

Characterization of Amniotic Stem Cells

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Abstract

The amnion membrane is developed from embryo-derived cells, and amniotic cells have been shown to exhibit multidifferentiation potential. These cells represent a desirable source for stem cells for a variety of reasons. However, to date very few molecular analyses of amnion-derived cells have been reported, and efficient markers for isolating the stem cells remain unclear. This paper assesses the characterization of amnion-derived cells as stem cells by examining stemness marker expressions for amnion-derived epithelial cells and mesenchymal cells by flow cytometry, immunocytochemistry, and quantitative PCR. Flow cytometry revealed that amnion epithelial cells expressed CD133, CD 271, and TRA-1-60, whereas mesenchymal cells expressed CD44, CD73, CD90, and CD105. Immunohistochemistry showed that both cells expressed the stemness markers Oct3/4, Sox2, Klf4, and SSEA4. Stemness genes' expression in amnion epithelial cells, mesenchymal cells, fibroblast, bone marrow-derived mesenchymal stem cells (MSCs), and induced pluripotent stem cells (iPSCs) was compared by quantitative reverse-transcription polymerase chain reaction (RT-PCR). Amnion-derived epithelial cells and mesenchymal cells expressed *Oct3/4*, *Nanog*, and *Klf4* more than bone marrow-derived MSCs. The sorted TRA1-60-positive cells expressed *Oct3/4*, *Nanog*, and *Klf4* more than unsorted cells or TRA1-60-negative cells. TRA1-60 can be a marker for isolating amnion epithelial stem cells.

Introduction

THE AMNION IS A FETAL origin tissue and is composed of a single layer of epithelial cells on a thicker basement membrane and spongy collagen layer containing mesenchymal cells that are derived from the inner cell mass (ICM) in the blastocyst. It has been reported that embryonic stem cells (ESCs) derived from blastocysts have normal karyotypes, express high levels of telomerase activity, express all embryonic stem cell markers, and can develop to all three germ layers (Thomson et al., 1998). Amnion membrane-derived cells are also reported to be multipotent cells that can replicate as undifferentiated cells as they express stem cell genes, such as *Oct3/4*, *Sox2*, and *c-Myc* and that have the potential to differentiate into various tissue (Bilic et al., 2008; Diaz-Prado et al., 2010; Izumi-Yoneda et al., 2009; Murphy et al., 2010; Nagura et al., 2013; Nogami et al., 2012; Otaka et al., 2013; Takashima et al., 2004; Toda et al., 2007; Tsuno et al., 2012; Wei et al., 2003, 2009; Zhao 2005). In addition, they do not express human leukocyte antigen (HLA) class II and

secrete HLA-G and CD59, which are immunologic suppression factors (Adinolfi et al., 1982; Akle et al., 1981; Kamiya et al., 2005; Wolbank et al., 2007). It has also been shown that the conditioned medium of amnion-derived cells have immunosuppressive activity (Cargnoni et al., 2014). Moreover, they do not attract ethical concern because they are usually discarded after parturition. Thus, amnion-derived cells are anticipated to be a valuable cell source for cell therapy (Corgnoni et al., 2009; De Coppi et al., 2007; Hu et al., 2009; Murphy et al. 2010; Parolini et al., 2009, 2010).

However, few molecular biological analyses have been performed to characterize amnion-derived cells. Here we report a comparison analysis of human amnion-derived epithelial (HAE) cells and human amnion-derived mesenchymal (HAM) cells. Although amnion-derived cells have stem cell characteristics and differentiation potency for several cell types, they are a heterogeneous cell population that includes stem cells, progenitors of certain cells, and differentiated cells. It has been shown that they have multidifferentiation potential, but their differentiation efficiency is low. If the

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stem cells are isolated from the heterogeneous population, the differentiation efficiency may increase and those cells could represent a better cell source for cell therapy. TRA1-60 is known to be one of the markers of ESCs (Thomson et al., 1998). Also, it is known that some amnion cells express TRA1-60. Thus, the isolation of stem cells from the heterogeneous population using TRA1-60 as a marker was attempted. The analysis of the isolated cells showed a higher expression of stemness genes relative to unsorted cells.

Materials and methods

Cell isolation

The amniotic membrane was mechanically peeled from the chorion of a placenta obtained, with informed consent, after an uncomplicated cesarean section. The study and the use of the amnion membrane were approved by the Research Ethics Committee of the University of Toyama as described previously (Wei et al., 2003). The tissue was minced and treated with trypsin (2 mg/mL) at 37°C for 20 min to isolate HAE cells. After repeating this treatment several times, the epithelial cells were completely removed. The tissue pieces were placed in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing collagenase (0.75 mg/mL) and DNase (0.075 mg/mL) and were incubated at 37°C for 60 min to isolate HAM cells. The dispersed HAE or HAM cells were collected by filtration of the mixture through gauze and centrifugation.

