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Importance of CD44 on umbilical cord mesenchymal stem cells for expansion of hematopoietic cells

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Abstract

Human umbilical cord mesenchymal stem cells (hUCMSCs) have important functions on the expansion of hematopoietic stem cells (HSCs) through providing the essential microenvironment for hematopoiesis. In order to test whether CD44 on hUCMSCs could have a key function for the ability of hUCMSCs to expand human HSCs, the soluble anti-CD44 antibody was added to the co-cultures of hUCMSCs and cord blood (CB) $CD34^+$ cells, which blocked the ability of hUCMSCs to expand CB $CD34^+$ cells significantly. Long-term culture initiating cell (LTC-IC) assay revealed that the ability of multipotent differentiation of CB $CD34^+$ cells co-cultured with CD44 knockdown hUCMSCs could only retain lasting at most for 5 weeks *in vitro*. *In vivo* assay, based on non-obese diabetic/severe combined immunodeficient disease (NOD/SCID) mice, revealed that the hematopoietic reconstitution potential of CB $CD34^+$ cells co-cultured with CD44 knockdown hUCMSCs is significantly reduced. The hematopoietic supporting ability of hUCMSCs *in vivo* and *in vitro* is reduced upon the knockdown of CD44. CD44 has important functions on the ability of hUCMSCs to expand human HSCs in the cell- extrinsic control.

Key words: CD44, umbilical cord mesenchymal stem cells, hematopoietic supporting.

Introduction

As a crucial constituent of the essential niche for hematopoiesis, human mesenchymal stem cells (MSCs) are critical for the survival, growth, and differentiation of hematopoietic stem cells (HSCs) (1, 2) through the adhesion and direct cell-to-cell contact with HSCs. More and more data have shown that MSCs from human umbilical cord (hUCMSCs) could be used *in vitro* as a stromal holder for supporting and expanding HSCs through cell-to-cell contact (3, 4, 5). The membrane-bound and secreted molecules from hUCMSCs have important functions for the hematopoietic supporting process.

CD44 was initially identified as the lymphocyte homing receptor (6, 7). CD44 is one kind of transmembrane glycoproteins and is commonly expressed in embryonic (8), hematopoietic (9), mesenchymal (10), epithelial and cancer (11, 12, 13, 14) stem cells. Wagner *et al* have shown that human HSCs constitutively expressed CD44 highly, which mediated the interaction of HSCs and their niche (15). Moreover, CD44 also affected the differentiation and apoptosis of human leukemia cells in the cell-intrinsic control (16, 17). CD44 is also a common constituent of the hematopoietic stem cell microenvironment and usually exists as a standard isoform. While the important functions of CD44 on HSCs have been studied in considerable detail, our knowledge about the roles of CD44 from HSC microenvironments on hematopoiesis remained limited. Brahmananda *et al* identified Lin-Sca1-OPN+CD166+CD44+CD90+ osteoblasts as osteoblastic lineage cells, which can promote the expansion and reconstitution potential of hematopoietic progenitor and stem cells *in vitro* and *in vivo* (18). Oliver *et al* found that CD44 on stromal cells supports the homing and adhering of mouse HSCs (19). Marc *et al* showed that CD44 on stromal cells is also involved in the adhesion and maturation of mouse HPCs (20). These data all indicated that CD44 on stromal cells is an important regulator of hematopoiesis. But most of the studies were performed in murine models and the relevance of hematopoietic supporting of CD44 in stromal cells, especially in hUCMSCs, to human HSCs still remains unsubstantiated.

In this study, we first identified that CD44 is expressed by hUCMSCs highly, which is coincident with the former reports. The ability of hUCMSCs to expand CB $CD34^+$ cells was significantly blocked after the soluble anti-CD44 antibody was added to the co-cultures of hUCMSCs and CB $CD34^+$ cells. The result is confirmed by the co-culture of CB $CD34^+$ cells and CD44 knockdown hUCMSCs. These data indicated that CD44 on hUCMSCs play a key role for the expansion of primitive HSC/ HPC *in vitro*. Long-term culture initiating cell (LTC-IC) assay revealed that the ability of multipotent differentiation of CB $CD34^+$ cells co-cultured with CD44 knockdown hUCMSCs could only retain lasting at most for 5 weeks *in vitro*. *In vivo* assay, based on non-obese diabetic/severe combined immunodeficient disease (NOD/SCID) mice, revealed that CB $CD34^+$ cells co-cultured with CD44 knockdown hUCMSCs can differentiate to the tested hematopoietic lineages

but the hematopoietic reconstitution potential of CB $CD34^+$ cells is significantly reduced after co-cultured with CD44 knockdown hUCMSCs *in vivo*. CD44 knockdown hUCMSCs may altered the hematopoietic niche, which specifically promotes the efficient transition of primitive to committed differentiated-HSCs and then promote the continuous and successive production of mature blood cells.

In summary, our data suggested that CD44 from hUCMSCs has important functions on the ability of hUCMSCs to support the expansion of $CD34^+$ cells from CB *in vitro*. CD44 in hUCMSCs was also necessary for long-term supporting hematopoiesis *in vitro* and *in vivo*.

