plate was dried over night, and (iv) the MPC polymer-coated culture plate was sterilized by UV irradiation for an adequate time. Therefore, the resulting MPC unit density on the plate was 0, 1, 2, 5, and 10% MPC unit composition.

Surface elemental analysis of the MPC polymer-coated PS plate was carried out by X-ray photoelectron spectroscopy (XPS, AXIS-His, Shimadzu/KRATOS, Kyoto, Japan). The X-ray source used for XPS measurements was Mg Ka source. The take-off angle of the photoelectrons was fixed as 90°. At least 5 points of the sample were measured by XPS and these intensities were averaged before the following calculation. The surface compositions of the MPC units was calculated as follows. The ratio of signal intensity at 133 eV based on the phosphorus atom attributed to the MPC units over that at 285 eV based on the carbon atoms attributed methyl groups and methylene groups in both BMA and MPC units was determined. The calibration was carried out using the ratio obtained from the XPS spectra of both poly(BMA) and poly(MPC) coated PS plate as 0 % and 100 % of MPC unit, respectively.

MSC preparation and selection by MPC polymer-coated plates

All procedures for the present experiments were approved by the ethics committee or institutional committee for animal research of the University of Tokyo Hospital (ethics permission #622). Figure 1(a) indicates the experimental design. Human MSCs were obtained from the femur of osteoarthritic patients who underwent total hip replacement at the University of Tokyo Hospital, after informed consent. Cells in bone marrow aspirates (100 μL/φ 2.2 cm dish) were seeded on MPC polymer-coated culture plates with various MPC unit compositions as 0 -10%, and cultured using the hMSC bullet kit (Cambrex, East Ruatherford, NJ) in a 37°C/5% CO_2 incubator. Rat MSCs were collected from six-week-old male Sprague-Dawley rats (Nisseizai, Tokyo, Japan). After the epiphyses of the tibias were removed, the marrow was flushed out by using a syringe filled with

medium and filtered through a 70-μm nylon mesh. The obtained bone marrow materials (100 μL/φ 2.2 cm dish) were plated and cultured in the same manner as human MSCs.

The cells were harvested by treatment using trypsin-EDTA solution. After the cell harvest of the primary culture from the MPC polymer-coated plates, the cells were re-seeded onto the conventional PS culture plates at a density of 5.0×10^3 cells/cm^2. Passages were performed when the cells were approaching confluence. The medium was changed three times/week. The cell numbers were counted by a haematocytometer, while the viability of the cells was checked by trypan blue staining. Cell proliferation was also colorimetrically measured by cell counting kit-8 (Dojin, Kumamoto, Japan), one week after cell seeding.

Flow cytometric analysis

Cells were harvested using trypsin-EDTA solution, centrifuged at 1500 x g for 5 min, and resuspended at 5×10^6 cells/ml in phosphate-buffered saline (PBS) containing 3% fetal bovine serum. Aliquots containing 10^5 cells were incubated with individual primary antibodies or control IgG for 30 min at room temperature. The cells were washed in PBS containing 3% fetal bovine serum and incubated with a fluorescent conjugated secondary antibody for 30 min at room temperature. Samples were analyzed using a FACS LSL II (BD, Franklin Lakes, NJ). The following monoclonal antibodies were used: mouse monoclonal antibodies against human CD29 (integrin β1, BD), human CD34 (Chemicon, Victoria, Australia), human CD44 (hyaluronan receptor, Ancell, Bayport, MN), human CD45 (LCA, Cymbus, Chandlers Ford, UK), human CD105 (Endoglin, Ancell), CD166 (ALCAM, Ancell), normal mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and fluorescein isothiocyanate (FITC)-conjugated rabbit antibody against mouse IgG (Santa Cruz Biotechnology).

Osteogenic and chondrogenic induction for MSCs

The osteogenic[1] or chondrogenic[17, 18] differentiation was induced in MSCs according to previously reported procedures with some modifications. For the osteogenic differentiation, cells were seeded at 4.0 x 10^4 cells per 2.2 cm plates and maintained for 21 days in DMEM supplemented with 10% fetal bovine serum, 10mM β-glycerophosphate, 100nM Dexamethasone, and 50 μg/mL ascorbic acid-2-phosphate. For the chondrogenic differentiation, cells were seeded at 2 x 10^5 cells per 15 ml plastic centrifuge tube and maintained in 2 mL of serum-free a-MEM supplemented with 3500 μg/mL glucose, 6.25 μg/mL insulin, 6.25 μg/mL transferrin, 6.25 ng/mL selenite, 5.33 μg/mL linolate, 1.25 mg/mL bovine serum albumin, 10 ng/mL transforming growth factor-β3, 100 nM dexamethasone and 50 μg/mL ascorbic acid-2-phosphate. The cells were cultured under the chondrogenic status for 21 days. The medium was changed three times/week.