Flow cytometric analysis and cell sorting

Cells were blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich) in phosphate-buffered saline (PBS) for 30 min at room temperature and stained with antibodies at a concentration of 20 μ L/1 \times 10⁶ cells at room temperature for 1 h. Antibodies against CD14, CD29, CD34, CD45, CD49f, CD105, HL-DR (Beckman Coulter, Brea, CA, USA), CD24, CD44, CD73, TRA1-60, TRA1-81, SSEA3, SSEA4 (BD Pharmingen, Franklin Lakes, NJ, USA), CD90 (Immune tech, Cedex, France), CD133, or CD271 (Miltenyi Biotec, Bergisch Gladbach, Germany) were used. Flow cytometry was performed on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) using Cell Quest software, and data were analyzed using WinMDI ver 2.9.

MACS separation (Miltenyi Biotec) was used for cell sorting according to the manufacturer's protocols. Anti-TRA1-60-FITC at a concentration of 20 μ L/1 \times 10⁶ cells was used for selection.

Immunocytochemistry

Cells were seeded in a 24-well plate at a density of 1 \times 10⁵ cells/well and cultured for 7 days. Cells were fixed with 4% paraformaldehyde and treated with 0.3% Triton-100. After washing with 0.05 M PBS, cells were stained with primary antibodies against vimentin (1:200), Oct 3/4 (1:200), c-Myc (1:200), Sox-2 (1:50), Klf4 (1:200), Nanog (1:200), SSEA-3 (1:200), SSEA-4 (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), TRA1-60 (1:200), TRA1-81 (1:200), and pan-CK (1:200) (Millipore, CA, USA) overnight at 4°C. Cells were incubated with Alexa Fluor 488 anti-mouse immunoglobulin M (IgM), IgG, rabbit IgG, or rat IgM (Life Technologies, CA, USA) (1:1000 in PBS) for 30 min at room

temperature. Then, cells were counterstained with Hoechst 33342 solution (Wako, Okasa, Japan) (1:1000 in PBS) and examined with a fluorescence microscope (Zeiss, Oberkochen, Germany) with Axiovision LE.

Reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cells using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Aliquots of 1 μ g of total RNA were treated with 0.1 U/ μ L deoxyribonuclease I (DNase I, Sigma-Aldrich, Inc.) at room temperature for 15 min. cDNAs were synthesized using 0.5 μ g of DNase I-treated RNA by using a Rever Tra Ace qPCR RT Kit (Toyobo Co., Ltd., Osaka, Japan). The resulting cDNA was subjected to quantitative PCR using Brilliant II Fast QPCR Master Mix (Agilent Technology, Santa Clara, CA, USA) on MxP3000 (Agilent) with primers for *Oct3/4*, *c-Myc*, *Sox2*, *Klf4*, *Nanog*, *Lin28* (Nagura et al., 2013), and β 2-microglobin (*B2M*) (Takara Bio, Tokyo, Japan) or primers for matrix metalloproteinase 1 (*MMP1*), leukemia inhibitory factor (*LIF*), *MGP*, or apolipoprotein D (*APOD*) (Applied Biosystems, Carlsbad, CA, USA) (Igarashi et al. 2007; Ishii et al., 2005). Primers of *B2M* were as follows: forward, 5'-cgggcattctgaagctga-3'; reverse, 5'-ggatggtgaaaccagacacatag-3'. The levels of a given mRNA were normalized to the internal control gene *B2M* mRNA level.

Statistical analysis

All values are expressed as mean \pm standard error. Comparison parameters for more than three groups were made by Student's *t*-test using statistical software (SPSS Statistics ver. 20 for Mac; IBM, Tokyo, Japan).

Results

The characteristics of HAE and HAM cells were analyzed. Morphologically, cultured HAE cells are small and round in shape, and cultured HAM cells are spindle-shaped (Fig. 1). To confirm that we had obtained epithelial cells and mesenchymal cells, HAE and HAM cells were stained with antibodies against the epithelial marker pan-CK or mesenchymal marker vimentin, respectively. More than 90% of HAE cells expressed the epithelial marker pan-CK, whereas less than 5% of HAM cells did. On the other hand, less than 5% of HAE

