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Showa Univ J Med Sci **27**(2), 83～91, June 2015

Original

Improved Long-term Culture of Epidermal Stem Cells Utilizing CD200R-expressing Feeder Cells

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Abstract: Tissue stem cells have great potential as a source of tissue for regenerative medicine. Epidermal stem cells (EpSCs) are the most accessible population of tissue stem cells that can regenerate the specialized cell types of tissues including the epidermis, smooth muscle and the sciatic nerve. However, the difficulties in isolation of the high numbers of EpSCs and their long-term culture have hampered the development of wider clinical applications of EpSCs. Here, we present a novel approach to EpSC culture that utilizes a feeder layer of Swiss 3T3 cells expressing the putative EpSC niche-specific molecule, CD200R. The colony forming efficiency of $CD34^+$, α_6-$integrin^+$ EpSCs was increased on CD200R-expressing Swiss 3T3 feeder cells compared with normal Swiss 3T3 feeder cells. Furthermore, treatment with glycogen synthase kinase (GSK)-3 inhibitor, an activator of Wnt signaling, synergistically enhanced the proliferation of EpSCs. These results raise the possibility that an artificial microenvironment equivalent to *in vivo* niches will enable the persistent culture of EpSCs, thereby increasing the utility of EpSCs for tissue engineering and regeneration.

Key words: epidermal stem cell, cell culture, CD200R, niche, Wnt signal

Introduction

Regenerative medicine has great potential to cure a variety of diseases and injuries that are caused by the loss of tissues or their functions. Several lines of studies have led to striking breakthroughs in the establishment of pluripotent stem cells such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs)[1]. Both ESCs and iPSCs are expected to provide rich sources of cells for therapy to replace dysfunctional tissues. However, there are serious safety concerns with these cells, including their tumorigenic capacity, susceptibility to host immune responses, and the possibility of differentiation into unexpected cell lineages. In addition, there are ethical issues associated with the use of ESCs, which have led to legal constraints on the establishment and utilization of human ESCs for clinical applications in Japan.

The use of tissue stem cells could provide an effective means of overcoming these difficulties because they are unaffected by transplant rejection and are not subject to ethical considerations. Especially, epidermal stem cells (EpSCs) have particular advantages compared to other tissue

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stem cells. They reside at the bulge region of the follicular epidermis in the skin, the largest organ of body, which effectively yields a high number of stem cells[2, 3]. The enrichment and isolation of EpSCs is facilitated because they express the readily identifiable CD49f (integrin α_6) and CD34 cell surface markers[4]. Furthermore, under appropriate culture conditions, EpSCs can differentiate into multiple types of cells including keratinocytes, melanocytes, neuronal cells and smooth muscle cells[5, 6]. One difficulty with EpSCs can be the maintenance of cells in culture without spontaneous transformation. Therefore, it is necessary to develop an improved culture method that maintains the stem cell characteristics of EpSCs in vitro so that they can be used clinically. The *in vitro* culture method for epidermal keratinocytes including its stem cells was developed by Rheinwald and Green in 1975[7]. This standard protocol, Green's method, is characterized by the usage of a feeder layer of 3T3 cells and fetal calf serum. Green's method remains the optimal protocol to culture EpSC to date, although several alternative procedures that dispense with feeder cells and serum have been developed. Thus, in this study, we attempted to modify and improve the culture protocol for EpSC based on Green's method.

The specific microenvironments where stem cells are located are referred to as stem cell niches. They are essential for maintaining stem cell characteristics or "stemness". For example, the anterior ovariolar somatic cells, termed Caps cells, act as a germ line stem cell niche in Drosophila[8]. CXCL12-expressing reticular cells surround hematopoietic stem cells (HSC) and are required for the *in vivo* maintenance of HSC and progenitor cells in the bone marrow of mice[9]. The fate of stem cells is regulated by several factors within the niche including cell-to-cell interactions via adhesion molecules, association with the extracellular matrix, and the presence of secreted proteins such as growth factor and cytokines. Thus, we hypothesized that artificial environments equivalent or similar to the stem cell niches will permit the long-term *in vitro* culture of EpSC. In the current study, we established feeder layer cells that express the niche-specific gene and cultured EpSC on these feeder cells. CD200 is preferentially and highly expressed in the bulge keratinocytes containing EpSC in both human and mouse skin[10]. $CD200^+$ keratinocytes exhibit high growth potential and have stem cell characteristics. CD200 is a cell-surface glycoprotein that interacts with the CD200 receptor (CD200R). This suggested that CD200R could function as a niche-specific gene, which would maintain the stemness of EpSCs through binding to CD200. Therefore, we introduced the CD200R gene into the Swiss 3T3 feeder cells and evaluated the effectiveness of these CD200R-expressing feeder cells for EpSC culture.

