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Differentiation of Mouse P19 Embryonic Carcinoma Stem Cells Injected into an Empty Zebrafish Egg Chorion in a Microfluidic Device

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Mouse P19 embryonic carcinoma (EC) stem cells were xenotransplanted into the emptied chorion, the transparent envelope of a fertilized zebrafish egg (rather than mouse native zona pellucida) combined with a microfluidic device to study P19 EC cell differentiation in the chorion biomaterial. A distilled-water jet was used to remove the innate yolk and perivitelline inner mass from the chorion. P19 EC cells were injected into the emptied chorion using a micropipette, and they were subsequently cultured until the inner space of the chorion became completely occupied by cells. A simple microfluidic device was used for handling convenience and effective experiment. At d15, we found neural cells in the outer layer of the cell mass and beating cardiomyocytes in the inner layer of the large embryoid body. We propose that even though the species are different, the external innate membranes developed for embryo protection represent a useful type of ECM.

Key words: extracellular matrix; zebrafish chorion; mouse P19 EC cell; microfluidic device; cardiomyocytes

While various strategies for *in vitro* differentiation of embryonic stem cells have been described in the recent reviews of Heng *et al.*,[1,2] the potential of extracellular matrices (ECMs) has received relatively little attention as an important factor in directed stem cell differentiation. But, the very first cell differentiation at the transition from an eight-cell embryo to a blastocyst occurs within an ECM (the zona pellucida) during early mammalian embryonic development.[3] The zona pellucida joins mammalian early development from the oocyte through to the zygote and morula, and finally to the blastocyst, as the only natural ECM available. The role of the zona pellucida terminates just before blastocyst implantation on the uterus wall (the second natural ECM) to form the placenta during embryogenesis. Therefore, some important early cellular behaviors including zygote cleavage, morula compaction, and the formation of the trophectoderm and inner cell mass occur inside the zona pellucida. Though this thin natural membrane is not a definitive element for blastogenesis, since a zygote can differentiate *in vitro* into a blastocyst without the membrane, differentiation efficiency is naturally higher in cultures that include the membrane.[4] Hence we considered the zona pellucida to be one of the best candidate ECMs for stem cell cultures, if it could be clearly separated. In addition, the natural three-dimensional spherical structure of the zona pellucida would also be effective for embryoid body (EB) formation. But, to obtain the zona pellucida in its original spherical form without splitting of the membrane is quite difficult due to its small size (less than 100 μm in diameter). In this study, we used the outer membrane of a zebrafish egg, called the chorion,[5] as an alternative to the zona pellucida for mouse P19 embryonic carcinoma (EC) stem cells due to its handling convenience (about 1 mm in diameter) (Fig. 1A).[6] Generally the chorion of carp-like zebrafish is transparent and contains many penetrating nanopores,[7] (500–700 μm in diameter) (Fig. 1B). Moreover, the membrane protein of the zebrafish chorion (ZP2) is known to exhibit a degree of homology similar to the mouse zona pellucida proteins ZP1 and ZP2.[8] The chorion is also collected relatively easily because zebrafish eggs require just 2d to develop from embryos to fry. To make it easier to handle the tiny chorion, a simple microfluidic device, with a fluid channel structure,[9] was constructed from polydimethylsiloxane (PDMS) and SU-8 photoresist, as shown in Fig. 2.

Materials and Methods

Microfluidic device fabrication. A microfluidic device (4 cm × 3 cm) was assembled with four polymer layers: cover, culture-well, microhole, and bottom-channel (Fig. 2A). Three of them (the cover, culture-well, and

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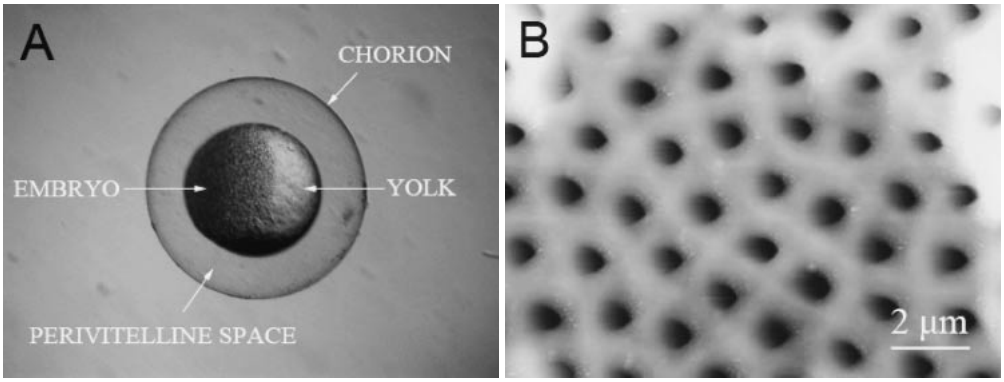

