様々なGPI-アンカー型蛋白質や、膜貫通型蛋白質がSST法により同定できた。

以上の同定蛋白質のなかで、機能の解析の行われていない分子でしかも、ノックアウトマウスが胎生致死であるPTK7について、検討を開始した。

④ PTK7は膜貫通型蛋白質であり、細胞―基質間接着に関係する。
PTK7はtyrosin kinase ドメインを有する１回膜貫通型蛋白質であるが、tysoine kinase 活性はなく、細胞外ドメインにはイムノグロブリン様ドメインを持っていることがアミノ酸の１次構造、２次構造予測からわかった。PTK7 cDNA全長を得た後にEGFPをカルボキシ末端に付加して発現させる発現ベクターを構築した。これを血管内皮細胞に発現させて局在を検討したところ、予想通り膜貫通型の局在パターンを示した。また、PTK7蛋白質の発現を低下させることにより、細胞での機能を検討した。PTK7のノックダウンにより、細胞外マトリックスとの結合が抑制されており、インテグリンのシグナルに不可欠な分子である可能性が示唆された。

D. 考察

今年度の研究で、骨髄間葉系細胞が分泌するシグナルシークエンス分子を多数同定することが可能であった。シグナルシークエンストラップ法は、分泌蛋白質を決定するためにはとても、重要で迅速なアッセイ方法であることが明らかになった。
骨髄間葉系細胞がstemnessを維持するために必要な細胞膜表面分子、あるいは、分化に必要な因子を同定するためには、今後も分化誘導後に出現してきた因子や受容体などを同様にSST法で突き止めていくことが必要になるが、今回は初回のすくリンーニングとして、本SST法の妥当性・有用性を示すことができた。

SST-Rexで得られた３００以上のクローンのうち、殆どがコラーゲンなどの細胞外マトリックス分子であった。これは、骨髄間葉系細胞が細胞―基質間接着が重要であることを示している。他の細胞でも同様な結果がSST-Rexを用いた場合に報告されているが、コラーゲンα１鎖が９０%以上をしめた。これは、この細胞に特異的な結果とも考えられる。骨髄間葉系細胞は多分化能（多能性分化能）をもつが、分化する細胞とそのまま多分化能を維持している細胞が重要である。このstemnessの維持機構としてに不可欠な因子生体内ではnicheに存在することが考えられている。特に骨髄内のmesenchymal cellは細胞間接着があることでそのstemnessを維持していると考えられる。基質間接着も重要な、nicheの分子と考えられた。

機能の未知分子 PTK7に着目して同分子の機能を明らかにする研究に着手した。PTK7分子のノックアウトマウスは胎生致死であり、情報伝達あるいは分子の接着に不可欠の分子であることが予想された。特に、細胞接着分に認められる細胞外ドメインのイムノグロブリン様ドメインがあるために、細胞外ドメイン依存性の接着などが検討可能になる。インテグリン依存性の接着も制御している可能性も想定された。PTK7をノックダウンした細胞ではインテグリン依存性の細胞接着が抑制されていたからである。今後さらに、インテグリン依存性の細胞内シグナルの変化を詳細に検討していく予定である。

E. 健康危険情報

なし。

F. 結論

骨髄間葉系細胞が分泌する蛋白質あるいは、骨髄間葉系細胞の細胞表面に発現する分子をSST-Rex法により分離同定した。機能未知の分子も分離したので、その機能とstemness維持・分化誘導のメカニズムについて今後検討していく。

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2. 学会発表

特になし。

H. 知的財産権の出願・登録状況

特になし。

Ⅲ　研究成果に関する一欄表

別紙４

研究成果の刊行に関する一覧表

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Ⅳ　研究成果の刊行物・別冊

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Membranous osteogenesis system modeled with KUSA-A1 mature osteoblasts

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Abstract

Several stromal cells were established from murine bone marrow cultures. One of the KUSA subclones, KUSA-A1 cells, displays osteogenic characteristics in vitro and in vivo. The calcium deposition, osteocalcin release, and parathyroid hormone (PTH) responsiveness of KUSA-A1 cells indicate that they are mature osteoblasts or osteocytes. Bone had formed in subcutaneous tissue 1 week after subcutaneous injection of cells into immunodeficient mice. The osteogenesis by KUSA-A1 was not mediated by chondrogenesis and thus was considered to be membranous ossification. These unique characteristics of KUSA-A1 cells provide an opportunity to analyze the process of membranous ossification in detail.

Keywords: Membranous osteogenesis; Stromal cell; KUSA; Osteoblast; Gap junction

1. Introduction

The concept of regenerative medicine refers to the cell-mediated restoration of damaged or diseased tissue. Candidate cell sources for tissue regeneration include embryonic stem cells, fetal cells, and adult cells, such as marrow stromal cells [1], each of which has both advantages and drawbacks. Clinical trials with marrow stromal cells have been performed in patients with osteogenesis imperfecta [2] and osteoporosis [3,4], and marrow stromal cells are expected to be a good source of cell therapy [5]. Multipotent mesenchymal stem cells have been isolated from adult marrow and shown to differentiate into multiple cell types, such as osteoblasts, chondrocytes, adipocytes, myoblasts [1,5,6], cardiomyocytes [7,8], endothelial cells, and neuronal cells [9].

In the present study, we characterized a single cloned, immortalized stromal cell line, KUSA-A1, established from murine bone marrow cultures [6]. KUSA-A1 cells are capable of generating mature bone in vivo. They are a unique, mature osteoblast cell line and will serve as a very suitable model for in vivo osteogenesis.

2. Materials and methods

2.1. Cell culture

The stromal cell lines were isolated from long-term bone marrow cultures of C3H/He female mice and cultured as previously described [6,9–11]. Cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supple-

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mented with 20% fetal bovine serum (FBS) and penicillin (100 μg/ml)/streptomycin (250 ng/ml) at 37 °C in humid air with 5% CO_2. Immortalized cells were obtained by frequent subculture for over a year. Cell lines from different dishes were subcloned by limiting dilution. The murine stromal cell lines are summarized in Fig. 1. In vitro calcification assay, osteocalcin production, and evaluation of parathyroid hormone (PTH) response were performed as previously described [10].

2.2. Measurement of alkaline phosphatase (ALP)

KUSA/A1 and MHCTC-E1 cells were analyzed by ALP assay as described [20].

2.3. Communication assay

Intercellular transfer Fluorescent Lucifer Yellow CH (Sigma, St. Louis, Missouri) was measured after the direct microinoculation of the dye into a KUSA-A1 cell as previously described [12,13].

2.4. RNA extraction and Northern blotting

RNA was prepared by homogenizing the specimens in guanidinium isothiocyanate, followed by centrifugation over a cesium chloride cushion as previously described [6,14]. The RNA was then electrophoresed in a 1.0% agarose gel, transferred to a nylon filter, and hybridized with a cDNA insert labeled with ^{32}P-dCTP by the random-primer method at 65 °C for 14–16 h in a buffer containing 5× SSPE (1× SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4/Na_2HPO_4 (pH 7.4), and 1 mM EDTA), 5× Denhardt's solution (1× Denhardt's solution is 0.02% Ficoll polyvinylpyrrolidone and 0.02% BSA), 0.02% poly(A), and 1% SDS. The blots were washed with 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.4) containing 1% SDS at room temperature and 65 °C. Final washing was performed with 0.1× SSC containing 0.1% SDS at 65 °C.