Materials and methods

Cell cultures

Human umbilical cord mesenchymal stem cells (hUCMSCs) were obtained as described previously (21). Briefly, the cryopreserved primary hUCMSCs were thawed and plated in low-glucose Dulbecco's modified Eagle's medium (L-DMEM) (Hyclone, USA) complemented with 10% FBS (Gibco) at 37°C supplemented with 5% CO_2 as described previously (21). The experimental protocol was approved by the Institutional Authority for Laboratory Animal Care of Tianjin Central Hospital.

Human CB samples were obtained as described previously (21, 22). Briefly, the mononuclear cells (MNCs) from CB were firstly collected by the lymphocyte separation medium (1.077 g/ml) (TBD Biotech, Tianjing, China). $CD34^+$ cells were then immunomagnetically enriched through the MACS $CD34^+$ Cell Isolation Kit (Miltenyi Biotech Inc., Bergisch Gladbach, Germany). Flow cytometry (FCM) was used to assess the purity of isolated $CD34^+$ cells. This experiment was approved by the Ethics Committee of Tianjin Central Hospital. Before the studies, the objectives, requirements and procedures of the experiments were noticed to the subjects.

CD44 shRNA construction and transduction of hUCMSCs

The two CD44-specific small hairpin RNAs (KD1 and KD2) oligomers (12) were tested as described previously (21, 22). Briefly, the two designed oligomers were putted into the retroviral vector RNAi-pSIREN-RetroQ. The retroviral vector can drive shRNA production through the U6 promoter (Clontech). Before transduction the CD44 shRNA construction was sequenced (ABI PRISM 310 Genetic Analyzer, USA). The RNAi-pSIREN-RetroQ vectors including the scrambled sequences, which do not target any known mRNAs, were used as controls (CTRL).

The knockdown of CD44 was done as described previously (21). In brief, according to the manufacturer's instructions, the RNAi-pSIREN-RetroQ retroviral plasmid and the viral packaging plasmid co-transfected the Phoenix packaging cell line through Lipofectanine 2000 (Invitrogen). The viral supernatants, which were obtained at 48 or 72 hours after transfection, were used for further transfection of hUCMSCs. The viral supernatants including polybrene (8μg/ml) were added into culture plates when the density of hUCMSCs became 70%–80% confluent. After transfection for 5 hours at 37°C, the fresh medium substituted for the transfection supernatant. The puromycin (5μg/ml) could be added into the medium after cultured for 24 hours. The blocking anti-CD44 antibody was A3D8 (ab11358, Abcam), which recognizes a constant epitope on the CD44 receptor.

RNA extraction and real-time PCR

The total RNA was isolated through Trizol reagent (Invitrogen). The first-strand cDNA was synthesized by the M-MLV Frist Strand cDNA Synthesis Kit (OMEGA). Real-time PCR was analyzed with ABI7500 (Applied Biosystems) using the Super-Real PreMix Plus (SYBR Green) kit. β-actin (forward primer, 5′TCATGTTTGAGACCTTCAA; reverse primer, 5′GTCTTTGCGGATGTCCACG) was used as the endogenous control of CD44 (forward primer, 5′CCACATTCTACAAGCACAATCCA; reverse primer, 5′AGTCCATATCCATCCTTCTTCCT). All samples were done in triplicate. The relative expression was determined using the $\Delta\Delta C_T$ method.

The co-culture of human CB $CD34^+$ cells with hUCMSCs

The co-culture test was analyzed as described previously (22). Briefly, the CD44 knockdown hUCMSCs or control hUCMSCs were plated in 24-well plates (5.0×10^5/well) overnight after irradiated at a dose of 40Gy. $CD34^+$ cells (2.0×10^4/well) in Iscove's Modified Dulbecco's Medium (IMDM) (Bio-WHITTAKER) containing 10% FBS, 10^{-4}M 2-mercaptoethanol, 2mM L-glutamine, 5 mg/ml insulin, 100 U/ml penicillin, and 100 mg/ml streptomycin as well as a cytokine cocktail were added into the indicated hUCMSCs . After the indicated days of co-culture, the hematopoietic cells were collected gently, counted and analyzed further.

Cell cycle analysis

Cells were collected and washed twice with PBS. An equal volume of ethanol was added to the cells and kept overnight at 4°C. Th e cells were After washed twice, cells were resuspended in PBS and treated with 100 μ g/mL of RNase for 1 h at 37°C. Cells were incubated with 0.05 mg/mL PI for 30 min in the dark. Cell cycle phase distribution of nuclear DNA content was analyzed using a FACSCalibur flow cytometer. Data were analyzed with WINMDI 2.9 software (Phoenix Software, El Segundo, CA).

Long-term culture initiating cells (LTC-IC) assay

LTC-IC assay was done as described previously (21). Briefly, irradiated CD44 knockdown hUCMSC or control hUCMSC were co-cultured with 1.0×10^4 CB $CD34^+$ cells in LTC medium (Myelo-Cult, StemCell Inc., Vancouver, BC, Canada), which contained horse serum, foetal bovine serum, 2-mercaptoethanol and a-MEM supplemented with 10^{-6}M hydrocortisone sodium hemisuccinate (Sigma). The fresh medium substituted for the half medium every week. Both nonadherent and adherent hematopoietic cells were collected every week during 3–7 weeks of co-culture, and were then seeded in the complete methylcellulose medium at 37°C with 5% CO_2. After 14–16 days of co-culture, the LTC-IC activities were tested by the number of colonies with

greater than 50 cells.

