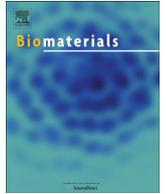




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An injectable spheroid system with genetic modification for cell transplantation therapy

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ABSTRACT

The new methodology to increase a therapeutic potential of cell transplantation was developed here by the use of three-dimensional spheroids of transplanting cells subsequent to the genetic modification with non-viral DNA vectors, polyplex nanomicelles. Particularly, spheroids in regulated size of 100- μ m of primary hepatocytes transfected with luciferase gene were formed on the micropatterned culture plates coated with thermosensitive polymer, and were recovered in the form of injectable liquid suspension simply by cooling the plates. After subcutaneously transplanting these hepatocyte spheroids, efficient transgene expression was observed in host tissue for more than a month, whereas transplantation of a single-cell suspension from a monolayer culture resulted in an only transient expression. The spheroid system contributed to the preservation of innate functions of transplanted hepatocytes in the host tissue, such as albumin expression, thereby possessing high potential for expressing transgene. Intravital observation of transplanted cells showed that those from spheroid cultures had a tendency to localize in the vicinity of blood vessels, making a favorable microenvironment for preserving cell functionality. Furthermore, spheroids transfected with erythropoietin-expressing DNA showed a significantly higher hematopoietic effect than that of cell suspensions from monolayer cultures, demonstrating high potential of this genetically-modified spheroid transplantation system for therapeutic applications.

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1. Introduction

Cell transplantation therapy has attracted considerable attention for the treatment of various intractable diseases. The therapeutic potential of cell transplantation is primarily dependent on the efficacy and longevity of bioactive factors secreted from the transplanted cells [1]. In this respect, genetic modification of transplanted cells by introducing transgene(s) using either viral or non-viral methods is a promising approach to modulate the secretion of bioactive factors [2]. In addition to endogenous factors,

transgenes expressing functional proteins and peptides such as growth factors and coagulation factors can further enhance the therapeutic potential of transplanted cells [3–5]. Furthermore, a scheme to maintain transplanted cells in optimal long-acting conditions is a key for successful treatment. Although the survival rate of cells varies depending on cell type and source, therapeutic effects are likely to be limited by the death of transplanted cells or the loss of cell activity due to unfavorable microenvironments such as ischemia, hypoxia, or inflammation [6].

Three-dimensional (3D) spheroid cell culture is a promising technique to improve cell survival and function by preserving cell-to-cell interactions. Several groups including ours have reported that 3D spheroid cultures could increase the survival rate of the cells and enhance innate functions such as albumin secretion from primary hepatocytes and multilineage differentiation of mesenchymal stem cells (MSCs) [7–12]. Recently, we introduced a procedure in genetically-modified cell transplantation using a 3D spheroid culture system on micropatterned culture plates (Cell-

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able™ multi-well plates; Transparent, Chiba, Japan) combined with gene transfection by polyplex nanomicelles [13]. The polyplex nanomicelle is a non-viral gene carrier composed of plasmid DNA (pDNA) and poly(ethylene glycol) (PEG)–polycation block copolymers, which possesses core–shell structure consisting of PEG shell and inner core of pDNA in a condensed state [14–16]. For the polycation, we developed poly[N'–[N-(2-aminoethyl)-2-aminoethyl] aspartamide] [PAsp(DET)]. This polycation possesses two distinguished properties: the efficient capability of endosomal escape and rapid biodegradability in the cytoplasm, allowing safe and effective gene introduction into various cells [17–19]. Using this polyplex nanomicelle system, we achieved high and prolonged transgene expression for more than one month from the spheroids of rat primary hepatocytes cultured on micropatterned plates [13]. The nanomicelle-treated spheroids also exhibited sustained albumin secretion at a level comparable with that exhibited by untreated spheroids, suggesting that this system allows safe gene transfection without impairing the innate function of hepatocytes.

In this study, the hepatocyte spheroids gene-transfected by the polyplex nanomicelles were transplanted into mice to obtain insight into their application in cell therapy. For transplanting spheroids in their intact 3D form, we introduced thermosensitive property to the micropatterned culture plate, which allows the recovery of spheroids simply by lowering the temperature of the plate. Then, hepatocyte spheroids transfected with luciferase expressing pDNA were transplanted to subcutaneous tissue to evaluate the efficiency of transgene expression in host animal. The advantages of this system were analyzed in detail by intravital imaging of transplanted cells in the host tissue. Finally, to examine therapeutic potential, hepatocyte spheroids receiving transfection with erythropoietin-expressing pDNA were transplanted, followed by evaluation of the hematopoietic effect in the host mice.

2. Materials & methods

2.1. Materials

Collagenase, dimethylsulfoxide (DMSO), dexamethasone, insulin, and L-proline nicotinamide were purchased from Wako Pure Chemical Industries (Osaka, Japan). Hank's buffered salt and L-ascorbic acid 2-phosphate (Asc-2P) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), trypsin inhibitor, and Pen–Strep–Glut (PSQ) were purchased from GIBCO (Frederick, MD, USA). Human epidermal growth factor (hEGF) was purchased from Toyobo (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Dainippon Sumitomo Pharma (Osaka, Japan). For the construction of plasmid DNA (pDNA) expressing luciferase, the protein-expressing segment of pGL4.13 plasmid (Promega, Madison, WI, USA) was cloned into pCAG-GS plasmid (RIKEN, Tokyo, Japan) to obtain expression under CAG promoter/enhancer. For pDNA expressing Gaussia luciferase (Gluc), the protein-expressing segment of pCMV-Gluc control plasmid (New England Biolabs, Ipswich, MA, USA) was cloned into pCAG-GS. For pDNA expressing mouse erythropoietin (mEpo), protein expressing segments of pCMV-X4 plasmid (OriGene, Rockville, MD, USA) were cloned into pCAG-GS. These pDNAs were amplified in competent DH5 α *Escherichia coli* and purified using a NucleoBond® Xtra Maxi Plus (Takara Bio, Shiga, Japan).