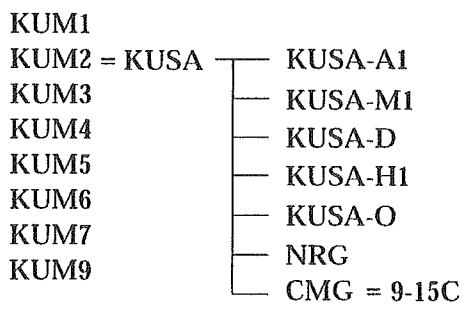

Fig. 1. The murine stromal cell lines. The clonal cells were designated KUM1 [6], KUM2/KUSA [6,9], KUM3-7 [6], and KUM9 [6,9]. The KUSA-A1 [6,11], KUSA-M1 [6], KUSA-D [6], KUSA-H1 [6], KUSA-O [6], and NRG [9] cells were subclones of KUSA cells. The CMG/9-15C cells are a subclone from KUSA cells that had been after exposed to 5-azacytidine [7,11]. The cell names are available at http://1985.jukuin.keio.ac.jp/umezawa/kum/kumh.html.

The blots were exposed to X-ray film at 80 °C with an intensifying screen. RNA blot analysis was carried out as previously described [6,14].

2.5. Transmission electron microscopy (TEM)

KUSA-A1 cells cultured in vitro for 14 days post-confluence were examined by TEM. The samples were fixed in 2.5% glutaraldehyde postfixed in 1% osmium tetroxide. They were rinsed in water, dehydrated, and embedded in epoxy resin. Ultrathin sections (70–90 nm) were cut and stained with 2% uranyl acetate and Reynold's lead citrate before being examined with a JEM-1200 EX microscope (JOEL Co., Japan) at 80 kV.

2.6. Inoculation of cells into mice

To determine the ability of KUSA, KUM3, KUM4, and NIH-3H3 cells to differentiate in vivo, freshly scraped KUSA-MTAg, KUM3-MTAg, KUM4-MTAg, and NIH3T3-MTAg cells (10^7 cells) were subcutaneously inoculated into Balb/c *nu/nu* mice (Sankyo Laboratory, Hamamatsu, Japan) as previously described [6]. Animals were sacrificed by cervical dislocation between 4 and 8 weeks after inoculation.

To determine the potential osteogenic activity of KUSA-A1 cells in vivo, freshly scraped KUSA-A1 cells (10^7 cells) were subcutaneously inoculated into severe combined immunodeficient (SCID) mice (Nippon CLEA, Hamamatsu, Japan). Subcutaneous specimens were resected between 1 and 4 weeks after inoculation and decalcified for a few days in formic acid. The implants were embedded in paraffin. Paraffin sections were deparaffinized, hydrated, and stained with hematoxylin and eosin.

All animals received human care incompliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research, and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resource and published by the US National Institute of Health (NIH Publication No. 86-23, revised in 1985). The operation protocols were accepted by the Laboratory Animal Care and the Use Committee of the National Research Institute for Child and Health Development, Tokyo, and Keio University School of Medicine.

3. Results

3.1. In vitro characterization of KUSA-A1 cells, single-cell-derived mature osteoblasts

In vitro, calcification by KUSA-A1 cells gradually increased during the culture period (Fig. 2A), and the amount of osteocalcin released into the culture medium also increased (Fig. 2B). The KUSA-A1 cells responded to PTH

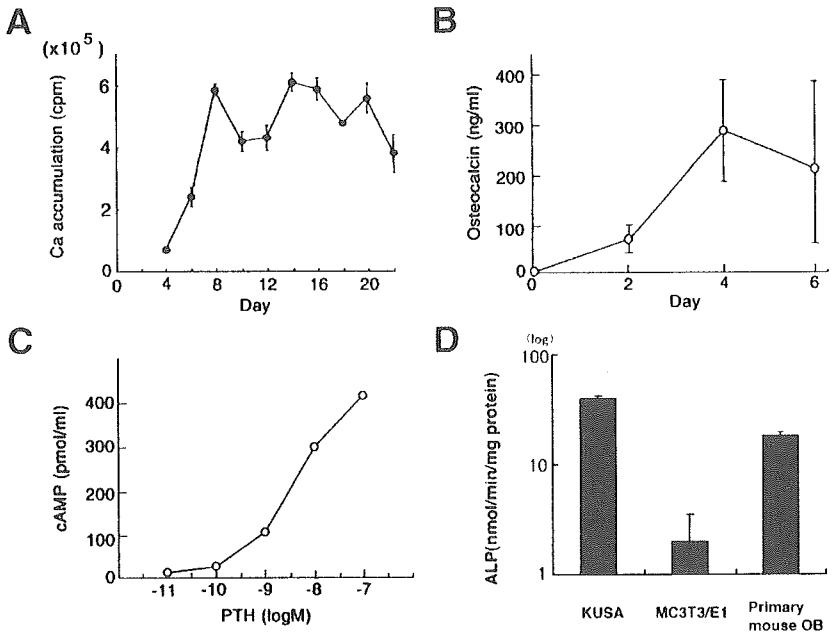

Fig. 2. Characteristics of KUSA-A1 cells as an osteoblast model. (A) Quantitative analysis of calcium deposition by KUSA-A1 cells. (B) Bone Gla protein (Osteocalcin) secretion by KUSA-A1 cells. (C) cAMP production after exposure to PTH. The KUSA-A1 cell response to PTH was assessed by measuring cAMP production. (D) ALP activity in KUSA-A1 cells and MC3T3-E1 cells at 7 days.

in a dose-dependent manner (Fig. 2C). The ALP activity of KUSA-A1 cells was approximately ten-fold higher than in MC3T3-E1 cells at 7 days (Fig. 2D). The calcium deposition and osteocalcin release indicated that KUSA-A1 cells are mature osteoblasts or osteocytes.

3.2. Morphology of KUSA-A1 cells: transmission electron microscopy (TEM) in vitro

Bone nodules [15] that had developed in KUSA-A1 cells cultured in medium supplemented with 10 mM beta-glycerophosphate were fixed and stained in situ by the von Kossa technique (Fig. 3A). The bone nodules consisted of an eosinophilic matrix containing ovoid cells resembling osteocytes, and the KUSA-A1 cells were arranged at the periphery of the nodule in the form of a periosteum-like cell layer. Mineral deposition by the KUSA-A1 cells demonstrated that they have the capacity to differentiate into osteoblasts, to deposit hydroxyapatite in well-developed bone matrix, and to express the differentiated state of osteoblasts.