Total RNA extraction and realtime RT-PCR

The total RNA was isolated from MSC using the chaotropic Trizol method (Nippon-gene, Tokyo, Japan). The total mRNA (1 μg) was reverse transcribed using the Super Script reverse transcriptase with a random hexamer (Takara Shuzo, Shiga, Japan). The full-length or partial-length cDNA of the target genes, including the PCR amplicon sequences, was amplified by PCR, cloned into pCR-TOPO Zero II or pCR-TOPO II vectors (Invitrogen, Carlsbad, CA), and used as standard templates after linearization. The QuantiTect SYBR Green PCR Master Mix (Qiagen, Hilden, Germany) was used, and the SYBR Green PCR amplification and real-time fluorescence detection were performed with an ABI 7700 Sequence Detection system (Foster City, CA). All reactions were run in quadruplicate. The sequences of the primers were 5'-CTCCTCGCTTTCCTTCCTCT-3' and 5'-GTGCTAAAGGTGC CAATGGT-3' for COL1A1; 5'-GAGTCAAGGGTGATCGTGGT-3' and 5'-CACCTTGGTCT CCAGAAGGA-3' for COL2A1; 5'-AGGAATGCCT GTGTCTGCT T

-3' and 5'-ACAGGCCTACCCAAACATGA-3' for COL10A1; 5'-GACCCTTGACCCCCACAAT-3' and 5'- GCTCGTACTGCATGTCCCCT-3' for ALP; 5'-CATG AGCGAGGG CACTCC-3' and 5'-TCGCTTCAGGTCAGCCTTG-3' for Sox9; 5'-GAAG GTGAAGGTCGGAGTCA-3' and 5'-GAAGATGGTGATGGGATTTC-3' for GAPDH.

Enzyme activity for ALP

The enzyme activity was histochemically detected in the MSCs in which the osteogenic differentiation was induced. For ALP enzyme histochemistry, the cells were incubated with a mixture of 5 mg naphthol AS-BI phosphate (Sigma, St. Louis, MO) as a substrate and 18 mg of fast red violet LB salt (Sigma) diluted in 30 mL of 0.1 mol/L Tris-HCl buffer (pH 8.5). The images were taken by the digital camera, while the enzyme activity was quantitatively measured by histomorphometrical approaches using the software Scion Image alpha 4.0.3.2 (Scion, Frederick, MD).

RESULTS

Selection using MPC polymer-coated plates

Polymer coating of PS culture plate with the PMB30/poly(BMA) mixed solution was proceeded well and the surface of the plate was covered with these polymers completely. When the surface composition of MPC units on the plates was calculated from the XPS results, it was found that the MPC unit composition at the surface increased in parallel with that in the polymer mixed solution

containing poly(BMA) and PMB30 used in a single dip coating as shown in Figure 1(b). We confirmed that the surface composition the MPC units could be controlled.

With these plates, we first selected some subpopulations of the MSCs according to the degrees of the adhesiveness on the culture plates coated with different compositions of the MPC unit. Human bone marrow aspirates (approximately 0.1 mL) was seeded onto the culture plates with a 2.2 cm diameter coated with 0, 1, 2, 5 and 10% MPC unit compositions. For 3 days, the number of adherent cells on the plate surface had plateaued on all plates. At 3 days, the medium was changed together with the floating cells and were replaced by another medium. The adherent cells continued to be cultured for 4 more days on the same MPC polymer-coated plates, and then were harvested for cell counting. The cells attached on the plate surface were observed to have a higher density on the dishes treated with a 0% MPC unit composition, compared with those of increasing the MPC unit composition, at 7 days [Fig. 2]. The number of cells harvested from the plates had significantly decreased according to the increase in the density of the MPC polymer coating [Fig. 2 (graph)]. The cell numbers on the MPC polymer-coated dishes with 2% or 10% MPC units were approximately half or quarter of 0%, respectively.

