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Letters

Using the Chorions of Fertilized Zebrafish Eggs as a Biomaterial for the Attachment and Differentiation of Mouse Stem Cells

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The development of proper biomaterials is critical for the success of cell therapy and modern tissue engineering. Here, we extruded the yolk and remaining inner mass from fertilized zebrafish eggs and used the resulting chorions as a biomaterial for the differentiation and attachment of mouse P19 embryonic carcinoma (EC) cells. Cells inserted into the chorion showed the spontaneous formation of embryoid body due to the repulsive cell adhesion of the chorion and differentiated specifically into neural cells and cardiomyocytes. In contrast, dissolved chorion extracellular matrix (ECM) conferred enhanced cell attachment on it, suggesting that a unique property of the zebrafish chorion with nanoporous structure appears to be responsible for the simple and controllable embryoid formation for stem cell differentiation. These results indicate that chorions from fertilized zebrafish eggs may be used as an extracellular matrix alternative and applied for stem cell differentiation to specific cell lineages.

The extracellular matrix is vital to living cells; interactions between cells and the ECM are closely related to cell attachment, motion, growth, and apoptosis[1] as well as the activation of cytokines,[2] growth factors,[3] and intracellular signaling.[4] The ECM is also vital to tissue engineering and cell-based biotechnology because it offers a specific environment where the fate-determining signaling pathways of stem cells can be regulated.[5] Various ECMs have been used for tissue and cell engineering. The most common of these are collagen, fibronectin, and laminin, which contain a specific cell-binding tripeptide (arginine-glycine-aspartic acid, RGD) known to be essential for cell adhesion.[6] However, there may be additional factors related to cell culture such as the microenvironment. For example, the mouse blastocyst offers both a substrate and a micromilieu favoring the differentiation of cells within the embryo and also allows the incorporation of transplanted stem cells such as embryonic stem cells, EC cells, and others.[7] The blastocyst consists of early

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epithelial cells mechanically supported by a transparent ECM called the zona pellucida, which is equivalent to the zebrafish egg chorion. The chorion of most vertebrates consists of mainly polysaccharides and proteins showing a natural affinity for living cells.[8] Thus, we hypothesized that a zebrafish chorion emptied of the normal yolk and inner mass might function as an artificial egg case for stem cell differentiation.

The zebrafish chorion, a porous membrane (1.5−2.5 μm in thickness) surrounding the fertilized egg[9] (1−1.5 mm in diameter; Figure 1a), has two structural advantages for stem cell culture. First, the inner volume is approximately 1−3 μL, which is 1000 times smaller than the required amount for dish-based conventional cell culture systems for the differentiation of P19 EC cells.[10] Second, the membrane has an intrinsic, well-defined nanopore structure. The pores, which have a diameter of 500−700 nm and are arranged at intervals of 1.5−2.0 μm,[11] allow free nutrient supply from the outside. Like other ECMs, the chorion is primarily composed of several types of *N*-linked glycoproteins[12,13] such as collagen, gelatin, and fibronectin. Thus, the chorion surface might be mainly hydrophilic to cells and generate a suitable environment for cellular proliferation. However, cell attachment to the natural chorion would be limited because the porous surface offers insufficient area for the stable attachment of cell surface molecules, which will become concentrated at the focal adhesion sites of the cell.[14] Each cell should move easily along the porous surface, promoting the cell−cell adhesion and embryoid body (EB) formation necessary for the differentiation of stem cells. These characteristics seem to make the zebrafish chorion an attractive candidate for use in the stem cell microculture.

