adhere to the bottom of the dish, resulting in the loss of cell recovery. To overcome this problem, we here demonstrate an improved explant method that involves the use of a stainless steel mesh (Cellamigo®; Tsubakimoto Chain Co.) to protect the tissue from floating, after the minced fragments are aligned at regular intervals in culture dishes. This improved explant method is simple, fast, and feasible for use in clinical settings.

Materials and Methods

Improved explant method

The present study was approved by the Ethics Committee of the Institute of Medical Science, University of Tokyo and the NTT Medical Center Hospital, Japan. UCs were collected after informed consent was obtained from pregnant women planning to have cesarean sections. The UC surface was immersed in phosphate-buffered saline (Saibou Kagaku) containing 50 μg/mL of gentamicin (Eltacin; Fuji Pharma) and 0.25 μg/L amphotericin B (Fungizon; Bristol-Myers Squibb Company). A flow chart illustrating the conventional and improved explant method is shown in Figure 1. UCs were minced into 1–2-mm^3 fragments and divided into two groups for explant isolation with and without Cellamigo. Minced fragments (2 g) were aligned at regular intervals in 10-cm culture dishes and semidried until the fragments were firmly attached to the bottom of the dish for at least 30–60 min. Then, the culture medium, consisting of the minimum essential medium-α (α-MEM) supplemented with 10% fetal bovine serum (FBS), gentamicin, and amphotericin B, was gently poured into the dishes. On the other hand, in the improved explant method with Cellamigo, the minced fragments were aligned at a density of about 2 g/10-cm dish and covered with Cellamigo, and the medium was immediately poured into the dishes. The cells were incubated at 37°C with 5% CO_2, and the culture medium was refreshed 5 days after plating and then every 2 days until the fibroblast-like adherent cells reached 80–90% confluence. Within 9 to 12 days, the adherent cells and tissue fragments were harvested using 10% trypsin solution (rTE; Cell Science & Technology Institute) and washed with α-MEM supplemented with 10% FBS. The cells and tissue fragments were removed with Cell Lifter (Corning) and filtered to remove the tissue fragments with a 100-μm cell strainer (BD Falcon).[10] The harvested cells, other than those undergoing further analysis, were immediately cryopreserved in the Stemcell Banker freezing medium (Zenoaq). The cells used for further analysis were plated at 4500 cells/cm^2, the medium was refreshed every 2 days, and the cells were expanded after 4 days of culture.

Tissue staining on the plastic plate

To determine the proportion of $CD105^+$ cells in the minced UC tissue, the UC was cut segmentally and cultured on the plastic dish using the explant method, as described above. The segments were gently peeled off from the bottom of the dish, fixed with 4% formaldehyde, and paraffin sections were prepared. The sections were histologically stained with alkaline phosphatase-conjugated anti-human CD105 mouse monoclonal antibodies (mAbs, Novocastra; Leica Biosystems). Corresponding fragments were stained with hematoxylin and eosin (HE; Sigma).