2.2. Animals

Balb/c nude mice (female; 7 weeks old) and Wistar rats (male; 5 weeks old) were purchased from Charles River Laboratories (Yokohama, Japan). Transgenic Sprague–Dawley (SD) rats (male; 5 weeks old) expressing EGFP in all tissues under the control of CAG promoter/enhancer (EGFP–SD rats) were purchased from Japan SLC (Shizuoka, Japan). All animal studies were conducted with the approval of the Animal Care and Use Committee of the University of Tokyo, Tokyo, Japan.

2.3. Isolation and culture of primary hepatocytes

Rat hepatocytes were isolated using a modified two-step collagenase digestion process as previously reported [20,21]. In brief, after the rat liver was perfused from the hepatic portal vein with a special solution described below, the collagenase solution was recirculated through the liver to obtain hepatocytes. The perfusion medium (pH 7.2) was composed of 8 g/L sodium chloride (NaCl), 400 mg/L potassium chloride (KCl), 78 mg/L sodium dihydrogen phosphate dehydrate (NaH₂PO₄·2H₂O), 151 mg/L disodium hydrogen phosphate 12-water (Na₂HPO₄·12H₂O), 2.38 g/L 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), 190 mg/

L ethylene glycol tetraacetic acid (EGTA), 350 mg/L sodium hydrogencarbonate (NaHCO₃), and 900 mg/L glucose. The collagenase solution (pH 7.2) was composed of 500 mg/L collagenase, 9.8 g/L Hank's buffered salt, 2.38 g/ml HEPES, 556 mg/ml calcium chloride hydrate (CaCl₂·H₂O), 350 mg/L NaHCO₃, and 50 mg/L trypsin inhibitor. To preserve the function of hepatocytes under *in vitro* conditions, a special medium comprising DMEM supplemented with 10% FBS, 1% PSQ, 1% DMSO, 10^{–7} mol/L dexamethasone, 0.5 μ g/ml insulin, 10 mmol/L nicotinamide, 0.2 mmol/L Asc-2P, and 10 ng/ml hEGF was used for cell culture [22].

2.4. Recovery and transplantation of the cells from spheroid and monolayer cultures

The micropatterned architecture was constructed on thermosensitive cell culture plates (UpCell™, CellSeed Inc., Tokyo, Japan) to prepare the thermosensitive micropatterned plates, in which cell adhesion sites of a 100- μ m diameter are regularly arrayed surrounded by a non-adhesive area. Primary hepatocytes were seeded onto 12- or 96-well culture plates at densities of 4 \times 10⁵ cells/well or 4 \times 10⁴ cells/well, respectively. The spheroid cells were recovered as a suspension for transplantation studies by lowering the temperature without any damage to the structure of the spheroids. Cells on monolayer culture plates were recovered by trypsinization followed by centrifugation at 200 \times g for 3 min. The recovered suspension from a spheroid and monolayer culture was transplanted to the subcutaneous tissue of Balb/c nude mice by injection using 23-gauge needles. The number of cells to be transplanted per mouse was adjusted at the stage of seeding the cells onto the plate, to be 2 \times 10⁵, 4 \times 10⁵, and 1.2 \times 10⁶ for the transplantation to forelimb, abdomen, and earlobe, respectively. Because the number of recovered cells per well in 12-well plate was determined to be (4.2 \pm 0.6) \times 10⁵, and (4.8 \pm 0.7) \times 10⁵ (means \pm SD) in spheroid and monolayer culture respectively, the number of transplanted cells per mouse were comparable between these two groups.

2.5. Gene introduction using polyplex nanomicelles

PEG–PAsp(DET) block copolymer and PAsp(DET) homopolymer were synthesized as previously reported [17]. PEG used in this study had a molecular weight (Mw) of 12,000, and the polymerization degree of the PAsp(DET) segment was determined to be 59 by ¹H-NMR. The polymerization degree of the PAsp(DET) homopolymer determined by ¹H-NMR was 55.

Polyplex nanomicelles were prepared as described in our previous report [13]. In brief, the nanomicelle was formed by mixing polymer and pDNA solutions in 10 mM Hepes buffer (pH 7.3). For preparing the polymer solution, we recently revealed that the combined use of two polymers, PEG–PAsp(DET) block copolymer and PAsp(DET) homopolymer, was advantageous to achieve both effective PEG shielding and functioning of PAsp(DET) to enhance endosomal escape [23]. Thus, in this study, nanomicelles were prepared by mixing pDNA solution with a premixed solution of the two polymers at the equal molar ratio of residual amino groups at the N/P ratio (residual molar ratio of total amino groups in the two polymers to phosphate groups in pDNA) of 10. The diameter of the resulting nanomicelles was determined to be approximately 70 nm by dynamic light scattering (DLS) [24]. In 12-well plate, a total of 10 μ g of pDNA was added to 1 ml of culture medium for each well, and in 96-well plate, 1 μ g of pDNA was added to 100 μ l of culture medium.

2.6. In vivo and in vitro measurement of luciferase expression

In vivo luciferase expression after transplantation was measured using an IVIS™ Imaging System (Xenogen Corp., Alameda, CA, USA) after intravenous injection of D-luciferin (150 mg/kg, Sumisho Pharmaceuticals International, Tokyo, Japan).