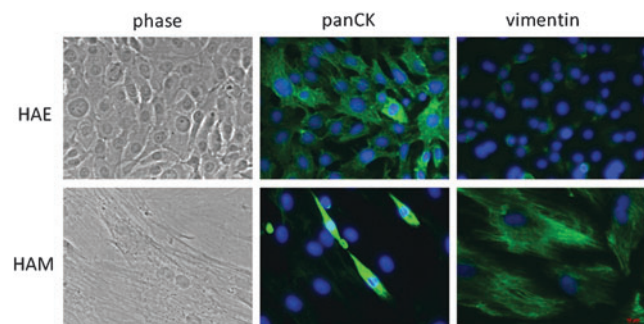


FIG. 1. Morphology of HAE and HAM cells. HAE and HAM cells are stained with anti-pan-CK or vimentin antibodies, respectively (green). Nuclear staining was performed with Hoechst (blue).

cells expressed vimentin, the mesenchymal marker, whereas over 90% of HAM cells expressed it (Fig. 1). These results confirmed that we had obtained epithelial cells and mesenchymal cells, respectively.

Cell-surface marker expression

We analyzed by flow cytometry the HAE and HAM cells with mesenchymal stem cell (MSC) markers CD105, CD90, CD73, and CD44; hepatopoietic markers CD45, HLA-DR, CD14, and CD34; mesenchymal cell-related markers, CD24, CD133, and CD271; and stemness markers, TRA1-60, TRA1-81, SSEA3, and SSEA4 (Fig. 2).

Almost none of the HAE cells expressed hematopoietic markers CD34, CD45, HLA-DR, or CD14, but surprisingly 3% of HAM cells expressed CD34, 17% of HAM cells expressed CD45, 14% of HAM cells were positive for HLA-DR, and 10% were positive for CD14. Less than 1% of HAE cells expressed CD105, CD90, or CD44, less than 5% of HAE cells expressed CD24 or CD133, and 69% of HAE cells expressed CD73, 89% were positive for CD29, 91% for CD49f, 38% for CD271, 7.3% for TRA1-60, 2.8% TRA1-81, 0.6% for SSEA3, and 61% for SSEA4, respectively. Less than 10% of HAM cells expressed CD105, CD44, CD24, or CD133, whereas 20% of HAM cells were positive for CD90, 43% for CD73, 99% for CD29, 69% for CD49f, 50% for CD271, 0.5% for TRA1-60, 0.5% for TRA1-81, 3.6% SSEA3, and 43% for SSEA4, respectively (Table 1). Both HAE and HAM cells expressed CD73, CD29, CD49f, CD271, SSEA3, and SSEA4. Only HAE cells expressed

TABLE 1. MARKER EXPRESSION BY HAE AND HAM CELLS

	HAE cells	HAM cells
CD 105	0%	8% ± 2.2%
CD 90	0%	20% ± 9.5%
CD 73	69% ± 2.2%	43% ± 16%
CD 44	0%	3% ± 0.2%
CD 34	0%	3% ± 0.9%
CD 45	0%	17% ± 3.9%
HLA-DR	0%	14% ± 2.7%
CD 14	0%	10% ± 1.9%
CD 24	2% ± 2%	8% ± 2.1%
CD 29	89% ± 12%	99% ± 1%
CD 49f	91% ± 14%	65% ± 14%
CD 133	3.2% ± 5%	2% ± 0.2%
CD 271	38% ± 20%	50% ± 9.1%
TRA1-60	7.3% ± 3.3%	0.5% ± 0.2%
TRA1-81	2.8% ± 1.4%	0.5% ± 0.2%
SSEA3	0.6% ± 0.5%	3.6% ± 0.5%
SSEA4	61% ± 19%	43% ± 7.2%

HAE cells, human amnion-derived epithelial cells; HAM cells, human amnion-derived mesenchymal cells.

TRA1-60 and TRA1-81. Only HAM cells expressed CD105, CD90, and CD44.

HAE and HAM cells were stained with antibodies against the stemness markers Oct3/4, Sox2, Klf4, c-Myc, Nanog, TRA1-60, TRA1-81, SSEA3, and SSEA4, respectively. Both HAE and HAM cells expressed all stemness markers, Oct3/4, Sox2, Klf4, c-Myc, TRA1-60, TRA1-81, SSEA3, and SSEA4,