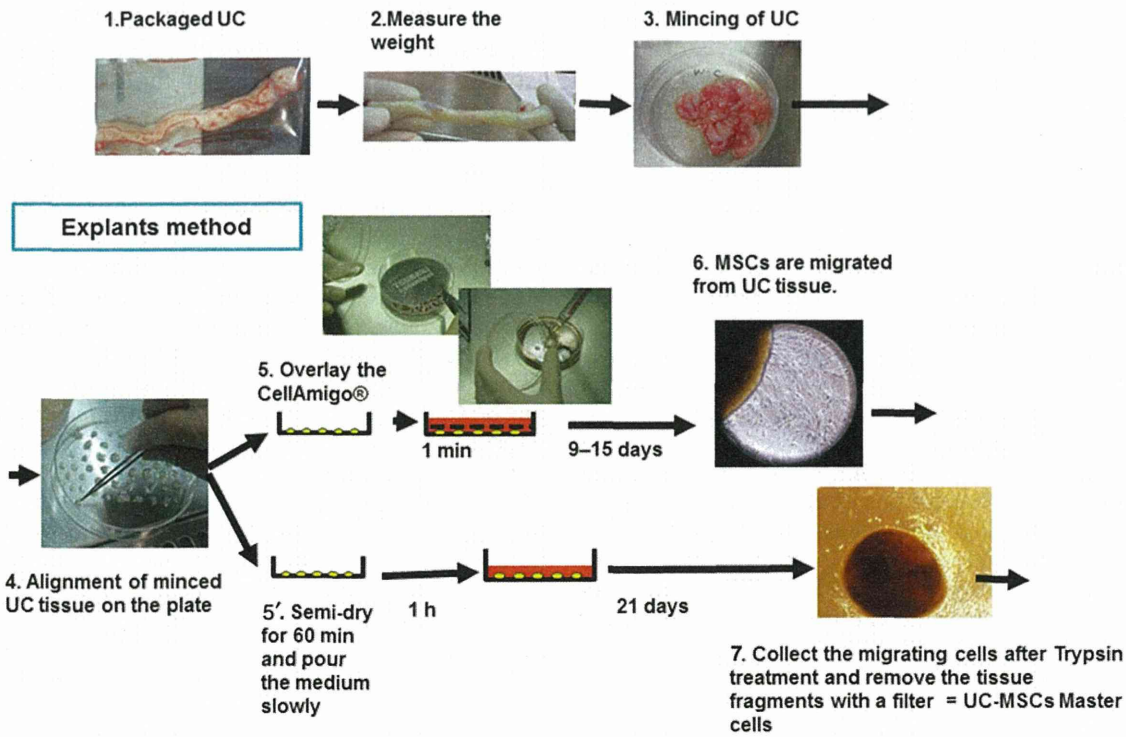

FIG. 1. Isolation of mesenchymal stem cells (MSCs) with the conventional and improved explant methods. The flow chart illustrates the isolation process for umbilical cord-derived mesenchymal stem cells (UC-MSCs) by the explant methods with or without the Cellamigo® stainless steel mesh. The first obtained UC-MSCs are defined as the master cells. Color images available online at www.liebertpub.com/tec

Flow cytometry analysis and cell sorting

Standard flow cytometry techniques were used to determine the defined MSC markers for UC-MSCs, as described elsewhere.[11] UC-MSCs were stained with the following mouse mAbs: phycoerythrin (PE)-conjugated anti-human CD73, HLA-ABC, CD14, CD19, and CD44 (BD Biosciences); fluorescein isothiocyanate (FITC)-conjugated anti-human CD90, CD105 (eBioscience), CD34, and HLA-DR (BD Biosciences); allophycocyanin (APC)-conjugated anti-human CD45 (BD Biosciences); and FITC-, PE-, and APC-conjugated anti-mouse IgGs (BD Biosciences).

Mixed lymphocyte reactions

Mixed lymphocyte reaction (MLR) was performed on UC-MSCs and control cells. Carboxyfluorescein succinimidyl ester (CFSE)-labeled human CB-derived mononuclear cells, as the responder cells, were mixed with irradiated (50 Gy) human autologous or allogeneic CB-derived dendritic cells (DCs), as stimulator cells, in a 10:1 ratio in the presence of low-dose anti-CD3 antibody. CFSE-labeled responder cells were stained with APC-conjugated anti-human CD4 or CD8 mAbs on the day of harvest. The stained cells were acquired with a FACSCanto II flow cytometer (BD Biosystems) and analyzed using FlowJo software (Tomy Digital Biology, Co. Ltd.). In CFSE histograms, when the responder cell divides, the fluorescence should be apportioned equally to daughter cells to reflect half the parental intensity, indicating the proliferation of the responder cells.

