Isolation and Characterization of Trophoblast Stem Cells-like Cells Derived from Human Term Placenta

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ABSTRACT : The trophectoderm is one of the earliest cell types to differentiate in the forming placenta. It is an important for the initial implantation and placentation during pregnancy. Trophoblast stem cells (TBSCs) develop from the blastocyst and are maintained by signals emanating from the inner cell mass. However, several limitations including rarity and difficulty in isolation of trophoblast stem cells derived from blastocyst still exist. To establish a model for trophoblast differentiation, we isolated TBSCs from human term placenta (≥38 weeks) and characterized. Cell cycle was analyzed by measuring DNA content by FACS analysis and phenotype of TBSCs was characterized by RT-PCR and FACS analysis. TBSCs have expressed various markers such as self-renewal markers (Nanog, Sox2), three germ layer markers (hNF68, alpha-cardiac actin, hAFP), trophoblast specific markers (CDX-2, CK7, HLA-G), and TERT gene. In FACS analysis, TBSCs isolated from term placenta showed that the majority of cells expressed CD13, CD44, CD90, CD95, CD105, HLA-ABC, cytokeratin 7, and HLA-G. Testing for CD31, CD34, CD45, CD71, vimentin and HLA-DR were negative. TBSCs were shown to decrease the growth rate when cultured in conditioned medium without FGF4/heparin as well as the morphology was changed to a characteristic giant cell with a large cytoplasm and nucleus. In invasion assay, TBSCs isolated from term placenta showed invasion activities in *in vivo* using nude mice and *in vitro* Matrigel system. Taken together, these results support that an isolation potential of TBSCs from term placenta as well as a good source for understanding of the infertility mechanism.

Key words : Trophoblast stem cells, Placenta, Trophoblast, Invasion.

INTRODUCTION

At early human implantation, the close networking between embryonic trophectoderm and apical luminal epithelium of maternal myometrium leads to the successful pregnancy (Moore & Persaud, 2008; Horne et al., 2000). Differentiation of trophectoderm-derived syncytiotrophoblasts, which is invasion into the underlying stroma and endometrial vessels, make dynamic progress during implantation (Benirshke & Kaufmann, 2000). These differentiation events of trophoblasts through various microenvironment factors including maternal blood circulation and fibrin or other extracellular matrixes on the endometrial stroma of maternal myometrium make an alteration to a specialized trophoblast from primitive trophectoderm such as trophoblastic stem cells (Douglas et al., 2009). Differentiated trophoblasts are to mediate the process of implantation and invasion of the conceptus into the uterus, otherwise, insufficiency invasion of trophoblasts, consequently failure of differentiation of trophoblasts cause developing for high-risk pregnancy resulting in recurrent miscarriage, fetal retardation, intrauterine growth retardation (IUGR), preeclampsia and so on (Craven et al., 1998; Lyall, 2005; Blomberg et al., 2008). However, understanding on the mechanism for trophoblast differentiation or the function of trophoblast in embryo implantation still limits because these events happen to under the early pregnancy. Although a few trophoblast cell lines have been generated from first

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or second trimester placenta, choriocarcinoma which is a malignant neoplasm composed exclusively of trophoblasts, or transformed using various gene transductions as an *in vitro* model are significantly lacking for conducting experiments of trophoblasts differentiation. (King et al., 2000).

In accordance with the progression of the technology of stem cells, many researchers have been reported the feasibility of conducting experiments with trophoblasts differentiation using embryonic stem cells. (Xu et al., 2002; Gerami-Naini et al., 2004; Peiffer et al., 2007; Schenke- Layland et al., 2007; Golos et al., 2006). However, it is difficult to regulate the differentiation of trophoblast derived from embryonic stem cells because the mechanism of differentiation of embryonic stem cells has not yet been explored. Also, the low efficiency of trophoblast-like cells derived from embryonic stem cells may be due to the origin of embryonic stem cells from the inner cell mass of blastocysts excluded trophectoderm (Schulz et al., 2008). So, human trophoblast stem cells as renewable and convenient sources derived from term placenta has been required to serve *in vitro* model system for understanding the mechanisms of implantation and placentation. In the present study, we isolated and characterized trophoblast stem cells derived from human term placenta.

MATERIALS AND METHODS

1. Placenta Collections

Pregnancies were considered normal when there was no evidence of medical, obstetrical or surgical complications and they resulted in a term delivery (≥37 gestational weeks). All women provided written, informed consent prior to the collection of samples. The collection of samples and their utilization for research purposes were approved by the Institutional Review Boards of CHA General Hospital, Seoul, Korea.