However, there are no previous studies of zebrafish as a potential surrogate for the differentiation of mammalian early embryonic or ES cells, although mammalian surrogate zona pellucida has been used in efforts to multiply embryonic cells.[15] Here, we tested whether zebrafish chorion support mouse stem cell differentiation as a natural biomaterial. A microinjection pipet was gently inserted into the chorion of each fertilized zebrafish egg, and a forceful injection of distilled water was used to extrude the yolk and remaining inner mass of the early embryo (Figure 1a). The original volume of the chorion before injection increased up to 2-fold after extrusion, providing evidence that the chorion is naturally elastic. P19 EC cells were then introduced through the injection pore, and a tiny EB was successfully formed inside the chorion by day 4 (Figure 1b). Each EB grew continuously until it completely occupied the entire inner space. Interestingly, on day 7 (Figure 1c), the chorion shrank down to 75−90% of its original volume (depending on the cell proliferation activity within the chorion), pulling the chorion membrane into close proximity to the P19 EC cell mass. A crescent-shaped cavity was clearly visible inside the cell mass at this time. These observations likely indicate that the medium supply through the pore structure could not match the required amount for further cell growth. Although restricted by the limited nutrient supply, cell growth continued until the original chorion volume was recovered (Figure 1d). Beyond day 10, further cell growth stretched the chorion in a manner reminiscent of mouse blastocyst expansion, leading to thinning of the chorion and complete filling of the empty space. Finally, cells overflowed through the injection opening at day 15 (Figure 1e), similar to the phenomenon seen during assisted hatching in mouse blastocysts.[16] Although there was a 10% volume expansion of the chorion during cell growth and the cells overflowed through the tiny surface opening (50 μm in diameter), membrane splitting was not observed.

Cells picked from the cell mass at day 15 showed evidence of both neural cell lineages with elongated filopodia (Figure 1f) and periodically beating cardiomyocytes (Figure 1g). To verify these observations, we analyzed the cell mass using various cell lineage markers: nestin[17] for neural stem cells, MAP2b[18] for neurons, GATA-4[19,20] for cardiac-restricted transcription, and cardiac troponin T (cTnT)[21] for cardiac cells. 4′,6-Diamidino-2-phenylindole dihydrochloride hydrate (DAPI) staining of each sample was added for nuclei visualization. As shown in Figure 2, the expression of nestin (Figure 2a and b) and MAP2b (Figure 2c and d) was found extensively on the outer layer of the cell mass, whereas GATA-4 (Figure 2e and f) and cTnT (Figure 2g and h) were highly expressed inside the cell mass. The expressions of the early progenitor cell lineage markers of neural stem cells (nestin) and cardiac precursors (GATA-4) in the outer layers and mature cell lineage markers of neurons (MAP2b) and cardiomyocytes (cTnT) in the inner mass suggest that the EB within the empty chorion follows a developmental program similar to that observed during normal mouse development.[22] Collectively, these morphological and immunocytochemical observations demonstrate that neural cell and cardiomyocyte lineages are capable of differentiating within the nanoporous chorion.

This novel technique has a number of advantages over the more traditional bacteriological dish systems.[23] For example, no cell transfer is required during differentiation culture, and fresh medium changes are required only every 48 h once cells are injected in the empty chorion. In conventional methods, 10^5−10^6 cells are loaded in bacteriological dishes;[23] this high cell concentration is required for effective EB formation because of the large surface area of the culture dish. However, the volume of a chorion is just 1−3 μL and allows cells to be densely packed, thus inducing interactions that likely promote specific cell differentiation without diluting various cellular short-