In vitro analyses were performed using Gluc-expressing pDNA. Expressed Gluc is secreted into the culture medium and remains stable for more than a week [25]. In this study, to trace the real-time activity of transgene expression, the culture medium was replaced with fresh medium precisely 24 h before each indicated measuring point. 24 h after the replacement, the culture medium was collected to quantify Gluc secretion during the last 24 h, using a Renilla Luciferase Assay System (Promega) and GloMax® 96 Microplate Luminometer (Promega) following the manufacturer's protocol.

2.7. Quantification of transplanted cells and transgene and gene expression in host tissue

At 24 h after hepatocyte transplantation into the forelimb of mice, total DNA and mRNA in whole of the cutaneous and subcutaneous tissue in the forelimb were extracted from the transplantation site using DNeasy Blood & Tissue Kits (Qiagen, Hilden, Germany) and RNeasy Mini Kits (Qiagen), respectively, according to the manufacturer's protocol. Using an ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City, CA, USA), quantitative real-time PCR (qRT-PCR) was performed. Because hepatocytes from male rats were transplanted to female mice, the number of transplanted cells in the host tissue was proportional to the copy number of SRY genes on Y chromosomes, which was amplified using the following primer pair: forward, CATCGAAGGGTTAAAGTGCCA; reverse, ATAGTGTGTAGGTTGTGTCC, with standardization by quantifying pDNA copies of mouse β -actin (Mm00607939, Applied Biosystems). The number of transgenes (luciferase-expressing pDNA) in the host tissue was quantified using the following primer pair: forward,

TGCAAAAGATCCTCAACGTG; reverse, AATGGGAAGTCACGAAGGTG. The mRNA level of albumin expression was quantified and standardized with the mRNA level of β -actin using TaqMan[®] Gene Expression Assays (albumin: Rn00592480_m1, β -actin: Mm00607939, Applied Biosystems).

2.8. Intravital microscopic imaging of transplanted cells

The hepatocytes were isolated from EGFP–SD rats, and cultured as described above. The distribution of the cells was evaluated 24 h after transplantation to mouse earlobes using intravital real-time confocal laser scanning microscopy [26]. One hour before imaging, Evans Blue dye (2.5 mg/kg; Wako), which binds to serum albumin, was intravenously injected to visualize blood vessels. All picture acquisitions were performed using a Nikon A1R confocal laser scanning microscope system attached to an upright ECLIPSE FN1 microscope equipped with a Plan Apo λ 20X NA 0.75 objective lens (Nikon, Tokyo, Japan). The pinhole diameter was set to result in a 10- μ m optical slice. For fluorescent imaging of EGFP and Evans Blue, 488 nm and 640 nm excitation lasers and band-pass emission filters of 525/50, and 700/75 nm was used, respectively. Acquired data were further processed using Nikon NIS Elements software.

3. Results

3.1. Transgene expression in host mice after transplantation of genetically-modified hepatocytes

Uniform spheroids from rat primary hepatocytes with a 100- μ m diameter were prepared using micropatterned culture plates, in which cell adhesion areas of 100- μ m diameter are regularly arrayed in a two-dimensional manner surrounded by non-adhesive areas coated by PEG matrix (Cell-able[™] multi-well plate; Transparent,

Chiba, Japan) [7]. The cell adhesion areas were coated with a polymer, poly(*N*-isopropylacrylamide) (PIPAAm), which is widely used in the temperature-responsive cell recovery system (Fig. 1a) [27–29]. After placing the plate on ice followed by the addition of cold phosphate-buffered saline (PBS), PIPAAm becomes hydrophilic, resulting in the detachment of spheroid from culture plates simply by pipetting. Thus, spheroids with uniform size of 100 μ m were recovered in the form of an injectable suspension without disrupting their 3D structure (Fig. 1b).

The recovered spheroids were subcutaneously transplanted to mouse abdomen simply by injection with 23-gauge needles. It is confirmed that the spheroid size and structure were kept almost constant after injecting through 23-gauge and 27-gauge needles, whose inner diameters were 400 μ m and 230 μ m, respectively (Fig. S1). Transgene expression in host mice was evaluated after transplantation of spheroids transfected with luciferase-expressing pDNA. For the control, identical numbers of suspended hepatocytes prepared from a monolayer culture system were transplanted by injection. Luciferase expression in host mice was measured using an IVIS[™] imaging system. After subcutaneous transplantation of hepatocytes (spheroids or isolated suspensions) in the abdominal region one day after receiving transfection with luciferase-expressing pDNA, the spheroids showed approximately 10 times higher levels of luciferase expression than those in hepatocytes in suspension form over a period of one month except 4 h after transplantation (Fig. 2). The increase of luciferase expression from