Ultrastructurally, the matrix was electron-dense and was clearly produced by the cells in the bone nodules (Fig. 3B). The extracellular matrix produced by KUSA-A1 cells was

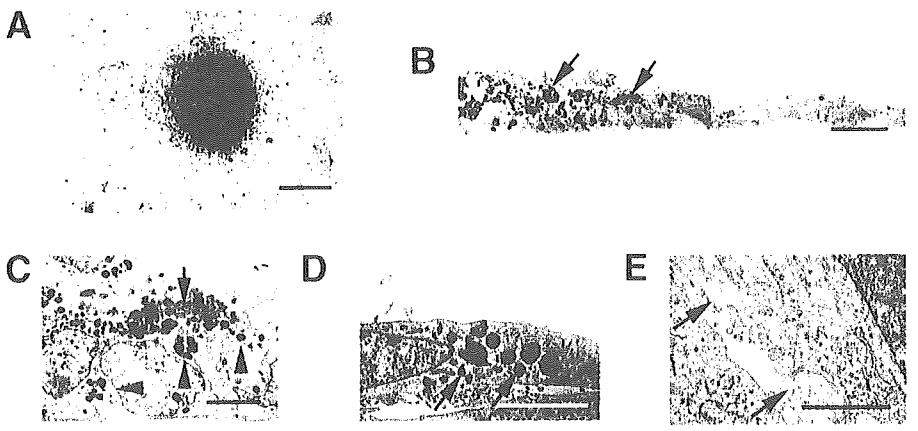

Fig. 3. Ultrastructural analysis of KUSA-A1 cells in culture. (A) KUSA-A1 cells 30 days after confluence. KUSA-A1 cultured in medium supplemented with beta-glycerophosphate was stained in situ by the von Kossa technique. Discrete mineralized nodules are seen. (B) Transmission electron micrograph (TEM) of a nodule present in a KUSA-A1 culture 14 days post-confluence. The extracellular matrix produced by KUSA-A1 cells was observed on the cell surface [arrow]. (C) High-power view of panel B. TEM revealed that the sphere of extracellular matrix produced by KUSA-A1 cells contained a small granular material [arrowhead]. (D) High-power view of a KUSA-A1 cell. Many lysosomal myelin-like figures [arrow] and rough endoplasmic reticulum are seen in its cytoplasm. (E) High-power view of KUSA-A1 cells. Abundant collagen fibrils are visible in the intercellular spaces [arrow], and the cytoplasm contains rough endoplasmic reticulum. Scale bars: 1 mm (A), 20 μm (B), 5 μm (C, D), and 1 μm (E).

observed on the cell surface (Fig. 3C). The cytoplasm of the spindle-shaped cells contained extensive, dilated, and rough endoplasmic reticulum and myelin-like lysosomes (Fig. 3D). Lysosome-rich cells were found in the bone nodules. These cells are probably similar to those observed in vivo [16] and in the primary bone cell cultures [17]. The cells produce abundant, orthogonally oriented collagen fibrils in the intercellular spaces (Fig. 3E), and these fibrils were also found in membrane folds.

3.3. Gap-junctional communication in KUSA-A1 cells

Gap-junctional communication is required for the osteoblast maturation process in culture [18,19], and we used the dye-transfer method to assess the gap-junctional communication between KUSA-A1 cells. When Fluorescent Lucifer Yellow CH was injected into a KUSA-A1 cell, the dye was rapidly transferred to the neighboring cells (Fig. 4A and B), implying that KUSA-A1 cells constitutively communicate with each other via gap junctions.

The expression of gap-junction genes by KUSA cells was investigated by blot hybridization of stromal cell RNA with a connexin 43 cDNA probe. H-1/A of the marrow preadipocyte cell line is a positive control. Distinct 3.0-kb mRNA bands were observed in all of the marrow stromal cell lines when tested with the connexin 43 cDNA probe (Fig. 4C), but no bands were detected in any of the cells when tested with a connexin 32 probe (data not shown).

3.4. Microscopic examination of large subcutaneous masses

NIH3T3, KUM3, KUM4, and KUSA cells transfected with MTAg were designated NIH3T3-MTAg, KUM3-MTAg, KUM4-MTAg, and KUSA-MTAg cells, respectively. Four weeks after subcutaneous inoculation of 10^7 NIH3T3-MTAg, KUM3-MTAg, KUM4-MTAg, KUSA-MTAg, and untransfected KUSA-A1 cells [6] into immunodeficient mice, masses had formed in the subcutaneous tissue. The masses were of three types histologically. Sarcoma-type masses were induced by NIH3T3-MTAg, KUM3-MTAg, and KUM4-MTAg cells and diagnosed as fibrocytic sarcoma; they did not contain bone. The tumors consisted of pleomorphic mesenchymal cells, including multinucleated bizarre giant cells (Fig. 5A and B). The second type of masses was sarcomas with complete bone formation. KUSA-MTAg cells induced sarcomas, most of which contained well-defined complete bone. These sarcomas exhibited an irregular woven pattern of pleomorphic spindle cells that included multinucleated tumor giant cells (Fig. 5C–E). The third type of masses consisted of complete bone and bone cavities with trilineage hematopoiesis. Untransfected KUSA cells formed bone (Fig. 5F–H). Untransfected KUSA-A1 cells, a subclone of the KUSA cells, also formed complete bone 4 weeks after inoculation (Fig. 5I and J). No sarcomatous cell proliferation was observed.

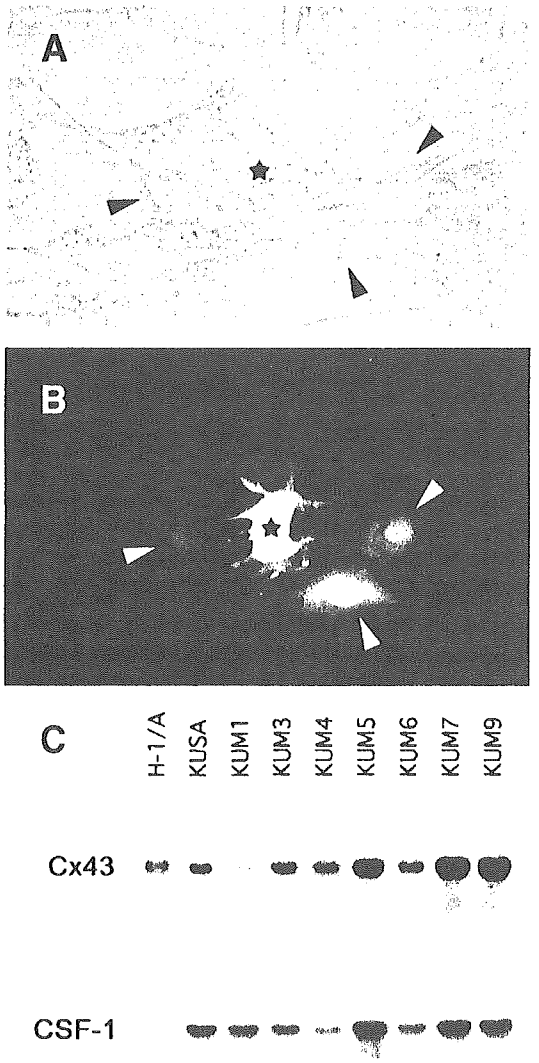

Fig. 4. Dye transfer in KUSA-A1 cells at semiconfluence. Intercellular communication was assessed by the dye transfer method. A and B: Phase-contrast micrograph (A) and fluorescence photograph (B) of KUSA-A1 cells. A KUSA-A1 cell [asterisk] was injected with Lucifer Yellow CH, and the dye was transferred to several adjacent cells. The dye has spread to three first-order-neighboring cells [arrowheads]. (C) Analysis of connexin 43 (Cx. 43) expression by KUSA, KUM1, KUM3, KUM4, KUM5, KUM6, KUM7, and KUM9 cells at semiconfluence. The same blot, which was rehybridized with the CSF-1, is shown for reference [6] in the lower panel.