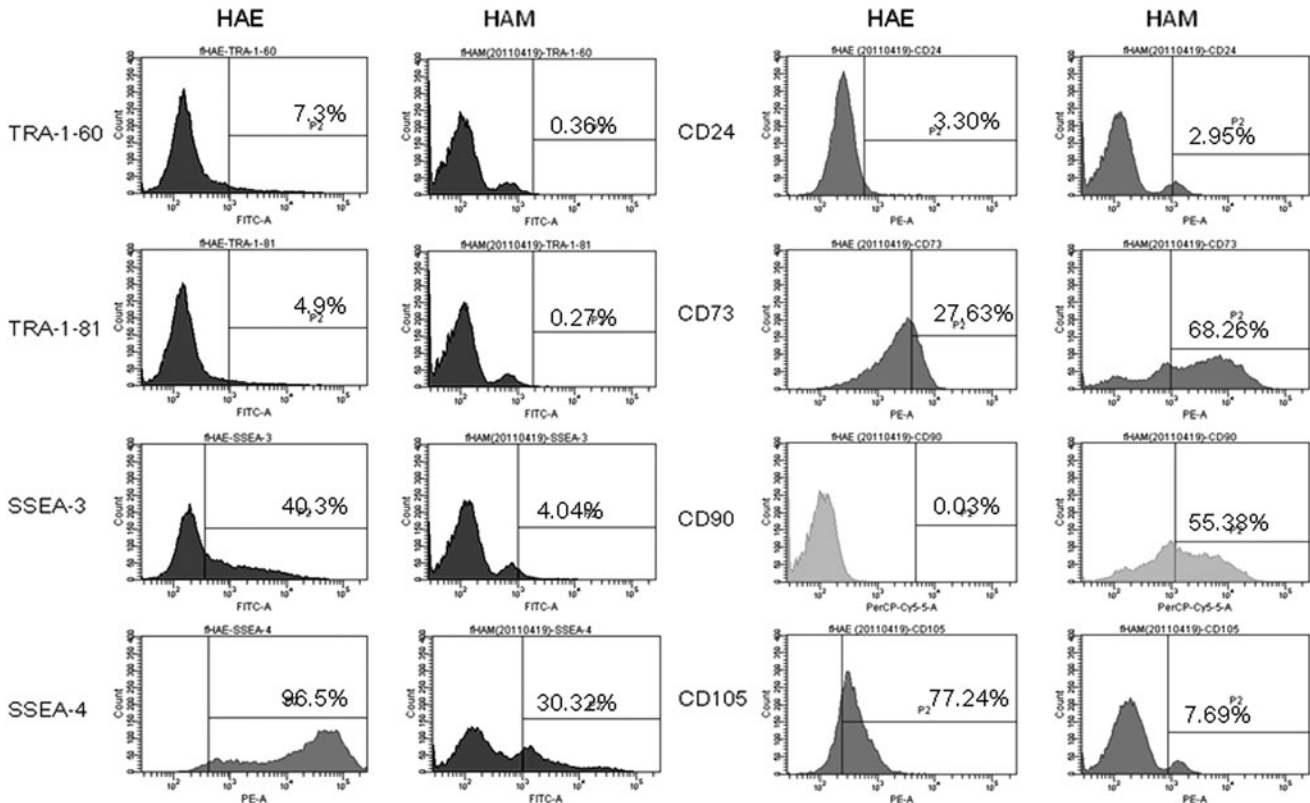


FIG. 2. Histograms of flow cytometric analysis against TRA-1-60, TRA-1-81, SSEA3, SSEA4, CD24, CD73, CD90, and CD105 using HAE and HAM cells.

but not all of the cells were stained with antibodies against stemness markers. Overall, more HAE than HAM cells expressed stemness markers, as more than 80% of HAE cells expressed Oct3/4, Sox2, Klf4, and SSEA4. Nearly 70% of HAE cells expressed c-Myc. More than 90% of HAM cells expressed Oct3/4. About 75% of HAM cells expressed Sox2 and Nanog. Klf4 positive HAMS were about 60% and c-Myc positive and SSEA4 positive HAMS were 30%, respectively (Fig. 3). From the fact that not all the cells expressed markers, it is suggested that they are heterogeneous, including stem cells that express stemness markers.

MSC marker expression was measured by quantitative RT-PCR. MMP1 and ApoD are negative MSC markers and are supposed to be expressed more in fibroblasts than in MSC (Ishii et al., 2005). The expression of MMP1 was lower in all bone marrow (BM)-MSCs, induced pluripotent stem cells (iPSCs), HAE cells, and HAM cells than in fibroblasts. Interestingly, iPSCs expressed ApoD as well as fibroblasts, but HAE and HAM cells expressed less than fibroblasts, about 100-fold lower than BM-MSCs. LIF and MGP are positive MSC markers and are supposed to be expressed higher in BM-MSC than in fibroblasts. The expression of LIF in BM-MSCs was only 1.2-fold higher than that in fibroblasts, although iPSCs expressed it 7.5-fold more. HAE cells expressed LIF 0.4-fold that of fibroblasts and HAMS 0.8-fold. BM-MSCs expressed MGP 44-fold compared with fibroblasts, whereas iPSCs expressed MGP 0.4-fold, HAEs 0.004-fold, and HAMS 0.02-fold (Fig. 4A). These results suggest that HAE cells, HAM cells, and BM-MSCs have different characteristics.