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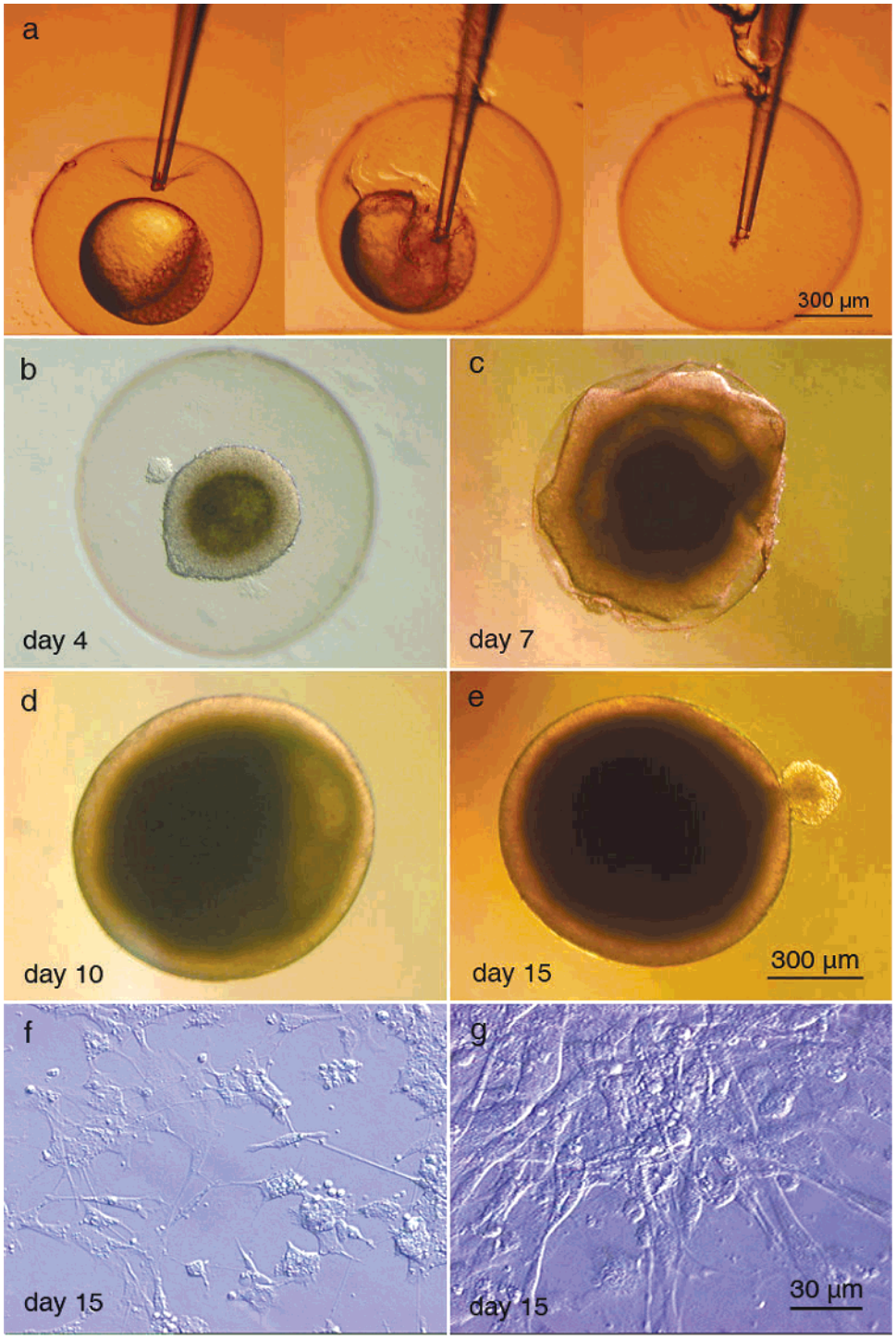

Figure 1. Optical micrographs showing P19 EC cell culture inside an empty zebrafish chorion. (a) From the left, intrusion of a micropipet into a zebrafish egg to create an opening for the release of the yolk and remaining inner mass. During the process, the chorion expands to more than twice its original volume without any surface splitting. (b) By day 4, an embryoid body (EB) was successfully formed inside the chorion by the adhesions among P19 EC cells. A carefully controlled micropipet can deliver the required number of cells into the chorion for one EB formation. (c) By day 7, membrane shrinkage is likely visible because of a deficiency of medium supply inside the chorion. A crescent-shaped cavity is clearly observable inside the cell mass. (d) By day 10, the shape is recovered by continued cell proliferation. (e) The complete occupation of the whole inner space can be observed by day 15, and proliferating cells can be seen extruding through the injection opening. The fluid-filled cavity is obscured by the dense cell mass. (f) Optical micrograph of neural cells with elongated filopodia recovered on day 15. (g) Beating cardiac cell masses were found on day 15. The scale bar in frame g is also applicable to frame f.

range ligands (a major drawback of cell culture in large polymer dishes). With continued cellular proliferation, the cell mass requires more and more space; the chorion can adapt to this need through its natural elasticity and also through softening caused by digestion with unknown hydrolases secreted from the growing EB. In addition, the natural antibacterial property of the chorion[24] helps the cells survive longer without microbial contamination. Finally, cell culture in the chorions does not require any