In order to examine the proliferation ability of MPC polymer-selected MSCs, the cells harvested from each MPC polymer-coated plate were re-seeded onto the conventional PS plates (φ 2.2 cm) with the same cell number of 1.9×10^4 in the second passage (passage 2), and then cultured for 7 days. The cells were equally proliferated during this period, while the total cell number after a 7 day-culture had not significantly changed among the cells derived from the different MPC polymer-coated plates [Fig. 3 (cell count)]. The result was represented by the experiment using the cell counting assay [Fig. 3 (cell counting assay)].

Surface epitopes of cells selected by MPC polymer-coated plates

We next examined the surface epitopes of the cells selected by the MPC polymer-coated plates (passage 1). It is known that CD29 (integrin β1), CD44 (hyaluronan receptor), CD105 (Endoglin) and CD166 (ALCAM) were expressed in MSC, but that CD34 and CD45 (LCA) were markers specific for heamatopoietic stem cells. Although the hematopoietic stem cell markers were negative in all cells selected by the plates coated with the 0, 1, 2, 5 or 10% of MPC unit composition, CD29, CD44, CD105 and CD166 were detectable in the cells of all MPC unit compositions. The levels of the MSC markers in the cells selected by the 1-10% MPC unit composition were almost similar to those in cells of 0% that corresponds to the control MSC, implying that the MPC polymer-selected cells belong to the category of MSC on the surface epitopes [Table 1].

Osteogenic and chondrogenic potential of MPC polymer-selected cells

After the culture on the MPC polymer-coated plates (passage 1), the cells were cultured on the conventional PS culture plates for a long term with repeated passages. By passage 5, the cell numbers had expanded by approximately 1000-fold in the cells of each MPC unit composition (0-10%). Under the osteogenic condition, the cells selected by the MPC polymer coated-plates and cultured in the conventional PS plate ones for a single time (passage 2) more highly expressed the COL1A1 mRNA in the 2-5% MPC than in the 0%, but those by the 1 or 10% MPC polymer coated-plates did not show any significant increase in the COL1A1 expression. The promotion effects of the COL1A1 expression in 2% MPC unit composition continued even at passage 5, although the cells at passage 2 were more sensitive for the osteogenic differentiation than those at passage 5. ALP also peaks at 2-5% MPC unit composition for both passages, although no statistical difference of the ALP expression was

detected in passage 2 [Fig. 4(a)]. The ALP enzyme activity was also significantly higher in 5% MPC unit composition than others at passage 2 [Fig. 4(b)].

The expression of the chondrocyte markers in the MPC polymer-selected cells under the chondrogenic conditions was also enhanced in the 2-5% MPC unit composition, as observed during osteogenesis. Responding to the chondrogenic induction, the cells began to express COL2A1, COL10A1 and Sox 9, and especially cells selected by the 2% MPC unit composition showed a prominent expression of all chondrocyte markers not only at passage 2, but even at passage 5 [Fig. 5].

DISCUSSION

The adhesion capacity seems to have some association with the cellular activities and functions. Specific adhesion to the laminin and type IV collagen coated on the surface of the culture dishes could select the myogenic cells of the embryonic mouse thigh from fibroblastic cells. Over a brief time period (10-20 min), myoblasts from the embryonic mouse thigh muscle had adhered faster to the laminin than did the fibroblasts from the same tissue, while the latter adhered faster to the fibronectin than the former.[19] Laminin-1 also enriched the osteoblast progenitor cells from rat calvarial cells when they were seeded on the culture wells coated with it. The laminin-1 inhibited cell attachment of the rat calvarial cells, but could select the highly osteogenic lineage according to the difference in the cell adhesiveness to that of the molecule.[20] Thus, through the selection of the cell adhesion to some molecules, a specific cell subpopulation that possesses a high differentiation potency would be concentrated from heterogeneity of the cell sources.

