REVIEW

Development of Cell-processing Systems for Human Stem Cells (Neural Stem Cells, Mesenchymal Stem Cells, and iPS Cells) for Regenerative Medicine

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Regenerative medicine using human stem cells is one of the newest and most promising fields for treating various intractable diseases and damaged organs. For clinical applications, choosing which human stem cells to use, i.e. according to tissue of origin and progenitor type, is a critical issue. Neural stem/progenitor cells (NSPCs) hold promise for treating various neurological diseases. We have shown that the transporter protein ABCB1 is predominantly expressed in immature human fetal NSPCs, and thus could be used as a phenotypic marker to investigate and monitor NSPCs in culture. We describe our proposed model for the *in vitro* proliferative process of aggregated human NSPCs and show that neurosphere enlargement and NSPC proliferation are mutually reinforcing. We have established that human neurospheres contain a heterogeneous cell population, knowledge that will contribute to the development of human neurospheres with desirable characteristics for clinical applications. Furthermore, decidua-derived mesenchymal cells (DMCs), which we isolated from human placenta, have unique properties as mesenchymal stem cells. They also generate a pericellular matrix (PCM-DM) that supports the growth and pluripotency of human embryonic stem cells and induced pluripotent stem cells (hiPS) cells. The newly developed re-programming techniques for generating hiPS cells should greatly contribute to cell therapies using human pluripotent stem cells, including those derived from DMCs. Our DMC-derived hiPS cells are a promising candidate source of allogeneic hiPS cells for clinical applications. We hope our findings will contribute to the development of cell-culture systems for generating human allogeneic stem cells for clinical use in regenerative medicine. (Keio J Med 59 (2) : 35–45, June 2010)

***Keywords*: neural stem cells, mesenchymal stem cells, iPS cells, cell processing system, regenerative medicine**

Introduction

Regenerative medicine, which involves advanced techniques using human stem cells that are processed *ex vivo*, is one of the newest and most promising fields for treating various intractable diseases and damaged organs. Many preclinical and clinical studies using stem cells from various somatic tissues have reported encouraging results,[1–3] and the first clinical trial using cells derived from human embryonic stem (hES) cells[4] for the treatment of spinal cord injuries has been launched.

For clinical applications, choosing which human stem cells to use, i.e. according to tissue of origin and progenitor type, is a critical issue.[1–3] Regenerative treatments using patient-derived autologous somatic stem cells are the most desirable for reasons of safety and ethics.[1–3]

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Recently developed re-programming techniques to generate human induced pluripotent stem (hiPS) cells may make the ideal cell therapy, the use of autologous pluripotent stem cells, a reality.[5] On the other hand, the transplantation of allogeneic human fetus-derived stem cells[1,2] or hES cells[4] is also a hopeful strategy, and the establishment of cell banking systems will increase the feasibility of using these cell sources.

In addition, the preparation and storing of human stem cells for clinical use requires time-consuming and difficult techniques. Such cells are strictly prepared using aseptic processing procedures to prevent contamination with microbiological and hazardous materials. Methods are still being developed for cell-processing and cell-banking systems to generate and maintain the various human stem cells intended for clinical use.

Neural Stem Cells (NSCs)

NSCs are characterized as self-renewing and multipotent progenitors of neurons, astrocytes, and oligodendrocytes.[1,2,6,7] During development, NSCs inhabit the ventricular zone and subventricular zone (SVZ) of the brain.[1,2,6,7] Postnatally, they reside in only particular regions of the brain, including the SVZ of the lateral ventricles, the dentate gyrus of hippocampus, and the olfactory bulb, and they can be found in all of these places throughout life in rodents.[1,2,6,7] *In vitro*, NSCs of rodent or human origin can be selectively expanded in serum-free culture medium supplemented with fibroblast growth factor 2 (FGF-2) and/or epidermal growth factor (EGF) as free-floating aggregates termed 'neurospheres,' which contain a population of immature neural progenitor cells (neural stem/progenitor cells; NSPCs).[8–10] Many preclinical studies indicate that the transplantation of cultured NSPCs holds promise as a treatment for various neurological diseases that could aid in functional recovery.[11–14] Furthermore, the first clinical trial using human NSPCs, defined by their expression of CD133,[15] to treat neuronal ceroid lipofuscinosis (NCL, often called Batten disease) has begun.[16] Although several studies have already described some of the biological properties of *in-vitro*-expanded human NSPCs and the molecules involved in the maintenance, proliferation, and/or differentiation of human NSPCs,[9,17] many others remain to be investigated.

The transporter protein ABCB1 is predominantly expressed in human NSPCs

Neurospheres are very heterogeneous cell aggregates consisting of stem cells, progenitor cells, and more-differentiated cells.[17–19] Because of their heterogeneity and the potential for tumorigenesis or other unexpected effects of transplantation, it is very important to establish strict standards for determining and controlling the quality of the human NSPCs used for investigations and therapies.

ABCB1 (MDR1/PGY1) is a member of the ATP-binding cassette (ABC) transporter superfamily and was originally characterized by its ability to confer a multidrug resistance (MDR) phenotype on cancer cells.[20–22] In the mammalian central nervous system, ABCB1 is expressed in cells at the blood-brain barrier, where it presumably helps to transport compounds that cannot be removed by diffusion out of the brain.[21,23] It is highly expressed in hematopoietic stem cells, which ABCB1 may protect from toxins. ABCB1 is thought to play important roles in various immature stem cells, and it is recognized as a functional marker molecule for some stem cells.[22,24] We previously reported that functional ABCB1 is expressed in human NPSCs *in vitro*, and not in differentiated cells.[22,25] Recently we performed a detailed examination of ABCB1's expression on human NSPCs *in vitro* and in human fetal brain tissues, and analyzed the cellular properties of the human NSPCs expressing ABCB1.[26]

In our analysis, we used four different phenotypic markers (ABCB1, CD133,[15] Nestin,[27,28] and CD24[15]) and showed that most cells (over 90%) in human neurospheres express Nestin, suggesting that the neurosphere-forming cells are immature. About 68.6% (34.8/34.8+15.9) of the ABCB1-positive NSPCs co-expressed CD133 on their cell surface (**Fig. 1**).[26] In the opposite analysis, about 73.1% (34.8/34.8+12.8) of the CD133-positive NSPCs also co-expressed ABCB1 on their cell surface (**Fig. 1**).[26] This analysis revealed that two thirds of the cells expressing ABCB1 also express CD133 and *vice versa*, and the remaining one-third expresses either ABCB1 or CD133. Although about 24.5% (14.5/14.5+45) of the ABCB1-positive NSPCs co-expressed CD24 on their cell surface, its level was relatively low compared to the ABCB1 non-expressing NSPCs and the expression of CD24 in human NSPCs was in contrast to that of ABCB1 (**Fig. 1**).[26] This finding showed that cells expressing both ABCB1 and CD24 make up a minor population in neurospheres. Thus, the four phenotypic markers used in our analysis are applicable to the quantitative analysis of heterogeneity in human neurospheres (**Fig. 1**). Furthermore, the predominant expression of ABCB1 in immature human fetal NSPCs *in vitro* indicates that ABCB1 might serve as a good functional marker for human NSPCs.