The expression of *Oct3/4*, *Sox2*, *Klf4*, *c-Myc*, *Nanog*, and *Lin28* genes in HAE and HAM cells was analyzed by quantitative RT-PCR. Compared to human fibroblasts, HAE

and HAM cells expressed eight-fold and 13-fold more *Oct3/4*, whereas BM-MSCs expressed 0.7-fold, and iPSCs expressed 34,535-fold. Similar results were seen with *Nanog*, as HAE cells expressed 13-fold, HAM cells expressed 26-fold, BM-MSCs 0.9-fold, and iPSCs 3601-fold. They also showed similar expression patterns with the *Lin28* gene. HAE and HAM cells expressed 12-fold, MSCs 0.9-fold, and iPSCs expressed 232-fold compared to fibroblasts. Both HAE and HAM cells expressed *Sox2* about the same as fibroblasts, whereas BM-MSCs expressed 0.7-fold and iPSCs 1445-fold. As for *Klf4*, HAE cells expressed 25-fold and HAM cells 15-fold, whereas BM-MSCs expressed 0.3-fold and iPSCs expressed 23-fold (Fig. 4B). HAE and HAM cells expressed *Oct3/4*, *Sox2*, *Klf4*, *c-Myc*, *Nanog*, and *Lin28* more than BM-MSCs. Especially, they expressed *Klf4* as much as iPSCs.

These results imply that HAE and HAM cells are heterogeneous cell populations containing stem cells, progenitors, and differentiated cells. Isolation of stem cells was attempted. HAE cells were sorted with anti-TRA1-60 antibody using magnetic-activated cell sorting (MACS) beads. TRA1-60-positive cells were concentrated up to 72% by sorting twice (Fig. 5A). Quantitative PCR of stemness genes was performed to compare their expression levels between the TRA1-60-positive cell population, TRA1-60-negative population, and unsorted population (Fig. 5B). TRA1-60-positive cells expressed the *Oct3/4* gene 6.9-fold higher than unsorted cells and 33-fold higher than TRA1-60-negative cells. TRA1-60-positive cells expressed the *Sox2* gene 7.8-fold higher than unsorted cells and 18-fold higher than TRA1-60-negative cells, the *Klf4* gene 1.8-fold that of unsorted cells and two-fold of TRA1-60-negative cells, the *c-Myc* gene 3.7-fold that of unsorted cells, 5.6-fold of TRA1-60-negative cells, the *Nanog*

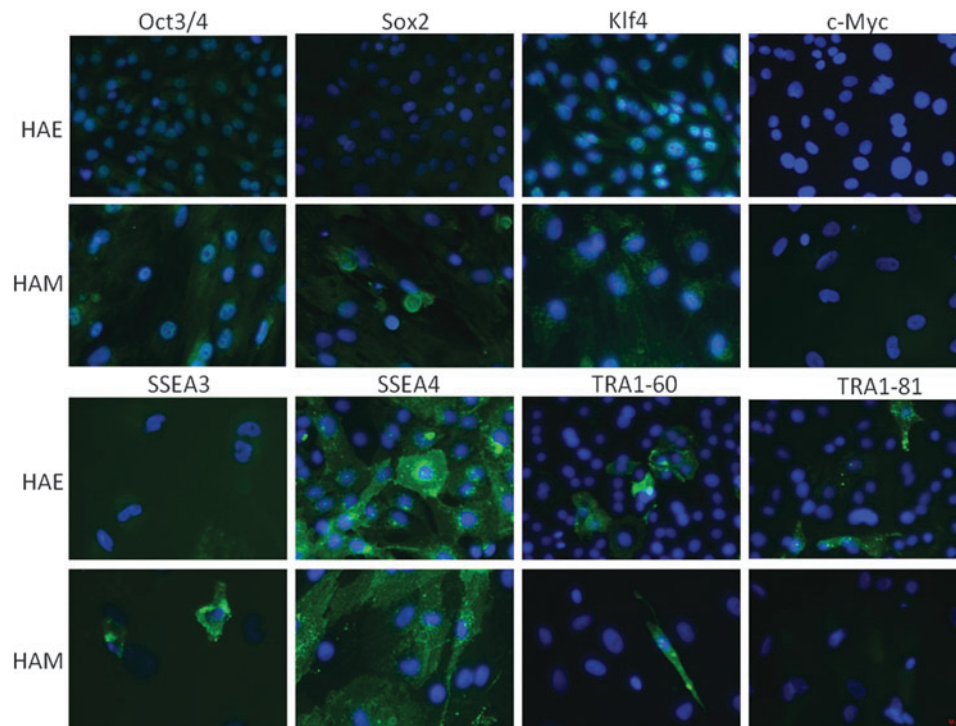


FIG. 3. HAEs and HAMS are stained with anti-Oct3/4, Sox2, Klf4, c-Myc, SSEA3, or SSEA4, antibodies, respectively (green). Nuclear staining was performed with Hoechst (blue).