MSC expresses many adhesion-related molecules, like the integrin subunits α4, 5, 6, 8, 9, v/β1, 3, 5, ICAM-1, ALCAM, VCAM-1, SCF, fibronection, E-cadherin and hyaluronan receptor[21-23] and can be bound to various ligands including laminin and E-cadherin to play biological roles through the cell-to-cell or cell-to-matrix contacts. As examples of the cell-to-cell contact with MSCs in vivo,

homing functions for the hematopoietic cells of MSCs should be discussed. Through the cell-to-cell contacts with hematopoietic stem cells mediated by VCAM-1, fibronectin, SCF, E-cadherin, or ICAM-1, MSCs secrete extracellular matrix proteins, produce secreted/membrane-bound cytokines and regulate hematopoiesis.[22] MSCs are also recruited and adhered to the damaged tissues in order to participate in tissue repair. These cells can provide cell sources for tissue repair in bone, cartilage, and even skeletal muscle or myocardium that do not directly make contact with bone marrow. Once muscles are injured, the MSCs are delivered to the degenerative muscles from the circulation, are adhered to the lesion, take part in the regenerative process, and provide fully differentiated muscle fibers.[24] In the murine model of cardiac repair following ischemic injury, MSCs were mobilized from bone marrow, homed and generated cardiac myocytes. Among the adhesion molecules of the MSC such as integrin α4, 6, 8, 9, and β1, blockade of the integrin β1 by the neutralizing antibody reduced the total number of MSCs in the infracted myocardium, suggesting that MSCs utilized integrin β1 for cell adhesion to the myocardium and its regeneration.[23]

Thus, MSCs can be bound to various partners via many kinds of adhesion molecules to exert physiological and pathological functions. Although the adhesiveness to some ligands likely selects a cell subpopulation with a high differentiation potency of a certain lineage, [19,20] such a specific selection may have the risk to reduce the multipotency in MSCs. Therefore, we applied the selection system based not on the adhesiveness to specific molecules, but the general adhesion ability to the MPC polymer-coated plates. As a result, we could enrich the cells to have a high potency of both osteogenesis and chondrogenesis from the crude MSCs.

It has yet remained unknown why the strength of the adhesion ability in MSCs could enhance not the proliferation rate of the cells, but the differential potential for both osteogenesis and chondrogenesis. Speculating that such multipotent cells may show a stronger adhesion than fibroblastic cells in bone marrow, the MPC polymer-selection due to cell attachment could exclude the fibroblastic ones that possess a lower differentiation potential. This selection probably enriched the

cells with high differentiation potential. It implied not that the MPC polymer-coated plates did not induce the phenotype changes in each cell, but that they purified the cell populations by the elimination of fibroblastic cells from the total populations of bone marrow adhesive cells. Therefore, the difference in osteogenic and chondrogenic ability was maintained during the repeated passaging, and the MPC polymer selection could improve cellular potential even after recultivation on PS plates. However, as we do not currently possess the methods to exactly distinguish MSCs from fibroblastic cells using cell surface epitopes, it may be hard to prove that the MPC selection could concentrate the multipotent MSCs from a mixture of the MSCs with fibroblast, by flow cytometry that can exactly exclude the hematopoietic lineage from the MSCs.

MSC can be differentiated into a variety of tissues including bone, cartilage, tendon, fat, heart, muscle or brain, in vitro and in vivo.[1, 8] Autologous MSCs have advantages over embryonic stem cells, regarding the teratocarcinoma formation, immune rejection, or ethical problems. The cell sources have already been used for the treatment of osteogenesis imperfecta, bone/cartilage defects, myocardiac infarction, or skin ulcer.[25-28] On the other hand, the MPC polymers have also been already applied in the clinical field for the surfaces of intravascular stents, intravascular guide wires, soft contact lenses, and the artificial lung, all of which were authorized by the United States Food and Drug Administration.[13, 14] Thus, the biocompatible polymer is regarded to be approved for safe clinical use.

The MPC selection is as simple as to culture MSCs with MPC polymer-coated plates in the first passage, which would reduce the risks of contamination or mismanagement during the culture procedure. The improvement of the MSCs in purity and multipotency by the MPC polymer-selection would provide promising technologies for the next generation-cell therapy that can be applied for more severe and other various diseases. The clinical application of the MPC polymer-selected MSCs is now underway.

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Figure legends

Figure 1. (a) The experimental design. Cells in bone marrow aspirates were seeded on MPC polymer-coated plates at the composition of 0-10% MPC unit, at passage 1, while the adhesion ability of MSCs to the MPC polymer-coated plates and the surface epitopes of MPC-selected cells were evaluated. Although cells were cultured on the MPC polymer-coated plates at passage 1, the cells were seeded onto the conventional PS plates thereafter. The proliferation of cells (passage 2) was measured by cell counting, while the differentiation potential for osteogenesis and chondrogenesis was examined at passages 2 and 5. (b) Relationship between MPC unit composition at the surface on PS plate after coating and that in polymer coating solution.