Effect of cell aggregation on the growth of human NSPCs

Neurospheres can form from the clonal growth of a single NSC, under suitable culture conditions (**Fig. 2A**), and it has been long assumed that neurospheres typically arise as such clones.[8,9] Furthermore, in many studies, the self-replication and neurosphere-forming potential of

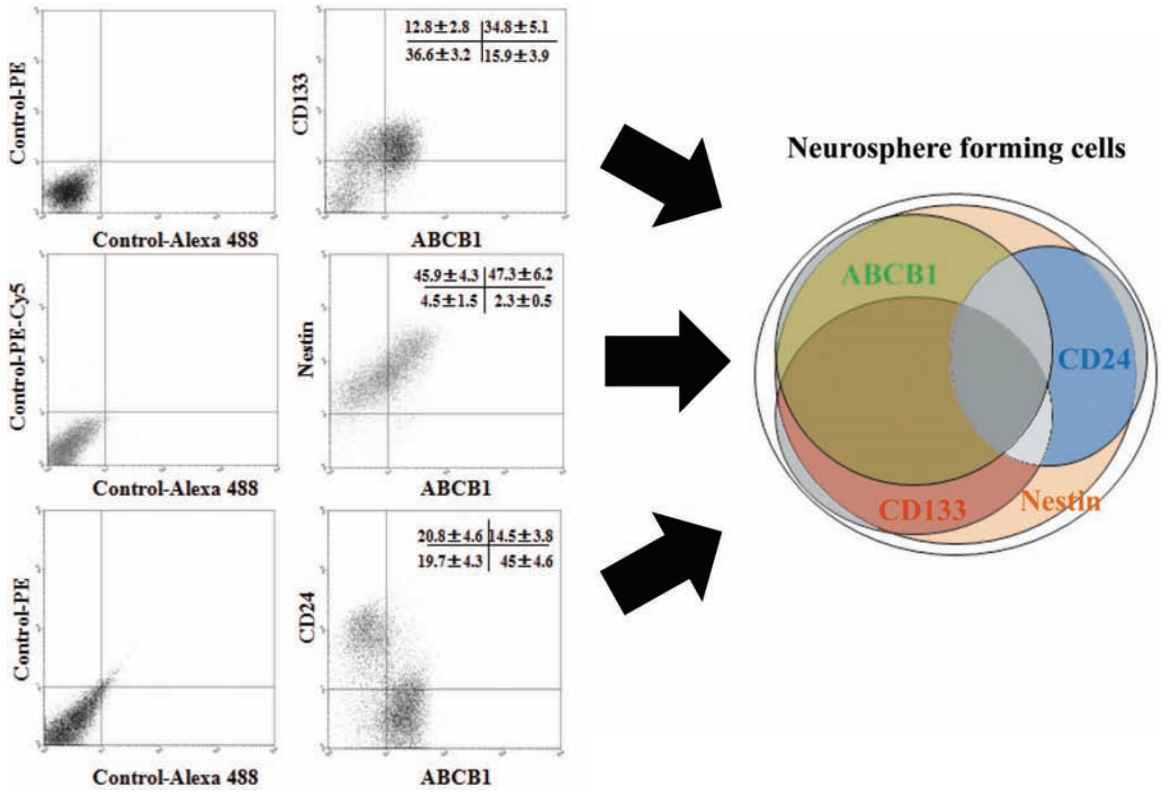

Fig. 1 Expression of ABCB1 on the cell surface of human NSPCs from neurospheres.
(Left) Flow cytometry analysis of cells immunolabeled for ABCB1 and Nestin, CD133, or CD24 expression. Single-cell suspensions were prepared from neurospheres using enzymatic dissociation and stained with an anti-ABCB1 antibody (Ab) (MRK16, Kyowa, Tokyo, Japan) and phycoerythrin (PE)-conjugated CD133/1 (AC133) Ab (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), PE-conjugated CD24 Ab (BD Biosciences), or anti-human Nestin Ab (Nakamura, *et al*., 2003). After several washes, the samples were incubated with AlexaFluor 488-conjugated goat anti-mouse IgG Ab (Molecular Probes, Eugene, OR) and/or PE-Cy5 (R-phyco erythrin-cyanine) -conjugated goat anti-rabbit IgG Ab (Santa Cruz Biotechnology Inc., Santa Cruz, CA) as appropriate. The stained samples were analyzed on a FACS Calibur cytometer (BD Biosciences). All the results show the mean ± S.D. from three independent experiments (Reproduced with revision from Yamamoto, *et al*., J Neurosci Res 2009; 87: 2615–2623). (Right) Venn diagram showing the relative proportions of the cells in neurospheres labeled by each marker, indicating the heterogeneity of human neurospheres. Our findings do not prove the presence of some cells that co-express several markers, and their proportions in neurospheres are uncertain; these hypothetical populations are shown in gray in the diagram (Reproduced with revision from Yamamoto, *et al*. J Neurosci Res 2009; 87: 2615–2623).

dissociated cells have been regarded as indicators for the presence of NSCs, and the number of neurospheres generated in a primary heterogeneous culture has been accepted as representing the number of NSCs plated.[9] However, it is inefficient and time-consuming to use single human NSCs to form neurospheres. For example, Uchida, *et al*. established neurospheres from human NSCs plated as single cells, but only after an 8-week culture period.[15] In our hands, in contrast, large human neurospheres form in cultures of dissociated cells in only a few days, even though the proliferation of human NSPCs is very slow compared with rodent cells.[10] These findings call into question whether the cells in a neurosphere are indeed typically clones of a single NSC.

