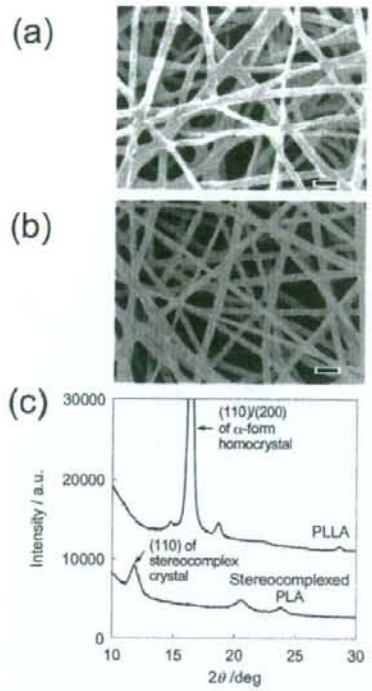

Figure 1. SEM images of (a) PLLA and (b) stereocomplexed PLA nanifibers and (c) wide-angle X-day diffraction patterns of PLLA and stereocomplexed PLA nanofibers. Scale bars = 1 μm. PLLA nanofiber shows diffraction peaks at 2θ = 15.1, 16.5 (assigned to (110)/(200)), and 18.1° that are assigned to α-form homocrystal of PLA. On the other hand, stereocomplexed PLA nanofiber showed diffraction peaks at 2θ = 12.0 (assigned to (110)), 20.8, and 24.1° that are aasigned only to stereocomplex crystal of PLA.

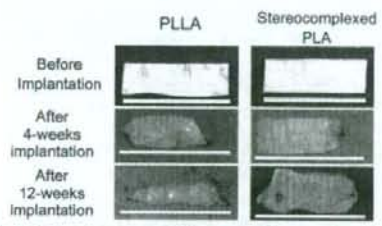

Figure 2. Bulk appearances of the nanofiber mats of PLLA and stereocomplexed PLA (a) before implantation, (b) after 4 weeks of implantation and (c) after 12 weeks of implantation. Scale bars = 3 cm.

This shows that the stereocomplexed PLA nanofiber consists of only the stereocomplex crystal and does not contain homocrystal of PLLA and PDLA at all.

The bulk appearances of the nanofiber mats were observed before and after removing the surrounding tissues. Figure 2 shows the photographs of nanofiber mats before and after 4 week and 12 week implantations, respectively. Significant reduction in the size of the PLLA nanofiber mat was recognized with the increasing period of implantation. In particular, the PLLA nanofiber mat after a 12 week implantation was densely covered with the surrounding tissues and only small fragments of the nanofibers mat were recovered. On the other hand, the stereocomplexed PLA nanofiber mat showed a less degree of the reduction in size than the PLLA nanofiber mat. This suggests

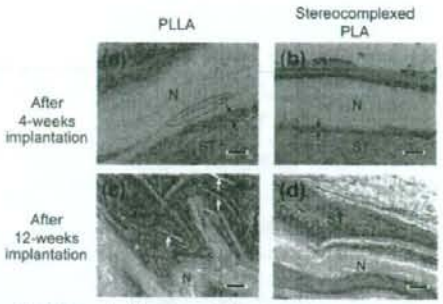

Figure 3. Histological images of PLLA and stereocomplexed PLA nanofibers before and after 4 weeks of implantation. (a) PLLA, before implantation; (b) stereocomplexed PLA, before implantation; (c) PLLA, after implantation; (d) stereocomplexed PLA, after implantation. Tissues were stained with hematoxylineosin. Nuclei of the inflammatory cells are stained blue. The width of inflammatory cells is indicated by the arrows and lines in (a) and (b). Ellipsoid region in (a) and white arrows in (c) indicate the infiltration of surrounding tissues and fragmented nanofibers, respectively. ST: surrounding tissues; N: nanofiber mats. Scale bars = 50 μm.

that the in vivo degradation of the stereocomplexed PLA nanofiber mat occurs slower than the PLLA nanofiber mat.

Histological Observation of Nanofiber Mats with the Surrounding Tissues. Histological observations of the nanofiber mats were performed to investigate the degree of inflammatory reactions and penetration of the surrounding tissues into the nanofiber mats. Figure 3 shows the phase contrast images of ultrathin sections of the explanted nanofiber mats stained by hematoxylin-eosin. The nuclei of inflammatory cells were stained blue by the hematoxylin dye and their presence is an indication of tissue response toward the implanted nanofiber mats. As indicated by the arrows and lines in Figure 3a, a thick layer of inflammatory cells was accumulated at the interface between the PLLA nanofiber mat and the surrounding tissues. In contrast, the layer of accumulated inflammatory cells was thinner for the stereocomplexed PLA nanofiber mat, as shown in Figure 3b. This indicates that the stereocomplexed PLA nanofiber mat causes smaller degree of inflammatory reactions than the PLLA nanofiber mat.

Furthermore, delamination (indicated by the ellipsoid in Figure 3a) occurred on the surface of the PLLA nanofiber mat, and hence, the infiltration of the surrounding tissues was observed. However, no infiltration of the surrounding tissues was observed for the stereocomplexed PLA nanofiber mat. After 12 weeks of implantation, while the PLLA nanofiber mat was significantly fragmented (white arrows indicate the fragmented nanofiber mat), the stereocomplexed PLA nanofibers retained the mat-like bulk morphology. These trends are well correlated with the bulk appearances of the nanofiber mats and support the observation that the in vivo degradation of the stereocomplexed PLA nanofiber mat proceeds slower than the PLLA nanofiber mat.

SEM Observation. SEM observation was performed for the nanofiber mats before and after 4 weeks and 12 weeks of implantation. Figure 4 shows the SEM images of nanofiber mats before implantation, after implantation and incubation at different periods of time. As for PLLA, cleavage of each strand of nanofiber occurred after 4 weeks. Furthermore, after 12 weeks, a decrease in the density of the nanofiber mat was observed. This is consistent with the histological image showing the fragmentation of the PLLA nanofiber mat. On the other hand, no cleavage of the stereocomplexed PLA nanofibers was observed even after 12 weeks of implantation.