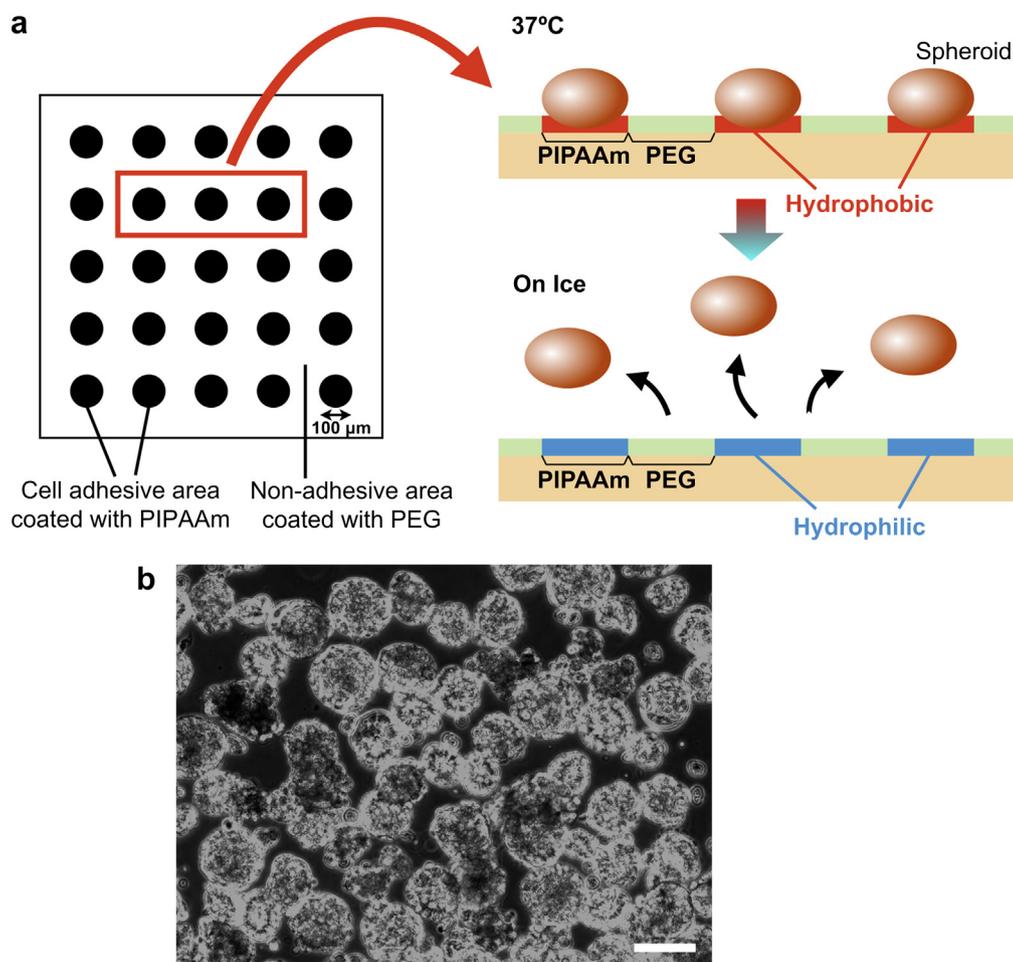


Fig. 1. Preparation of spheroids in the form of homogeneous suspension using thermosensitive micropatterned plate. (a) Scheme of micropatterned plate and mechanism of spheroid recovery. By placing the plate on ice followed by the addition of cold phosphate-buffered saline (PBS), the spheroids were recovered in an injectable suspension form without disrupting their 3D structure. (b) Microscopic image of hepatocyte spheroids dispersed in PBS. Scale bar: 100 μ m.