Figure 2. The adhesion of cells in human bone marrow aspirates onto the culture plates coated with MPC polymers with various compositions of MPC unit. The number of cells that were attached on the MPC polymer-coated plates at day 7 of the cell culture decreased according to the density of the MPC unit. All values are presented as mean plus standard deviation of 5 samples per group. Statistics were assessed using Dunnett's test (*: $P<0.01$ vs 0% MPC unit composition).

Figure 3. Proliferation of the cells that had been selected by the plate coated MPC polymer with various MPC uinit compositions. The cells cultured on the MPC polymer-coated plates were harvested and then re-seeded onto the conventional PS plates. The numbers of human cells were counted at 7 days of culture (cell count). All values are presented as mean plus standard deviation of 5 samples per group. Statistics were assessed using Dunnett's test. No significant difference was seen among the proliferation of the cells harvested from each MPC polymer-coated plate (0-10% MPC unit composition). The dashed line indicats the number of cells originally seeded on the plate (1.9×10^3 cells). The result was represented by the experiment using the cell counting assay in the rat MSCs

(cell counting assay). All values are presented as mean plus standard deviation of 3 measurements per group. No significant difference (Dunnett's test) was seen among each groups.

Figure 4. (a) Gene expression of COL1A1 and ALP in the osteogenic induction. Signficant expression of COL1A1 gene was found in human MSCs selected by the MPC polymer-coated plates (2-5% unit composition) at passage 2, while the high expression level in the 5% MPC unit composition continued by passage 5. Also, in the ALP expression, the promotion effect was observed in 2-5% MPC unit composition, expecially at passage 5. All values are presented as mean plus standard deviation of 5 samples per group. Statistics were assessed using Dunnett's test (*: $P<0.01$ vs 0% MPC unit composition). (b) The enzyme activity for ALP in the osteogenic induction. The ALP enzyme activity was also significantly higher in 5% MPC unit composition than others in the rat MSCs at passage 2. All values are presented as mean plus standard deviation of 3 measurements per group. Statistics were assessed using Dunnett's test (*: $P<0.01$ vs 0% MPC unit composition).

Figure 5. Gene expression of COL2A, COL10A1 and Sox9 during the chondrogenic induction. The expressions of COL2A1, COL10A1 and Sox9 genes peaked at 2-5% MPC unit composition not only at passage 2, but also at passage 5. All values are presented as mean plus standard deviation of 5 samples per group. Statistics were assessed using Dunnett's test (*: $P<0.01$ vs 0% MPC unit composition).

