green color after staining with fluorescein isothiocyanate (FITC)-conjugated antibody (Fig. 3E). The expression of marker proteins: pPAR gamma for adipocyte (Fig. 3B), osteocalcin for osteocyte (Fig. 3D), and nestin for neuro progenitor (Fig. 3G) was detected by Western blot. Thus, the MSCs from our culture system had the potential to transdifferentiate to other lineages under the specific conditions.

Discussion

In our study, a cell colony proliferated on a large multinuclear cell which was surrounded by spindle-shaped cells (Fig. 2A-b). This structure might be related correlated to the hypothesis of Wilson and Trumpp [4]. That is, the quiescence stored stem cells would anchor in the center of the niche, whereas self-renewing stem cells would be located close to the border separating the niche from the non-niche microenvironment, which could provide signals that would induce differentiation and/or cell division. Our data also indicated that the center cells were more primitive than the spindle cells around them (Fig. 2B-a–c). Moreover, the FACS result (Table 1) indicated that the suspending cells which produce by that central cell colony could contain more primitive cells than the spindle-shaped cell population. We suspected that there was some contamination in the spindle-shaped cell population by the round cells from the unremoved colonies. However, as our results have

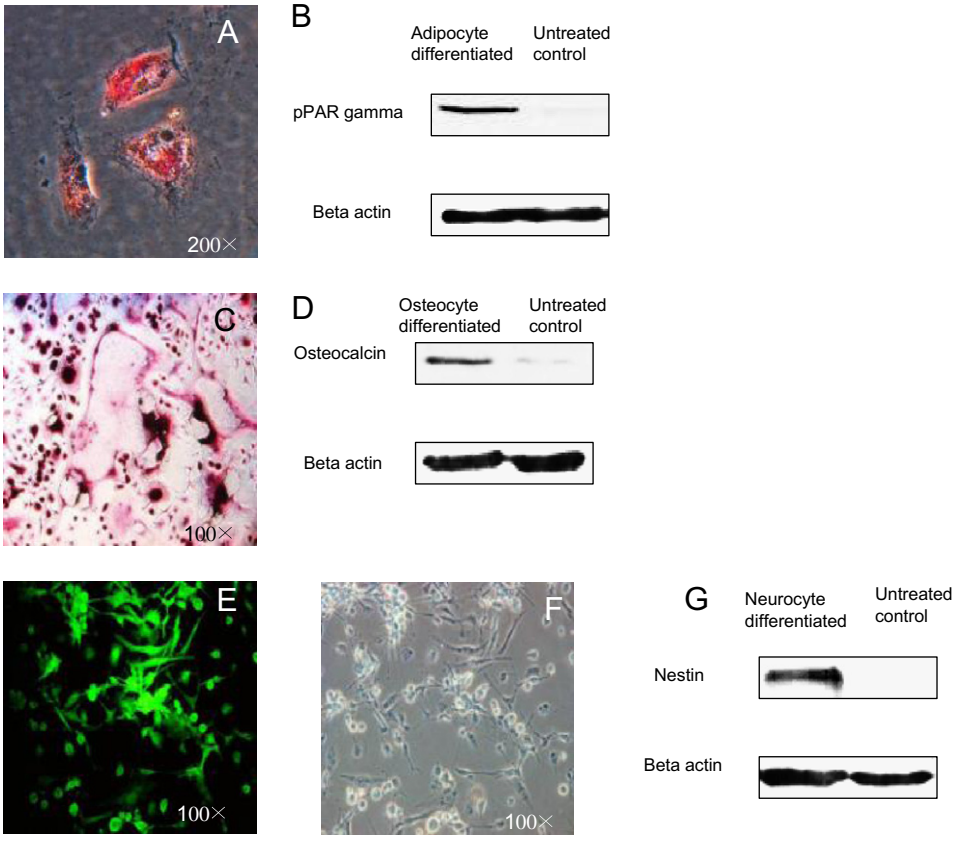

Fig. 3. Differentiation ability of the floating cells derived from the colony. (A) Oil-red O stain for adipocyte differentiation. (B) Adipocyte specific protein pPAR gamma expression was shown by Western blotting after two weeks of treatment with adipocyte differentiation medium. (C) von Kossa staining (red brown) for osteogenesis. (D) Osteocalcin expression was shown in the cells treated with osteocyte differentiation medium. (E) GFAP was shown with green color in the cells that underwent neuron differentiation. (F) Optical image of the cells after neurogenetic differentiation. (G) Nestin expression was shown in the cells treated with neuronal lineage differentiation medium. (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

shown, even with a mixture with the round- shaped cell, there are still differences of the two populations.

A similar structure had been described by Soboleva et al. [13], using the blood from patient with moderate hyperlipidemia. They also characterized the large multinuclear cells with TRAP staining and considering those cells as osteoclasts.

However, we could rule out that the small round-shaped cells were lymphocyte, but rather they were stem cells due to the fact that these cells had negatively expressed T cell marker (CD4) and B cell marker (CD20) and had the multi-differentiation abilities of osteogenesis, adipogenesis, and neurogenesis. The osteoclast cells should come from its progenitor cells, which could attach to the vessel and promoted by human mesenchymal stem cells since osteoclast committed progenitors, which were thought to have been derived from their $CD34^{+}$ primary progenitors, could have lost the expression of CD34 and existed in the $CD34^{-/low}$ MSC population [14]. In the niche-independent culture regime, the niche-like structure could not be found though the large multinuclear cells also emerged. The different outcome of the two culturing regimes might due to the frequent medium change schedule. It also might indicate interaction between cells might play a critical role in the formation of a niche-like structure.

The well-studied stem niche has been the hematopoietic stem cell niche. The effect of osteoblasts has been extensively discussed [15,16]. Stojkovic et al. [17] and Xu et al. [18] mentioned that the autogenic osteoblast-like cells could efficiently support the growth of undifferentiated human embryonic stem cells as a feed layer or by its conditioned medium. However, regarding the dynamic equilibrium of osteoblast and osteoclast, the latter might also play an important role in the development and maintenance of stem cells. The works of Ge et al. [19] and Yaccoby et al. [20] had provided a clue of this possibility. This was also consistent with our findings that a physical contact between osteoclast and cord blood stem cells was necessary for the proliferation of the latter.

Though the in vivo conditions are more complex than ex vivo cultures, the roles of osteoclasts on the progress of myeloma and breast cancer metastasis indicated the osteoclasts might be more valuable in the research of stem cell biology [21]. Osteoclasts, usually, are considered as playing a negative role under the control of osteoblasts, but the fact might be reversed. Indeed, long-term coculture of normal CD34 expressing HSC resulted in loss of CD34 expression, suggesting a common mechanism for osteoclast-induced myeloma and HSC plasticity [22].

The signaling pathways and their integration might depend on a successful model in vitro. In addition, the requirement on the large scale of stem cells for research or clinical use demanded an efficient system for expanding stem cells. Our study produced the specific cellular structure for the proliferation of stem cells; it might be regarded as a niche for UCB-MSCs in vitro. Although it might not accurately recapitulate the in vivo functions of niches, this stem niche-like structure might provide a model for the study on stem cell niches in vitro or in vivo. It might also be helpful for expanding stem cells through their niches.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.03.072.

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