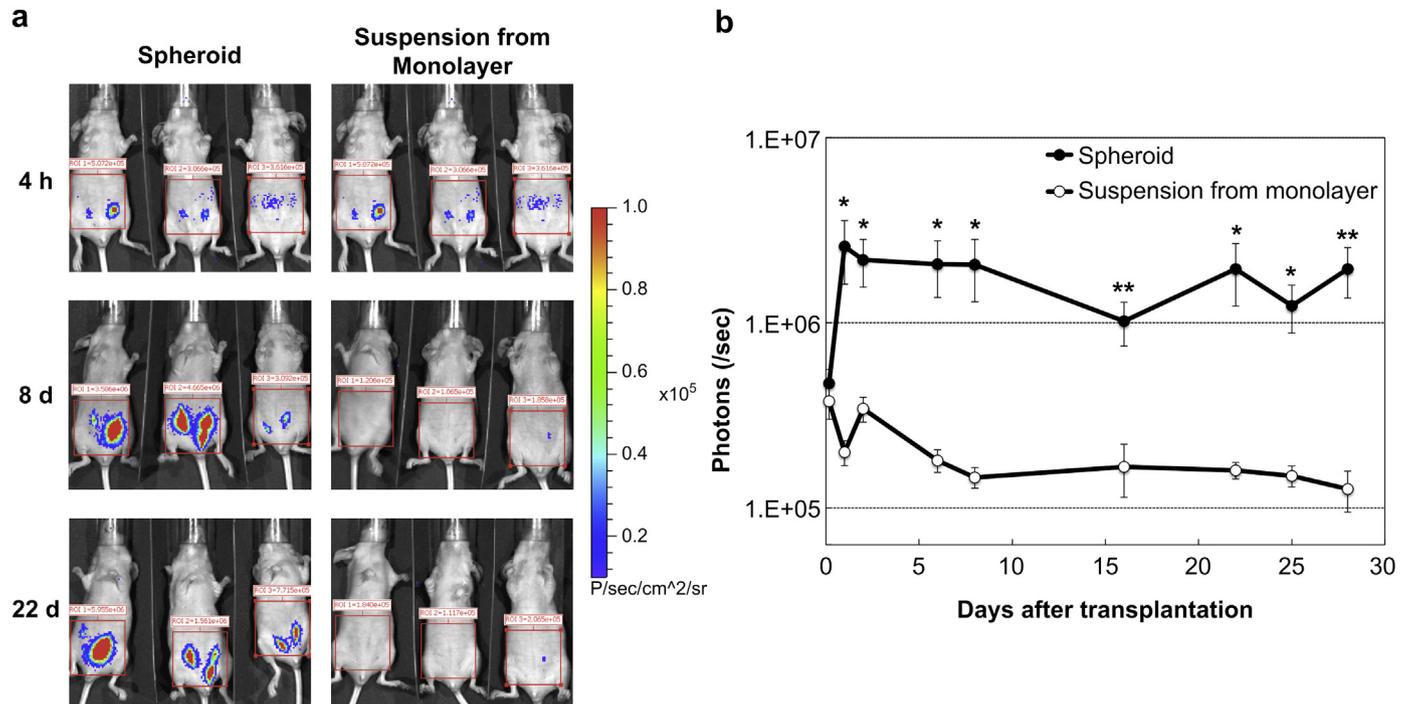


Fig. 2. Luciferase expression in host mice after hepatocyte transplantation. After 24 h of transfection with luciferase-expressing pDNA, hepatocyte spheroids and single-cell suspension from monolayer cultures were transplanted into the subcutaneous tissue of the abdominal region. Luciferase expression in host mice was evaluated using an IVIS™ Imaging System. (a) Representative images of the indicated time point after transplantation, (b) quantification of luminescence intensity. Data are presented as the mean ± standard error of the mean (s.e.m.) ($N = 7$). Statistical significance was assessed by 2-tailed Student's *t*-test, * $p < 0.05$, ** $p < 0.01$ versus hepatocytes from monolayer cultures.

4 h to 24 h after transplantation is likely to reflect the time interval required for intracellular processing of pDNA before its expression, such as endosomal escape and nuclear entry. A similar result was obtained for cells transplanted into the subcutaneous tissue of the forelimbs, in which the spheroids yielded higher transgene expression than that in the suspension form 24 h after transplantation, while both showed comparable expression at 4 h (Fig. S2). Thus, the difference in transgene expression between spheroid and monolayer cultures after transplantation became obvious over time. It is interesting to note that hepatocytes in both spheroid and monolayer culture systems exhibited comparable transgene expression of luciferase in *in vitro* culture (Fig. 3). Therefore, the decreased luciferase expression in transplanted hepatocytes in suspension form over time is suggested to be because of a gradual decrease in cell survival or functionality after transplantation, which is in a sharp contrast to the spheroids showing sustained transgene expression.

3.2. Cell survival and distribution in host tissue after transplantation

To obtain more insight into the prolonged transgene expression of cells in spheroids, we examined the survival and function of transplanted hepatocytes in host tissue. The number of vital cells in host tissue was evaluated using quantitative real-time polymerase chain reaction (qRT-PCR) measurements of the copy number of SRY genes on the Y chromosome after collecting total DNA from the host tissue. Using hepatocytes from male rats for cell transplantation into female mice, the number of transplanted cells was determined by discriminating them from host cells [30]. As shown in Fig. 4a, the number of hepatocytes in the host tissue 24 h after transplantation was comparable between the groups of spheroids and single-cell suspension. The transgene copy numbers (luciferase-expressing pDNA) were also comparable between these two

groups (Fig. 4b), and even the cells prepared from the monolayer cultured sample showed a relatively high value. Consequently, the enhanced transgene expression in spheroids, as seen in Fig. 1, was not explained by the difference in survival rate of the cells after transplantation.

The albumin expression in host mice was evaluated as a marker of innate hepatocyte function by collecting total mRNA from the transplantation site (forelimb) 24 h after cell transplantation, followed by qRT-PCR analyses to calculate the mRNA expression of

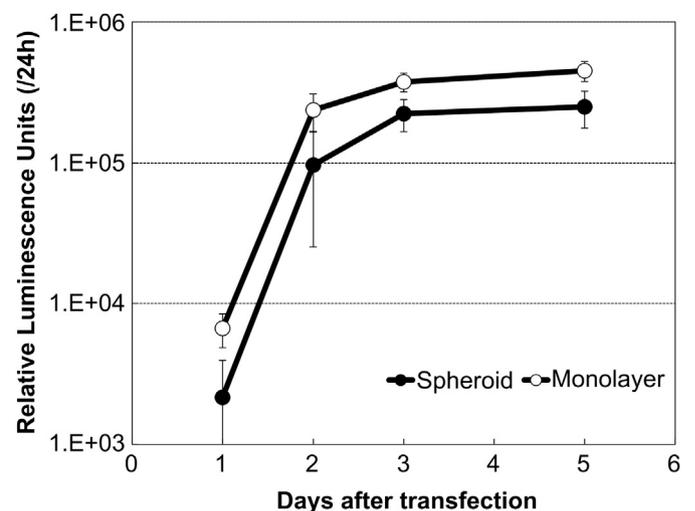


Fig. 3. Luciferase expression after *in vitro* transfection. Hepatocytes in spheroid or monolayer cultures were transfected with a secretory form of luciferase (Gaussia luciferase; Gluc)-expressing pDNA. The values indicate Gluc expression in the last 24 h of each time point because the culture medium was replaced precisely 24 h prior to the measurement. The data are presented as the mean ± standard error of the mean (s.e.m.) ($N = 6$). RLU, relative luminescence unit.