PLA.[18] In particular, we have first succeeded in processing sterecomplexed PLA nanofiber in which the racemic crystal is only the crystalline polymorph.[19] The formation of racemic crystal was performed by annealing the as-spun nanofiber at 100 °C, which is 80 °C lower than those in previously reported studies.

The degradation behavior of PLA nanofibers has been investigated by using various specimens and conditions.[20−22] However, the previous reports were all limited to in vitro conditions. There have been no reports on the biocompatibility and in vivo degradation behavior of stereocomplexed PLA nanofibers.

In this work, tissue responses and degradation behavior of PLLA and stereocomplexed PLA nanofibers in vivo were investigated by subcutaneously implanting these nanofibers in rats. The tissue response against the nanofibers has been investigated by means of histological observation. The changes in structure and properties of the nanofibers during subcutaneous implantation has been investigated using scanning electron microscopy (SEM), wide-angle X-ray diffraction (WAXD), gel permeation chromatography (GPC), and mechanical tensile testing. The relation between tissue response and degradation behavior of nanofibers is discussed in terms of the structural and property changes of the nanofibers.

Experimental Section

Materials. PLLA with a M_n of 4.7 × 10^5 and M_w/M_n of 1.8 was purchased from Polysciences, Inc. and used as received. PDLA with a M_n = 2.2 × 10^5 and M_w/M_n of 1.5 was synthesized according to the following procedure. The D-lactide monomer, obtained from Purac, was recrystallized from anhydrous ethyl acetate. Bulk polymerizations were carried out in glass ampoules containing a magnetic stirring bar at 130 °C. Stannous octanoate in petroleum ether was used as the catalyst for the ring-opening polymerization. The ampoules were evacuated using a high vacuum pump and repeatedly flushed with high purity nitrogen to remove volatile impurities, solvents, and oxygen. Then the ampoules were sealed with a blowtorch and heated to the reaction temperature. The products in the ampoules were dissolved in chloroform, precipitated in the excess of methanol, filtered, and dried.

Electrospinning. Solutions of PLLA and PDLA (1 wt %) were prepared using 1,1,1,3,3,3-hexafluoro-2-propanol, HFIP, as the solvent. For the preparation of PLA stereocomplex nanofibers, equal volume of PLLA and PDLA solutions were mixed for several seconds by vortex mixer. Nanofibers were prepared using an Esprayer ES-2000 electrospinning device by Fuence, Co. Ltd. Dope solutions were extruded with a speed of 2.4 mL/h from a syringe needle with an inner diameter of 0.5 mm. Electrical voltage of 15 kV was applied to the syringe. Nanofibers were deposited onto a 10 × 10 cm^2 aluminum substrate placed perpendicular to the needle. To ensure sufficient thickness of nanofiber mats, the substrate was covered with a template made by a 51.4 μm thick Kapton film on which a 3 × 3 cm^2 window was opened. Distance between the needle tip and the substrate was set to 15 cm. The atmosphere of the spinning chamber was kept at less than 30% of relative humidity. PLLA and PLA stereocomplex nanofibers were then annealed in an oven at 100 °C for 8 h. Each electrospun PLA nanofiber mats was then cut into two different dimensions measuring 1 × 1 cm^2 and 1 × 3 cm^2, respectively. To prevent contamination, all scaffolds were sterilized overnight with ethylene oxide at 40 °C and kept in sealed bags until use.

Subcutaneous Implantation in Rat and Retrieval. Two 12-week old male Wistar rats were used for implanting the scaffolds; one for scaffolds measuring 1 × 1 cm^2, while the other for scaffolds measuring 1 × 3 cm^2. The experimental protocol had been approved by the Animal Care Committee of the National Cardiovascular Center, Osaka, Japan. The implantation of nanofiber mat was performed under anesthesia using diethyl ether. The 1 × 1 cm^2 scaffolds were implanted subcutaneously at one side of the backbone while the 1 × 3 cm^2 scaffolds were implanted subcutaneously at the backbone. The grouping of the rats was based on the duration of observation for 4 weeks and 12 weeks.

Upon explantation, the nanofiber mats measuring 1 × 1 cm^2 were excised with the surrounding tissues and stored in 2.5% glutaraldehyde in phosphate buffer saline (PBS) with a pH of 7 until further preparation of ultrathin section for histological observation. The retrieved 1 × 3 cm^2 nanofiber mats were treated with 1.25 wt % trypsin solution to remove the surrounding tissues. They were then kept in tubes containing PBS at 4 °C until further use. Trace amount of sodium azide was added to avoid the decay of the specimens. After trypsin treatment, surrounding tissues were manually removed as much as possible. The nanofiber mats were repeatedly washed using milli-Q water and dehydrated using ethanol series. Finally, the dehydrated nanofiber mats were dried overnight using vacuum desiccator at room temperature.

In Vitro Degradation. Nanofiber mats with the size of 1 × 3 cm^2 were incubated in 5 mL of PBS with a pH of 7.27 for 4−12 weeks at 37 °C. The medium was changed every 2 weeks. After 4 and 12 weeks of incubation, the nanofiber mats were washed thoroughly with distilled water, vacuum-dried at room temperature, and then subjected to SEM observation.

Histological Observation. The surrounding tissues were excised together with the implanted nanofiber mats and fixed with 2.5% glutaraldehyde in PBS with a pH of 7. A small piece of the tissue was then embedded in paraffin before subjecting it to microtome sectioning. Hematoxylin and eosin (HE) were used for staining the tissues. The tissue response to nanofiber mats was evaluated from the coloration observed with a phase-contrast microscope.

Scanning Electron Microscopy (SEM). Nanofiber mat was placed on a stub and then coated with Au. The thickness of Au coat was about 15 nm. SEM images of nanofibers were obtained using a field emission scanning electron microspope (JSM-6330F, JEOL, Co. Ltd.) operating at an acceleration voltage of 5 kV of and an emission current of 12 μA. For estimating the average diameter of nanofibers, diameter was measured at more than 60 points on the printed SEM image.