Recently, several groups including ours reported that neurospheres can form not only from the clonal progeny of a single NSC, but also from aggregated cells or neurospheres (**Fig. 2B**).[17,29–31] Therefore, we have proposed a new model for the proliferative process of human NSPCs that includes *in vitro* aggregation (**Fig. 2B**).[17] In this model, human NSPCs *in vitro* first increase their cell number by clonal growth. Meanwhile, cell-cell interactions initiated between human NSPCs in small aggregates (i.e. small neurospheres), signal the cells to divide. Subsequently, each neurosphere becomes larger both through cell division and agglomeration with other neurospheres (**Fig. 2B**).

Interestingly, each human NSPC in an aggregate contributes to its own proliferation through the formation of neurospheres.[17] To examine the human neurosphere formation process, we conducted an *in vitro* culture of human NSPCs focusing on the cell proliferation and the molecular mechanisms involved, using a combination of cell biological assays and morphological image analysis.

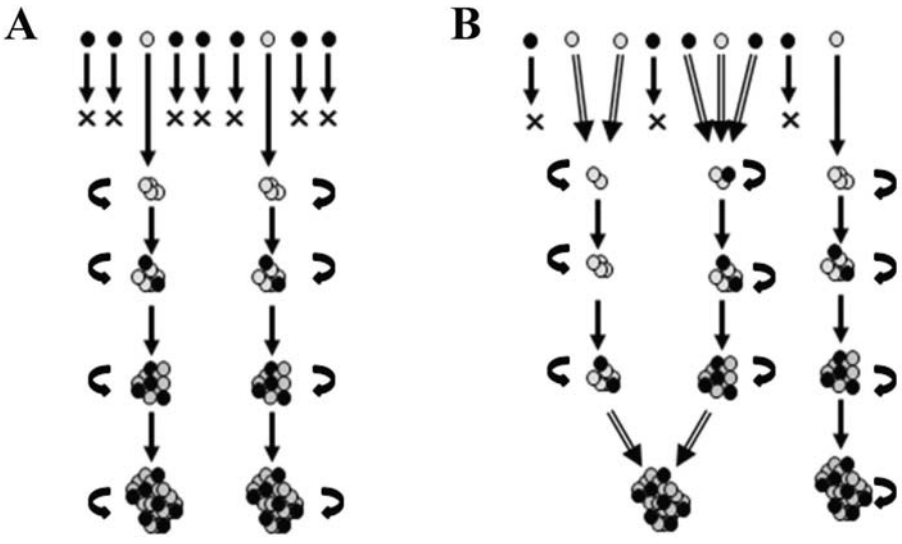

Fig. 2 Proposed models of the *in vitro* proliferative process of human NSPCs during neurosphere formation.
(A) Original concept of neurosphere formation and growth. All neurospheres are clones derived from a single neural stem cell (NSC), which divides to produce NSCs (gray circles) and/or other progenitor cells (black circles). As the NSCs divide (curved arrows), the size of the neurosphere increases with the proliferation of the clonally related NSCs. (B) Proposed model. Neurospheres can form not only from the clonal progeny of a single NSC (gray circles) (shown at right), but also by the aggregation of cells into neurospheres (left and middle). In this model, several human NSCs divide (curved arrows), increasing their cell number by clonal growth (right). Meanwhile, other human NSCs and progenitor cells (black) begin to divide in response to a signal derived from cell-cell interactions that is initiated by the aggregation of NSPCs (open arrows) to form small neurospheres. Subsequently, each neurosphere becomes larger, either through cell division (right) or through the combination of cell division and agglomeration with other neurospheres (left and middle).

We cultured human NSPCs under two different conditions: in flat-bottomed 96-well plates (the control) and in plates with U-bottomed wells designed to promote cell aggregation (**Fig. 3A**).[17]

In the flat-bottomed wells, the NSPCs formed small neurospheres (less than 50 μm) that consisted of a few cells, possibly resulting from clonal cell division and/or spontaneous sphere aggregation at a low frequency. These neurospheres grew gradually, reaching a maximum diameter of less than 100 μm during the 144-hour culture (**Fig. 3B**).[17] In contrast, more and larger neurospheres formed in the U-bottomed wells, and they reached a diameter of approximately 250 μm after only 24 hours in culture (**Fig. 3A, B**).[17] The maximum diameter of the neurospheres in the U-bottomed wells was greater than in the control cultures throughout the experiment and reached more than 300 μm by 144 hours (**Fig. 3B**).[17] A comparison of the proliferation profiles in both culture systems showed that human NSPC proliferation in the U-bottomed wells was significantly better than in the flat-bottomed ones. That is, at 144 hours the number of viable cells in the U-bottomed wells was double that in the flat-bottomed ones. Furthermore, the population doubling time in the U-bottomed wells was approximately 60 hours, which was two-thirds of the value in the flat-bottomed wells.

Finally, this effect of aggregation in the U-bottomed wells involved cell-cell signaling molecules of the Notch1 pathway, as shown by the higher levels of Hes1 and Hes5, target genes of the Notch signal, express by the cells in the U-bottomed wells compared with those in the control flat-bottomed wells.[17] These results suggest that the three-dimensional architecture of NSPC aggregates creates a microenvironment that promotes the proliferation of human NSPCs, and that as neurospheres grow larger, the proliferative signals increase.

Many researchers report the usefulness of sphere formation *in vitro* for the acquisition and maintenance of the physiological properties and functions of various somatic cells. Examples include the neural differentiation of ES cells,[32] the promotion of albumin production from hepatocytes,[33] and the expression of insulin and glucagon by pancreatic islet cells.[34] In the case of neurospheres, our findings suggest that the structure of the neurosphere itself may contribute to the proliferation of human NSPCs and the maintenance of their multipotency. We also speculate that cell motility affects neurosphere growth and/or maintenance. A recent study showed that β1-integrin mediates the migration, differentiation, and proliferation of NSPCs.[35,36] Moreover, we have detected several phenomena that suggest cell migration occurs in neurospheres.[37]

Considering all these observations, we speculate that a neurosphere is not simply a static cell cluster resulting from the clonal proliferation of a single NSC, but a dynamic three-dimensional biological structure that can