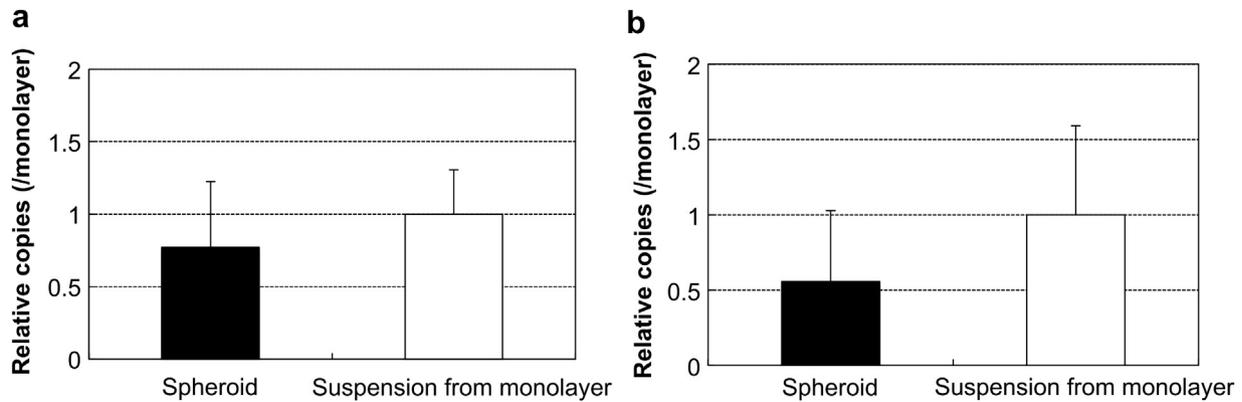


Fig. 4. Evaluation of the cell number of transplanted hepatocytes and copy number of transfected luciferase pDNA in the host tissue. After 24 h of transfection with luciferase-expressing pDNA, hepatocyte spheroids and a single-cell suspension from a monolayer culture were transplanted into the subcutaneous tissue of mouse forelimbs. At 24 h after transplantation, total DNA was extracted from the transplantation site for the following analyses: (a) The number of transplanted hepatocytes was measured using quantitative real-time PCR (qRT-PCR) analysis of the SRY gene on the Y chromosome, (b) copy number of luciferase pDNA was measured using qRT-PCR. The data are presented as the mean \pm standard error of the mean (s.e.m.) ($N = 7$).

albumin. As shown in Fig. 5, the hepatocyte spheroids showed significantly higher albumin expression in the host tissue compared with that in hepatocytes from the monolayer culture. This result of preserved albumin secretion in spheroids is consistent with the previous *in vitro* study results showing sustained albumin secretion in the culture medium from hepatocyte spheroids [7,13], indicating that spheroid formation essentially contributes to the maintenance of innate functions, such as albumin secretion of hepatocytes in both *in vitro* culture and *in vivo* transplantation.

To further analyze cell behavior in the host tissue, we performed imaging of the transplanted cells in the tissue using intravital confocal microscopy [26]. Hepatocytes from transgenic rats stably expressing enhanced green fluorescent protein (EGFP) were transplanted into the earlobes of mice without EGFP expression in the form of either spheroids or a single-cell suspension, followed by *in vivo* imaging 24 h after transplantation. Fig. 6a, b shows typical

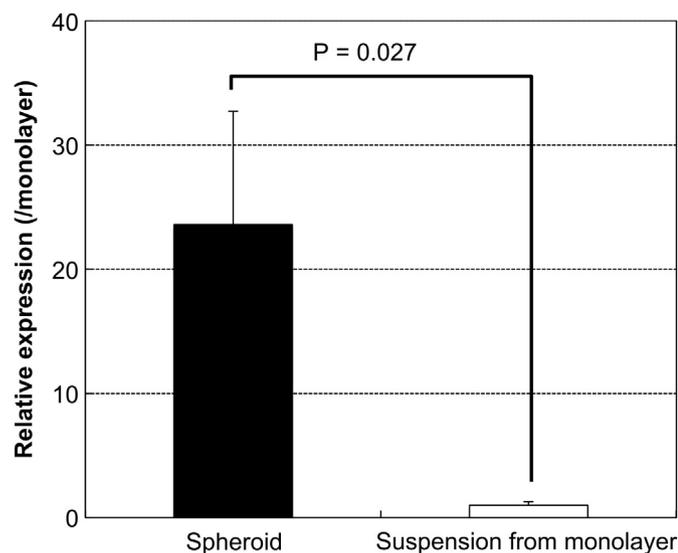


Fig. 5. Albumin expression from transplanted hepatocytes in the host tissue. After 24 h of transfection with luciferase-expressing pDNA, hepatocyte spheroids and single-cell suspensions from monolayer cultures were transplanted into the subcutaneous tissue of mouse forelimbs. At 24 h after transplantation, mRNA expression levels of rat albumin in the forelimb were measured using quantitative real-time PCR (qRT-PCR). The data are presented as the mean \pm standard error of the mean (s.e.m.) ($N = 8$). Statistical significance was assessed by 2-tailed Student's *t*-test.

images exhibiting the distribution of transplanted EGFP-positive cells. Of interest, in the spheroid-transplanted mice, the EGFP fluorescence was obviously aligned with blood vessels. The proximity of spheroids to the vessels was confirmed in high-resolution images with co-staining of the vessels by Evans blue (Fig. 6c). In contrast, the distribution of hepatocytes from monolayer cultures showed no specific pattern (Fig. 6b.) Although the detailed mechanism of the difference in cell distribution in the host tissue is yet to be clarified, the localized alignment in the vicinity of blood vessels is definitely an advantage of the spheroid systems for providing a favorable microenvironment to preserve cell activity.

