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<td>組織分化模倣型細胞外マトリックスで修飾したPLGA-コラーゲン複合メッシュの作製及び幹細胞機能への影響</td>
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Abstract

Extracellular matrix (ECM) is a supramolecular assembly which consists of proteins, proteoglycans and glycosaminoglycans. The matrix composition provides a niche responsible for maintenance of cell activity such as cell adhesion, migration and differentiation because in vivo cells are surrounded and interact with their ECMs. Understanding the interaction between ECMs and cells is important for tissue engineering and regenerative medicine. Recently, various strategies have been developed to investigate the interaction between ECMs and cells, including isolation of ECM components and decellularization of tissue and organs. Isolated ECM components such as collagen, laminin, fibronectin have been used to coat cell culture plates and tissue engineering scaffolds. The components of isolated ECMs can be well controlled, but it is hard to mimic the complex components and structures of native ECM microenvironment. In contrast, decellularized ECM is an alternative approach for ECM preparation. Decellularized ECM from tissues and organs has been broadly explored and used for tissue engineering. However, ECM is dynamically changed and remolded during tissue development. Decellularized tissue ECM cannot reflect the dynamic remodeling composition of ECM because there is difficulty to obtain tissues of different development stages from the same animal. Motivation to tackle these problems has initiated the development of cultured cells-derived ECMs. In vitro culture of stem cells can be differentiated to a specific phenotype and controlled at different stage of differentiation. In previous reports, human mesenchymal stem cells (hMSCs) have been well controlled at different differentiation stages of osteogenesis, adipogenesis and osteogenesis-co-adipogenesis to deposit biomimetic ECMs on two-dimensional (2D) cell culture substrates. However, 2D biomimetic ECMs are different from the three-dimensional (3D) ECMs surrounding cells in vivo. Furthermore, 3D ECM scaffolds are required for tissue engineering applications. Therefore, it is highly desirable to develop 3D ECM scaffolds from cultured cells to mimic in vivo ECM microenvironment.

3D ECM scaffolds need reinforcement by other biomaterials because their mechanical strength is too low. In our previous work, PLGA-collagen hybrid mesh has shown high mechanical property and good biocompatibility. It is anticipated that PLGA-collagen hybrid
mesh can be used as a good mechanical supporting skeleton for deposition of cell-derived ECMs to prepare tissue development-mimicking 3D ECM scaffolds. Therefore, in this work, PLGA-collagen hybrid mesh was prepared by hybridization of collagen solution and a PLGA knitted mesh and used for deposition of cell-derived ECMs (Figure 1).

![Figure 1. Preparation scheme of PLGA-collagen hybrid mesh.](image)

Deposition of cell-derived ECMs was conducted by culturing hMSCs and inducing their differentiation at different stages. The hMSCs were seeded in the PLGA-collagen hybrid mesh and controlled at stem stage, early-stage and late-stage of adipogenesis or osteogenesis to mimic the stepwise adipogenesis or/and osteogenesis. After deposition of ECMs and further decellularization, stepwise tissue development-mimicking ECM-deposited PLGA-collagen hybrid meshes (PLGA-collagen-ECM hybrid meshes) were prepared. The biomimetic hybrid meshes were used for culture of hMSCs to investigate their influence on the adhesion, proliferation and differentiation of hMSCs. In part I, PLGA-collagen-ECM hybrid meshes mimicking stepwise adipogenesis ECM were prepared and used for investigation of their influence on adipogenic differentiation of hMSCs (Figure 2a). In part II, PLGA-collagen-ECM hybrid meshes mimicking stepwise osteogenesis ECM were prepared and used for investigation of their influence on osteogenic differentiation of hMSCs (Figure 2b). In part III, an in vitro 3D model that reflects the dynamic remodeling of ECMs during simultaneous osteogenesis and adipogenesis of hMSCs was developed. The biomimetic ECM scaffolds were used for 3D culture of hMSCs to investigate how they balance osteogenic and adipogenic differentiation of hMSCs (Figure 2c).