Wide-Angle X-ray Diffraction (WAXD). WAXD patterns of nanofiber mats were acquired under ambient condition using Rigaku RINT-2500 system operating at 40 kV and 200 mA. Measurements were performed on a Bragg−Brentano type $2\theta/\theta$ goniometer in a reflection mode. Ni-filtered Cu Kα radiation (λ = 0.15418 nm) was collimated with a 1/2 deg divergence slit, 1/6 deg scatter slit and 0.15 mm receiving slit. Scans were performed three times in a 2θ range of 10−40° with a scan rate of 0.5°/min and 0.05° step.

Gel Permeation Chromatography (GPC) Analysis. The molecular weight analysis of the nanofiber mats was performed with gel-permeation chromatography at 40 °C, using a Shimadzu LC-10A GPC system equipped with a RID-10A refractive index detector and Shodex K-806 M and K-802 columns. Chloroform was used as the eluent at a flow rate of 0.8 mL min^{-1}. The calibration curve was prepared by using monodisperse polystyrene standards.

Results

Changes in the Appearance of Nanofiber Mats During Subcutaneous Implantation. Figure 1a,b shows SEM images of the PLLA and stereocomplexed PLA nanofibers, respectively. Both nanofibers possess similar morphology with the average fiber diameter of about 300 nm. However, totally different crystalline structure is formed in these nanofibers, as seen from the WAXD profiles in Figure 1c. PLLA nanofiber shows diffraction peaks at 2θ = 15.1, 16.5 (assigned to (110)/(200)), and 18.1° that are assigned to α-form homocrystal of PLA.[12] On the other hand, stereocomplexed PLA nanofiber showed diffraction peaks at 2θ = 12.0 (assigned to (110)), 20.8, and 24.1° that are assigned only to stereocomplex crystal of PLA.[12]

In Vivo Tissue Response and Degradation Behavior of PLLA and Stereocomplexed PLA Nanofibers

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Biocompatibility of PLLA and stereocomplexed PLA nanofibers was evaluated by subcutaneous implantation in rats for 4−12 weeks. Characterization of the nanofibers was performed by GPC, SEM, wide-angle X-ray diffraction, and optical microscopy of hematoxylin-eosin stained ultrathin sections of explanted nanofibers. Stereocomplexed PLA nanofiber showed slower degradation than PLLA nanofiber and thus retained their shape after prolonged implantation. Furthermore, stereocomplexed PLA nanofiber caused milder inflammatory reaction than PLLA nanofiber. These results offer the potential use of PLLA and stereocomplexed PLA nanofibers as a biomaterial for short-term and long-term tissue regeneration, respectively. Stereocomplexed PLA nanofiber after in vitro degradation showed smaller degree of swelling than PLLA nanofiber. Taking the results of in vivo degradation together with in vitro degradation into consideration, bioabsorption mechanism of the in vivo degradation of the nanofibers is proposed.

Introduction

In the field of medical sciences, the method of tissue engineering has been extensively studied to overcome the problems of conventional methods such as organ transplantation and usage of artificial organs.[1] In tissue engineering, the proliferation and differentiation of cultured cells for deficiency repair has to be artificially controlled. The development of scaffold materials on which cells proliferate and differentiate has been a major concern in tissue engineering. Conventionally, collagens and gelatins extracted from animals have been used to produce scaffolds. However, the usage of these animal-origin materials is shrinking for fear of infectious diseases. Alternatively, the usage of biodegradable and biocompatible polymers that do not contain infectious substances such as endotoxins and prions has been explored.

Recently, as a novel method for producing scaffolds, formation of nanofibers with the diameter ranging from several tens to hundreds of nanometers is extensively studied.[2−4] Nanofiber scaffolds have fine pores and grooves as small as a few micrometers wide. Such fine structural features facilitate the adhesion and proliferation of cells. It is required for nanofiber scaffolds to sustain sufficient strength to support regenerating tissue cells and to be degraded after the tissue regeneration is completed. To meet these demands, various kinds of biodegradable and biocompatible polymers have been processed into nanofibers. Furthermore, the fiber morphology, crystalline structure, and degradation behavior of the nanofibers have been investigated.[5−7]

Poly(lactide) (PLA) is one of a few polymers that is practically applied as various medical materials such as implants and sutures.[8] PLA possesses mechanical properties sufficient to endure the mechanical load applied in human body. However, it is readily hydrolyzed both in enzymatic and nonenzymatic conditions.[9] The high susceptibility of PLA toward hydrolysis becomes a shortcoming when the long-time storage under physiological conditions is required. Various efforts to overcome this shortcoming have been attempted. One of such efforts is the formation of stereocomplex in PLA materials.

Stereocomplexed PLA is a characteristic crystalline form of PLA.[10,11] A sterically stable racemic crystal of stereocomplexed PLA is formed by complexing poly(L-lactide) (PLLA) and poly(D-lactide) (PDLA) that take molecular conformations of left-handed and right-handed helices, respectively.[12] As a result, stereocomplexed PLA has a melting temperature of 230 °C, that is 50 °C higher than PLLA and PDLA.[10] Furthermore, it has been reported that stereocomplexed PLA is more stable against hydrolysis than PLLA.[13−15] This finding offers the possibility for controlling the hydrolytic behavior of PLA material by the formation of stereocomplex. Although various methods have been proposed and investigated for the formation of stereocomplex within PLA materials,[16,17] PLA materials that contain only racemic crystal has not yet been processed. Furthermore, the conventional processes involve long-time annealing at elevated temperatures as high as 180 °C and repeated stretching. To form stereocomplexed PLA more conveniently, electrospinning has recently been applied to the formation of stereocomplexed