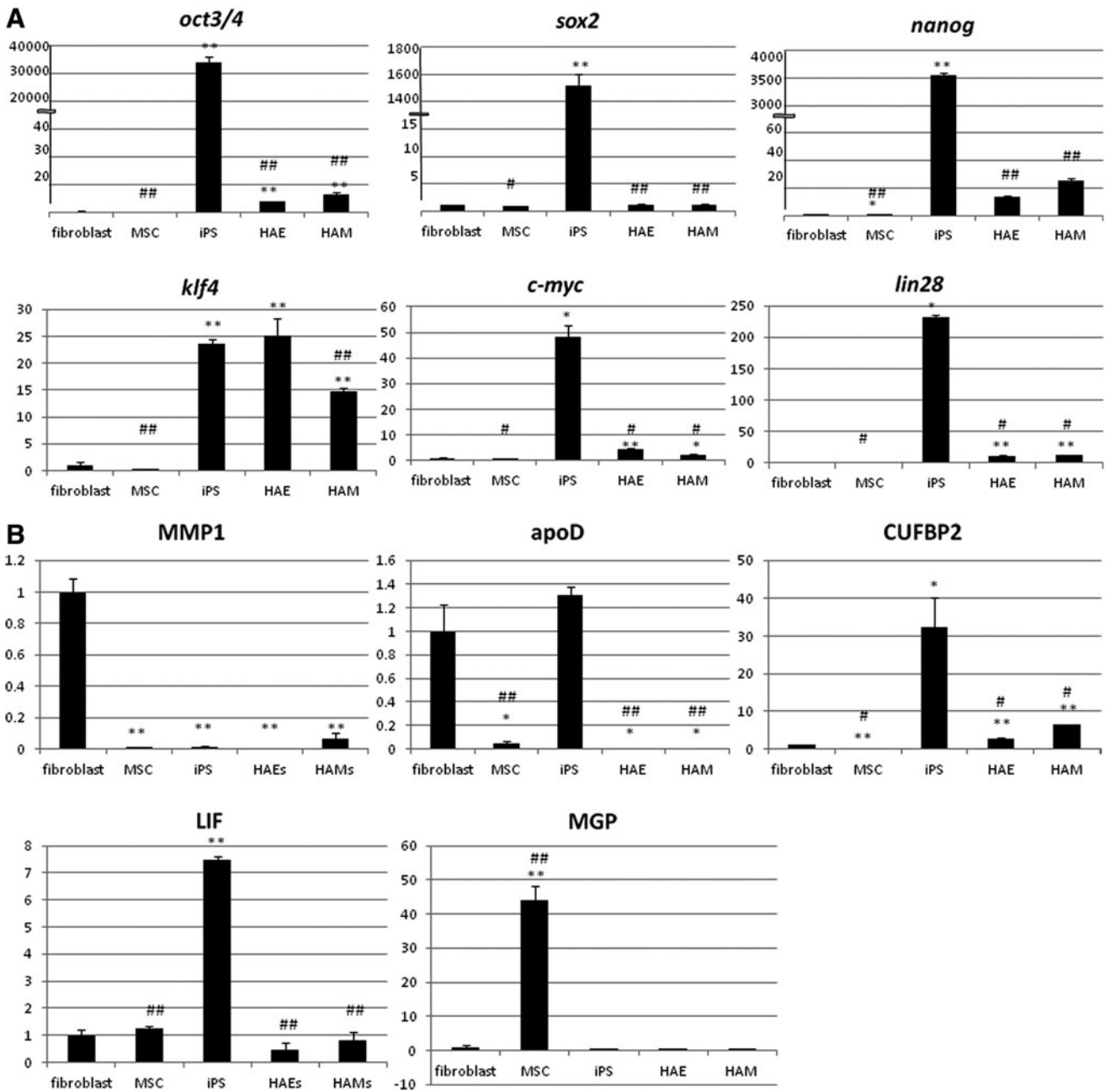


FIG. 4. Quantitative RT-PCR was performed using cDNAs from fibroblasts, MSCs, iPSCs, HAE cells, or HAM cells. Relative expression patterns compared with the expression level of fibroblasts are shown. Bars, mean \pm standard error (SE). Statistical significance: (*) $p < 0.05$, (**) $p < 0.01$ compared to fibroblasts, (#) $p < 0.05$, (##) $p < 0.01$ compared to iPSCs are shown. (A) MSC markers, MMP1, ApoD, LIF, or MGP. (B) Stemness markers Oct3/4, Sox2, Klf4, c-Myc, Nanog, or Lin28.