3.5. Ossification by KUSA-A1 is membranous

To determine whether the ossification by KUSA-A1 is membranous or enchondral, we followed the time-course of KUSA-A1 ossification in the subcutaneous tissue of SCID mice after injecting cells 7 days post-confluence. The injected cells had produced a meshwork of collagen fibers and amorphous ground substance (osteoid matrix) at 1 week (Fig. 6A), and the fibers and ground substance markedly increased and became larger. The matrix was highly calcified at 4 weeks (Fig. 6B and C), and marrow cavities had formed inside the KUSA-A1 bone.

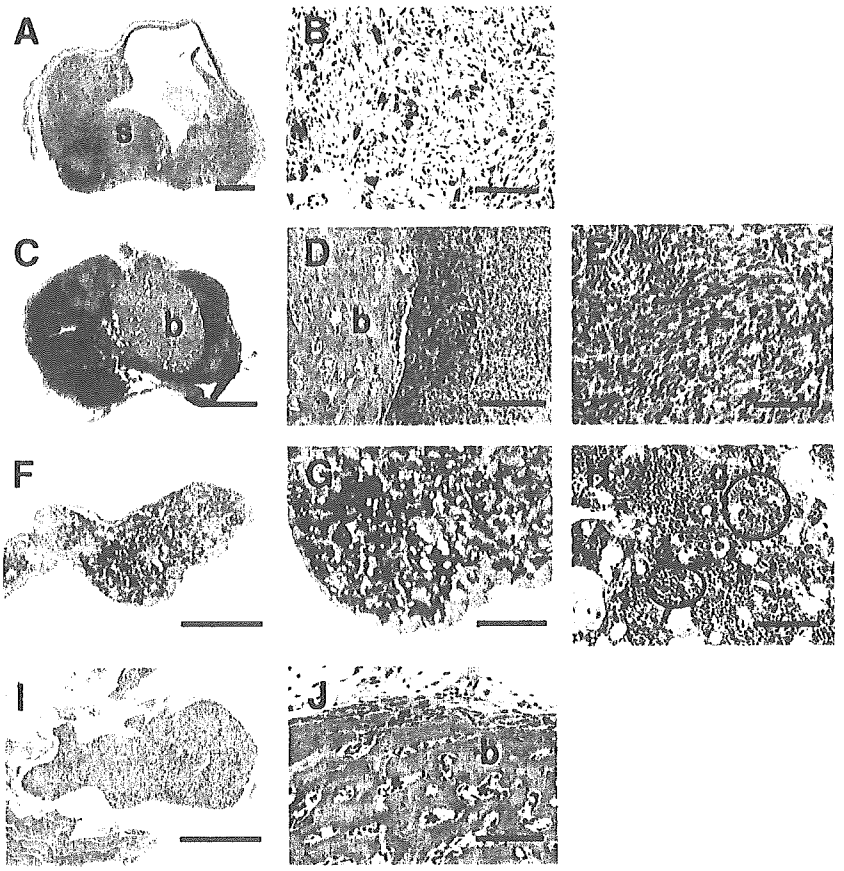

Fig. 5. Microscopic appearance of subcutaneous masses 4 weeks after subcutaneous inoculation of NIH3T3-MTAg (A, B), KUSA-MTAg (C, D, E), KUSA (F, G, H), and KUSA-A1 cells (I, J). (A) Mass of the first type. It is a sarcoma [s] without bone. (B) High-power view of a part of panel (A). The tumor consists of pleomorphic mesenchymal cells, including multinucleated bizarre giant cells. (C, D) Mass of the second type. The mass is composed of sarcoma [s] and well-defined bone [b]. (E) High-power view of part of the tumor in panel (D). The tumor shows proliferation of pleomorphic spindle cells, including giant multinucleated tumor cells. (F, G) Mass of the third type. The mass is composed of complete bone with bone cavities [asterisks], but there is no sarcomatous proliferation. (H) High-power view of part of the mass in panel (G). Trilineage hematopoietic cells (granulocytes [g], erythrocytes [e], and megakaryocyte [arrow]) in the bone cavities inside the mass are shown. (I) Mass of the third type. The mass is composed of complete bone with bone cavities [asterisks], but there is sarcomatous proliferation. (J) High-power view of panel (I). H–E stain. Scale bars: 2 mm (A, C, F, K), 0.5 mm (G), 200 μm (D), and 100 μm (B, E, H, J).

Histological examination of complete bone mass revealed highly dense bone trabeculae, and hematopoietic cells were observed in the bone marrow, as reported previously [6,10]. No cartilage had formed in any of the KUSA-A1-produced bones, indicating that the osteogenesis by KUSA-A1 is membranous ossification rather than enchondral ossification. Osteogenesis was monitored radiographically at scheduled times after the inoculation of $3-5\times10^7$ KUSA-A1 cells, and the results showed a gradual increase in newly formed bone 2 weeks after the injection, and complete bone density 4 weeks after the inoculation.

We then monitored the fate of KUSA-A1 bone transplanted into subcutaneous tissue and the abdominal cavity and found that the ectopic KUSA-A1 bone remained unchanged in size and shape for 12 months at both sites. Histological examination revealed complete functional hematopoiesis by ectopic KUSA-A1 bone in the subcutaneous tissue, but not in the abdominal cavity.

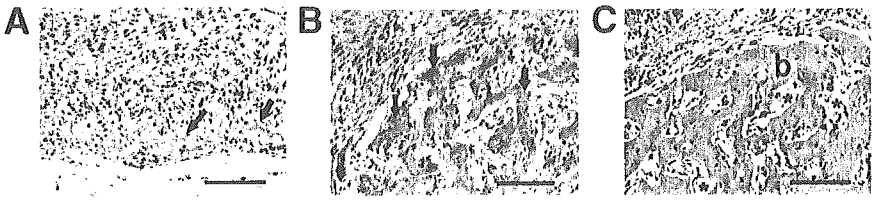

Fig. 6. The process of osteogenesis by KUSA-A1 cells in vivo. (A) One week after the inoculation of 10^7 KUSA-A1 cells, bone formation [arrows] was observed around the periphery of the mass at all inoculation sites. It is noteworthy that no chondrogenesis was observed during early osteogenesis. (B) Two weeks after inoculation. There is more bone matrix [arrows] at week 2 than at week 1. (C) Four weeks after inoculation, the mass consists of complete bone tissue [b] and bone cavities [asterisks]. H–E stain. Scale bars: 100 μm.

4. Discussion

4.1. KUSA-A1 cells can be used as a model for developmental bone formation and abnormal ossification

The sequence of KUSA-A1 bone formation is as follows: deposition of matrix by KUSA-A1 cells that subsequently become mineralized, deposition of bone as a network of immature or woven trabeculae, and formation of bone marrow or conversion of the spongiosa into primary cortical bone by filling of spaces between the trabeculae. This process results in the formation of cancellous bone and bone marrow. Importantly, the osteogenesis by KUSA-A1 cells was irreversible and reproducible, and the transplanted KUSA-A1 cells never transformed into malignant cells, formed any abnormal extracellular matrices, or induced any significant inflammatory reactions. It is noteworthy that the osteogenesis by KUSA-A1 cells was not mediated by chondrogenesis, and it was therefore considered to be membranous ossification. Thus, the unique characteristics of KUSA-A1 cells provide an opportunity to analyze the process of membranous ossification in an experimental system in detail.