Materials and methods

Cell culture

Swiss 3T3 cells were grown in Dulbecco's minimal essential medium (DMEM) containing 10% fetal calf serum (FCS) at 37°C in 5% CO_2. The two days before seeding EpSCs, Swiss 3T3 cells were cultured for 2 h in DMEM containing 10 mg/ml mitomycin C. The mitomycin C-treated Swiss 3T3 cells were detached with trypsin and seeded onto a 6-well plate at 2.0×10^5 cells/well. EpSCs were added to the plate and cultured in either epidermal stem cell growth medium

(ESC) consisting of a 1:3 mixture of F12 and DMEM with 15% FCS, 0.18 mM adenine, 5 μg/ml insulin, 4.5 ng/ml EGF, 0.5 μg/ml dexamethasone, 0.1 nM cholera toxin (CT), 2 nM triiodothyronine and 5 μg/ml transferrin or LFAM (low calcium F12 and MEM) medium consisting of a 1:3 mixture of F12 and calcium-free MEM supplemented with 10% dialyzed FCS, 10 ng/ml EGF, 5 mg/ml insulin, 0.5 mg/ml hydrocortisone and 0.1 nM CT.

Establishment of CD200R-expressing Swiss 3T3 cells

The full-length murine CD200R1 cDNA was amplified by PCR from a mouse keratinocyte cDNA library using the following primers 5'-ttaCTCGAGggcggaaaaccagaaaacc-3' and 5'-attGCGGCCGCgtagtccttccattgtcgaa-3', where the upper case letters represent the recognition sites of the XhoI or NotI restriction enzymes, respectively. The PCR amplification product was digested with XhoI and NotI, and ligated into the XhoI-NotI site of CSII-CMV-MCS-IRES2-Bsd lentivirus backbone vector (CSII-CD200R1). This vector drives the expression of the inserted cDNA using the CMV promoter. HEK293T cells were co-transfected with CSII-CD200R1 vector as well as the pCMV-VSV-G-RSV-Rev and pCAG-HIVgp vectors to generate the CD200R1-expressing lentivirus vector (Lenti-CD200R). Stable clones of CD200R1-expressing cells were established by infection of Swiss 3T3 cells with Lenti-CD200R. The expression of CD200R1 was confirmed by immunoblotting with the CD200R1-specific antibody (#MCA2281GA, Serotec, UK).

Isolation of EpSC cells and measurement of colony-forming efficiency

The back skin with all follicles in the resting stage of the hair cycle was removed from euthanized 7 to 10-week-old C3H/HeN mice. The skin was incubated in a solution of 0.2% trypsin and 100 U/ml dispase I (EIDIA Co., Ltd., Japan) initially at 4℃ for 15 h and subsequently floating in the solution at 37℃ for 3 h. The epidermis was scraped off the dermis and was suspended in 0.025 % trypsin/0.02 % EDTA in PBS and was repeatedly aspirated by pipette to disperse the epidermal cells as single cells. Living cells were sorted by MACS cell separation system (Miltenyi Biotec, San Diego, CA, USA) according to the manufacturer's instructions to isolate $CD34^+$, $CD49f^+$ keratinocytes. Briefly, the cell suspension in MACS buffer (2 % FCS, 2 mM EDTA in PBS) was incubated with anti-CD34 antibody (#553751, BD) for 15 min at 4℃. After repeated washing with MACS buffer, magnetic micro beads-conjugated with anti-Rat IgG (Miltenyi) were added to the cell suspension and incubated for 15 min. Following extensive washing to remove the unbound secondary antibody, the cells were subjected to column purification using MACS MS separation columns. Subsequently, FITC-conjugated anti-CD49f antibody (#555735, BD) was incubated with the purified $CD34^+$ cell suspension, followed by the addition of microbead-conjugated anti-FITC antibody. The microbead-bound $CD49f^+$ cells were purified by MACS separation columns as described above. The fractionated $CD34^+$, $CD49f^+$ epidermal cells were seeded onto mitomycin C-treated Swiss 3T3 feeder cells at a density of 1500−2500 cells per well of a 6-well plate and cultured at 32℃ in 6.5% CO_2. Seventeen days after seeding, the colonies were fixed with 10% formalin/PBS and stained with 1% Rhodamine B. The colony numbers were either counted manually or using ImageJ software.