gene five-fold of unsorted cells, 29.4-fold of TRA1-60-negative cells, and *Lin28* gene 9.4-fold that of unsorted cells and 37.9-fold of TRA1-60-negative cells, respectively. TRA1-60-negative cells expressed all stemness genes less than unsorted cells.

All stemness gene expression was higher in TRA1-60-positive cells compared to unsorted HAE cells or TRA1-60-negative cells. The small difference in expression level of *Klf4* between TRA1-60-positive cells, TRA1-60-negative cells, and unsorted cells would be because the expression level of *Klf4* was originally quite high. This result suggests sorting HAE cells using anti TRA1-60 antibody concentrated the epithelial stem cells.

Discussion

We have shown here the stemness marker expression in HAE and HAM cells. Our immunohistochemistry results showed that both HAE and HAM cells express all stemness markers, Oct3/4, Sox2, Klf4, c-Myc, Nanog, and SSEA-4. Some cells were stained strongly with antibodies against marker proteins, some weakly, and some were not stained among both HAE and HAM cells. This that suggests HAE and HAM cells are heterogeneous populations consisting of stem cells, progenitor cells, and differentiated cells. Flow cytometric analysis showed that HAE and HAM cells

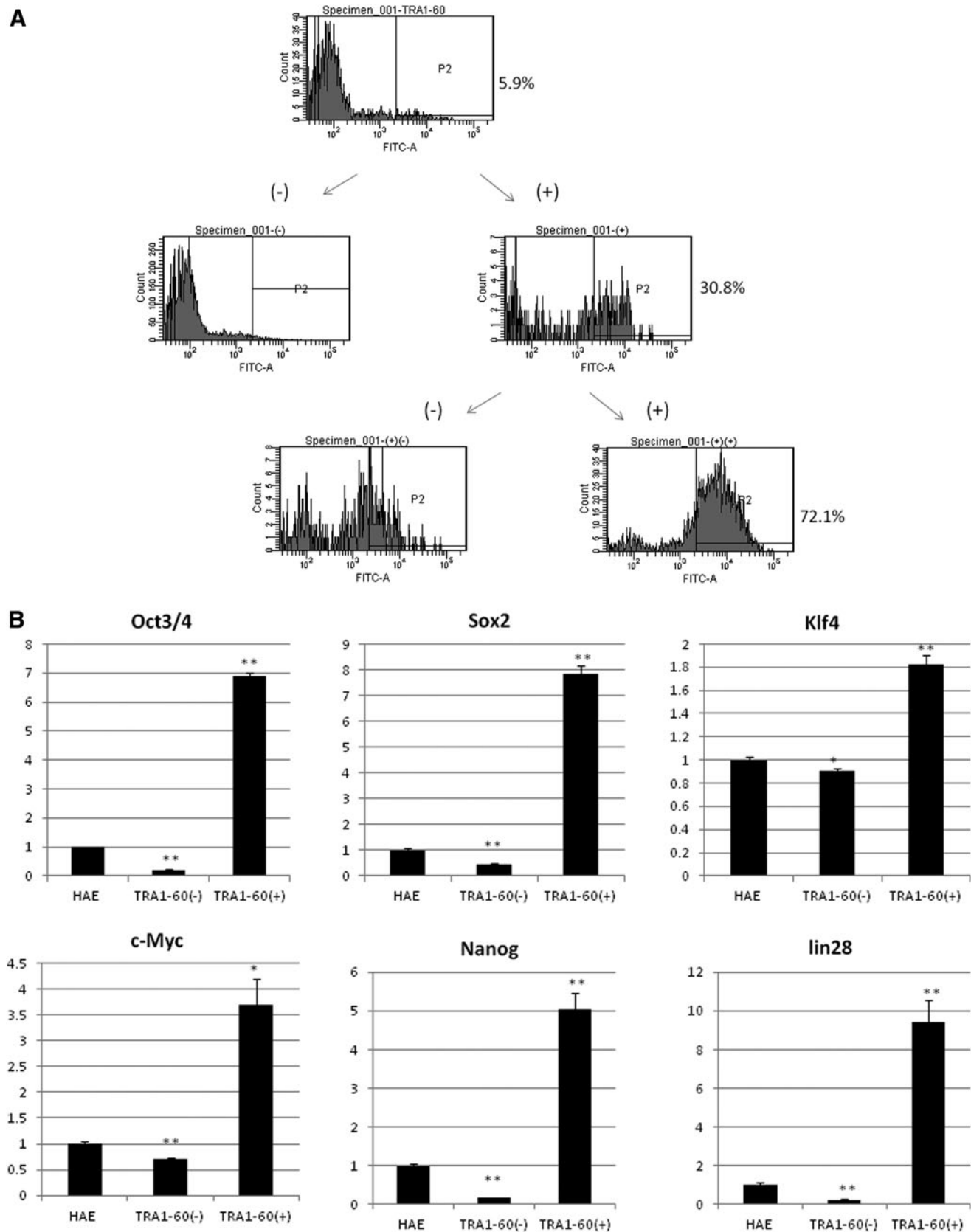


FIG. 5. (A) HAEs were sorted by MACS beads twice using anti-TRA1-60 antibody. (B) Quantitative RT-PCR was performed using cDNAs from HAEs, TRA1-60-negative cells, and TRA1-60-positive cells. Relative expression patterns compared with the expression level of HAEs are shown. (*) $p < 0.05$, (**) $p < 0.01$ compared to unsorted cells are shown.

expressed CD73, CD29, CD49f, and SSEA-4. The expression patterns of CD 24, CD133, and SSEA4 are consistent with the results of Miki et al. (2005, 2006, and 2007). Because only HAM cells expressed CD105, CD90, and CD44, MSCs would be enriched in this population.

HAE and HAM cells expressed stemness marker genes *Oct3/4*, *Klf4*, *c-Myc*, and *Nanog* more than BM-MSCs. This suggests that they are closer to iPSCs than BM-MSCs as stem cells. HAE and HAM cells expressed *Klf4* as much as iPSCs. This suggests that their stem cell characters are closer to that of iPSCs than BM-MSC, thus supporting the idea that amnion-derived cells being more efficiently reprogrammed than fibroblasts (Easley et al., 2012). One means of efficiently reprogramming amnion-derived cells is through introduction of Oct4, Sox2, and Nanog (Zao et al., 2010).