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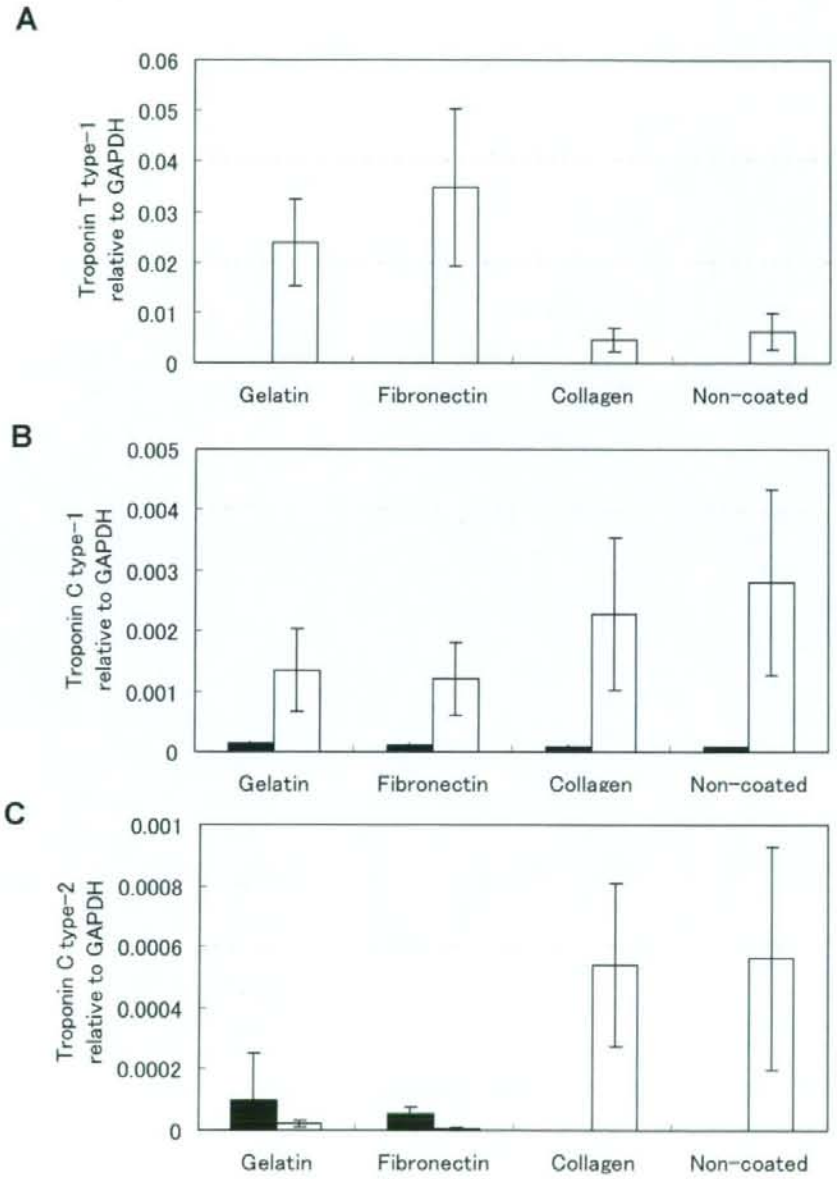

Fig. 3. Expression of cardiac markers in P19.CL6 cells treated with α-MEM containing 1 % DMSO on various dishes (white bar) or with a control α-MEM for 11 days (black bar)

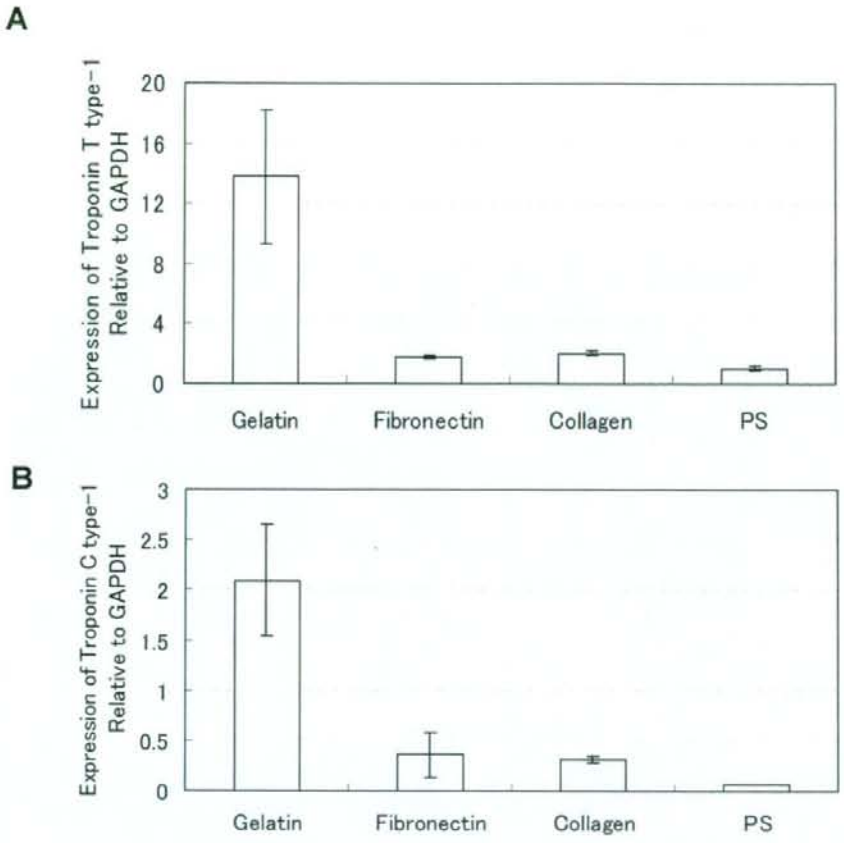

Fig. 2. Expression of cardiac markers in isolated cardiomyocytes after cultivation for 4 weeks on various types of ECM.