Results

Optimization of culture conditions for EpSC cells

The optimization of the culture medium for EpSC culture was a primary focus of this study because of the contrastive optimal medium requirements for EpSC and Swiss 3T3 feeder cells. The presence of calcium ions is required for Swiss 3T3 feeder cells, whereas calcium ions induce the terminal differentiation of keratinocytes. Therefore, we assessed the effect on colony-forming efficiency (CFE) of EpSCs using three different media formulations for EpSC culture. Each medium had a different calcium ion concentration in accordance with previously proposed condition[11]. The CFE reflects the clonal expansion of the stem cells and depends on both cell growth and attachment. A mixed cell population containing keratinocytes were obtained from the back skin of adult mice. EpSCs were enriched from this population using the MACS cell separation system to isolate $CD34^+$, $CD49f^+$ keratinocytes (Fig.1A). The enriched EpSC population was seeded onto mitomycin C-treated wild-type Swiss 3T3 cells and cultured at 32°C in 6.5% CO_2 in the three different medium formulations. The highest CFE for EpSC was found for FAM medium that contained a low calcium ion concentration (0.075 mM) (Fig. 1B). On the other hand, EpSC did not form colonies in ESC medium, which was based on a mixture of DMEM and F12 medium and had the highest calcium ion concentration (0.85 mM). In addition, EpSC grown in FAM medium containing an intermediate calcium ion concentration (0.3 mM) had a low CFE. Thus, we selected the FAM medium with a low calcium ion concentration, termed LFAM, to culture EpSC.

Establishment of CD200R-expressing feeder cells

To assess the effects of feeder layer on growth and the maintenance of EpSCs stemness, the mouse CD200R-expressing Swiss 3T3 cells was established. The expression of CD200R1 protein in Swiss 3T3 cells infected with CD200R1-expressing lentivirus vector was confirmed by immunoblotting (Fig. 2A). Swiss 3T3 cells expressing CD200R1 exhibited a more spindle-shape morphology compared with wild-type Swiss 3T3 cells (Fig. 2B).

CD200R-expressing feeder cells increase colony-forming efficiency of EpSCs

We examined the effect on EpSCs CFE of CD200R expression in Swiss 3T3 feeder cells. Viable EpSCs were seeded at the same density onto normal Swiss 3T3 cells or CD200R-expressing Swiss 3T3 cells (3T3/CD200R). Approximately 2-fold more colonies were formed on 3T3/CD200R than on normal Swiss 3T3 cells (Fig. 3A). No significant difference was observed in the size of individual colonies between the control Swiss 3T3 and 3T3/CD200R feeder cells (Fig. 3B). This suggests that CD200R affected the adherence to the feeder cells rather than the growth capability of EpSC.

GSK3β inhibitor enhances EpSCs colony-formation efficiency

EpSCs grown on the 3T3/CD200R feeder layer were evaluated for their survival after passage.