1. Preparation of stepwise adipogenesis-mimicking ECM-deposited PLGA-collagen hybrid meshes and their influence on adipogenic differentiation of hMSCs

The PLGA-collagen hybrid mesh was prepared by forming web-like collagen microsponges in the void spaces of a PLGA knitted mesh. SEM image at a high magnification indicated that the fibers of collagen microsponges and the fiber bundles of PLGA mesh were physically locked (Figure 3a). hMSCs were cultured in the hybrid mesh and controlled at the stem cell stage (SC), the early (EA) and late (LA) stages of adipogenesis. The stepwise adipogenic differentiation of hMSCs in the PLGA-collagen hybrid mesh was confirmed by Oil Red O staining (Figure 3b) and real-time PCR analysis. After cell culture, cellular components
such as cell nuclei and membranes were removed by decellularization to obtain the adipogenesis-mimicking 3D ECM hybrid scaffolds (Figure 3c). They were defined as SC-ECM scaffold, EA-ECM scaffold and LA-ECM scaffold. SEM images of the ECM scaffolds showed that they had similar porous structure to that of PLGA-collagen hybrid meshes (Figure 3d). The influence of ECM scaffolds on adipogenic differentiation of hMSCs was investigated by reseeding hMSCs in the 3D ECM hybrid scaffolds.

The results of stained lipid vacuoles showed that hMSCs cultured in the EA-ECM scaffolds had the highest amount of lipid droplets (Figure 3e). The highest gene expression level of PPARγ, CEBPA, LPL and FABP4 was shown in the stem cells when being cultured in EA-ECM scaffolds, while the expression of these genes in hMSCs cultured in the SC-ECM and LA-ECM scaffolds was significantly lower than that in cells cultured in PLGA-collagen scaffold (Figure 3f). The results indicated that EA-ECM scaffolds promoted adipogenic differentiation of hMSCs. However, the SC-ECM and LA-ECM scaffolds had an inhibitory effect on adipogenic differentiation of hMSCs.
Figure 2. Preparation scheme of stepwise adipogenesis-mimicking ECMs-deposited PLGA-collagen hybrid meshes (a). The PLGA-collagen-ECM hybrid meshes mimicking stepwise osteogenesis (b). Preparation of PLGA-collagen-ECM hybrid meshes by decellularizing hMSCs/PLGA-collagen hybrid mesh constructs. The hMSCs were cultured and simultaneously induced at stepwise stages of osteogenesis-co-adipogenesis (c).

Figure 3. SEM image of PLGA-collagen hybrid mesh (a). Oil Red O staining of hMSCs after being cultured in the hybrid mesh at different conditions (b). DNA quantification of the hMSCs/PLGA-collagen hybrid mesh constructs before and after decellularization (c). SEM images of the ECM-deposited PLGA-collagen hybrid meshes (d). Adipogenic differentiation of hMSCs in the stepwise adipogenesis-mimicking ECM-deposited PLGA-collagen hybrid meshes in adipogenic induction medium. Representative images of Oil Red O staining of hMSCs in the scaffolds (after 14 days of culture) (e). Real-time PCR analysis of PPARγ, CEBPA, LPL, FABP4 genes (f). Data represent means ± S.D. (n = 3). *, P < 0.05; **, P < 0.01; ***, P < 0.001. N.S., no significant difference.