The hematopoietic reconstitution potential assay

After co-cultured with irradiated CD44 knockdown hUCMSC or control hUCMSCs for 4 weeks, the hematopoietic cells from the initial 5.0×10^4 CB $CD34^+$ cells were collected, and injected intravenously (i.v.) into 6 8-week-old, sublethally irradiated (3.5 Gy) NOD/SCID mice per group (22). The peripheral blood was obtained through nonlethal eyebleeds under anesthesia with isoflurane. After the breeding of 12 weeks, the grafted mice were sacrificed. The mononuclear cells from bone marrow were collected and analyzed through flow cytometry. The hematopoietic reconstitution potential of human cells was analyzed based on the expression of CD45 (FITC; 555482), CD34 (PE; 550761), CD36 (PC7; 563666) and glycophorin A (APC; 551336) antibodies. Mouse IgG1 was used as the isotype controls. The hematopoietic reconstitution potential of human cells was further analyzed by PCR amplification of the human 17α-satellite gene. The experiments about the animals were done upon the agreement of Institutional Authority for Laboratory Animal Care of Tianjin Central Hospital.

Statistical analysis

The data are shown as the mean ± standard deviation (SD). Statistical comparisons were analyzed based on a two-tailed Student's *t* -test.

Results

CD44 from hUCMSCs is important for the expansion of CB $CD34^+$ cells

We first investigated the expression of CD44 on hUCMSCs by flow cytometry. As shown in Figure 1A, hUCMSCs constitutively expressed CD44 highly, which is coincident with prior work (23, 24). To investigate whether CD44 is important for hematopoietic-supporting activity of hUCMSCs, we examined the expansion of CB $CD34^+$ cells with hUCMSCs in the presence of soluble anti-CD44 antibody. As expected, the expansion ability of CB $CD34^+$ cells, including $CD34^+CD38^-$ and $CD34^+CD38^+$ populations, was significantly blocked upon the addition of soluble anti-CD44 antibody to the co-cultures (Figure 1B). The expansion of $CD34^+CD38^+$ cells is raised after adding the soluble isotype IgG1, although the mechanism by which the expansion of $CD34^+CD38^+$ cells is promoted upon the addition of soluble isotype IgG1 is not yet clear, which is similar with the results from our previous work (21). The results give the possibility that the addition of antibody to the culture altered the culture milieu, which prefers to promote or inhibit $CD34^+$ cell expansion, which depends on the effects on interactions between hUCMSCs and CB $CD34^+$ cells. Based on the results above, CD44 from hUCMSCs may be important for the ability of hUCMSCs to expand CB $CD34^+$ cells.

The expansion of CB $CD34^+$ cells upon co-cultured with CD44 deficient hUCMSCs

In order to further confirm the function of CD44 in hUCMSCs on hematopoiesis supporting, CD44 knockdown hUCMSCs were produced through retro-

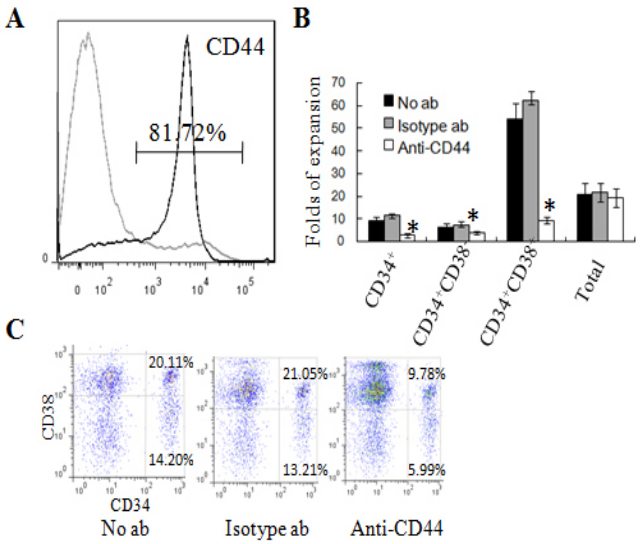

Figure 1. CD44 is required for hematopoietic supporting activity of hUCMSCs. (A) Flow cytometry analysis of CD44 expression in hUCMSCs. The hUCMSCs were stained with the FITC-conjugated monoclonal antibody to CD44 and analyzed by flow cytometry. Gray histogram: isotype control; black histogram: specific antibody. Data shown are from one representative experiment of three reproducible experiments. (B) The irradiated confluent hUCMSCs were co-cultured with 2.0 x 10^4 per well CB $CD34^+$ cells in the presence of soluble anti-CD44 or isotype antibody (5 μg/ml) in six-well plates in triplicate for 7 days, and the cells were harvested, counted, stained PE-conjugated mAb to CD34 and FITC-conjugated mAb to CD38, and analyzed by flow cytometry. Values indicate the fold increase compared with the initial number of cells. The results are given as mean ± standard deviation (SD) of three separate experiments. The data shown are from one of two reproducible experiments. *$P<0.05$, as compared with the cultures with isotype mAb. (C) Flow cytometric analysis of CB $CD34^+CD38^-$ /$CD34^+$ $CD38^+$ cell expansion in a representative experiment upon addition of specific antibodies.