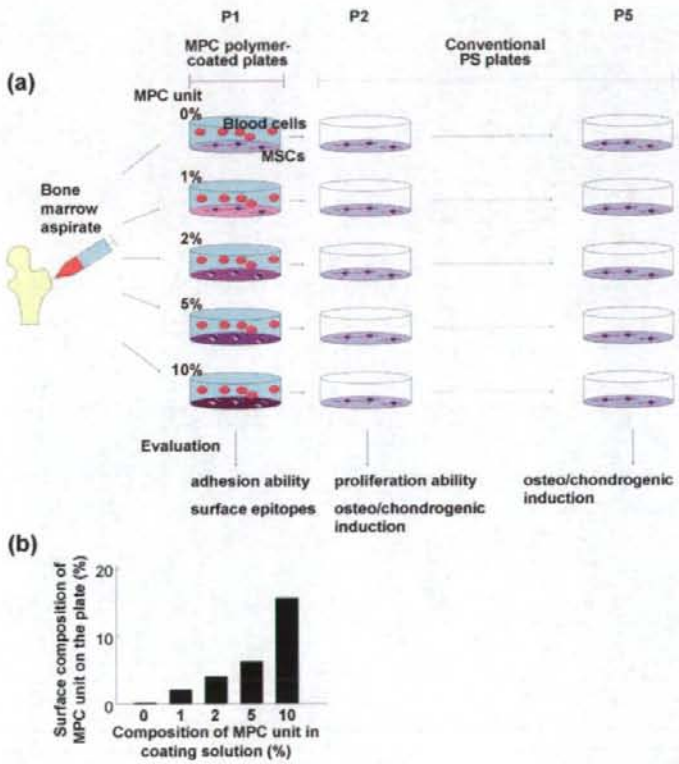

Figure 1

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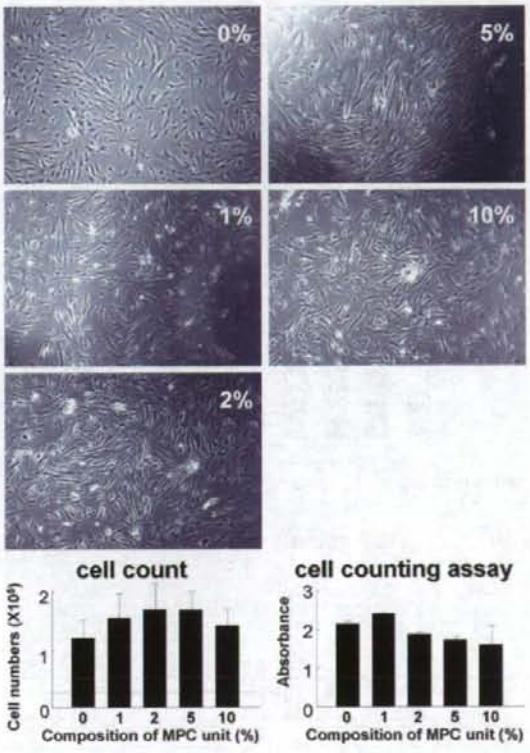

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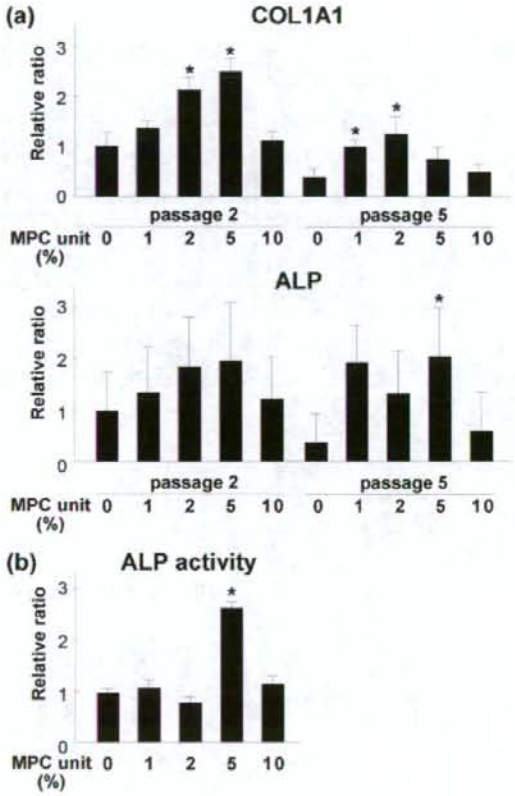

Figure 4

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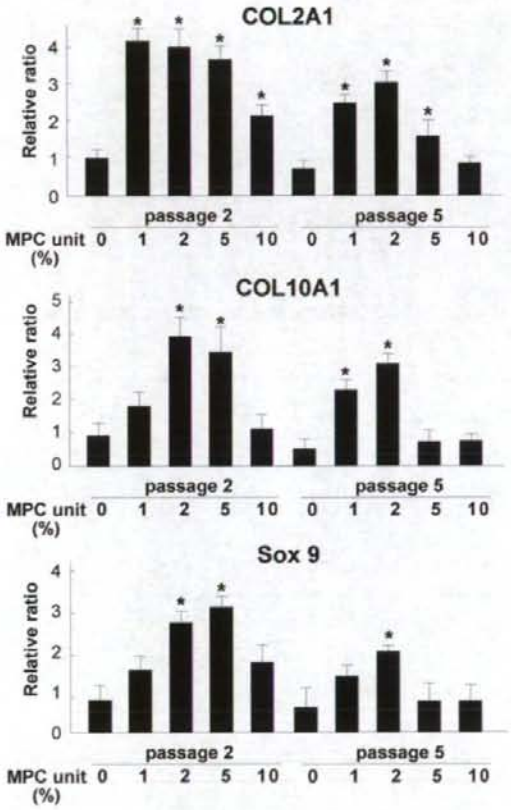

Figure 5

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