筑波大学審査学位論文（博士）**博士論文の要約**

Development of Biomimetic Matrices from Cultured Cells
(培養細胞由来のマトリックス材料の作製)

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Abstract

Cells are surrounded by the extracellular matrix (ECM) which plays an important role in regulation of cell functions and tissue development. The main function of ECM on cells is to provide biochemical and biomechanical signals that regulate cell behaviors such as cell attachment, proliferation, migration, differentiation and apoptosis. ECM is a dynamic and complex environment characterized by biophysical, biomechanical and biochemical properties specific for each tissue. The composition of specific matrices not only varies with the type of tissue, but also alters according to the tissue's development stage and pathological state. Therefore, precisely controlled ECM model is necessary for regulation of cell functions and effective regeneration of functional tissues.

A detailed characterization of ECM constituents is now considered essential for understanding cell behaviors in the context of tissue and organ development. Therefore, precisely controllable ECM model is required to figure out the role of ECM. Generally, chemical coating of ECM, tissue decellularization and cell-derived ECM are used to mimic the native ECM. However, chemical coating method is hard to achieve the molecular complexity of matrices. Although some decellularization tissues are used in pre-clinical research due to the preserved tissue architecture, most of the decellularization tissues are xenogenic matrices that may have potential risks of pathogen transmission and provocation of undesirable inflammatory and immunological reaction. The decellularization tissue matrices cannot mimic the development process of the tissues either. Similar to decellularization tissues and organs, ECMs derived from cultured cells represent a complex meshwork with a composition and organization of native ECM, which can mimic the development process of tissues. The properties of cell-derived ECMs can be controlled by manipulation of specific stimuli making them suitable for specific application. Cell-derived ECM also can obtain a tunable architecture by deposition of ECM on template surface.

Stem cells are one of the most promising cell sources for tissue engineering and regeneration. When stem cells differentiate into somatic cells, they pass through stepwise stages of maturation. During the stepwise differentiation, the ECM produced by the cells dynamically alters to regulate the stem cell proliferation and differentiation. The properties of cell-derived ECM, especially biochemical composition, are highly dependent on the source cell population. Meanwhile, as disease states are often reflected by alteration of cell phenotypes, investigation of the properties of matrices derived from diseased cell populations should provide favorable models for better understanding the interaction between cells and ECM during disease progression.

In this study, biomimetic stepwise matrices were prepared by cell culture method. At first, a novel type of ECM stepwise and simultaneously mimicking osteogenesis and adipogenesis was developed to mimic the dynamic change of ECM during bone tissue development by culturing human bone marrow-derived mesenchymal stem cells (MSCs). The effect of ECM on MSCs differentiation was further investigated. Secondly, osteosarcoma

microenvironment associated cells (MSCs, fibroblasts, osteoblasts and MG63 cells) derived matrices were prepared and used for investigation of their effects on MSCs and cancer cell (MG63) attachment and proliferation. Finally, three-dimensional (3D) ECM scaffolds mimicking chondrogenesis of MSCs were prepared and the role of "stepwise chondrogenesis-mimicking 3D scaffold" on regulation of chondrogenic differentiation of MSCs was investigated.