In fetal life, primary ossification centers form by one of two processes: endochondral ossification or membranous ossification. Endochondral ossification refers to bony replacement of cartilage and is the mode of formation of the long bones. During membranous ossification, mesenchymal cells form membranes within which ossification occurs, and this is the mode of formation of the scapula and skull and, in part, of the clavicle and pelvis. After birth, bone growth continues by both endochondral and membranous ossification. Further endochondral ossification occurs in the physes and results in continuous longitudinal growth of the long bones until skeletal maturity. KUSA-A1 cells were obtained from a long bone the femur, but formed bone by membranous ossification. There are also cells responsible for the periosteal membranous ossification in tubular bones, which model the diaphyseal cortex, and KUSA-A1 cells may be derived from such a minor cell population in long bones that is responsible for periosteal bone formation or callus.

The process of ossification by KUSA-A1 cells may also serve a model for ectopic or heterotopic ossification, such as soft tissue ossification, ligament ossification, and heterotopic bone formation in a number of disorders, including central nervous system and spinal cord disorders, probably as a consequence of immobilization, a model of myositis ossificans progressiva, which often develops after a traumatic event, and a model of ossification of the posterior longitudinal ligament of the spine, which is characterized by the presence of a linear band of ossification along the posterior margin of vertebral bodies and intervertebral discs, especially in the cervical spine.

4.2. Possibility of implanted-cell transformation

The lack of tumor formation or in vivo transformation after implantation of KUSA-A1 cells into immunodeficient mice does not mean that the donor cells are incapable of transforming after implantation, at least in mice. The observation period after cell implantation into mice is usually less than 1 year because the life span of mice is approximately 2 years. By contrast, since patients who receive cell-based therapy may survive for decades, the possibility of implanted-cell transformation cannot be ignored. Care should be exercised when using donor cells transfected with certain genes for the therapeutic purposes, because zero risk of implanted-cell transformation cannot be achieved, even though human non-tumor cells seldom transform in vitro or in vivo without gene transfection.

4.3. Can cancellous bone grafting be replaced by osteoblast-based therapy?

The transplantation of bone from one site to another usually promotes osteogenesis or provides structural stability. Grafts may be used to fill bone defects, promote union, or provide material for arthrodesis. The donor sites most frequently used for grafts are the iliac crest, tibia, and fibula. Depending on circumstances, the patient or animal may receive either a cancellous bone graft or a cortico-cancellous graft. Cancellous bone grafts are the gold standard for bone defects. They have greater capacity to induce new bone formation and, thus, are considered to be generally much more successful in inducing new bone formation than osteoblast-based therapy. Osteoblast-based therapy is poorer than cancellous bone grafts in providing structural stability.

However, complications occur after cancellous bone grafting and include fracture at the donor site, intraoperative bleeding, and postoperative pain. Graft failure may lead to progressive bone resorption and, ultimately, to disappearance of the graft. Follow-up radiographs show healing of iliac donor sites with sclerosis at the margins of the bone defects. Painful excrescences of the bone may develop at the donor site. Loss of the sharp margins between the graft and host bone on radiographs generally signifies graft healing, and the persistence of a thin residual radiolucent area between the graft and the host bone suggests fibrous union.

It would be interesting to assess the possibility of using mature osteoblasts as a therapeutic agent. The inoculation of isolated mature osteoblasts into a bone defect or fracture site would be a more efficient means of accelerating bone fusion with minimal invasion than inoculation of unfractionated marrow cells into fracture sites. The critical step in realizing osteoblast-based therapy will be the isolation of human counterparts to KUSA-A1 cells and growing them in sufficient numbers in culture. The separation of osteoblasts from human marrow stroma [21] and inoculation of the cells with an appropriate scaffold will provide new methods of

osteogenesis engineering without any of the complications associated with cancellous bone grafts.

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Characterization and gene transfer in mesenchymal stem cells derived from human umbilical-cord blood

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It has been shown that the stromal-cell population found in bone marrow can be expanded and differentiated into cells with the phenotypes of bone, cartilage, muscle, neural, and fat cells. However, whether mesenchymal stem cells (MSCs) are present in human umbilical-cord blood (UCB) has been the subject of ongoing debate. In this study, we report on a population of fibroblastlike cells derived from the mononuclear fraction of human UCB with osteogenic and adipogenic potential, as well as the presence of a subset of cells that have been maintained in continuous culture for more than 6 months. These cells were found to express CD29, CD44, CD90, CD95, CD105, CD166, and MHC class , but not CD14, CD34, CD40, CD45, CD80, CD86, CD117, CD152, or MHC class II. We also compared gene expression after gene transfer using lenti- and adenoviral vectors carrying the green fluorescence protein to the MSCs derived from UCB because a reliable gene-delivery system is required to transfer target genes into MSCs, which have attracted attention as potential platforms for the systemic delivery of therapeutic genes. The lentiviral vectors can transduce these cells more efficiently than can adenoviral vectors, and we maintained transgene expression for at least 5 weeks. This is the first report showing that UCB-derived MSCs can express exogenous genes by way of a lentivirus vector. These results demonstrate that human UCB is a source of mesenchymal progenitors and may be used in cell transplantation and a wide range of gene-therapy treatments. (J Lab Clin Med 2005;146:271-278)

Abbreviations: adeno-GFP = recombinant adenovirus expressing the green fluorescent protein gene Ax1CAGFP; BBS = BBS 50 mmol/L BES, 280 mmol/L NaCl, 1.5 mmol/L Na_2HPO_4; BM = bone marrow; DMEM = Dulbecco's modified Eagle medium; EDTA = ethylenediaminetetraacetic acid; FACS = fluorescence-activated cell sorting; FBS = fetal bovine serum; FITC = fluorescein isothiocyanate; GFP = green fluorescent protein; IU = infectious unit; lenti-GFP = lentivirus expressing the green fluorescent protein; MNC = mononuclear cell; MSC = mesenchymal stem cell; MSCGM = MSC growth medium; PBS = phosphate-buffered saline solution; TBST = Tris-buffered saline solution containing Tween-20; UCB = umbilical-cord blood

Human UCB has been reported to contain stem/progenitor cells at concentrations greater than or equal to those in bone marrow and adult peripheral blood.[1] UCB-cell transplantation for various blood diseases has recently been successful, with a lower incidence of graft-vs-host disease than that seen in many conventional treatments.[2] In these clinical applications, hematopoietic stem cells can differentiate to

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mature blood cells. It has been reported that CD34-positive cells, well-known stem-cell markers, have the capacity to differentiate into all blood-cell lineages.[3] After birth, UCB contains hematopoietic precursors, and it has become an important alternative source of hematopoietic stem cells for transplantation.