viral vectors. The CD44 expression in the hUCMSCs after transfection was analyzed by real-time RT-PCR. The obvious down-modulation of CD44 was observed in KD cells in comparison to that in the CTRL cells (Figure 2A and B). Based on the function of shRNA on CD44 expression, we chosen KD1 for further analysis. We further examined the hematopoietic supporting activity of hUCMSCs after CD44 knockdown. As shown in Figure 2C, CB $CD34^+$ cells were co-cultured with irradiated KD1 or primary hUCMSCs or in the absence of stromal cells (cytokine-conditioned medium alone). After 7 days of co-culture, the number of total cells, $CD34^+$ cells, $CD34^+CD38^-$ cells, and $CD34^+CD38^+$ cells was counted, respectively. As shown in Figure 2C, the total nucleated cells and $CD34^+CD38^+$ cells were dramatically expanded by ~ 80-fold and ~ 90-fold respectively in the presence of hUCMSCs with or without CD44 knockdown. Importantly, the activity of CD44 deficient hUCMSCs for the expansion of $CD34^+$ cells and $CD34^+CD38^-$ cells is significantly reduced compared with that of CTRL ($CD34^+$ cells: 11.33- versus 18.99-folds; $CD34^+CD38^-$ cells: 5.56- versus 15.76-folds). These results indicated that CD44 in hUCMSCs appeared to be important for the proliferation of primitive HSC/ HPC *in vitro*. The cell cycle status of hematopoietic cells co-cultured with CTRL or KD1 cells were tested by flow cytometry. As shown in

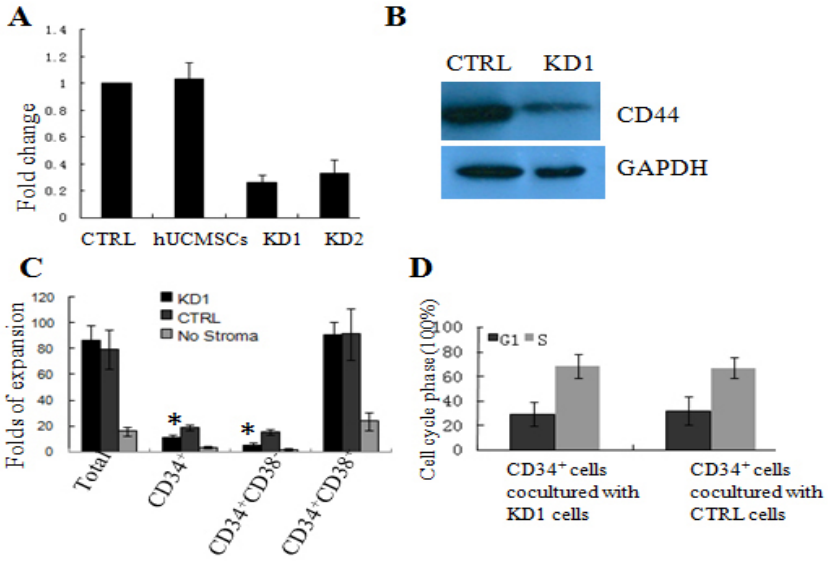

Figure 2. Ex vivo expansion of CB CD34⁺ cells co-cultured with CD44 deficient hUCMSCs. (A) Real-time RT-PCR was performed to evaluate the expression level of CD44 in hUCMSCs after retrovirally transduced with vectors for CD44 knockdown (KD1 or KD2), or control (CTRL). Each reaction was performed in triplicate. (B) The expression of CD44 protein was evaluated by western blot in KD1 cells. (C) The irradiated confluent CD44 deficient hUCMSCs were co-cultured with 2.0 x 10^4 per well CB CD34⁺ cells in six-well plates in triplicate for 7 days, and the cells were harvested, counted, stained with PE-conjugated mAb to CD34 and FITC-conjugated mAb to CD38, and analyzed by flow cytometry. Values indicate the fold increase compared with the initial number of cells. The results are given as mean standard deviation (SD) of three separate experiments. * P<0.05, compared between KD1 and CTRL cells (n=3) (Student's *t*-test). (D) The percentage of cells in G1 phase or S phase from CB CD34⁺ cells cocultured with KD1 cells and CB CD34⁺ cells cocultured with CTRL cells. Data are representative of three independent experiments.