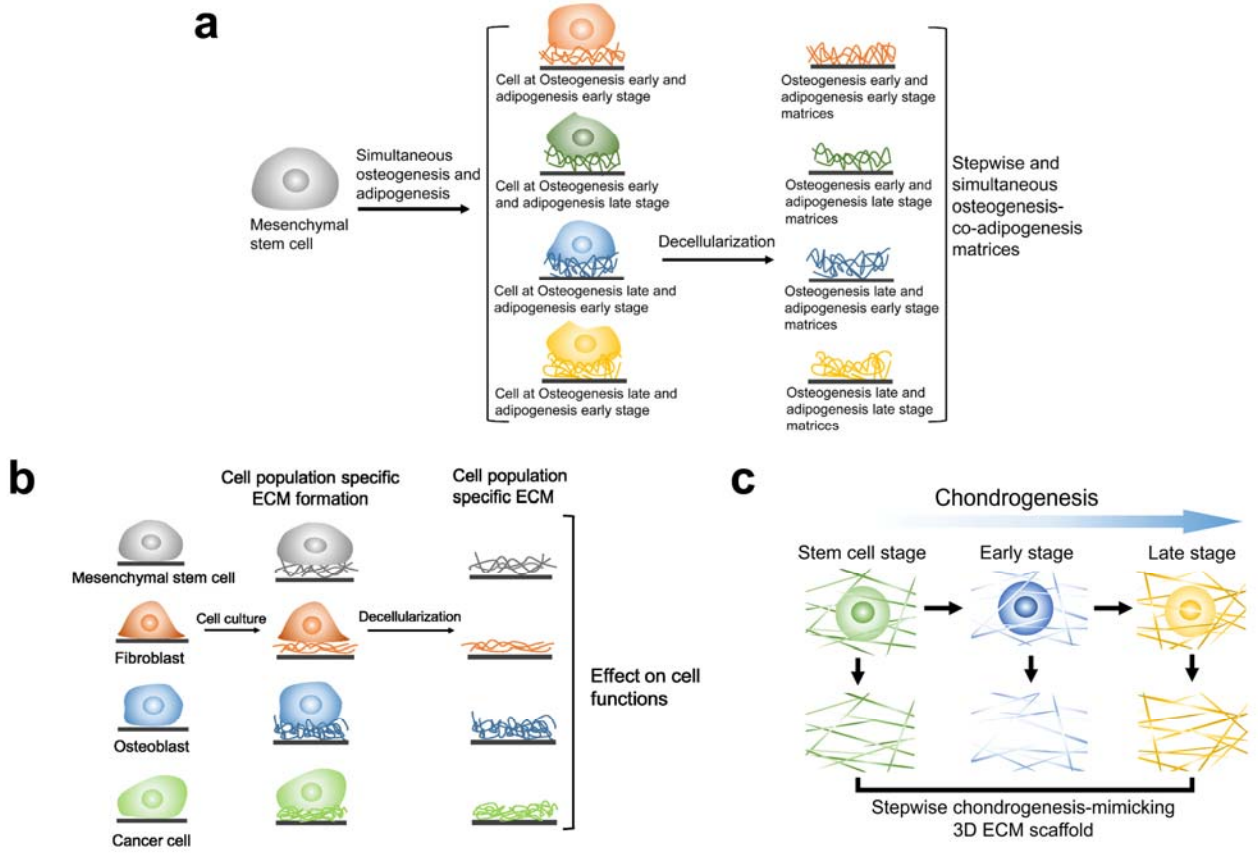

Figure 1. (a) Preparation strategy of stepwise and simultaneous osteogenesis-co-adipogenesis-mimicking matrices. (b) Preparation strategy of matrices derived from MSCs, fibroblasts and cancer cells for investigation of their effects on cancer cell behaviors. (c) Preparation strategy of stepwise chondrogenesis-mimicking 3D matrices.

1. Development of stepwise and simultaneous osteogenesis-co-adipogenesis-mimicking matrices

In normal developmental stages, the ECM surrounding bone marrow-derived mesenchymal stem cells (MSCs) is remodeled according to the different stages of differentiation. Abnormal ECM dynamics are documented in clinical studies of many diseases. For example, the ECM in pathological cases such as osteoporosis has different characteristics compared with the normal tissues. The ECM in osteoporosis cases hase a mixture composition derived from osteogenesis and adipogenesis of MSCs. So far, ECM during either osteogenesis or adipogenesis have been examined to understand their roles in pathological cases which disrupt the balance of osteogenesis and adipogenesis. Although decellularized osteoporotic bone tissues have been used, the stages of osteogenesis and adipogenesis are different from each patient. Decellularized osteoporotic bone tissues are not suitable for the investigation of ECM roles in the diseases caused by disruption of bone homeostasis. To solve this problem, ECM models which possess the composition of both osteogenic and adipogenic states and the stages of osteogenesis and adipogenesis are strongly desirable. In this part, a novel type of extracellular matrices that could mimic the dynamic variation of ECM was prepared from simultaneous osteogenesis and adipogenesis of human

bone marrow-derived MSCs. Four types of osteogenesis-co-adipogenesis-mimicking matrices were prepared and used to investigate their effect on the osteogenic and adipogenic differentiation of MSCs. The simultaneous osteogenesis and adipogenesis of MSCs were induced by culturing MSCs in the mixture medium of osteogenic medium and adipogenic medium for different culture period. The four types of matrices are early osteogenesis and early adipogenesis (OEAE) matrices, early osteogenesis and late adipogenesis (OEAL) matrices, late osteogenesisans and early adipogenesis matrices (OLAE) matrices and late osteogenesis and late adipogenesis (OLAL) matrices (Figure 2a-b). The matrices had different compositions. They supported adhesion and proliferation of MSCs and showed different effects on adipogenic and osteogenic differentiation of MSCs. OLAE and OLAL matrices promoted osteogenic differentiation but not adipogenic differentiation of MSCs. OEAE matrices promoted adipogenic differnation but not osteogenic differentiation of MSCs. OEAL did not promote either osteogenic or adipogenic differentiation of MSCs (Figure 2c-d). The stepwise osteogenesis-co-adipogenesis-mimicking matrices will provide a novel tool for investigation of the ECM effects on stem cell differentiation and some diseases.