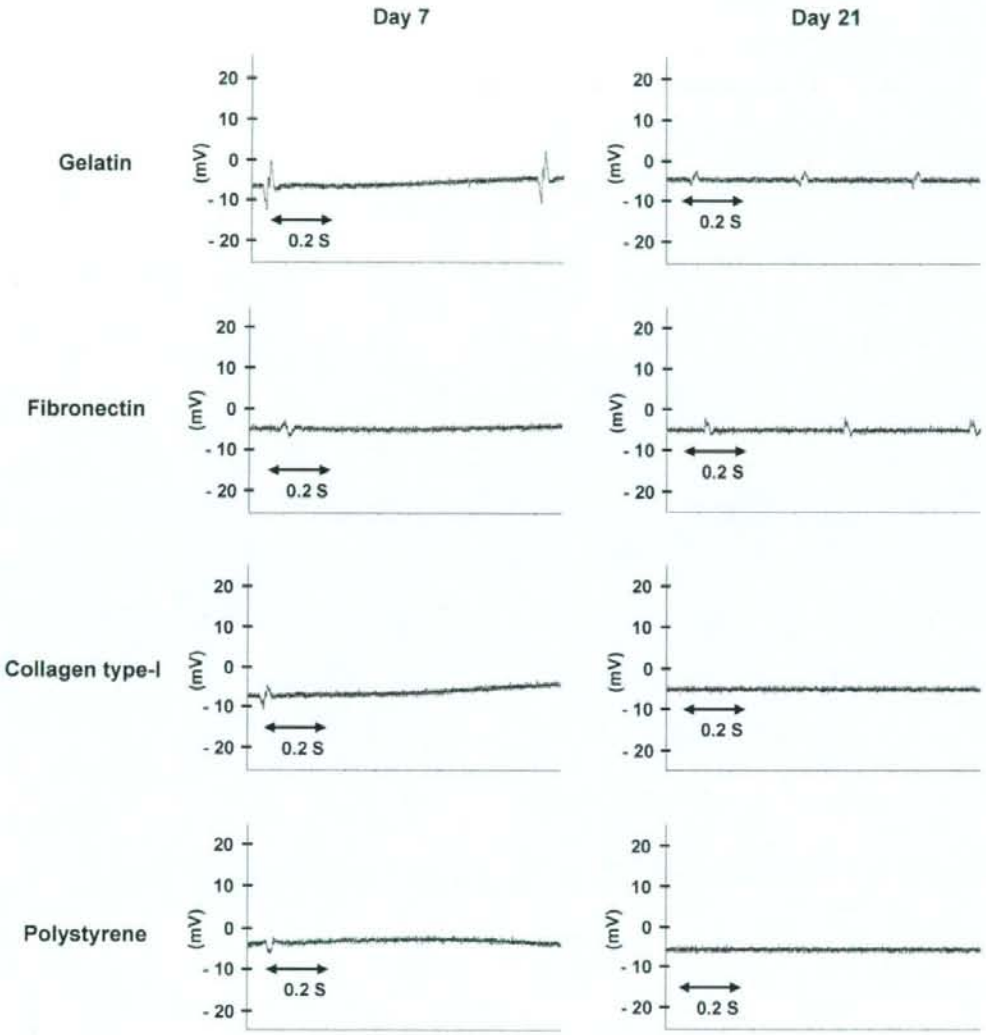

Fig. 1. Electrophysiological assessment on isolated cardiomyocytes after 7 and 21 days cultivation.

Tab. 1. PCR primers used in this study.

Genes	Sens	Anti-sens
Troponin T type-1	5'-GAAACAGGATCAACGACAACCA-3'	5'-CGCCCGGTGACTTTGG-3'
Troponin C type-1	5'-GATCTCTTCCGCATGTTTGACA-3'	5'-TGGCCTGCAGCATCATCTT-3'
Troponin C type-2	5'-AGATCGAATCCCTGATGAAGGA-3'	5'-CATCTTCAGAAACTCGTCGAAGTC-3'
GAPDH	5'-CTACCCCCAATGTATCCGTTGT-3'	5'-TAGCCCAGGATGCCCTTTAGT-3'

Tab. 2. Summary of voltage potential in several types of ECM-coated dish.

Substrate	Action potential (mV) [Beating rate (Hz)]		
	Day 7	Day 14	Day 21
Gelatin	6.7 ± 0.49 [1.2 ± 0.05]	6.6 ± 1.26 [1.3 ± 0.01]	3.1 ± 0.21[2.8 ± 0.03]
Fibronectin	1.1 ± 0.97 [1.1 ± 0.30]	6.9 ± 1.15 [1.3 ± 0.42]	2.8 ± 0.11 [2.0 ± 0.11]
Collagen type-I	2.6 ± 0.35 [0.8 ± 0.02]	1.7 ± 0.03 [2.3 ± 0.05]	ND
Polystyrene	2.0 ± 0.75 [0.3 ± 0.04]	ND	ND

Tab. 3. Number of beating colonies 11 days after induction with 1% DMSO.

Substrate	Average number of beating colony per dish
Gelatin	13 ± 7
Fibronectin	9 ± 5
Collagen type-I	5 ± 2
Polystyrene	3 ± 1

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5. Conclusion

The physical and biological properties of substrate were the important factor not only for maintaining the cardiac functions but also for leading to the cardiac differentiation of P19.CL cells. Gelatin was promissing ECM protein to this end *in vitro*.

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binding domain of collagen, resulting in fibrilogenesis [21]. It was also reported that the fibronectin bound to gelatin more strongly than to collagen [20-21]. Therefore, the high production of fibril might be occurred on gelatin- more effectively than on fibronectin- or collagen-coated dish. These connecting elastic fibers might regulate the motion of cardiomyocytes during contraction and recoil as suggested by Ahumada, G.G et al. [30]. The highly elastic feature of the matrices of gelatin, fibronectin bound onto gelatin [18,22], and developed fibrin matrices may allow easier contraction of cardiomyocytes, which results in the larger action potential and longer beating duration on gelatin.

P19.CL6 cells were used for studying the effect of culture substrates on the cardiogenesis *in vitro*. Since A.H. Ohkubo reported that, P19.CL6 differentiated into cardiomyocytes in 10 days after DMSO treatment, observation of differentiation continued for 11 days. The first beating colony was found on 9^{th} day on gelatin-coated dish, while on 10^{th} day on fibronectin-coated dish and 11^{th} day on collagen type I-coated dish and non-coated polystyrene dish. The expressions of troponin T type-1 on gelatin- and fibronectin-coated dishes were significantly higher than the collagen type I-coated dish and non-coated polystyrene dish. High expression of troponin C type-2 in collagen type I-coated dish and non-coated polystyrene dish indicated that the differentiation of P19.CL6 cells was delayed as compared to gelatin- and fibronectin-coated dish [13].