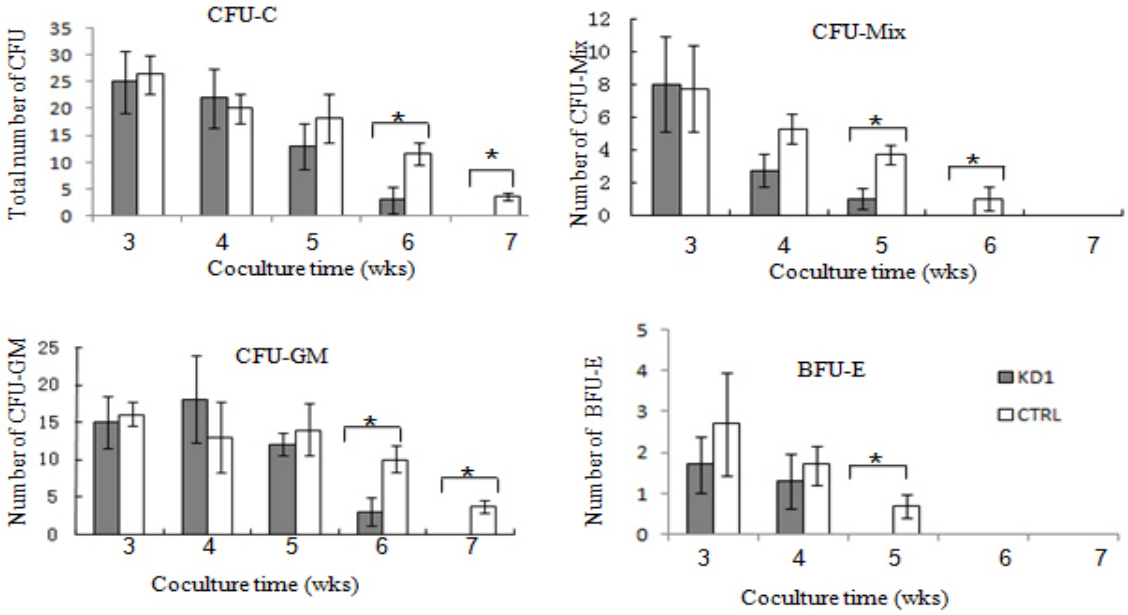

Figure 3. The effect of CD44 knockdown in hUCMNCs on long-term culture initiating cells activity of CB CD34⁺ cells. 1.0 x 10^4 CB CD34+ cells were co-cultured with KD1 cells or CTRL cells for 3–7 weeks and then subject to CFU assay. After 14–16 days of culture, the colonies, including BFU-Es, CFU-GMs, and CFU-Mix's, with greater than 50 cells were counted. The results are expressed as mean ± SD (n=5). * P<0.05, compared between KD1 and CTRL cells (Student' *t*-test).

Figure 2D, no significant difference in percentage of cells in G1 phase or S phase was observed between CB CD34⁺ cells co-cultured with KD1 cells and those co-cultured with CTRL cells.

The function of CD44 knockdown in hUCMSCs on LTC-IC activity of CB CD34⁺ cells

In order to examine the function of CD44 in hUCMSCs on the self-renewal and differentiated potential of HSC, a long-term culture initiating cells (LTC-IC) assay was done. After co-cultured with CD44 knockdown hUCMSCs or control cells in LTC-IC medium for 3–7 weeks, the CB CD34⁺ cells were collected and subject to a CFU test. After 14–16 days of culturing, the colonies containing over 50 cells were counted. As shown in Figure 3, the total number of CFCs and the number of CFU-GMs from the CB CD34⁺ cells co-cultured with CD44 in hUCMSCs for 3–5 weeks showed similar with that from the CB CD34⁺ cells co-cultured with control cells. However, the numbers of total CFCs and CFU-GMs from the CB CD34⁺ cells co-cultured with CD44 knockdown hUCMSCs reduced quickly at 6 weeks and then gradually lost their differentiated potential along the time of culture, because CFCs and CFU-GMs were almost undetectable at 7 weeks of co-culture. Consistently, although the numbers of CFU-

Mix's and BFU-Es from the CB CD34$^+$ cells co-cultured with CD44 knockdown hUCMSCs cells for 3–4 weeks showed similar with those from the CB CD34$^+$ cells co-cultured with control cells the numbers of CFU-Mix's and BFU-Es from the CB CD34$^+$ cells co-cultured with CD44 knockdown hUCMSCs cells reduced quickly at 5 week. In comparison to CB CD34$^+$ cells co-cultured with control cells, which kept the ability of differentiated potential lasting at least for 6 weeks, CB CD34$^+$ cells co-cultured with CD44 knockdown hUCMSCs could only keep the ability of differentiated potential lasting at most for 5 weeks because the numbers of CFU-Mix's and BFU-Es from these cells could be undetected at 6 weeks. All these investigations suggested that CD44 from hUCMSCs have important functions on the capacity of umbilical cord MSCs for supporting long-term hematopoiesis *in vitro*.

Hematopoietic reconstitution of CD34$^+$ cells co-cultured with CD44 knockdown hUCMSCs