Fig. 1. A New Source of Biomaterial for Mouse Stem Cell Culture.
A, Optical micrograph of a single fertilized zebrafish egg consisted of an embryo, a yolk, perivitelline materials, and a chorion. B, AFM (atomic force microscopy) image of the chorion inner surface showing the innate pore structure.

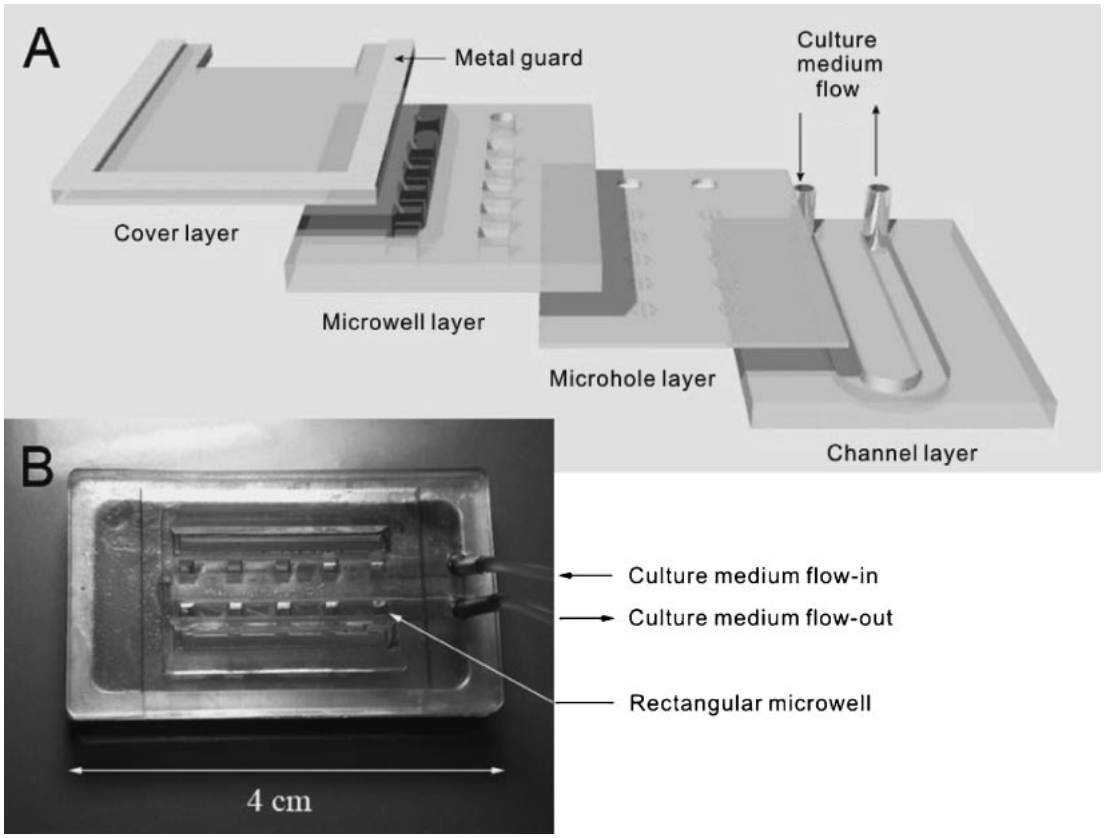

Fig. 2. A Microfluidic Device for Living Cell Cultures Consists of 10 Rectangular Culture Wells and One Microchannel for Culture-Medium Flow, Which Will Be Integrated with the New Biomaterial, Zebrafish Chorions.
A, Schematic illustration of the device components: cover layer, microwell layer, microhole layer, and channel layer, from the top. B, Assembled final unit.