Differentiation of P19.CL6 on gelatin- and collagen type I-coated dish was very different despite of their similar unit structure. The fast differentiation on gelatin- was possibly because of slow cell proliferation on high dynamic storage modulus substrate [18]. Growth of P19.CL6 on gelatin- was about half of that on collagen type I-coated dish (data not shown). Walsh et. al. suggested that, the proliferation of mesenchymal stem cell was suppressed during the differentiated to osteoblast *in vitro* [31]. The fast differentiation on fibronectin-coated dish may also relate to the elasticity of the substrate [22].

This finding will offer a bright future for the myocardial patch scaffold. It has been reported that the probability of cardiac differentiation of adipose tissue derived mesenchymal stem cells after transplantation to infracted rat heart is quite low [32]. In the present study, the gelatin-based niche is found to be preferable for cardiac differentiation and for beating function of cardiomyocytes. We will be applying these results on the cardiac differentiation of mesenchymal stem cells in order to prepare the allogeneic beating cardiomyocytes.

cardiac differentiation [14]. Expression of troponin T type 1 on gelatin- and fibronectin-coated dishes was higher than the other dishes as shown in Fig. 3A. However the expression of troponin C type-2 in collagen type I-coated dish and non-coated polystyrene dish were higher than gelatin- and fibronectin-coated dish. The high expression of troponin C type-2 on collagen-coated dish and non-coated polystyrene dish on day 11 was possibly because of delayed differentiation of P19.CL 6 cell to cardiomyocytes-like cells. Stoutamyer et al. reported that expression of troponin C tyepe-2 was only expressed at day 3, reach maximum expression at day 5, and decreased by day until no expression at day 11 during the development of quail heart *in ovo* [14]. Stoutamyer et al. also reported that the expression of troponin C type-1 was detected at day 2 and decreased during the increasing of troponin C type-2 and reached constant after that [14].

These results demonstrated that differentiation of P19.CL6 cells to beating cells on gelatin-coated dish and fibronectin-coated dish was faster and effective more than collagen type I-coated dish and non-coated polystyrene dish.

4. Discussion

In the present study, enhanced action potential and elongated beating duration of neonatal cardiomyocyte were observed on gelatin-coated dish compared to collagen type I-coated dish. Possible differences between gelatin and collagen were 1) collagen posses triple helical conformation, 2) gelatin has wide molecular distribution depending on its preparation process, 3) gelatin is easily hydrolyzed by protease [17], 4) dynamic storage modulus of gelatin was higher than collagen [18], and 5) gelatin binds with fibronectin with higher ability [19-21]. There was already a report that the fibronectin is a very elastic substrate [22].

Mechanism of the elongated beating duration on gelatin-coated substrate is unclear but the mechanical property (elasticity) and biological activity of the substrates might affect it. It has been reported that the mechanical properties of culture matrices affect various cellular properties such as morphology of embryonic stem cell [23-24], collagen production of to fibroblasts [25-26], or differentiation of mesenchymal and neural stem cells [27-28].

Fibrinectin produced by culture cells is known to associate with the fibronectin

not detected on collagen type I-coated dish and polystyrene dish.

Beating rate of cardiomyocyte was also affected by the ECM proteins. After 7 days culture, the beating rate of cardiomyocytes was 1.2 ± 0.05 Hz on gelatin-coated dish, 1.1 ± 0.3 Hz on fibronectin-coated dish, 0.8 ± 0.02 Hz on collagen type I-coated dish and 0.3 ± 0.04 Hz on non-coated polystyrene dish. After 14 days culture, the beating rate became 1.3 ± 0.01 Hz on gelatin-coated dish, 1.3 ± 0.42 Hz on fibronectin-coated dish, and 2.3 ± 0.05 Hz on collagen type I-coated dish. After 21 days, beating rate was 2.8 ± 0.03 Hz on gelatin-coated dish and 2.0 ± 0.11 Hz on fibronectin-coated dish. Whilst, cardiomyocytes cultured on polystyrene dish and collagen-coated dish did not beat well and stopped at the early stage of cultivation. These results indicated that, gelatin could maintain the beating behavior of cardiomyocytes for a longer period of time compared to fibronectin or collagen type I.

3.2 Expression of troponin T type-1 and troponin C type-1

Cardiac troponin T type-1 and troponin C type-1, which are known to be cardiomyocytes markers and important structure of muscle tissue, and to play a role in contraction of muscle cells [13]. After 4 weeks of culture, expression of troponin T type-1 in cardiomyocytes on gelatin-coated dish was 7, 6, and 12 times higher than those on fibronectin-coated, collagen-coated, and non-coated dishes (Fig. 2A). Expressions of troponin C type-1 on gelatin-coated dish were also 5, 6, and 32 times higher than those on fibronectin-, collagen type I-coated, and non-coated dishes (Fig. 2B). These results were in proportional with the result of electrophysiological study (Fig. 1), in which the beat of cardiomyocytes still could be detected on gelatin- and fibronectin-coated dish after 3 weeks cultivation.

3.3 Differentiation of P19.CL6 cells

Beating colonies were found on gelatin-coated dish in 9 days with α-MEM medium containing 1% DMSO. This was followed by cells cultured on fibronectin-coated dish after 10 days culture, and collagen type I-coated dish after 11 days culture. The average number of beating colonies found on the first day of detection were 13 ± 7 colonies per dish on gelatin-coated dish, 9 ± 5 colonies per dish on fibronectin-coated dish, 5 ± 2 colonies on collagen type I-coated dish, and 3 ± 1 colonies on polystyrene dish (Tab. 3).

As described earlier, troponin T type-1 and troponin C type-1 are known to be markers of cardiomyocytes [13] and troponin C type-2 is known to be markers of

TOYOBO Co., Ltd., Osaka, Japan) were added into the mixture and RT reaction was extended at 37 °C for 1 hour. Afterward, the reaction was heated at 94 °C for 5 minutes to inactivate the enzyme and cooled at 4 °C for 15 minutes. The RNase (DNase-free, 0.5 μg, Roche Diagnostics GmbH, Mannheim, Germany) was added into the mixture and incubated at 37 °C to remove the template of RNA.