Izumi et al. showed that 17- to 19-week amnia expressed Nanog and Sox2 more than term amnia (Izumi et al., 2009). Because our HAE and HAM cells were isolated from a 35- to 36-week amnion membrane, it is possible that cells would express stemness markers more than our HAE and HAM cells if isolated from the amnion membrane at an earlier stage. Also amnia at an earlier stage might contain more stem cells than amnia at later stages or at term. However, ethical problems would arise from using earlier-stage amnia because they are a consequence of abortion. Considering the ethical issues, HAE and HAM cells appear to be the best amniotic cell source at this point.

It was interesting that iPSCs highly expressed MSC-negative markers, ApoD, or expressed less of the MSC-positive marker MGP. We assumed that somatic stem cells such as MSCs and ESCs or iPSCs have different characteristics that do not have one direction of stemness. This idea is supported by previous studies demonstrating that iPSCs and ESCs have different signatures of gene expression (Chin et al., 2009; Kim et al., 2008; Maguire et al., 2013; Marchetto et al., 2009).

To isolate stem cells from the heterogeneous population of amnion epithelial cells, anti-TRA1-60 antibody was used. TRA1-60-positive cells expressed stemness markers more than unsorted cells. TRA1-60-negative cells expressed all stemness marker genes less than unsorted cells. The expression of *Klf4* was only 1.5-fold that of unsorted cells, because *Klf4* expression is as high as in iPSCs in unsorted HAE cells. This suggests that TRA1-60-positive HAE cells have more stem cell-like characteristics. Thus, TRA1-60-positive amnion-derived epithelial cells are the concentrated stem cell population. We concluded that TRA1-60 can be a marker for isolating stem cells from amnion epithelial cells. More studies will be needed to identify the markers for amniotic MSCs.

Conclusion

We analyzed the characteristics of HAE and HAM cells. Both HAE and HAM cells expressed the stemness markers Oct3/4, Sox2, *Klf4*, *c-Myc*, Nanog, TRA1-60, TA1-81, SSEA3, and SSEA4. Especially, the relative expression of the *Klf4* gene in HAE cells was as high as in iPSCs. TRA-1-60-positive HAE cells expressed those stemness markers more than unsorted cells and TRA-1-60-negative cells. Our results suggested that HAE and HAM cells have stem cell characteristics and TRA-1-60 can be a marker for isolating HAE stem cells.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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