2. Isolation and Cultivation of Trophoblast Stem Cells Derived from Human Term Placenta

Trophoblast stem cells (TBSCs) from human chorionic villi using the Percoll gradient method, from placentas collected after neonatal delivery. Briefly, chorionic villi were dissected, rinsed, and minced for cell isolation. Minced villi were incubated in HBSS containing 0.125% trypsin (GibCO, New York, NY, USA), 25 mN HEPES, 50 U DNase I (Sigma, St. Louis, MO, USA) for 35 minutes with agitation. After tissue sedimentation, the supernatant was taken and filtered by cheeth gauze. Cell suspensions were carefully layered over a discontinuous Percoll Gradient (70% to 5%, in 10% steps) and centrifuged at 2,000 rpm for 20 minutes. The middle layer (30-45%) was removed and washed with DMEM. Cells were diluted to 1×10^6 cells/㎖ with basal medium (DMEM supplemented with 2 mM glutamine, 10% FBS, 25 mM HEPES, 100 UI/㎖ penicillin, and 100 ㎍/㎖ sterptomycine) supplemented with FGF-4 (25 ng/㎖) and heparin (1 ㎍/㎖, Sigma-Aldrich), then plated in T25 flask and incubated. The culture media were changed every day.

3. Karyotype Analysis

The chromosome analysis was performed according to standard methods with minor modifications. Three days after seeding, trophoblast stem cell-like cells were incubated with 100 ㎕ colcemid (Gibco) for 3 hours at 37℃ in a CO_2 incubator and then trypsinized. After hypotonic solution treatment (1% citrate buffer), lysed cells were fixed in methanol: glacial acetic acid (3:1). G-banding was performed for identification of chromosomes.

4. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Cells were homogenized and lysed in 1 ㎖ of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was extracted with 200 ㎕ of chloroform and precipitated with 500 ㎕ of 80% (v/v) isopropanol. A reverse transcription reaction was performed with 1 ㎍ purified total RNA using Superscript Ⅲ reverse transcriptase (Invitrogen). Synthesized cDNA was amplified by PCR. Primers used

Table 1. Sequence of primers used for RT-PCR and length of fragments

Genes	Sequences	Tm (˚C)	Size (bp)
Oct4	F: 5'-ACA CTC GGA CCA CGT CTT TC-3' R: 5'-CGT TCT CTT TGG AAA GGT GTT C-3'	54	300
Nanog	F: 5'-TTC TTG ACT GGG ACC TTG TC-3' R: 5'-GCT TGC CTT GCT TTG AAG CA-3'	54	300
Sox2	F: 5'-GGG CAG CGT GTA CTT ATC CT-3' R: 5'-AGA ACC CCA AGA TGC ACA AC-3'	52	200
NF-68	F: 5'-GAG TGA AAT GGC ACG ATA CCT A-3' R: 5'- TTT CCT CTC CTT CTT CTT CAC CTT C-3'	58	500
Cardiac	F: 5'-GGA GTT ATG GTG GGT ATG GGT C-3' R: 5'-AGT GGT GAC AAA GGA GTA GCC A-3'	58	500
AFP	F: 5'-AGC TTG GTG GAT GAA AC-3' R: 5'-TCC AAC AGG CCT GAG AAA TC-3'	50	200
CDX-2	F: 5'-GCA GTC CCT AGG AAG CCA AGT GA-3' R: 5'-CTC TCG GAG AGC CCA AGT GTG-3'	58	300
CK7	F: 5'-ACA GAG CTG CAG TCC CAG AT-3' R: 5'-GTA GGT GGC GAT CTC GAT GT-3'	57	500
HLA-G	F: 5'-GCG GCT ACT ACA ACC AGA GC-3' R: 5'-GCA CAT GGC ACG TGT ATC TC-3'	58	900
TERT	F: 5'-GAG CTG ACG TGG AAG ATG AG-3' R: 5'-CTT CAA GTG CTG TCT GAT TCC AAT G-3'	55	300
β-Actin	F: 5'-TCC TTC TGC ATC CTG TCA GCA-3' R: 5'-CAG GAG ATG GCC ACT GCC GCA-3'	58	300

for RT-PCR analysis are presented in Table 1. The amplification conditions followed several steps; 5 minutes at 95 ℃, followed by 24-31 cycles, at 94℃ for 30 seconds, 48-60℃ for 1 minute, and at 72℃ for 1 minute. PCR products were visualized by electrophoresis on a 1.2% (w/v) agarose gel (Cambrex, Rockland, ME, USA) containing 0.5 ㎍/㎖ ethidium bromide (Promega, Madison, WI, USA), and were visualized using a video image analyzer (Bio-Rad; Hercules, CA, USA).