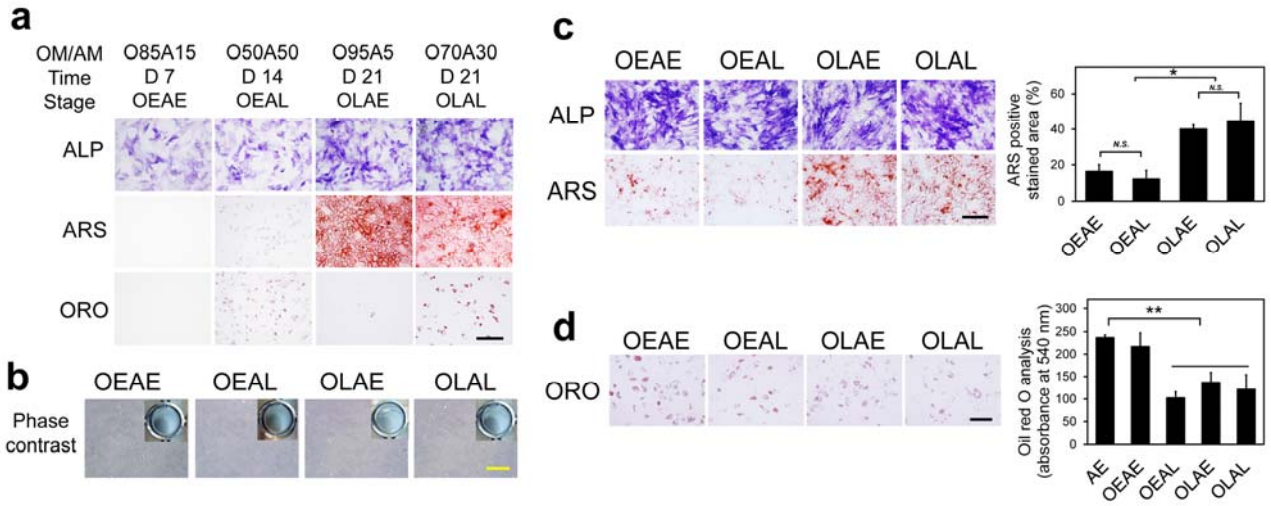

Figure 2. Development of stepwise and simultaneous ostogenesis-co-adipogenesis-mimicking matrices and the effect of the matrices on osteogenic differentiation and adipogenic differentiation of MSCs. (a) Histological stainings were used to define the stepwise stages of simultaneous osteogenesis and adipogenesis of MSCs. ALP, ARS and ORO represent alkaline phosphatase, Alizarin red S and Oil red O staining, respectively. Scale bar, 500 μm. (b) Phase-contrast images of decellularized matrices. Insets show Coomassie Brilliant Blue staining indicating proteins remained after decellularization. Scale bar, 500 μm. (c) Histological stainings (ALP and ARS) and quantification of the percentage of ARS positively stained area were used to investigate the effect of the matrices on osteogenic differentiation of MSCs. Scale bar, 500 μm. (d) Oil red O staining and quantification of cellular oil droplets (n = 3) were used to investigate the effect of the matrices on adipogenic differentiation of MSCs. Scale bar, 200 μm. All data represent means ± S.D.. *N.S.*, no significant difference; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

2. Investigation of the effect of osteosarcoma involved cells derived ECM on MSCs and cancer cell behaviors.

Tumors are made of multiple cell types and components. Altered ECM properties have been associated with numerous pathological conditions including cancers. In all disease cases, changes to the ECM are not simply symptoms of disease but are contributes to the pathogenic process. In cancer, alteration in ECM composition and organization has been implicated in the progression of malignant tumors. Cancer progression is regulated by a

complex interplay of genetic and epigenetic changes, evolving interactions between tumor cells and the surrounding ECM. In this part, osteosarcoma microenvironment associated cells such as MSCs, fibroblasts (FB), osteoblasts (OB) and MG63 cells were cultured for preparation of cell-derived ECM (MSC-ECM, FB-ECM, OB-ECM and MG63-ECM) (Figure 3a). These ECMs showed different components and different effects on MSCs and MG63 cells attachment, spreading and proliferation. MSC-ECM and FB-ECM highly enhanced the initial attachment and spreading of both MSCs and MG63 cells (Figure 3b-c). OB-ECM slightly enhanced the initial attachment of MSCs and MG63 cells. On contrast, MG63-ECM obviously inhibited the initial attachment and spreading compared to TCPS. The matrices will be useful for investigation of the effect of ECM on cancer cells functions.