In order to further verify the expansion and LTC-IC assays *in vitro*, the hematopoietic reconstitution potential of CB CD34$^+$ cells co-cultured with CD44 knockdown hUCMSCs or control cells in NOD/SCID mice was examined through temporally monitoring the peripheral blood and bone marrow of engrafted mice for 12 weeks. 5.0 × 10^4 CB CD34$^+$ cells co-cultured with irradiated CD44 knockdown hUCMSCs (KD1) or CTRL cells for 4 weeks were injected into the sublethally irradiated NOD/SCID mice. The percent of CD45$^+$ cells from the PB of mice, which were engrafted with CB CD34$^+$ cells after co-cultured with control umbilical cord MSCs, was increased gradually. But the positive percent of CD45 cells from CB CD34$^+$ cells co-cultured with CD44 knockdown umbilical cord MSCs was highest during 2 and 4 weeks and then decreased (Figure 4A). The hematopoietic reconstitution potential of engrafted CB CD34$^+$ cells were tested at 12 weeks post-transplant by flow cytometry based on the CD marker expression including CD45$^+$, CD45$^-$CD36$^+$ and CD36$^-$GPA$^+$, and CD45$^+$CD34$^+$ on the bone marrow mononuclear cells of the graft mice. At 12 weeks, in comparison to that of the mice transplanted with CB CD34$^+$ cells co-cultured with control cells, the mice transplanted with CB CD34$^+$ cells co-cultured with KD1 showed the significantly lower percentage of total human cells (Figure 4B; p=0.0155). The statistical comparison was analyzed between the mice transplanted with CB CD34$^+$ cells co-cultured with CD44 knockdown hUCMSCs and those transplanted with CB CD34$^+$ cells co-cultured with control cells. The