It is also widely accepted that MSCs, as well as hematopoietic stem cells, are present in BM. MSCs can differentiate into multiple lineages, including osteoblasts, chondrocytes, and adipocytes. This rare characteristic has resulted in speculation about the possibility of using BM-derived MSCs as a source of cell therapy for more than 5 years.[4–7] Recently human UCB cells were also reported to differentiate into a variety of cell types, such as hepatocytes and nerve and muscle cells, as well as bone, cartilage, and fat cells from the nonhematopoietic cell fraction that appears to comprise MSCs.[8–11] However, controversy continues as to whether human UCB contains MSCs.[12,13]

A reliable gene-delivery system is required to facilitate the transfer of target genes into MSCs that express therapeutic proteins, which cause tissues of mesenchymal origin to express gene products essential for tissue regeneration and repair. Previous studies involving nonviral vectors and replication-deficient recombinant adenovirus and adenovirus-associated viral vectors have demonstrated highly efficient gene transfer to MSCs.[14–19] Recently lentivirus has received considerable attention as a possible vector in the field of gene therapy. Although lentivirus is one of the subfamilies of retrovirus species, lentivirus vectors based on the human immunodeficiency virus genome have many advantages over retrovirus vectors, particularly their ability to transduce nondividing cells.[14–19] Furthermore, lentivirus vectors can integrate in the host genome and replace the natural glycoprotein envelope with G-glycoprotein of vesicular stomatitis, allowing the viral particles to infect a broad range of host-cells types. Therefore exogenous gene expression in target cells would be extended to much longer periods after gene transfer, regardless of the type of target organ, tissue, or cell. The lentivirus vector may therefore be considered ideal for long-term exogenous gene expression, especially for stem cells. Although the lentivirus vector has been proposed as a potential vector for gene transfer, and high-level gene expression of transduced stem cells of various types by viral and nonviral vectors has also been reported, transgene expression efficiency of MSCs derived from UCB infected with lentivirus vector has remained low.

In this study, we investigated the presence of multipotent progenitor cells in human UCB, similar to the cells derived from BM. We found that the mesenchymal progenitors in UCB do not express hematopoietic stem-cell or mononuclear-cell markers CD34, CD14, or CD45 and that they may be cultured and divided for periods of longer than 6 months. We also examined exogenous gene expression in UCB-derived MSCs, using lentivirus and adenovirus vectors because have been no reports as to whether lentivirus vector can transduce exogenous genes, yield enough gene expression to affect cell phenotype, or cause expression level in UCB-derived MSCs to persist. We hypothesize that lentiviral vectors facilitate extended periods of gene expression in MSCs. This is considered a useful characteristic for therapeutic applications because progenitor cells with human mesenchymal stem-cell characteristics may be used for both cell transplantation and gene therapy.

METHODS

Cell isolation and culture. Human UCB, 30 to 150 mL, was collected by way of venous puncture of the umbilical vein at the time of full-term delivery. Equal volumes of UCB and 6% hydroxyethyl starch (NIPRO, Osaka, Japan) were mixed in sterile centrifuge tubes and left to stand for 90 minutes. Red blood cells were allowed to settle by way of gravity. Nucleated cells were then obtained from the supernatant. After being washed twice with sterilized PBS, isolated UCB cells were primarily cultured in MSCGM (Cambrex Bio Science, Walkersville, Md). The cells were seeded at a density of 1×10^6 to 10^7 cells/cm^2, and the medium was changed after 5 days. Nonadherent cells were discarded. Thereafter, half of the medium was changed at weekly intervals. Cells were harvested with the use of 0.25% trypsin and 1 mmol/L EDTA when they reached 60% to 70% confluence, then replated at a density of 1000 to 2000 cells/cm^2. We assessed the structure of adherent cells with the us eof a phase-contrast microscope (Olympus, Tokyo, Japan). Cells were first harvested by means of cytospin centrifugation and then stained with May-Giemsa stain (Wako Pure Chemical Industries, Osaka, Japan). This research was carried out in accordance with the principles of the Declaration of Helsinki and under the approval of the ethical-review board of the National Center for Child Health and Development.

FACS analysis. We trypsinized the cells and then incubated them with fluorochrome-conjugated antibody, on ice, in the dark, for 20 minutes; rinsed them twice with cold PBS; and fixed them with cold, freshly prepared 2% paraformaldehyde (Sigma-Aldrich, St Louis, Mo). FACS analysis was carried out with the use of a Becton-Dickinson Immunocytometry System and a FACS Calibur cytometer (Becton Dickinson, San Jose, Calif) with a minimum of 10,000 events counted. The following human antibodies were used: CD14-phycoerythrin, CD29FITC, CD40-FITC, CD44-FITC, CD80-PE, CD86-FITC, CD95-FITC, CD117-PE, CD152-PE, MHC class I–FITC, MHC class II–phycoerythrin (Becton-Dickinson), CD34-phycoerythrin, CD45-phycoerythrin (Miltenyi Biotec, Germany), CD90-phycoerythrin, CD105-phycoerythrin, and CD166-phycoerythrin (Serotec, Oxford, UK).

Osteogenic potential. We plated cells at a concentration of 1500 cells/cm^2 in the growth medium. Osteogenesis medium (Cambrex) containing 0.1 μmol/L dexamethasone, 0.05 mmol/L ascorbic acid-2-phosphate, 10 mmol/L β-glycerophosphate) was applied 24 hours after plating. The medium was refreshed every 3 to 4 days. Osteogenesis was assessed after on day 21 of culture. The expression of collagen type I was detected with the use of cytochemial staining. The cells were fixed in equal volumes of methanol and acetone for 1 minute at room temperature, washed with TBST (Dakocytomation, Kyoto, Japan), incubated with 3% hydrogen peroxide (Wako Pure Chemical Industries), and blocked with 10% normal rat serum at room temperature. Washed cells were incubated with mouse anti–human collagen type I monoclonal antibody (Sigma-Aldrich Japan, Tokyo, Japan) at a 1:50 dilution with the use of Dako antibody diluent (Dakocytomation) for 30 minutes at room temperature. Antimouse peroxidase-conjugated IgG (Sigma-Aldrich Japan) was used as secondary antibody at a 1:100 dilution, and 3-3′-diaminobenzidine in chromogen solution (Dakocytomation) was applied to the cells, which were counterstained with vector methyl-green nuclear countersolution (Vector Laboratories, Burlingame, Calif). We detected the expression of alkaline phosphatase by means of cytochemical staining with an alkaline phosphatase staining solution (0.1mg/mL Nafthol AS-MX phosphatase, 0.6mg/mL Fast-blue BB salt, 0.5% *N*-dimetyl-foramide, 2 mmol/L $MgCl_2$, 0.1 mol/L Tris-HCl; pH 8.8). A blue precipitate denoted a positive reacion. The in vitro mineralization was detected with the use of Alizarin red S (40 mmol/L, pH 4.2) staining after fixation in ice-cold ethanol (70%) for 1 hour.

Adipogenic potential. Cells were grown in adipogenic induction medium (Cambrex) containing h-insulin, L-glutamine, dexamethasone, indomethacin, and 3-isobuty-l-methyl-xanthine for 14 days. Adipocytes were visualized after fixation in formaldehyde buffer (4%) for 10 minutes followed by washing with isopropanol (3%). The cells were then stained with fresh oil red O (Sigma-Aldrich).