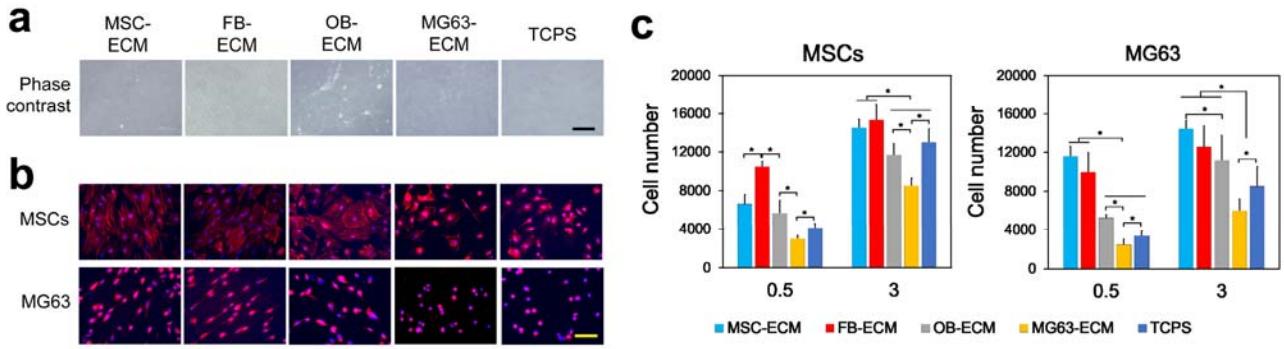

Figure 3. Preparation of MSCs, fibroblasts (FB), osteoblasts (OB) and MG63 cells derived ECM and investigation of their effects on MSCs and MG63 cells behaviors. (a) Phase contrast images of MSC, FB, OB and MG63 cells derived ECM. Tissue culture polystyrene plates (TCPS) were used as a control group. Scale bar, 500 μm. (b) Cell morphology of MSCs and MG63 cells on the matrices after 1 day of culture. Scale bar, 200 μm. (c) Cell numbers of MSCs and MG63 cells attached on the matrices after 0.5 and 3 hours of culture (n = 3). All data represent means ± S.D.. *, $p < 0.05$, indicates significant difference.

3. Development of stepwise chondrogenesis-mimicking 3D matrices scaffold

Besides osteogenesis and adipogenesis, MSCs also can differentiate into chondrocytes. In this part, 3D stepwise chondrogenesis-mimicking ECM scaffolds were prepared from mesenchymal stem cells (MSCs) by controlling the stages of chondrogenic differentiation. ECM scaffolds mimicking the early stage and late stage of chondrogenesis were obtained when MSCs cultured in the chondrogenic medium for 1 and 3 weeks, respectively (Figure 3a). The ECM scaffolds had different compositions. Stem cell (SC)-ECM scaffold was rich in collagen I and biglycan, early stage chondrogenesis-mimicking (CE)-ECM scaffold was rich in collagen II, while the late stage chondrogenesis-mimicking (CL)-ECM scaffold was rich in collagen II and aggrecan. These three ECM scaffolds had different effects on chondrogenesis of MSCs. The CE-ECM scaffold facilitated chondrogenesis, however, the CL-ECM scaffold remarkably inhibited chondrogenesis of MSCs (Figure 3b-d). These ECM scaffolds not only provide new 3D ECM models to investigate the effects of ECM on MSCs functions, but also can be used as favorable ECM scaffolds for tissue engineering.