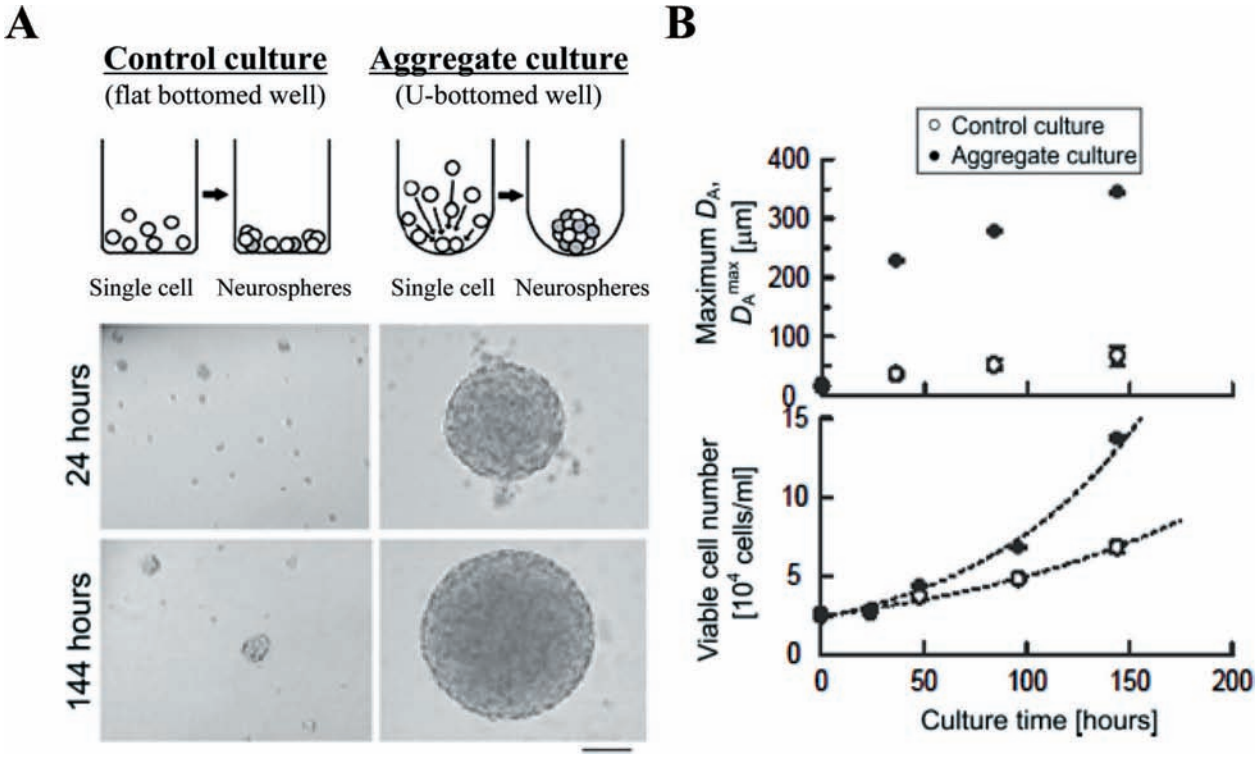

Fig. 3 Effect of cell aggregation on the growth of human NSPCs.
(A) Human NSPCs were cultured as aggregates either in plates with U-bottomed wells (spheroid culture plate, Sumitomo Bakelite Co. Ltd., Tokyo, Japan), to promote aggregation, or in plates with flat-bottomed wells (NUNC, Roskilde, Denmark), as the control. The NSPCs were seeded as a single-cell suspension of 2.5×10^4 cells/ml and cultured for 0, 24, 48, 96, or 144 hours in the DMEM/F-12(1:1)-based defined medium supplemented with EGF (20 ng/ml), FGF2 (20 ng/ml), B27 (Invitrogen), and leukemia inhibitory factor (LIF) (10 ng/ml; Chemicon, Temecula, CA), as described previously.[10] Typical images of neurospheres taken at 24 and 144 hours. Scale bar: 100 μm (Reproduced with revision from Mori, *et al*. J Neurosci Res 2006; 84:1682–1691). (B) Human NSPC proliferation is up-regulated by aggregation. Time-courses showing numbers of viable cells and maximum neurosphere diameters (D_A^{max}). The data represent the mean value ± S.E.M. from six independent cultures for each culture condition (Reproduced with revision from Mori, *et al*. J Neurosci Res 2006; 84:1682–1691).

function like a physiological micro-incubator for human NSPCs. The findings presented here describe some fundamental biological properties of neurosphere formation that will contribute to establishing new strategies for culturing human NSPCs intended for clinical applications.

The regional distribution of cells in human neurospheres

As the size of a neurosphere increases, so do the number of cell-cell interactions and the proliferation of NSPCs through Notch signaling.[17] To inspect the cellular heterogeneity of neurospheres, we developed an algorithm for image cytometry to quantify the area of immunolabeling in living neurospheres cultured from human NSPCs. We also investigated the localization of specific neural cell types and the regional fluctuation of cell density in neurospheres.[17,19]

To carry out this quantification, we captured images of neurospheres and measured the diameter of each. To obtain the total number of cells in each sphere, we used an ATP assay.[10,17] The viable cell number (N) determined by the ATP assay correlated fairly well with the diameter (D) of the spheres, and we derived the following equation to describe this relationship: $N=1.05\times10^4\ D^{3.18}$ ($r = 0.997$) (**Fig. 4A**).[17] Thus, we were able to estimate the total number of viable cells in human neurospheres from the sphere diameter alone. Next, neurospheres with different diameters were sectioned, labeled with 1 μM TO-PRO-3 iodide (Invitrogen, Carlsbad, CA), and the distribution and density of the nuclei were measured.[19] The radial distance from the periphery of each neurosphere section (d) was adopted as the variable of regional dimension, and the regional cell density ($X(d)$) was recorded as the total number of cells (TO-PRO-3 labeled nuclei) per unit volume.[19] As shown in **Figure 4B**, a similar tendency in the regional variation of total cell density was observed in terms of the examined d values. The $X(d)$ values were approximately 1.0×10^6 cell/mm^3 near the periphery of neurospheres, which decreased gradually to d = 50 μm. Beyond this point, i.e. d=50-180 μm, the value was constant at approximately 6.0×10^5 cell/mm^3 (**Fig. 4B**).[19]