bottom-channel layers) were fabricated with polydimethylsiloxane (PDMS), and a thin microhole layer was fabricated with SU-8 photoresist material by MEMS (micro- electro- mechanical system) technology. The culture-well layer included 10 culture wells (2 mm × 2 mm × 2 mm), each with enough space for a single chorion complex. Culture medium flowed in the bottom-channel layer, which was 3 mm wide and positioned right below each culture well *via* a thin microhole layer (100 μm thick). The culture-well layer held a chorion in each culture well and supplies nutrient through nine microholes (300 μm in diameter) (Fig. 2B). Because the device was fabricated from a flexible polymer, a metal guard ring was attached on the cover layer to ensure structural rigidity.

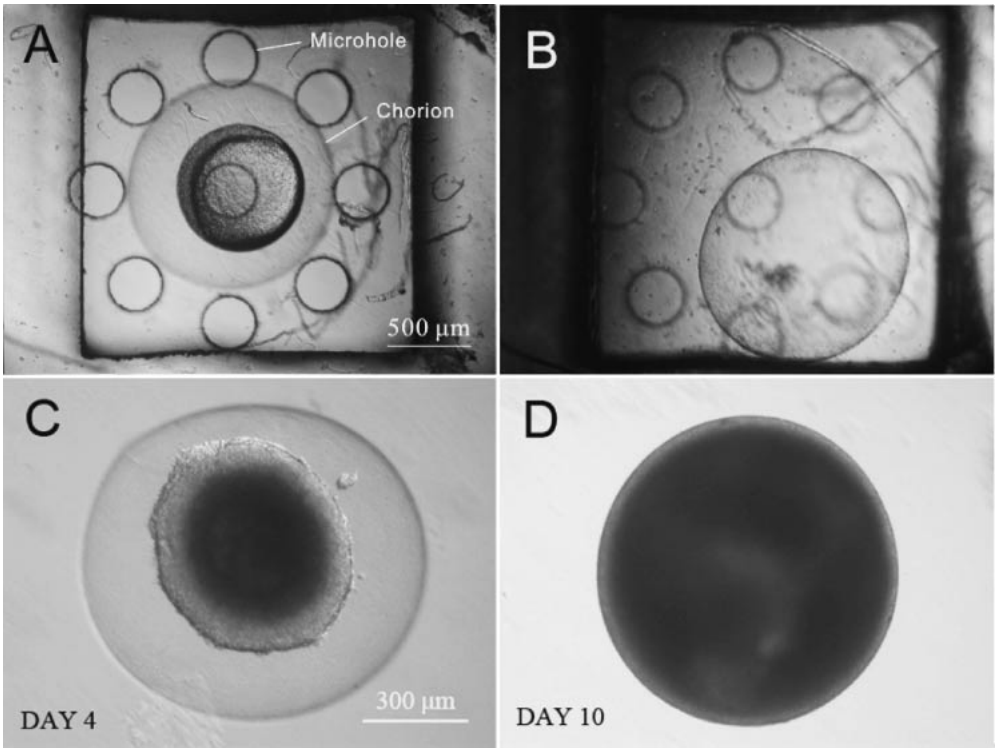

Fig. 3. Culture of P19 EC Cells Inside a Chorion Located in a Well of a Microfludic Device.
A, Optical micrograph of a single zebrafish egg located inside a culture well filled with culture medium. B, Optical micrograph of a single chorion whose yolk and inner mass were removed with a distilled-water jet. C, Successful formation of an EB of P19 EC cells at day 4 inside an emptied chorion. D, Complete occupation of the chorion inner space by P19 EC cells showing variations in cell density, which indicates the formation of blastocoels inside the cell mass by day 10.

Cell culture. Before mouse P19 EC cells (American Type Culture Collection, Manassas, VA) were injected into a chorion, the inside of a single fertilized zebrafish egg including yolk and perivitelline inner mass was evacuated through a micro-opening (50 μm in diameter) using a strong jet of distilled water from a micropipette (Fig. 3A, B). The emptied chorion was subsequently washed with distilled water to eliminate residual microbes, and examined as strictly as possible using 20 μg/ml 4′,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI, Sigma-Aldrich, Korea) for nuclear staining prior to use. Approximately 5,000 P19 EC cells were then injected into the emptied chorion through the same micro-opening. Cells in the chorion was subsequently cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS, Gibco Invitrogen, USA) at 37 °C in 5% CO_2 in air. The culture medium was changed every 48 h for up to 15 d of culture.