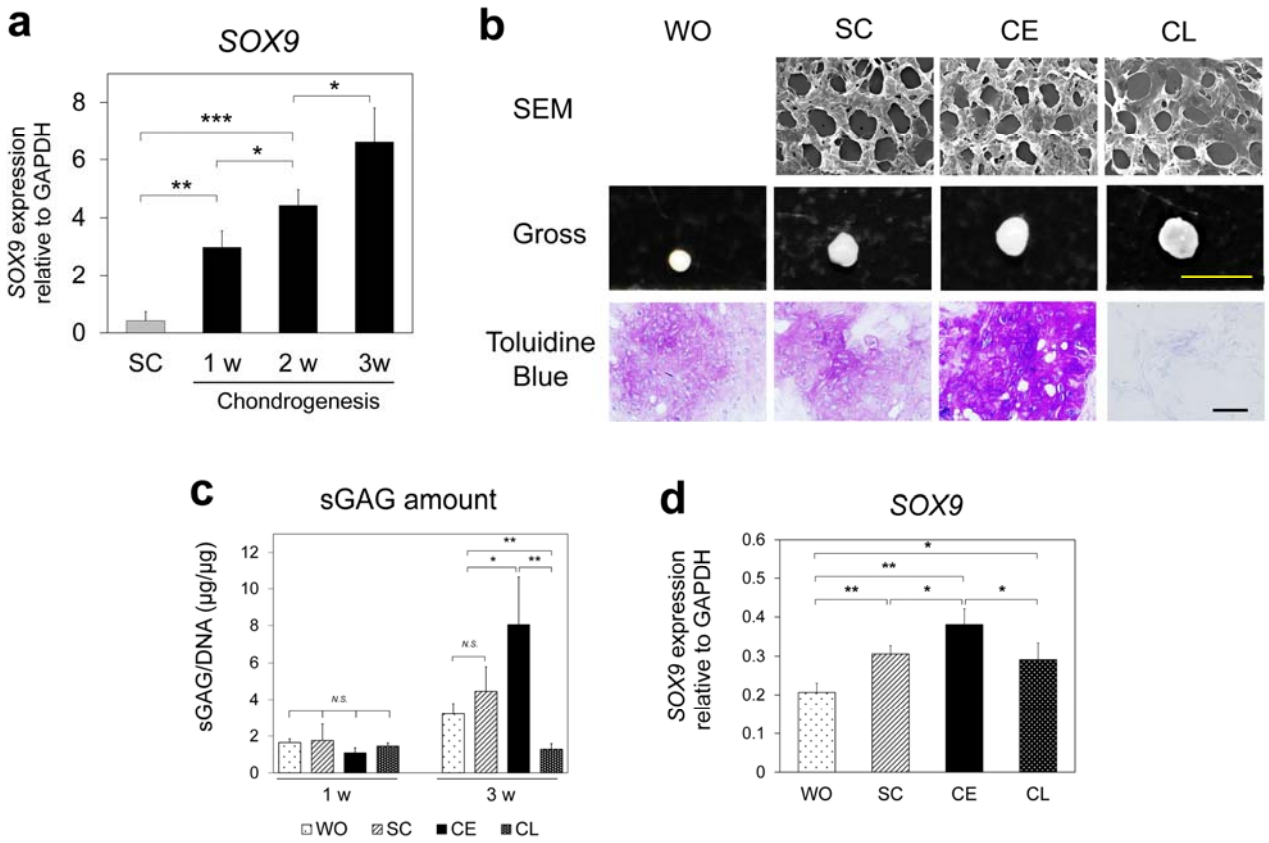

Figure 3. Development of stepwise chondrogenesis-mimicking matrices and the effect on chondrogenic differentiation of MSCs. (a) Expression level of *SOX9* (the key transcription factor of chondrogenesis) in cells that were cultured on PLGA templates in the presence of chondrogenic medium for 1, 2 and 3 weeks or cultured in the normal cell culture medium (SC) was used to define the different stage of chondrogenesis (n = 3). (b) Scanning electron microscope images of stepwise chondrogenesis-mimicking matrices after decellularization and removal of PLGA templates. Gross appearance of MSCs/matrices constructs after 3 weeks of culture. Toluidine blue staining of cartilage specific matrices in MSCs/matrices constructs after 3 weeks. WO, SC, CE and CL indicate no matrices (conventional pellet culture), stem cell stage matrices, chondrogenesis early stage matrices and chondrogenesis late stage matrices, respectively. Scale bar, 100 μm. (c) sGAG amount in the MSCs/matrices constructs after 1 and 3 weeks of culture (n = 3). (d) Expression level of *SOX9* in the MSCs/matrices constructs after 3 weeks of culture (n = 3). All data represent means ± S.D.. *N.S.*, no significant difference; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

In summary, cell-derived matrices were demonstrated to be a good alternative to investigate the interaction between cells and ECM and the role of ECM on regulation of stem cells. “Stepwise development-mimicking matrices” provided well controlled ECM models for mimicking the differentiation process of stem cells. According to the different cell populations related to the diseases, diseased cells ECM could also be obtained. In addition, cell-derived matrices not only could be deposited on two-dimensional substrates, but also could be formed in 3D ECM scaffolds by using 3D scaffold templates. All the matrices and ECM scaffolds could mimic the dynamic ECM variation during osteogenesis and adipogenesis of MSCs. The advantage of cultured cell-derived ECMs is their best controllability due to the easy availability and controllable culture conditions. Specific ECMs could be developed for regulating cell functions in a desirable way. Therefore, the future of cultured cell-derived ECM in tissue regeneration is promising.