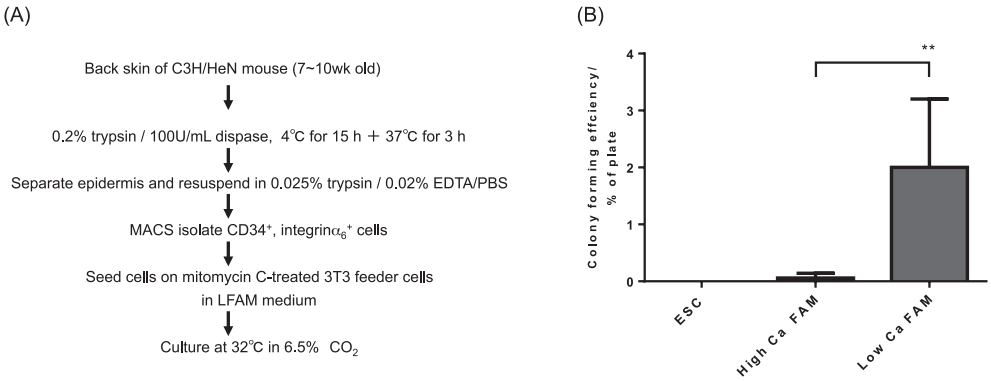

Fig. 1. Optimization of culture medium for mouse EpSC cells. (A) Schematic procedure of isolation of EpSC cells from mouse back skin. $CD34^+$ and $CD49f^+$ epidermal cells were isolated from the epidermis of C3H/HeN mouse back skin using the MACS system. The isolated cells were seeded onto a Swiss 3T3 feeder cell layer in culture medium optimized for EpSC cells. (B) Comparison of colony-forming efficiency (CFE) of EpSCs cells cultured in medium with different composition. Seeded cells were cultured in ESC, FAM medium with high (high calcium ion concentration, 0.3 mM or low calcium medium (low calcium ion concentration, 0.075 mM for 17 days, and were stained with 1 % Rhodamine B and counted. The CFE is shown as the mean and standard deviation of the triplicate samples. Statistical significance was analyzed by the Student's t-test, $^{**}P < 0.05$.

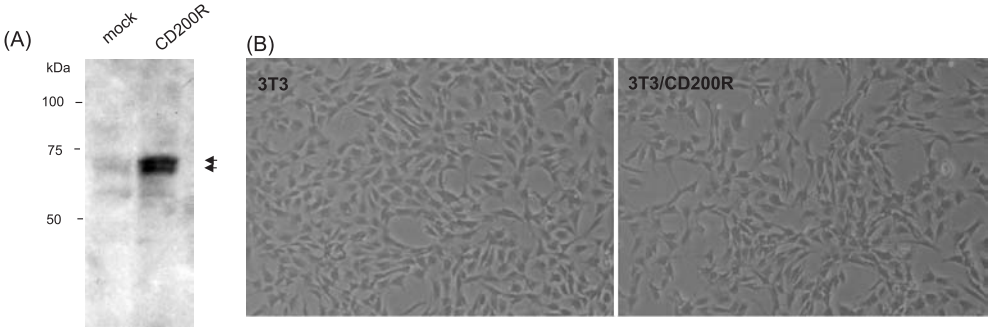

Fig. 2. Ectopic expression of CD200R1 in Swiss 3T3 feeder cells. (A) Swiss 3T3 cells were infected with either lentivirus vector containing mouse CD200R1 (CD200R) or the control empty vector (mock). Expression of CD200R1 protein was detected with immunoblotting using anti-CD200R1 antibody. Arrows indicate the specific CD200R1 bands. (B) Morphology of Swiss 3T3 cells expressing CD200R1. Representative bright field images of CD200R1-expressing or non-expressing Swiss 3T3 cells are shown.

EpSCs were detached using TrypLE™ Select enzyme and re-seeded onto either Swiss 3T3 or 3T3/CD200R feeder cells. EpSCs had a higher CFE when cultured on 3T3/CD200R cells than on Swiss 3T3 (Fig. 4A, left panel).

Wnt/β-catenin signaling has been implicated in the regulation of stem cells via its action as a niche factor that maintains stemness[12, 13]. Glycogen synthase kinase 3 (GSK3) is a serine/threonine kinase that inhibits Wnt signaling by phosphorylation and subsequent degradation of