Immunostaining. The cell masses in the chorion at day 15 of culture were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min. To inactivate the aldehyde groups, the cells were 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 cell mass was immunostained with appropriate primary antibodies, followed by secondary antibodies as described above. The mass was then also stained with DAPI for nuclear staining and washed several times in PBS containing 0.02% NP-40 (Sigma-Aldrich, Korea). Staining images were obtained under a fluorescence microscope (Axoplan, Zeiss, Germany) with a digital camera (Nikon, Japan).

Results and Discussion

Generally, dimethylsulfoxide (DMSO),[10] or retinoid acid,[11] is added to induce differentiation of embryonic stem cells or EC cells to EBs and muscle cells (cardiomyocytes or skeletal muscle cells). In the present study, however, we did not add any external differentiation agents so as to evaluate only the efficacy of the emptied chorion. At day 4, we found a well-developed, round EB inside the chorion, as shown in Fig. 3C. The EB grew continuously until the internal space of the chorion was completely occupied by cells. Figure 3D shows a chorion completely filled with cells at day 10, which illustrates variations in the cell density within the cell mass. The formation of such a blastocoel-like structure normally identifies a cell mass at the blastocyst or gastrulation stage, and indicates that cells inside the chorion have differentiated into other cell types. At day 15, the hollow space disappeared and some of cells burst

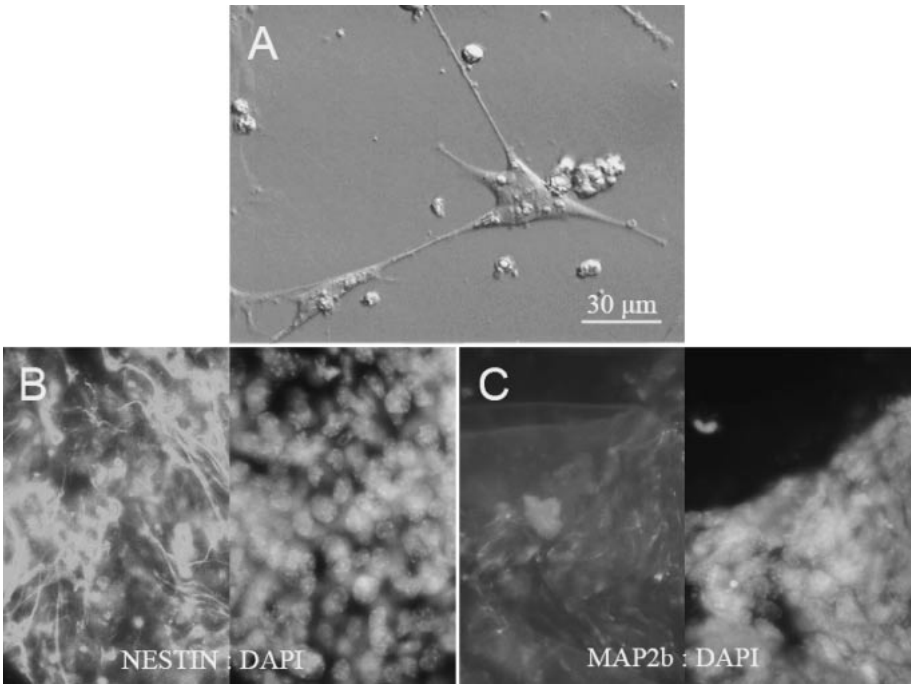

Fig. 4. Lineage-Specific Differentiation of P19 EC Cells into Neural Cells and Immunocytochemical Analysis with DAPI Staining for Comparison with the Lineage-Specific Markers.

A, Optical micrograph of two neural cells connected by a common axon found on day 15 from outside the cell mass. B, Immunostaining of neural cells with nestin (left) and DAPI (right). C, Immunostaining with MAP2b (left) and DAPI (right).