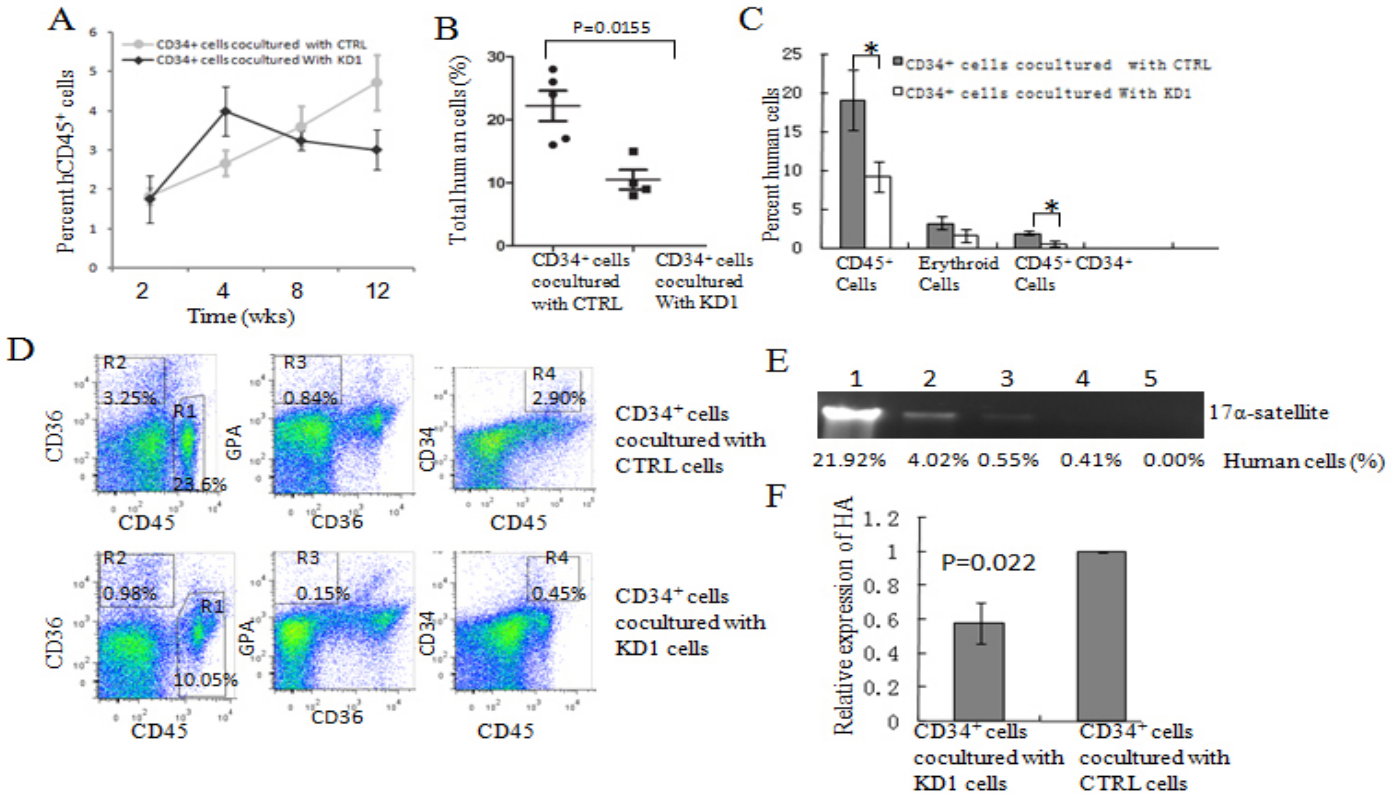

Figure 4. Effect of CD44 knockdown in hUCMNCs on repopulation of CB CD34$^+$ cells in NOD/SCID mice. (A) Kinetic analysis of peripheral blood engraftment by cells from the NOD/SCID mice injected with CD34$^+$ cells co-cultured with KD1 and those injected with CD34$^+$ cells co-cultured with CTRL (Student's *t*-test) (n=6 per group). (B) The level of total human cell engraftment was shown. The mice were sacrificed 12 weeks after transplantation and the mononuclear cells from bone marrow were analyzed by flow cytometry. P=0.0155 compared between the KD1 group and CTRL group (Student's *t*-test). (C) The fraction of CD45$^+$, erythroid and CD45$^+$CD34$^+$ population among the engrafted human cells was shown. *p<0.05, compared between KD1 group and CTRL group (Student's *t*-test). (D) Flow cytometric analysis of human CB CD34$^+$ cell repopulation in a representative primary NOD/SCID mouse after co-culture with KD1 or CTRL cells. Mononuclear cells from bone marrow of engrafted NOD/SCID mice were examined for assessment of human CD45$^+$ cells (R1) and erythroid cells including CD45$^-$CD36$^+$ (R2) and CD36$^-$GPA$^+$ (R3) population and CD45$^+$ CD34$^+$ cells (R4). (E) The mononuclear cells from bone marrow containing the different percentage of human cells (lanes 1-4) from the engrafted mice at 12 weeks were analyzed for human-specific 17α-satellite DNA by PCR. The human-specific 17α-satellite gene was detected when the human cells were over 0.41% (lanes 1-3) whereas it was undetectable at a percentage of 0.41% (lane 2). Lane 5, one mouse without transplants; lane 1-4, mice receiving transplants of CD34$^+$ cells co-cultured with CTRL or KD1 cells. (F) Real-time RT-PCR was performed to evaluate the expression level of HA in CB CD34$^+$ cells after co-cultured with KD1 or CTRL. Each reaction was performed in triplicate.

differentiated potential of transplanted CB $CD34^+$ cells was further analyzed. In comparison to the percent from the control group, a significantly lower positive percent of CD45 cells was observed in KD1 group. What's more, in comparison to that of the control group, a significantly lower positive percent of CD45CD34 cells was also observed in the KD1 group (Figure 4C). The differentiated potential of transplanted CB $CD34^+$ cells co-cultured with CD44 knockdown hUCMSCs verified the expansion and LTC-IC results *in vitro* to some extent. To comparison to that of mice transplanted with CB $CD34^+$ cells co-cultured with control hUCMSCs, the percent of $CD45^-CD36^+$ cells and $CD36^-GPA^+$ cells from CD44 knockdown hUCMSCs mice, which were transplanted with CB $CD34^+$ cells co-cultured with CD44 knockdown hUCMSCs, showed a tendency to be lower but the statistical comparisons between the two groups showed no significant difference. The hematopoietic reconstitution potential of transplanted CB $CD34^+$ cells was also assayed by PCR to examine the expression of human-specific 17α-satellite gene in the transplanted mice from the different groups. When the positive percent of human cells was more than 0.41%, the human-specific 17α-satellite gene could be detected by PCR amplification (Figure 4E, lanes 1-3). When the positive percent of human cells was less than 0.41%, the human-specific 17α-satellite gene could be undetected (Figure 4E, lane 4). In order to test the possible reason for the poor engraftment of CB $CD34^+$ cells co-cultured with KD1 cells, we checked the expression level of hyaluronic acid (HA) in CB $CD34^+$ cells after co-cultured with KD1 or CTRL. As shown in Figure 4F, the expression of HA in CB $CD34^+$ cells after co-cultured with KD1 became significantly reduced compared with that in CB $CD34^+$ cells after co-cultured with CTRL, which may induce the poor homing ability of CB $CD34^+$ cells after co-cultured with KD1. All of these verified the *in vitro* results and indicate that CD44 from umbilical cord MSCs also has important functions on the capacity of umbilical cord MSCs to keep the hematopoietic reconstitution and differentiated potential of CB $CD34^+$ cells *in vivo*.

Discussion

Human umbilical cord MSCs (hUCMSCs) have been successfully used *in vitro* for stromal support and expansion of HSCs through creating a niche suitable for stem cell expansion. CD44 is a crucial constituent of hematopoietic stem cell microenvironment and expressed by many hematopoietic supporting stromal cells highly including hUCMSCs. However, the function of CD44 in hUCMSCs on short-term expansion of human HSCs or long-term hematopoietic supporting *in vitro* or *in vivo* remains largely unclear.

In this study, we firstly tested and verified that CD44 in hUCMSCs has important functions on the ability of hUCMSCs to expand CB $CD34^+$ cells for short-term. The expanding ability of $CD34^+$ cells induced by hUCMSCs, especially $CD34^+CD38^+$ cells <Figure 1B> is significantly decreased upon adding the soluble anti-CD44 antibody to the co-cultures of hUCMSCs and CB $CD34^+$ cells. We also confirmed the previous work that CD44 is constitutively expressed by hUCMSCs highly. These results suggested that, in addition to be necessary in the cell-intrinsic control of HSC function (9, 15), CD44 also has important functions on the ability of hUCMSCs for expanding HSCs in the cell- extrinsic control.