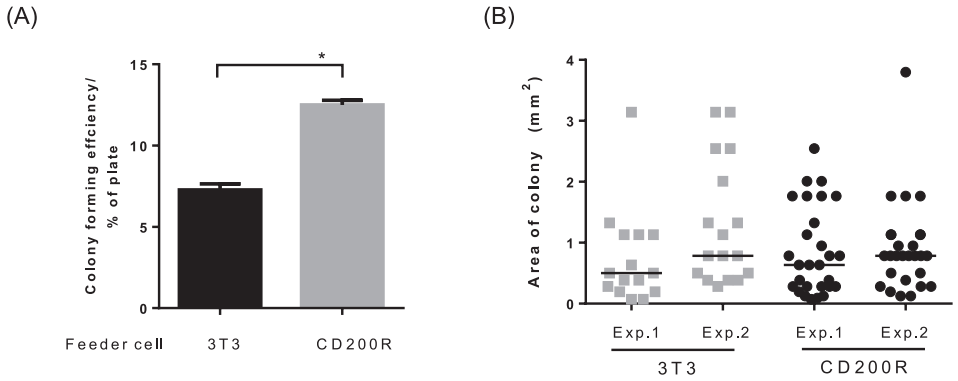

Fig. 3. CD200R1-exressing feeder cells increase EpSC colony formation. Isolated EpSC cells were plated on wild-type Swiss 3T3 (3T3) or CD200R1-expressing Swiss 3T3 feeder cells (CD200R) and cultured for 17 days. (A) EpSCs were cultured in FAM with low calcium ion concentration (LFAM). Values represent means of colony-forming efficiency +/− SD. (B) Area of colonies formed on Swiss 3T3 or CD200R1-feeder cells. The size range of individual colonies from two different experiments (Exp. 1 or Exp. 2) is shown. The area was measured using ImageJ software. Statistical significance was analyzed by the Student's t-test, $^{*}P < 0.001$.

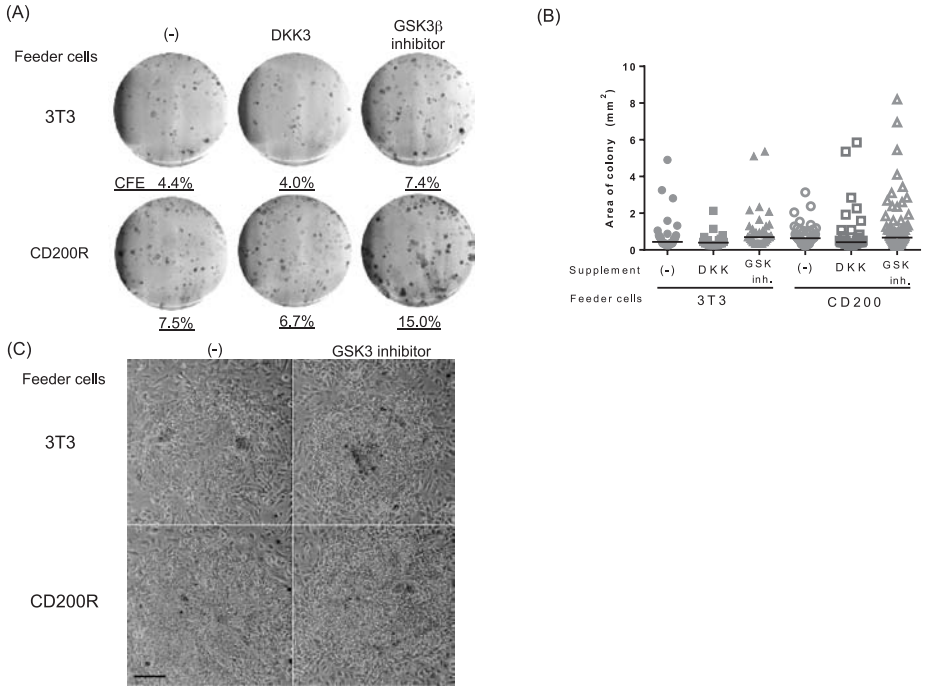

Fig. 4. GSK3β inhibitor treatment accelerates EpSC colony formation. Isolated EpSC cells were seeded on CD200R1-expressing Swiss 3T3 feeder cells and cultured for 3 weeks. The cell colonies that grew were dispersed with Tryp LE Select cell dissociation enzyme and $CD34^{+}$ cells were sorted and isolated. The $CD34^{+}$ cells were plated on wild type Swiss 3T3 (3T3) or CD200R1-feeder cells (CD200R) in the presence or absence of 0.5 μg/ml DKK3 or 1 μM GSK3β inhibitor IX. (A) Colony-forming efficiency of the second passage of EpSC cells. Values represent the colony formation efficiency (CFE) under each culture condition. (B) Size range of individual colonies in (A) measured using ImageJ software. (C) Phase contrast micrographs of representative colonies. Scale bar = 500 μm.