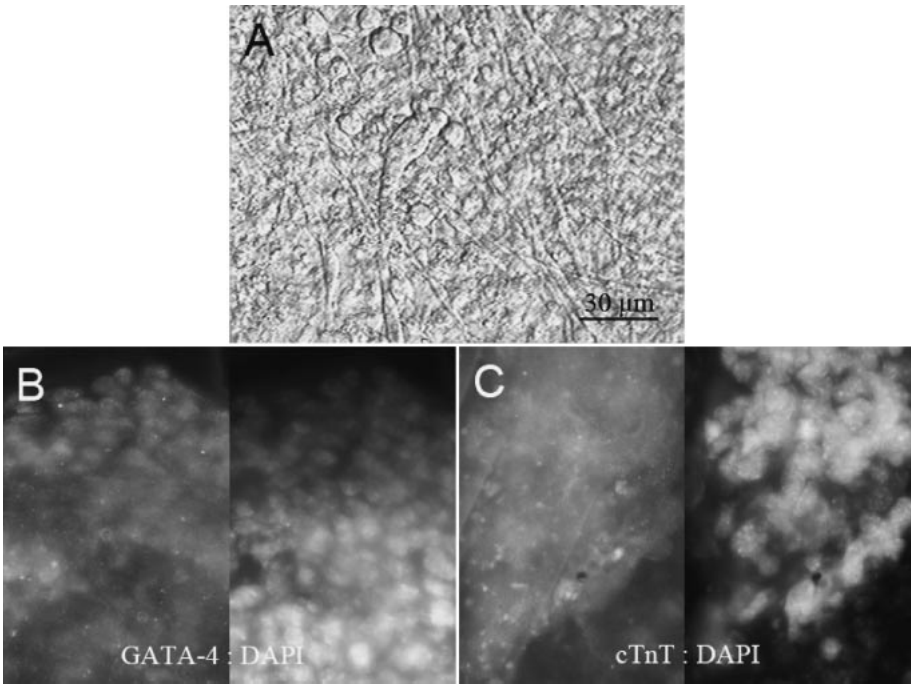

Fig. 5. Lineage-Specific Differentiation of P19 EC Cells into Cardiomyocytes and Immunocytochemical Analysis with DAPI Staining for Comparison with the Lineage-Specific Markers.

A, Beating mass of cardiomyocytes found on day 15 from inside the cell mass, showing the specific vascular structure. B, Immunostaining of cardiomyocytes with GATA-4 (left) and DAPI (right). C, Immunostaining with cardiac troponin T (cTnT, left) and DAPI (right).

out from the hole through which the original P19 EC cells were injected. The embryoid body inside the chorion was immunostained as a whole and some of cells were collected for image observations of differentiated cells, but without further culture. This revealed two discrete cell types, neural cells and cardiac muscle