As expected, knockdown of CD44 in hUCMSCs decreased the ability of hUCMSCs to expand CB $CD34^+$ cells, especially $CD34^+CD38^-$ cells <Figure 2C>. The expansion of $CD34^+CD38^+$ population is reduced after the addition of anti-CD44 antibody comparing to isotype IgG1 while the knockdown of CD44 in hUCMSCs did not affect the expansion of $CD34^+CD38^+$ cells. The possible reasons are that, except for inhibiting CD44 function from hUCMSCs, the addition of anti-CD44 antibody to the co-culture may also inhibit the function of CD44 on hematopoietic cells directly. Moreover, the hematopoietic long-term supporting ability of hUCMSCs for CB $CD34^+$ cell expansion is also significantly reduced <Figure 3>. After co-cultured with control hUCMSCs, the CB $CD34^+$ cells can keep the ability of multipotent differentiation lasting at least for 6 weeks but the CB $CD34^+$ cells co-cultured with CD44 knockdown hUCMSCs could only retain the capacity of multipotent differentiation lasting at most for 5 weeks. The reason for the difference between the CFU observed between KD1 and CTRL after 3 weeks of co-culture and the hematopoietic cells yielded after co-cultured with KD1 comparing to CTRL in Fig 2C after 1 week is possible from the different co-culture medium used in short-term culture and long-term culture. It is known that CD44 play an important role for the interaction between the stem cells and their stromal cells from microenvironment (25, 26). It is possible that the CD44 deficient hUCMSCs created a hematopoietic niche, which is not suitable for stem cell expansion. The knockdown of CD44 on hUCMSCs may promote the efficient transition of primitive to committed differentiated-HSCs. The efficient and continuous production of mature blood cells from CB $CD34^+$ cells after co-cultured with the hUCMSCs may be speeded upon the knockdown of CD44. These investigations also verified the conclusion that the adhering molecules from the hematopoietic stromal cells have important functions on the interaction between HSCs and their niche stromal cells (27).

The data from the LTC-IC test were further verified by hematopoietic reconstitution potential assay *in vivo*. To comparison to that from the control group, the percent of $CD45^+$ cells or $CD45^+CD34^+$ cells from CD44 knockdown hUCMSCs group became significantly lower. To comparison to that of mice transplanted with CB $CD34^+$ cells co-cultured with control hUCMSCs, the percent of $CD45^-CD36^+$ cells and $CD36^-GPA^+$ cells from CD44 knockdown hUCMSCs mice, which were transplanted with CB $CD34^+$ cells co-cultured with CD44 knockdown hUCMSCs, showed a tendency to be lower but the statistical comparisons between the two groups showed no significant difference. The percent of $CD45^+$ cells from the PB of mice, which were engrafted with CB $CD34^+$ cells after co-cultured with control umbilical cord MSCs, was increased gradually. But the positive percent of CD45 cells from CB $CD34^+$ cells co-cultured with CD44 knockdown umbilical cord MSCs was highest during 2 and 4 weeks and then decreased.

All of the investigations indicated that, after co-cultured with CD44 knockdown umbilical cord MSCs, the hematopoietic reconstitution potential of CB CD34$^+$ cells became higher than that of CB CD34$^+$ cells, which were co-cultured with control umbilical cord MSCs during the first 4 weeks after transplant. The reduced hematopoietic reconstitution potential of CB CD34$^+$ cells co-cultured with CD44 knockdown hUCMSCs *in vivo* is possibly due to, at least in part, an inefficient implanting. The successful engraftment of human hematopoietic cells *in vivo* needs proper adhesion between hematopoietic cells and the bone marrow niche, as well as the appropriate shift between bones. It is known that, as a cell-surface receptor, CD44 is expressed by stromal cells and essential for niche structural organization. Within the complicated milieu of the HSC microenvironment, the interactions between HA and CD44 modulate the processes including HSC specification and differentiation. Comparing with those of CB CD34$^+$ cells co-cultured with control umbilical cord MSCs, the expression levels of HA from CB CD34$^+$ cells, which were co-cultured with CD44 knockdown hUCMSCs, became lower possibly, which further caused the improper interaction between the transplanted hematopoietic cells and their niche. The data resulted from the bone marrow also exclude the possibility that the CB CD34$^+$ cells upon co-cultured with CD44 knockdown hUCMSCs have decreased ability for homing and proliferating in the bone marrow, which is coincident with the conclusion from the engraftment of CD49f$^-$HSCs (Notta et al., 2011). All of these indicate that CB CD34$^+$ cells co-cultured with CD44 knockdown hUCMSCs can grow up to all tested hematopoietic lineages but their long-term capacity of expansion and engraftment is significantly reduced, suggesting that upon CD44 knockdown the capacity of umbilical cord MSCs for expanding HSCs for long-term is decreased. It is possible that the knockdown of CD44 on hUCMSCs creates a microenvironment that is improper for the growth of HSCs. The expression of HA in CB CD34$^+$ cells became significantly reduced upon co-culture with KD1 compared with that in CB CD34$^+$ cells after co-cultured with CTRL, which is the possible reason for the reduced hematopoietic reconstitution potential of CB CD34$^+$ cells after co-cultured with KD1.. Based on the investigations and former results, we put forth the conclusion that CD44 from umbilical cord MSCs has important functions for the hematopoietic development.

In conclusion, the investigation suggested the possible contribution of CD44 from umbilical cord MSCs to the function on the expansion of human HSCs and HPCs *in vitro* and *in vivo*. Our study demonstrated that, in addition to be necessary in the cell-intrinsic control of HSC function (9, 15), CD44 may also has important functions on the capacity of hUCMSCs for expanding HSCs in the cell- extrinsic control.

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