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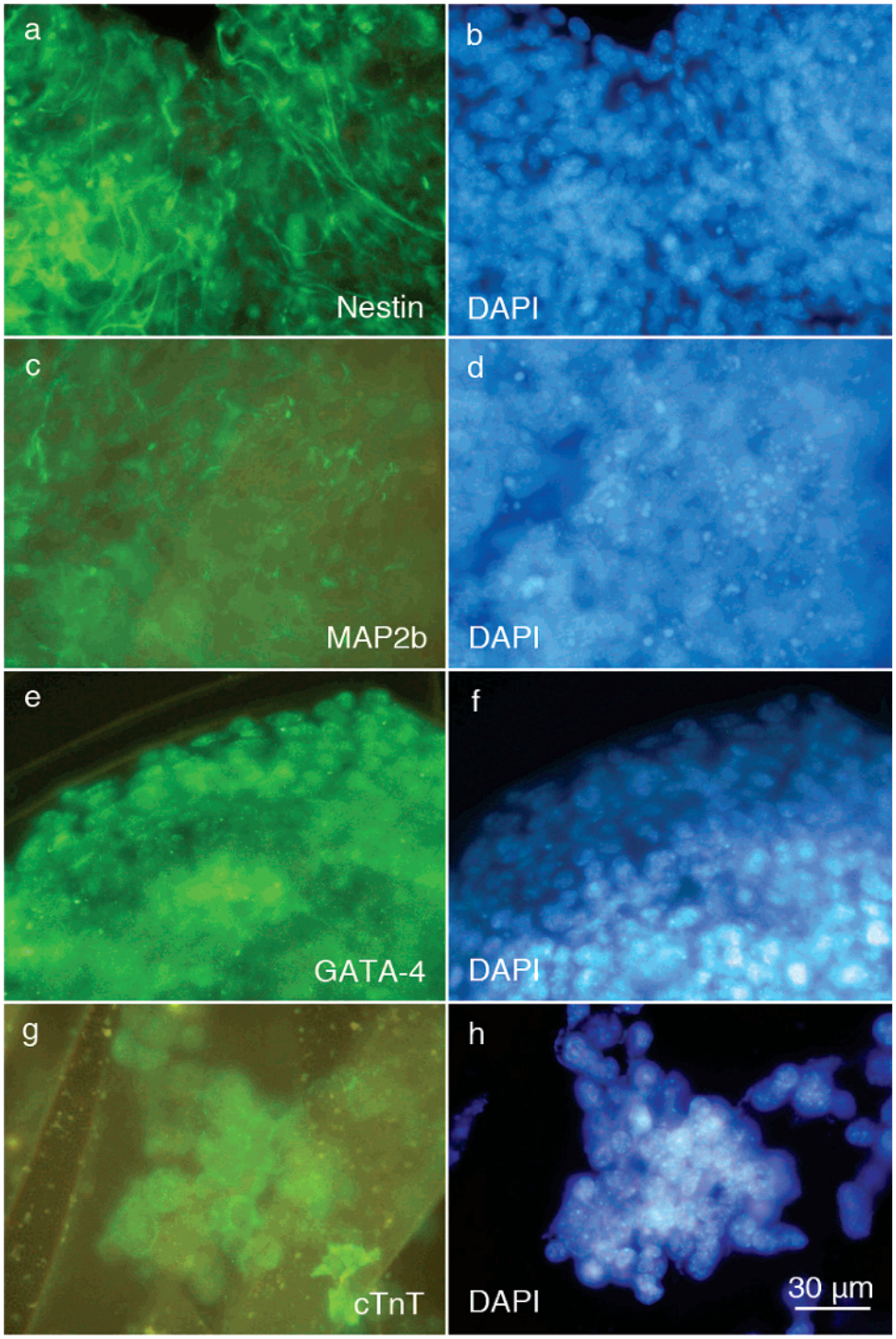

Figure 2. Expressions of lineage-specific markers in the differentiating P19 EC cells of embryoid bodies. P19 EC cells differentiated into neural cells and cardiomyocytes on day 15. The images in the right column show nuclei stained with DAPI, and paired images are shown in the left column. Expression of the neural cell lineage markers, nestin (a) and MAP2b (c), was seen in the ectodermal layer. Expression of the cardiac cell lineage markers, GATA-4 (e) and cardiac troponin T (cTnT, g), was found inside the cell mass. The cross section of the chorion and the boundary of the cell mass can be seen in frame e, whereas frame g shows the folded chorion as a dark rod on the left.