2. PLGA-collagen-ECM hybrid meshes mimicking stepwise osteogenesis and their influence on osteogenic differentiation of hMSCs

Bone marrow cell-derived ECM can provide a favorable microenvironment for the maintenance of human mesenchymal stem cell (hMSC) activity, controlling cell adhesion and osteogenic differentiation. On the other hand, cells can mediate the remodeling of ECMs
through secretion of bioactive molecules such as matrix metalloproteinases (MMPs) and large amounts of ECMs. The regulation of hMSC behavior mainly relies on ECM-cell interactions. Recently, investigations of human bone marrow cell-derived ECMs and how ECM elements interact with hMSCs to modulate their functions have attracted broad attention. In this part, a PLGA-collagen hybrid mesh was used as a mechanical supporting skeleton for the cell culture and deposition of osteogenesis-mimicking ECMs. The hMSCs were seeded in the PLGA-collagen hybrid meshes and controlled at the stem cell stage, the early and late stages of osteogenesis. Alkaline phosphatase (ALP) staining, alizarin red S staining and detection of the gene expression of bone sialoprotein 2 (IBSP) were used to confirm the stepwise osteogenic differentiation of hMSCs. ALP is an early-stage marker of osteogenesis, while calcium deposition and IBSP are late-stage markers of osteogenesis. The results of staining analysis showed that after 7 days of culture in osteogenic medium, the hMSCs/PLGA-collagen hybrid mesh constructs showed obvious positive staining for ALP but no obvious calcium deposition (Figure 4a, EO). When the cells were cultured in the PLGA-collagen hybrid meshes for 21 days, obvious calcium deposition was observed in the constructs after alizarin red S staining (Figure 4a, LO). Both ALP staining and calcium deposition were negative when hMSCs were cultured in the PLGA-collagen hybrid meshes in basal medium for 7 days (Figure 4a, SC). The stepwise differentiation of the cells was further confirmed by analysis of the expression of the ALP and IBSP genes with real-time PCR. The expression of ALP was high in the cell/PLGA-collagen hybrid mesh constructs after 7 days of culture in osteogenic medium (Figure 4b). On the other hand, the expression of IBSP was not high until 21 days of culture in osteogenic medium. Based on these results, the early stage (EO) and late stage (LO) of osteogenesis were defined as 7 days and 21 days culture of hMSCs in the PLGA-collagen hybrid meshes with osteogenic medium.

After the stepwise osteogenesis of hMSCs in PLGA-collagen hybrid meshes, the cell/hybrid mesh constructs were decellularized. To evaluate the decellularized efficiency, the nuclei and actin skeleton of cells located in the constructs were visualized by fluorescence staining. The staining analysis showed that actin filaments and cell nuclei could be observed before decellularization but that positive staining was not observed after decellularization (Figure 4c). SEM observations showed that the cells proliferated and secreted ECM, filling the void spaces in the hMSCs/PLGA-collagen hybrid mesh constructs before decellularization. After decellularization, cellular components were removed, leaving cell-derived ECMs in the scaffolds and the scaffolds became more porous (Figure 4d). The porous structure of the PLGA-collagen-ECM hybrid meshes were different from that of the PLGA-collagen hybrid mesh because ECMs were deposited on the PLGA-collagen hybrid mesh and caused the pores becoming slightly smaller.

After hMSCs were cultured in the PLGA-collagen-ECM hybrid scaffolds in osteogenic medium for 21 days, the expression of osteogenesis-related genes was analyzed. The results indicated that the hMSCs cultured in the EO-ECM scaffold showed the highest gene expression of RUNX2, SP7, IBSP and SPP1 (Figure 4e). When the cells were cultured in the LO-ECM scaffold, only IBSP gene expression was significantly upregulated, while the expression of RUNX2, SP7 and SPP1 was not significantly different from that in the cells cultured in the PLGA-collagen hybrid mesh. In contrast, the expression of RUNX2 and IBSP in the hMSCs cultured in the SC-ECM scaffold was significantly lower than that in the cells cultured in the
PLGA-collagen scaffold. The results indicated that the osteogenic differentiation of the hMSCs in the EO-ECM scaffolds was increased, while an inhibitory effect was observed when hMSCs were cultured in SC-ECM scaffolds. The LO-ECM scaffolds showed a moderate effect on the promotion of the osteogenic differentiation of hMSCs.