Adipogenic and chondrogenic differentiation

UC-MSCs were plated at a density of 2×10^4 cells/well in 12-well plates and were induced to differentiate into adipocytes with a culture medium supplemented with 100 μM indomethacin (Sigma-Aldrich), 0.5 mM IBMX (Sigma-Aldrich), and 10 μg/mL insulin (Sigma-Aldrich)[10] and stained with Oil Red O (Sigma-Aldrich).

To evaluate the chondrogenic differentiation ability of the UC-MSCs, we used a pellet culture system in the chondrogenic differentiation medium. The pellet cells, consisting of 2.5×10^5 UC-MSCs, were cultured in StemMACS™ ChondroDiff Media (Miltenyi Biotec GmbH) in 15-mL conical tubes. The medium was refreshed every 3 days, and after 24 days, the cells were fixed with 4% formaldehyde and stained with the hematoxylin–eosin solution and 0.05% Toluidine blue (Sigma-Aldrich).

Statistical analyses

Differences between groups were analyzed with JMP 6.0.2 software (SAS Institute). Statistical analyses were performed with Student *t*-tests, and a *p*-value of 0.05 was regarded as statistically significant.

Results

Proportion of CD105$^+$ cells in the UC tissue fragments during explant culture

UC-MSCs that migrated from the adherent fragment were observed under the microscope (Fig. 2). UC tissue fragments were stained with anti-human CD105 mAbs to detect the CD105-positive MSCs contained in the fragment cultured by the explant method. CD105-positive cells were observed among the endothelial cells of the vessels before culture. We only found the CD105-positive cells on the side of the fragment that was adherent to the plastic plate after culture when using the explant method (Fig. 2).

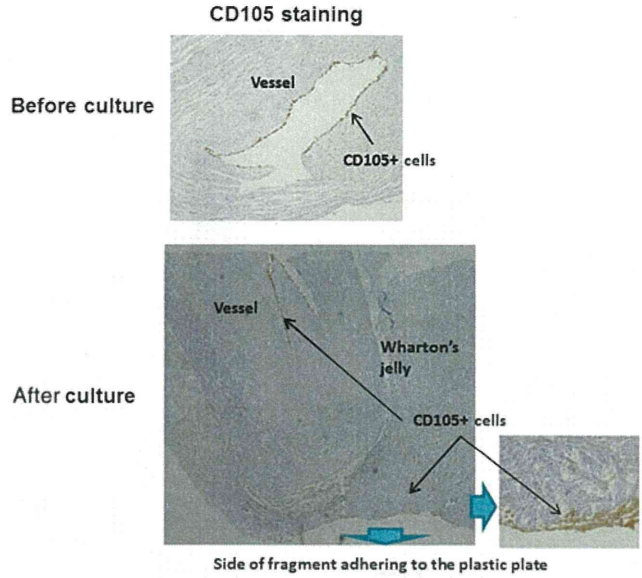

FIG. 2. CD105-positive cells in the UC tissue fragment during the explant method culture. The UC fragment was stained with anti-human CD105 monoclonal antibodies to detect the CD105-positive MSCs. CD105-positive cells were observed at the endothelial cells of the vessels before the culture. The CD105-positive cells appeared on the side of the fragment that adhered to the plastic plate, but not on the nonadherent sides, after culture by the explant method. CD105-positive cells are indicated by *arrows* (brown cells).

Numbers of UC-MSCs isolated with the improved and conventional explant methods

A significantly higher number of UC-MSCs were isolated by the explant method when using Cellamigo ($2.9\pm1.4\times10^6$ cells/g) compared to those obtained with the conventional method ($0.66\pm0.53\times10^6$ cells/g) ($n=6$, $p<0.01$) when the cells cultured with Cellamigo reached 80–90% confluence on day 11 (range 9 to 12 days) (Fig. 3A). The incubation time was also significantly reduced in the improved explant method with Cellamigo compared to the time required to reach an equivalent number of cells with the conventional explant method. It took only 10.3 ± 1.0 days ($n=12$) to obtain $2.83\pm1.2\times10^6$ cells/g by the explant method with Cellamigo, while 20.1 ± 4.4 days ($n=12$) were required to obtain 2.28×10^6 cells/g with the conventional explant method.