β-catenin. GSK-3 inhibitor maintains ES cells in their undifferentiated state and also maintains expression of pluripotent state-specific transcription factors by activating Wnt signaling[14]. Thus, we finally investigated the effects of GSK3 inhibitor IX on the CFE of EpSCs. Treatment with GSK3 inhibitor IX the markedly elevated CFE as shown in Fig. 4A (right panel). The CFE increased by at least 3 fold in the presence of GSK3 inhibitor, when EpSCs were cultured on 3T3/CD200R feeder cells compared with Swiss 3T3 cells in the absence of GSK3 inhibitor. The median colony size was not significantly changed by GSK3 inhibitor treatment. But some colonies were much larger when cultured on 3T3/CD200R feeder cells in the presence of GSK3 inhibitor (Fig 4B). Phase contrast micrographs showed that GSK3β inhibitor-treated EpSC colonies contained highly proliferative, tightly packed, small round cells of undifferentiated epidermal stem cells (Fig. 4C). In contrast, treatment of EpSCs with the secreted inhibitor of Wnt signaling, Dickkopf-related protein-3 (DKK3), decreased the CFE. These findings suggest that the combination of GSK3 inhibitor and CD200R-expressing feeder cells enhances the proliferation of EpSCs while maintaining stemness.

Discussion

In this study, we showed that CD200R1-expressing Swiss 3T3 feeder cells increased the colony-forming ability of EpSCs. In addition, treatment with a GSK3β inhibitor further enhanced the proliferation of EpSCs.

Tissue stem cells are a useful source of cells in the regeneration of tissues and organs, because they show no transplant rejection and have a reduced risk of tumors unlike ESC and iPSc. Therefore, it is worthwhile isolating and preserving the tissue stem cells from individuals before disease and injuries arise. Therefore, it is proposed a clinical strategy isolating and preserving tissue stem cells from individuals before disease and injuries arise. EpSCs were originally identified as the stem cells of the epidermis, the hair follicles and the sebaceous gland. Recently, it has been indicated that they can differentiate into a wide range of cell lineages including blood vessels and neural tissue[4]. Amoh *et al.* found that implanted hair follicle stem cells can form Schwann cells that support the repair of severed peripheral nerves[5]. These observations underline the importance and utility of EpSCs.

Stem cell niches are essential microenvironments for maintaining stemness *in vivo*, and the feeder layer used to culture stem cells is a potential substitute for these niches *in vitro*. We have shown that CD200R-expressing Swiss 3T3 feeder cells accelerate EpSC colony formation. The molecular mechanism of this process was not elucidated in this work, but CD200 was shown to exert anti-apoptotic effects through association with CD200R[16]. The interaction between these molecules may act to preserve EpSC stemness.

GSK3β inhibitor synergistically increases the EpSC CFE together with CD200R1-expressing Swiss 3T3 feeder cells (Fig. 4A). This finding indicates that the induction of Wnt signaling stimulates EpSC growth. In the skin, the canonical Wnt signaling pathway plays complex roles in stem cell maintenance, proliferation and differentiation. Many studies of EpSC regulation by the Wnt pathway have been reported but their findings remain controversial. Wnt/β-catenin sig-

naling was classically regarded as an inducer of EpSC differentiation into hair follicles, but not a stem cell maintenance factor. Forced expression of β-catenin in the skin of mice stimulates hair morphogenesis[17]. Deletion of Lef-1 or β-catenin as well as overexpression the Wnt inhibitor Dkk1 results in a paucity of hair follicles[12, 18]. Our findings appear to contradict these observations. Recently, however, two studies revealed that autocrine Wnt/β-catenin signaling contributes to proliferation and self-renewal of EpSC *in vivo*[19, 20]. Wnt/β-catenin signaling induces the division of EpSC to supply progenitor cells for hair follicles as well as for self-renewal that follows the induction of hair morphogenesis. Our preliminary data supports this proposition. The GSK3β inhibitor induced an approximately 2-fold increase in $CD34^+$ cells growing on both normal and CD200R-expressing feeder cells (No treatment : 19.4%, GSK3β inhibitor treatment : 36.6% on Swiss 3T3 cells, No treatment : 20.4%, GSK3β inhibitor treatment: 39.6 % on CD200R-expressing 3T3 cells). Conversely, the Wnt signaling inhibitor, DKK3, significantly decreased the formation of $CD34^+$ cells (No treatment : 19.4% , DKK3 treatment : 3.2% on Swiss 3T3 cells, No treatment : 20.4%, DKK3 treatment : 2.0% on CD200R-expressing 3T3 cells).

Our results show that an artificial environment suitable to the stem cells, which resembles the niche, enables stem cells to proliferate without losing stemness. In addition to CD200R, expression of other specific molecules in the EpSC niche might help the maintenance and self-renewal of EpSCs, which would provide a significant increase in stem cells for clinical applications.

Conflict of interest disclosure

The authors have declared no conflict of interest.

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[Received January 15, 2015 : Accepted January 28, 2015]