Production of lentiviral and adenoviral vectors. A lentivirus vector expressing GFP was produced by means of transient transfection into 293T cells in accordance with a calcium phosphate transfection protocol. We seeded 1×10^7 293T cells in 15-cm-diameter dishes and treated them with 0.01% poly-L-lysine (Wako) for 24 hours before transfection in DMEM (Invitrogen, Carlsbad, Calif) with 10% FBS (Invitrogen) and 75 mg/L kanamycin (Meji Seika, Tokyo, Japan) in a 5% CO_2 incubator at 37° C. We used 33.3 μg of plasmid DNA for the transfection in each of the dishes, which contained 15.3 μg of transfer vector plasmid pCS-CDF-CG-PRE (a gift from Dr Miyoshi, RIKEN; containing the GFP gene); 9 μg of packaging plasmid, pMDLg/p; 4.5 μg of Rev plasmid, pRSV-Rev; and 4.5 μg of envelope plasmid, pMD.G. To achieve precipitation, we used the following procedures. First we added the plasmids to a final volume of 3645 μL of sterilized water and 135 μL of 2.5 mol/L $CaCl_2$ and mixed them well. Next we added 1350 μL of 2×BBS, then mixed the DNA solution and incubated it for 20 minutes at room temperature. We resuspended the DNA solution by means of inversion and added it in drops to the culture dish, which we incubated in a 3% CO_2 incubator at 37° C. After 12 to 16 hours of incubation, the medium was replaced with 15 mL of DMEM containing 10 μmol/L forskolin (Wako), and the dish was then placed in a 5% CO_2 incubator at 37°C. The conditioned medium was collected after an additional 48 hours of incubation and filtrated through 0.45-μg cellulose acetate filters. The conditioned medium was ultracentrifuged twice, after which the virus pellet was resuspended in PBS and frozen at −80° C until it was needed for use in the experiment. The 293T cells were infected overnight in 6-well plates with serial dilutions of the conditioned medium from the 293T transient transfectants or with the concentrated viral stocks in the culture medium that was used for titration. The lentivirus titers were derived from the quantitative FACS (FACSort; Becton-Dickinson, San Jose, Calif) analysis performed with the 293T cells and expressed as IUs. Adeno-GFP was provided by the RIKEN BioResource Center.

Transduction of cells. MSCs (passage 2) grown in a flask for 1 week were detached with the use of trypsin-EDTA and replated in 6-well plates at a density of 5×10^4 cells in 1 mL of MSCGM per well. The medium was aspirated when the cells reached subconfluence. We added adeno-GFP and lenti-GFP diluted medium to the cells to achieve a multiplicity of infection of 20 (adeno-GFP) or 10 (lenti-GFP). After incubation at 37° C for 24 hours, we replaced the transduction medium with fresh MSCGM. We maintained the transduced MSCs by changing the medium every 3 days and replating the cells when they reached subconfluence. Cells were then analyzed for long-term expression of the transgene. The cells were kept for 5 weeks, with medium changes and passage before FACS was carried out.

RESULTS

Cell structure and proliferation. We cultured whole mononuclear fractions of UCB. Over the first 3 to 4 weeks, almost all of the stem cells died, leading to a very small viable cell population. The hematopoietic component of the culture survived for 4 to 6 weeks. The MSCs were identifiable as colonies of adherent cells with a fibroblastlike appearance, similar to those observed in bone marrow (Fig 1, *A* and *B*).

MSCs were replated at a density of 1000 cells/cm^2 by means of trypsinization when the cells reached 70% to 80% confluence. After repeated subcultures, the cultures contained a homogenous layer of fibroblastlike cells (Fig 1, *C* and *D*). These cells can be maintained in continuous culture for more than 6 months and 10 passages. Initially the growth rate was slow; it gradually increased between passages 2 and 7 and then decreased after passage 9, corresponding to 180 days of culture. We extrapolated a total expansion over 10 passages of 10^{11} cells (Fig 2).

Flow-cytometric characterization. We determined the immunophenotype of MSC to be monolayered single colony-derived adherent cells from UCB and compared

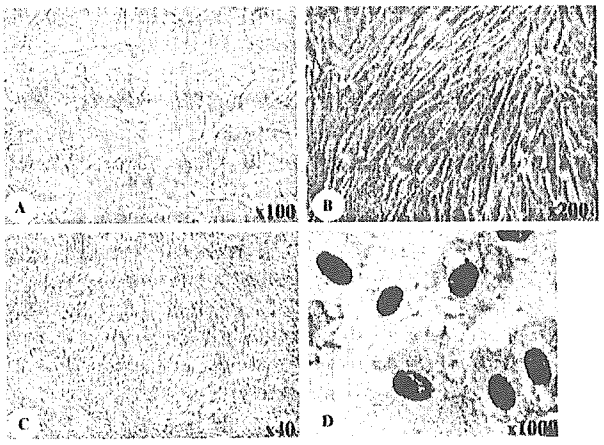

Fig 1. Structure of MSCs obtained from human UCB. Established, confluent MSCs in the culture displayed a typically homogeneous fibroblastlike pattern. (**A**) Phase-contrast view of MSCs cultured with MSCGM at passage 2, original magnification 100×. –; (**B**) same cells, original magnification 200×; (**C**) at day 60, as a confluent colony, original magnification 40×. (**D**) Cytospin of a trypsinized MSC colony stained with May-Giemsa stain. Data are representative of 3 independent experiments.

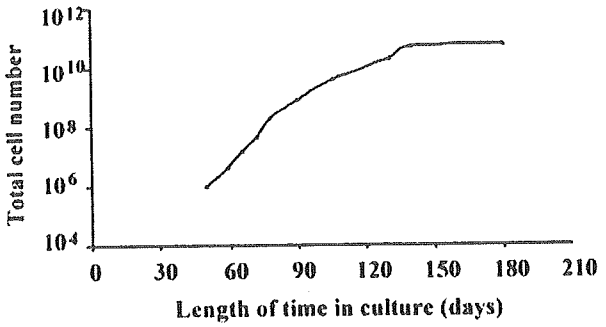

Fig 2. Growth curve of UCB-derived MSCs. Fifty days after we started the culture of UCB, the adherent fibroblast cells obtained from UCB reached subconfluence. We extrapolated the growth of MSCs from the number of cells counted during subculture of the cells (1000 cells/cm^2). Total extrapolated growth over time demonstrates different rates of growth during the 50 to 180 days after culture.

it with that of UCB-derived MNCs by means of flow cytometry. The MSCs were larger than lymphocytes, requiring significant adjustment of the flow cytometry gating established for lymphocytes (data not shown). The immunophenotypical profile of the MNC fraction changed significantly after the culture period, turning to typical MSC immunophenotypes that were positive for adhesion molecules, including β-1 integrin (CD29), hyaluronate receptor (CD44), Thy-1 (CD90), endoglin (CD105), and activated leukocyte cell adhesion molecule (CD166), as shown in Fig 3. Significantly, the MSCs were all CD45 negative, consistent with a nonhematopoietic origin and confirming that either hematopoietic or mononuclear cells had been depleted from the culture. In line with this finding, we detected no macrophages (CD14-positive cells) in the culture. Finally, the MSCs were all found to be negative for CD34 and stem cell–factor receptor (CD117). With respect to the markers known to participate strongly in immune activation, we found that MSCs were positive for MHC class I and Fas (CD95) but that they did not express MHC class II, one of the TNF receptor–superfamily members (CD40), B7-1 (CD80), B7-2 (CD86), cytotoxic lymphocyte–associated protein-4, or CTLA4 (CD152), as shown in Fig 3.

Osteoblstic differentiation potential of UCB-derived MSCs. In the presence of an osteogenic induction medium containing β-glycerophosphate, ascorbate, and dexamethasone, the UCB-derived MSCs exhibited clear changes in structure, from spindle-shaped to cuboidal, as they differentiated and mineralized. These cells stained positive for osteoblastic markers: collagen type I and alkaline phosphatase (Fig 4, *A* and *B*). When the confluent cells were incubated in osteogenic induction medium for 2 to 3 weeks, the UCB-derived MSCs formed a mineralized matrix in vitro, demonstrated by positive staining with Alizarin red S (Fig 4, *C*).