3.3. Hematopoiesis after transplantation of erythropoietin-introduced hepatocyte spheroids

To investigate the feasibility of spheroid transplantation for therapeutic purposes, hepatocyte spheroids receiving transfection with erythropoietin-expressing pDNA were transplanted into the subcutaneous tissue of mice, followed by the evaluation of the hematopoietic effect induced by erythropoietin [31]. A single-cell suspension of hepatocytes from a monolayer culture was used as the control. After the transplantation of erythropoietin-expressing hepatocyte spheroids, the hematocrit and hemoglobin levels showed significant increases in host mice on days 22 and 28 (Fig. 7). In contrast, hepatocytes from monolayer cultures induced only marginal increases in hematocrit and hemoglobin levels, suggesting that spheroid transplantation was more beneficial than transplantation of cells from monolayer cultures to obtain therapeutic efficacy by transgene expression.

4. Discussion

In this study, we demonstrated an effective cell transplantation for therapeutic purpose by combining two of our original technologies, non-viral gene transfection vector, and micropatterned spheroid culture plates. Using the micropatterned plates, the size of spheroids was controlled in a narrow range around 100 μm to maintain functionality and survival of the cells in spheroids. Indeed, in the previous reports, optimal size of hepatocyte spheroids to obtain maximal secretion of albumin in cultured condition was determined to be 100 μm , while spheroids with the size of more than several hundred μm resulted in the necrosis of inner core [32–34]. By using micropatterned culture plates, we succeeded in the preparation of spheroids with uniform size of 100 μm , whereas

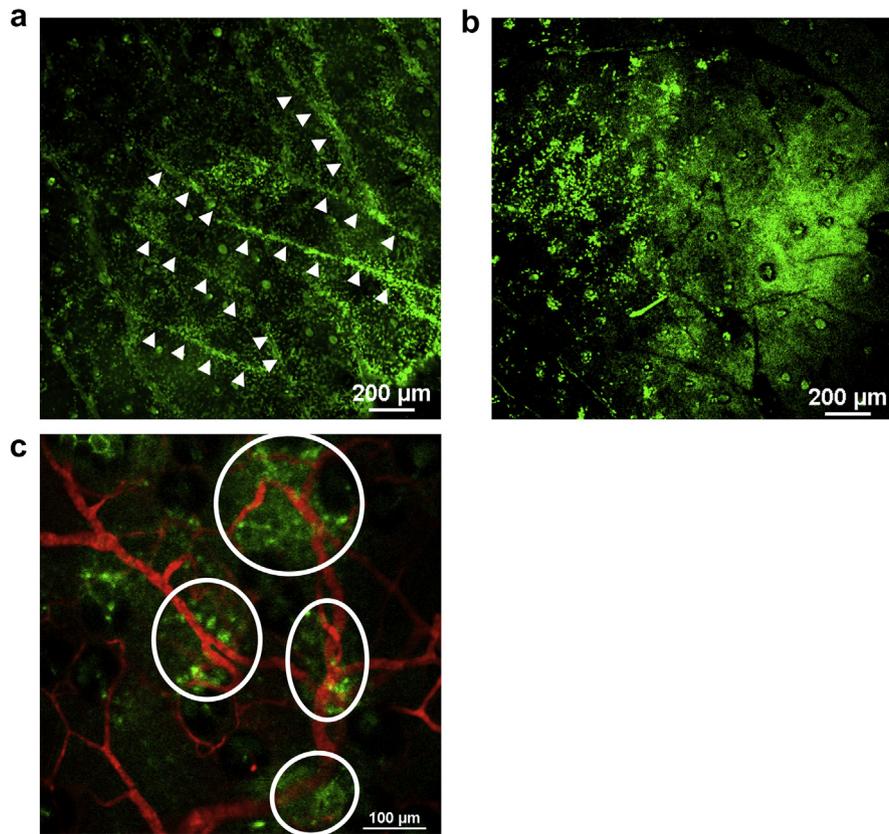


Fig. 6. Intravital microscopic imaging of transplanted hepatocytes in the host tissue. Hepatocytes stably expressing enhanced green fluorescence protein (EGFP, green) were used to track the transplanted cells. After 24 h of transfection with luciferase-expressing pDNA, the hepatocytes were subcutaneously transplanted into mouse earlobes. At 24 h after transplantation, earlobes were observed using an intravital confocal laser scanning microscope. (a, b) Broad field images after the transplantation of (a) hepatocyte spheroids and (b) single-cell suspension from a monolayer culture. Arrowheads indicate alignment of transplanted cells along blood vessels. (c) High-resolution images with co-staining of blood vessels by intravenous injection of Evans blue (red) after the transplantation of hepatocyte spheroids. Circles indicate association of transplanted cells with the blood vessels. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

precise control of spheroid size was difficult in other commonly used methods, such as those using spinner flasks, and non-adhesive culture plates, and hanging-drop methods [10,35–37]. It should be emphasized that the uniform size of spheroids with relatively small

diameter of 100 μm makes it possible to transplant the spheroids by commonly used injection needle. The ease of spheroid preparation even in a large-scale using the micropatterned plates (maximum of 2,500 spheroids per cm^2 culture plate) and the practicability of