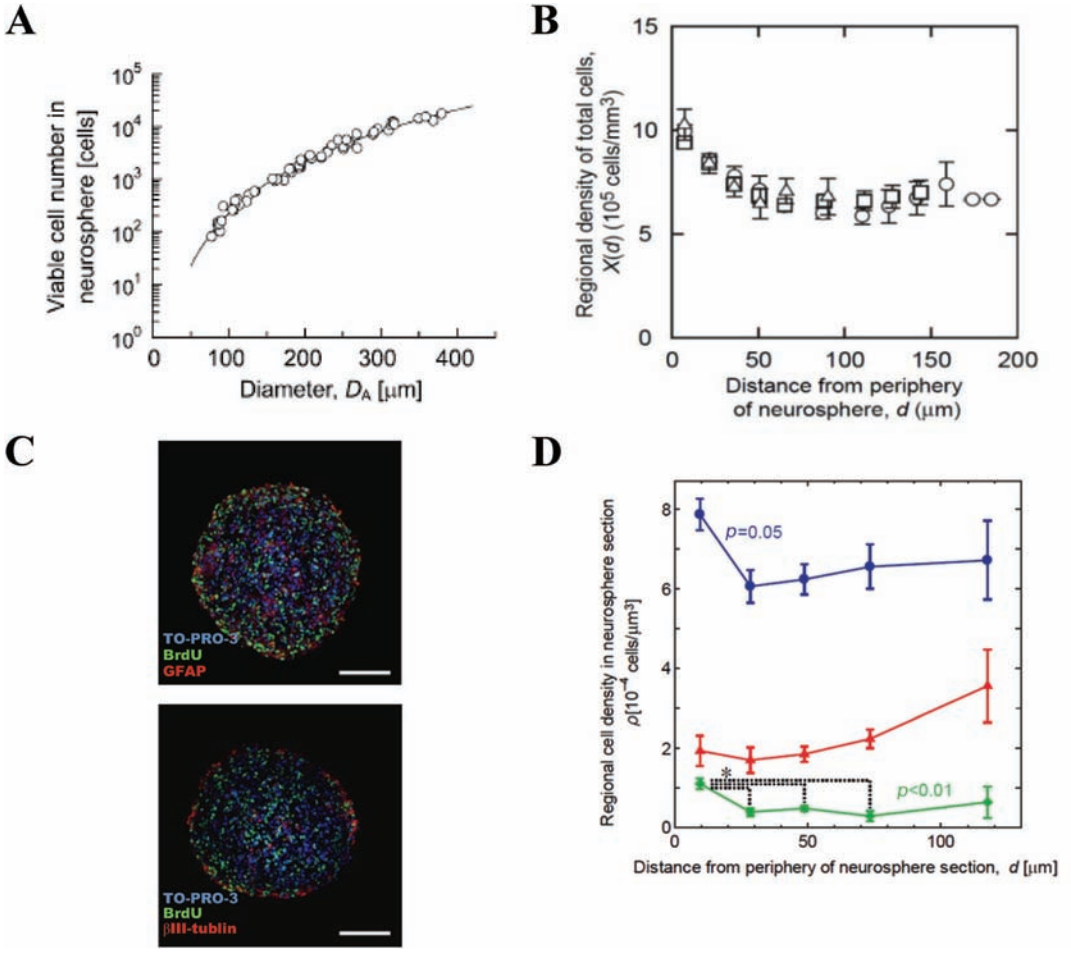

Fig. 4 Analysis of the regional distribution of cells inside human neurospheres.
(A) Relationship between viable cell number and neurosphere diameter (D_A). Images of neurospheres were captured and the projected area (A_A) of each neurosphere and the number of neurospheres were analyzed. The size of each neurosphere was defined as an equivalent circle diameter, $D_A = 2(A_A/\pi)^{1/2}$. The number of viable human NSPCs was determined indirectly by an ATP assay (Reproduced with revision from Mori, *et al*. J Neurosci Res 2006; 84:1682-1691). (B) Regional distribution of total cell density ($X(d)$) in neurospheres of varied sizes. Symbols indicate neurosphere diameter (D): triangle, D=100-200 μm; square, D=200-300 μm; circle, D=300-400 μm (Reproduced with revision from Mori, *et al*. J Biosci Bioeng 2007; 103: 384–387). (C) Immunofluorescent images of human neurosphere sections. Sections were immunostained with a proliferation marker (anti-BrdU: green) and anti-glial fibrillary acidic protein (GFAP) (red) or a neuronal cell marker (anti-βIII-tubulin: red), and counterstained with TO-PRO-3 (blue). Scale bar: 100 μm (Reproduced with revision from Mori, *et al*. J Biosci Bioeng 2007; 103: 384–387). (D) Regional changes in the densities of proliferating and differentiated cells in neurospheres. Blue: BrdU-positive cell. Red: BrdU-negative/GFAP-positive cell. Green: BrdU-negative/βIII-tubulin-positive cell (Reproduced with revision from Mori, *et al*. J Biosci Bioeng 2007; 103: 384–387).

When neurospheres were cultivated in medium containing BrdU (5-bromo-2'-deoxyuridine; 10 mM) for 72 hours to label proliferating cells, cells with three different molecular profiles were identifiable: BrdU-positive ($BrdU^+$), BrdU-negative and βIII-tubulin-positive ($BrdU^-$ βIII tub^+), and BrdU-negative and GFAP-positive ($BrdU^-$ $GFAP^+$) (**Fig. 4C**).[19] The $X(d)$ values for $BrdU^+$ (**Fig. 4D**, blue) and $BrdU^-$βIII tub^+ cells (**Fig. 4D**, green) showed a significant decrease from the periphery to $d = 28$ μm and were nearly constant thereafter. On the other hand, the $X(d)$ value for $BrdU^-$ $GFAP^+$ cells (**Fig. 4D**, red) did not show a significant difference from its value in the peripheral region.[19]

These findings revealed that there were differences in the distributions of proliferating and differentiated cells within human neurospheres, which should help characterize the nature of the human NSPCs they contain.