5. Flow Cytometry

Cells were dissociated with cell dissociation buffer (Gibco) and washed with PBS containing 2% (v/v) FBS. Cells (1×10^6/㎖) were fixed in 70% ethanol for 2 hours at 4℃ and resuspended in 1 ㎖ of freshly prepared propidium iodide/RNase solution for cell cycle analysis. Also, isolated cells (1×10^5/㎖) were incubated with isotype control or antigen-specific antibodies for 30 minutes. And then, the cells were incubated with various fluorescence-conjugated goat anti-mouse IgG1 (Vector Laboratories, Burlingame, CA, USA) for 15 minutes. After washing, propidium iodide staining (5 ng/㎖, Sigma-Aldrich) was used to identify nonviable cells. Cell cycle distribution and surface marker expression were performed using a FACS vantage Flow Cytometer (BD Bioscience, San Jose, CA, USA). Based on the intensity of the propidium iodide fluorescence, the flow cytometry program will separate resting cells with one copy of each chromosome (G0/G1), cells that have replicated and contain double DNA content and thus double intensity of fluorescence (G2/M) and cells in S phase.

6. Differentiation Assay

TBS cells was cultured with the basal medium supple-

mented with FGF-4 (25 ng/㎖) and heparin (1 ㎍/㎖, Sigma-Aldrich) for suppression the spontaneous TBSCs differentiation in cultures on plastic ware. To induce differentiation, 5×10^4 TBS cells per well were seeded and cultured in basal medium without FGF-4 and heparin for 2 weeks. The medium was changed daily.

7. *In vitro* and *in vivo* Invasion Assays

To induce invasiveness of TSCs on matrigel, 5×10^4 cells were then plated on Matrigel (Becton Dickinson, MA), incubated at 37℃ for 4 days, and analyzed using a phase contrast microscope at 100× (Nikon, Eclipse TE 2000-U, Japan). For *in vivo* invasion assay, SCID nude mice (6 weeks old) were maintained in an air-conditioned animal house with specific pathogen-free conditions, and were subjected to a 12:12-h daylight/darkness and allowed unlimited access to chow and water. The morphological and behavioral changes of mice were monitored every day. Trophoblast stem cells were given at a dose of 1×10^6 cells per mice. In order to trace of transplanted trophoblast stem cells in SCID nude mice, trophoblast stem cells were stained using the PKH-26 Fluorescent Cell Linker kit (Sigma-Aldrich). PKH-26 stained trophoblast stem cells were directly transplanted by hypodermic subcutaneous injection into the mice. After the transplantation, PKH-26 stained trophoblast stem cells were traced using IVIS Imaging System 200 (Xenogen Corporation, California, USA) and the skins of mice were collected after 4 weeks. The samples were determined by histopathological examination. We conducted all animal experiments using approved protocols and according to the National Institutes of Health guidelines.

8. Immunofluoresecence

Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes. After treatment with 1% normal goat serum for 30 minutes at room temperature, the cells were incubated with a mouse anti-human cytokeratin 7 primary antibody (DAKO Cytomation, Denmark) for 24 hours at 4℃. Cells were washed and then incubated with a Rhodamine- conjugated or FITC-conjugated (Molecular Probes Inc., Eugene, OR, USA) secondary antibody for 1 hour. After being washed with PBS, the stained slides were mounted with a glycerol-based mounting solution containing DAPI (Sigma-Aldrich).

Mice were sacrificed and skin tissues were collected on 4 weeks after the transplantation. For immunofluorescent staining to trace human trophoblast stem cells, the skin samples were embedded in O.C.T. compound (TISSUE-TEK® 4583, Sakura Finetek USA Inc., Torrance, CA, USA), frozen, and cut into 8 ㎛-thick sections at −20℃. The slides were stained with anti-cytokeratin 7 antibody. The positive signals for cytokeratin 7 were visualized with FITC-conjugated anti-mouse IgG secondary antibodies (Molecular Probes Inc). All images were acquired using by fluorescence microscope (Carl Zeiss Inc, Germany).