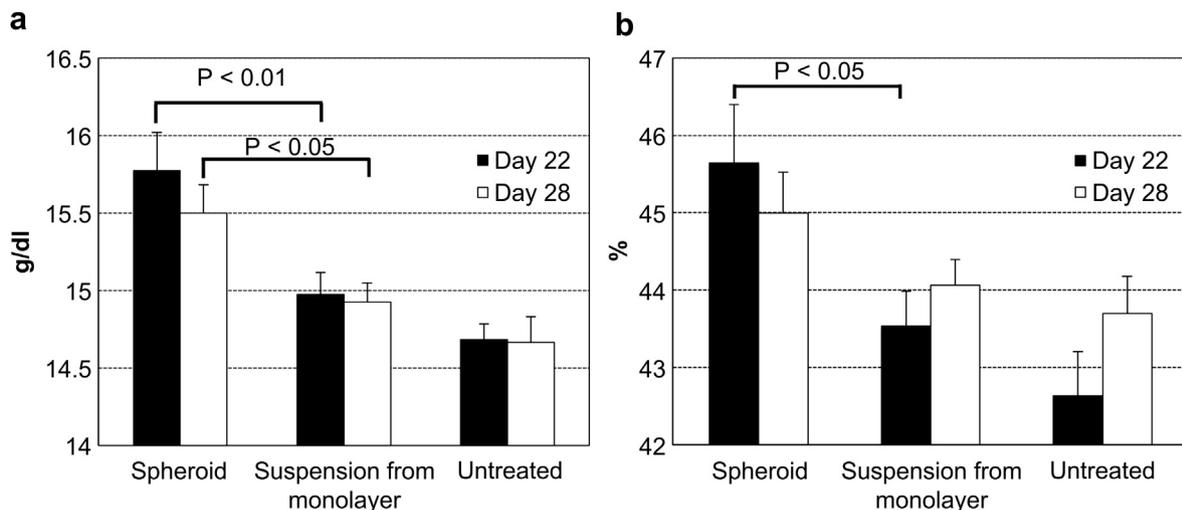


Fig. 7. Hematopoiesis after transplantation of erythropoietin-introduced hepatocytes. Hepatocytes in spheroid and monolayer cultures were transfected with erythropoietin-expressing pDNA. At 24 h of transfection, these spheroids and single-cell suspensions from monolayer cultures were subcutaneously transplanted into mouse abdomens. At 22 and 28 days after transplantation, hemoglobin (a) and hematocrit (b) levels were measured from blood samples. The data are presented as the mean \pm standard error of the mean (s.e.m.). ($N = 12$ for spheroid and monolayer groups, and $N = 6$ for untreated controls). Statistical significance was assessed by 2-tailed Student's *t*-test.

transplantation by injection needles with maintaining the spheroid structure is particularly useful for future clinical application.

The other aspect of this system is the genetic modification of the spheroids by gene introduction using polyplex nanomicelles. The transgene expression from the spheroids exceeded 10 folds compared with the hepatocytes from monolayer cultures in host mice after transplantation (Fig. 2). To confirm the potential of this system for therapeutic application, we used erythropoietin-expressing pDNA for genetic modification. Erythropoietin is a systemically-secreted hormone produced by kidney peritubular interstitial cells in adult mammals, stimulating the production of red blood cells [31]. Recombinant erythropoietin is clinically used as a hematopoietic factor [38]; however, the short duration of action of recombinant erythropoietin may necessitate multiple doses to obtain a continuous effect on hematopoiesis. In this study, a single transplantation procedure for erythropoietin-introduced hepatocyte spheroids was demonstrated to induce a hematopoietic effect in host mice for more than a month. The effect was significantly higher than that produced by cell transplantation from a monolayer culture (Fig. 6). Thus, it was revealed that the sustained manner of transgene expression from the spheroids was beneficial to obtain a therapeutic effect by secretion of bioactive factors such as erythropoietin from the transplanted cells into host mice.

It is interesting that in the *in vitro* study, transgene expression was comparable between the spheroid and monolayer culture groups (Fig. 3). In addition, the total cell number surviving in the host tissue after transplantation was comparable between these two groups (Fig. 4). We assumed that the increased transgene expression from the spheroids was attributed to the enhanced functionality of individual cells in the spheroids. Indeed, albumin expression as a marker for the innate function of transplanted hepatocytes significantly increased in spheroid systems (Fig. 5). For further investigation of the mechanism, we performed intravital imaging of the transplanted cells in the host tissue. As shown in Fig. 6, the cells transplanted as spheroid form had a tendency to locate in the vicinity of blood vessels, while the cells from the monolayer culture showed no specific distribution pattern. Thus, it is likely that the transplantation in spheroid form affected the subsequent cell behavior in the host tissue to accumulate near the blood vessels, resulting in prolonged secretion of erythropoietin. Regarding the unique image shown in Fig. 6a, c that the transplanted hepatocytes accumulated to the vicinity of the vessels, it is still unclear whether these cells migrate as the group of spheroids or individual cells from the disintegrated spheroids direct to the vessels. Although the detailed mechanism is yet to be clarified, the accumulation of transplanted hepatocytes into the vicinity of the vasculature is apparently beneficial to exert their proper functionality. Presumably, the molecule regulating cell adhesion such as CXCR4, which were reported to be upregulated in MSC spheroids compared with that in cells in monolayer culture [39], may play a crucial role to regulate the cell-to-cell interaction with the host cells and the extracellular matrix in the host tissue.

5. Conclusion

We established an effective platform for cell transplantation by a combined use of micropatterned spheroid culture on thermosensitive plates with a non-viral gene introduction system of polyplex nanomicelle. The hepatocyte spheroids could be recovered simply by lowering temperature with maintaining the spheroid structure after gene introduction. The spheroids were injectable directly into host animals by needles, where the transgene expression as well as innate functionality of hepatocytes represented by secretion of albumin was effectively preserved in host

tissue for more than a month. The transplantation of hepatocyte spheroids receiving erythropoietin-expressing pDNA provided sustained therapeutic effect of enhanced hematopoiesis in host animals, demonstrating the high potential of this system for therapeutic cell transplantation.

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Appendix A. Supplementary data

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