UC-MSCs isolated by both the conventional and improved explant methods met the minimal criteria of MSCs defined by the International Society for Cell Therapy.[12] UC-MSCs isolated with both methods showed a spindle-shaped, plastic-adherent, phenotype with positive CD105, CD73, and CD90 expression, and negative CD45, CD34, CD14, CD19, and HLA–DR expression (Fig. 3B).

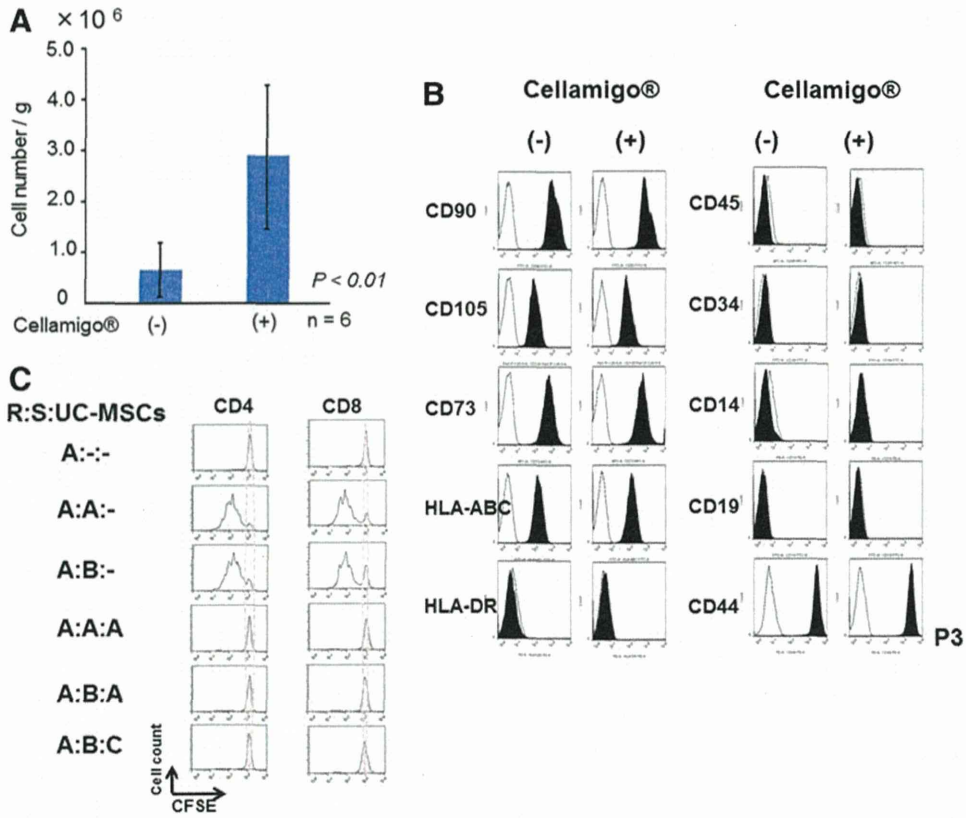

FIG. 3. Characterization of UC-MSCs isolated by the improved explant method with Cellamigo. **(A)** Comparison of the number of UC-MSCs isolated by the conventional and improved explant methods. **(B)** UC-MSCs isolated with both methods showed positive CD105, CD73, and CD90 expression, and negative CD45, CD34, CD14, CD19, and HLA–DR expression. **(C)** UC-MSCs isolated by the improved explant method with Cellamigo showed the inhibition of CD4- and CD8-positive T-cell proliferation in the autologous and allogeneic setting of the mixed lymphocyte reaction (MLR). Carboxyfluorescein succinimidyl ester (CFSE)-labeled mononuclear cells (R) were cultured in different conditions, A:-:- indicates R only, A:A:- or A:B:- indicates coculture with autologous or allogeneic dendritic cells (S), A:A:A indicates all autologous cells, A:B:A indicates allogeneic stimulation, but autologous UC-MSCs, and A:B:C indicates allogeneic stimulation and allogeneic (third party) UC-MSCs. The ratio of the mixture of R:S:UC-MSCs for the MLR was 10:1:1. Color images available online at www.liebertpub.com/tec