RESULTS

1. Isolation and Cultivation of Trophoblast Stem Cell-like Cells from Normal Term Placenta

Morphology of trophoblast stem cell-like cells derived from placenta was similar to epithelioid cells, called trophoblast (Fig. 1A). The cell cycle of trophoblast stem cell-like cells show to decreased G1 phase and increased S/G2 phase indicating elevated proliferation activity (Fig. 1B). A specific marker of trophoblast, cytokeratin-7, was detected in the isolated trophoblast stem cell-like cells (Fig. 1C). Their karyotype was normal as a XY, 46 (Fig. 1D). A specific marker of embryonic stem cells (Oct-4) was not detected in the trophoblast stem cell-like cells. However, three stem cell markers (Nanog, Sox2, and TERT), three germ layer markers [NF68, cardiac muscle, and α-fetoprotein (AFP)], and an immunomodulator gene (HLA-G) were all expressed in the trophoblast stem cell-like cells. These results suggest that the trophoblast stem cell-like cells isolated from term placenta have the potential

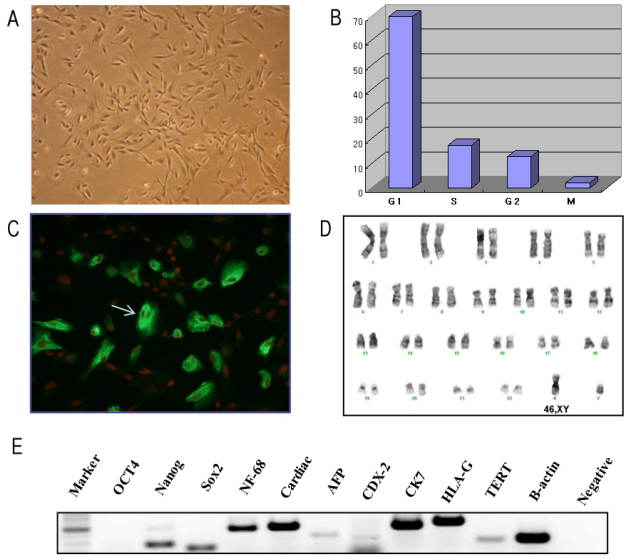

Fig. 1. Characterization of trophoblast stem cell-like cells derived from term placenta. Trophoblast stem cell-like cells were assessed at passage numbers 3 through 5. (A) The morphology of trophoblast stem cell-like cells was similar to the epithelioid cells (×100). (B) Cell cycle using FACS analysis. (C) Expression of cytokeratin 7 (CK7) by immunofluoresecence. Arrow means CK7 positive cells. Nucleus: PI. (×400). (D) Chromosome analysis. (E) RT-PCR analysis for stem cell markers in trophoblast stem cell-like cells.

for self-renewal and the capacity to differentiate into multiple lineages.

2. Analysis for Surface Phenotypes of Trophoblast Stem Cell-like Cells using FACS Analysis

To confirm the surface phenotypes of the trophoblast stem cell-like cells, we performed FACS analysis using various mouse anti-human antibodies (Fig. 2). The phenotypes of the trophoblast stem cell-like cells were negative for hematopoietic markers such as CD45, CD71, and HLA-DR; otherwise, positive for non-hematopoietic markers, including CD13, CD44, CD90, CD95, CD105, and HLA-ABC. Furthermore, the surface expressions of cytokeratin 7 and HLA-G, which are trophoblast markers, were detected in the trophoblast stem cell-like cells isolated from term placenta.

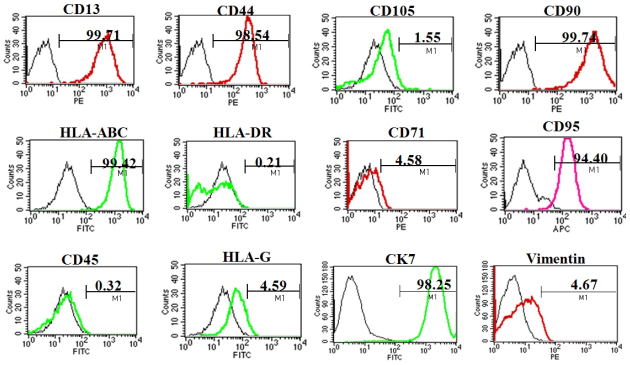

Fig. 2. FACS analysis of the expression of surface markers in trophoblast stem cell-like cells derived from term placenta. The percentages are indicated along with the fluorescence intensities.