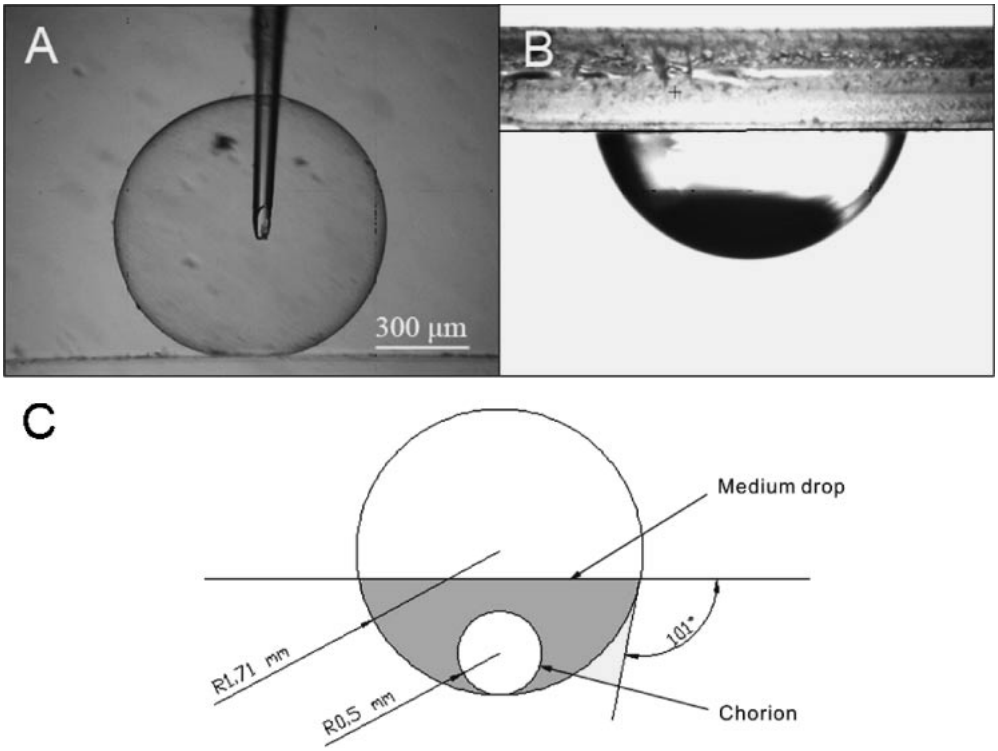

Fig. 6. Scale Comparison between Chorion and Hanging Drop.

A, Optical micrograph of a single empty chorion, of which the yolk and perivitelline inner mass were removed with a microneedle. B, A single drop of culture medium hanging on the surface of a polystylene Petridish. C, Schematic illustration of a chorion and a medium drop. The gray area indicates the drop of 8 μl culture medium. The small white circle represents a single chorion.

cells, as shown in Fig. 4A and 5A. Figure 4A shows a neural cell body (right) with two axons and two dendrite arms. One of the axons is connected to the axon of another cell (left). The cells in Fig. 5A show a vascular structure specific to beating cardiac muscle. The immunostaining results of the differentiated cells using various cell lineage markers are shown in the figures: nestin[12] and MAP2b[13] for the neural cells in Fig. 4B, C, and GATA-4[14] and cardiac troponin T (cTnT)[15] for the cardiomyocytes in Fig. 5B, C. To identify nuclei and further evaluate the immunostaining results, DAPI staining was also applied. As Fig. 4B, C shows, nestin and MAP2b were extensively expressed in the outer layer of the cell mass. In contrast, GATA-4 and cTnT were mostly expressed inside the cell mass (Fig. 5B, C). These results, which were obtained without the addition of any inducing agents or differentiation stimulants, imply that the development of a cell mass in the emptied chorion is similar to that of a normal mouse embryo.[16]

The above results indicate that a nanoporous chorion constitutes a new type of cell culture system. This integration of new biomaterial with a microfluidic system exhibits higher efficacy than traditional dish-based cell-culture systems. For example, the diameter of the chorion is approximately 1 mm, and the cells are forced to slide down to the bottom from the peripheral wall (Fig. 6A). Similarly, in the so-called hanging drop (HD) system, EBs were also formed from the bottom of the culture medium hanging from an inverted Petridish (Fig. 6B),[17] but the diameter of the controllable medium drop (volume $> 5\,\mu l$) was much larger than that of a chorion (Fig. 6C). The bottom of each drop does not appear to be round from the viewpoint of a cell. Therefore, the HD system requires more cells to form a single discrete EB. Of course, in the HD method a much smaller drop can also be realized by careful pipette control, but this results in problems such as fast drying of the culture medium and the risk of the drop falling due even to weak vibrations in the surrounding atmosphere. In our method, cells in the emptied chorion immersed in culture medium are supplied continuously with culture medium through the nanoporous membrane on the chorion surface. Also, the cells in the chorion are not exposed to the risk of dropping. The volume of a chorion is just 1–3 μl, which results in a densely packed cell mass and hence improved intercellular differentiation signaling without the dilution of short-range ligands. In addition, the original volume of the chorion can increase by two to three times by digestion with unknown hydrolases secreted from the growing EB. This property allows many more cells to be accommodated inside the chorion. Additionally, the chorion membrane is antibacterial,[18] which lengthens cell survival without microbial contamination.

In summary, we propose that the external membrane of a fertilized zebrafish egg is useful as a type of ECM

for culturing mouse stem cells, even though the zebrafish is a different species from the mouse. Presently we are attempting to transform the natural chorion into various forms of normal ECM such as collagen, gelatin, and fibronectin, and we are also focusing on additional biochemical functions of the innate zona pellucida besides induction of the acrosome reaction.[19] These investigations are expected to provide additional information on the roles of the ECM in the expansion and differentiation of stem cells.

Acknowledgments

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