In the MLR, CFSE-labeled responder T cells proliferated in response to autologous or allogeneic stimulations. Autologous- or third party-derived UC-MSCs isolated by the improved explant method with Cellamigo efficiently inhibited the proliferation of responder T cells triggered by allogeneic or autologous DCs as the stimulator (Fig. 3C).

Adipocyte and chondrocyte differentiation

To confirm the differentiation ability of the UC-MSCs isolated by the improved explant method with Cellamigo, we induced UC-MSCs at passage 3 into adipocytes and chondrocytes. Using the adipocyte differentiation induction

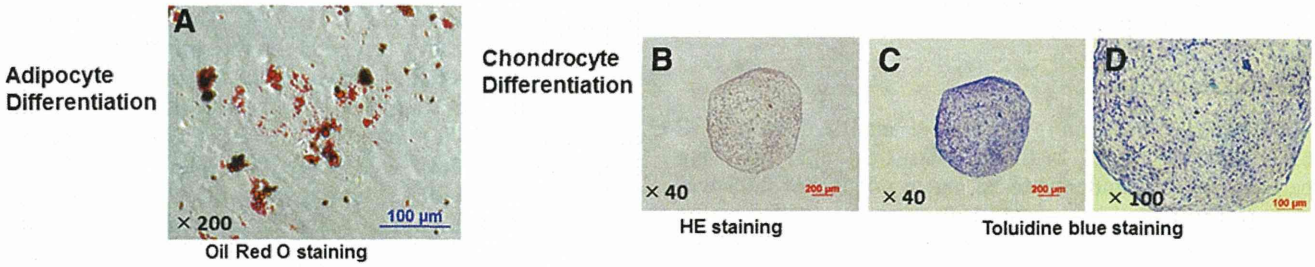

FIG. 4. Adipocyte and chondrocyte differentiation. UC-MSCs isolated by the improved explant method with Cellamigo were differentiated into adipocytes (*left panel*) and chondrocytes (*right panel*). In adipocyte-differentiated cells **(A)**, the accumulation of Oil Red O-stained lipid drops was observed (*red droplets*; magnification, 200×). For chondrogenic differentiation, the pellet culture system was used. **(B, C)** The elastic firm pellet was observed in the differentiation medium after 3 weeks of culture (magnification 40×). Hematoxylin–eosin staining and toluidine blue staining revealed the presence of extracellular matrix with metachromasia in histological sections **(C, D:** magnification of 40× and 100×, respectively). Color images available online at www.liebertpub.com/tec

medium, we observed the accumulation of Oil Red O-stained lipid drops in UC-MSCs (Fig. 4A). The pellet culture system was used to evaluate the differentiation of cells in the chondrogenic differentiation medium. Elastic firm pellets with diameters of 1–3 mm were observed in cells derived from UC-MSCs. HE staining and Toluidine blue staining revealed the presence of an extracellular matrix with metachromasia in histological sections of cells grown in the chondrogenic induction medium (Fig. 4B–D).