addition of inducing agents or differentiation stimulants such as retinoic acid[25] or dimethyl sulfoxide.[26]

To examine whether the chorion of which the innate structure was intentionally deconstructed confers a similar repulsive cell adhesion property found inside the empty chorion, chorions were dissolved chemically and transformed into a form similar to that observed in traditional ECM materials such as gelatin, collagen, and poly-L-lysine (PLL)[27] using a 1% sodium dodecyl sulfate (SDS) and 8 M urea solution including 10 mM β-mercaptoethanol and 2 mM ethylenediamine tetraacetic acid. SDS (1%) deconstructs the 3D protein network by creating negative

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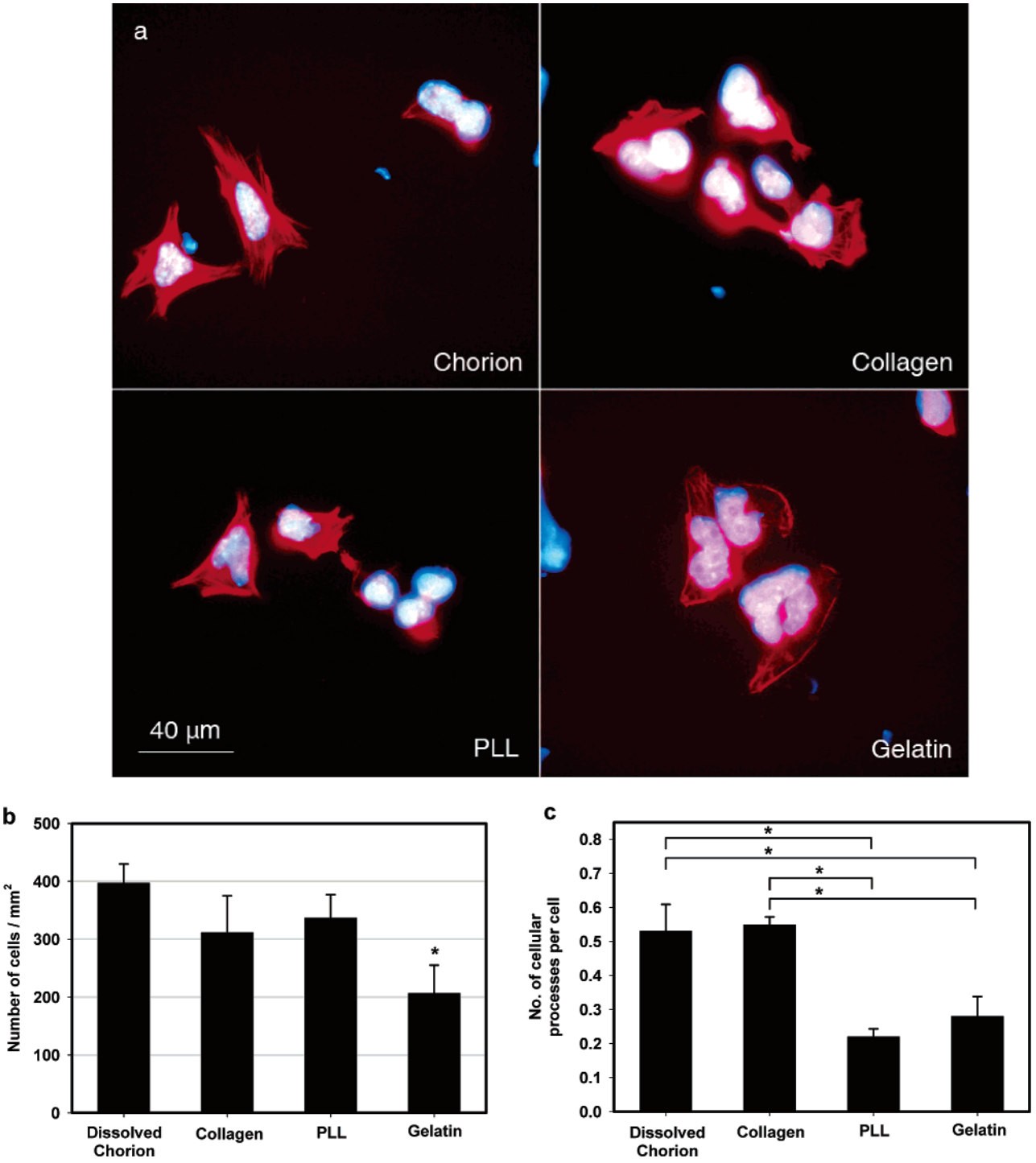