2.5 Real-time quantitative PCR

Real-time quantitative PCR was conducted with SYBR Green. Primers for PCR analysis for troponin T type-1, troponin C type-1, and troponin C type-2 were designed using Primer Express software (Perkin-Elmer Applied Biosystems, Cheshire, UK). Primer sequences are shown in Tab. 1. Reaction mixtures contained 23.74 μl distilled water, 25 μl SYBR Green Realtime PCR master mix (TOYOBO Co., Ltd, Osaka, Japan), 100 nM of each primers, and 0.26 μl cDNA. The thermal profile for PCR was 50 °C for 2 minutes, followed by 95 °C for 10 minutes, followed by 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. The 0.26 μl distilled water was used as negative control PCR reaction to ensure the absence of template contamination in PCR reagents. The average threshold cycle (Ct) values of triplicate measurements were used for all subsequent calculations on the basis of the delta Ct method.

3. Results:

3.1 Beating behavior of isolated cardiomyocytes

One week after culture, action potential of cardiomyocytes on gelatin-coated dish was higher than the other conditions (Fig. 1), and its beating duration was also longer than the other conditions. Action potential and beating rates on each matrix were summarized in Tab. 2. After 7 days culture, the action potential was around 6.7 ± 0.49 mV, for cardiomyocytes cultured on gelatin-coated dish, 1.1 ± 0.97 mV on fibronectin-coated dish, 2.0 ± 0.35 mV on collagen type I-coated dish, and 2.0 ± 0.75 mV on non-coated polystyrene dish. These results indicate that the beating rate on fibronectin-, collagen type I-coated dish and polystyrene dish were 84, 61, and 70% lower than gelatin-coated dish after 1 week cultivation.

After 14 days culture, the action potential became 6.6 ± 1.26 mV on gelatin-coated dish, 6.9 ± 1.15 mV on fibronectin-coated dish, and 1.7 ± 0.03 mV on collagen type I-coated dish. No beating was observed on polystyrene dish after 2 weeks cultivation. After 21 days culture, the action potential was 3.1 ± 0.21 mV on gelatin-coated and 2.8 ± 0.11 mV on fibronectin-coated dish. The action potential was

2.2 Differentiation of P19.CL6 cells

Differentiation of P19.CL6 cells was performed as described by Ohkubu A.H with modifications [12]. Briefly, P19.CL6 cells were plated at a density of 3.7 x 10^5 cells on 60 mm gelatin-, fibronectin-, collagen type I-coated dish and non-coated polystyrene dish with a α-MEM supplemented with 10 % (v/v) FBS containing 1 % dimethyl sulfoxide, (DMSO, Wako, Ltd., Osaka, Japan). As a control experiment, P19.CL6 cells were cultured with α-MEM supplemented with 10 % (v/v) FBS without 1 % DMSO. The medium was changed every 2 day.

2.3 Measurement of action potential

Cultured plate on which beating colonies appeared was placed on stage of an inverted phase-contrast optical microscope (ZEISS, Axiovert 135, Munich, Germany) and action potentials were measured immediately by conventional microelectrode. The measurements were conducted after 1, 2, and 3 weeks cultivation. Silicon coated Ag wire (A-M system, Inc., Carlsborg, Washington, USA, 250 μm bare, 330 μm coated) was used as microelectrode. The microelectrode was set at micromanipulator system (MON-202D, Nikon Narishige Co., Ltd., Tokyo, Japan) and connected with bioelectric amplifier (AB-621G, Nihon Kohde Co., Osaka, Japan). The sensitivity and time constant of bioelectric amplifier were set at 0.1 mV / div and 0.003 s. For the measurement, microelectrode was adjusted using micromanipulator until it was attached to the membrane of beating cells. The voltage difference was amplified with bioelectric amplifier, as well as displayed and recorded with chart 5 AD Instrument software (AD Instrument, Bella Vista, Australia).

2.4 Total RNA isolation and reverse transcription

Total RNAs of cardiomyocytes and DMSO-treated P19.CL6 cells cultured on various dishes were extracted by QuickGene RNA cultured cell kit S (Fujifilm Life Science, Tokyo, Japan) 4 weeks after culture and 11 days after culture, respectively.

First-strand cDNAs were synthesized using a mixture of oligo$(dT)_{18}$ primer. Total cellular RNAs (200 ng) were incubated with 2.5 μM oligo$(dT)_{18}$ primer at 70 °C for 10 minutes to denature RNA secondary structure and then incubated at 4 °C to let the primer anneal to the RNA. A givent amount of 5X RT buffer (TOYOBO Co., Ltd., Osaka, Japan) and 2.5 mM dNTP mixture (Takara Bio Inc., Shiga, Japan) (4 μl) were added and incubated at 37 °C for 5 minutes. The reverse transcriptase (100 Units,

type IV, heparan sulfate proteoglycans and entactin), and Cardiogel™ (mixture of collagen type I and III, glycoproteins, laminin, fibronectin and proteoglycans) on cell viability, proliferation rate, and cardiomyocyte gene expression were reported [10-11]. However, the cardiomyocytes beating behavior were not fully discussed.

In the present study, differentiation to beating cardiomyocytes and the beating duration of the cardiomyocytes were studied using two types of model cells. Murine embryonal carcinoma (EC) stem cells (P19.CL6) [12], which are widely used for investigating cardiac differentiation were treated with differentiation medium containing 1 % dimethyl sulfoxide (DMSO) on various ECM proteins (collagen, gelatin, and fibronectin), and their differentiation efficiency was evaluated. The effect of these substrates on the beating duaration of rat neonatal cardiomyocytes was also investigated and intracellular cardiac marker genes (troponin T type-1 and troponin C type-1) [13], and cardiac differentiation marker gene (troponin C type-2) [14] were also investigated. These fundamentals information would be important for cardiac differentiation of various stem cells including autologous BMSCs.