Discussion

The human UC is a promising source of MSCs because the UC is considered a medical waste and the collection procedure is a noninvasive process; access to UC-MSCs is not encumbered with ethical challenges. Several protocols and culture methods have been developed for the isolation of MSCs from various compartments of the UC, including the WJ, veins, arteries, subamnion, and perivascular regions.[13] Among the various isolation methods available, two major methods are commonly used to obtain MSCs from the UC: the explant method and the enzyme digestion method. We previously reported that there were no significant differences in the number of MSCs isolated from the UC when using the collagenase and explant methods on day 21,[10] and no differences in MSC phenotypes derived from WJ, veins, or arteries were observed.[9] When using the explant method, it is critical that the UC tissue fragments tightly adhere to the plastic plate to obtain MSCs consistently and efficiently. This is because MSCs can only migrate from the adherent UC tissue fragments and not from floating fragments. In fact, we demonstrated that only the adherent part of the cells in UC tissues showed positive CD105 expression. However, we could not clarify whether the $CD105^+$ cells migrated from the vessels, perivascular region, or intra-WJ. The exact origin of $CD105^+$ cells has not been elucidated, but we clearly demonstrated that the MSCs could be obtained from various compartments of the UC, including the WJ, veins, arteries, UC lining, and subamnion, and perivascular regions.[13,14]

We therefore developed a stainless steel mesh with 1-mm-diameter pinholes (Cellamigo) to enable the UC tissue fragments to adhere to the plastic plate. With this method, we could still observe the migrating cells from the fragments under the microscope through the pinhole windows. Moreover, the use of Cellamigo accelerated the rate of UC-MSC migration, resulting in a significant increase in the number of MSCs isolated with a reduced processing and incubation time.

Cellamigo is made of stainless steel and therefore autoclavable; no evidence of bacterial contamination was found during culture (data not shown). The diameter of the Cellamigo plate was adjusted to match that of the conventional culture dish and different sized plates and pinholes are available. The phenotypic characteristics of the UC-MSCs obtained with and without Cellamigo were similar. As previously reported, UC-MSCs have immunosuppressive ability.[15,16] In this study, we demonstrated that the UC-MSCs obtained with Cellamigo showed immunosuppressive ability in the MLR even when used as an autologous and a third-party donor. The MSCs obtained also showed differentiation ability into both adipocytes and chondrocytes.

Conclusively, our results demonstrate that Cellamigo is useful for explant culture for the isolation of UC-MSCs. In addition, Cellamigo might be applicable for isolating other cell types using the same explant method. The isolation of UC-MSCs with the proposed improved explant method is simple and feasible for clinical use and should facilitate the use of UC-MSCs as an alternative MSC source for immunotherapy and regenerative medicine.

Acknowledgments

This study was supported by the research fund (Cord Blood and Cord-derived MSC banking) of Ministry of Health, Labor, and Welfare of Japan. The authors thank the staff of the NTT Medical Center Hospital, Tokyo for assistance with collection of UC and CB tissues.

Disclosure Statement

Research expenses for the development of Cellamigo were jointly provided by the University of Tokyo, Institute of Medical Science, and the Tsubakimoto Chain Company. Cellamigo has been registered as a brand name by the Tsubakimoto Chain Co. The patent for Cellamigo is pending. Mr. J. Ohshimo is an employee of Tsubakimoto Chain Co. No other competing financial interests exist.