Figure 3. Attachment of mouse P19 EC cells on four different extracellular matrixes (ECMs). (a) Merged fluorescent micrographs of the attached cells stained with phalloidin (red) and nuclei (blue) on four different ECMs including dissolved chorions, gelatin, collagen, and poly-L-lysine (PLL). Five thousand cells were plated on each ECM and cultured in DMEM (Dulbecco's modified Eagle's medium) containing 10% fetal bovine serum for 6 h. (b) The numbers of attached cells on the dissolved chorions and two other ECMs were significantly greater than those on gelatin (*: $p < 0.05$). The number of cells attached per unit area was quantified after DAPI staining of cell nuclei. The results were obtained from experiments replicated more than 100 times. (c) The numbers of elongated filopodia per cell were significantly higher in dissolved chorion and collagen than in the other ECMs (*: $p < 0.001$). Taken together, these results indicate that the cells on dissolved ECM follow normal cellular responses to the novel extracellular matrix. Significant differences were indicated by asterisks (*), and the error bars reflect the standard deviation.

charges on the protein surfaces and leading to repulsion among double helices. Then, urea dissolves each protein complex further into nearly molecular units. This chorion dissolution method was designed to remove zebrafish genetic materials (i.e., sperm remnants) as well as microbes possibly present in the egg-laying tank and may cause developmental problems in P19 EC cells, while preserving protein components.

As shown in Figure 3, the organization of actin filaments (Figure 3a) and degree of cell attachment property (Figure 3b) were comparable to or slightly better than those of the other ECMs used. Because the cellular organization of

the abundant actin filaments is sensitive to fluid and solid cellular environments,[28,29] we tried to find any difference in the array of actin filaments. In addition, the number of elongating filopodia of cells on each ECM were scored.[30] However, no different staining pattern was found among the cells on the four different types of ECMs, and mean numbers of filopodia of the cell on the dissolved chorion are quite similar to that of collagen but slightly higher than those of PLL and gelatin ($p < 0.001$) (Figure 3c), suggesting that the dissolved chorion ECM might not adversely affect the major cellular components. Figure 3b shows that the number of cells on the dissolved chorion ECM was also found to be slightly higher than that of the other ECMs, although a significant difference was found between gelatin and the rest of the ECMs ($p < 0.05$). Taken together, these results show that the dissolved zebrafish chorion ECM may offer a suitable matrix for general cell culture without the deterioration of cellular activity.

Our peptide mapping and subsequent amino acid sequencing (data not shown) of the dissolved chorion solution showed that one of the major components was highly glycosylated zona pellucida protein 2 (ZP2).[13] This leads us to speculate that this protein could be partly responsible for the suitability of the chorion as an ECM. For example, ZP2 recognizes approaching sperm and works as a receptor during fertilization,[8] and cell attachment is probably understood to follow similar processes.

We also split a chorion into small fragments and loaded cells on each fragment for 6 h; however, enhanced cell attachment found above on the dissolved chorion ECM was not observed (data not shown), whether the cells were loaded on the outer or inner surface of a chorion. The result suggests that the unique property of repulsive cell adhesion found in the chorion depends on the specific nanopore structure.

To date, glass and various polymers including poly(dimethylsiloxane) and poly(methyl methacrylate) have been widely employed to demonstrate biocompatibility and normal cellular activities in both tissue engineering and micro/nanodevices. However, no material has been shown to be effective in stem cell differentiation to our knowledge, partly because of the innate complexity of biological functions. Because the controlled differentiation of stem cells is crucial for the advancement of cell replacement therapy and regenerative medicine, we hope that our findings of zebrafish chorion with unique nanopore structure and glycoconjugates as a biomaterial for stem cell culture will provide a direction toward the development of more suitable artificial materials for both future research and clinical tissue engineering.