Figure 4. Alkaline phosphatase (ALP) and alizarin red S staining of hMSCs after being cultured in the hybrid mesh under different conditions (a). Actin cytoskeleton (green fluorescence) and cell nucleus (blue fluorescence) staining of the hMSCs/PLGA-collagen constructs before and after decellularization (b). SEM images of the hMSCs/PLGA-collagen constructs before and after decellularization (d). Real-time PCR analysis of the RUNX2, SP7, IBSP and SPP1 genes (e). Data represent means ± S.D. (n = 3). *, P < 0.05; **, P < 0.01; ***, P < 0.001; N.S., no significant difference versus the PLGA-collagen group.

3. PLGA-collagen-ECM hybrid meshes functionalized with biomimetic extracellular matrix secreted by mesenchymal stem cells during stepwise osteogenesis-co-adipogenesis

The development of an in vitro 3D model that reflects the dynamic remodeling of ECMs during simultaneous osteogenesis and adipogenesis of hMSCs can provide a useful tool to mimic the process and to investigate how 3D ECMs balance the osteogenic and adipogenic differentiation of hMSCs. In this part, hMSCs were cultured in the PLGA-collagen hybrid mesh and their osteogenic differentiation and adipogenic differentiation were simultaneously controlled at early or late stages. The ECMs secreted by the cells were deposited in situ on the hybrid mesh to prepare PLGA-collagen-ECM hybrid meshes mimicking osteogenesis-co-adipogenesis of hMSCs. Biomimetic ECM scaffolds were used for 3D culture of hMSCs to
investigate how they balance the osteogenic and adipogenic differentiation of hMSCs. Stepwise osteogenic-co-adipogenic differentiation was controlled at five different stages: stem cell stage, early osteogenesis-co-early adipogenesis stage (EOEA), early osteogenesis-co-late adipogenesis stage (EOLA), late osteogenesis-co-early adipogenesis stage (LOEA) and late osteogenesis-co-late adipogenesis stage (LOLA). Four types of stepwise differentiation ECMs were prepared after decellularization: ECMs secreted by hMSCs at early stages of osteogenesis and adipogenesis (EOEA-ECMs), hMSCs at early stages of osteogenesis and late stages of adipogenesis (EOLA-ECMs), hMSCs at late stages of osteogenesis and early stages of adipogenesis (LOEA-ECMs) and hMSCs at late stages of osteogenesis and late stages of adipogenesis (LOLA-ECMs). The stages of osteogenic-co-adipogenic differentiation of hMSCs were confirmed by measuring the early and late stage markers of osteogenesis and adipogenesis. ALP activity and calcium deposition were used as early and late stage markers of osteogenesis, respectively. The early stage gene markers of osteogenesis, ALP, and late stage gene markers of osteogenesis, IBSP, were also analyzed to characterize stepwise osteogenesis. Gene expression of lipoprotein lipase (LPL) and formation of lipid vacuoles served as early and late stage markers of adipogenesis, respectively. ALP staining was positive in the EOEA, EOLA, LOEA and LOLA groups, while only the LOEA and LOLA groups showed positive staining for calcium deposition (alizarin red S staining). The results of Oil Red O staining showed that lipid vacuoles were only observed in the EOLA and LOLA groups (Figure 5a). During osteogenic-co-adipogenic differentiation of hMSCs, hMSCs proliferated and secreted ECMs filling the void spaces in the PLGA-collagen hybrid meshes. The hMSCs/PLGA-collagen-ECM constructs were decellularized according to our previous method. Measurement of DNA content in the constructs before and after decellularization showed that more than 98.9% of DNA was removed after decellularization. The decellularized scaffolds were defined as SC-ECM, EOEA-ECM, EOLA-ECM, LOEA-ECM and LOLA-ECM scaffolds. SEM observation showed that all the PLGA-collagen-ECM scaffolds were highly porous (Figure 5b). The deposited ECMs had different compositions that were dependent on the different stages of osteogenesis and adipogenesis. To understand the role of osteogenesis-co-adipogenesis mimicking ECMs in balancing adipogenic and osteogenic differentiation of hMSCs, their effect on adipogenic and osteogenic differentiation of hMSCs was compared. hMSCs were first cultured in PLGA-collagen-ECM hybrid meshes with adipogenic medium for 14 days to evaluate their adipogenic differentiation capacity. hMSCs cultured in PLGA-collagen hybrid mesh was used as a control. Lipid vacuoles formed in the cells were stained with Oil Red O solution. Quantification of the Oil Red O staining showed that cells cultured in EOEA-ECM scaffolds and PLGA-collagen hybrid mesh showed significantly higher staining intensity than did cells cultured in the other groups. Furthermore, cells cultured in SC-ECM and EOLA-ECM scaffolds showed the lowest staining intensity (Figure 5c). The influence of the ECM scaffolds on the osteogenic differentiation of hMSCs was evaluated by culturing hMSCs in ECM scaffolds with osteogenic medium for 21 days.
Figure 5. ALP, alizarin red S and Oil Red O staining of the constructs after stepwise differentiation (a). SEM images of PLGA-collagen-ECM scaffolds (b). Quantitative analysis of the lipid vacuoles dye after 14 days of culture in adipogenic medium (c). Quantitative analysis of the calcium deposits after 21 days of culture (d).