Mesenchymal Stem Cells (MSCs)

MSCs comprise a heterogeneous subset of stromal stem cells that can differentiate into cells of the mesodermal linage, such as osteoblasts, chondrocytes, and adipocytes.[3,38] MSCs are typically isolated from the non-hematopietic mononuclear cells of the bone marrow (bone marrow derived-mesenchymal stem cells; BM-

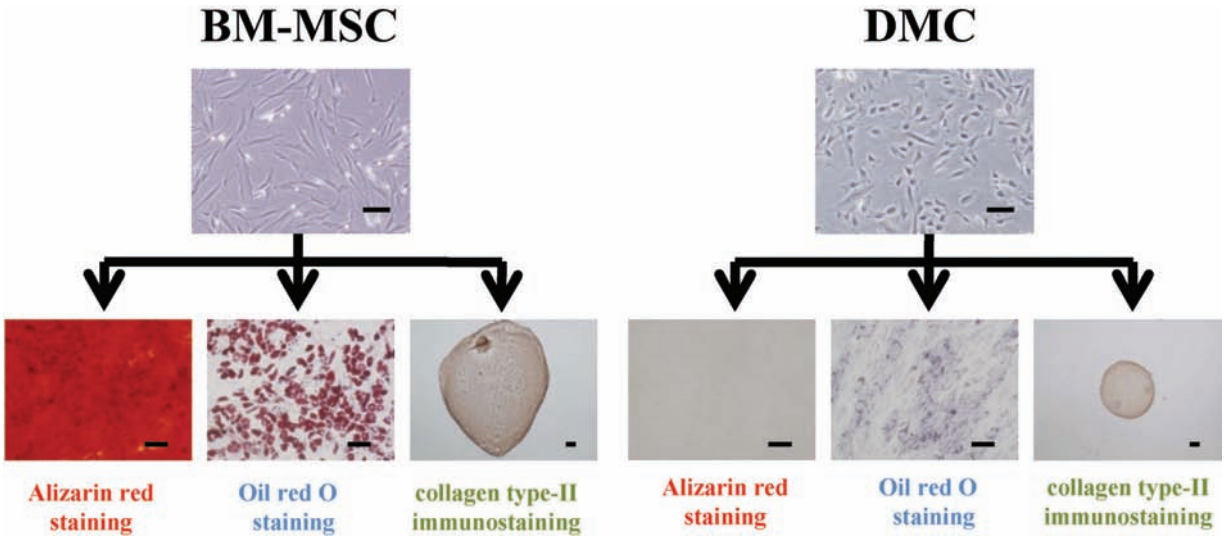

Fig. 5 *In vitro* differentiation of bone marrow derived-mesenchymal stem cells (BM-MSCs) and decidua-derived mesenchymal cells (DMCs).
To induce osteogenic differentiation, cells were cultured with dexamethasone (Dex; 0.2 μM), β-glycerol phosphate (10 mM), ascorbic acid 2-phosphate (100 mM), and 10% fetal bovine serum (FBS) for 28 days. To evaluate the mineralized matrix, cells were stained with 0.1% Alizarin red S. To induce adipogenic differentiation, cells were cultured with Dex (1 mM), 3-isobutyl-1-methylxanthin e (IBMX) (500 mM), indomethacin (200 mM), recombinant human insulin (20 mg/ml), and 10% FBS for 28 days. To evaluate the presence of cytoplasmic inclusions of neutral lipids, cells were stained with Oil red O and counterstained with Mayer hematoxylin. To induce chondrogenic differentiation, cells were pelleted in a polypropylene tube and then cultured with Dex (0.1 μM), ascorbic acid 2-phosphate (170 μM), sodium pyruvate (1 mM), proline (350 μM), recombinant human transforming growth factor-β3 (TGF-β 3) (10 ng/ml), and ITS+Premix (BD Biosciences) for 28 days. To evaluate chondrogenic differentiation, sections of the chondrogenic cell pellet were incubated with anti-collagen type-II Ab and signals were detected by the avidin-biotin complex (ABC) method with a metal-enhanced DAB Kit (Pierce, Rockford, IL). Scale bar: 100 μm.

MSCs) or they can be also isolated from various human fetal and adult tissues.[3,38]

Isolation of decidua-derived mesenchymal cells (DMCs)

The fetal adnexa is composed of the placenta, fetal membrane (FM), and umbilical cord. These are ephemeral organs that connect the developing fetal tissues to the uterine wall, supplying the fetus with maternal nutrients, allowing fetal waste to be disposed of via the maternal kidneys, and contributing to fetal-maternal immune tolerance. Many recent studies have shown that fibroblast-like adherent cells isolated from various placental components are phenotypically similar to BM-MSCs.[39–42] Therefore, the fetal adnexa may prove to be a useful source of human MSCs.

We isolated decidua-derived mesenchymal cells (DMCs) from human FMs at term[43] and examined how their cellular properties compared with those of BM-MSCs. The DMCs adhered to plastic dishes and exhibited a typical fibroblast-like morphology. They showed excellent potential for *in vitro* expansion, because they maintained their morphology for more than 30 population doublings (**Fig. 5**). The DMCs had an F-actin cytoskeleton that extended throughout the entire cytoplasm, and they strongly expressed a mesenchymal cell marker, vimentin, but not cytokeratin 19 or HLA-G.[43] *In vitro* differentiation assays showed that the DMCs differentiated well into chondrocytes, and moderately into adipocytes, but hardly at all into osteoblasts, as compared with BM-MSCs (**Fig. 5**). Although DMCs are not superior to BM-MSCs in multipotency, their greater proliferative ability requires less maintenance in cell preparation, and their derivation from the maternal portion of human FMs, which are otherwise discarded, would resolve many ethical concerns. These unique properties of DMCs have several advantages for clinical use and make these cells an attractive alternative to allogeneic MSCs for use in regenerative medicine.