References

1. Gnecchi, M., and Melo, L.G. Bone marrow-derived mesenchymal stem cells: isolation, expansion, characterization, viral transduction, and production of conditioned medium. Methods Mol Biol **482,** 281, 2009.
2. Bieback, K., and Kluter, H. Mesenchymal stromal cells from umbilical cord blood. Curr Stem Cell Res Ther **2,** 310, 2007.
3. In't Anker, P.S., Scherjon, S.A., Kleijburg-van der Keur, C., de Groot-Swings, G.M., Claas, F.H., Fibbe, W.E., and Kanhai, H.H. Isolation of mesenchymal stem cells of fetal or maternal origin from human placenta. Stem Cells **22,** 1338, 2004.
4. Gruber, H.E., Deepe, R., Hoelscher, G.L., Ingram, J.A., Norton, H.J., Scannell, B., Loeffler, B.J., Zinchenko, N., Hanley, E.N., and Tapp, H. Human adipose-derived mesenchymal stem cells: direction to a phenotype sharing similarities with the disc, gene expression profiling, and coculture with human annulus cells. Tissue Eng Part A **16,** 2843, 2010.
5. Tong, C.K., Vellasamy, S., Tan, B.C., Abdullah, M., Vidyadaran, S., Seow, H.F., and Ramasamy, R. Generation of mesenchymal stem cell from human umbilical cord tissue using a combination enzymatic and mechanical disassociation method. Cell Biol Int **35,** 221, 2011.
6. Salehinejad, P., Alitheen, N.B., Ali, A.M., Omar, A.R., Mohit, M., Janzamin, E., Samani, F.S., Torshizi, Z., and Nematollahi-Mahani, S.N. Comparison of different methods for the isolation of mesenchymal stem cells from human umbilical cord Wharton's jelly. In Vitro Cell Dev Biol Anim **48,** 75, 2012.
7. Han, Y.F., Tao, R., Sun, T.J., Chai, J.K., Xu, G., and Liu, J. Optimization of human umbilical cord mesenchymal stem cell isolation and culture methods. Cytotechnology **65,** 819, 2013.
8. Margossian, T., Reppel, L., Makdissy, N., Stoltz, J.F., Bensoussan, D., and Huselstein, C. Mesenchymal stem cells derived from Wharton's jelly: comparative phenotype analysis between tissue and *in vitro* expansion. Biomed Mater Eng **22,** 243, 2012.
9. Ishige, I., Nagamura-Inoue, T., Honda, M.J., Harnprasopwat, R., Kido, M., Sugimoto, M., Nakauchi, H., and

Tojo, A. Comparison of mesenchymal stem cells derived from arterial, venous, and Wharton's jelly explants of human umbilical cord. Int J Hematol **90,** 261, 2009.
10. He, H., Nagamura-Inoue, T., Tsunoda, H., Yuzawa, M., Yamamoto, Y., Yorozu, P., Agata, H., and Tojo, A. Stage-specific embryonic antigen 4 in Wharton's jelly-derived mesenchymal stem cells is not a marker for proliferation and multipotency. Tissue Eng Part A **20,** 1314, 2014.
11. Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D., and Horwitz, E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy **8,** 315, 2006.
12. Horwitz, E.M., Le Blanc, K., Dominici, M., Mueller, I., Slaper-Cortenbach, I., Marini, F.C., Deans, R.J., Krause, D.S., Keating, A., and International Society for Cellular, T. Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. Cytotherapy **7,** 393, 2005.
13. Nagamura-Inoue, T., and He, H. Umbilical cord-derived mesenchymal stem cells: their advantages and potential clinical utility. World J Stem Cells **6,** 195, 2014.
14. Sarugaser, R., Lickorish, D., Baksh, D., Hosseini, M.M., and Davies, J.E. Human umbilical cord perivascular (HUCPV) cells: a source of mesenchymal progenitors. Stem Cells **23,** 220, 2005.
15. Prasanna, S.J., Gopalakrishnan, D., Shankar, S.R., and Vasandan, A.B. Pro-inflammatory cytokines, IFNgamma and TNFalpha, influence immune properties of human bone marrow and Wharton jelly mesenchymal stem cells differentially. PLoS One **5,** e9016, 2010.
16. Santos, J.M., Barcia, R.N., Simoes, S.I., Gaspar, M.M., Calado, S., Agua-Doce, A., Almeida, S.C., Almeida, J., Filipe, M., Teixeira, M., Martins, J.P., Graca, L., Cruz, M.E., Cruz, P., and Cruz, H. The role of human umbilical cord tissue-derived mesenchymal stromal cells (UCX(R)) in the treatment of inflammatory arthritis. J Transl Med **11,** 18, 2013.