2. Materials and methods

2.1 Cardiomyoctes

Cardiomyocytes were isolated from neonatal SD rat heart (1 to 2-day-old) by the collagenase digestion method with modifications [15-16]. Institutional guidelines for the care and use of laboratory animals have been observed. The hearts were removed and carefully minced with a scalpel blade into fragment and rinsed several times with Hanks' balanced salt solution (Sigma-Adrich Inc, St. Louis) to remove blood and cellular debris. The minced hearts were gently stirred in 50 ml collagenase solution (0.15 M NaCl, 5.63 mM KCl, 0.02 M HEPES, 0.02 M $NaHCO_3$, 3.74 mM $CaCl_2 \cdot 2H_2O$, and 6.5 x 10^4 U collagenase (Wako, Ltd., Osaka, Japan, Lot no: 06032W)) at 37 °C for 30 minutes. The resulting cell suspension was filtered through nylon cell strainer (BD Falcon™, BD Biosciences, Bedford) with a 40 μm pore size and centrifuged at 1000 rpm for 3 minutes.

Isolated cardiomyocytes (1.0 x 10^5) were cultured in minimum essential medium alpha medium (α-MEM, Gibco®, Invitrogen Co., Grand Island, NY) supplemented with 10 % (v/v) fetal bovine serum (FBS, MP Biomedicals Inc., Eschwege, Germany, Lot no: 7297H), and 100 IU/L penicillin-streptomycin (Wako, Ltd., Osaka, Japan) on 60 mm gelatin- (IWAKI, Asahi Glass Co.,LTD., Tokyo, Japan), fibronectin- (BD Falcon™, BD BioCoat, New Jersey), collagen type I-coated dish and non-coated polystyrene dish (IWAKI, Asahi Glass Co.,LTD., Tokyo, Japan).

than that on collagen type I-coated dish or polystyrene dish 11days after induction. These results indicate that gelatin-coated surface has high ability not only to maintain the cardiac phenotype but also to enhance the cardiac differentiation.

Keywords: Extracellular matrix, Cardiomyocyte, Beating, Differentiation

1. Introduction

Cardiac tissue engineering such as cardiomyocyte transplantation for patients with ischemic heart disease or dilated cardiomyopathies are of great potential therapeutic option to enhance the contractile function of the failing heart. In this decade, fetal or neonatal rat cardiomyocytes were reported to form mature cardiac tissue in syngeneic heart, acutely injured myocardium, and granulation tissue in the heart [2]. However, the best cell sources for clinical cardiomyocyte transplantation are still under debate. In general, three types of potential cell sources were proposed [1]. One is the allogeneic source including human embryonic stem cells or fatal allogenic cardiomyocytes, but there still remain ethical issues. Another one is transgeneic source. Genetically engineered animal cardiomyocytes have been studied for reducing the rejection reaction *in vivo*, which takes still long period time to secure the safety.

Most promising cell source is autogeneic one. Isolating cardiomiocytes from the patient hearts is unrealistic at present, and autologous skeletal muscle precursors, fibroblast, or mesenchymal stem cells have been studied so far [3]. However, since beating cardiomyocytes are more promising [4], we have been trying to differentiate bone marrow mesenchymal stem cells (BMSCs) into "beating" cardiomyocytes. There is no certain induction method for BMSCs differentiation into beating cardiomyocytes. Many researchers observed cardiac gene expression in MSCs treated with various inducers [6-8] or passage number [9], but they do not beat spontaneously. Wakitani, S. et al and Makino, S. et al. reported that murine BMSCs were differentiated to beating cardiomyocytes-like cells *in vitro* by exposing to DNA-demethylating agent 5-azacytidine [6-7]. This is in contrast with a report stated that the functional cardiac cells and gene expression were not obtained by treating with 5-azacytidine [5].

Producing autologous beating cardiomyocytes is then attractive issue for cell-based therapy. The most crucial part is how to differentiate them to cardiomyocytes *in vitro* and how to maintain the beating feature. Various microenvironments surrounding the cells (niche) which play important roles not only in cell proliferation but also in cell differentiation. The effect of ECM proteins such as collagen type I, collagen type IV, gelatin, laminin, fibronectin, Matrigel™ (mixture of laminin, collagen

Beating behavior of primary neonatal cardiomyocytes and cardiac-differentiated P19.CL6 cells on different ECM matrices

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Abstract

Stem cell-based therapy in cardiac tissue engineering is the emerging field that shows great potential for treating heart diseases. Nevertheless, even preliminary issues, such as ideal niche for cardiomyocytes have not been clarified so far. In the present study, effect of extracellular matrix (ECM) components on beating duration of neonatal rat cardiomyocytes (RCMs) and on cardiac-differentiation of P19.CL6 carcinoma stem cells were studied. RCMs were cultured on gelatin-, fibronectin-, and collagen type I-coated, and non-coated polystyrene dishes, and their beating rate, beating duration, and cardiac gene expression were evaluated. The beating period and the expression of troponin T type-1, troponin C type-1 of cardiomyocytes cultured on gelatin-coated dish were longer and higher than the others. For cardiac differentiation of P19.CL6 cells, troponin T type-1 expression on gelatin- or fibronectin-coated dish was 5 times higher

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AU19: Please clarify what "would related" is supposed to say.
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AU21: Please clarify what "decreased lower than 60%" means.
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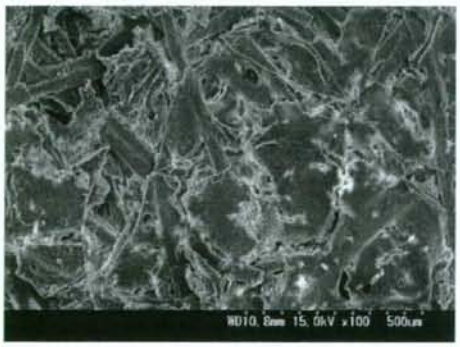
AU24: " As the polyphenol reversibly leaves the cell membrane with time, and the cell cycle resumes." Is not a sentence. Please check and correct.
AU25: Is Disclosure statement OK? If not, please rephrase.

SUPPLEMENTARY DATA 1. Turbid cell suspension **(A)** became clear in 2 h when perfused at the flow rate of 84.2 mL/min **(B)**.



SUPPLEMENTARY DATA 2. Scanning electron microscopy picture of hepatocytes cultured in a three-dimensional bioreactor for 1 week.