Pericellular matrix of DMCs (PCM-DM) is a potent human-derived substrate for the maintenance culture of human pluripotent stem cells

Unlike mouse ES cells, hES and hiPS cells are maintained on either feeder cells or special culture substrates such as Matrigel.[44] However, to expand hES/hiPS cells for clinical applications, it is desirable to minimize animal-derived materials in the culture for safety reasons. We therefore tested the pericellular matrix prepared from human DMCs (PCM-DM) for its ability to support hES cell growth and pluripotency. DMCs were cultured on

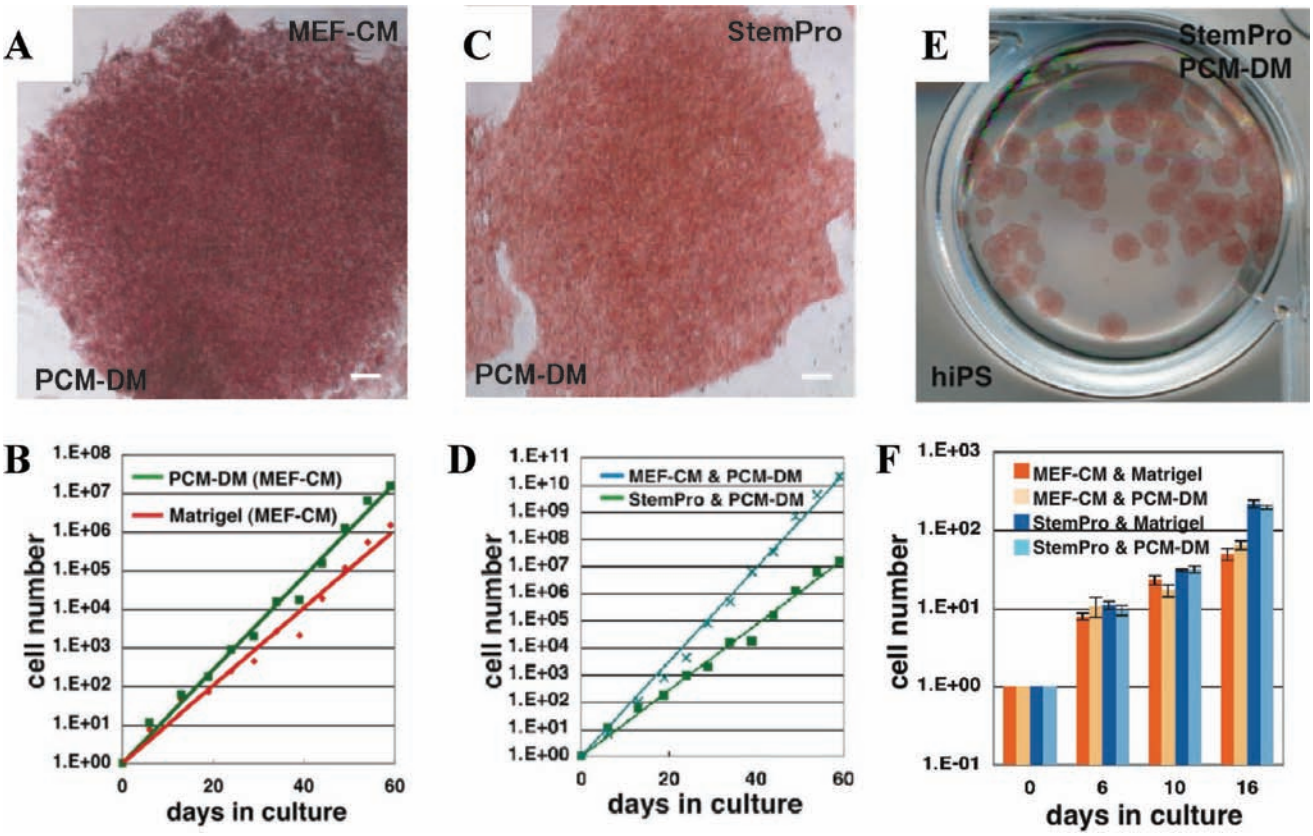

Fig. 6 Pericellular matrix of decidua-derived mesenchymal cells (PCM-DMs) is a potent human-derived substrate for the maintenance culture of human pluripotent stem cells.
Undifferentiated hES cells (KhES-1) and hiPS cells (253G4) were maintained on a feeder layer of mouse embryonic fibroblasts (MEFs) in DMEM/F12 supplemented with 20% Knockout Serum Replacement (KSR), glutamine (2 mM), nonessential amino acids (0.1 mM), recombinant human FGF-2 (5 ng/ml), and 2-mercaptoethanol (0.1 mM). MEF-conditioned medium (MEF-CM) was prepared by incubating the maintenance culture medium with confluent MEFs for 24 hours. (A) Staining of alkaline phosphatase in hES cells cultured on PCM-DM in MEF-CM. Scale bar: 100 μm. (B) Propagation efficiency of hES cells cultured in MEF-CM. PCM-DM supported propagation better than Matrigel. (C) Alkaline phosphatase activity of hES cells cultured in StemPro on a 4-well PCM-DM plate. Scale bar: 100 μm. (D) Propagation efficiency of hES cells. StemPro supported the propagation of hES cells. (E) hiPS colonies plated on a PCM-DM 4-well plate showing alkaline phosphatase activity. (F) hiPS cells cultured under various conditions. StemPro medium and PCM-DM supported the efficient growth of hiPS cells. (Reproduced with revision from Nagase, *et al*. Dev Dyn 2009; 238: 1118–1130).

gelatin-coated plastic culture dishes and lysed by deoxycholate to prepare the PCM-DM.[43] The PCM-DM was then used for hES cell culture in mouse embryonic fibroblast (MEF)-conditioned medium (MEF-CM), and its maintenance-supporting activity was compared with that of two conventional matrices, fibronectin and Matrigel.[44] During multiple passages, PCM-DM supported the growth of human ES cells with an efficiency similar to, or slightly better than, that of Matrigel, an animal-derived substrate, in MEF-CM (**Fig. 6A, B**).[43]

Since MEF-CM itself contains mouse-derived materials, we next examined whether PCM-DM-based hES cell culture was compatible with unconditioned serum-free medium. Human ES cell clumps were seeded on PCM-DM in StemPro hESC SFM (Invitrogen), which supports hES cell maintenance on Matrigel-type matrices. We found that the PCM-DM-based culture was compatible with non-conditioned commercial defined medium, and with the maintenance of dissociated hES cells (**Fig. 6C, D**).[43] We next tested whether hES cells maintained on PCM-DM in StemPro retained their pluripotency after multiple passages, and found that they did.[43] Notably, the maintenance-supporting activity of PCM-DM was stable and could be preserved for at least eight months after preparation, by keeping the plate in the refrigerator under semi-dry conditions.[43] Moreover, we tested whether PCM-DM was applicable to the maintenance culture of hiPS cells. Cells from the hiPS line 253G4[45] were cultured as cell clumps on PCM-DM or Matrigel in MEF-CM or StemPro. In both media, the PCM-DM supported hiPS cell growth as efficiently as Matrigel (**Fig. 6E, F**).[43]