Methods

Cell Culture for Embryoid Body (EB) Formation. To culture P19 EC stem cells (American Type Culture Collection, Manassas, VA) inside a chorion, a micro-opening (50 μm in diameter) was created on the surface of a single fertilized zebrafish egg chorion using a micropipet, and the yolk and remaining inner mass were expelled through the opening with a strong injection of distilled water. The empty chorion was then washed with distilled water to eliminate any residual microbes. To identify contamination and determine whether further washes were necessary, we examined the empty chorions as strictly as possible using 20 μg/mL 4′,6-diamidino-2-phenylindole dihydrochloride hydrate for nuclear staining (DAPI, Sigma) prior to use. Once the chorions were judged to be free of contaminating materials, 5000 P19 EC cells were loaded into each empty chorion through the micro-opening for EB formation. Though the number of loaded cells inside a chorion was 5000 for fast EB formation, a lower cell concentration (1000 cells) also showed efficient EB formation experimentally inside the chorion.

Chorion-containing P19 EC cells were subsequently cultured in Dulbecco's modified Eagles' medium (DMEM) (Gibco Invitrogen) supplemented with 10% fetal bovine serum (Gibco Invitrogen) (DMEM + FBS) at 37 °C in 5% CO_2 in air. The medium was changed at 48 h intervals for up to 15 days of culture.

Preparations of Dissolved Chorion ECM and Chorion Fragments. Isolated chorions were treated with 1% SDS (Sigma Chemical Co., St. Louis, MO) for 3 min for the removal of cytoplasmic remnants and other components. After SDS treatment, the chorions were washed three times with zebrafish embryo culture medium and distilled water, respectively. Then, they were treated in 8 M urea solution containing 10 mM β-mercaptoethanol and 2 mM EDTA (Sigma) for 8 h at 37 °C. The urea and other chemicals were then separated from the dissolved chorion ECM by centrifugation at 14 000*g* for 4 h with additions of distilled water to fill the Microcon (YM-3, Millipore Co., MA) tube every hour. The dissolved chorion ECM was stored at 4 °C before being spotted on coverslips for later experiments. To prepare chorion fragments, the empty chorions were manually passed several times through a finely pulled Pasteur pipet with a smaller diameter than that of the chorion over a flame. The number of chorion fragments was adjusted to be equivalent to the concentration used for the dissolved chorion ECM.

Cell Attachment to Different ECMs. For the cell attachment experiment, the prepared chorion fragments as a negative control, dissolved chorion ECM (20 μg protein/mL), gelatin (0.1%), collagen (20 ug/mL) and PLL (10 ug/mL) were coated on coverslips. To determine cell attachment, 5000 P19 EC cells were plated on five different matrixes including the chorion fragments and were cultured in DMEM + FBS for 6 h.

Staining of Cells. At the end of cell culture, EBs in the chorion or coverslips were fixed in 4% paraformaldehyde in PBS for 30 min. To inactivate the aldehyde groups, they were then incubated in 0.26% NH_4Cl in PBS for 20 min prior to blocking with 10% normal goat serum in PBS for 1 h. The EBs and cells were immunostained with appropriate primary antibodies, followed by secondary antibodies for EB experiments described above. To visualize actin filaments in cell attachment experiments, cells were extracted in 25 mM HEPES, 60 mM PIPES, 10 mM EGTA, and 2 mM $MgCl_2$ pH 6.9 buffer containing 0.5% Triton X-100 for 5 min and stained with phalloidin conjugated to tetramethyl rhodamine B isothiocyanate (TRITC; Sigma) at 1:1000 dilution. Both the EBs and the attached cells on coverslips were also stained with and 20 μg/mL 4′,6-diamidino-2-phenylindole dihydrochloride hydrate for nuclear staining (DAPI, Sigma). The stained EBs or cells were analyzed under a Zeiss Axoplan fluorescence microscope (Zeiss, Germany) and recorded with a Nikon digital camera (Nikon Co., Japan) after washing several times in PBS containing 0.02% NP-40 (Sigma).

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