3. Differentiation of Trophoblast Stem Cell-like Cells

To test the potential of the trophoblast stem cell-like cells to trophectoderm lineages, we induced trophoblastic differentiation of the trophoblast stem cell-like cells. As shown in Fig. 3, the trophoblast stem cell-like cells were

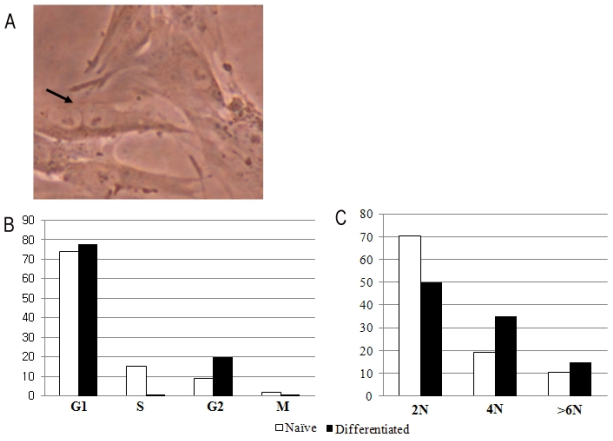

Fig. 3. Differentiation of trophoblast stem cell-like cells derived from term placenta. (A) The morphological changes of trophoblast stem cell-like cells derived from term placenta after differentiation. Arrow means differentiated trophoblast cells by cell-cell fusion (×400). (B) Cell cycle analysis of trophoblast stem cell-like cells depends on differentiation. (C) DNA content analysis of trophoblast stem cell-like cells in between naïve and differentiation. White bar and block mean undifferentiated and differentiated trophoblast stem cell-like cells, respectively.

changed in cell morphology and DNA content. The morphology of trophoblast stem cell-like cells shows indicative of cells that are changing to giant cells with expansive cytoplasm and large polyploidy nuclei (Fig. 3A). Also, we analyzed to measure cell cycle and ploidy in differentiated trophoblast stem cell-like cells using FACS. The cell cycle was to decreased S/G2 phase indicated proliferative activity (Fig. 3B) and the high diploid DNA content was increased in differentiated trophoblast stem cell-like cells (Fig. 3C). These results suggest that differentiated trophoblast stem cell-like cells have aneuploid DNA content via cell to cell fusion.

4. Invasion Activities of Trophoblast Stem Cell-like Cells using *in vitro* and *in vivo* Systems

To confirm the invasion activity of the trophoblast stem cell-like cells, we performed *in vitro* invasion assay using materigel system and *in vivo* invasion assay using SCID mice. PKH-26 stained trophoblast stem cell-like cells engrafted into the skin of SCID mice were detected by Xenogen and localized into hypodermic subcutaneous of the mice (Fig. 4A, B). Also, the trophoblast stem cell-like cells engrafted into the skin of SCID mice were expressed cytokeratin 7 (Fig. 4C). In addition, invasion activity of the trophoblast stem cell-like cells cultured on Matrigel more increased than HTR-8 SV/neo trophoblast cell lines (Fig. 4D).

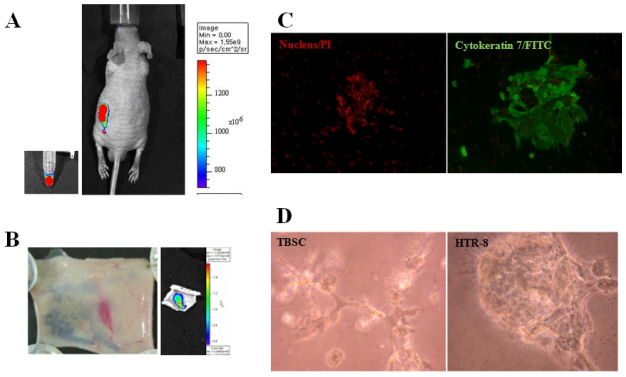

Fig. 4. Invasion assays of trophoblast stem cell-like cells into SCID mice and on Matrigel. (A) PKH-labeled trophoblast stem cell-like cells engrafted into SCID mice. (B) Engrafted trophoblast stem cell-like cells into the hypodermic subcutaneous of the mice. (C) Analysis of trophoblast stem cell-like cells engrafted into subcutaneous of the mice tissues using immunohistochemistry of human-specific cytokeratin 7. PI was used as a nuclear stain. (×100). (D) *In vitro* invasion assay of trophoblast stem cell-like cells on Materigel. HTR-8/SVneo trophoblast cells used as a control (×40).