Calcium deposition was quantified using the QuantiChrom™ calcium assay Kit (BioAssay). Quantification of the calcium deposition showed that LOEA-ECMs and LOLA-ECMs had higher level of calcium deposition than did the other scaffolds (Figure 5d). In conclusion, EOEA-ECMs scaffold showed promotive effect on adipogenic differentiation of hMSCs. EOLA-ECM scaffold had suppression effect on both adipogenic and osteogenic differentiation of hMSCs, while promotive effect on hMSCs proliferation. LOEA-ECMs and LOLA-ECMs scaffolds exhibited promotive effect on osteogenic differentiation and a moderate effect on adipogenic differentiation of hMSCs.

In summary, 3D PLGA-collagen-ECM hybrid meshes were prepared as a platform for investigation of ECM-cell interactions. In this system, the PLGA-collagen hybrid mesh was used as a porous support because of its high mechanical property and good biocompatibility. hMSCs were cultured in the PLGA-collagen hybrid mesh and their osteogenic, adipogenic or osteogenic-co-adipogenic differentiation were controlled at early or/and late stages. After cell
culture, the cells/PLGA-collagen constructs were decellularized to remove cellular components. Thus, ECMs secreted by the cells were deposited in situ on the PLGA-collagen hybrid meshes and formed PLGA-collagen-ECM hybrid meshes. Biomimetic ECM scaffolds were used for 3D culture of hMSCs to investigate ECM-cell interaction. The ECM scaffold mimicking the early stage of adipogenesis enhanced the adipogenic differentiation of hMSCs. The ECM scaffold mimicking the early stage of osteogenesis promoted the osteogenic differentiation of hMSCs. However, the ECM scaffold mimicking the stem cell stage of hMSCs exhibited an inhibitory effect on both adipogenic and osteogenic differentiation of hMSCs. Depending on the stage of osteogenesis-co-adipogenesis of hMSCs, the ECM scaffolds had different compositions. The influence of ECM scaffolds on adipogenic and osteogenic differentiation of hMSCs was also dependent on ECM type. EOEA-ECM scaffold had a high effect on promotion of adipogenesis while a low effect on promotion of osteogenesis of hMSCs. EOLA-ECM scaffold had a low effect on promotion of both adipogenesis and osteogenesis of hMSCs. LOEA-ECM and LOLA-ECM scaffolds showed a high effect on promotion of osteogenesis while a moderate effect on promotion of adipogenesis of hMSCs. The results of this study offer a useful method to mimic the dynamic ECM remodeling during cell development and the biomimetic ECM scaffolds are useful for investigation of ECM-cell interaction.