Since DMCs can be prepared and expanded in large quantity, PCM-DM is a practical human-derived substi-

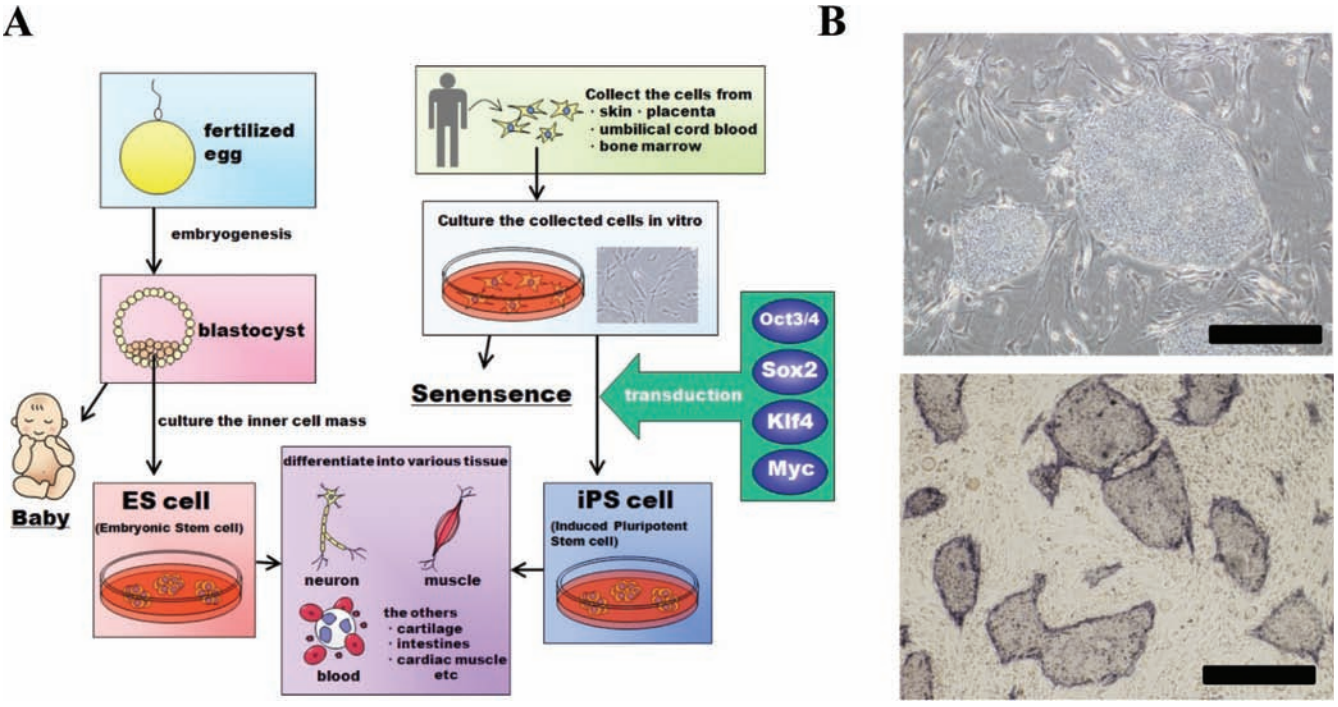

Fig. 7 Generation of Human iPS cells.
(A) The reprogramming of human somatic cells to generate human iPS cells has been achieved by the forced expression of exogenous Oct4, Sox2, Klf4, and c-Myc. (B) Human iPS cells prepared from decidua-derived mesenchymal cell (DMCs). Upper: Phase-contrast view of DMC-derived human iPS cell colonies. Lower: Alkaline phosphatase activity. Scale bar: 100 μm.

tute for the animal-derived substrates currently in use for the clinical-grade culture of hES/hiPS cells.

Human iPS Cells

The reprogramming of human somatic cells into iPS cells has been achieved by the forced transduction of Oct4, Sox2, Klf4, and c-Myc.[5] These iPS cells closely resemble ES cells in their gene expression pattern and epigenetic profile, and the transduced transcription factors restore the pluripotency of the iPS cells *in vitro* and *in vivo* (**Fig. 7A**).[5] Regenerative treatments that use patient-derived autologous somatic stem cells or iPS cells are the most desirable for reasons of safety and ethics. Human iPS cells will greatly contribute to making the clinical use of autologous pluripotent stem cells a future reality.

On the other hand, the transplantation of allogeneic human somatic stem cells derived from hES cells or hiPS cells is also a promising approach, especially for the treatment of acute diseases and injures (e.g. stroke, spinal cord injury) in which cell transplantation must be performed in the acute to sub-acute stage.[11,13] We have successfully derived hiPS cells from DMCs and are examining their biological properties (**Fig. 7B**). We think DMC-derived hiPS cells are likely to have properties that are virtually equivalent to those of allogeneic hiPS cells. The establishment of hiPS cell-banking systems will increase the feasibility of using allogeneic stem cells for clinical applications.

Conclusions

We found that ABCB1 is predominantly expressed in immature human fetal NSPCs, and thus could be used as a phenotypic marker to investigate and monitor NSPCs in culture. We have proposed a new model for the *in vitro* proliferation of human NSPCs in which cell aggregation increases cell-cell interactions that activate Notch and promote proliferation. Therefore, the proliferation of new NSPCs and the increase in neurosphere size are mutually reinforcing. We also found that neurospheres contain a heterogeneous, regionally biased cell population, which may help improve our understanding of how to isolate neurospheres with desired properties.

To improve cell culture processes and increase the number of cell sources available, we compared DMCs isolated from term human FMs with BM-MSCs and found that the two stem-cell populations had nearly identical properties. We also found that the pericellular matrix made by the DCMs, PCM-DM, supported the growth and pluripotency of hES/hiPS cells. These unique properties of DMCs suggest that they may be particularly appropriate for clinical use, and we expect

that future studies will show them to be an attractive alternative to allogeneic MSCs in regenerative medicine.

Recent advances in the re-programming techniques used for generating iPS cells should also greatly contribute to the development of cell therapies using human pluripotent stem cells. We have successfully derived hiPS cells from DMCs, and these hiPS cells may be an ideal candidate cell type for allogeneic hiPS cells in clinical applications. We hope these findings will promote the development of cell-processing systems to generate human allogeneic stem cells for clinical use in regenerative medicine.

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