DISCUSSION

In the present study, we isolated and characterized trophoblast stem cell-like cells derived from normal term placenta. Generally, trophectoderm is specialized from outer part of blastomeres and various trophoblasts including extravillous cytotrophoblast and syncytiotrophoblast differentiated from trophectoderm component of term placenta (Benirshke & Kaufmann, 2000). Trophoblast cells proliferate and differentiate along two lineages for villous and extravillous trophoblast. Villous cytotrophoblast cells form the outer epithelial layer of the chorionic villi as well as multinucleated syncytiotrophoblasts via cell fusion. Their major function is to facilitate the exchange of fetal and maternal metabolites including hormones, nutrients, wastes, and gas. Otherwise, extravillous trophoblast cells, which have a potential for invasion, migrate into the decidua and the basal matrix on the maternal endometrium (Knofler et al., 2008). These cells finally induce remodeling of uterine arteries in the endometrium. Several substances are metabolized and these metabolic products are released into maternal and fetal circulations. (Gude et al., 2004).

During the trophoblast differentiation, this outer layer is divided into two different cell types such as cytotrophoblast and syncytiotrophoblast when they begin differentiation. The cytotrophoblast as a single nucleate cell is located in the inner layer of the trophectoderm and exerts a strong proliferative activity thereby classifying it as a trophoblast stem cell (Oda et al., 2006; Rossant, 2007; Douglas et al., 2009). These cells also migrate into the syncytiotrophoblast to increase the population of finally

differentiated syncytiotrophoblasts through fusion and losing of their cell membrane. Otherwise, the syncytiotrophoblast known as syncytium is a mutilnucleate cell, which covers the surface of the placental villi and forms as a result of the differentiated trophoblast via multiple process including fusion of the between underlying cytotrophoblasts during placental developmnet. The syncytiotrophoblast grows into the endometrial stroma and plays a role as a barrier function of the placenta as well as produces several hormones. The syncytiotrophoblast secretes human chorionic gonadotrophin (hCG) to maintain the endocrine activity of the corpus luteum, which secretes estrogen and progesterone to sustain the pregnancy (Schumacher et al., 2009). Therefore, the balance between proliferation and differentiation of trophoblasts is physiologically important for the processes of trophoblast differentiation and placental development.

Although the studies for trophoblasts function according to classification have reported, there are limitations for trophoblast differentiation because primary cytotrophoblast or syncytiotrophoblast derived from placenta already committed to trophoblast so that early lineage decisions cannot be addressed. Especially, primary trophoblast cells derived from first trimester placentas represent the unique characteristics (e.g. cell fusion, invasion) of trophoblast cells, however, they are not widely available for study because of their spontaneously differentiation into extravillous trophoblast (Aplin et al., 2006). Therefore, new system for the study of trophoblast differentiation was required. Although Schulz et al have shown that human embryonic stem cells (hESCs) can use as a model for trophoblast differentiation, however, the efficiency for establishment and maintenance of trophoblast derived from hESCs still low and they have obstacles for the study of the mechanism of trophoblast differentiation (Schulz et al., 2008).

However, trophoblast stem cell-like cells isolated from term placenta have advantages for the study of trophoblast differentiation comparing to traditional systems due to the potentials as a stem cells. Spitalieri et al reported that cytotrophoblast cells derived from first trimester chorionic villi have a multipotent (Spitalieri et al., 2009). But, this approach required aggressive strategies for collecting chorionic villi at first trimester. Otherwise, we can effectively isolate trophoblast stem cell-like cells from term placenta discarded after delivery. They have a self-renewal capacity as well as stemness in the medium with FGF-4 and heparin used in the present study. After differentiation using the condition medium without FGF-4 and heparin, the high diploid of trophoblast and the invasion activity of trophoblast were increased. It means that trophoblast stem cell-like cells isolated from term placenta can differentiate into functional trophoblast (e.g. syncytiotrophoblast).

In conclusion, these results suggested that trophoblast stem cell-like cells isolated from term placenta could serve as models for understanding differentiation mechanism of trophoblast as well as a good source for understanding of the infertility mechanism.

ACKNOWLEDGMENTS

We special thank Dr. Charles H. Graham (Queen's University, Canada) for HTR-8/SVneo cell lines. This work was supported by the Korea Healthcare Technology R&D Project, Ministry for Health Welfare & Family Affairs, Republic of Korea (A084923).

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(received 5 July 2010, received in revised from 2 August 2010, accepted 3 August 2010)