Adipogenic differentiation potential of UCB-derived MSCs. After 3 weeks of culture in the adipogenic induction medium containing h-insulin and dexamethasone, the UCB-derived MSCs formed adipocytes with lipid cytoplasm denoted by staining with oil red O (Fig 4, *D*).

Efficiency of gene transfer to UCB with the use of adenoviral and lentiviral vectors. We used efficient gene transfer involving adenoviral and lentiviral vectors containing the GFP gene for gene transfer into the primary UCB-derived MSCs. After infection with the adenoviral and lentiviral vectors, the MSC structure did not change. However, expression of the exogenous gene GFP was confirmed under fluorescence microscopy (Fig 5, *A–F*). We compared the efficiencies of expression of GFP 1, 3, and 5 weeks after infection by means of FACS analysis and analyzed the kinetics of gene expression of adeno-GFP and lenti-GFP. The percentage of adeno-GFP positive cells decreased noticeably after 1 week of infection. The percentage of GFP-positive cells infected with lenti-GFP was more than the percentage of adeno-GFP–positive cells after 5 weeks (Fig 6). The lentiviral vectors have a significant advantage over adenoviral vectors in the long-term stability of transgene expression in human UCB–derived MSCs.

DISCUSSION

Stem-cell transplantation represents a promising therapy for several degenerative and necrotic diseases.[4–7]

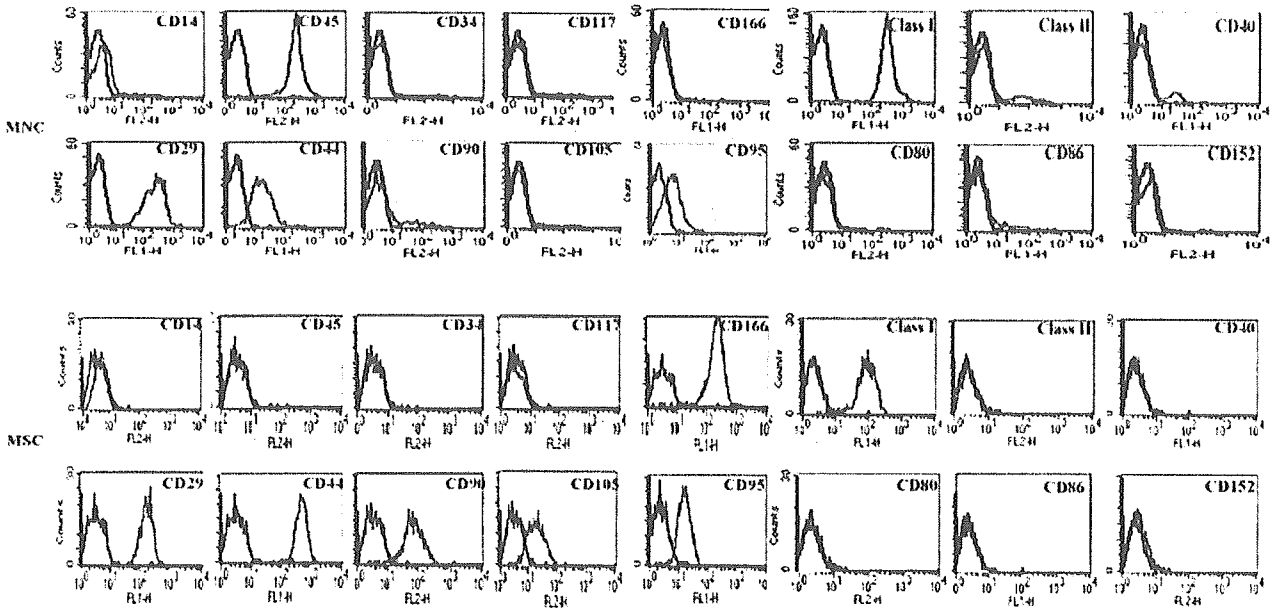

Fig 3. Immunophenotyping of MNC fraction and MSCs derived from UCB. Cells were labeled with FITC- or phycoerythrin-conjugated antibodies and examined by means of flow cytometory. Histograms demonstrating the expression of surface molecules were plotted against control (anti-IgG). The immunophenotypical profile of the MNC fraction changed significantly after 4 weeks of culture, to that of MSCs. MSCs expressed CD29, CD44, CD90, CD95, CD105, CD166, and MHC class I but not CD14, CD34, CD40, CD45, CD80, CD86, CD117, CD152, or MHC class II. Data are representative of 3 independent experiments.

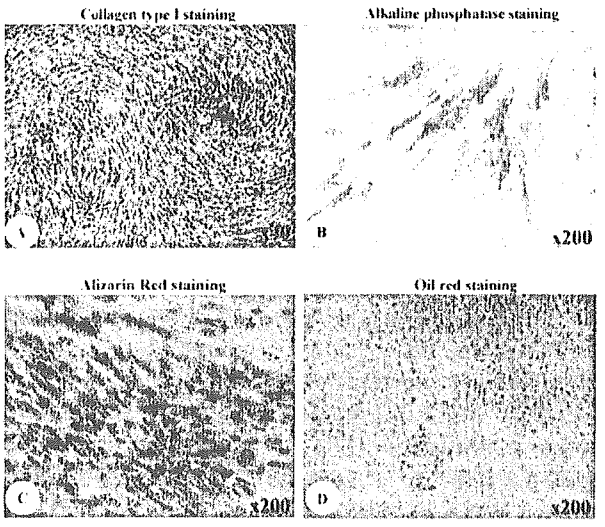

Fig 4. Expression of bone and fat phenotypes after exposure of MSCs to differentiation medium. MSCs were successfully differentiated along osteogenic and adipogenic lineages. Osteogenic differentiation was confirmed by means of (**A**) immunocytochemical staining for collagen type I (original magnification 40×), (**B**) cytochemical staining for alkaline phosphatase (original magnification 200×), and (**C**) the formation of a mineralized matrix stained with Alizarin red S (original magnification 200×). (**D**) Lipid-filled adipocyte detected with the use of oil red O staining (original magnification 200×). Data are representative of 3 independent experiments.

Many different types of stem cells reside in a range of tissue types. Among them, pluripotent stem cells have received a great deal of attention because of their capacity for multilineage differentiation. MSCs are fibroblastlike cells characterized by their capacity for rapid growth. They are also considered pluripotent stem cells.[20] MSCs are present in adult BM; they account for a small population but can be expanded exponentially under favorable conditions.[21,22] Some reports have also suggested that MSC may be effectively separated from many other tissues.[23–27]

Convenience of collection makes blood superior to other tissues as the source of therapeutic cells. In particular, UCB is considered as one of the best sources of therapeutic cells because the collection of these cells does not require invasive surgery. Hematopoietic stem cells are known to be present in UCB, but whether MSCs are also present in UCB remains a matter of dispute. Some reports have shown that UCB does not contain MSCs,[12,13,28–31] but the authors of many recent studies have reported that MSCs may be separated from UCB.[8,9,11,32,33] In addition, Lee et al[33] have demonstrated that immunophenotypes of clonally expanded cells derived from fresh UCB are similar to those of BM-derived MSCs. In spite of these reports, the presence of MSCs in UCB has yet to be unequivocally demonstrated.