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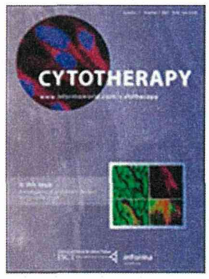
Received: June 30, 2014
Accepted: September 3, 2014
Online Publication Date: January 6, 2015



Serum- and Xeno-free Cryopreservation of Human Umbilical Cord Tissue as Mesenchymal Stromal Cell Source

Journal:	*Cytotherapy*
Manuscript ID:	CYTH-2014-0240.R2
Article Type:	Original Paper
Date Submitted by the Author:	n/a
Complete List of Authors:	Shimazu, Takahisa; Research hospital, The Institute of Medical Science, The University of Tokyo, Dep. of Cell Processing and Transfusion Mori, Yuka; Research hospital, The Institute of Medical Science, The University of Tokyo, Dep. of Cell Processing and Transfusion Takahashi, Atsuko; Research hospital, The Institute of Medical Science, The University of Tokyo, Dep. of Cell Processing and Transfusion Tsunoda, Hajime; NTT Medical Center Tokyo Hospital, Department of Obstetrics Tojo, Arinobu; Research hospital, The Institute of Medical Science, The University of Tokyo, Dep. of Cell Processing and Transfusion & Tokyo Cord Blood Bank Nagamura-Inoue, Tokiko; Research hospital, The Institute of Medical Science, The University of Tokyo, Dep. of Cell Processing and Transfusion
Keywords:	Cryopreservation, umbilical cord, mesenchymal stromal cell, immunosuppression, cryoprotectant

Manuscript ID: CYTH-2014-0240. Re-Revise version

Serum- and Xeno-free Cryopreservation of Human Umbilical Cord Tissue as Mesenchymal Stromal Cell Source

Running Title: Cryopreservation of human umbilical cord tissue

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ABSTRACT (249/250WORDS)

Background: Human umbilical cord (UC) has become a notable source for mesenchymal stromal cells (MSCs) that can migrate to areas of inflammation and damaged tissue, and can suppress excess immune reactions and to repair, respectively. Although UC is a solid tissue, there are several advantages including repeatable uses from the same donor sample when needed, and the possibility of future explorations for cells with unknown potential, if we could cryopreserve the UC as a living tissue material. However, since the cryoprotectants in the previous reports included animal- or allogeneic human-derived serum, or no serum, the frozen-thawed UC-MSCs were inferior to fresh UC-MSCs in cell proliferation. The object of this study was to find a suitable cryopreservation method of UC for clinical use.

Methods: The UC was cut in cross-section and incised longitudinally, immersed in the cryoprotectant and frozen slowly. Later, it was thawed and minced rapidly, and the fragments of UC were cultured by improved explant method.

Results: The highest yield of cells was obtained from frozen-thawed UC with serum- and xeno-free cryoprotectant, STEM-CELLBANKER®, when compared with others. The cells derived from frozen-thawed UC stored in STEM-CELLBANKER® expressed the phenotypes of MSCs, retained the immunosuppressive properties in allogeneic mixed lymphocyte

reactions and the differentiation potentials (into adipocyte and chondrocytes) comparable to those derived from fresh UC.

Discussion: UC can be cryopreserved in serum- and xeno-free cryoprotectant as a living tissue while keeping its growth and functions equivalent to fresh UC. Our method is simple and feasible for clinical use.

Key words: Cryopreservation; umbilical cord; mesenchymal stromal cell; immunosuppression

Highlights:

- Human umbilical cord (UC) is a rich source for mesenchymal stromal cells (MSCs).
- Umbilical cord tissue can be cryopreserved for subsequent use.
- Storage in STEM-CELLBANKER ® yields highest number of MSCs.
- Frozen UC-derived MSCs are similar to freshly derived MSCs in growth and functions.

Abbreviations:

UC, umbilical cord

MSCs, mesenchymal stromal cell

BM, bone marrow

CB, cord blood

DMSO, dimethyl sulfoxide

Xeno-free, xenogeneic animal origin reagents-free

CFSE, carboxyfluorescein diacetate, succinimidyl ester

DC, dendritic cell

GVHD, graft versus host disease